GLYCOPROTEIN K OF HERPES SIMPLEX VIRUS (HSV): ROLE IN VIRAL EGRESS AND HSV-INDUCED CELL-CELL FUSION

By

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ABSTRACT

The fusion of cellular and viral membranes induced by herpes simplex virus type 1 (HSV-1) is essential for many stages of the replication cycle including virus penetration into host cells, nucleocapsid envelopment, virus egress and transmission of virus from infected to uninfected cells. Wild-type infections also induce low levels of cell-cell fusion, and syncytial mutants of HSV cause cells to fuse extensively. Previous studies have demonstrated that a large fraction of syncytial viruses contain mutations in the HSV-1 UL53 gene, indicating that UL53 likely plays a central role in HSV-induced membrane fusion. Consequently, the objectives of this thesis are as follows: 1) Identify and characterize the UL53 gene product produced by wild-type and syncytial strains of HSV-1, and 2) Investigate the role of this protein in HSV-induced membrane fusion and in HSV-1 replication.

A single 40-kDa protein containing unprocessed, high-mannose N-linked oligosaccharides was detected in cells infected with wild-type and syncytial strains of HSV-1. This protein was designated gK, the ninth HSV-1 glycoprotein to be described. gK is unique among HSV-1 glycoproteins since all other HSV glycoproteins examined to date exist as two proteins species; an immature protein with unprocessed oligosaccharides and a mature form containing complex N-linked oligosaccharides and O-linked oligo-saccharides acquired during post-translational processing in the Golgi apparatus. The gK protein also exhibited signs of heat induced aggregation, an attribute which is commonly observed in proteins spanning the membrane multiple times.

Low levels of gK were expressed in HSV-infected cells relative to HSV

glycoproteins with a direct role in membrane fusion, which is more consistent with a regulatory role for gK in the membrane fusion process. Wild-type gK, but not other HSV glycoproteins, inhibited cell-cell fusion induced by HSV-1 strains encoding a mutant form of gK (gK^{syn}). As such, the recessive phenotype displayed by gK^{syn} may be symptomatic of a loss-of-function mutation, which disrupts a regulatory function governing membrane fusion.

Glycoprotein K was not detected in HSV-1 virions and immunofluorescence microscopy demonstrated that gK is not transported to the surfaces of cells infected with either wild-type or syncytial HSV. Instead, gK accumulates in the perinuclear and nuclear membranes of cells, and is unlikely to reach the Golgi apparatus because gK oligosaccharides remained sensitive to endoglycosidase H, These findings are in contrast to the behaviour of all other HSV glycoproteins described to date, which are expressed on the cell surface and incorporated into the virion envelope. Therefore gK must influence membrane fusion indirectly, since the protein is absent from the virion and does not reach the plasma membrane. Furthermore, these results imply that syn mutations in gK induce fusion between the surface membranes of HSV-infected cells by deregulating some aspect of virus replication.

Resident proteins are maintained within intracellular compartments (eg. Golgi membranes) by one of three mechanisms: retrieval signals, retention through oligomerization, or retention through membrane thickness. Increasing gK protein synthesis by 10-20 fold did not overcome the block in cell surface transport, and this property is typical of membrane proteins maintained within intracellular compartments by retention signals. Furthermore, gK expressed in the absence of other HSV proteins by using a recombinant adenovirus vector, exhibited the traits and subcellular distribution of gK

expressed in HSV-infected cells. These observations indicate that gK contains the targeting signals responsible for gK retention in the endoplasmic reticulum (ER) and nuclear envelope. Although the process controlling gK retention is still uncertain, gK oligomeric structures were observed in cells overexpressing the gK protein, suggesting that the formation of oligomeric assemblies may contribute to gK retention in the ER. In addition, sequence analysis identified a tyrosine-based motif (YTK[FILM]) conserved by the gK homologs of eight different alphaherpesviruses, which may have the potential to act as an ER retrieval signal.

To further investigate the role of gK in membrane fusion and HSV replication, a gK-negative mutant (F-gKß) was constructed. Since gK was found to be essential for virus replication, F-gKß was propagated on complementing cells which can express gK. F-gKß produced normal plaques characterized by rounded cells when plated on complementing cells, and F-gKß produced syncytia on cells expressing low levels of gK. In contrast, F-gKß formed microscopic plaques (consisting of 3-6 infected cells) on gK-negative cell lines and these plaques were reduced by 10^2 to 10^6 in number. In the absence of gK, large quantities of unenveloped capsids accumulated in the cytoplasm of HSV-infected cells and virus particles did not reach the cell surface. The few enveloped particles that were assembled displayed a reduced capacity to enter cells and initiate infection. Overexpression of gK also caused defects in virus egress, although virions accumulated in the perinuclear space, and large multilamellar membranous structures juxtaposed with the nuclear envelope were observed. Together these results demonstrate that gK regulates or facilitates HSV egress from cells. In addition, these findings raise the possibility that defects in gK may influence cell-cell fusion by altering the cell surface transport of viral particles, or other viral and/or cellular components which govern the fusion process.

ACKNOWLEDGEMENTS

Many people deserve thanks, for I could not have completed this research without their input. Dr. David Johnson had a profound impact during my doctoral studies, challenging me to think like a scientist and to always strive hard in my pursuit of scientific knowledge. Dr. Jim Smiley was integral to the success of this thesis and provided important insight. I also valued the advice and comments of Dr. John Capone. Dr. Frank Graham and Andy Bett participated in helpful discussions and the Ad vector technology they have developed was indispensable. McMaster Faculty members, (Drs. Bill Orr, Silvia Bacchetti, Hara Ghosh, Mark Hatton, Peter Whyte, Richard Haslam, Ken Rosenthal & Dennis Schneider) also supplied guidance and suggestions. I am extremely fortunate to have had Cindy Roop-Beauchamp, Kim Goldsmith, and Mark Whaley as co-workers and friends during the course of my Doctoral studies. Encouragement from fellow students, Craig Brunetti and Pieter Yugovich, was much appreciated. In addition, I would like to thank all of the many labmates that I have encountered over the years (Steve, Veronica, Mojgie, Mike, Tom, Ian, Kevin, Jesicca, Roman & Cathy) for making lab-life both enjoyable and educational. Other colleagues made valuable contributions to my studies at McMaster, and include Craig Smibert, Phil Kibler, Richard Tapping, Geoff Worestuik, and Scott Galachan. Further, I am greatly indebted to Josie Maljar, Laura Kendry and Ketty Sartorio both for their humour and for their technical expertise. I thank the Medical Research Council of Canada for supporting this research. Finally, I would like to thank Mary Tang, and my parents Jack and Nonie Hutchinson for inspiring me to persevere and also for their loving support.

DEDICATION

This thesis is dedicated to my parents, Jack and Norma Hutchinson, and to my dearest Mary for their unwavering support, understanding and encouragement throughout my academic studies.

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GENERAL INTRODUCTION

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LIST OF ABBREVIATIONS

a.a.	= amino acid
alphaherpesviruses	= HSV-1, HSV-2, VZV, PRV, BHV-1, EHV-1
betaherpesviruses	= HCMV, HHV-6, HHV-7
BHK cells	= baby hampster kidney cells
BHV-1	= bovine herpesvirus type 1
CG	= cis-Golgi cisternae
CHO	= chinese hamster ovary cells
CCV	= channel catfish herpesvirus
CNS	= central nervous system
CV-1 cells	= african green monkey kidney cells
DNA	= deoxyribonucleic acid
C-terminus	= carboxy terminus
E or ß	= HSV early gene product
EHV-1	= equine herpesvirus type 1
EBV	= Epstein Barr virus
FACS	= Fluorescence activated cell sorting
gammaherpesviruses	= EBV, HVS, EHV-2,
GPI	= glycosylphosphatidylinositol
HA	= hemagglutinin (influenza - orthomyxovirus)
HCMV	= human cytomegalovirus
HEL cells	= human embryonic lung cells
HEp-2 cells	= human epithelial cells
HHV-6	= human herpesvirus type 6
HIV	= human immunodeficiency virus
HSV-1	= herpes simplex virus type 1
HVEM	= herpesvirus entry mediator
HVS	= herpes virus saimiri
IC	= intermediate compartment
IE or α	= HSV immediate early gene product
ILTV	= infectious laryngotracheitis virus (host = chicken)
H-particle	= Heavy particle - infectious herpesvirion
L-particle	= Light particle - a non-infectious virus-like particle made by herpesvirus infected cells which lack a capsid and virus DNA
L, τ_1 or τ_2	= leaky late (τ_1) or true late (τ_2) HSV gene product
M6PR	= mannose-6-phosphate receptor $M6PR^{cd}$ - cation dependent $M6PR^{ci}$ - cation independent

MAbs	= monoclonal antibodies
MDCK cells	= Madin-Darbin canine kidney cells
MDBK cells	= Madin-Darbin bovine kidney cells
MDV	= Marek's disease virus (host = turkey) (Avian herpesvirus, Gallid herpesvirus)
MG	= medial-Golgi cisternae
MHCII	= major histocompatibility complex type II
MHV	= Murine gammaherpesvirus 68
MOI	= multiplicity of infection
NDV	= Newcastle disease virus (paramyxovirus)
NGF	= nerve growth factor
N-terminus	= amino terminus
ORF	= open reading frame
PAA	= sodium phosphonoacetate
PM	= plasma membrane
PRV	= pseudorabies virus (Aujesky's disease)(host = pig)
R970 cells	= human osteosarcoma cells
RER	= rough endoplasmic reticulum
roe	= rate of entry mutation
SDS-PAGE	= SDS-polyacrylamide gel electrophoresis
syn-gB, gB^{syn}	= HSV-1 gB protein containing a syncytial mutation
syn-gK, gK^{syn}	= HSV-1 gK protein containing a syncytial mutation
TG	= trans Golgi cisternae
TGN	= trans Golgi network
TNF	= tumour necrosis factor
ts	= temperature sensitive
Vero cells	= african green monkey kidney cells
VZV	= varicella zoster virus
VSV	= vesicular stomatitis virus (rhabdovirus)
wt	= wild-type
wt-gB or gB^{wt}	= glycoprotein B encoded by wild-type HSV-1
wt-gK or gK^{wt}	= glycoprotein B encoded by wild-type HSV-1

Acknowledgement of contributions made by others

This thesis is based on material which has been published in peer-reviewed journals and these manuscripts make up the bulk of Chapters 2, 3, 4, and 5 (see below)

Chapter 2. Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F.L. Graham, and D.C. Johnson. 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. 66:5603-5609.

Chapter 3. Hutchinson, L., F.L. Graham, W. Cai, C. Debroy, S. Person, and D.C. Johnson. 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology 196:514-531.

Chapter 4. Hutchinson, L., C. Roop and D.C. Johnson. 1995a. Herpes simplex virus glycoprotein K which is known to influence fusion of infected cells, yet is not transported to the cell surface. J. Virol. 69: 4556-4563.

Chapter 5. Hutchinson, L., and D.C. Johnson. 1995b. Herpes simplex virus glycoprotein K promotes egress of virus particles. J. Virol. 69:5401-5413.

These chapters are preceded by an introduction to the HSV-1 lytic replication cycle with an emphasis on membrane fusion process required for virus entry, envelopment, egress, cell-to-cell transmission and cell-cell fusion. The chapters are followed by a Discussion, which unifies the concepts and issues raised in the thesis and which considers applications and further research.

The thesis candidate wrote the initial draft of each manuscript and subsequent revisions were made in conjunction with my thesis supervisor, Dr. D.C. Johnson. Chapters 2 and 3 are modified versions of the initial drafts, which Dr. Johnson and myself revised to create the published manuscript. Chapters 4 and 5 included the published manuscripts. Chapters 2, 3 and 4 also include the efforts of several coauthors and their contributions are outlined below.

Dr. D.C. Johnson and myself collaborated on the design of peptides used to generate the anti-UL53 antibodies mentioned throughout this thesis. Dr. D.C. Johnson was largely responsible for the production of anti-UL53 sera and the thesis candidate was assigned the job of characterizing the antisera. Dr. Hara Ghosh and his graduate student Dan Snoddy supplied a plasmid used by the thesis candidate in the *in vitro* translation experiments described in Chapter 2 (Figures 2 & 3). Ms. Kim Goldsmith, in collaboration with the thesis candidate constructed the plasmids required to create the adenovirus vectors, AdgH, AdgL, and AdgK. Dr. F.L. Graham and his technician John Rudy assisted in the rescue and propagation of adenovirus vectors used in Chapters 2 & 3. Dr. Weizhong Cai, Dr. Chitrita Debroy and Dr. Stanley Person performed the transient transfection experiment, Chapter 3 (Fig. 1). Mrs Cindy Roop-Beauchamp and myself performed the endoglycosidase H assay displayed in Chapter 4 (Fig. 5). Finally, David C. Johnson assisted with the immunofluorescence photography displayed in Chapter 4.

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General Introduction:

1.0 The Herpesviridae

Herpesviruses are widely distributed in nature, with most animal species yielding at least one example (Roizman, 1996). Assignment of newly discovered viruses to the family Herpesviridae has been based on the characteristic herpes virion architecture (ie. a large icosahedral capsid containing DNA, surrounded by an amorphous layer and enclosed by a membrane) and a common replication cycle (eg. DNA synthesis, capsid assembly and envelopment occur in the nucleus) which destroys the infected cell (Roizman et al., 1981; Roizman et al., 1992; Roizman, 1996). Furthermore, these traits extend to the genetic level, at least for the herpesviruses of higher vertebrates (birds & mammals), and recent DNA sequencing studies have identified a subset of genes conserved by herpesvirus genomes which constitute 25 to 70% of all genes within a particular virus (Nicolas, 1996). However, the unifying attribute of the Herpesviridae is their propensity to remain latent after the acute phase of infection and then reactivate episodically over the lifetime of the host to sustain a periodic production and shedding of infectious particles (Roizman, 1996). Strategies to establish latency differ greatly among individual herpesviruses and the effects of reactivation events may be subclinical, or the basis for a recurring disease which is generally less severe than the primary infection.

1.1 Subfamilies of the Herpesviridae: The Alpha- Beta- and Gammaherpesvirinae

Biological criteria (eg. host range, site of latency) have been used to define three subfamilies in the Herpesviridae, the Alpha-, Beta-, and Gammaherpesvirinae (Roizman et

al., 1981; Roizman et al., 1992; Roizman, 1996). Subsequent genetic analysis has confirmed that these subfamilies correspond to three major lineages in the vertebrate herpesviruses (Albrecht et al., 1992; Baer et al., 1984; Chee et al., 1990; Davison & Scott, 1986; Gompels et al., 1995; McGeoch et al., 1988; Telford et al., 1992, 1995) and consequently genetic properties (eg. content, organization and protein homology) have also been applied to herpesvirus taxonomy.

Humans are host to herpesviruses from all three subfamilies: herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and varicella-zoster virus (VZV) belong to the Alphaherpesvirinae (Arvin, 1996; Whitley, 1996), human cytomegalovirus (HCMV), human herpesvirus types 6 and 7 (HHV-6, HHV-7) belong to the Betaherpesvirinae (Britt & Alford, 1996; Frenkel & Roffman, 1996; Pellet & Black, 1996), and Epstein-Barr virus (EBV) as well as the newly discovered human herpesvirus type 8, have been placed in the Gammaherpesvirinae (Moore et al., 1996; Rickinson & Kieff, 1996).

The Alphaherpesvirinae are characterized by a variable host range, epidermo-neurotropism, a short growth cycle with rapid spread and efficient destruction of the infected cell, as well as the ability to remain latent and reactivate, primarily but not exclusively, within sensory ganglia (Mettenleiter, 1994; Roizman, 1996; Roizman & Sears, 1996). In the natural environment alphaherpesvirus infections are normally confined to the species of origin but the experimental host range of these viruses can be broad, as is the case for HSV, or narrow, with respect to VZV (Arvin, 1996; Cohen & Strauss, 1996; Roizman & Sears, 1996; Whitley, 1996). Other herpesviruses in the alphaherpesvirus subfamily include economically important animal pathogens including equine herpesvirus 1 and 4 (EHV-1, EHV-4), gallid herpesvirus 1 also known as Mareks Disease virus (MDV), bovine herpesvirus 1 (BHV-1), and suid herpesvirus 1 also designated pseudorabies virus

(PRV) (McGeoch & Cook, 1994). These viruses are responsible for a wide variety of clinical diseases ranging from respiratory, genital, and ocular disorders, to abortion, and less frequently encephalitis or generalized systemic infections which result in high mortality rates among young animals (for review see Mettenleiter, 1993, 1994; Jacobs, 1994; Crabb & Studdert, 1995, Tikoo et al., 1995; Zelnik, 1995).

The Betaherpesvirinae are typified by a narrow host range, a long reproductive cycle in culture with slow virus spread, the marked enlargement of infected cells (cytomegalia) with the presence of cytoplasmic and nuclear inclusions, as well as the capacity to establish a latent infection in secretory glands, lymphoreticular cells, kidneys and other tissues (Mocarski, 1996; Roizman, 1996). Human cytomegalovirus (HCMV) is a Betaherpesvirinae prototype (Chee et al., 1990; Britt & Alford, 1996; Mocarski, 1996). Human herpesviruses 6 and 7 (HHV-6, -7) also belong to this subfamily but are somewhat unusual because they exhibit a trophism for $CD4^+$ T-cells during productive infection, both *in vivo* and *in vitro* (Frenkel et al., 1990; Gompels et al., 1995; Nicolas, 1996; Pellet & Black, 1996; Frenkel and Roffman, 1996).

The Gammaherpesvirinae is comprised of viruses that have a very narrow host range, both *in vitro* and *in vivo* (ie. limited to family or order of the natural host). Such viruses which tend to replicate and persist in B- or T-lymphocytes of a single species, are often associated with a lymphoproliferative disease (Roizman, 1996). This subfamily contains two genera: the B-lymphotropic human Epstein Barr virus represents the gammaherpesvirinae subgroup 1 (Lymphocryptovirus) and the predominantly T-lymphotropic specific herpesvirus saimiri (HVS) is the prototype of subgroup 2 (Rhadinovirus) (Albrecht et al., 1992; Kieff, 1996; Rickinson & Kieff, 1996). Human herpesvirus 8 has also been assigned to the Rhadinovirus genera (Moore et al., 1996).

1.2 Disease associated with the human herpesviruses

Both of the human alphaherpesviruses (HSV and VZV) produce vesicular mucocutaneous eruptions during the primary and reactivation stage, but despite these shared properties their clinical symptoms are easily distinguished (for reviews see Meier & Straus, 1992; Gurevich, 1992; Liesegang, 1992; Arvin, 1996; Whitley, 1996) .

VZV belongs to the Varicellovirus genus of the Alphaherpesvirinae and like other members of this subgroup (eg. EHV-1, BHV-1) uses the respiratory route as the predominant form of virus spread (Liesegang, 1992; Crabb & Studdert, 1995; Tikoo et al., 1995; Arvin, 1996; Roizman, 1996). Most people contract VZV as children and this highly contagious infection produces widespread mucosal and skin lesions (varicella or chickenpox) as a result of viremia and subsequent dissemination. This diseases (varicella) is usually uneventful but, in newborns or immunosuppressed children and adults, complications are common (eg. nodular or interstitial pneumonia; general systemic infection). Symptomatic reactivation of VZV is uncommon and rarely occurs more than once. Furthermore, VZV recurs predominantly in the elderly and presents as Herpes zoster or shingles (vesicular rash), a painful disease that spreads to involve much of the cutaneous dermatome (reviewed by Meier & Straus, 1992; Gurevich, 1992; Liesegang, 1992; Arvin, 1996).

HSV is the prototype of the Alphaherpesvirinae genus, Simplexvirus, and the initial HSV infection is spread by contact with the epithelial or mucous membranes of the oral cavity or genitalia (Roizman et al., 1992; Roizman, 1996). Type 1 HSV is the major cause of infections above the waist (ocular and facial) and HSV-2 is the major cause of genital herpes (Corey & Spear, 1986). The clinical features of primary HSV infections range from asymptomatic to fatal dissemination but usually manifest as gingivostomatitis

or genital ulcers and less frequently as ocular infections (mild conjunctivitis to stromal keratitis) which can produce corneal scarring and blindness (reviewed by Whitley, 1996). Dissemination of the infection is rare except in the immunologically immature (infants) or immunocompromised host (eg. organ transplant, AIDS, or chemotherapy patients) (Tang & Shepp, 1992; Whitley & Lakeman, 1995; McGrath & Newman, 1994). One of the more disturbing aspects of HSV infections has been the discovery that latent HSV DNA is present in a high proportion (28 - 46%) of autopsied human brains (eg. olfactory bulbs, amygdala, hippocampus, brain stem and trigeminal ganglia) (Becker, 1995; Sanders et al., 1996). Although there is no clear evidence to link HSV-1 to a chronic neurological syndrome, it has been suggested that the presence of HSV-1 in human brain tissue may lead to learning or behavioral deficiencies or, like HHV-6, contribute to the pathology of a neurological disorder (eg. multiple sclerosis) (Becker, 1995; Pellet & Black, 1996; Sanders et al., 1996). Reactivation of latent HSV can occur at regular intervals and may be characterized by an asymptomatic mucocutaneous infection or a small number of tightly clustered vesiculo-ulcerative lesions which are usually less severe and confined to the same anatomic location as the primary episode (eg. herpes labialis) (Meier & Straus, 1992). These conditions typically produce mild discomfort and lead to the inevitable shedding of infectious HSV-1 particles. There are two important exceptions to this generalization: (1) reactivation of latent virus in the immunosuppressed patient can involve a widespread infection of epithelial tissues and occasionally dissemination to vital organs (eg. lungs, liver, central nervous system [CNS]) leading to significant morbidity and mortality (Tang & Shepp, 1992; McGrath & Newman, 1994; Whitley, 1996), and (2) herpes simplex encephalitis which can affect the normal host (all age groups) during an acute infection or a reactivation event and often results in death or neurological

abnormalities (Whitley & Lakeman, 1995).

Infections with the betaherpesvirus HCMV, are a relatively common occurrence, but illness is frequently mild or subclinical in the immunologically normal host. However, in those individuals with immature of defective cell-mediated immunity, infection with HCMV has been associated with a variety of severe and life-threatening diseases including cytomegalic inclusion disease of neonates, or infectious mononucleosis which can lead to complications in the immunocompromised patient, including interstitial pneumonia, hepatitis or CNS involvement. In addition, there have been reports that HCMV, by itself, can act as an immunsuppressive agent, thereby increasing a patient's susceptibility to HCMV-mediated or other forms of infectious disease (reviewed by Britt & Alford, 1996). HHV-6 is a $CD4^+$ T-lymphotropic betaherpesvirus which infects up to 90% of the general population prior to 1 year of age (for review see Pellet & Black, 1996), and the symptoms of this infection can range from subclinical to exanthem subitum (mild skin rash) (Yamanishi et al., 1988) with occasional severe or fatal complications (Hall et al., 1994). Secondary, reactivated HHV-6 infections have also been implicated in lymphoproliferative disorders, post-allograft complications (eg. pneumonitis and bone marrow suppression) (Carriagan et al., 1991; Drobyski et al., 1993; Pellet & Black, 1996) and in the progression of latent HIV infection to the development of AIDS and may contribute to this disease by accelerating the destruction of $CD4^+$ cells (Lusso et al., 1989; 1991; Fairfax et al., 1994; Gompels et al., 1994). Although HHV-7 exhibits characteristics similar to HHV-6 and may have similar disease associations, the primary infection occurs later, but typically prior to 2 years of age (reviewed by Frenkel & Roffman, 1996).

The Epstein Barr virus is a ubiquitous human gammaherpesvirus which is frequently acquired in early childhood (prior to the age of three) and is almost always

asymptomatic. EBV infections can manifest as infectious mononucleosis when the onset is delayed until adolescence, have been associated with diffuse lymphoproliferative disorders (eg. lymphoblastic, Burkitt's lymphoma, Hodgkin's disease) and linked to the development of undifferentiated nasopharyngeal carcinoma. Reminiscent of many herpesvirus conditions described above, immunocompromised individuals (eg. congenital, post-transplant, AIDs patients) have a greater risk of acquiring EBV-associated lymphomas than do immunocompetent individuals (for review see Rickinson & Kieff, 1996). Very little is known about the epidemiology or the clinical symptoms caused by human herpesvirus type 8 (HHV-8) infections. Since HHV-8 can only been detected in patients with Kaposi's sarcoma or a subset of AIDS-related non-Hodgkin's lymphomas it has been suggested that HHV-8 has an epidemiology similar to HSV-2, rather than that of other human herpesviruses which are ubiquitous among the adult human population (Moore et al., 1996).

2.0 The Infectious HSV-1 particle

All herpesviruses produce infectious virions which share a common fundamental architecture that consists of four distinct ultrastructural elements. They include a central core containing DNA surrounded by three concentric structures; namely the icosahedral nucleocapsid which encloses the core, an amorphous layer of protein known as the tegument and an outer lipid envelope modified by viral membrane glycoproteins (Roizman 1990). Although capsid dimensions for the Herpesviridae fall within a narrow range (ie. 100 - 110 nm in diameter) the overall particle size for individual herpesviruses has been reported to fluctuate from 120 to nearly 300 nm (Roizman & Furlong, 1974). Some of this variation can be attributed to a difference in tegument thickness (eg. HSV-1 versus

HHV-6) but it is likely that the dimensions at the upper end of this scale are an artifact caused by damage to the virion envelope (Roizman et al., 1992).

2.1 A noninfectious HSV-1 particle

In addition to the infectious virion, herpesviruses also produce a second type of non-infectious virus-like particle, which have been termed light or L-particles in HSV-infected cells, aberrant viral forms in VZV-infected cells, and dense bodies in CMV-infected cells (Szilagyi & Cunningham, 1991; McLauchlan & Rixon, 1992; Talbot & Almeida, 1977; Irmiere & Gibson, 1983; for review see Nii, 1992). L-particles lack capsid proteins and viral DNA, but contain a full spectrum of envelope and tegument proteins. As yet, no role has been assigned to L-particles in natural infections, however; L-particles can supply functions that facilitate herpes simplex virus type-1 (HSV-1) infections (McLauchlan et al., 1992) and contain proteins missing from the infectious virion (Szilagyi & Cunningham, 1991; McLauchlan & Rixon, 1992; Yang & Courtney, 1995). These observations have lead to the suggestion that L-particles may possess a supplementary activity that contributes to HSV replication *in vivo* (McLauchlan et al., 1992).

2.2 HSV-1 genome

Each infectious HSV-1 virion contains a single linear double-stranded DNA molecule of approximately 152 kbp (Kieff et al., 1971; Wadswoth et al., 1975). The genome consists of a long (L) and short (S) component that are covalently linked, and each of these components possess a long (UL) or short (US) region of unique sequences, flanked by two inverted repeats (repeat long [RL] or repeat short [RS]) (Sheldrick &

Berthelot, 1975). This arrangement permits the L and S components to invert relative to each other and gives rise to four isomeric forms of the genome (Hayward et al., 1975). At least 78 different open reading frames have been identified in the viral genome; 60 within the UL segment, 14 in the US sequences and 4 inside the inverted repeats (McGeoch et al., 1986; McGeoch et al., 1988; Chou & Roizman, 1986; Chou et al., 1990; Dolan et al., 1992; Liu & Roizman, 1991; Barker & Roizman, 1992; Barnett et al., 1992; Georgopoulou et al., 1993; Lagunoff & Roizman, 1994; Baradaram et al., 1994; Ward et al., 1996; Carter & Roizman, 1996). Of the 78 proteins specified by HSV-1 more than 30 are known to be structural components of the virion (Spear and Roizman, 1972; Heine et al., 1974; Cassai et al., 1975; Powell and Watson, 1975; Marsden et al., 1976)

2.3 HSV-1 Capsid

Three types of nucleocapsids, designated A, B, and C or empty, intermediate, and full, respectively, have been detected in HSV-infected cells (Gibson & Roizman, 1974; Schaffer et al., 1974). Type B capsids, the precursor of type A and type C capsids (Addison et al., 1990; Sherman and Bachenheimer, 1988), contain at least seven proteins. Four proteins are shell components (UL19-VP5, UL38-VP19C, UL18-VP23, UL35-VP26), two act as internal scaffolding proteins (UL26-VP21, UL26.5-VP22a) which are removed when the viral DNA is packaged, and UL26-VP24 is thought to fulfil a dual role, both as a structural protein and as the protease which releases the internal scaffolding proteins from the capsid (Cohen et al., 1980; Dargan, 1986; Gibson & Roizman, 1972; Heine et al., 1974; Matusick-Kumar et al., 1995). A recent report also indicates that the UL6 protein is also a minor component of all three capsid types (Patel & Maclean, 1995). Type C capsids contain DNA, become enveloped and mature into infectious virions whereas the

type A capsids are thought to be products of abortive DNA packaging (Addison et al., 1990; Sherman and Bachenheimer, 1988).

Like the capsids of all herpesviruses, HSV-1 capsids consist of 150 hexavalent capsomeres (hexons) and 12 pentavalent capsomeres (pentons) (Dargan, 1986; Roizman, 1990; Newcomb et al., 1993; Steven et al, 1986; Booy et al, 1996). UL19-VP5 is the predominant structural subunit of hexons and pentons (Newcomb et al., 1993; Steven et al., 1986). UL35-VP26, the other component of hexons, resides on the outer tips of the structure (Booy et al., 1994). The other structural proteins, UL38-VP19 and UL18-VP23, form hetero-trimers that sit on the capsid floor and connect each of the capsomers together (Newcomb et al., 1993).

2.4 HSV-1 Tegument

About half of the proteins species present in the infectious virion are located in the tegument, a fibrous protein coat surrounding the capsid and underlying the envelope. Unlike the virion capsid, the size and appearance of the tegument varies widely between members of the herpesvirus family, presumably because tegument the proteins supply important functions during the early stages of infection, in addition to playing a structural role (Nazerian & Witter, 1970; McCombs et al., 1971; Nii, 1991). For example, proteins in the HSV-1 tegument trans-activate immediate-early viral gene transcription (Batterson and Roizman, 1983) and shutoff host protein synthesis (Fenwick & Walker, 1978; Read & Frenkel, 1983).

The tegument proteins are thought to be capable of self-assembly, in part because UL48-VP16 and UL49-VP22 form tegument bodies in the absence of other HSV proteins (Elliott et al., 1995), and also because tegument integrity does not require the presence of

capsid or envelope (Rixon et al., 1992; McLauchlan & Rixon, 1992). Of the major structural proteins within the tegument, UL36-VP1/2, UL46-VP11/12, UL47-VP13/14, UL48-VP16 and UL49-VP22 (Spear & Roizman, 1972), only VP16 is known to be absolutely essential for virus assembly and perhaps tegument formation (Weinheimer et al., 1992; Ace et al., 1988; Zhang & McKnight, 1993; McNabb & Courtney, 1992; Batterson et al., 1983; Barker & Roizman, 1990; Liang et al., 1995). Recent studies have placed UL25, an essential protein implicated in virus penetration and capsid maturation, in the tegument, but the relative abundance of this protein has not been determined and it is not known if UL25 is involved in virus assembly and/or tegument formation (Ali et al., 1996; Addison et al., 1984). The other tegument proteins (eg. UL11, UL13-protein kinase, UL37, UL41-virion host shutoff, UL56, US9 and US10) are minor components of the virion and deletion mutants lacking these proteins are viable, suggesting that none of these proteins make a major contribution to tegument structure or play an essential role in its formation (Maclean et al., 1989; Cunningham et al., 1992; Coulter et al., 1993; McLauchlan et al., 1994; Read & Frenkel, 1983; Fenwick & Everett, 1990; McLauchlan et al., 1992; Kehm et al., 1994; Rosen-Wolff et al., 1991; Frame et al., 1986).

2.5 HSV-1 Virion Envelope

The virion envelope is a membrane lipid bilayer derived from the host cell, into which 14 or more different viral membrane proteins are embedded (see Fig. 2.1). These proteins act at critical stages in the virus life cycle including adsorption to, fusion of, entry into, and egress from cells, and play an active role in host immune evasion during productive infections (for review see Spear, 1993; Dubin et al.,1992; Baines et al., 1991; Rodriguez et al., 1993). Of the membrane proteins characterized in HSV-1, positional and

functional homologs for UL27-gB, UL22-gH, UL1-gL, UL10-gM, UL49.5, UL34 and UL11 have also been identified in all other herpesvirus subfamilies (ie. alpha, beta, and gamma) (Albrecht et al., 1992; Baer et al., 1984; Chee et al., 1990; Davison & Scott, 1986; Gompels et al., 1995; McGeoch et al., 1988; Nicholas, 1996; Telford et al., 1992, 1995; Spear, 1993; Klupp et al., 1994; Hutchinson et al., 1992; Kaye et al., 1992; Spaete et al., 1993; Yaswen et al., 1993; Liu et al., 1993; Forghani et al., 1994; Lehner et al., 1989; Lawrence et al., 1995; Pilling et al., 1994; Baines & Roizman, 1993; MacLean et al., 1991, 1993; Barnett et al., 1992; Liang et al., 1993; Jons et al., 1996; Barker & Roizman, 1992; Riggio & Onions, 1993).

The majority of proteins in the HSV-1 envelope fall into three structural classes: (1) glycoproteins UL27-gB, UL44-gC, US6-gD, US8-gE, US4-gG, UL22-gH, US7-gI, and UL49.5 are type 1 membrane proteins that feature an N-terminal signal peptide, a hydrophillic ectodomain modified by N-linked and/or O-linked glycosylation, a hydrophobic membrane anchor, and a C-terminal cytoplasmic domain that ranges from 11 to 109 amino acids, (Spear, 1992; Spear, 1976; Para et al., 1980; Frame et al., 1986a; Richman et al., 1986; Buckmaster et al., 1984; Roop et al., 1993; Barker & Roizman, 1992; Barnett et al., 1992; Liang et al., 1996; Jons et al., 1996) (2) glycoprotein M, and the UL20 product are integral membrane proteins which contain multiple (four to eight) transmembrane domains (Baines & Roizman, 1993; MacLean et al., 1993: Ward et al., 1994) and (3) UL34 and UL45 are peripheral membrane proteins that lack N-linked glycosylation signals and possess a single hydrophobic domain responsible for membrane insertion or membrane association (Visalli & Brandt, 1993; Purves et al., 1992). The glycoprotein UL1-gL also contains a single hydrophobic domain, however this sequence almost certainly acts as a signal peptide that is cleaved and presumably UL1-gL is

associated with the virion envelope because UL1-gL forms a hetero-oligomer with UL22-gH, which is anchored in the membrane (Hutchinson et al., 1992; Roop et al., 1993; Dubin & Jiang, 1995). In addition, the UL11 gene encodes a myristylated protein thought to be situated in the inner surface of the virion envelope, given that UL11 is associated with membranes in the infected cell yet also resides in the tegument of the virus particle (MacLean et al., 1989; 1992; Baines & Roizman, 1992, 1995). At present there is no direct evidence to show that US5-gJ, a putative type 1 membrane protein, or UL43, a protein with 8 potential membrane-spanning domains, are components of the virion envelope (MacLean et al., 1991). In addition, recent attempts to detect the HSV-1 membrane protein, UL53, in the virion envelope have been unsuccessful (Hutchinson et al., 1995; Hutchinson & Johnson, 1995).

Little is known about the signals, if any, which direct HSV glycoproteins to the virion envelope. It has been suggested that interactions between the tegument and membrane glycoproteins are responsible for the integration and organization of herpesvirus membrane proteins in the viral envelope (Spear, 1985; Roizman and Sears, 1990). Consistent with this idea, virion membrane proteins UL27-gB, US6-gD, UL22-gH and UL49.5, (but not UL45-gC) are known to associate with tegument proteins including VP16 (Zhu & Courtney, 1994; Liang et al., 1996; Johnson et al., 1984). Moreover, each of the UL27-gB, US6-gD and UL45-gC glycoproteins exist as distinct structures within the viral envelope which are often arranged in clusters (Stannard et al., 1987; Zhu & Courtney, 1988; Kikuchi et al., 1990).

It is also possible that a subset of HSV-1 membrane proteins act as chaperones and interactions with these proteins direct the other HSV-1 membrane proteins to the virion envelope. Previous investigations have established that HSV-1 glycoproteins gH:gL and

gE:gI form hetero-oligomeric complexes (Johnson & Feenstra, 1987; Johnson et al., 1988b; Hanke et al., 1990; Hutchinson et al., 1992; Roop et al., 1993) and demonstrated that UL27-gB, UL44-gC, US6-gD (and possibly UL10-gM and UL49.5) can be found as homo-oligomeric structures (Sarmiento & Spear, 1979; Eberle & Courtney, 1982; Eisenberg et al., 1982; Gibson & Spear, 1983; Claesson-Welsh & Spear, 1986; Zhu & Courtney, 1988; 1994; Liang et al., 1996; Osterrieder et al., 1996). However, a recent study by Handler et al., (1996a,b) indicates that the interactions between the different envelope proteins may be much more extensive. Hetero-oligomers of gB-gC, gC-gD, gD-gB, gC,-gL and gD-gL but not gB-gL (presumably UL22-gH is present in the hetero-oligomers containing UL1-gL) were identified in HSV-1 virions treated with cross-linking reagents revealing that the arrangement of proteins in the virion envelope is not limited to clustered patches of individual glycoproteins (eg. gB or gD [Stannard et al., 1987]) but also includes a variety of membrane proteins in close proximity to each other (ie. within 11.4 Å) (Handler et al., 1996a,b). Consequently, the presence of envelope localization signals in a subset of HSV-1 membrane proteins may be sufficient for normal envelope assembly.

Conversely, other reports indicate that glycoproteins of herpesvirus and cellular origin do not require specific signals to be incorporated into the virion envelope (Feenstra et al., 1990; Muggeridge et al., 1990; Solomon et al., 1990, 1991; Liang et al., 1993, 1995; Dolter et al., 1994; Knox & Young, 1995). Instead, assembly of proteins into the virion envelope correlated with high levels of expression, Golgi processing, and cell surface transport (Liang et al., 1993; 1995b; Solomon et al., 1990;1991; Whealy et al., 1988; 1989). However, these studies examined a small number of proteins (ie. gC, gD, CD4, & MHCII) and other viral glycoproteins may possess/require a specific envelope

localization signal (Mettenleiter & Spear, 1994).

3.0 HSV-1 Lytic Replication Cycle

This introduction will focus on the lytic replication cycle of HSV-1 (see Fig. 1.1). The subject, establishment of HSV-1 latency in neurons and subsequent activation, is beyond the scope of this thesis.

3.1 HSV-1 attachment at the cell surface

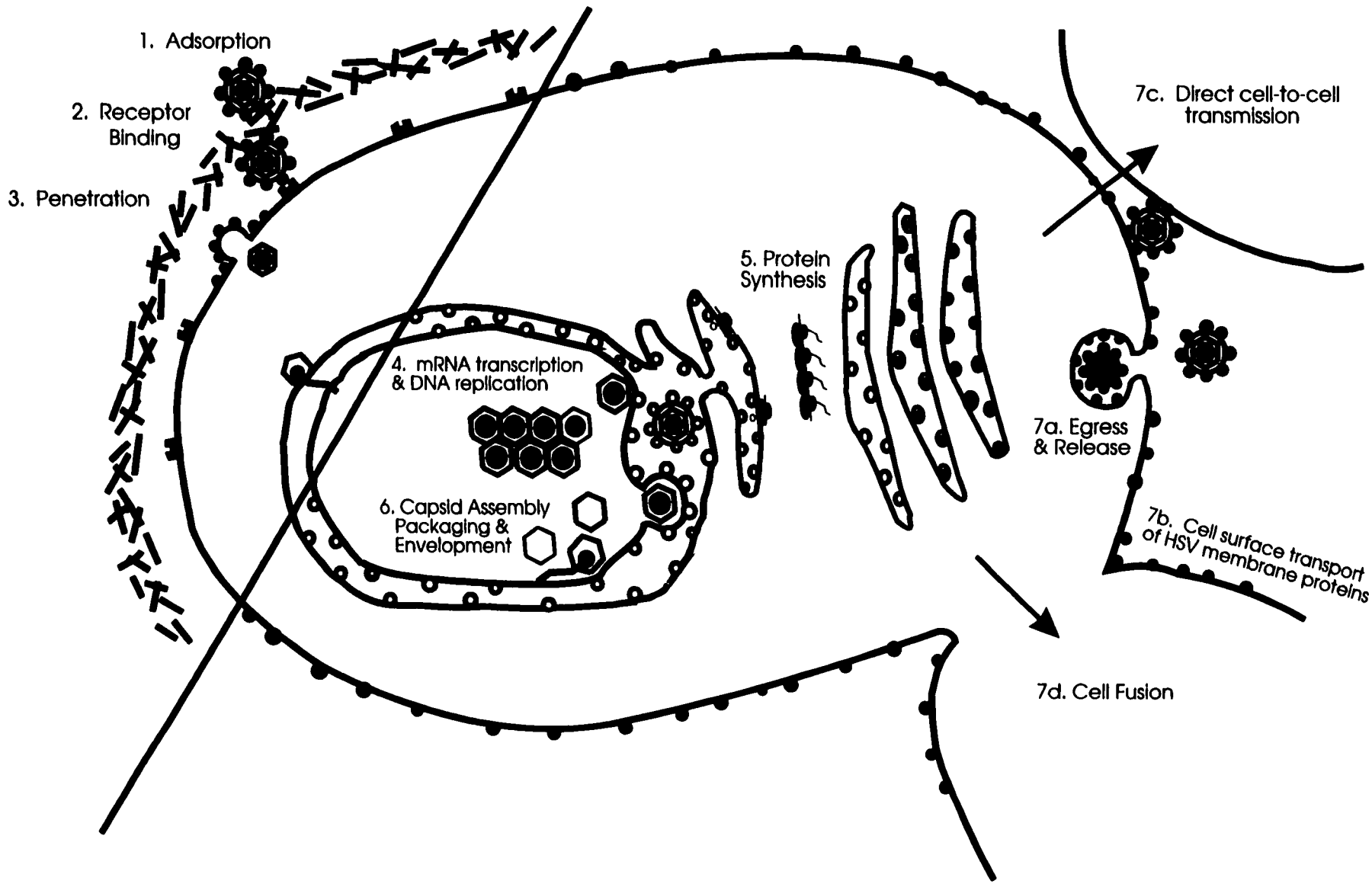
HSV-1 has the capacity to infect distinct cell types of divergent developmental lineages (eg. epithelial and neuronal) from a wide spectrum of different species (Roizman & Sears, 1996). From the standpoint of virus entry, this broad host range indicates, either that HSV-1 employs a ubiquitous cell surface component as a receptor, or that the virus can use multiple receptors: distinct molecules for individual cell types (eg. epithelial vs. neuronal) or multiple receptors to enter a single cell by several different routes. A large volume of evidence has accumulated which confirms that HSV-1 entry is a complex and multi-step process (Spear, 1992, 1993) involving at least two distinct phases: (1) binding or the attachment of viral particles to the cell surface and (2) penetration or release of capsids into the host cell cytoplasm (McClain & Fuller, 1994; Fuller & Spear, 1985, 1987; Fuller et al., 1989; Highlander et al., 1987, 1988; Cai et al., 1987; Johnson & Ligas, 1988; Forrester et 1992; Roop et al., 1993; Herold et al., 1994).

Furthermore, Johnson and Ligas (1988) and later Fuller & colleagues (Fuller & Lee, 1992; Lee & Fuller, 1993; McClain & Fuller; 1994) have hypothesized that two or more attachment steps take place prior to virus penetration, and subsequent studies have identified an intial adsorption stage which progresses to stable attachment (Ligas &

Figure 1.1. HSV-1 Lytic replication cycle.

The diagram, which is based on Ward & Roizman (1994) and Johnson & Ligas (1988), is simplified to emphasize steps involved in membrane fusion.

1. Envelope proteins displayed by the HSV-1 virion interact with, or adsorb to ubiquitous cell surface components (eg. heparan sulphate proteoglycans).
2. Specific binding by one or more glycoproteins, in the virion envelope, with high affinity cell surface receptors expressed by permissive cells.
3. Penetration is meditated by fusion of the virion envelope with the plasma membrane, and subsequent release of tegument proteins into the cytoplasm.
4. The capsid is transported to nuclear pores, and viral DNA is injected into the nucleus where mRNA transcription and DNA replication occur.
5. mRNA is transported to the cytoplasm and translated by ribosomes located in the cytoplasm, or ribosomes attached to the endoplasmic reticulum.
6. Structural proteins required for virion assembly are transported to the nucleus (eg. capsid proteins), or accumulate at patches associated with all cellular membranes. Capsid proteins are assembled into empty structures, DNA is packaged, and nucleocapsids bud from the inner nuclear membrane where they acquire a tegument and envelope proteins.
7. The following steps appear to take place during the same time frame and may be interrelated. Nevertheless, the time scale for each of these events has never been determined within the context of a single system based on standardized conditions, so a direct comparison cannot be made.
   a. Intracellular transport, maturation and release of HSV-1 particles (Lee & Spear, 1980) from membrane vesicles at the cell surface, by way of the putative egress mechanisms described in Figure 3.1
   b. Concomittant post-translational processing and intracellular transport of HSV-1 membrane proteins to the plasma membranes (Person et al., 1982).
   c. Cell-to-cell spread mediated by viral proteins in the plasma membrane and/or extracellular virions?
   d. Cell-cell fusion mediated by viral proteins in the plasma membrane and/or extracellular virions? (Lee & Spear, 1980; Person et al., 1976)


1. Adsorption
2. Receptor Binding
3. Penetration
4. mRNA transcription & DNA replication
5. Protein Synthesis
6. Capsid Assembly Packaging & Envelopment
7a. Egress & Release
7b. Cell surface transport of HSV membrane proteins
7c. Direct cell-to-cell transmission
7d. Cell Fusion

Johnson, 1988; Johnson et al., 1990). In the initial adsorption stage, glycoproteins UL44-gC and UL27-gB (Campadelli-Fiume et al., 1990; Fuller & Spear, 1985; Herold et al. 1991, 1994; Johnson et al., 1984; Kuhn et al., 1990; Langeland et al., 1990; Svennerholm et al., 1991; Tal-Singer et al., 1995) interact with the glycosaminoglycan moieties (GAGs), primarily heparan sulfate (HS) and in some circumstances dermatan sulfate (Nahmias & Kibrick, 1964; Banfield et al., 1995a; WuDunn & Spear, 1989; Shieh et al., 1992; Herold et al., 1995). Adsorption is followed by a stable binding step that involves glycoprotein D (Fuller & Spear, 1985; Johnson & Ligas, 1988; Johnson et al., 1990; Fuller & Lee, 1992; Lee & Fuller, 1993; McClain & Fuller, 1994) and a limited number of specific cell surface receptors (Johnson et al., 1990). The events surrounding the entry of related alpha-herpesviruses (eg. HSV-2, pseudorabies virus [PRV], bovine herpesvirus type 1 [BHV-1] and varicella zoster virus [VZV]) appear to be very similar, but not identical to those of HSV-1 (Liang et al., 1991, 1993; Okasaki et al., 1991; Byrne et al., 1995; Li et al., 1995; Thaker et al., 1994; Chase et al., 1990; Mettenleiter et al., 1990; Karger et al., 1995; Karger & Mettenleiter, 1993, 1996; Zsak et al., 1991; Schreurs et al., 1988; Mettenleiter et al., 1988; Peeters et al., 1992a; Petrovskis et al., 1988; Sawitzky et al., 1990; McClain & Fuller, 1994; Subramanian et al., 1994; Davison & Scott, 1986; Cohen & Seidel, 1994; Zhu et al., 1995a; Campadelli-Fiume et al., 1990b; Langeland et al., 1990; Gerber et al., 1995; Johnson & Ligas, 1988; Johnson et al., 1990).

3.1.1 Primary Adsorption of HSV-1 to the cell surface

As primary receptors for HSV-1, the ubiquitous nature of cell surface glycosaminoglycans (GAGs) is consistent with the broad tropism displayed by HSV-1 and in the absence of GAG synthesis HSV-1 infection of mouse fibroblasts is reduced (up to

300 fold) (Gruenheid et al., 1993; Banfield et al., 1995a,b) or blocked completely in CHO cells (Shieh et al., 1992). Nevertheless it is unlikely that GAGs are absolutely essential or sufficient for HSV-1 entry, at least *in vitro* (WuDunn & Spear, 1989; Shieh et al., 1992; Gruenheid et al., 1993; Subramanian et al., 1994; Banfield et al., 1995a, 1995b; Montgomery et al., 1996). For instance, Banfield et al. (1995) used a more stringent assay (ie. plaque formation) to assess the role of HS-GAGs in HSV-1 entry, yet they report that GAG-deficient mouse cells exhibit partial and not complete resistance to HSV-1 infection. Furthermore, recent studies by Montgomery et al. (1996), have demonstrated that the cell type (ie. CHO cells) used by Shieh et al. (1992) lack a non-GAG receptor involved in the HSV-1 entry process and therefore defects in GAG synthesis may have a greater impact on the infectivity of CHO cells than cell types which possess a full complement of HSV-1 receptors.

Similarly, UL44-gC, the HSV-1 protein which plays a predominant role in virus adsorption to HS-GAGs (Herold et al., 1991, 1994) is nonessential (Heine et al., 1974; Toh et al., 1993; Hidaka et al., 1990, 1991), although some (but not all) reports indicate that gC-negative virions exhibit a 10-fold decrease in the specific infectivity (Herold et al., 1991; Tal-Singer et al., 1995). However, subsequent studies have established that UL27-gB mediates HSV-1 attachment to HS-GAGs in the absence of UL44-gC (Herold et al., 1994; 1995), indicating the adsorption stage is important for efficient attachment and entry into cells, if not mandatory for HSV infection (WuDunn & Spear, 1989; Shieh et al., 1992; Gruenheid et al., 1993; Banfield et al., 1995a,b; Tal-Singer et al., 1995). Since, UL27-gB and UL44-gC are among the molecules which project the longest distance from the virion envelope (Stannard et al., 1987) it has been suggested that they tether the virus to the cell surface and by doing so, reduce a three-dimensional search for the second

HSV-1 receptor to two dimensions (Spear, 1992, 1993; Haywood, 1994; Herold & Spear, 1994; Banfield et al., 1995b). In other herpesviruses (ie. BHV-1 and human cytomegalovirus [HCMV]) homologs of UL27-gB, which comprise the most highly conserved group of glycoproteins within the Herpesviridae, are reputed to bind a secondary cell surface receptor (Li et al., 1995; Adlish et al., 1990; Taylor & Cooper, 1990; Nowlin et al., 1991; Wright et al., 1994; Compton, 1995), however, there is no evidence that HSV-1 gB binds to cell surface molecules other than heparan-sulfate proteoglycans (Ligas & Johnson, 1988; Johnson et al., 1990; Forrester et al., 1992; Lee and Fuller, 1993; Herold et al., 1994).

3.1.2 Stable attachment of HSV-1 to the cell surface

In the subsequent attachment step, HSV-1 interactions with the cell surface become resistant to heparin elution and entail binding to a saturable cell surface receptor implicated in virus penetration, but not required for adsorption to the cell surface (Johnson & Ligas, 1988; Johnson et al., 1990; McClain & Fuller, 1994). US6-gD is required for this attachment step which has been termed "stable binding" (Johnson & Ligas, 1988; Fuller & Lee, 1992; Lee & Fuller, 1993). The first sign that gD might be a receptor binding protein emanated from studies which demonstrated that cell lines expressing gD can be resistant to infection by HSV-1 and HSV-2 (Campadelli-Fiume et al., 1988, Johnson & Spear, 1989). However, two interpretations have been put forward to explain this phenomenon: (1) cell-associated gD sequesters a gD-specific cell surface receptor or (2) interactions between cell-associated gD and components of the virion [eg. gD] prevent HSV entry, and there is evidence for both types of gD-mediated interference (Campadelli-Fiume et al., 1990a; 1991; Brandimarti et al., 1994; Dean et al., 1994, 1995; Roller &

Roizman, 1994). Perhaps the best indication that it is US6-gD itself which binds to a non-HS receptor comes from the studies which showed that: (1) soluble gD protein and wild-type HSV virions (< 5000 particles/cell), but not gD-negative virions, can block penetration by a homologous challenge virus (Johnson & Ligas, 1988; Forrester et al., 1992; Fuller et al., 1993), and (2) attachment of wild-type HSV virions to the cell surface is more resistant to heparin elution than the adsorption of gD-negative virions (Fuller et al., 1992). Since, the putative US6-gD receptor is limited in number (Ligas and Johnson, 1988) and because US6-gD binding at the cell surface is saturable (4-5 x 10^6 molecules bound/cell) (Johnson et al., 1990), it is doubtful that these observations result from interactions between cell-associated gD and a virion component.

The presence of US6-gD in the HSV virion is an absolute requirement for HSV entry (Ligas & Johnson, 1988) and evidence has been presented indicating that disruption of US6-gD interactions at the cell surface inhibit virus penetration by up to 95% (Johnson et al., 1990; Tal-Singer et al., 1995; Nicola et al., 1996). Therefore, it is tempting to suggest that the receptor binding ability of US6-gD constitutes an essential function. Furthermore, the quantity of gD and gH in the virion envelope is at least 10 times greater than UL27-gB and UL44-gC implying that US6-gD receptor binding may be more important to virus penetration than the HS-GAG interactions mediated by UL27-gB and UL44-gC, at least *in vitro* (Handler et al., 1996). Conversely, US6-gD may be multifunctional, like many other HSV proteins, and one of these functions may be essential for HSV-1 infectivity. Several groups have reported that gD-specific MAbs suppress HSV-1 plaque formation (ie. cell-to-cell spread & cell-cell fusion), but they found no correlation between the anti-fusion activity and the ability of MAbs to neutralize extracellular virus and block subsequent entry, suggesting that gD fulfils more than one

function (Minson et al., 1986; Highlander et al., 1987). However, this information must be interpreted cautiously, since MAbs may have the capacity to neutralize extracellular virions through multiple mechanisms which may not be related to gD function. Nevertheless, Nicola et al. (1995) have demonstrated that mutant gD molecules which do not support HSV entry (Chiang et al., 1994) can still bind to cells and inhibit subsequent virus infection, reinforcing the idea that US6-gD may have more than one essential function (Roizman & Sears, 1990).

Recently, Brunetti et al. (1994) showed that mannose-6-phosphate (M6P) residues on US6-gD mediate binding to cell surface mannose-6-phosphate receptors (M6PRs). As such the M6PRs represent a potential receptor for HSV, although the significance of this finding for viral entry remains unclear (Brunetti et al., 1995; Sodora et al., 1991). M6PR appears to act as species specific receptor which facilitates penetration and cell-to-cell spread of HSV-1 in primate cells (Brunetti et al., 1995). By contrast, M6PR does not act as an HSV-1 receptor in mouse cells, even though US6-gD is crucial for virus penetration into the same mouse cells (Tal-Singer et al., 1994; Banfield et al., 1995b, Brunetti et al., 1995). Moreover, M6P-negative HSV-1 virions are fully infectious both in mouse and primate cells (Sodora et al., 1991; Brunetti et al., 1995) clearly indicating that the M6PR pathway cannot be the only route by which virus enters cells. These results and new evidence suggesting that a member of the TNF/NGF receptor family (HVEM for herpesvirus entry mediator) employs a gD-specific mechanism to enhance HSV entry, has led to the proposal that US6-gD can interact with several different receptors (Brunetti et al., 1995; Montgomery et al., 1996). In addition, studies which revealed that mutations in US6-gD can overcome a number of distinct host-cell restrictions to infection, also lend support to the hypothesis that US6-gD binds to several different receptors, perhaps one or

more related proteins belonging to a family of cell surface receptors (Dean et al., 1994; Brandimarti et al., 1995).

3.1.3 Potential HSV-1 cellular receptor-binding proteins

Furthermore, HSV-1 encodes at least 15 different envelope proteins and although many are not required for virus replication in a number of cultured cells (ie. US8-gE, US4-gG, US7-gI, US5-gJ, UL10-gM, UL49.5, and UL45, see Table 1.1 & Fig. 2.1) they may interact with tissue-specific receptors *in vivo*, given the broad tropism displayed by HSV-1 (MacLean et al., 1993; Neidhardt et al., 1987; Meignier et al., 1988; Rajcani et al., 1990; Balan et al., 1994; Dingwell et al., 1994, 1995).

For instance the HSV-1 glycoproteins US8-gE and US7-gI, which form a functional hetero-oligomer that binds to immunoglobulin (IgG), (Johnson & Feenstra, 1987; Johnson et al., 1988b; Bell et al., 1990; Dubin et al., 1990, 1994; Hanke et al., 1990; Basu et al., 1995) are also required for efficient cell-to-cell spread in normal diploid cells, epithelial and neuronal tissues (Neidhardt et al., 1987; Balan et al., 1994; Dingwell et al., 1994, 1995). In addition, homologs of US8-gE and US7-gI play a comparable role in the replication of pseudorabies virus (PRV) (Zuckermann et al., 1988; Card et al., 1991, 1992; Whealy et al., 1993; Zsak et al., 1992; Mulder et al., 1994; Jacobs, 1994), a related alphaherpesvirus, and mutations in the extracellular domain of PRV-gE exert the same effect on virus growth and neurovirulence as deleting the entire gE protein (Jacobs et al., 1993a, 1993b), again consistent with a possible role for gE in receptor binding.

UL10-gM negative HSV-1 mutants exhibit a growth phenotype which is similar, but not identical to US8-gE or US7-gI negative viruses, and in a mouse model these mutants are impaired for growth at the periphery and subsequent spread to and/or growth

within the nervous system (MacLean et al., 1991, 1993; Baines & Roizman, 1991, 1993). Furthermore, the UL10-gM homolog of equine herpes virus type 1 (EHV-1), another member of the alphaherpesvirus subfamily, has been implicated in virus penetration and cell-to-cell spread (Osterrieder et al., 1996). Genes that encode glycoproteins homologous to UL10-gM have been found in all Herpesviridae examined to date (except the channel catfish herpesvirus (CCV) [Davison, 1992]), and between the alpha- beta- and gammaherpesviruses represent the second most highly conserved group of herpesvirus glycoproteins, surpassed only by the gB homologs (Gompels, 1995; Nicolas, 1996). Moreover, gM homology ranks first ahead of values for gB and gH, among glycoproteins conserved within beta- or gammaherpesvirus subfamilies (Gompels, 1995; Telford, 1995; Nicolas, 1996) and third within the alphaherpesvirus glycoproteins (McGeoch et al., 1988; Klupp & Mettenleiter, 1991; Telford et al., 1992; Spear, 1993; Baumeister et al., 1995). These observations suggest an important role for UL10-gM in herpesvirus replication, perhaps as a receptor binding protein.

UL22-gH is another envelope glycoprotein preserved by the alpha- beta and gammaherpesviruses (except CCV, [Davison, 1992]) and between subfamilies of the Herpesviridae these homologs constitute the third most highly conserved group of membrane glycoproteins (Gompels, 1995; Nicolas, 1996; Klupp & Mettenleiter, 1991). All of the glycoprotein H homologs which have been characterized (ie. HSV-1, HCMV, EBV, HHV-6, VZV, PRV, BHV-1, MDV) form a physical association with homologs of HSV-1 UL1-gL and this interaction seems to be a universal requirement for gH processing, transport to plasma membranes and incorporation into the virion envelope (Gompels & Minson, 1989, Forrester et al., 1991, 1992; Foa-Tomasi et al., 1991; Roberts et al., 1991; Hutchinson et al., 1992a; Roop et al., 1993 Cranage et al., 1988; Kaye et al.,

1992; Spaete et al., 1993; Heineman et al., 1988; Yawsen et al., 1993; Li et al., 1995; Liu et al., 1993a, 1993b; Forghani et al., 1994; Duus et al., 1995; Klupp et al., 1994; Yoshida et al., 1994; Khattar et al., 1996). In addition, gH homologs are required for herpesvirus entry into cells and appear to play an essential, undefined role in membrane fusion events (Buckmaster et al., 1984; Gompels & Minson, 1986; Fuller et al., 1989; Desai et al., 1988; Forrester et al., 1992; Fuller & Lee, 1992; Roop et al., 1993; Wilson et al., 1994; Davis-Poynter et al., 1994; Peeters et al., 1992b; Rasmussen et al., 1984, 1985; Cranage et al., 1988; Keay et al., 1989; Keay & Baldwin, 1991; Baldwin et al., 1996; Strnad et al., 1984; Miller & Hutt-Fletcher., 1988; Haddad & Hutt-Fletcher, 1989; Li et al., 1995a; Foa-Tomasi et al., 1991; Keller et al., 1984; Montalvo & Grose, 1986; Keller et al., 1987; Rodriguez et al., 1993; Forghani et al., 1994; Duss et al., 1995; Baranowski et al., 1993, 1995). Together, these observations predict a common function for gH homologs in the viral replication cycle. There is evidence to believe that this function may involve interactions between UL22-gH and a saturable, non-heparan sulfate receptor. Binding of inactivated, wild-type HSV to cells can block the subsequent penetration, but not the binding of HSV or PRV challenge virus, whereas gH-negative HSV virions bind to cells but possess only partial blocking activity (Forrester et al., 1992; Lee & Fuller, 1993). Recent studies suggest that a 92.5 kDa cell surface glycoprotein has a role in host cell penetration by human cytomegalovirus (HCMV), but not adsorption of virus to the cell surface, possibly as the putative "fusion" receptor for HCMV-gH (Keay et al., 1989, Keay & Baldwin, 1991, 1992; Baldwin et al., 1996). However, this receptor was defined using anti-idiotype IgM antibodies, which have a history of producing artifacts and therefore the importance of this 92.5 kDa protein remains unclear. Nevertheless, it is possible that gH homologs may participate in receptor binding and in membrane fusion, two events which

are not mutually exclusive.

3.2 HSV-1 penetration at the plasma membrane.

To penetrate the host cell an enveloped virus must bind to one or more cell surface receptors, induce a cellular membrane to fuse with the virion envelope and deliver the virus nucleocapsid into the cytoplasm (see Table 1.1 for a summary of the functions supplied by HSV-1 membrane proteins). One or more envelope glycoproteins encoded by the virus mediates the fusion event. Many enveloped viruses including the orthomyxoviruses (eg. influenza), rhabdoviruses (eg. vesicular stomatitis virus, [VSV]), and togaviruses (eg. Semiliki Forest), take advantage of receptor-mediated endocytosis to enter cells (Lamb & Krug, 1996; Wagner & Rose,1996; Schlesinger & Schlesinger,1996). Exposure to the acidic pH in the endosomal compartment triggers the viral fusion protein to undergo a conformational change which results in perturbation of membranes and fusion of the viral envelope with endosomal membranes (Stuart, 1996). On the other hand, members of the paramyxovirus subgroup (eg. Sendai, Newcastle disease virus [NDV], measles), and some retroviruses (eg. human immunodeficiency virus [HIV]) possess envelope proteins which mediate membrane fusion at neutral pH (White, 1990; Lamb, 1993; Luciw, 1996). These viruses can induce fusion at the plasma membrane, however, it is not yet known if the productive infection stems from penetration at the plasma membrane or requires internalization of the virus within an endosomal vesicle, followed by pH-independent fusion.

HSV-1, most probably enters the cell by direct fusion with the plasma membrane. The evidence supporting this view is as follows. (1) Inhibition of endocytosis or endosome acidification did not affect HSV penetration but did prevent the entry of other

viruses (eg. VSV, Semiliki Forest virus [SFV] (Koyama & Uchida, 1987; Wittels & Spear, 1991; Perez & Carrasco, 1994). (2) A mutant cell line with a defect in endocytosis, is able to support HSV-1 entry at the nonpermissive temperature (C. Brunetti, personal communication). (3) In the absence of protein synthesis HSV envelope glycoproteins are transferred to the plasma membrane shortly after virus penetration (Para et al., 1980). (4) HSV virions are observed fusing with the plasma membrane, but not with endosomes, in electron microscopy studies (Morgan et al., 1968; Para et al., 1980) and neutralizing antibodies block this fusion (Fuller & Spear, 1987; Fuller et al., 1989). (5) HSV-1 membrane proteins in the plasma membrane, or virion particles at the cell surface, can induce the formation of multinucleated cells (polykaryons). This phenomenon, which is also referred to as cell-cell fusion or syncytium formation, is common to viral fusion proteins with activity at neutral pH (White, 1990, 1992; Lamb, 1993). In contrast, viral fusion proteins activated by the low pH of endosomes will only form syncytia when infected cells are exposed to acidic medium (Lamb & Krug, 1996). Since HSV-1 entry and cell-cell fusion are inhibited by mild acidic pH conditions, and this effect is completely reversed when neutral conditions are restored, it is doubtful that HSV-1 virions enter the endosome and fuse in a pH-independent fashion (Rosenthal et al., 1989; Baghian et al., 1992). In addition, HSV-1 virions accumulate in the endosomes of transfected cells expressing US6-gD, but this process results in a nonproductive infection and virus degradation (Campadelli et al., 1988).

At least four envelope glycoproteins, UL27-gB, US6-gD, the non-covalent hetero-oligomer UL22-gH:UL1-gL, and one tegument protein, UL25, supply functions essential for virus penetration at the host cell plasma membrane (see Table 1.1) (Sarmiento et al., 1979; Little et al., 1981; Cai et al., 1987; 1988; Highlander et al., 1988,1989; Ligas &

Johnson, 1988; Showalter et al., 1981; Para et al., 1985; Fuller & Spear, 1987; Highlander et al., 1987; Desai et al., 1988; Forrester et al., 1992; Buckmaster et al., 1984; Gompels & Minson, 1986; Fuller et al., 1989; Hutchinson et al., 1992; Roop et al., 1993; Addison et al., 1984; Ali et al., 1996). As such HSV-1 is fairly unique, since many virus families (eg. rhabdoviruses, orthomyxoviruses, paramyxoviruses, retroviruses) require one, or at most two, proteins to penetrate host cell membranes (White, 1992; Lamb, 1993). Furthermore, HSV-1 penetration has resisted the sort of analysis which divided virus attachment into two distinct phases governed by the glycoproteins C and D (ie. gC-adsorption, gD-stable binding, see above). This limited ability to assign individual proteins to specific events during virus penetration, and the overall complexity of HSV-induced membrane fusion, has led to the widespread view that gB, gD, and gH:gL may linked together in a viral fusion complex. Cross-linking studies which examined the organization of gB, gC, gD, and gH:gL in the virion envelope during virus penetration, lend credence to this belief (Handler et al., 1996a,b). Prior to virus entry homo- and hetero-oligomeric forms of gB, gC, gD, and gH:gL (eg. gB-gB, gC-gC, gD-gD, gB-gC, gC-gD, gD-gB, gH-gL, gC-gL and gD-gL) were detected in the virion envelope and may represent a fusion complex. Even more notable was the discovery that oligomers containing gB, gD, or gH:gL diminish as virus penetration proceeds, while oligomeric gC levels remain unchanged (Claesson-Welsh & Spear, 1986; Zhu & Courtney, 1988, 1994; Handler et al., 1996a,b). Handler et al., (1996b) have suggested that gB, gD, and gH:gL become inaccessible to cross-linking because they form a fusion complex that is "buried" in the plasma membrane when it forms the fusion pore, whereas gC serves as an attachment protein which is not involved in penetration and thus would not be included the fusion pore. Alternatively, changes in the structural conformation of UL27-gB, US6-gD

TABLE 1.1 Deletion of HSV membrane proteins: Effect on replication, entry and cell fusion

Functional Unit	No. of infectious progeny[a]	No. of intracellular virions	Specific infectivity	Required for entry	Required for fusion	Potential functions
gB	severely reduced	normal	severely reduced	+	+	1. adsorption 2. fusion protein
gC	normal or slightly reduced[c]	normal	normal or slightly reduced[c]	-	-	1. adsorption 2. binds complement
gD	severely reduced	slightly reduced	severely reduced	+	+	receptor binding
gE:gI	normal	normal	normal	-	-/+[d]	1. Fc receptor 2. cell-to-cell spread
gH:gL	severely reduced	normal	severely reduced	+	+	entry (expansion of fusion pore?)
gK	reduced	normal	reduced	-/+[c]	?	1. egress 2. virion assembly
gM	slightly reduced	?	?	-	+[d]	unknown small plaque phenotype
UL20	slightly reduced to reduced [b]	?	?	-	-*	host range egress defect *deletion confers syn phenotype
UL45	normal or reduced [c]	?	?	-	+[d]	unknown small plaque phenotype

[a] This table which is based on Spear (1990) is simplified to emphasize membrane proteins with a role in HSV entry or cell fusion. The yield of infectious progeny was determined in one step growth experiments and is a reflection of the number of physical particles assembled and the specific infectivity of those virions. The relative contribution of these factors is included in the next two columns.
Normal = wt yields; slightly reduced = 10 to 100% of wt yields; reduced = 1 to 10% of wt yields; severely reduced = 0.1% of wt yield.
[b] Virus yields were "slightly reduced" on cell lines supporting UL20 egress & "reduced" on those cell lines which do not.
[c] UL45: normal (Haanes et al., 1994); reduced (Visall et al., 1991). gC: normal (Tal-Singer et al., 1995); slightly reduced (Herold et al., 1991)
[d] Protein required for syn-3 (UL27-gB) phenotype. [e] Specific infectivity reduced 25-50 fold.

and UL22-gH induced by the formation of a fusion complex, could have reduced the capacity of anti-sera to recognize the individual proteins, and might explain the reduction (albeit less dramatic) in monomeric proteins (eg. UL27-gB and perhaps US6-gD) that was observed as virus penetration progressed. This possibility is further emphasized by the observation that denaturing conditions drastically reduce the ability of anti-gH polyclonal sera to recognize UL22-gH in HSV-1 virions (Handler et al., 1996a).

Although the precise role of each protein in virus penetration has yet to be defined, a general consensus with respect to a probable function gB, gD and gH:gL has developed from the comparison of HSV proteins with the homologs of other herpesviruses, and studies which examine the effect of specific mutations or monoclonal antibodies on virus entry, membrane fusion, and cell-to-cell spread.

3.2.1 UL27-gB, the HSV-1 fusion protein?

Most herpes virologists consider UL27-gB to be the envelope glycoprotein with fusogenic activity, whereas US6-gD, and UL22-gH:UL1-gL are thought to play auxiliary roles in the membrane fusion process. For instance, MAbs directed against the extracellular domain of UL27-gB and mutations in the same region, have been described that alter the rate at which HSV-1 virions can penetrate cells (Deluca et al., 1982, 1984; Bzik et al., 1984b; Highlander et al., 1988; 1989) or block entry altogether (Sarmiento et al., 1979; Cai et al., 1987, 1988b; Navarro et al., 1992). Moreover, the number of gB molecules in the virion envelope can modify the entry kinetics of HSV-1 virions (Desai et al., 1994). In addition, mutations in the cytoplasmic tail of UL27-gB may be sufficient to confer HSV-1 virions with the ability to bridge cells and induce cell-cell fusion in the absence of viral protein synthesis (fusion-from-without) (Ruyechan et al., 1979; Weise et

al., 1987; Falke et al., 1985; Walev et al., 1991a, b).

Between alpha-, beta-, and gammaherpesviruses, the UL27-gB homologs are more highly conserved than any other group of membrane proteins (Gompels, 1995; Nicolas, 1996; Spear, 1993) and the gB deletion mutants of different herpesviruses appear to share analogous defects in virus penetration (Cai et al., 1987, 1988; Rauh & Mettenleiter, 1991; Rauh et al., 1991; Peeters et al., 1992a; Herrold et al., 1996). In many instances gB homologs from one herpesvirus (eg. PRV-gB, BHV-1-gB) can replace gB function in another herpesvirus (eg. HSV-1) and gB homologs often share common antigenic eptiopes (Felser et al., 1987; Rauh et al., 1991; Misra & Blewett, 1991; Kopp & Mettenleiter, 1992; Mettenleiter & Spear, 1994; Otsuka & Xuan, 1996), whereas homologs of some other herpesvirus glycoproteins have failed to do so (Snowden & Halliburton, 1985; Borchers et al., 1990; Whealy et al., 1989; Liang et al., 1991a; Muggeridge et al., 1990). Like the fusion proteins of other enveloped viruses [eg. influenza hemagglutinin (HA) (Gething et al., 1986; Copeland et al., 1986; Whiley & Skehel, 1987; Garten et al., 1989; Stieneke-Grober et al., 1992), the Sendai glycoprotein (F) (Tashiro et al., 1990; Sechoy et al., 1987; Morrison & Portner, 1991), and the human immunodeficiency virus envelope protein gp160 (HIV-gp160) (McCune et al., 1988; Earl et al., 1990, 1992; Bosch & Pawlita, 1990; Weiss et al., 1990; Hallenberger et al., 1992), for reviews see White, 1992; Doms et al., 1993; Klenk & Garten, 1994], gB molecules must assemble into homo-oligomers to acquire biological activity (Sariento & Spear, 1979; Claesson-Welsh & Spear, 1986; Chapsal & Pereira, 1988; Cai et al., 1988b; Zhu & Courtney, 1988, 1994; Highlander et al., 1991; Desai et al., 1994; Handler et al., 1996a,b; for HSV-1 review see Laquerre et al., 1996; Eberle & Courtney, 1982; Whealy et al., 1990; Britt & Vugler, 1992). In addition, like the fusion proteins of other enveloped viruses precursor maturation in the Golgi

apparatus includes cleavage of the primary translation product by a cellular protease, to yield two subunits covalently linked by disulphide bonds (ie. PRV, [Luckas et al., 1985; Wolfer et al., 1990], VZV [Montalvo & Grose, 1987], BHV-1 [van Drunen Littel-van den Hurk et al., 1989], EHV1 [Sullivan et al., 1989; Whalley et al., 1989], EHV-4 [Meredith et al., 1989], MDV [Ross et al., 1989], HCMV [Spaete et al., 1988; Britt & Vugler, 1989; Vey et al., 1995], MCMV [Loh, 1991], HHV-6 [Ellinger et al., 1993; BHV-4 [Glotz et al., 1994], but not HSV-1 [Sarmiento & Spear, 1979; Haffey & Spear, 1980; Pereira et al., 1981, 1982; Chapsal & Pereira, 1988], EBV or HVS [Gong et al., 1987]). Interestingly, proteolytic cleavage is a common mechanism used to activate the fusogenic domain in many viral fusion proteins (White, 1992; Doms et al., 1993, Klenk & Garten, 1994), however, the analogous cleavage event does not occur in HSV UL27-gB (Sarmiento & Spear, 1979; Haffey & Spear, 1980; Pereira et al., 1981, 1982; Chapsal & Pereira, 1988) and is not required for gB function by other herpesviruses (eg. PRV, BHV-1, HCMV), at least *in vitro* (Brucher et al., 1990; Blewett & Misra, 1991; Kopp et al., 1994; Yoshida et al., 1994; Vey et al., 1995). Instead, Reschke et al. (1996) have proposed that an intramolecular hydrophobic domain neighbouring the actual membrane anchor for UL27-gB (Rasile et al., 1993; Gilbert et al., 1994) serves as the putative fusogenic peptide, because the primary sequence is highly conserved between gB homologs and bears a striking resemblance to the fusogenic peptides of other viral fusion proteins (White, 1992). Although, no obvious fusogenic peptide is proteolyticaly activated in gB homologs, the protease recognition site is conserved by the majority of herpesviruses and it is possible that the conformational alteration induced by gB cleavage may influence overall infectivity and/or the host cell range, when the virus is subjected to *in vivo* conditions (Tashiro et al., 1990, 1992; Kopp et al., 1994).

3.2.2 The role of UL22-gH:UL1-gL in HSV-1 penetration.

There is evidence to suggest that HSV-1 UL22-gH (Desai et al., 1988; Forrester et al., 1992; Roop et al., 1993; Wilson et al., 1994; Davis-Poynter et al., 1994; Buckmaster et al., 1994; Gompels & Minson, 1986; Fuller & Lee, 1992) and the other gH homologs (Keay et al., 1989; Keay & Baldwin, 1991; Baldwin et al., 1996; Miller & Hutt-Fletcher, 1988; Haddad & Hutt-Fletcher, 1989; Foa-Tomasi et al., 1994; Montalvo & Grose, 1986; Rodriguez et al., 1993; Duss et al., 1995; Peeters et al., 1992b) participate in the membrane fusion process during virus penetration, and Fuller & Lee (1992) have proposed that UL22-gH facilitates expansion of a fusion bridge (presumably created by UL27-gB) between the virion envelope and the host cell plasma membrane. Fuller and Lee (1992) came to this conclusion when they treated virions with US6-gD or UL22-gH neutralizing antibodies and examined the distribution of virus particles in various stages of virus entry, however, these studies were based on electron microscopy which can not differentiate between infectious and non-infectious viruses. That rate of entry (roe) mutations, like those observed for UL27-gB, have not been reported for UL22-gH is in agreement with the notion that UL22-gH plays a secondary role in membrane fusion. In addition, Handler et al. (1996a) report that the molar ratio for UL27-gB, UL44-gC, US6-gD and UL22-gH in the HSV-1 virion envelope is approximately 1:1.5:12.5:12.5, respectively. One interpretation of this data might be that UL27-gB catalyzes the initial fusion event, a process which could require only a few molecules, whereas UL22-gH activities may involve receptor binding and/or enlarging the fusion pore, functions that could entail a large number of molecular interactions.

However, UL22-gH also has the potential to act as the primary fusion protein. Like the fusion proteins G1:G2 of Uukunemi virus and E1:E2:E3 of Semiliki forest virus

(White, 1992; Kielian et al., 1990; Lobigs & Garoff, 1990; Persson & Pettersson, 1991), the gH:gL complex is a non-covalent hetero-oligomer, and contains a membrane-spanning subunit (UL22-gH) with a central domain reminiscent of a fusion peptide (ie. hydrophobic but not membrane spanning)(Gompels & Minson, 1989; Roberts et al., 1991; Hutchinson et al., 1992; Forrester et al., 1991, 1992; Roop et al., 1993; Spear, 1993; Dubin et al., 1995). That the central hydrophobic domain in UL22-gH may have the capacity to associate with membranes has been suggested by studies employing a baculovirus expression vector (BacgH) to produce a soluble form of UL22-gH which is transported across the ER and lacks the membrane anchor and cytosolic domain (Tomazin et al., 1996). When BacgH microsomes were subjected to proteolysis in the absence of detergent, much of the gH protein was resistant to proteolysis, however, a reasonable fraction of the total gH protein had apparently inserted into the membrane and was sensitive to proteolysis (Tomazin et al., 1996). In similar studies, the fusion peptides from paramyxovirus F proteins have converted a soluble protein to a membrane bound derivative, by acting as a transmembrane anchor (Paterson & Lamb, 1987).

Since UL22-gH must form a complex with UL1-gL to undergo posttranslational processing and transport to the cell surface, it is conceivable that UL1-gL masks the central hydrophobic domain in gH, thereby preventing aggregation and retention of gH in the ER. According to this model, the appropriate stimulus may cause gL to expose the hydrophobic domain in gH, allowing this region to act as a fusogenic peptide (Pelham, 1989; Rose & Doms, 1988; Fuller et al., 1995). However, it has also been suggested that gL is a functional chaperone (Hutchinson et al., 1992; Kaye et al., 1992; Roop et al., 1993), which serves only to facilitate authentic folding and cell-surface transport of gH and also that gL does not contribute to membrane fusion events (Duss et al., 1995, 1996).

Both of these possibilities should be amenable to experimental examination. Since HSV-1 gH and gL form noncovalent hetero-oligomers, UL1-gL could be tagged with an ER retrieval signal (eg. KDEL) to determine if: (1) gH:gL interactions in the ER and Golgi compartments are sufficient for UL22-gH activity or (2) gL must interact with gH molecules in the plasma membrane to produce a functional HSV-1 fusion complex. A similar strategy has been used to show that the ß-2-microglobulin subunit is not a functional component of the class I major histocompatibility complex. Instead, ß-2-microglobulin acts as an ER chaperone by allowing the active component (class I heavy chain) to fold correctly, acquire peptide, and migrate from the ER to the cell surface (Solheim et al., 1995). If gL is required for fusogenic activity, it would be interesting to map the site in UL22:gH which binds to UL1:gL. Given that protein structure can change irreversibly when the fusogenic activity of a viral fusion protein is triggered (Stuart, 1994; Lamb, 1993; Wahlberg & Garoff, 1992), one could also examine the structure of gH:gL molecules transferred to the plasma membrane during virus penetration (Para et al., 1980), to determine if they have lost the conformational epitopes exhibited by gH:gL molecules within the virion envelope (and formed only when UL22-gH and UL1-gL are co-expressed in cells, eg. those recognized by MAbs LP11 & 53S) (Gompels & Minson, 1989; Gompels et al., 1991; Hutchinson et al., 1992; Roop et al., 1993).

Similar studies with conformation specific MAbs raised against UL27-gB (Marlin et al., 1986; Chapsal & Pereira, 1988; Highlander et al., 1988, 1989; Navarro et al., 1992) might provide useful information concerning the role of UL27-gB in the membrane fusion process. The discovery that tsB5 virions (which contain a ts-mutation in the extracellular domain of UL27-gB) made at the permissive temperature (34°C) suffer a permanent loss of infectivity when incubated at a non-permissive temperature (45°C) (Manservigi et al.,

1977; Sarmiento et al., 1979; Deluca et al., 1984) indicates that the conformation of fully mature gB molecules is open to an irreversible transformation, as one would expect for a viral fusion protein.

3.2.3 US6-gD, the role of receptor binding in virus penetration.

The role US6-gD plays in binding to a secondary cell surface receptor is generally considered to be the essential contribution US6-gD makes to HSV-1 penetration (see section 2.1.2 above; Ligas & Johnson, 1988; Johnson & Ligas, 1988; Johnson et al., 1990). According to this hypothesis, the interactions between US6-gD and cell surface receptors are required to bring the virion envelope into close proximity with the plasma membrane so that the membrane fusion protein(s) can proceed with virus penetration. Since binding avidity is increased by multiple interactions (Haywood, 1994) the observations that (1) US6-gD is a major component of the virion envelope relative to UL27-gB and UL44-gC (Handler et al., 1996a), and (2) penetration efficiency (roe) is dependent on the amount of US6-gD in the virion envelope (Fehler et al., 1992), would also be consistent with this hypothesis. In addition, single amino acid substitutions in the extracellular domain of US6-gD can produce a cell-type-dependent increase or reduction in HSV penetration, which suggests that these mutations alter the ability of US6-gD (or another HSV receptor binding protein influenced by the structure of gD) to interact with cell specific factors and do not exert a global effect on gD function (eg. on an undiscovered fusogenic activity) (Dean et al., 1994). Furthermore genes that encode glycoproteins homologous to US6-gD have been found in the alphaherpesviruses (eg. PRV, BHV-1 and EHV-1 but not VZV) but not the beta- or gamma-herpesviruses (Davison & Scott, 1986; Petrovskis et al., 1986; Tikoo et al., 1990; Telford et al., 1992;

Gompels et al., 1995; Nicolas, 1996). Consequently, the notion that US6-gD plays an auxillary role in virus penetration as a receptor binding protein seems plausible, because it has been suggested that proteins directly involved in virus penetration are conserved between alpha- beta- and gammaherpesviruses (Miller & Hutt-Fletcher, 1992; Gage et al., 1993; Qingxue et al., 1995; Laquerre et al., 1996). Since the herpesvirus subfamilies can infect different tissues (eg. alpha-[neurons], gamma-[B lymphocytes]), it is probable they have evolved unique receptor binding proteins which are adapted for virus attachment to different tissue types (eg. EBV gp350/220 mediates attachment to B lymphocytes).

Early studies had suggested that US6-gD might have the capacity to induce membrane fusion or more specifically cell-cell fusion (syncytium formation) (Campadelli-Fiume et al., 1988a; Butcher et al., 1989; Tikoo et al., 1990). However, reports of this nature are rare and this phenomenon may be restricted to certain cell types because a survey of the literature reveals multiple examples illustrating that gD expression is not sufficient to induce syncytium formation (Johnson & Smiley, 1985; Caras et al., 1987; Johnson & Spear, 1989; Lisanti et al., 1989; Hutchinson et al., 1993; Zurzolo et al., 1993; Dean et al., 1994, 1995; Chase et al., 1990; Petrovskis et al., 1988) including those cell lines which complement the growth of gD-negative herpesvirus mutants (Fehler et al., 1992; Liang et al., 1995 Rauh & Mettenleiter, 1991; Peeters et al., 1992). Furthermore, gD fusogenic activity was observed on cell lines expressing low but not high levels of gD, implying that this phenomenon may depend more on the availability of a cellular gD-receptor located on adjacent cells, than on a fusion activity intrinsic to gD.

Perhaps the best evidence that gD plays a modulatory role, and not a direct role in virus entry, stems from studies showing that the gD homologs of BHV-1 and PRV are essential for host cell penetration, yet dispensable for cell-to-cell transmission (Wathen &

Wathen, 1984; Eloit et al., 1988; Rauh & Mettenleiter, 1991; Peeters et al., 1992a; Okazaki et al., 1986; Fehler et al., 1992; Liang et al., 1995b). PRV gD-negative mutants form syncytial plaques in tissue culture comparable to the wild-type virus (the primary mode of PRV cell-to-cell spread *in vitro* [Zsak et al., 1992])(Rauh & Mettenleiter, 1991; Peeters et al., 1992a), are fully virulent in the mouse animal model (Babic et al., 1993; Peeters et al., 1993) and only slightly less virulent in its natural host, the pig (Heffner et al., 1993; Mulder et al., 1996). Because syncytium formation involves membrane fusion Peeters et al. (1992a) concluded that, except for a few cell types (Mulder et al., 1996), PRV gD is not required for membrane fusion.

On the other hand, gD-negative BHV-1 mutants exhibit an intermediate phenotype and form much smaller plaques on noncomplementing gD^- cells than the wild-type parental virus, illustrating that gD can facilitate cell-to-cell spread of BHV-1 virions (Liang et al., 1996). Subsequent analysis demonstrated that BHV-1 gD molecules, anchored by glycosyl-phosphatidylinositol (GPI) to the outer leaflet of the virion envelope, are sufficient for BHV-1 host cell penetration and virus replication (Liang et al., 1996). Since replacement of an authentic membrane anchor with GPI has been shown to yield viral fusion proteins that can induce hemi-fusion (ie. fusion between the outer leaflets of lipid membrane bilayers) but not complete fusion (Weiss & White, 1993; Kembel et al., 1994), it is doubtful that fusogenic activity constitutes an essential function of BHV-1 gD in virus entry. This technique could also be useful in studies designed to determine the contribution UL27-gB and UL22-gH make to virus penetration and membrane fusion.

It is probable that gD homologs are at least in part, functionally similar because the presence of PRV or BHV-1 gD at the cell surface can also block HSV-1 penetration (see section 2.1.2 above; Petrovskis et al., 1988; Chase et al., 1990; Lee & Fuller, 1993) and

for this reason it may be valid to suggest that HSV-1 US6-gD (like PRV and BHV-1 gD) is not a fusion protein. However, this conclusion must be viewed with caution for at least two reasons: (1) the amino acid sequence identity between gD homologs of HSV-1, PRV and BHV-1 is very low (HSV vs PRV or BHV-1 = 11%, PRV vs BHV-1 = 32.8%) (Spear, 1993) and (2) because US6-gD is extremely important if not indispensable for cell-to-cell spread in cultured cells (Ligas & Johnson, 1988; Brunetti et al., 1995) and obligatory for inter-cellular spread in the mouse animal model (Dingwell et al., 1995), whereas PRV gD and perhaps BHV-1 gD are not (Rauh & Mettenleiter, 1991; Peeters et al., 1992a; Babic et al., 1993; Peeters et al., 1993).

In addition to binding a cellular receptor, US6-gD molecules may fulfil another function required for virus penetration. This premise is based on the observation that mutant forms of US6-gD do not rescue a gD-negative virus, yet they are transported to the cell surface, incorporated into extracellular virions and retain the ability to bind to cells and block HSV-1 penetration (Chiang et al., 1994; Nicola et al., 1996). US6-gD is in close proximity (ie. within 11.4 Å) to a variety glycoproteins in the virion envelope (eg. gB, gC, gH:gL) and may form oligomeric structures with many of these proteins (Handler et al., 1996). Thus it is possible that receptor binding provokes a conformational change in US6-gD, allowing gD to interact with other envelope protein(s) thereby shifting the HSV fusion protein into an active state receptive to the fusion trigger. For instance, the binding interactions of a gD-specific MAb increases the infectivity of HSV-2 three to five fold (Minson et al., 1986) and mutations mapping to the same epitope confer resistance to gD-mediated interference in HSV-1 (Campadelli-Fiume et al., 1990a; Brandimarti et al., 1994; Dean et al., 1994, 1995).

Support for this idea is also provided by data suggesting that GPI-gD on the cell

surface (but not soluble of wild-type forms of BHV-1 gD) can facilitate penetration of a gD-negative BHV-1 mutant, at a step between virus binding and membrane fusion (Liang et al., 1995). Furthermore, there is a precedence for this mechanism. For example, both the hemagglutinin (HN) and fusion protein (F) of some paramyxoviridae (eg. Newcastle disease virus [NDV]) are required to induce membrane fusion, and interactions between HN and the viral receptor, sialic acid, are thought to induce conformational changes in HN and in F which are required to expose the fusogenic peptide in F (Lamb, 1993).

3.2.4 The role of tegument protein, UL25, in HSV-1 penetration.

Very little is known about the role UL25, an essential tegument protein, plays in virus penetration (Addison, et al., 1984; Ali et al., 1996). Evidence that UL25 is involved in this process stems from the analysis of a temperature-sensitive mutant (ts1204) which contains a defect in the UL25 gene (Addison et al., 1984). ts1204 virions made at the permissive temperature (31°C) can attach to the cell surface but fail to penetrate the plasma membrane at the nonpermissive temperature (38.5°C), unless the cells are treated with a fusogenic agent such as polyethylene glycol (PEG) or the temperature is shifted back to 34°C. The ts1204 virions are thought to bind primary and secondary HSV receptors at the non-permissive temperature since these virions prevent subsequent infection of cells by wild-type HSV-1 (Addison et al., 1984). In addition, UL25 belongs to a cluster of genes which include UL22-gH and UL24, proteins which have been implicated in HSV-induced membrane fusion, and this cluster of genes is conserved by all herpesvirus families (Gompels et al., 1995; Nicolas, 1996; Davis-Poynter et al., 1994; Wilson et al., 1994; Sanders et al., 1982; Jacobson et al., 1989). Consequently, all UL25 homologs may play an essential role in herpesvirus penetration.

Interestingly, a recent cross-linking study by Zhu and Courtney (1994) identified oligomeric complexes in HSV virions containing the envelope proteins (gB, or gD, or gH but not gC) and four different tegument proteins including UL48-VP16 (65-kDa). Although the molecular weight of UL25 (60-kDa) does not coincide with that of tegument proteins detected by Zhu & Courtney (1994), it is still conceivable that interactions between the cytoplasmic tail of UL27-gB, or UL22-gH, or US6-gD and UL25 (or another tegument protein influenced by UL25) could have an impact on the fusion process required for virus penetration. For instance, mutations in the cytoplasmic tail of UL27-gB, US6-gD, or UL22-gH, influence virus-induced membrane fusion (Deluca et al., 1982; Bzik et al., 1984; Cai et al., 1988a,b; Weise et al., 1987; Goodman & Engel, 1991; Engel et al., 1993; Baghian et al., 1993; Gage et al., 1993; Wilson et al., 1994; Feenstra et al., 1988) alter the rate at which HSV-1 enters cells (roe mutation) (Gage et al., 1993), or abolish penetration altogether (Cai et al., 1988b; Desai et al., 1994; Dean et al., 1995) and these mutations could be interpreted as modifications that alter binding of a regulatory protein(s). By combining the ts lesion in UL25 and the mutations described above into a single virus, it might be possible to determine whether UL25 exerts a positive or negative effect on virus penetration.

3.3 HSV-1 induced cell-cell fusion

Virus families which possess envelope glycoproteins with activity at physiological pH also induce cell-cell fusion or syncytium formation (ie. creation of multi-nucleated cells or polykaryons) (Lamb, 1993; Luciw, 1996; Holmes & Lai, 1996) and herpesviruses including HSV-1 are no exception (Zsak et al., 1992; Rodriguez et al., 1993; Navvarro et al., 1993; Frenkel et al., 1990; Honess, 1984; Hoggan & Roizman, 1959). Although

polykaryocytes are characteristic of herpetic lesions *in vivo*, fresh isolates of HSV-1 usually elicit little or no cell-cell fusion in cultured cells (Spear, 1992). Instead, the typical wild-type plaque observed in tissue culture is characterized by cell rounding and aggregation (Hoggan & Roizman, 1959; Ejercito et al., 1968). However, the plaque phenotype of wild-type HSV-1 strains can vary somewhat on different cell types. For example 20% of the human embryonic lung cells (HEL) infected with wild-type HSV-1 form polykaryons, and this fusion occurs over a brief period, ending abruptly two hours after it begins (Person et al., 1976; Read, 1980; Bzik & Person, 1981). This observation is reminicent of the report that varicella zoster virus (VZV), a related alphaherpesvirus, exhibits a syncytial phenotype on monolayers of human melanoma cells and the nonsyncytial phenotype on normal human fibroblasts (Grose et al., 1979; for review see Rodriguez et al., 1993).

In contrast, mutant strains of HSV-1 have been isolated which cause extensive cell-cell fusion in a wide variety of cultured cells and these mutants, which have been designated "syn" because they form syncytia, arise with considerable frequency (Hoggan & Roizman, 1959; Nii & Kamahora, 1961; Brown et al., 1973; Person et al., 1976; Read et al., 1980; Bond et al., 1982). Early studies focused primarily on HSV-1 mutants with syncytial mutations in two loci commonly referred to as syn-1 and syn-3 (Ruyechen et al., 1979). The cell-cell fusion kinetics of the syn-1 and syn-3 mutants differs substantially from wild-type viruses and fusion, once it begins, will often continue in an uncontrolled fashion until the entire monolayer becomes a single multi-nucleated cell (Hoggan & Roizman, 1959; Person et al., 1976; Kousoulas et al., 1978; Read et al., 1980; Bond et al., 1982). In addition, the syncytial phenotype of HSV-1 mutants is often recessive in mixed infections with wild-type virus, although dominance of the wild-type phenotype is

sometimes cell type dependent (Roizman, 1962; Keller, 1976a; Manservigi et al., 1977; Little et al., 1980; Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982). Together, these observations led to the suggestion that wild-type strains of HSV-1 possess a fusion factor and a fusion inhibitor, a combination which yields a small amount of fusion, whereas defects in the latter allow fusion to continue unabated in cells infected with syncytial mutants (Ruhlig & Person, 1977; Manservigi et al., 1977; Kousoulas et al., 1978; Read et al., 1980; Lee & Spear, 1980).

Because cells infected with syn-1 mutants fuse with neighbouring uninfected cells and syn-mutant infected cells but do not fuse efficiently with cells infected by wild-type HSV-1, Keller (1976a) concluded that the membranes of wt-infected cells are modified by the fusion inhibitor to prevent cell-cell fusion (Keller, 1976a; Read et al., 1980; Lee & Spear, 1980). For example, a newly synthesized viral protein such as US6-gD could bind to a cellular receptor during cell surface transport and block the receptor interactions required for cell-cell fusion. Lee and Spear (1980) extended this work and demonstrated that the viral factor(s), which prevent syncytial mutants from recruiting wild-type HSV-infected cells into polykaryocytes do not result from true-late gene expression. However, Lee and Spear (1980) went on to show that inhibition of cell-cell fusion between cells infected with wild-type HSV-1 is related more to the suppression of HSV-1 fusogenic activity (ie. the ability of wt-infected cells to cause cell-cell fusion), than to a modification in the plasma membrane which reduces the capacity of wt-infected cells to be recruited into polykaryocytes. Based on studies of a syn-1 mutant, Lee and Spear (1980) attributed this inhibitory activity to a late gene product. These results led to the conclusion that syncytia formation involves a complex series of events which are controlled by at least one viral fusion protein, several regulatory proteins and an undetermined number of

cellular factors.

3.3.1 Mutations which confer the syncytial phenotype

Subsequent studies have demonstrated that a large number of viral factors contribute to HSV-induced cell-cell fusion, some that have a direct role in membrane fusion and others which regulate the fusion process in some unknown manner (see Table 1.1 and Fig. 2.1).

Syncytial mutations can arise in at least four different viral genes including: (1) the UL20 gene which specifies an integral membrane protein required for virus egress from some cell types but not others (Maclean et al., 1991; Baines et al., 1991; Avitabile et al., 1994; Ward et al., 1994), (2) the UL24 gene (syn-5 locus) encoding a cytosolic protein of unknown, but important, function given that UL24 insertion mutants experience a severe reduction in virus replication (ie. 50-200 fold) (Sanders et al., 1982; McGeoch et al., 1988; Jacobson et al., 1989; Tognon et al., 1990; Spear, 1992), (3) the UL27 gene (syn-3 locus) which encodes gB an essential glycoprotein required for virus entry and cell-cell fusion (see sections 3.1.1 & 3.2.1; Manservigi et al., 1977; Ruyechan et al., 1979; Sarmiento et al., 1979; Deluca et al., 1982; Bzik et al., 1984a,b; Cai et al., 1987; 1988), and (4) the UL53 gene (syn-1 locus) which specifies a hydrophobic protein with four potential membrane spanning domains (Ruyechan et al., 1979; Bond & Person, 1984; Pogue-Geile et al., 1984; Debroy et al., 1985; Pogue-Geile & Spear, 1987; McGeoch et al., 1988; Spear, 1992).

Another syncytial locus, syn-6, resides within a region of the HSV-1 genome flanked by genes encoding ICP0 and ICP34.5, but the affected gene has not yet been identified (Romanelli et al., 1991). Interestingly, characteristics of the syn-6 mutation are

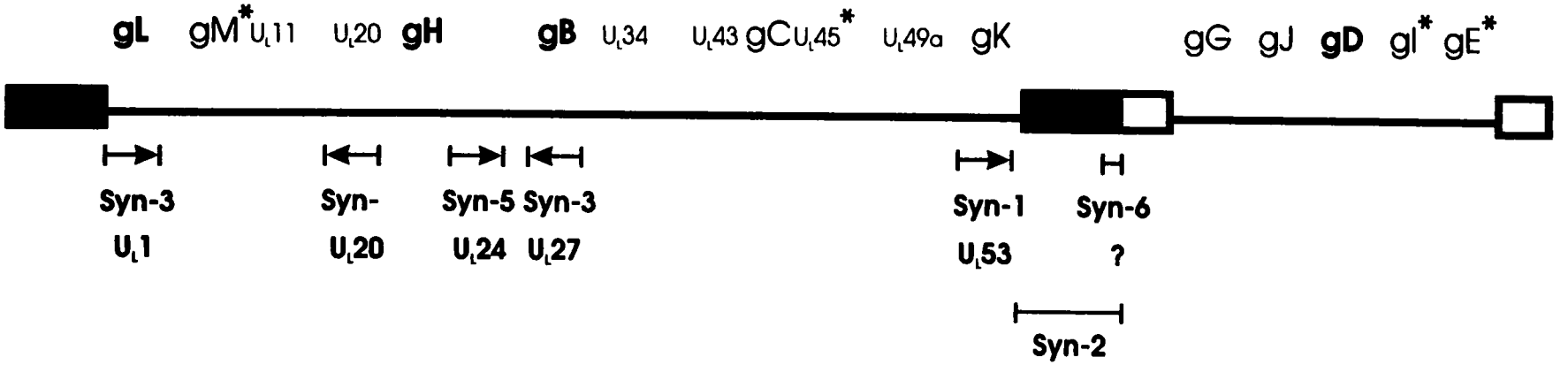


Figure 2.1 HSV genome: Location of membrane proteins, and site of syncytial mutations.

The HSV-1 genome is represented as unique long (U_L) and short (U_S) segments flanked by inverted repeats (filled and open boxes). The position of genes encoding HSV-1 membrane proteins are indicated above the genomic map. Glycosylated membrane proteins are given the "gP" designation. Mutations in the U_L20, U_L24, U_L27-gB, U_L53-gK genes and an unknown locus (?) (indicated below the map) can produce the syncytial phenotype (eg. Syn-1). The syn phenotype (~~Syn-~~4) originally ascribed to a mutation in UL1-gL has now been attributed to UL53-gK. The Syn-2 mutation is situated within map units 0.75 to 0.83 and the Syn-6 mutation is located between map units 0.81 and 0.82, but neither mutation has been assigned to a specific gene (?). In addition, the current status of Syn-2 (Ruyechan et al., 1979) is uncertain, since this locus may influence plaque size without producing cell-cell fusion (Pogue-Geile et al., 1984). The UL1-gL, UL22-gH, UL27-gB, and US6-gD glycoproteins (boldface type) are essential for HSV-1 replication in cultured cells, cell fusion, and virus entry. The HSV genes encoding UL10-gM, US7-gI, US8-gE, and UL45 membrane proteins are also required for virus-induced cell fusion associated with a syncytial mutation in gB (asterisks).

reminiscent of the locus Ruyechan et al. (1979) designated syn-2 and Pogue-Geile et al. (1984) later classified as a plaque size mutation. Both the syn-2 and syn-6 loci map to similar regions of the HSV genome (ie. syn-6 = map units 0.81 to 0.85, syn-2 = map units 0.70 to 0.83) and have the capacity to augment HSV-1 fusion of HEp2 cells whereas mutations in syn-1 (UL53), syn-3 (UL27-gB) or syn-5 (UL24) do not (Ruyechan et al., 1979; Haffey & Spear, 1980; Lee & Spear, 1980; Bzik & Person, 1981; Pogue-Geile et al., 1984; Tognon et al., 1991). Previously, marker transfer experiments had suggested that UL1-gL may also be the site of a syn mutation (syn-4) (Little et al., 1981), but subsequent studies have demonstrated that this mutation actually maps to the UL53 gene (Hutchinson et al., 1993; Roop et al., 1993; Novotny et al., 1996).

In the absence of UL20 or UL24 gene expression HSV mutants exhibit the syncytial phenotype indicating that these proteins are not required for cell-cell fusion, whereas their loss disturbs a facet of viral replication that influences the normal regulatory mechanism controlling HSV-1 fusogenic activity (Jacobson et al., 1988; Baines et al., 1991). Very little is known about the role of UL24 in virus replication, however UL20 has been well characterized and is known to facilitate the transport of enveloped virions from the perinuclear space, at least in cell types which possess a fragmented Golgi apparatus (Baines et al., 1991). Although UL20 is incorporated into the virion envelope it is not expressed on the cell surface and therefore it is unlikely that UL20 interacts directly with components of the viral fusion complex to inhibit cell-cell fusion (Ward et al., 1994). Interestingly, Avitabile et al. (1994) report that transport of viral glycoproteins (eg. UL44-gC and US6-gD) from the trans-Golgi to the plasma membrane is impaired in Vero cells infected with a UL20-deletion mutant, suggesting UL20 mutations may affect cell surface transport of the fusion complex or regulatory proteins. As such, defects in UL20 may

have a differential effect on the cell surface transport of the putative "fusion inhibitor" or the transport of viral proteins known to mediate fusion and by doing so create an imbalance that results in cell-cell fusion. Still, it is not clear if UL20 has a direct role in viral protein transport. Similar traits have been observed in other syncytial mutants, and consequently the reduction in cell surface transport may be an indirect result of the syncytial phenotype (Person et al., 1982).

In contrast, to the gross mutations in UL20 and UL24 which result in cell-cell fusion, amino acid substitutions in gB confer the syncytial phenotype and these mutations are confined to essential and highly conserved hydrophillic, alpha-helical domains in the cytoplasmic tail (DeLuca et al., 1982; Bzik et al., 1984b; Weise et al., 1987; Cai et al., 1988a,b; Goodman & Engel, 1991; Baghian et al., 1993; Engel et al., 1993; Gage et al., 1993). Rate of entry (roe) mutations have also been identified in these cytoplasmic domains, providing a direct molecular link between cell-cell fusion and virus penetration (Cai et al., 1988; Gage et al., 1993). Since HSV-infected cells require mature UL27-gB for cell-cell fusion, just as HSV-1 virions require UL27-gB for virus penetration (see section 3.2.1 above; Manservigi et al., 1977; Sarmiento et al., 1992 Cai et al., 1987, 1988a; Highlander et al., 1988; Navarro et al., 1992) it seems likely that UL27-gB is a direct participant in the membrane fusion events which lead to cell-cell fusion.

In addition, several reports have claimed that HSV-variants with syn-3 mutations in UL27-gB have the ability to induce fusion-from-without (ie. extracellular virions introduced to apical membranes bridge uninfected cells to induce cell-cell fusion) and fusion-from-within (ie. newly synthesized viral fusion protein(s) located in the plasma membrane of infected cells, or newly made virions released at the cell surface, induce cell-cell fusion) (Ruyechan et al., 1979; Weise et al., 1987; Falke et al., 1985; Walev et al.,

1991a,b). By contrast, HSV-1 variants with syn mutations in UL20, UL24 (syn-5), or UL53 (syn-1) must infect cells and synthesize viral proteins to induce cell-cell fusion (fusion-from-within) (Keller, 1976b; Person et al., 1976; Kousoulas et al., 1978; Ruyechan et al., 1979; Walev et al., 1991a,b). Walve et al. (1991) also showed that cyclosporin A inhibits the fusion-from-within phenotype of syn-1 and syn-5 mutants, yet fails to suppress both categories of fusion caused by syn-3 mutants, indicating once again that the underlying basis for the cell-cell fusion induced by syn-3 mutants is distinct from that of the other syncytial mutants (McKenzie et al., 1987). These results are consistent with previous work illustrating that the ability of mutants with distinct syn loci (eg. syn-3 vs syn-6) to produce syncytia on different cell types can overlap, but will not always be identical (Ruyechan et al., 1979; Haffey & Spear, 1980; Lee & Spear, 1980; Bik & Person, 1981; Toggon et al., 1991; Romanelli et al., 1991). Based on results such as these, Cai et al. (1988a) have proposed that the cytoplasmic domain of UL27-gB has a modulatory role in membrane fusion and suggest that the other HSV proteins which carry syn mutations exert a regulatory influence on UL27-gB activity through this domain, perhaps by direct molecular interactions (Gage et al., 1993). By this model syn-3 mutations in gB disrupt the normal regulatory process governing membrane fusion.

Because all herpesvirus families possess homologs of UL24 and UL27-gB it seems reasonable to suggest that both of these proteins have a fundamental role in the mechanism controlling cell-cell fusion caused by alpha and betaherpesvirus infection (Zsak et al., 1992; Rodriguez et al., 1993; Navvarro et al., 1993; Frenkel et al., 1990; Honess, 1984; Hoggan & Roizman, 1959; Gompels et al., 1995; Nicolas, 1996). By contrast, the UL20 and UL53 genes are restricted to the alphaherpesvirinae (Baer et al., 1984; Davison & Scott, 1986; Davison & Taylor, 1987; Chee et al., 1988; McGeoch et al., 1988; Debroy,

1990; Albrecht et al., 1992; Telford et al., 1992, 1995; Zhao et al., 1992; Ren et al., 1994; Baumeister et al., 1995; Gompels et al., 1995; Nicolas, 1996). Therefore it is somewhat surprising that syn mutations occur infrequently in the UL20, UL24, and UL27-gB genes, at least in some strains, and map consistently to the UL53 gene (Keller, 1976a; Little et al., 1981; Bond et al., 1982; Bond & Person, 1984; Read et al., 1980; Roop et al., 1993; Dolter et al., 1994). Of the syn-1 mutations in UL53 which have been characterized, all were of the missense type, 80% were located in the N-terminal ectodomain and the other 20% in a conserved region upstream of hydrophobic domain 4 and thirty residues from the C-terminus (Debroy et al., 1985; Pogue-Geile et al., 1987; Dolter et al., 1994). Residue 40 of UL53, which lies in a motif (C[I/V]YA) conserved by alphaherpesvirus homologs, was found to be altered in 50% of these mutants (ie. Ala to Val, or Ala to Thr) indicating this region may be especially critical to the role UL53 plays in cell-cell fusion. The exact role for UL53 in cell-cell fusion has yet to be established. Although some have suggested that UL53, like UL27-gB, may be essential for cell-cell fusion because syn-1 mutations in UL53 are limited to missense mutations (Dolter et al., 1994; Wilson et al., 1994) it is equally possible that this constraint reflects a requirement for UL53 in HSV-1 replication and not the need for UL53 in cell-cell fusion. For instance, the recessive phenotype associated with many UL53 syn-1 mutations could signify a loss-of-function and therefore specific domains of UL53 may have a modulatory effect on membrane fusion, presumably within the category of "fusion inhibition" (Roizman, 1962; Keller, 1976a; Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982).

3.3.2 Role for HSV-1 glycoproteins which are required during virus penetration (ie. gB, gD, gH:gL), in cell-cell fusion

There is overwhelming evidence that HSV-1 membrane proteins which are obligatory for host cell penetration also participate in cell-cell fusion given that US6-gD and UL22-gH:UL1-gL, like UL27-gB, are mandatory for the membrane fusion events which mediate HSV penetration and promote the fusion of HSV-infected cells (Noble et al., 1983; Minson et al., 1986; Ligas & Johnson, 1988; Forrestor et al., 1992; Buckmaster et al., 1984; Gompels & Minson, 1986; Roop et al., 1993; Novotny et al., 1996; for review see Davis-Poynter et al., 1994). A few reports have claimed that expression of any one of these proteins (ie. UL27-gB, US6-gD, UL22-gH:UL1-gL or one of their homologs) is sufficient to induce cell-cell fusion, but this effect tends to be much less extensive than the cell-cell fusion induced by HSV-infected cells and appears to be cell-type dependent, which may indicate that cell specific or unidentified viral factors can substitute for the missing HSV-1 gene products (eg. the action of a retrovirus or vaccinia virus fusion protein) (HSV-Ali et al., 1987; BHV-Fitzpatrick et al., 1990; CMV-Tugizov et al., 1994; HSV-Campadelli-Fiume et al., 1988a; Butcher et al., 1989; BHV-Tikoo et al.,1990; Duus et al., 1995; Spear, 1993). Together these results led to a general belief among herpesvirus virologists that HSV-induced cell-cell fusion and virus penetration are very similar phenomena. However, there is a growing body of evidence which indicates that the two fusion processes have different requirements. For instance, neutralization of HSV-1 infectivity by MAbs specific for UL27-gB or US6-gD does not correlate with their capacity to block cell-cell fusion (Gompels & Minson, 1986; Minson et al., 1986; Highlander et al., 1987; Navvaro et al., 1992). Mutations in the cytoplasmic tail of UL22-gH and US6-gD suppress cell-cell fusion but have little or no effect of virus penetration (Feenstra et al., 1990; Wilson et al., 1994). The discovery that a related alphaherpesvirus, pseudorabies virus (PRV), requires gD for virus entry but not for cell-cell fusion also

serves to emphasize the difference between virus penetration and cell-cell fusion (Peeters et al., 1992a; Rauh & Mettenleiter, 1991). If one takes into consideration that the virion envelope and plasma membrane are likely to have different properties, then virion-cell fusion (penetration) and cell-cell fusion might be expected to have different requirements. The lipid and protein content, organization of these molecules and the curvature of the virion envelope are likely to differ from the infected cell-plasma membrane and all of these properties can influence the fusion process (Kohn et al., 1980; Spear, 1993; Lamb, 1993; Chernomordik et al., 1995).

3.3.3 Role of HSV-1 membrane proteins which are dispensable in cultured cells, in cell-cell fusion and direct cell-to-cell transmission.

HSV-1 plaque formation is controlled by two phenomena: the rate at which extracellular virions are released from cells and then attach to and penetrate adjacent cells, as well as the rapidity of direct transmission (cell-to-cell spread), a process which occurs even in conditions that block the movement of cell-free virions (ie. under agarose or the in the presence of neutralizing Ab) (Farham, 1958; Hoggan et al., 1960; Zsak et al., 1992; Spear et al., 1992; Spear, 1993; Dingwell et al., 1994). The mechanism which sustains cell-to-cell spread in these conditions has not been identified, but several models have been put forward to explain these observations. For instance, cell-associated virions may bridge the distance between infected and uninfected cells (eg. through virion movement in the basolateral plane, or virion diameter may be sufficient). According to this model, fusion events analogous to virus penetration are responsible for cell-to-cell spread, ie. between the envelope of cell-associated virions and the plasma membrane of neighbouring cells (Dingwell et al., 1994). On the other hand, cell-to-cell transmission of HSV-1

virions may involve formation of an intercellular bridge or channel between the plasma membranes of an infected cell and an uninfected neighbour, permitting the direct transfer of HSV virions across such a junction (Zsak et al., 1992).

Recently it has become apparent that the characteristics of cell-cell fusion have more in common with cell-to-cell transmission of HSV-1 virions, than with virus penetration at the cell surface (ie. with respect to the envelope proteins needed for each of these events). This information has prompted those authors who support the hypothesis that cell-to-cell spread is comprised of limited fusion between apposing plasma membranes, to suggest that the extensive cell-cell fusion caused by syncytial variants of HSV-1 is an uncontrolled version of the same process (Davis-Poynter et al., 1994; Wilson et al., 1994). Nevertheless, cell-cell fusion and cell-to-cell spread may be distinct from virus penetration, and still entail cell-associated virions, if for example, these events are restricted primarily to the basolateral surface of polarized cells (Tashiro et al., 1992). The apical and basolateral membranes of polarized cells are equally susceptible to HSV-1 infection, yet there is evidence to suggest that the envelope protein UL44-gC is required for HSV-1 entry at apical membranes, and dispensable both for penetration and subsequent cell-to-cell spread of gC^- virions exposed to basolateral membranes (Sears, 1991; Spear, 1993; Tugizov et al., 1996). In contrast, UL44-gC, like many HSV-1 envelope proteins (eg. gE:gI, gG, gJ, gM, UL45), can be removed altogether with only a marginal impact on HSV-1 replication in nonpolarized cell lines (Baines & Roizman, 1991; 1993; MacLean et al., 1991, 1993; Spear, 1993; Visalli et al., 1991, 1993; Baanes et al., 1994; Balan et al., 1994; Dingwell et al., 1994; Tal-Singer et al., 1995). Therefore, it is conceivable that a different subset of "nonessential" envelope proteins (eg. gE:gI) mediate receptor interactions required for virus penetration, cell-to-cell spread and cell-cell fusion at the

basolateral surface of polarized cells (Dingwell et al., 1994). In nonpolarized cells infected with HSV-deletion mutants the accessibility of "apical" receptors may permit the remaining envelope proteins (eg. gB, gC, gD, gH:gL) to compensate for the loss of other membrane proteins (eg. gE:gI), and allow cell-to-cell spread to proceed.

Although UL27-gB, US6-gD, and UL22-gH:UL1-gL are required for all forms of membrane fusion, additional HSV-1 membrane proteins, which are not essential for virus penetration, have also been implicated in cell-cell fusion and cell-to-cell spread. Little or no cell-cell fusion is detected when cells are infected with deletion mutants containing a syncytial form of gB but lacking US8-gE, US7-gI, UL10-gM or UL45 (Balan et al., 1994; Davis-Poynter et al., 1994; Haanes et al., 1994). Similarly, the gE:gI hetero-oligomers and gM proteins of HSV-1 (and other alphaherpesviruses) facilitate efficient cell-to-cell spread (Zsak et al., 1992; Balan et al., 1994; Dingwell et al., 1994, 1995; MacLean et al., 1991, 1993; Baines & Roizman, 1991, 1993; Osterrieder et al., 1996). In addition, HSV-1 mutants that lack UL45 form tiny plaques (on Vero & Hela cells) relative to the wild-type virus, indicating that UL45 could also have a role in HSV-1 transmission (Visalli & Brandt, 1991). However, there are reports that syncytial mutants lacking gE (Neidhart et al., 1987) or gI (Ligas & Johnson, 1988; Dingwell et al., 1994) retain the syncytial phenotype, implying that these deletion mutants possess syn mutations in a gene other than UL27-gB (eg. UL53), or possess secondary mutations which enhance cell-cell fusion (eg. in US6-gD, UL10-gM, etc.), relieving the need for gE:gI in this process (Neidhart et al., 1987; Izumi & Stevens, 1990; Yuhasz & Stevens, 1993). Furthermore, the requirement for gE:gI hetero-oligomer in cell-cell fusion may be influenced by cellular factors because the reduction in plaque size exhibited by gE and gI null-mutants is more severe in polarized cells than in nonpolarized cell lines (Zsak et al., 1992; Balan et al., 1994; Dingwell, 1994).

Together these observations suggest that US8-gE, US7-gI, UL10-gM and UL45 have a modulatory impact on membrane fusion. Since US8-gE, US7-gI, UL10-gM and UL45 are found in the virion envelope and on the plasma membrane, they may act as tissue specific, receptor binding proteins (see section 3.1.3), or they may interact directly with the HSV-1 proteins which mediate membrane fusion to influence fusogenic activity, or fulfil an as yet unidentified function required for cell-to-cell spread and cell-cell fusion (Para et al., 1980; Baines & Roizman, 1993; MacLean et al., 1993; Spear, 1993; Visalli & Brandt, 1993).

In contrast to the gE:gI hetero-oligomer which is necessary for efficient cell-to-cell transmission yet has no discernable role in virus entry (Neidhardt et al., 1987; Balan et al., 1994; Dingwell et al., 1994), deletion of UL44-gC can reduce HSV-1 infectivity, presumably by reducing virus adsorption to cells (Herold et al., 1991; 1994; Sears et al., 1991) but does not inhibit cell-cell fusion. Nevertheless, many syn mutants are also defective in the synthesis of UL44-gC (Heine et al., 1974; Zezulak & Spear, 1984; Cassai et al., 1976; Weise et al., 1987; Goodman & Engel, 1991). Although an early study did suggest UL44-gC might be a fusion inhibitor (Manservigi et al., 1977) subsequent marker rescue experiments have ruled out this possibility (Lee et al., 1982; Weise et al., 1987). Others have suggested that gC function is somewhat incompatible with the syncytial phenotype and therefore secondary mutations arise which block gC expression (Spear, 1984; Spear, 1993; Novotny et al., 1996). Support for this idea stems from reports that spontaneous gC-negative variants can arise in gC-positive syncytial populations (Zezulak & Spear, 1984; Weise et al., 1987). However, there is another alternative: loss of UL44-gC function might occur first and the syncytial mutation second.

The spread of wild-type HSV-1 depends on two mechanisms: (1) direct cell-to-cell transmission and (2) release of extracellular virions followed by attachment and

penetration of neighbouring cells (Spear et al., 1992; Spear, 1993; Balan et al., 1994; Dingwell et al., 1994). Since UL44-gC plays a major role in virus adsorption to the cell surface, the extracellular spread of gC-negative virions is likely to be impaired (Herold et al., 1991; 1994). Consequently, those gC-negative strains which acquire secondary mutations of a type that augment the alternative mode of virus spread, direct cell-to-cell transmission, may have a selective advantage. Syn mutations may provide this type of advantage. The following evidence provides support for this hypothesis: (1) Cell-cell fusion may represent an enhanced form of cell-to-cell spread since both phenomena have similar requirements. (2) Cell-to-cell transmission of a gC^- syn-1 mutant, HSV-1 (MP), is more efficient than the intercellular spread of wild-type strains derived from the same genetic background, and the number of infectious progeny produced by HSV-1 (MP)-infected cells is small relative to wild-type strains (Lee & Spear, 1980). (3) Conditions designed to select gC-negative mutants (ie. serial passage in the presence of heparin, a potent inhibitor of HSV infectivity) yielded gC-negative mutants with a syncytial phenotype (Goodman & Engel, 1991; Engel et al., 1993). (4) Marker transfer of wild-type gB^{KOS} sequences to a syn-3 (gB) mutant (ANGpath) created recombinants which possess MAb epitopes specific to wt-gB^{KOS} and 70% of these viruses reverted to the wild-type plaque morphology (Weise et al., 1987). In contrast, all wt-gB^{KOS} positive recombinants retained the syncytial phenotype when a gC^- variant of the parental syn-3 (gB) mutant (C18-ANGpath) was used in these experiments. After restoration of UL44-gC expression, subsequent transfers of the wt-gB^{KOS} sequence to C18-ANGpath produced recombinants which form plaques with a wild-type morphology, demonstrating that C18-ANGpath does not contain a second syn mutation absent in the parental strain ANGpath. In regards to direct transmission, the preceding results imply that gC^- strains with a syncytial phenotype

have a selective advantage over gC$^-$ strains that possess a wild-type plaque morphology.

It should be noted however, that the genetic background of a parental virus may determine if a gC$^-$ mutant will acquire the syncytial phenotype, since 101 of 103 gC$^-$ mutants generated from HSV-1 (KOS) retained a wild-type plaque morphology (Holland et al., 1984; Homa et al., 1986). This result may reflect the selection conditions used in this study to isolate gC$^-$ strains. On the other hand, a shift to a syncytial phenotype may benefit only those gC$^-$ strains which have additional mutations that reduce the normal cell-to-cell spread of HSV virions (eg. in gE:gI or gM), or mutations that alter virus assembly. This view is supported by studies of a different alphaherpesvirus, PRV, which showed that gE$^-$ or gC$^-$ mutants display wild-type virulence in the mouse animal model, whereas PRV gC$^-$gE$^-$ double mutants are almost completely avirulent (Mettenleiter et al., 1987, 1988). Presumably, gC$^-$ and gE$^-$ mutants retain most of their virulence since they can be transmitted either by direct cell-to-cell contact (when gE is present) or by extracellular virus (when gC is present), whereas double mutants contain defects in both types of viral spread causing a severe reduction in virulence (Zsak et al., 1992).

3.3.4 Cellular factors involved in HSV-induced cell-cell fusion

Cellular factors can also influence the fusion of infected cells. That specific host factors are required for cell-cell fusion first became evident because HSV-1 mutants form syncytial plaques on some cell types but not on others, yet the same cells which are refractory to cell-cell fusion can often be used to propagate virus (Ruyechan et al., 1979; Haffey & Spear, 1980; Lee & Person, 1980; Bzik & Person, 1980). The ability of HSV-1 mutants to express the syncytial phenotype in a mixed infection with wild-type virus is also cell type dependent and this was best exemplified by studies of the syn-1 mutant

HSV-1 (MP) (Roizman, 1962; Keller, 1976a; Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982). The syncytial phenotype of HSV-1 (MP) is dominant in mixed infections of HEL cells, co-dominant in CV-1 cells, and recessive to wild-type virus in HEp-2 cells (Roizman, 1962; Bzik & Person, 1981).

To determine if the cellular factors which contribute to cell-cell fusion influence the capacity of infected cells to induce cell-cell fusion (ie. to recruit neighbouring cells into polykaryons) or instead affect the capacity of cells (themselves unable to induce fusion) to be recruited into polykaryocytes, several labs have employed a modified version of the infectious centre assay to examine cell lines which vary in their susceptibility to HSV-induced cell-cell fusion (Lee & Spear, 1980; Bzik & Person, 1981). Essentially, cells were infected with a syn-1 or syn-3 mutant, labelled with [^{3}H]-thymidine, then mixed with uninfected cells under conditions which prevented transmission of the viral infection, followed by autoradiography and quantification of the fusion events between labelled and unlabelled cells. These studies illustrated that host-cell restrictions to cell-cell fusion can be attributed both to cell-specific differences in the expression, post-translational processing or cell-surface transport of a viral protein(s) involved in cell-cell fusion, and to cell-specific differences in the cell-surface characteristics which determine if cells can be recruited into polykaryocytes (eg. nature or abundance of molecules that bind a viral receptor).

To date, only a few of the cell factors which may influence HSV-induced cell-cell fusion have been identified. Inhibitors of glycosylation suppress cell-cell fusion induced by a syn-1 mutant (Keller, 1976b, Knowles & Person, 1976; Holland & Person, 1977; Kousoulas et al., 1978; 1983; Campadelli-Fiume et al., 1982), but it is not clear if the absence of cell fusion is caused by a change in cell-surface characteristics or a change to

the processing or cell surface transport of the viral glycoproteins responsible for membrane fusion (Johnson & Spear, 1982; Norrild & Pedersen, 1982; Peake et al., 1982; Glorioso et al., 1983; Cheung et al., 1991; Chatterjee & Sarkar, 1992; Ghosh-Choudhury et al., 1987, 1994). However, cell lines with less severe defects in glycosylation were unable to support the syn-1 phenotype and it is doubtful that the cell surface transport of viral glycoproteins is substantially reduced in these cell lines (Serafini-Cessi et al., 1983). Furthermore, exposure of cells to neurominidase inhibits cell-cell fusion induced by a syn-1 mutant (Serafini-Cessi et al., 1983). In addition, cell-to-cell spread by wild-type HSV-1 is negligible on a cell line with defects in the phosphorylation of mannose residues, and by extension this modification could be required for cell-cell fusion since both events are closely related phenomena (Brunetti et al., 1995; see section 3.3.3. above). Although mannose-6-phosphate residues acquired by US6-gD (and perhaps other HSV glycoproteins) were shown to augment virus spread, neither Brunetti et al. (1995) or Serafini-Cessi et al. (1983) excluded the possibility that mannose-6-phosphate or the composition of the N-linked oligosaccharides on cell-surface constituents also affect cell-to-cell spread and cell-cell fusion. In a perfect world, the modified infectious centre assay described by Lee & Spear (1980) would have been used to distinguish the contribution made by viral oligosaccarides to cell-cell fusion, from the role of cellular oligosaccharides in this process.

In a recent study, Shieh & Spear (1994) presented evidence indicating that cell surface glycosaminoglycans (GAGs) of the heparan sulfate (HS) type are necessary for the cell-cell fusion induced by HSV-1 (MP) a gC^- syn-1 mutant. Since HS-GAGs mediate HSV-1 adsorption, it is conceivable that HS-GAGs contribute to cell-cell fusion by acting as cell adhesion molecules (Herold et al., 1991, 1994; Shieh et al., 1992; see section

3.1.1.). However, the addition of soluble heparin restored expression of the syn-1 phenotype to HS-deficient cells leading Shieh & Spear, (1994) to suggest that viral interactions with cell-surface GAGs may alter the conformation of a heparin-binding envelope protein (probably UL27-gB), and trigger an activity required for membrane fusion. Therefore Shieh and Spear have also proposed that HS-GAGs may have a role in HSV-induced membrane fusion equivalent to the role of endosomal acidification, a process which triggers a pH-induced conformational change required for the fusogenic activity of many viral fusion proteins. Moreover, there is precedence for this type of mechanism because sialic acid is used a neutral pH trigger by the envelope proteins which mediate virus penetration and cell-cell fusion for human parainfluenza virus 3 (HPIV-3) (Moscona & Peluso, 1991; 1992).

Although the HS-GAG trigger hypothesis is very attractive, it is doubtful that HS-GAGs are sufficient to activate the HSV-1 fusion complex because HSV-1 virions require additional binding steps (eg. between gD and a cellular receptor) for penetration (Ligas & Johnson, 1988; Johnson & Ligas, 1988; Johnson et al., 1990; McClain & Fuller, 1994). Similarly, other viral fusion proteins which induce membrane fusion at neutral pH (eg. HIV-gP160), have been shown to require multiple receptor interactions before they acquire fusogenic activity (Maddon et al., 1986; Dalgleish et al., 1984; Claphan et al., 1989; Maddon et al., 1986; Doranz et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Yu et al., 1996). As such multiple binding steps may operate as a regulatory mechanism to prevent premature activation of the viral fusion complex when newly assembled virus is released from infected cells (Campadelli-Fiume et al., 1991).

UL27-gB is an obvious candidate for the fusion stimulus rendered by HS-GAGs since glycoprotein B has the characteristics of a viral fusion protein (see section 3.2.1

above) and HSV-1 (MP) does not express UL44-gC, the only other envelope protein known to bind heparin under physiological conditions (Heine et al., 1974; Manservigi et al., 1977; Pogue-Geile et al., 1984; Herold et al., 1991). If UL27-gB is the HS-GAG target then it seems unlikely that HS-GAGs are an absolute requirement for HSV-induced membrane fusion because the putative N-terminal heparan binding domain (Spear et al., 1992) is removed by proteolytic cleavage in Vero cells (Pereira et al., 1981, 1982, Chapsal & Pereira, 1988), yet HSV-1 syn mutants form syncytia with high efficiency on Vero cells (Ruyechan et al., 1979; Haffey & Spear, 1980; Bzik & Person, 1981). In support of this view, Banfield et al. (1995b) report HSV-1 (MP) can induce cell-cell fusion in a mouse L cell mutant (sog9) which fails to synthesize any glycosaminoglycans.

To rationalize this result one could argue that mouse L cells possess a cell surface receptor which can substitute for HS-GAGs, and this receptor may be missing on CHO cells. Support for this hypothesis is provided by Montgomery et al., (1996) who demonstrated that CHO cells lack a cell surface receptor (HVEM) which augments HSV-1 entry. However, it is possible that HS-GAG-negative CHO and mouse L cells exhibit distinct fusion properties for another reason. For instance, HSV-infected CHO and mouse L cells may differ in their ability to recruit neighbouring uninfected cells into polykaryocytes. Mouse L cells support HSV replication whereas CHO cells used by Shieh and Spear (1994) are nonpermissive and exhibit defects in HSV-1 late gene synthesis. Since viral proteins involved in HSV-induced membrane fusion include late gene products (eg. UL27-gB, US6-gD, UL22-gH), this defect is likely to reduce the inherent capacity of HSV-1 infected CHO cells to recruit adjacent cells into polykaryocytes. Therefore the contribution made by HS-GAGs to HSV-1 syncytia formation may become apparent only when another cellular factor required for this process is absent, indicating that the HS-

GAGs are likely to play an auxiliary role and not an essential role in HSV-induced cell-cell fusion. To resolve this question and determine if HS-GAGs are indeed obligatory for HSV-induced fusion of uninfected CHO cells, Vero cells (which exhibit superior cell fusion recruiting ability) could be substituted for HSV-infected CHO cells in the modified infectious centre assay described by Lee & Spear (1980).

3.4 HSV-1 transcription, DNA replication and Encapsidation

3.4.1 HSV infection prior to viral protein synthesis: capsid transport and tegument function

After penetration by fusion, the HSV-1 capsid is transported through the cytoplasm to the nuclear envelope, by a microtubular dependent mechanism (Morgan et al., 1968; Kristensson et al., 1986). Viral capsids dock at the nuclear pore complex and a viral function, likely to involve tegument-penton interactions, delivers the viral DNA into the nucleus (Toggon et al., 1981; Batterson et al., 1983; Newcomb et al., 1994; Zhou et al., 1994). In addition, uncoating of the virus particle takes place in the cytoplasm and at least two of the structural proteins released from the tegument (UL41-vhs and UL48-VP16) have a significant regulatory role during the initial stages of infection (Batterson et al., 1983; Schek & Bachenheimer, 1985; McLauchlan et al., 1992).

The rapid cessation of host protein synthesis associated with HSV-1 infection is caused by the virion host shut-off (UL41-vhs) protein which can mobilize an "RNAase" activity that degrades cytoplasmic mRNA during the first stage of the infectious cycle (Read and Frenkel, 1983; Fenwick & McMenamin, 1984; Kwong & Frenkel, 1987; Oroskar & Read, 1987; 1989; Jones et al., 1995; Pak et al., 1995; Zelus et al., 1996; for review see Lam et al., 1996; Schmelter et al., 1996; Roizman & Sears, 1996). Although

UL41-vhs does not discriminate between viral and cellular mRNA (Fenwick & McMenamin, 1984; Kwong & Frenkel, 1987; Oroskar & Read, 1987, 1989; Fenwick & Owen, 1988), recent evidence indicates that an interaction with UL48-VP16 serves to repress the activity of newly synthesized UL41-vhs permitting viral protein synthesis to continue unabated late in the infectious cycle (Fenwick & Everett, 1990ab; Fenwick & Owen, 1988; Smibert et al., 1994; Lam et al., 1996; Schmelter et al., 1996). The rapid turnover of mRNA mediated by UL41-vhs facilitates the admission of newly synthesized viral mRNA to host cell translational machinery and has the potential to expedite the switch from one regulatory class of viral protein synthesis to the next (discussed below).

UL48-VP16 (also termed Vmw65, alpha-TIF, and ICP35) is brought into cells by the infecting virion, enters the nucleus and initiates a transcriptional cascade which results in the temporal synthesis of viral proteins according to their function (Honess & Roizman, 1974, 1975; Batterson et al., 1983; Campbell et al., 1984; Pellet et al., 1985; for review see Thompson and McKnight, 1992). The HSV-1 genes are sequentially activated and repressed in a process which is tightly controlled both at the level of transcription and translation, in part by the combinatorial interactions of viral and cellular regulatory factors (Roizman & Sears, 1996). Consequently, viral genes are expressed at different times and fall into three general classes which are defined by mRNA kinetics, expression requirements and function: the immediate early (IE or alpha [α]) genes, the early (E or beta [ß]) genes and the late (L or gamma [γ]) genes (Roizman & Sears, 1996).

3.4.2 HSV-1 transcription cascade

Transcription of herpes DNA begins within the nucleus at preexisting replication sites adjacent to nuclear domain 10 (Maul et al., 1996). UL48-VP16 is transported to this

site and triggers viral IE gene expression by recognizing the consensus sequence TAATGARAT (R stands for purine) common to the regulatory elements of all IE genes (Post et al., 1981; Mackem & Roizman, 1982a,b, reviewed in Thompson & McNight, 1992). However, UL48-VP16 does not bind this sequence directly (Kristie & Sharp, 1990) but instead forms a multicomponent complex which includes Oct-1 (McKnight et al., 1987; Gerster & Roeder, 1988; Preston et al., 1988; O'Hare & Goding, 1988; O'Hare et al., 1988; Kristie et al., 1989), a cellular DNA-binding protein which interacts with the TAATGARAT elements (Stern et al., 1989; Kristie & Roizman, 1988; Kristie & Sharp, 1990), and at least one other cellular protein, HCF, (also designated VCAF-1, CFF, or C1) which binds to VP16 and stabilizes the entire complex (Kristie et al., 1989, 1995; Katan et al., 1990; Xiao & Capone, 1990; Kristie & Sharp, 1990, 1993; Wilson et al., 1993). Assembly of this complex on the IE promoter places the activation domain of UL48-VP16 (Sadowski et al., 1988; Triezenberg et al., 1988; Greaves & O'Hare, 1989) in an environment suitable for subsequent interactions with components of the transcriptional apparatus (Stringer et al., 1990; Lin et al., 1991; Goodrich et al., 1993; Ge & Roeder, 1994; Xiao et al., 1994).

Expression of the IE proteins does not require prior protein synthesis and one engaged the replication cycle is self-sustaining, because the presence of IE proteins is sufficient to maintain IE transcription and induce expression of the viral early and late genes (O'Hare and Hayward, 1985; Ace et al., 1989; Cai and Schaffer, 1989, 1992; McFarlane et al., 1992). Six alpha genes have been identified: α0-ICP0, α4-ICP4, US1-ICP22, US1.5, UL54-ICP27, and US12-ICP47 (Carter & Roizman, 1996; for review see Roizman & Sears, 1996). All of these genes with the exception of US12-ICP47 have regulatory functions which influence the synthesis of early and/or late gene products

(Watson & Clements, 1980; DeLuca & Schaffer, 1985; Sears, et al., 1985; McCarthy et al., 1989; Cai & Schaffer, 1992). IE proteins contribute to the temporal control of virus replication by acting on upstream promoter-regulatory elements and adjacent noncoding sequences (in a coordinated fashion) to have a positive and/or negative effect on viral gene expression (Everett, 1984; DeLuca & Schaffer, 1985, 1988; O'Hare & Haywood, 1985, Gelman & Silverstein, 1986; Sekulovich et al., 1988; Shepard et al., 1989; Koop, 1993; Purves et al., 1993; Desai et al., 1994; Yao & Schaffer, 1994; Zhu et al., 1994; Gu et al., 1995; Mullen et al., 1995; Samaniego et al., 1995; Zhu & Schaffer, 1995; Carrozza & DeLuca, 1996; Cook et al., 1996; for review see Roizman & Sears, 1996), through phosphorylation of RNA polymerase II (Rice et al., 1995) or by altering posttranscriptional processes such as mRNA splicing and 3' processing (Hardy & Sandri-Goldin, 1994; Brown et al., 1995; McGregor et al., 1996).

The IE proteins activate ß (or early) gene transcription, however, the accumulation of individual ß proteins can be regulated by different subsets of the IE gene products (DeLuca & Schaffer, 1985,1988; Cai & Schaffer, 1992; Desai et al., 1994; Samaniego et al., 1995; Uprichard & Knipe, 1996; McGregor et al., 1996). Regulatory differences of this type may be the basis for the two groups of ß proteins ($ß_1$ & $ß_2$) which are observed in HSV-1 infected cells: $ß_1$ proteins such as UL39-ICP6 (the large subunit of ribonucleotide reductase) and UL29-ICP8 (the major DNA binding protein) appear very early, whereas $ß_2$ proteins such viral thymidine kinase (TK) appear later (Roizman & Sears, 1996). Early (β) gene products are critical for the onset of viral DNA replication and include enzymes involved in nucleotide metabolism, DNA repair, and the proteins dedicated to viral DNA synthesis (Ward & Roizman, 1994).

γ (or late) gene transcription is dependent on the prior synthesis of functional IE

and ß proteins, and this includes ß protein activities which are not related to DNA synthesis. (Godowski & Knipe, 1985, 1986; DeLuca & Schaffer, 1985; Sacks et al., 1985; Sears et al., 1985; Rice & Knipe, 1990; Gao & Knipe, 1991; Cai & Schaffer, 1992; Purves et al., 1993; Roizman & Sears, 1996). However, the γ (or late) genes form a heterogeneous group and can be subdivided into genes which are partially (eg. γ_1 genes) or completely (eg. γ_2 genes) repressed by inhibitors of viral DNA synthesis (Honess & Roizman, 1974; 1975; Holland et al., 1979, 1979; Conley et al., 1981; Silver & Roizman, 1985; Mavromara-Nazos & Roizman, 1987). The γ_1 and γ_2 genes possess different expression kinetics and under normal conditions, γ_1 genes (eg. major capsid protein UL19-VP5, tegument protein UL48-VP16) are expressed earlier than strict γ_2 genes (eg. membrane glycoprotein C) (Holland et al., 1979; 1980). These proteins constitute the structural elements of the HSV-1 virion and include envelope glycoproteins, capsid proteins, as well as components of the tegument (Ward & Roizman, 1994).

Like other viral infections, HSV-1 commandeers the host cell mechanism responsible for mRNA transcription (eg. RNA polymerase II), mRNA translation (eg. transfer RNA & ribosomes), and post-translational modification and employs this cellular machinery in viral protein synthesis. Consequently, viral proteins are synthesized in the cytoplasm and transported to their site of action (eg. capsid proteins are targeted to the nucleus). The HSV membrane proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER) and follow two transport routes: immature HSV membrane proteins are (1) conveyed to the inner nuclear membrane, the site of nucleocapsid envelopment or (2) through the ER and Golgi apparatus where they acquire post-translational modifications (eg. oligosaccharide processing) *en route* to the plasma membrane (for review see Olofsson, 1992; Spear, 1985, 1993).

3.4.3 HSV-1 DNA Replication

Viral DNA replication, maturation and encapsidation occur in the infected-cell nucleus (Jacob et al., 1979; Ladin et al., 1980). Upon entry into the nucleus the HSV-1 genome circularizes and the early stages of HSV-1 DNA replication may involve a theta mechanism (Challberg & Kelly, 1989). Since the newly synthesized viral is also found as large head-to-tail concatemers, which are subsequently cleaved to genome-length monomers and packaged into capsids, the rolling circle mechanism is also likely to play a role in HSV replication (Ben-Porat et al., 1976; Jacob et al., 1979; Ladin et al., 1980).

The viral genome contains three cis-acting origins of DNA replication; a diploid oriS located in the short repeats (RS) and oriL, which is bordered by the UL29 and UL30 genes (Vlazny & Frenkel, 1981; Stow, 1982; Weller et al., 1985; Lockson & Galloway, 1988). Seven viral proteins (UL5, UL8, UL9, UL29, UL30, UL42, UL52) supply the minimum viral machinery needed for origin-dependent DNA replication (Challberg, 1986; Wu et al., 1988). ATP-dependent cooperative binding of UL9 to specific origin DNA sequences induces a conformational change in DNA which is thought to facilitate DNA replication (Olivio et al., 1991; Gustafsson et al., 1994; Elias et al., 1990). UL9 also acts as an ATP-dependent 3'->5' helicase, that unwinds DNA ahead of the replication fork (Bruckner et al., 1991; Fierer & Challberg, 1992; Boehmer et al., 1993). UL30 and UL42 comprise a two-subunit DNA polymerase in which UL30 is the catalytic domain (Dorsky & Crumpacker, 1988) and UL42 is a processivity factor (Gallo et al., 1989). UL5, UL8 and UL52 form a three-subunit helicase-primase (Crute et al., 1989) that exhibits ATP/GTP dependent 5'->3'helicase activity (Crute & Lehman, 1991) and sequence-dependent primer synthesis (Tenney et al., 1995). Presumably, this complex functions in leading- and lagging-strand replication, both as a helicase and by supplying oligo-

ribonucleotide primers for continuous strand DNA synthesis from the origin of replication and discontinuous DNA synthesis on the lagging strand (Klinedinst & Challberg, 1994). UL29-ICP8 is a single-strand DNA binding protein (Powell et al., 1981; Weller et al., 1983) attributed with multiple functions that affect DNA replication, including the ability to; 1) melt partially duplex DNA in the absence of ATP and magnesium (Boehmer & Lehman, 1993a), 2) directly or indirectly enhance the actions of UL9 helicase (Fierer & Challberg, 1992; Boehmer & Lehman, 1993b; Boehmer et al., 1994), the helicase-primase complex (Tenney et al., 1995) and DNA polymerase (O'Donnell et al., 1987; Hernandez & Lehman, 1990) and 3) direct DNA polymerase and other proteins to nuclear replication compartments (Bush et al., 1991; Albright & Jenkins, 1993).

Other viral and cellular proteins also contribute to HSV-1 genome replication, as enzymes involved in DNA precursor metabolism, or by interacting directly with the HSV replication machinery (Albright & Jenkins, 1993; Curtin & Knipe, 1993; Lee et al., 1995) and newly synthesized viral DNA (Ebert et al., 1994). The cellular enzyme [Human] topoisomerase II, for example, has been implicated in HSV replication and may be involved in the decatenation of newly synthesized viral DNA (Ebert et al., 1994; Hammarsten et al., 1996). Furthermore, viral enzymes such as thymidine kinase and ribonucleotide reductase greatly augment viral DNA synthesis, and in some growth environments are required for HSV replication (Dubbs & Kitt, 1964; Goldstein & Weller, 1988a,b; Jacobson et al., 1989; Jamieson et al., 1974).

3.4.4 HSV-1 DNA maturation and encapsidation

Because the HSV DNA is synthesized as large head-to-tail concatomers, maturation into genome-length monomers is required for encapsidation and involves site-specific

cleavage at sequences located within the "a" sequence (Davison & Wilkie, 1981; Mocarski & Roizman, 1982; Spaete & Mocarski, 1985; Varmuza & Smiley, 1985; Stow et al., 1983; Vlazny & Frenkel, 1981; Vlazny et al., 1982). Six HSV-1 genes (UL6, UL15, UL25, UL28, UL32, and UL33) have been identified whose products are required for DNA cleavage and subsequent packaging into capsids (Patel & Maclean, 1995; Patel et al., 1996; Baines et al., 1994; Addison et al., 1984; Addison et al., 1990; Cavalcoli et al., 1993; Tengelsen et al., 1993; Schaffer et al., 1974; Sherman & Bachenheimer, 1987; Chang et al., 1996; Kobaisi et al., 1991). Mutations that inactivate any one of these proteins, yield viruses that synthesize near wild-type levels of DNA yet fail to cleave or package the concatemeric viral DNA, and consequently B capsids that still contain scaffolding proteins UL26-VP21 and UL26.5-VP22a accumulate in the nucleus. The inability to separate DNA cleavage from packaging has made it difficult to study individual functions of these proteins and led to the conclusion that genome maturation is intrinsically linked to encapsidation.

3.5 HSV-1 Capsid Assembly

Disruptions to capsid assembly also abolish DNA cleavage, maturation and concomitant packaging (Desai et al., 1993; Preston et al., 1983; Gao et al., 1994; Rixon et al., 1988; Matusick-Kumar et al., 1994; Pertuiset et al., 1989), however, it is not known if this effect is an indirect result of aberrant capsid assembly or because capsid proteins participate directly in DNA processing

Three of the shell proteins (UL19-VP5, UL38-VP19C and UL18-VP23 but not UL35-VP26) in conjunction with one of the scaffold proteins (UL26.5-VP22a or UL26-VP21), are sufficient to produce capsid-like particles (Thomsen et al., 1994; Tatman et al.,

1994). In HSV infected cells, however, UL26.5-VP22a acts as the major scaffold protein, in part because UL26.5-VP22a is present in much greater abundance than VP21-UL26 (Thomsen et al., 1994; #s- Newcomb & Brown, 1989, 1991; Newcomb et al., 1993). During capsid assembly UL26.5-VP22a is responsible both for the transport of UL19-VP5 into the nucleus, and the formation of a protein core around which the outer shell proteins condense to create properly dimensioned capsids (Matusick-Kumar et al., 1995b; Desai et al., 1994; Thomsen et al., 1995; Thomsen et al., 1994; Tatman et al., 1994). Presumably, core protein UL26.5-VP22a interactions with the shell proteins UL19-VP5 and UL38-VP19C, are an important part of this process (Nicholson et al., 1994; Matusick-Kumar et al., 1994; Thomsen et al., 1995; VP19C interactions - Desai et al, 1994; Newcomb et al., 1993).

The UL26.5-VP22a coding region (329 a.a.) overlaps and is in frame with the UL26 gene (635 a.a.), and initiates at a methionine that corresponds to residue 307 of the UL26 open reading frame (McGeoch et al., 1988; Liu & Roizman, 1991a). The UL26 gene encodes a protease-scaffold protein which undergoes auto-cleavage between residues 610 and 611 (maturation site) and between residues 247 and 248 (release site) (Dechman et al., 1992; DiIanni et al., 1993; Liu & Roizman, 1991b, 1992, 1993; McCann et al., 1994) to yield the capsid proteins VP21 and VP24 and a 25-a.a. C-terminal peptide (Person et al., 1993; Weinheimer et al., 1993). VP24 represents the N-terminal 247 a.a. of the UL26 protein and contains the catalytic domain of the protease while VP21 (Liu & Roizman, 1993; Weinheimer et al., 1993) encompasses a.a. 248 through 610 of the UL26 protein (Person et al., 1993; DiIanni et al., 1993; Davison et al., 1992). The UL26 protease also recognizes the cleavage site in UL26.5-VP22a identical to the 610/611 in UL26-VP21 and removes the C-terminal a.a. of UL26.5-VP22a (Deckman et al., 1992; Liu

& Roizman, 1991b, 1992, 1993).

UL26-VP22a and UL26.5-(VP21 & VP24) processing occurs during or subsequent to capsid assembly and binding to UL19-VP5 is thought to prevent premature cleavage of these proteins (Gao et al., 1994; Preston et al., 1983; Robertson et al., 1996; Thomsen et al., 1994, 1995). Proteolytic cleavage of the UL26.5 and UL26 proteins is necessary for capsid maturation (Thomsen et al, 1995; Gao et al., 1994; Matusick-Kumar et al., 1995a), and since DNA encapsidation entails concomitant expulsion of the protein core, cleavage is likely a prerequisite for removal of scaffold proteins UL26.5-VP24 and UL26-VP21 (Gibson & Roizman, 1972; Rixon, 1993; Thomsen et al., 1995; Gao et al., 1994). In addition, UL26-VP24 remains inside the capsid after DNA encapsidation (Gibson & Roizman, 1972; Rixon, 1993) as a structural protein (Robertson et al., 1996) and release of the protease VP24 from UL26-VP21 is essential for DNA packaging to follow (Matusick-Kumar et al., 1995a).

3.6 Nucleocapsid envelopment at the inner nuclear membrane

Following DNA encapsidation, HSV-1 nucleocapsids bud through the inner nuclear membrane into the perinuclear cisternae and by doing so, acquire an envelope containing immature viral glycoproteins which lack Golgi modifications (Morgan et al., 1954; Schwartz & Roizman, 1969; Compton and Courtney, 1984; Poliquin et al., 1985; Ali et al., 1987; HSV infections-Gilbert & Ghosh, 1993; Torrisi et al., 1992; Avitabile et al., 1994). The mechanism(s) by which the precursor glycoproteins are targeted to the inner nuclear membrane and integrated into the virion envelope are not yet known.

It has been suggested that the interactions between the tegument and membrane glycoproteins initiate capsid envelopment at the inner nuclear membrane (Spear, 1985;

Roizman and Sears, 1990; Zhu & Courtney, 1994; Liang et al., 1996). Although no single HSV membrane protein (gB, gC, gD, gE, gH, gI, gL, gM, UL11, UL20, UL45, or UL49.5) characterized to date is essential for this process, these studies have examined individual proteins and any determinant(s) which directs capsid envelopment would escape detection if redundant signal(s) are present in more than one HSV membrane protein (Spear, 1992; Maclean et al., 1991; MacLean et al., 1993, Baines & Roizman, 1993; MacLean et al., 1992; Baines & Roizman, 1992; Baines & Roizman, 1991; Vissali & Brandt, 1991; Pyles et al., 1992; Hutchinson & Johnson, 1995; Roop et al., 1993). For instance, the myristylated virion protein UL11 may contain an envelopment signal (Maclean et al., 1989). In cells infected with a UL11 negative mutant the proportion of capsids juxtaposed to the inner lamellae of the nuclear membrane increases at least four fold, indicating that UL11 may influence the rate, or the incidence of capsid envelopment (Baines & Roizman, 1992). The discovery that UL11 is associated with the inner nuclear and cytoplasmic membranes of infected cells, yet also resides in the tegument of mature virions, has led to the suggestion that UL11 may act as a connection between the virion envelope and tegument proteins (Maclean et al., 1992; Baines et al., 1995).

In addition, proteins encoded by the UL12 and gamma1-34.5 genes (and/or ORF-P an anti-sense gene overlapping the gamma1-34.5 gene) also affect the egress of capsids from the nucleus, at least in certain cell types, and may be involved in capsid envelopment (Shao et al., 1993; Brown et al, 1994; Lagunoff & Roizman, 1994). Virus capsids which contain viral DNA are assembled by HSV-1 null mutants lacking UL12 or gamma1-34.5/ORF-P, however, these capsids are retained within the nuclei of infected cells (Shao et al., 1993; Brown et al, 1994). The UL12 gene encodes the enzyme, alkaline nuclease, which is transported to the nucleus where it probably participates in viral DNA maturation

(Shao et al., 1993). Since DNA processing is a prerequisite for capsid maturation, mutations in UL12 may result in the production of defective capsids that can not be enveloped (Shao et al., 1993). The deletion mutant used by Brown et al., (1994) lacks the gamma1-34.5 and the overlapping ORF-P gene, and thus it is not clear which of these two proteins has an impact on capsid envelopment, or how the absence of this protein leads to a decrease in capsid envelopment. For example, the gamma1-34.5 gene product (ICP34.5) is found in the cytoplasm, not the nucleus (Ackermann et al., 1986; MacKay et al., 1993; McKie et al., 1994), and acts a virulence factor which prevents the shut off of host cell protein synthesis in neurons and other cell types (Chou & Roizman, 1992, 1994; Chou et al., 1995; He et al., 1996). However, the defect in capsid envelopment does not appear to involve the shut off of viral protein synthesis (Brown et al., 1994, 1994a). ORF-P, on the other hand encodes a nuclear protein, however, expression of the ORF-P protein is blocked by ICP4 during lytic virus replication (Lagunoff & Roizman, 1996; Lagunoff et al., 1996) which argues against a role for ORF-P in capsid envelopment.

Other HSV proteins, such as UL48-VP16 and UL35-VP26 may also contribute to virion envelopment at the inner nuclear membrane although this possibility has not yet been addressed experimentally. UL48-VP16 is a major tegument protein which interacts with membrane proteins in the virion envelope (Zhu & Courtney, 1994; Liang et al., 1996) and is necessary for virion assembly (Weinheimer et al., 1992). Because UL35-VP26, a capsid protein that resides on the outer tip of each hexon, is required for virus replication (Ward & Roizman, 1994) but not for capsid integrity or assembly, several groups have suggested that the essential function of UL35-VP26 may be to link the capsid to the surrounding tegument (Booy et al., 1994; Thomsen et al., 1994; Trus et al., 1995; Zhou et al., 1995). Nevertheless, Rixon et al. (1992) have demonstrated that L-particle assembly

and egress from HSV-infected cells takes place in the absence of the capsid maturation, envelopment and egress. This result implies that the capsids proteins are not obligatory, either for the envelopment or egress mechanisms used by HSV-1, if one assumes that L-particles (non-infectious virus-like particles that lack a capsid and virus DNA) and HSV virions (H-particles) use the same pathway to exit cells (Rixon et al., 1992).

3.7 Egress of alphaherpesviruses

Although there is general agreement that the capsids of alphaherpesviruses bud through the inner nuclear membrane, acquire an envelope and pass into the perinuclear cisternae, there is considerable debate concerning the process by which these viruses transit across the cytoplasm to the extracellular space and at what stage they acquire a tegument and fully mature membrane glycoproteins. Several models have been put forward to describe virus egress from perinuclear space, subsequent maturation and passage to the extracellular space (see Fig. 3.1) (eg. Stackpole, 1969; Johnson & Spear, 1982; Jones & Grose, 1988; Torrisi et al., 1992; Whealy et al., 1991; Nii, 1992; Lazzaro et al., 1995; see below for more references). To further complicate matters, it has been suggested that the egress pathway employed by different members of the herpesvirus family is dependent both on the virus involved and the cell type which is infected (Brown et al., 1994; Komuro et al., 1989; Penfold et al., 1994; Harson & Grose, 1995; Cheung et al., 1991; Whealy et al., 1991)

3.7.1 Egress Model #1: The exocytic pathway

Johnson and Spear (1982) have proposed that vacuoles, derived from the outer nuclear membrane, carry enveloped virions from the perinuclear space to the Golgi

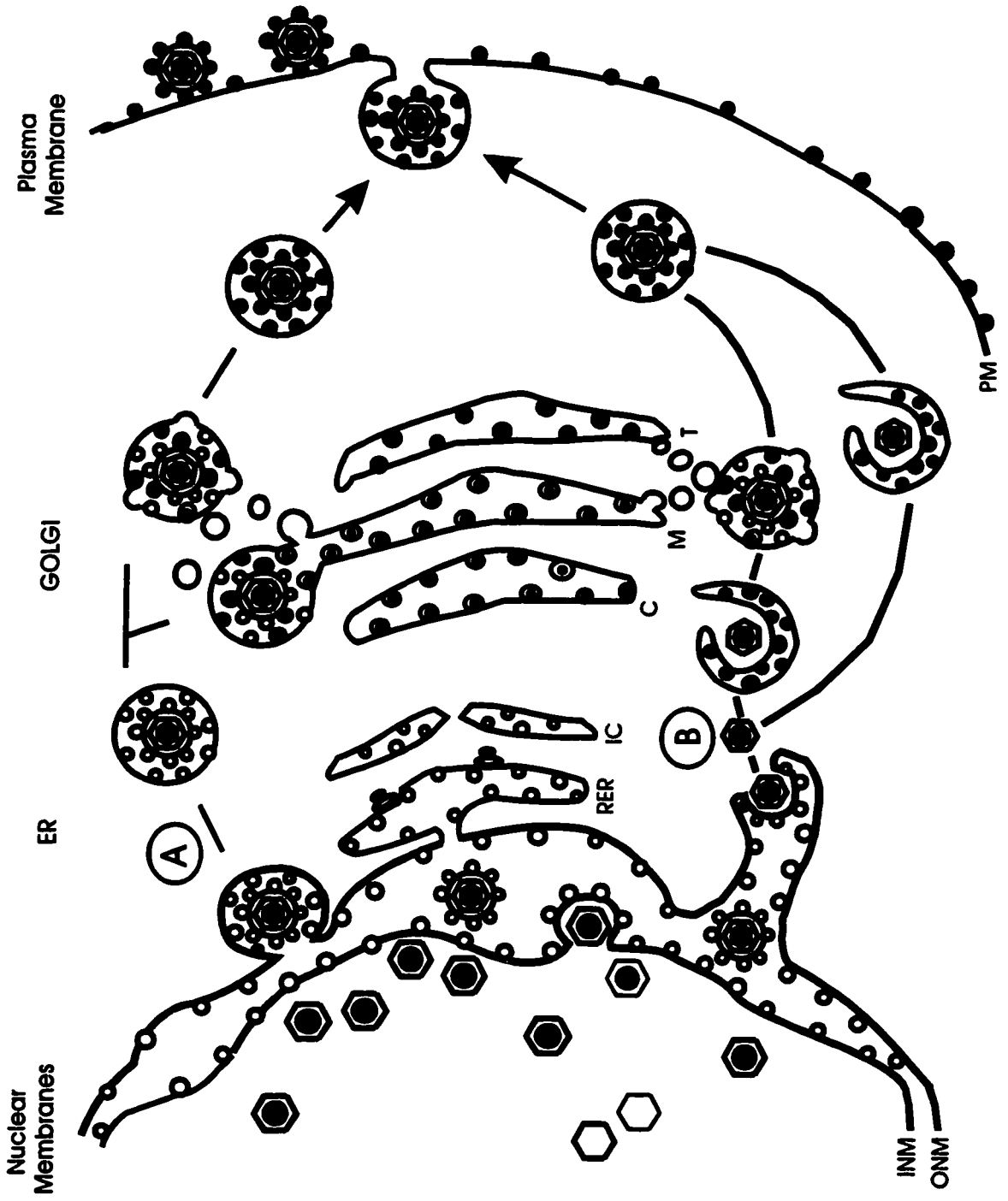

Plasma Membrane
GOLGI
ER
Nuclear Membranes
A
B
RER
IC
C
M
T
PM
INM
ONM

Figure 3.1 HSV-1 Egress Models.

The leading egress models with some potential variations are illustrated. In all models the nucleocapsid acquires an envelope containing immature high-mannose glycoproteins (open symbols) when it buds through the inner nuclear membranes and enveloped virions accumulate in the perinuclear space. Immature glycoproteins can also be found in membranes of the inner (INM) and outer nuclear membranes (ONM), the rough endoplasmic reticulum (RER), and the intermediate compartment (IC).

A) According to the exocytic model of virus egress (upper pathway), virus particles retain the primary INM envelope and migrate through the cytoplasm enclosed in membrane bound vacuoles derived from the ONM or the RER. Maturation of the envelope glycoprotiens may proceed by one of two mechanisms. The virion transport vacuoles may fuse with Golgi membranes allowing envelope glycoproteins to be modified as the virion proceed through the lumen of the Golgi stack to the plasma membrane during exocytic vesicular transport. Alternatively, transport vesicles pinch off from the Golgi stacks, travel to to and fuse with virion-laden vacuoles to provide the Golgi enzymes required for post-translational modifications (Torrisi et al., 1992). Intermediate glycoproteins (ringed circle) in the cis- (C) and medial-Golgi (M) are defined by the trimming of mannose residues and the addition of N-acetylgalactosamine (GalNAc). Enzymes found in the trans-Golgi (T) substitute GalNac residues with galactose and sialic acid to produce the mature glycoproteins (closed symbols) observed on the plasma membrane (PM).

B) In the de-envelopment-re-envelopment model (bottom pathways) perinuclear virions shed the primary INM envelope by fusing with the outer nuclear membrane, releasing naked capsids into the cytoplasm. Naked capsids migrate and bud into membranes of the Golgi or a post-Golgi compartment to acquire their final envelope. The viral glycoproteins in trans-Golgi (T) membranes are processed via the normal exocytic pathway and if naked capsids are re-enveloped by these membranes as some authors have argued, HSV-1 particles would incorporate mature glycoproteins in a single step (Whealy et al., 1991; Gershon et al., 1994; Browne et al., 1995). If cytoplasmic capsids are re-enveloped by cis-Golgi membranes as other authors have suggested, the post-translational processing of viral glycoproteins is likely to follow one of the routes outlined in the exocytic egress model above (Lazzaro et al., 1995). Membranes of the virion transport vacuole (present in all models), fuse with the plasma membrane (PM) and enveloped virions emerge onto the surface of the infected cell.

apparatus and suggested that virus particles proceed in the lumen of the Golgi stacks to the plasma membrane, by employing a route similar to the exocytic pathway used by newly made membrane glycoproteins (see Fig. 3.1). In addition, other investigators have put forward aspects of this model in previous reports (Schwartz & Roizman, 1969; Roizman & Furlong, 1974; Poliquin et al., 1985). Because capsids or immature virions are rarely observed in the lumen of the Golgi apparatus (Avitabile et al., 1995; Campadelli et al., 1993; Jones & Grose, 1988; Whealy et al., 1991), others endorse a similar model, but postulate that enveloped virions remain inside a specialized vacuole dedicated to virus transport, and suggest that Golgi transport vesicles (Pfeffer & Rothman, 1987) fuse with membranes of the specialized vacuole introducing Golgi enzymes to the immature virions within (Campadelli-Fiume et al., 1991; Torrisi et al., 1992; Harson & Grose, 1995). The predictions which arise from these models include: (1) the expectation that the lipid composition of extracellular virions will be similar to that of the inner nuclear membrane, (2) all tegument and membrane proteins must be incorporated into virions when they bud from the inner nuclear membrane, and (3) because extracellular virions contain mature glycoproteins the envelope membrane proteins must be modified by Golgi-derived enzymes while resident in the virus envelope.

3.7.2 Egress Model #2: De-envelopment and re-envelopment

The competing hypothesis for alphaherpesvirus egress invokes an multi-step envelopment process that involves envelopment, de-envelopment, and re-envelopment of the nucleocapsid as the virus particle migrates from the nucleus to the plasma membrane (see Fig. 3.1) (Morgan et al., 1954; Morgan et al., 1954, 1959; Siminoff & Menefee, 1966; Nii et al., 1968; Nii, 1971, 1992; Rodriguez & Dubois-Dalcq, 1978; Stackpole, 1969;

Whealy et al., 1991). In this model, virus particles in the perinuclear cisterna are thought to lose their envelope by fusing with the outer nuclear membrane, and the naked capsids released into the cytosol obtain a "mature" envelope with fully modified membrane proteins, by budding into, or becoming wrapped in membrane vesicles of Golgi origin (Lazzaro et al., 1995). Although most of the researchers who favour this model maintain that the naked capsids acquire a final envelope containing mature glycoproteins from the trans-Golgi membranes (Whealy et al., 1991, 1992; Gershon et al., 1994; Zhu et al., 1995), more recent evidence indicates that re-envelopment would have to occur at cis-Golgi membranes, at least in non-neuronal cells infected with HSV-1 (Lazzaro et al., 1995). The latter hypothesis is suggested by the discovery that the herpesvirions within HSV-infected cells contain immature, intermediate and fully processed derivatives of the envelope glycoproteins (Lazzaro et al., 1995). One would expect the glycoproteins of enveloped virions to consist of immature or fully processed derivatives if re-envelopment is restricted to the trans-Golgi. Again, the membrane proteins would be processed *in situ*, as the enveloped virions travel inside transport vesicles to the plasma membrane where they are released from transport vesicles by exocytosis. Because tegument proteins are synthesized in the cytosol, they may bind to cytoplasmic capsids or the membranes which re-envelop the virus and by doing so become part of the mature virion, however, this model does not preclude the possibility that tegument proteins are transported to the nucleus where they bind to nucleocapsids prior to the initial envelopment step.

3.7.3 Evidence for two egress pathways

Much of the controversy surrounding the egress models has come about because electron microscopy has been used as the primary tool by most researchers when studying

herpesvirus egress and the static images produced by this technique are open to conflicting interpretations. In particular, the presence of unenveloped capsids juxtaposed to cytoplasmic vesicles can be viewed as a prelude to capsid re-envelopment by membranes of Golgi origin, or alternatively as the last step of a dead-end pathway in which de-envelopment results in the irreversible loss of the virion envelope.

The latter viewpoint is supported by observations which revealed that unenveloped capsids present in the cytoplasm frequently show signs of degradation (ie. eccentric or partially extruded DNA) (Campadelli-Fiume et al., 1991; Whealy et al., 1991), are found juxtaposed against a variety of cellular membranes and do not accumulate only in proximity of the Golgi. Moreover, plaque formation on cell types reputed to favour the de-envelopment-re-envelopment route of virus egress (Gershon et al., 1973, 1993), is reduced ten-fold compared to that of cell types in which vesicular transport of enveloped virions through the secretory pathway appears to be the dominant form of virus egress (Harson & Grose, 1995). Likewise, a single amino acid substitution in HSV-1 US6-gD, which permits large numbers of unenveloped capsids to accumulate in the cytoplasm, also caused a severe reduction in the yields of extracellular infectious virus, again supporting the position that de-envelopment is an aberrant process rather than an intermediate stage in virus egress (Campadelli-Fiume et al., 1991; Campadelli-Fiume et al., 1990). In addition, capsids in the process of nuclear envelopment are rarely observed, indicating that this step occurs rapidly. In contrast, partially enveloped capsids have been detected in the cytoplasm with relative ease which would imply that re-envelopment must be a slower and therefore distinct process, if this phenomenon does in fact represent a step in herpesvirus egress, and not a premature loss of the virion envelope.

The idea that herpesvirions acquire a single envelope and retain that envelope as

they travel through the exocytic compartments to the extracellular space, is attractive because it has the benefit of simplicity. Most of the evidence in support of this model has been based on the concept that any block to vesicular transport should cause enveloped virions to accumulate inside membrane-bound compartments, if the enveloped herpesvirions are carried to the cell surface inside transport vesicles derived from the secretory apparatus. Another prerequisite of this model is the complete absence of unenveloped capsids in the cytoplasm. Alternatively, this distribution might be observed if enveloped particles are retained inside a membrane-bound compartment following re-envelopment at the same site. Nevertheless, the following observations have been accepted as evidence that the enveloped herpesviruses in the perinuclear cisternae utilize the host cell exocytic machinery to exit cells: (1) envelope glycoprotein processing and maturation is impaired, (2) enveloped virions are retained within membrane-bound cytoplasmic compartments, and (3) release of extracellular virus is reduced in mutant cell lines that exhibit defects in protein secretion (Banfield & Tufaro, 1990; Whealy et al., 1992) or lack Golgi glycosyl transferase activities (Campadelli-Fiume et al., 1982; Serafini-Cessi et al., 1983) and in competent cells exposed to pharmacological agents which interfere with the glycosylation or exocytic machinery [for example, monensin (Johnson & Spear, 1982; Ghosh-Choudhury et al., 1987; Kousoulas et al., 1983b), brefeldin A (Cheung et al., 1991; Whealy et al., 1991; Chatterjee et al., 1992; Jensen et al., 1995), ammonium chloride (Holland & Person, 1977; Kousoulas et al., 1982,1983a; Koyama et al., 1984, 1989), tunicamycin, (Peak et al., 1982; Pizer et al., 1980; Norrild et al., 1982; Kuhn et al., 1988; Svennerholm et al., 1982), and 2-deoxy-D-glucose (Courtney et al., 1973, 1976; Glorioso et al., 1983; Spivack et al., 1982; Svennerholm et al., 1982; Knowles & Person, 1976; Ludwig et al., 1974)]. That herpesvirus egress

involves all aspects of the normal host cell exocytic pathway has been confirmed by the discovery that the membrane proteins within herpesvirus envelopes are converted from an immature form, to an intermediate class and finally to fully processed glycoproteins, as the enveloped virions migrate from the perinuclear space to cytoplasmic vesicles and then to the extracellular space (Torrisi et al., 1989; 1992; Avitabile et al., 1994; Lazzaro et al., 1995; Poliquin et al., 1985; Avitabile et al., 1994; Gershon et al., 1994; Jones & Grose, 1988; Montalvo et al., 1986). This result accommodates the premise that immature glycoproteins acquired at the inner nuclear membrane are retained in the envelope and processed as the virion exits the cell. However, these observations do not rule out the possibility that cytoplasmic capsids are re-enveloped at the cis-Golgi and transported to the cell surface by the exocytic machinery.

Perhaps the best evidence for de-envelopment and re-envelopment to come from electron microscope studies involved an HSV mutant (ts1201) that does not assemble mature virions (H-particles), (Preston et al., 1983, 1992) but does produce L-particles in quantities similar to wild-type HSV (Rixon et al., 1991). Rixon et al., (1991) observed what appear to be tegument bodies budding into cytoplasmic vacuoles and detected L-particles inside the cytoplasmic vacuoles, but not the perinuclear cisternae of infected cells. Although other groups have reported seeing enveloped "dense bodies" devoid of a capsid (L-particles) between the nuclear lamellae (Nii, 1992), capsid envelopment in the cytoplasm is at least a possibility if HSV virions (H-particles) share the egress pathway used by L-particles (McLauchan & Rixon, 1992; Rixon et al., 1991; Szilagyi & Cunningham, 1991; Yang & Courtney, 1995).

Some of the same studies which examined herpesvirus egress in the presence of drugs that block vesicular transport and/or glycosylation, have argued that de-envelopment

and re-envelopment are authentic steps in herpesvirus egress, in part because unenveloped capsids were detected in the cytoplasm and found immediately adjacent to the membrane-bound compartments in which the enveloped virions had accumulated (Whealy et al., 1991; Cheung et al., 1991; Peake et al., 1982). Biological and biochemical data derived from studies using mutant cell lines or pharmocological agents to alter protein transport and/or glycosylation, also lend support to the de-envelopment-re-envelopment model. The salient points to come from these studies are: (1) some envelope proteins are transported to the cell surface (eg. UL44-gC) but many are retained inside cells (eg. UL27-gB) (Cheung et al., 1991; Chatterjee et al., 1992; Jensen et al., 1995; Johnson & Spear, 1982; Ghosh-Choudhury et al., 1994; Norrild & Pedersen, 1982; Ali et al., 1987; Kousoulas et al., 1982; Glorioso et al., 1983), (2) the yield of infectious extracellular virus was reduced by a greater degree than the release of physical particles (Cheung et al., 1991; Chatterjee et al., 1992; Ghosh-Choudhury et al., 1987; Koyama et al., 1989; Kuhn et al., 1988), (3) the specific infectivity of intracellular virus appeared to be greater than that of extracellular virus (Johnson & Spear, 1982; Ghosh-Choudhury et al., 1987; Kuhn et al., 1988; Pizer et al., 1980; Peake et al., 1982; Kousoulas et al., 1983a), and (4) essential glycoproteins (UL27-gB, US6-gD) were detected in the envelopes of intracellular virions (Kousoulas et al., 1983b; Ghosh-Choudhury et al., 1987; Peake et al., 1982; Kousoulas et al., 1983a; Courtney et al., 1973), but not the envelopes of extracellular particles (Chatterjee et al., 1992; Ghosh-Choudhury et al., 1987; Peake et al., 1982; Kuhn et al., 1988). These results suggest that the specific infectivity of intracellular and extracellular virions differ because they contain a different subset and/or quantity of the envelope proteins involved in HSV-1 entry. Furthermore, these findings are consistent with the hypothesis that the extracellular virions are derived from capsids which are re-enveloped at membranes lacking a full

complement of the virion envelope proteins (eg. early endosomal cisternae), as a result of the block in viral protein transport. Of particular interest, Ghosh-Choudhury et al. (1987) have reported that cells treated with monensin produce intracellular virions that have the envelope glycoproteins UL27-gB, UL44-gC, US6-gD, and US4-gG, but release extracellular virions which contain only US4-gG. Likewise, infectious intracellular virions are assembled by HSV strains (tsQ26, SC16-gH^{KKXX}) which possess mutations that prevent cell surface transport of glycoprotein H. However, extracellular virions which exit from cells infected with HSV-1 mutants, tsQ26 and SC16-gH^{KKXX}, were non-infectious and lack UL22-gH (Desai et al., 1988; Browne et al., 1996). In addition, mutations in UL27-gB have been described which permit the intracellular virions to acquire gB, yet exclude gB from extracellular virus (Desai et al., 1994).

While the results above appear to support an egress model which includes de-envelopment and re-envelopment, they should be viewed with caution. For instance, UL22-gH carried by HSV-1 strain SC16-gH^{KKXX} was tagged with an ER retrieval signal in an effort to concentrate UL22-gH in the ER compartment and differentiate HSV-1 envelopment at the inner nuclear membrane, from re-envelopment at trans-Golgi membranes (Browne et al., 1996). Nevertheless, the ER retrieval signal (KKXX) carried by UL22-gH^{KKXX} also reduced the specific infectivity of intracellular SC16-gH^{KKXX} virions (≈10-50 fold), although the magnitude of this reduction was slightly less dramatic than the one observed for extracellular SC16-gH^{KKXX} virions (≈139 fold). Supplementary experiments will be needed to: (1) verify that gH^{KKXX} is transported to the inner nuclear membrane and incorporated into the envelope of intracellular virions and, (2) rule out the possibility that gH^{KKXX} migrates to cis-, medial- or trans-Golgi membranes (eg. endoglycosidase H, O-glycanase, or neurominidase digestion), before the gH^{KKXX} data can

be used as evidence that virion de-envelopment & re-envelopment constitutes an authentic mode of HSV-1 egress. A report by Roop et al. (1993) also underscores the need for immuno-electron microscopy studies which can ascertain the intracellular location of mutant gH and gB molecules. Interestingly, Roop et al. (1993) have also demonstrated that HSV-1 gL-negative mutants assemble non-infectious intracellular (& extracellular) virions lacking UL22-gH, suggesting that the loss of intracellular infectivity experienced by gL^- and gH^{KKXX} mutants may have a common basis (eg. a defect in gH transport to the inner nuclear membrane). Furthermore, the mutant UL27-gB molecules described above were concentrated in the lighter off-peak fraction of intracellular virions, indicating that these particles may arise from a different process (eg. L-particle assembly). Consequently, mutations which disrupt the transport of viral proteins may have unforseen effects on the production of intracellular virions and therefore complicate the interpretation of egress experiments.

Another indication that the perinuclear virions may lose their initial envelope, stems from the finding that extracellular viral envelopes have a different phospholipid composition than that of host cell nuclear membranes (Van Genderen et al., 1994, Steinhart et al., 1981). The envelopes of extracellular virions have an intramembrane particle (IMP) density which is three times greater than the average densities for nuclear, cytoplasmic and plasma membranes. In contrast, perinuclear virions have an IMP density similar to that of the inner nuclear membrane at 16 hours post-infection, however, the perinuclear and extracellular virions do exhibit comparable IMP densities by 48 hours post-infection (Rodriguez & Dubois-Dalcq, 1978). Finally, electron microscopic examination of cells infected with the betaherpesvirus, human herpesvirus 6 (HHV-6), revealed that enveloped virions within the cytoplasm or on the surface of cells contain a

distinct tegument, whereas enveloped particles found in the perinuclear cisternae lack a discernable tegument (Yoshida et al., 1989; Ni et al., 1990; Rothman et al., 1990; Gompels et al., 1995).

These reports have argued (1) that the retention of an envelope protein within the ER should not reduce the amount of this protein in the virus envelope (Whealy et al., 1991; Desai et al., 1988; Browne et al., 1996) and (2) that the composition of extracellular and perinuclear virions should be identical, with respect to tegument and phospholipid composition (Nii et al., 1992; van Genderen et al., 1994), if the final envelope is obtained at the inner nuclear membrane. Although alternative explanations have not yet been ruled out in the studies listed above (eg. loss of glycoproteins by proteolysis?, do virions bud from domains enriched in specific phospholipids?, were the envelope proteins transported to the inner nuclear membrane?), taken together these results suggest that herpesvirus egress involves a process of membrane exchange (Desai et al., 1988; Browne et al., 1995; Van Genderen et al., 1994; Pessin & Glaser, 1980; Luang et al., 1995).

3.7.4 Viral proteins involved in egress

In recent years, a growing number of viral proteins have been identified which influence the transit of enveloped virions from the perinuclear cisternae to the extracellular space, implying that virus egress is directed and not merely a default process which relies entirely on host cell exocytic machinery. It is clear however, that elements of the host cell secretory apparatus are essential for virus egress (Serafini-Cessi et al., 1983; Campadelli-Fiume et al., 1982; Banfield & Tufaro, 1990; Whealy et al., 1992; Baines et al., 1991; Campadelli et al., 1993; Avitabile et al., 1994, 1995).

Mutations affecting glycoproteins D and H, UL11, UL20 and ICP34.5 have been

described which affect the egress of enveloped perinuclear virions, at least in certain cell lines (Baines et al., 1991; Campadelli-Fiume et al., 1991; Desai et al., 1988; Rodriguez et al., 1993; Baines & Roizman, 1992; Brown et al., 1994). To date, two phenotypes have emerged from the study of these mutants; (1) enveloped virions become trapped between the nuclear lamellae (ie. UL20 or ICP34.5 deletion mutants) and/or (2) unenveloped capsids accumulate in the cytoplasm (ie. UL11, UL20, and gD mutants).

Deletion of UL20 or ICP34.5 affects egress in some cells but not others and it has been suggested that each of these viral proteins replaces a cellular function missing in certain cell types (Baines et al., 1991; Brown et al., 1994). In particular, UL20 is thought to facilitate the exocytosis of virions from the perinuclear space in cells where the Golgi apparatus is fragmented and dispersed throughout the cytoplasm (Avitabile et al., 1994, 1995; Campadelli et al., 1993; Ward et al., 1994). Although HSV-1 viruses with mutant ICP34.5 or UL11 proteins exhibit defects in capsid envelopment (see above), the subsequent block in virus egress appears to be distinct. It is not known how the lack of ICP34.5 or UL11 leads to retention of, or failure to transport enveloped particles from the perinuclear space to the cell surface (Baines & Roizman, 1992; Brown et al., 1994; Baines et al., 1995). It has been suggested that the presence of US6-gD in cellular membranes blocks superinfection at the plasma membrane and premature de-envelopment of progeny virions en route to the extracellular space [with concomitant release of capsids into the cytoplasm] (Campadelli-Fiume et al., 1988,1991; Johnson & Spear, 1989). Certain mutations in gD are thought to abolish this function (Campadelli-Fiume et al., 1990a; Brandimarti et al., 1994), however it is equally possible that these mutations augment the ability of US6-gD to promote fusion between the virion envelope and intracellular membranes (Dean et al., 1994). Exactly how the temperature-sensitive defect in UL22-

gH^{tsQ26} blocks the egress of infectious gH^- HSV-1 particles, while permitting gH^- noninfectious particles to be assembled which are then able to exit cells, has not been determined (Desai et al., 1988). Nevertheless, these results are consistent with the observation that noninfectious gH^- virions are released from cells infected with HSV-1 gH^- and gL^- deletion mutants (Forrester et al., 1992; Roop et al., 1993).

Recently, Fuchs et al. (1996) reported that the UL3.5 gene of Pseudorabies virus (PRV) has a role in virus egress. The UL3.5 protein does not appear to be a virion component but does associate with cytoplasmic membranes, and unenveloped capsids accumulate in the vicinity of the Golgi apparatus when cells are infected with a PRV mutant lacking UL3.5 (Fuchs et al., 1996). Other alphaherpesviruses including varicella-zoster virus (VZV), equine herpesvirus 1 (EHV-1) and bovine herpesvirus 1 (BHV-1) contain UL3.5 genes, however their products share limited homology (ie. 20 to 31%) and no UL3.5 homolog has been detected in the genomes of herpes simplex virus types 1 and 2 (Dean & Cheung, 1993; Khattar et al., 1995; Davison & Scott, 1986; Telford et al., 1992; McGeoch et al., 1988, 1991). Since PRV and VZV egress is reputed to involve re-envelopment and de-envelopment, and many studies indicate that HSV-1 egress does not, Fuchs et al. (1996) have speculated that UL3.5 may be involved in the de-envelopment-re-envelopment route of virus egress (Jones & Grose, 1988; Gershon et al., 1994; Zhu et al., 1995; Whealy et al., 1991, 1992; Fuchs et al., 1996). If this theory proves to be correct, it would resolve much of the controversy surrounding alphaherpesvirus egress. However, it seems unlikely that members of the alphaherpesvirus subfamily use different routes to exit cells, given that PRV and HSV exhibit the same egress phenotype when propagated on a mutant cell line with defects in protein secretion (Banfield & Tufaro, 1990; Whealy et al., 1992; Michaelis et al., 1992) or on competent cells treated with "egress inhibitors"

(Whealy et al., 1991; Cheung et al., 1991; Johnson & Spear, 1982; Kerr & Pennington, 1984).

4.0 Thesis Objectives.

Membrane fusion events are absolutely essential for HSV replication and play an integral role in virus penetration, envelopment, egress and cell-to-cell transmission. An interest in the viral proteins which mediate these events was first sparked by a discovery made in the late 50's: HSV variants arise in cell culture which induce massive amounts of cell-cell fusion (Hoggan & Roizman, 1959). These mutants were designated syn, for the syncytial phenotype. It was not until the mid 70's when genetic mapping, complementation assays and the characterization of ts-mutants identified more than one syncytial locus (eg., syn-1 & syn-3) that herpes virologists attributed membrane fusion events to the activities of several different viral proteins (Person et al., 1976; Manservigi et al., 1977; Ruyechen et al., 1979; Sarmiento et al., 1979; Read et al., 1980). .

Two of the syncytial loci, syn-1 and syn-3, have been studied intensively, and in the mid 80's these syn genes were cloned and sequenced (Deluca et al., 1982; Bzik et al., 1984; Debroy et al., 1985; Pogue-Geile et al., 1987). Subsequent studies have demonstrated that the syn-3 locus encodes UL27-gB, an essential glycoprotein required for virus penetration, cell-to-cell spread and cell-cell fusion (Sarimiento et al., 1979; Cai et al., 1987; Cai et al., 1988a,b). The first laboratories to establish that the syn-1 locus resides in the UL53 gene, also went on to reveal that residue 40 in the UL53 gene product is altered within syn-1 mutants obtained from different sources (Hoggan & Roizman, 1959; Person et al., 1976; Bond & Person, 1984; Debroy et al., 1985; Pogue-Geile et al., 1984, 1987). Follow up studies, designed to characterize the UL53 gene product, were conducted in

several laboratories (eg. Person and colleagues, MacLean et al., 1991), but these efforts met with failure. Consequently, the extent of our knowledge concerning UL53 function, at the outset of this thesis came from complementation studies which demonstrated that the syncytial phenotype associated with UL53 mutations can be recessive to, co-dominant, or dominant over the wild-type phenotype (Roizman, 1962; Keller, 1976a, Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982). These results may reflect the wide range of genetic backgrounds exhibited by the syn-1 mutants and therefore may be unrelated to UL53 function. However, this data led to the supposition that the recessive mutations represent a loss-of-function suggesting that UL53 may be "fusion inhibitor", and also to speculation that the dominant and co-dominant syn-1 mutations represent a gain-of-function indicating that mutant UL53 induces fusion, two conclusions which are diametrically opposed (Person et al., 1976; Kousoulas et al., 1978; Read et al., 1980; Hutchinson et al., 1993, 1995; Dolter et al., 1994). Nevertheless, there was a general expectation that UL53 would contribute to membrane fusion during virus entry, perhaps as a regulatory protein. Therefore my objectives were as follows: (1) identify and characterize the UL53 gene product in HSV-1 infected cells, (2) determine the role UL53 plays in the virus replication cycle and (3) identify the contribution UL53 makes to membrane fusion events.

CHAPTER 2

PUBLISHED MANUSCRIPT

Lloyd Hutchinson, Kim Goldsmith, Dan Snoddy, Hara Ghosh, Frank L. Graham, and David C. Johnson. (1992) Identification and characterization of a novel Herpes simplex glycoprotein, gK, involved in cell fusion. J. Virol. 66:5603-5609.

The material in Chapter 2 has been published in a peer-reviewd journal. However, the text presented in Chapter 2 is a modified version of my initial drafts, which Dr. David C. Johnson in collaboration with myself, revised to create the final manuscript published in the J. of Virology.

PREAMBLE

The major objectives of this study were as follows: 1) to demonstrate that the UL53 gene encodes an HSV-1 glycoprotein, gK, the ninth to be described, 2) to examine the level of gK expression in HSV-infected cells, and explore gK properties (eg. heat-induced aggregation & oligomer formation) and 3) because the UL53 gene has been implicated in HSV-induced membrane fusion, to compare the properties of gK made by wild-type HSV-1, with that of mutant proteins produced by syncytial strains with a lesion mapping to the UL53 gene. The purpose of the final experiment was to provide additional information concerning the molecular mechanism by which syncytial mutations in UL53 induce cell-cell fusion.

The development of antipeptide sera specific for the UL53 gene represented a pivotal step required for the success of these studies. Expression vectors were constructed (eg. AdgK, *in vitro* system) and used to confirm the specificity of the antisera. Subsequently these anti-UL53 antibodies were used in immunoprecipitation assays to characterize the UL53 proteins made by wild-type HSV-1 and HSV-2, HSV-1 syncytial strains, and AdgK, as well as the proteins derived from UL53 RNA translated *in vitro*.

Prior to this publication, several laboratories had attempted to identify the UL53 gene product but despite intensive efforts were unsuccessful, partly because HSV-infected cells produce small amounts of gK relative to the other HSV glycoproteins, but also because they elected to generate anti-peptide sera against the C-terminus of gK (which appears to be nonimmunogenic) or failed to realize that gK is sensitive to heat-induced aggregation (MacLean et al., 1991; Dr. S. Person, personal communication, Dr. P.G. Spear, personal communication, Dr. T. Holland, personal communication).

Identification and Characterization of a Novel Herpes Simplex Virus Glycoprotein, gK, Involved in Cell Fusion

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Abstract. Antipeptide sera were used to identify a novel glycoprotein, encoded by the UL53 gene of herpes simplex virus type 1 (HSV-1). The UL53 gene product is thought to play a central role in regulating membrane fusion because syncytial mutations, which provoke HSV-1 variants to fuse cultured cells extensively, frequently map to the UL53 gene. Antipeptide sera detected a single 40-kDa protein, in cells infected with wild-type or syncytial strains of HSV-1, and labelled with [^{35}S]methionine and [^{35}S]cysteine. ^{3}H-glucosamine was incorporated into the 40-kDa protein (gK) and treatment of cells with tunicamycin increased the electrophoretic mobility of gK, demonstrating that the 40-kDa protein contains N-linked oligosaccharides. We have designated the 40-kDa protein, gK, the ninth HSV-1 glycoprotein to be described. With all other HSV glycoproteins studied to date, at least two glycosylated species, often differing substantially in electrophoretic mobility have been observed in HSV-infected cells; thus, gK is unusual in this respect. In addition, small quantities of gK were observed in HSV-infected cells relative to other HSV-1 glycoproteins (eg. gE & gI). The 40-kDa gK protein was also produced by cells infected with a recombinant adenovirus vector expressing the UL53 gene, and the anti-UL53-1 peptide sera recognized a 36-kDa protein in extracts from cells infected with HSV-2. The translation of UL53 RNA *in vitro*, with microsomes present, generated two glycosylated species of 39 and 41 kDa, and these proteins differed from gK produced by HSV-infected cells not only because they possessed different electrophoretic mobilities, but also because both of the *in vitro* proteins were highly susceptible to heat-induced aggregation.

Introduction. Herpes simplex viruses (HSV) enter cells by fusion of the virion envelope with the plasma membrane (Para et al., 1980; Johnson et al., 1984; Fuller & Spear, 1987). By what is thought to be an analogous process HSV can cause fusion of infected cells (Spear, 1993). This phenomenon is most readily observed when cells are infected with syncytial (syn) mutants which produce extensive cell-cell fusion so that large multinucleated cells (polykaryocytes) are created (Hoggan & Roizman, 1959, reviewed in Spear, 1993). In contrast, cells infected with wild type HSV primarily become rounded as single cells, although a small amount of fusion can be detected (≈20%), at least in some cell types (Person et al., 1976). Nonlethal mutations in any one of at least six different HSV-1 genes can manifest in a syncytial (syn) phenotype (reviewed in Spear, 1993). For instance, mutations which abolish the synthesis of proteins specified by the UL20 or UL24 genes can result in cell-cell fusion, indicating that these proteins are not a mandatory component of membrane fusion, but may act to suppress fusion events in cells infected with wild-type HSV-1 (Jacobson et al., 1989; Baines et al., 1991). Mutations affecting the cytoplasmic domain of glycoprotein B (gB) also give rise to syncytium formation (Bzik et al., 1984; Deluca et al., 1982). This observation coupled with the observation that a gB negative virus cannot enter cells or induce cell-cell fusion (Cai et al., 1987, 1988), suggests that gB is directly involved in the membrane fusion process, perhaps as the fusion inducing protein. Additional HSV glycoproteins (gD & gH) are required for virus entry and influence the extent of cell-cell fusion (Desai et al., 1988; Ligas & Johnson, 1988; Feenstra et al., 1990; Forrester et al., 1992). HSV-1 mutants lacking gD or gH adsorb to, but do not enter cells, and a gD$^-$ syncytial mutant exhibits the syn phenotype only when full-length, wild-type gD is supplied by the complementing cell line (Ligas & Johnson, 1988; Feenstra et al., 1990; Forrester et al., 1992). Evidence has accumulated

that gD acts as a receptor-binding protein (Campadelli-Fiume et al., 1988; Johnson & Ligas, 1988; Johnson & Spear, 1989; Johnson et al., 1990) and in this capacity may be responsible for the interactions which bring apposing membranes domains into close proximity, and create the environment necessary for subsequent fusion events.

Although syn mutations have been mapped to HSV-1 genes including UL1, UL20, UL24, and UL27-gB (Little & Schaffer, 1981; Bzik et al., 1984; McGeoch et al., 1988; Jacobson et al., 1989; Baines et al., 1991), the majority of syncytial mutations which have been characterized in detail appear to involve the UL53 gene (Read et al., 1980; Bond et al., 1982; Bond & Person, 1984; Pogue-Geile et al., 1984; Ruyechan et al., 1979). Furthermore, two syncytial strains, MP and syn-20, isolated by different laboratories possess contain mutations affecting a residue 40 in the UL53 open reading frame (Debroy et al., 1985; Pogue-Geile & Spear, 1987). Since the UL53 syncytial mutations identified to date are limited to missense mutations, the presence of UL53, like UL27-gB, may be a requirement of HSV-induced cell-cell fusion. In addition, other herpesviruses possess UL53 homologs, an observation which is consistent with the hypothesis that UL53 makes an important contribution to HSV-1 replication (Davison & Scott, 1986; McGeoch et al., 1988; Debroy, 1990; McGeoch et al., 1991; Telford et al., 1992; Zhao et al., 1992). Although there have been extensive efforts to identify the UL53 gene product, none to date has been successful (MacLean et al., 1991; S. Person, personal communication, P.G. Spear, personal communication). In this study, antipeptide sera was used to identify and characterize the UL53 gene product. The UL53 gene specifies a 40-kDa glycosylated protein which was designated gK, the ninth HSV-1 glycoprotein to be described.

Preparation of antibodies against the UL53 gene product, gK. The amino acid sequence

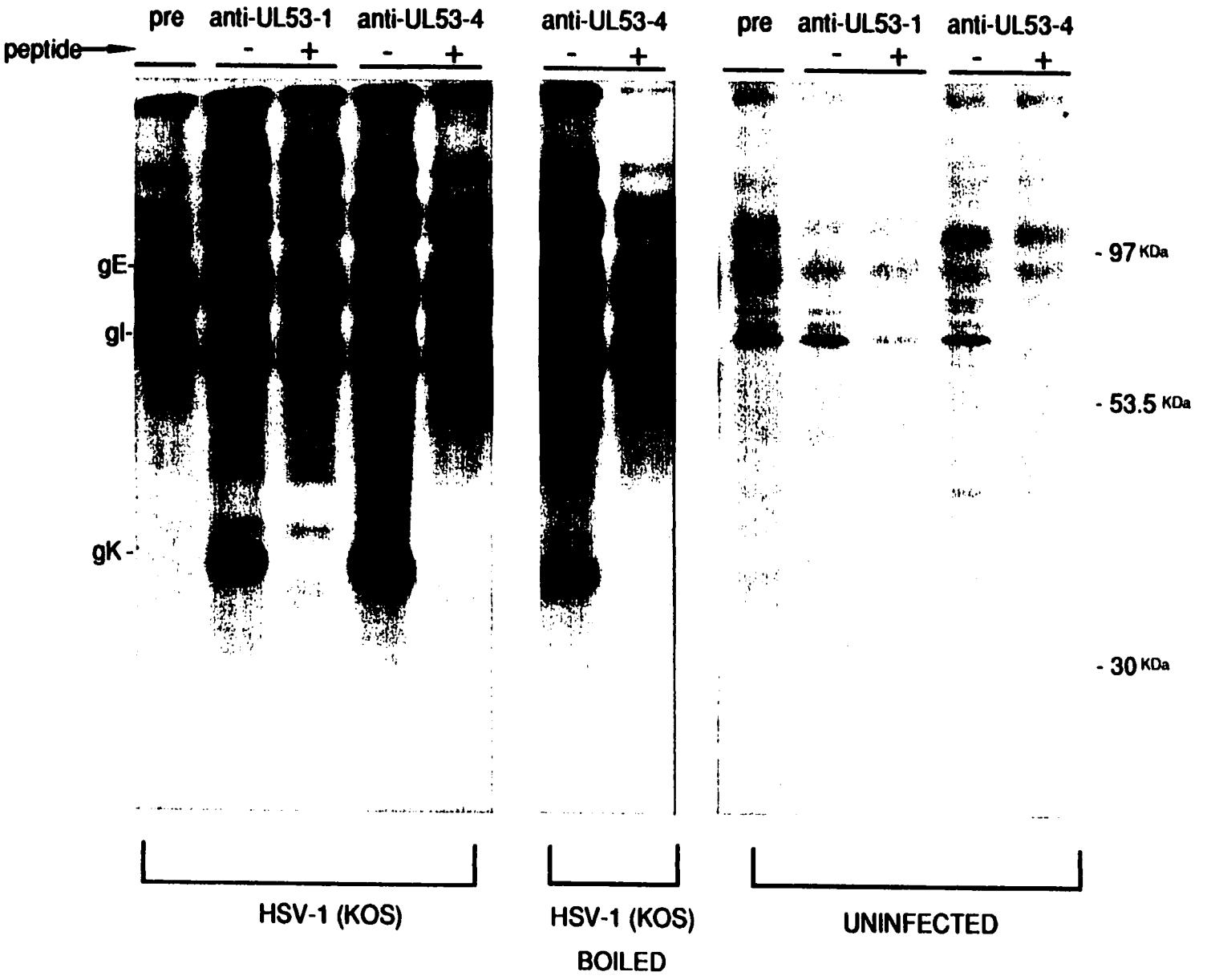
predicted from DNA sequence analysis of the UL53 gene suggests that the protein is relatively hydrophobic and is predicted to span the lipid membrane 3 or 4 times (Debroy et al., 1985; Pogue-Geile & Spear, 1987). In order to identify the UL53 gene product, four peptides derived from hydrophillic regions of the molecule were synthesized (Bachem Inc., Torrance CA), and used to generated peptide antisera. The synthetic peptides were named UL53-1 (includes residues 31 to 46), UL53-2 (includes residues 66 to 83), UL53-3 (includes residues 273 to 289), and UL53-4 (includes residues 89 to 104). Peptides were coupled to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) and injected into rabbits at 3-4 week intervals. Whereas uncoupled UL53-2 peptide was soluble, difficulties were experienced with UL53-2 peptide-protein conjugates which were insoluble, and we were unsuccessful in producing UL53-2 serum with these conjugates. Antipeptide sera were tested for immunoreactivity by using extracts of HSV-infected cells labelled with [^{35}S]cysteine & [^{35}S]methionine ([^{35}S]Cys-[^{35}S]Met) as the antigen in precipitation assays.

Radiolabelling of cells, immunoprecipitation assays, and gel electrophoresis have been described previously (Hutchinson et al., 1992) and were performed with the following modifications. Human R-970-5 cells (Rhim et al., 1975) were infected with HSV-1 using 30 PFU/cell and radiolabelled with [^{35}S]Cys-[^{35}S]Met (200 μCi of each per ml) from 4.5 until 10.5 h postinfection. Cell extracts were partially precleared by incubation with rabbit anti-gE:gI sera and protein A-Sepharose. Following this procedure, extracts (from approximately 1 x 10^5 to 2 x 10^5 cells) were mixed with antipeptide sera (20 μl anti-UL53-1, 10 μl anti-UL53-4) which in some circumstances had been preincubated in the presence or absence of peptide. Antigen-antibody (Ag/Ab) complexes were precipitated with protein A-Sepharose and washed using stringent conditions (Hutchinson et al., 1992).

Precipitated proteins were mixed with 2X sample buffer containing 2% sodium dodecyl sulfate (SDS) and 2% ß-mercaptoethanol, eluted at 37°C for 30 min or 100°C for 5 min, and analyzed using discontinuous SDS-polyacrylamide (12%) gel electrophoresis (Hutchinson et al., 1992; Laemmli, 1970).

Antisera from animals injected with the UL53-1 or UL53-4 protein-peptide conjugates precipitated one protein species of 40 kDa, which was not recognized by pooled sera from preimmune animals (Fig. 1.2). In other experiments the anti-UL53-3 sera also precipitated the 40-kDa protein, but in smaller quantities than the UL53-1 and UL53-4 antisera (not shown). A temperature of 37°C was used to elute the 40-kDa protein from protein A-Sepharose beads because preliminary studies conducted in our laboratory demonstrated that UL53 protein synthesized *in vitro* aggregates upon boiling (Snoddy et al., 1990), an observation consistent with the characteristics of other hydrophobic proteins (Semenza et al., 1990; Baines et al., 1991). Subsequently, Ramaswamy & Holland (1992) reported that the UL53 gene product produce *in vitro* aggregates after boiling. When the sample of UL53 protein precipitated from HSV-1 infected cells was divided in half and eluted at 100°C, rather than 37°C, we also observed a reduction in the quantity of 40-kDa protein. Nevertheless, a substantial fraction of the UL53 protein obtained from HSV-infected cells was able to enter polyacrylamide gels even after boiling (Fig. 1.2), whereas most or all of the UL53 protein translated *in vitro* remained in the stacking gel (Fig. 2.2). HSV immunoglobulin G (IgG) Fc receptors, composed of two glycoproteins, gE and gI, were also precipitated by rabbit IgG as previously described (Johnson & Feenstra, 1987; Johnson et al., 1988b), although the relative abundance of these proteins was markedly reduced by the preclearing step and stringent wash. In contrast to the UL53 protein, similar quantities of the gE and gI were observed in the samples heated at 37°C or 100°C (Fig. 1.2).

Fig. 1.2 Anti-peptide sera recognize gK produced in HSV-1 infected cells. Human R-970 cells were left uninfected or infected with wild type HSV-1 strain KOS. Cells were radiolabelled with [^{35}S]-methionine-[^{35}S]-cysteine and immunoprecipitations performed as described in the text. Cell extracts were mixed with pooled preimmune sera (pre), anti-UL53-1 preincubated in the presence (+) or absence (-) of peptide UL53-1, or anti-UL53-4 preincubated with (+) or without (-) peptide UL53-4. Antigen-antibody complexes were eluted in 2X sample buffer (50 mM Tris-HCl containing 2% SDS, 2% ß-mercaptoethanol, and 15% glycerol) at 37°C for 30 min or 100°C (BOILED) for 5 min and analyzed using discontinuous SDS-PAGE (12% gel). The positions of the 40 kDa gK species, HSV IgG Fc receptor proteins (gE:gI), and marker proteins of 97 kDa, 53.5 kDa, and 30 kDa, are indicated.


pre
anti-UL53-1
anti-UL53-4
anti-UL53-4
pre
anti-UL53-1
anti-UL53-4
peptide
- +
- +
- +
- +
- +
gE-
gI-
gK-
- 97 KDa
- 53.5 KDa
- 30 KDa
HSV-1 (KOS)
HSV-1 (KOS)
BOILED
UNINFECTED

In order to confirm the specificity of the antipeptide antibodies, immunoprecipitation reactions were performed using uninfected cell extracts and antipeptide sera pretreated with excess quantities of synthetic peptide. The 40-kDa protein was not detected in uninfected cells, or when antipeptide sera were preincubated with the correct peptide (Fig. 1.2). As expected, the 40-kDa protein was precipitated by anti-UL53 incubated with an unrelated peptide VHS-1, anti-UL53-1 preincubated with UL53-4 peptide and by anti-UL53-4 sera preincubated with UL53-1 peptide (data not shown). In some experiments, anti-UL53-1 and anti-UL53-4 precipitated small amounts of a 38-kDa viral protein, but the addition of peptide competitors (ie. UL53-1 or UL53-4) did not reduce the level of 38-kDa protein within the Ag/Ab complex, suggesting that it was unrelated to UL53 protein (see for example, Fig. 4.2).

Henceforth the 40 kDa protein species, will be referred to as gK, because subsequent experiments demonstrated that the protein is glycosylated (see below). Interestingly, the UL53 gene product, gK, was much less abundant than other HSV-1 glycoproteins such as gE and gI (Fig. 1.2) and gD (see Fig. 3.2). Increasing the volume of anti-peptide sera improved the signal emanating from Fc receptors, but had little effect on the amount of gK that was immunoprecipitated (results not shown), suggesting that antibodies to gK were not limiting. However, anti-UL53 peptide antibodies may have the ability to recognize denatured forms of gK, and still lack the capacity to bind gK molecules with a folded conformation. Since denatured forms of gK are likely to comprise a fraction of the total gK present in HSV-infected cells, the precipitation assays, which employed anti-UL53 peptide sera to quantify gK, may be inaccurate. The relative scarcity of gK in HSV-infected cells complicated experiments and made pulse-chase analysis especially difficult because the other, more abundant viral proteins frequently

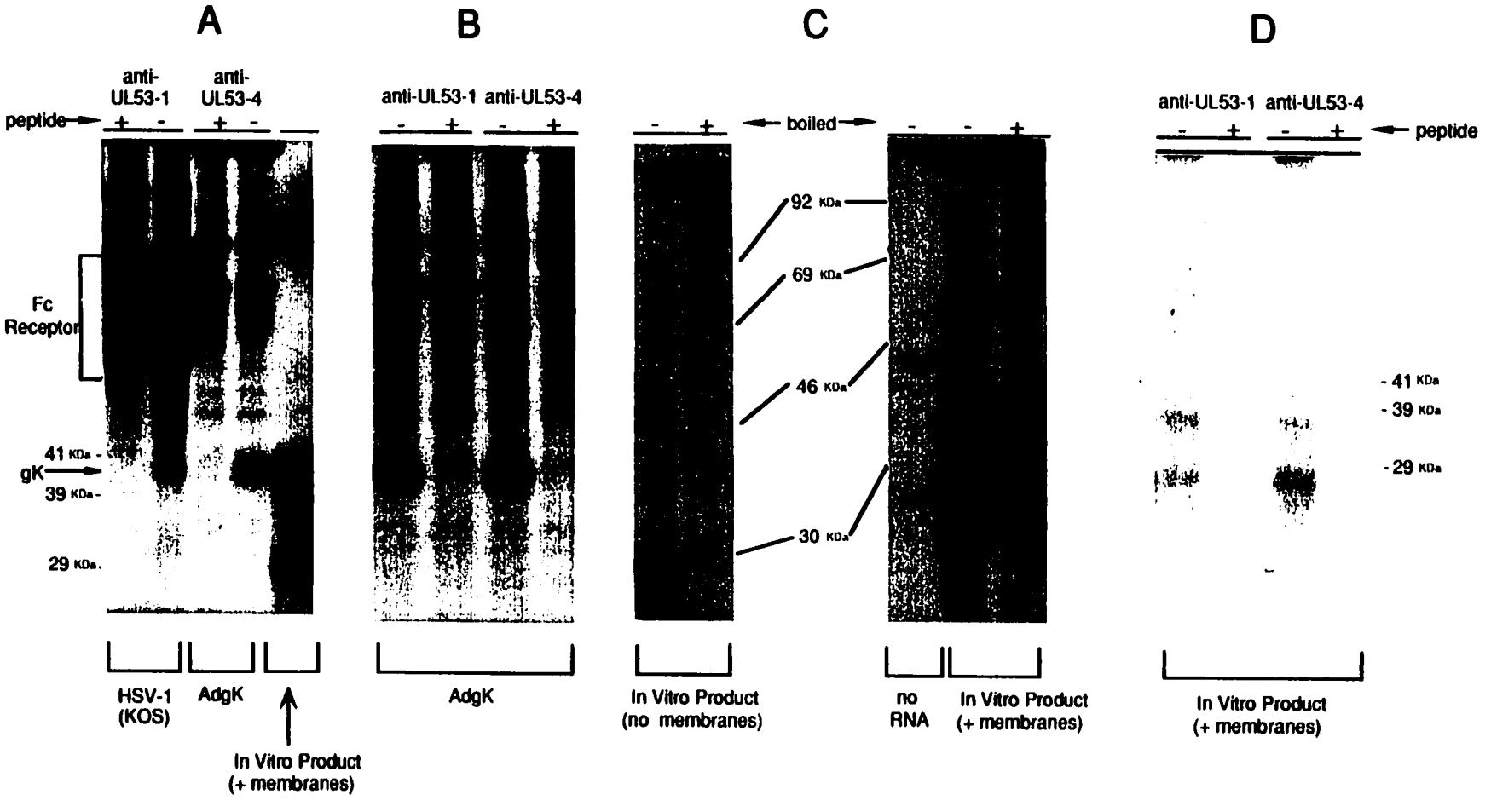
contaminate the immunoprecipitates. In other experiments the addition of phosphonoaceticacid (PAA), an inhibitor of HSV DNA replication (Mao et al., 1975), did not reduce the amount of gK synthesized by HSV-1 strain KOS but caused a severe decline in the production of a true late gene product, glycoprotein C, suggesting that gK is the product of an early or leaky late gene.

Expression of gK in cells infected with a recombinant adenovirus containing the gK gene. To further confirm the specificity of the anti-UL53 peptide sera, verify that the 40-kDa protein is derived from the UL53 gene, and study the characteristics of gK in the absence of other HSV-1 proteins, we constructed a recombinant adenovirus expression vector, AdgK. By using techniques similar to the protocols described in previous reports, an *Nae*I restriction fragment containing the UL53 gene derived from HSV-1 (KOS) (Goldin et al., 1981) was inserted into the E3 region of adenovirus type 5 (Ad5) (Johnson et al., 1990; Hanke et al., 1990; Johnson, 1991; Graham & Prevec, 1991). Initially, the plasmid pCMVsyn was generated by introducing the UL53-*Nae*I fragment into blunted *BstX*1 sites of pRc/CMV (Invitrogen, San Diego, CA). A *Hind*III/*Xho*I fragment from pCMVsyn was placed in the polylinker of pSV2XXX (L. Prevec, unpublished; Hanke et al., 1990) yielding pSV2UL53. pFG144gK, a plasmid comprised of the UL53 gene and the right end of Ad5 DNA, was created by cloning a 1.8 kb *Xba*I fragment from pSV2UL53 into the unique *Xba*I site in plasmid pFG144K3 (F. Graham, unpublished; Bett et al., 1993). Human 293 cells (Graham et al., 1977) were cotransfected with pFG144gK and the plasmid pFG173, which includes the entire Ad5 sequence except for a lethal deletion in E3 (75.9 to 85.0 map units) (Hanke et al., 1990). The recombinant Ad plaques, which appear 14 days after transfection, were isolated and the viral DNA was subjected to

restriction analysis in order to identify suitable vectors containing the correct UL53 insert. The recombinant virus was designated AdgK, and used in the following experiments. Human R-970-5 cells (Rhim et al., 1975) were infected with AdgK using 30 PFU/cell and radiolabelled from 24 until 30 h postinfection. Detergent extracts were prepared, and immunoprecipitation reactions were performed using the anti-UL53-1 or anti-UL53-4 sera, and a procedure based on the protocol outlined above except for some minor changes; ie. cell lysates were not precleared and Ag/Ab complexes were washed 3 times with NP40-DOC buffer (1% Nonidet P-40 [NP40, 0.5% sodium deoxycholate [DOC], 50 mM Tris-HCl [pH7.5], 100mM NaCl). Both the anti-UL53-1 and the anti-UL53-4 sera precipitated a 40-kDa protein from cells infected with AdgK, and this protein displayed an electrophoretic mobility identical to gK derived from HSV-infected cells (see Fig 2.2A and B). In addition, proteins with apparent molecular weights of approximately 80 and 120 kDa were detected in AdgK-infected cells by the UL53-1 and UL53-4 peptide sera, and we have evidence that they contain the gK protein (eg. a gK dimer) (Hutchinson & Johnson, unpublished data, see Fig.3.6 in the Thesis discussion).

***In vitro* translation and characterization of gK.** To study the properties of gK further, an *in vitro* expression system was used to generate UL53 RNA which was translated using a rabbit reticulocyte extract, in the presence or absence of microsomal membranes. A vector, pGEM4Zsyn, was constructed by inserting an HSV-1 (KOS) *Nae*I fragment containing the UL53 open reading frame into the *Sma*I site of plasmic pGEM4 plasmid (Promega Corp. Madison, WI), placing it downstream of an SP6 RNA polymerase promoter. Plasmid DNA was linearized with *Hind*III and transcribed with SP6 polymerase(Promega) as described by the supplier. RNA (5 μg/50 μl reaction) was

Fig. 2.2. Anti-UL53 peptide sera recognize gK expressed by the adenovirus expression vector (AdgK) or translated *in vitro*. Panels A and B: R970 cells were infected with HSV-1 (KOS) or AdgK. Infected cell extracts were immunoprecipitated with anti-UL53-1 sera in the presence (+) or absence (-) if UL53-1 peptide or with anti-UL53-4 sera in the presence (+) or absence (-) of UL53-4 peptide, and proteins were eluted in 2X sample buffer at 37°C for 30 min. Panel C: Linearized pGEM4Zsyn DNA was transcribed *in vitro* using SP6 polymerase (Promega Biotec) to produce RNA which was translated *in vitro* using a rabbit reticulocyte extract (Promega) containing [^{35}S]-Met in the presence (+ membranes) or absence of membranes (no membranes). In one sample, RNA was not added to the reaction (no RNA). SDS (2%) and ß-mercaptoethanol (2%) were added to the *in vitro* samples, which was follwed by a heating step at 37°C for 30 min (- boiled) or 100°C for 5 min (+ boiled), and then separated on 12% polyacrylamide gels. Panel D: RNA was translated *in vitro* with microsomes present and synthesized protein was immunoprecipitated using anti-UL53-1 or anti-UL53-4 sera preincubated in the presence (+) or absence (-) of the appropriate peptide and then eluted in 2X sample buffer at 37°C for 30 min. The position of the 40-kDa gK species produced by HSV-1 and AdgK, the *in vitro* 29-, 39- and 41-kDa species, HSV IgG Fc receptor proteins, and marker proteins of 92, 69, 46, and 30 kDa, are indicated.


A
B
C
D
anti-UL53-1
anti-UL53-4
peptide
Fc Receptor
41 KDa
gK
39 KDa
29 KDa
HSV-1 (KOS)
AdgK
In Vitro Product (+ membranes)
boiled
92 KDa
69 KDa
46 KDa
30 KDa
In Vitro Product (no membranes)
no RNA
In Vitro Product (+ membranes)
- 41 KDa
- 39 KDa
- 29 KDa

translated *in vitro* for one hour at 30°C, using a rabbit reticulocyte extract (Promega) containing 50 μCi [^{35}S]Met. Canine pancreatic microsomal membranes (kindly provided by Dr. David Andrews, Dept. of Biochemistry, McMasterUniversity) were added to some reaction mixtures to process the *in vitro* translated proteins (Andrews, 1989). After synthesis, the *in vitro* translated proteins were immunoprecipitated and/or analyzed directly with SDS-polyacrylamide gels.

The unprocessed form of *in vitro*-gK (no membranes) displayed an apparent molecular weight of approximately 29 kDa (Fig. 2.2C). This result was unexpected because an evaluation of UL53 DNA sequence data indicated that the unprocessed protein should have a molecular weight approaching 38 kDa (Debroy et al., 1985; Pogue-Geile & Spear, 1987; McGeoch et al., 1988). The reason for this difference remains unclear, but may reflect the presence of secondary structure if the protein was not fully denatured by the 30 minute incubation in 2X sample buffer at 37°C. Alternatively, gK may be proteolytically cleaved, as suggested by Ramaswamy & Holland (1992). When UL53 RNA was translated in the presence of dog pancreas microsomal membranes, 39- and 41-kDa protein species were observed, in addition to a 29-kDa protein with an apparent molecular weight identical to that of the unprocessed UL53 protein (Fig. 2.2C, Fig. 3.2C). The increase in molecular weight exhibited by 39- and 41-kDa proteins is indicative of post-translational processing and the 29-kDa protein is probably an unglycosylated species produced on free ribosomes, if for example, microsomal membranes were the limiting factor within *in vitro* reaction mixtures.

All three of the *in vitro* protein species assembled in the presence of microsomes were also recognized by anti-UL53-1, anti-UL53-4 and anti-UL53-3 antibodies (Fig. 2.2D; not shown), confirming that each of these proteins contain the gK polypeptide backbone.

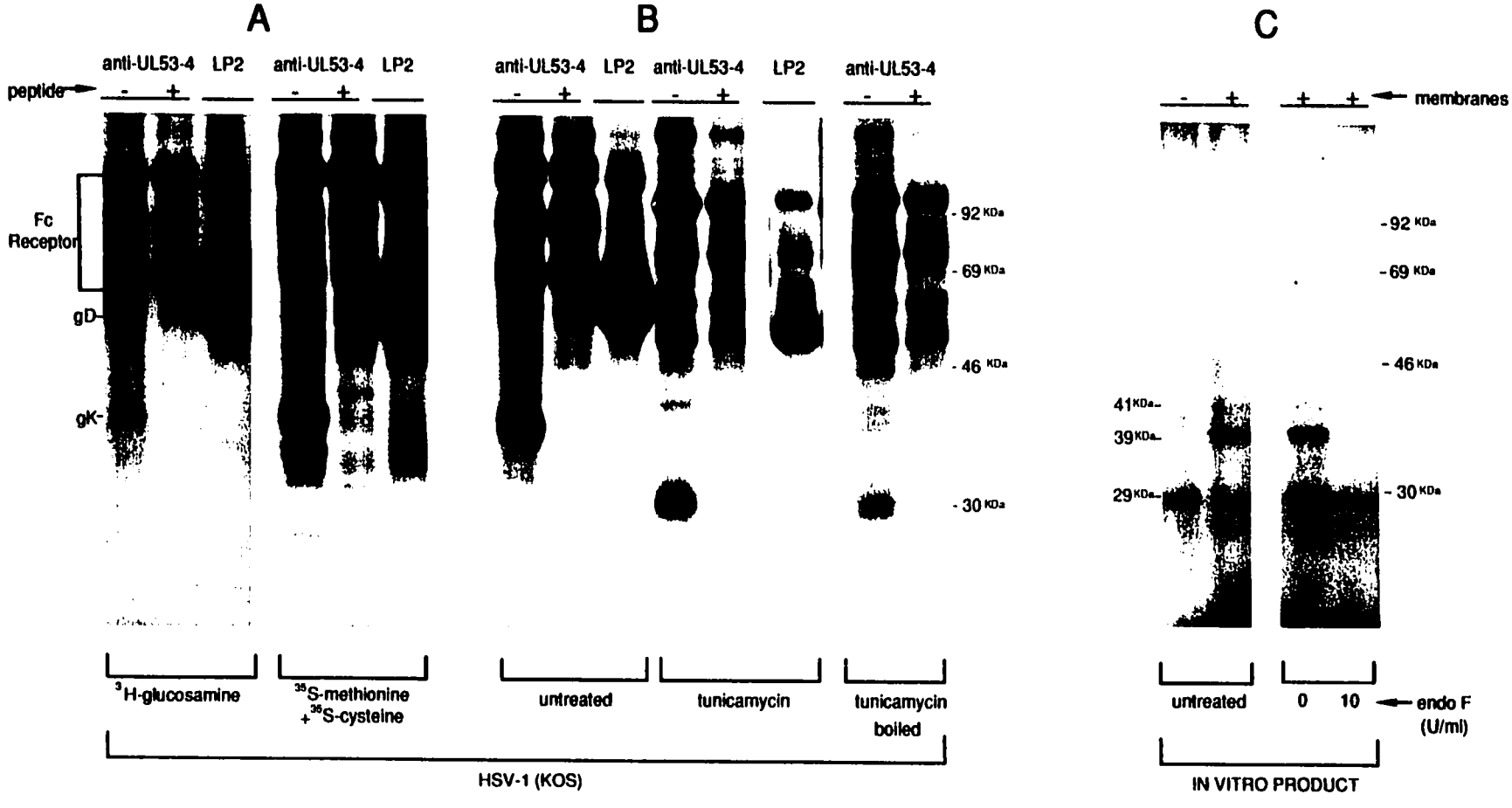
Nevertheless, UL53 proteins synthesized and processed *in vitro* (ie. the 39- and 41-kDA species) exhibit electrophoretic mobilities which differ from the 40-kDa gK protein detected in HSV-infected cells (Fig. 2.2A). This result suggests that there are major differences in the folding or processing of gK produced in HSV-infected cells, and the UL53 protein made *in vitro*. Another finding consistent with this hypothesis stems from the observation that the unglycosylated and glycosylated protein species produced *in vitro*, aggregate when the are heated at 100°C in 2X sample buffer. For instance, the 29-kDa protein species translated in the absence of microsomes disappeared from the resolving gel when the sample was heated at 100°C, and a concomitant increase in the quantity of protein located at the interface between the resolving and stacking gels was observed (Fig. 2.2C). The signal emanating from the 29-, 39-, and 41-kDa species, synthesized in the presence of microsomes, also declined dramatically when these samples were heated at 100°C and once again protein accumulated near the top of the resolving gel (Fig. 2.2C). In contrast, both unglycosylated and glycosylated forms of gK immunoprecipitated from HSV-infected cells were much less sensitive to heat-induced aggregation (Fig. 1.2, 3.2B).

Glycosylation of gK. In the lumen of the rough endoplasmic reticulum (RER) complete $(glucose)_3$-$(mannose)_9$-$(N\text{-}acetylglucosamine)_2$ complexes (N-linked oligosaccharides) are transferred to asparagine (Asn) residues from donor dolichol-oligosaccharide complexes (for review see Brandli, 1991). The gK polypeptide has two potential N-linked glycosylation signals at residues 48 and 58, as predicted from nucleotide sequence analysis (Debroy et al., 1985). To determine if gK is glycosylated protein, HSV-infected cells were radiolabelled with [^{3}H]-glucosamine (500 μCi/ml) in labelling media containing 0.3 mg of glucose per ml, or with [^{35}S]Met-[^{35}S]Cys in media lacking Met and Cys, from 6.5 h

to 17 h postinfection. A 40-kDa protein was immunoprecipitated from cells labelled with [^{3}H]-glucosamine which exhibited an electrophoretic mobility identical to gK labelled with [^{35}S]Met-[^{35}S]Cys, and disappeared when anti-UL53 antibodies were preincubated with the relevant peptide (Fig. 3.2A). Therefore gK is glycosylated. In addition, the anti-UL53 antibodies specifically recognized a minor 38-kDa species in cells labelled with [^{3}H]-glucosamine and [^{35}S]Met-[^{35}S]Cys (see Fig. 3.2A). The 38-kDa protein, which migrated slightly faster than the 40-kDa gK, may be the result of proteolysis caused by the extended labelling conditions used in this experiment (ie. 10.5 rather than 6 h). In support of this suggestion, we have observed that the 38-kDa species is more abundant in experiments which omitted the full spectrum of protease inhibitors (results not shown).

Tunicamycin, an efficient inhibitor of N-linked protein glycosylation, was also used to examine the properties of gK glycosylation. Tunicamycin inhibits formation of the lipid-linked intermediate in the glycosylation process by blocking synthesis of the N-acetylglucosaminyl-pyrophosphoryl dolichol (Heifetz et al., 1979). When HSV-infected cells were treated with tunicamycin, the apparent molecular weight of gK decreased from 40 kDa to 29 kDa, and this size is equivalent to that observed when the UL53 protein is produced *in vitro* without microsomes (compare Fig 2.2C and 3.2B). The electrophoretic mobility of internal controls, gE and gI, also increased in cells treated with tunicamycin, relative to gE and gI from untreated cells (Fig. 3.2B). gK sensitivity to tunicamycin and [^{3}H]-glucosamine incorporation, indicate that N-linked oligosaccharides are added to at least one site on gK. As such, an 11 kDa decrease in apparent molecular weight (ie. shift from unprocessed 29-kDa protein to processed 40-kDa form), induced by the removal of one or two N-linked oligosaccharides is relatively unusual, but not without precedent (Hay et al., 1987). Since the anti-UL53-1 antibodies are directed towards a region in gK

Fig. 3.2 Glycosylation of gK synthesized *in vitro* or in HSV-infected cells. Panel A: R970 cells were infected with HSV-1 strain KOS and labelled with [^{35}S]Met-[^{35}S]Cys or [^{3}H]-glucosamine. Panel B: Cells were infected with HSV-1 strain KOS in the presence of 0.5 µg/ml tunicamycin or its absence (untreated) and labelled with [^{35}S]Met-[^{35}S]Cys. Infected cell extracts were immunoprecipitated with MAb LP2 (Minson et al., 1986), specific for gD (as a positive control), or anti-UL53-4 sera with (+) or without (-) preincubation with UL53-4 peptide. Precipitated proteins were eluted from protein A-Sepharose in 2X sample buffer at 37°C for 30 min or 100°C (boiled) for 5 min and analyzed using discontinuous SDS-PAGE (12% gel). Panel C: *In vitro* gK protein synthesized in the presence (+) or absence (-) of microsomes was diluted in buffer (0.1 M $NaPO_4$ buffer (pH 7.5) with 0.1% SDS, 0.1% ß-mercaptoethanol, and 1.5 mM phenylmethylsulfonyl fluoride), heated for 5 min at 37°C, then octyl glucoside (1%) and endoglycosidase F (0 or 10 U/ml, Boehringer Mannheim) were added and samples were incubated at 37°C for 4 h. Untreated *in vitro* gK protein synthesized in the presence (+) or absence (-) microsomes and eluted at 37°C in 2X sample buffer for 30 min was included for comparison. The positions of HSV-1 Fc receptor, gD, gK, the 29-, 39-, and 41-kDa *in vitro* gK species and molecular markers of 30, 46, 68, and 97 kDa are marked.


A
B
C
anti-UL53-4
LP2
peptide
Fc Receptor
gD
gK
92 KDa
69 KDa
46 KDa
30 KDa
membranes
41 KDa
39 KDa
29 KDa
^{3}H-glucosamine
^{35}S-methionine
+ ^{35}S-cysteine
untreated
tunicamycin
tunicamycin boiled
HSV-1 (KOS)
0
10
endo F (U/ml)
IN VITRO PRODUCT

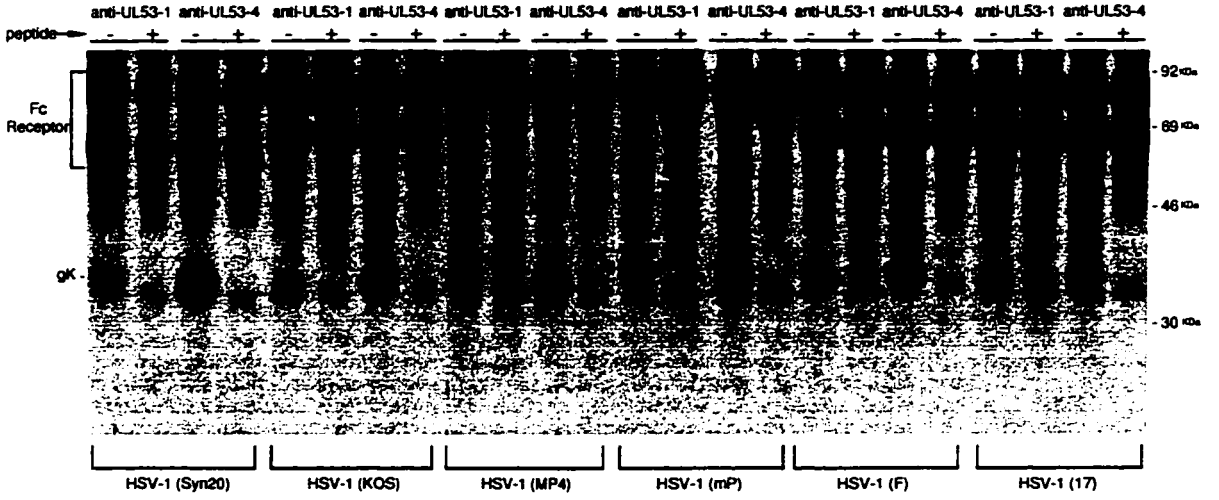
(residues 31 to 46) which flanks an N-linked glycosylation signal at residue 48 (NDT), this observation also raised the possibility that oligosaccharides attached to this site impede the efficacy of the anti-UL53-1 sera. Accordingly, small amounts of gK are precipitated from HSV-infected cells by 20 µl of anti-UL53-1 sera, compared to the quantities precipitated by 10 µl of anti-UL53-4 sera (see Fig. 1.2 & 4.2). Nevertheless, similar quantities of gK were precipitated by anti-UL53-1 sera from cells treated with tunicamycin and from the untreated controls (results not shown).

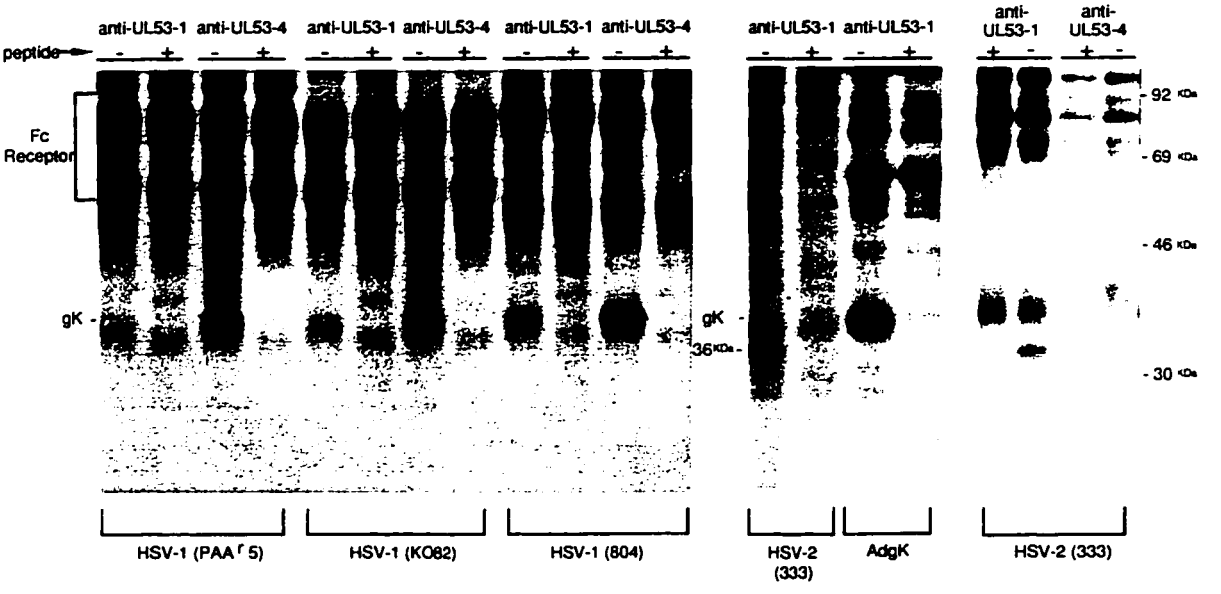
In addition, the 41- and 39-kDa gK proteins, obtained from *in vitro* translation of UL53 RNA in the presence of microsomes, were subjected to endoglycosidase F (endo F) digestion which has the ability to cleave N-glycans of the "high mannose" and the "complex" type. In these experiments samples were diluted in Endo F buffer (0.1 M $NaPO_4$ pH7.5, 0.1% SDS, O.1% ß-mercaptoethanol) containing 1.5 mM phenylmethyl-sulfonyl fluoride and heated at 37°C for 5 min. Finally, octyl glucoside was added to the reaction buffer (final concentration 1%), followed by 0 or 10 U of endo F per ml (Boehringer mannheim; 6 units/ml), and samples were incubated at 37°C for 4 hours. The endo F digestion converted the *in vitro* proteins to a 29-kDa species, confirming that the 41 and 39-kDa proteins also contain N-linked oligosaccharides (Fig. 3.2C). However, it is clear that the 39- and 41-kDa proteins have been exposed to different post-translational modifications. For example the 39- and 41-kDa species may differ because they consist of proteins modified by one and two N-linked oligosaccharides or because the signal peptide has been removed from the 39-kDa protein but not from the 41-kDa protein (Katz et al., 1977). We also suspect that oligosaccharide processing alters the electrophoretic mobility of gK produced in HSV-infected cells. For instance, the 40-kDa species observed in HSV-infected cells may possess either one or two N-linked oligosaccharides which have

been processed to complex oligosaccharides in the Golgi apparatus (Lingappa et al., 1977; Kornfeld & Kornfeld, 1985). Pulse chase analysis, or experiments which examine the sensitivity of gK produced by HSV-infected cells to digestion by endoglycosidase H (only removes high mannose N-linked glycans), may resolve this issue.

Expression of gK in cells infected with HSV-1 syncytial mutants. HSV-1 syncytial strains MP (Hoggan & Roizman, 1959) and syn-20 (Person et al., 1976) both contain substitution mutations at residue 40 in the UL53 gene (Debroy et al., 1985; Pogue-Geile & Spear, 1987). In order to examine the expression of gK in cells infected with these mutants, and with other virus strains, gK was immunoprecipitated from extracts of radiolabelled cells (as described above, Hutchinson et al., 1992). The wild-type gK protein (wt-gK) was detected in cells infected with wild type HSV-1 strains KOS (Smith, 1964), mP (Hoggan & Roizman, 1959), F (Ejercito et al., 1968), 17 (Brown et al., 1973), and PAA^{r}5 (Hall et al., 1985) (Fig. 4.2). We also observed a 40-kDa gK protein in cells infected with the HSV-1 mutants, strain 804 (Little & Schaffer, 1981), which has been reported to contain a syn mutation mapping to the UL1 gene (although we have data suggesting that the syn mutation actually maps to the UL53 gene [Roop, Hutchinson, & Johnson, 1993]), strain KO82, which is unable to express gB (Cai et al., 1987), strains syn-20 and MP (MP4) (Fig. 4.2). In this experiment the quantity of gK expressed by cells infected with syn 20 or MP-4 mutants was comparable to the levels observed in cells infected with the wild type HSV-1 strains. The anti-UL53-1 sera, which is directed towards residues 31 to 46, also precipitated gK from cells infected with HSV-1 syncytial strains (syn-20 and MP4), even though residue 40 is altered in the gK species (syn-gK) produced by these mutants.

Fig. 4.2 Expression of gK in cells infected with HSV-1 syncytial mutants. R970 cells were infected with wild type HSV-1 strains KOS, mP, F, 17 and PAA^{r}5, HSV-2 strain 333, AdgK or HSV-1 mutant strains including KO82 (which is unable to express gB), and syncytial strains 804 (putative UL1 mutation), MP-4 and syn-20 (which contain syn mutations in UL53). Infected cells were radiolabelled with [^{35}S]Met-[^{35}S]Cys and extracts were mixed with anti-UL53-1 sera preincubated in the presence (+) or absence (-) of peptide UL53-1, or anti-UL53-4 sera preincubated with (+) or without (-) peptide UL53-4. Protein A-sepharose was added and the precipitated proteins were eluted at 37°C in 2X sample buffer for 30 min and subjected to electrophoresis using 12% SDS-polyacrylamide gels. The positions of the HSV-1 40-kDa gK, HSV-2 36-kDa gK, HSV-1 IgG Fc receptor proteins, and marker proteins of 92, 69, 46, and 30 kDa are indicated.


anti-UL53-1 anti-UL53-4
peptide
Fc Receptor
gK
92 kDa
69 kDa
46 kDa
30 kDa
HSV-1 (Syn20)
HSV-1 (KOS)
HSV-1 (MP4)
HSV-1 (mP)
HSV-1 (F)
HSV-1 (17)


anti-UL53-1 anti-UL53-4
peptide
Fc Receptor
gK
36 kDa
92 kDa
69 kDa
46 kDa
30 kDa
HSV-1 (PAA r 5)
HSV-1 (KO82)
HSV-1 (804)
HSV-2 (333)
AdgK
HSV-2 (333)

Together these results imply that syncytial mutations in gK do not affect gK accumulation in HSV-infected cells (eg. through gross defects in gK folding which lead to proteolysis in the ER). Ideally, pulse chase analysis should be used to compare the relative stability of syn-gK with wt-gK. However, the meagre amounts of gK produced by HSV-infected cells have hampered these efforts.

The anti-UL53-1 and anti-UL53-4 sera were also used in immunoprecipitation assays to examine the properties of gK protein(s) produced by HSV-2 strain 333. Only the anti-UL53-1 antibodies recognized the HSV-2 gK protein, which displayed an apparent molecular weight of 36 kDa (Fig. 4.2). A comparison of the HSV-1 and HSV-2 UL53 genes revealed that the sequence contained within the UL53-1 peptide is identical in both viruses, whereas nine of 16 residues in the UL53-4 peptide differ between HSV type 1 and 2 (Debroy et al., 1985; Debroy, 1990). Therefore, the anti-UL53-4 sera should be considered type specific. Since HSV-1 gK and HSV-2 gK are almost identical (eg. 83% identity, 338 residues, conservation of N-linked glycosylation sites) it was a little surprising to find that these proteins exhibit different electrophoretic mobilities on SDS-polyacrylamide gels (ie. 40 kDa vs. 36 kDa). This result suggests that the folded conformation and/or post-translational processing of HSV-2 gK differs from that of HSV-1 gK.

Conclusions. We have identified an HSV-1 membrane glycoprotein specified by the UL53 gene, and designated this protein gK. Low levels of gK were immunoprecipitated from extracts of infected cells relative to the amount of HSV-1 Fc receptors (gE:gI) observed in the same extracts. In addition, gE and gI are considered to be minor components of the cellular membranes compared to other HSV-1 glycoproteins (eg. gB, gC, gD), further

emphasizing the scarcity of gK (Heine et al., 1974; Kousoulas et al., 1983; Spear, 1985). Since the UL53 gene is known to be involved in HSV-induced membrane fusion (Debroy et al., 1985; Pogue-Geile & Spear, 1987), the quantity of gK present in cells may be indicative of a regulatory role for gK in this process, rather than the structural role associated with many other HSV-1 glycoproteins (eg. gB, gD, gH) (Cai et al., 1988; Ligas & Johnson, 1988; Desai et al., 1988; Forrester et al., 1992). That cell-cell fusion was not inhibited by the anti-UL53 peptide antisera or any of the UL53 peptides, is also compatible with this hypothesis (results not shown), given that antibodies directed towards other glycoproteins (eg. gB, gD, gH) inhibit cell-cell fusion (Noble et al., 1983; Buckmaster et al., 1984; Gompels & Minson, 1986; Navarro et al., 1992). The gK protein was also expressed in cells infected with syncytial strains known to carry a syn mutation in the UL53 gene (ie. syn-20 and MP). Hence, mutant forms of gK synthesized by these syncytial viruses may be unable to regulate the fusion process. In support of the theory that gK regulates fusion in a negative fashion, we have recently found that transfected cell lines expressing wild-type gK or cell lines infected with AdgK can suppress fusion induced by syn20 and MP (see Chapter 3; Hutchinson et al., 1993). We also have evidence that gK is essential for HSV-1 replication (see Chapter 5; Hutchinson et al., 1995).

A single electrophoretic form of gK was detected in HSV-infected cells, and this protein is modified with N-linked oligosaccharides. In contrast, all the other HSV-1 glycoproteins described to date appear as two protein species (immature and mature forms), which differ in post-translational modifications (Johnson & Spear, 1983; Johnson & Feenstra, 1987; Johnson et al., 1988; Hutchinson et al., 1992). The immature HSV-1 glycoproteins acquire high-mannose N-linked oligosaccharides in the ER, and are

converted to mature glycoproteins containing complex N-linked oligosaccharides and O-linked oligosaccharides, during transit through the Golgi apparatus (Oloffson et al., 1981; Wenske et al., 1982; Johnson & Spear, 1983; Serafini-Cessi et al., 1988). Therefore gK may be processed differently than other HSV-1 glycoproteins. Another unusual result to come from these studies is the discovery that gK made in HSV-infected cells has different properties than gK synthesized *in vitro*. Two glycosylated protein species (39- & 41-kDa proteins) were observed *in vitro*, and both differed electrophoretically from gK produced in HSV-infected cells. Furthermore, the gK protein translated *in vitro* was much more sensitive to heat-induced aggregation than gK derived from HSV-infected cells. These observations suggest that *in vitro* gK synthesized in a reticulocyte lysate is folded or processed differently by pancreatic microsomes than the gK protein assembled by R-970 cells infected with HSV-1.

Recently, Ramaswamy and Holland (1992) reported that the UL53 gene product synthesized *in vitro* was modified with N-linked oligosaccharides, based on the finding that a single 36-kDa protein translated from full-length UL53 RNA and truncated forms of the protein were sensitive to endoglycosidase F. In addition, evidence compatible with the removal of a 30-residue signal peptide from gK molecules was presented. However, our observations indicate that several properties displayed by *in vitro* forms of gK, differ from the attributes of gK contained in HSV-infected cell and therefore it is not clear whether the latter conclusion can be extended to the gK protein produced by HSV-infected cells. It is interesting that the N-linked glycosylation signal at residue 48 includes an aspartic acid, which has been reported to reduce the use of this signal (Kornfeld & Kornfeld, 1985). Since gK contains two potential sites for N-linked glycosylation, this raises the possibility that only a single site may be utilized in HSV-infected cells. In this respect,

we note that the 41-kDa species that we observed *in vitro*, likely modified with two oligosaccharides, was less abundant than the 39-kDa species.

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CHAPTER 3

PUBLISHED MANUSCRIPT

Hutchinson, Lloyd, Frank L. Graham, Weizhong Cai, Chitrita Debroy, Stanley Person, and David C. Johnson. (1993) Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology. 196:514-531.

The material in Chapter 3 has been published in a peer-reviewed journal. However, the text presented in Chapter 3 is a predominantly a modified version of my initial drafts, which Dr. David C. Johnson in collaboration with myself, revised to create the final manuscript published in Virology.

PREAMBLE

When this work began, the HSV-1 glycoproteins B and K had been implicated in HSV-induced membrane fusion, and there was evidence to suggest that UL27-gB is a "fusion inducer", and UL53-gK a "fusion inhibitor" (Keller, 1976; Manservigi et al., 1977; Lee & Spear, 1980; Read et al., 1980; Bond et al., 1982). Several groups had demonstrated that mutations in either one of these proteins can produce the syncytial (syn) phenotype, defined by extensive cell-cell fusion and the creation of multinucleated cells. Furthermore, there had been some speculation in the field (mainly unpublished) that the UL53 gene product, gK, might regulate membrane fusion by interacting with the HSV-1 glycoproteins, (eg. UL27-gB) which promote cell-cell fusion.

Based on this information, these studies were designed to advance our understanding of the role gB and gK play in cell-cell fusion and provide additional information about the molecular mechanism by which syncytial mutations in UL27-gB and UL53-gB (gB^{syn}, gK^{syn}) induce cell-cell fusion. Specifically, we wished to determine if the syncytial phenotype produced by gB^{syn} or gK^{syn} is caused by gain-of-function, or loss-of-function mutations (ie. recessive to, or dominant over the wild-type protein). Since is has

been suggested that wild-type HSV-1 produces a both a fusion factor and a fusion inhibitor, the latter being defective in syn mutants (Ruhlig & Person, 1977; Manservigi et al., 1977; Read et al., 1980; Lee & Spear, 1980), we also examined the capacity for overexpression of wild-type gB, gC, gD, gE, gH:gL, gI, or gK to suppress cell-cell fusion induced by HSV-1 strains containing gB^{syn} or gK^{syn}. Transient transfections, stably transformed cell lines, and adenovirus expression vectors were constructed and used in conjunction with HSV-1 syn mutants to achieve these objectives.

Previously, mixed infections containing wild-type HSV-1 and strains with syn mutations in UL53, were used to assess gK's contribution to cell-cell fusion and indicated that syncytial mutations in UL53-gK can be recessive, co-dominant, or dominant over the wild-type protein (Roizman, 1962; Keller, 1976a; Manservigi et al., 1977; Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982). The most compelling theory to come from these results is the suggestion that gK is assembled into homo- or hetero-oligmers, and contains two or more functional domains, which would allow loss-of-functions mutations to generate recessive, co-dominant and dominant phenotypes (Hutchinson et al., 1992b; Davis-Poynter et al., 1994; see Discussion). In later years, however, it has become apparent that mutations in secondary loci (eg. gE:gI, gM, UL45), which are dispensable for HSV replication in cultured cells, can abolish cell-cell fusion by HSV-1 syncytial strains (Baanes et al., 1994; Davis-Poynter et al., 1994). In light of this discovery, it was not entirely clear what conclusions should be derived from the mixed-infection experiments described above. This study has resolved the dilemma, because a decline in cell-cell fusion could be directly attributed to specific HSV-1 proteins. In addition, we extended the analysis to include coexpression of wild-type gB and gB^{syn}, a condition which had never been examined systematically.

Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants

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Running title: HSV gB and gK inhibit cell fusion.

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Abstract. Herpes simplex virus type 1 (HSV-1) glycoproteins K and B (gK and gB) are intimately involved in virus-induced fusion of cells. Certain mutations in the UL27 (gB) and UL53 (gK) genes confer a syncytial (syn) phenotype characterized by extensive fusion of infected cells and giving rise to multinucleated cells. We have used HSV-1 syn mutants in conjunction with transfected cells or adenovirus vectors to overexpress wild-type gK or gB (gK^{wt}, gB^{wt}), in order to study the role of these proteins in virus-induced membrane fusion. Transient expression of wild-type gK inhibited fusion induced by HSV-1 with a mutant from of gK (gK^{syn}) but not by viruses with a mutant form of gB (gB^{syn}). Stably transformed cells expressing relatively high levels of gK, suppressed cell fusion induced by HSV-1 mutants with lesions in the gK gene but not the cell fusion associated with HSV-1 containing a syn mutation in the gB gene. In addition, there were marked reductions in the plagueing efficiencies and yields of HSV-1 on these cell lines. Cell fusion caused by HSV-1 Syn20, a mutant encoding gK^{syn}, was suppressed when cells were coinfected with an Ad vector, AdgK which expresses gK^{wt}. AdgK did not suppress fusion induced by HSV-1 KTTS.1, which expresses gB^{syn}. Conversely, cells coinfected with AdgB, an Ad vector expressing gB^{wt}, and HSV-1 KTTS.1 (gB^{syn}) were not fused; whereas, cells coinfected with AdgB and HSV-1 Syn20 (gK^{syn}) were fused. Expression of a number of other HSV-1 glycoproteins (gC, gD, gE, gH:gL, gI) using Ad vectors did not reduce membrane fusion induced by gK^{syn} or gB^{syn}. Together, these results support models in which gK and gB participate directly in the fusion of HSV-infected cells. Mutant forms of gB and gK apparently deregulate the fusion process; whereas, wild-type forms of gB and gK can act to suppress membrane fusion induced by their syn mutant counterparts.

Introduction

Membrane Fusion in HSV-infected cells. After binding to a host cell, herpes simplex virus type 1 (HSV-1) invades that cell by direct fusion of the virion envelope with the cell plasma membrane at neutral pH (reviewed in Spear 1992, 1993). HSV-1 virions also move from the infected cells to uninfected neighbours by a process involving direct intercellular transmission (cell-to-cell spread). This process occurs in the presence of neutralizing antibody and plays a critical role in virus spread and in viral pathogenesis (Balan et al., 1994; Dingwell et al., 1994; 1995). HSV-1 infections also induce infected and uninfected cells to fuse at neutral pH, through a mechanism analogous to virus penetration, and this event (cell-cell fusion) can also promote the spread of HSV-1 virions between infected and uninfected cells. In addition, cell-cell fusion may contribute to viral pathogenesis by reducing virus contact with the host immune system, given that large multinucleated cells (polykaryocytes) are a common feature of herpetic lesions observed *in vivo*.

Approximately 20% of cultured HEL cells fuse after infection with wild-type HSV-1, during a brief period from 4-6 hr until 6-8 hr after infection (Person et al., 1976). However, other cell types fuse less extensively (Hoggan & Roizman, 1959; Ruyechan et al., 1979; Lee & Spear, 1980; Bzik & Person, 1981). Instead, the majority of cultured cells infected with wild-type strains of HSV-1 round up and clump together (Spear, 1993). In contrast, cultured cells infected with syncytial (syn) variants or mutants of HSV-1 fuse much more extensively. Fusion begins 4-6 hr after infection and continues in an uncontrolled fashion until multinucleated cell sheets form (Hoggan and Roizman 1959, Person et al. 1976). Since the cell-cell fusion induced by wild-type strains and syncytial mutants of HSV begins simultaneously, but is suppressed shortly thereafter in cells infected with wild-type HSV, it has been suggested that wild-type strains of HSV-1

possess a fusion factor and a fusion inhibitor, the latter being defective in syn mutants. Additional evidence supporting the hypothesis that cell-cell fusion is actively inhibited, stems from the observation that syncytial mutants induce cultured cells to fuse with neighbouring uninfected cells, or cells containing syncytial mutants but not with cells infected with wild-type HSV-1 (Keller, 1976a; Lee & Spear, 1980; Read et al. 1980). Initially glycoprotein C (gC) was identified as a fusion inhibitor, but subsequent rescue experiments ruled out this possibility (Manservigi et al., 1977; Lee et al., 1982). To date, no one has identified the putative fusion inhibitor.

Viral Mutations which confer a Syncytial Phenotype. Mutations which confer the Syn phenotype have been mapped to at least 4 genes within the virus genome (UL20, UL24, UL27, UL53) and a region identified by Romanelli et al. (1991), located within the long terminal repeats. The UL20 gene encodes a membrane protein involved in virus egress (Maclean et al. 1991, Baines et al. 1991) and the UL24 gene encodes a cytosolic protein of unknown function (Sanders et al. 1982, Jacobson et al. 1988; McGeoch et al. 1988, Spear 1993). The UL27 gene (Bzik et al., 1984) encodes gB, a membrane glycoprotein essential for virus penetration, cell-to-cell spread and cell-cell fusion (Manservigi et al., 1977; Sarmiento et al., 1979; Cai et al. 1987, 1988). The UL53 gene encodes gK, an essential glycoprotein with four potential membrane spanning domains (Debroy et al., 1985; Pogue-Geile & Spear, 1987; Hutchinson et al. 1992b, see Chapter 5). Homologues of HSV gB and UL24 exist in herpesviruses from all subgroups (ie. the Alpha-, Beta-, and Gammaherpesvirinae) whereas homologues of UL20 and UL53-gK seem to be confined to the alphaherpesviruses (Baer et al., 1984; Bzik et al., 1984; Pellet et al., 1985; Davison & Scott, 1986; Cranage et al., 1986; Jacobson et al., 1988; McGeoch et al., 1988; Chee et al., 1990; Telford et al., 1992; Gompels et al., 1992). Marker rescue of the syncytial phenotype with purified viral DNA fragments had suggested that HSV-1 (804) carried a

syn mutation within the UL1 gene, which encodes the glycoprotein gL (Little and Schaffer 1981, Hutchinson et al. 1992a). More recently, marker transfer/rescue experiments with cloned DNA fragments have demonstrated that HSV-1 (804) has a syn mutation in the UL53-gK gene (Roop et al. 1993). Furthermore, we have demonstrated that HSV-1 (804) exhibits a wild-type plaque morphology when wild-type gK is provided in *trans* (this paper).

Mutations which abolish UL20 or UL24 expression produce the syncytial phenotype, but also cause a host range defect which impairs virus replication in a variety of cell lines (Baines et al. 1991; Jacobson et al. 1988). The UL20 and UL24 proteins are therefore, not required for membrane fusion and loss of either protein may deregulate some aspect of virus replication which serves to down regulate cell-cell fusion. In contrast, the syncytial mutations identified in gB have been restricted to single amino acid substitutions located within the carboxy-terminal cytoplasmic tail of this protein (Bzik et al. 1984, Cai et al. 1988b, Weise et al. 1987, Goodman and Engel 1991). In addition, mutations in the extracellular domain of gB affect the rate of virus entry into cells (DeLuca et al., 1982; Bzik et al., 1984; Highlander et al., 1989), or block virus entry and cell-cell fusion altogether (Cai et al., 1987, 1988). Interestingly, syn mutations in gB confer HSV-1 virions with the ability to bridge cells and induce cell-cell fusion (fusion-from-without), whereas HSV-1 virions with syn mutations in UL24 or UL53 must penetrate cells and initiate viral protein synthesis before cell-cell fusion is observed (fusion-from-within) (Keller et al., 1976b; Person et al., 1976; Kousoulas et al., 1978; Falke et al., 1985; Weise et al., 1987; Walev et al., 1991). The vast majority of syncytial mutations, at least in some strains of HSV-1 involve the UL53 gene (Read et al 1980, Bond et al., 1982; 1984, Pogue-Geile et al., 1984). Like UL27-gB, the syncytial mutations detected in UL53 have been limited to amino acid substitutions, and in two syncytial

mutants obtained from different sources, residue 40 within the N-terminal ectodomain has been altered (ala to val, or ala to thre) (Debroy et al. 1985, Pogue-Geile & Spear 1987). Together these results have raised the possibility that gB may play a direct role in membrane fusion events, perhaps as the fusion inducing factor, and gK may regulate membrane fusion by acting as a fusion inhibitor. By this model missense mutations in gK and gB may disrupt the regulatory mechanism which controls membrane fusion events occurring in HSV-infected cells (eg. during egress) or during virus penetration.

The Role of other HSV glycoproteins which participate in virus entry or cell-to-cell spread, in cell-cell fusion. Other HSV glycoproteins implicated in virus entry also influence virus induced cell fusion, including gD, gH:gL and possibly gC and gE:gI. Of these proteins homologues of gH and gL, which form a hetero-oligomer required for the proper folding, post-translational processing and cell surface transport of both proteins (Gompels & Minson, 1989; Hutchinson et al., 1992a), have been identified in the alpha-, beta- and gammaherpesviruses, whereas homologues of gC, gD, and gE:gI are confined primarily to the alphaherpesviruses (Baer et al., 1984; Davison & Scott, 1986; McGeoch et al., 1988; Cranage et al., 1988; Chee et al., 1990; Telford et al., 1992; Gompels et al., 1992; Kaye et al., 1992).

Since gD and the gH:gL complex are required for virus penetration, cell-to-cell transmission, and cell-cell fusion it seems likely that gD and gH:gL (like gB) are directly involved in the membrane fusion process (Buckmaster et al., 1984; Noble et al., 1983; Gompels & Minson, 1986; Minson et al., 1986; Desai et al., 1988; Ligas & Johnson, 1988; Forrester et al. 1992, Fuller et al. 1989, Gompels and Minson, 1986, Roop et al. 1993). HSV-1 gD is a receptor binding protein and may contribute to the fusion process by bringing viral and cellular membranes in close contact with each other (reviewed in Johnson et al., 1990). It has been suggested that the gH:gL complex sustains membrane

fusion by facilitating the expansion of the fusion pore (presumably created by UL27-gB), but as yet there is little direct evidence to support this position (Fuller & Lee, 1992).

Glycoprotein C (and B) appear to act early in the entry pathway by mediating virus adsorption onto cell surface glycosaminoglycans (WuDunn & Spear, 1989; Campadelli-Fiume et al., 1990; Herold et al., 1991; Shieh et al., 1992). gC antibodies inhibit adsorption of HSV-1 virions to cells (Fuller & Spear, 1985) and gC negative viruses exhibit defects in adsorption, penetration and specific infectivity (Herold et al., 1991). Nevertheless gC is dispensable for replication of herpes simplex virus in cell culture and is not required for cell-cell fusion (Manservigi et al., 1977; see above). Instead, Syn mutants often carry secondary mutations which prevent gC expression, implying that the absence of gC may actually enhance syncytia formation in some cell types (Heine et al., 1974; Cassai et al., 1976; Ruyechan et al. 1979, Weise et al., 1987; Goodman and Engel 1991; Spear, 1993).

The suggestion that gE:gI may have a role in cell-cell fusion stems from two observations: (1) gE and gI deletion mutants exhibit a small plaque phenotype on normal human fibroblasts (Dingwell et al., 1994) and (2) gE antibodies have been described which reduce HSV-1 plaque formation (Chatterjee et al., 1989). However, syncytial strains of HSV-1 exist which lack gE or gI, yet retain the capacity to produce syncytial plaques on at least some cell lines (Neidhardt et al., 1987; Ligas & Johnson, 1988; Johnson & Ligas, 1988).

Cellular factors also play a critical role in cell fusion. For instance, HSV-1 strains with syncytial mutations in different loci (eg. UL27-gB vs. UL53-gK), differ in their ability to induce cell-cell fusion on a panel of cell lines (Ruyechen et al., 1979; Lee & Spear, 1980; Bzik & Person, 1981; Toggon et al., 1990).

It is clear that HSV-induced membrane fusion is governed by a complex

mechanism involving multiple factors supplied by the HSV-1 virion and the host cell machinery. In an attempt to clarify the roles of gK and gB in HSV-1-induced membrane fusion, we expressed these proteins using transient transfection assays, in stably transformed cell lines or using recombinant adenovirus vectors. In addition, we also examined the ability of other HSV-1 glycoproteins (gC, gD, gE, gH:gL & gI) to suppress membrane fusion induced by HSV-1 strains with syncytial mutations in UL27-gB (gB^{syn}) or UL53-gK (gK^{syn}). Fusion induced by HSV-1 mutants containing lesions in the UL53-gK gene was suppressed when wild-type gK (gK^{wt}) was expressed in cells. In contrast, fusion caused by an HSV-1 gB^{syn} mutant was not suppressed by expressing gK^{wt} in the cells. Furthermore, expression of wild-type gB (gB^{wt}) inhibited fusion induced by gB^{syn} mutants but not by gK^{syn} mutants.

Materials & Methods

Cells and Viruses. HSV-1 wild-type and syncytial viruses were propagated and titrated on Vero cells which were grown in alpha-minimal essential medium (α-MEM) supplemented with 7% fetal calf serum (FCS), 1% penicillin-streptomycin (P/S) and 0.3% L-glutamine (L-glu) (GIBCO Laboratories, Burlington, Ontario, Canada). VS202 and gK-9 cells were maintained in Dulbecco's modified Eagle medium lacking histidine (DUL-his) supplemented with 0.3 to 0.5mM histidnol (Sigma Chemical Co., St. Louis, Mo.) 7% FCS, 1% P/S and 0.3% L-glu (GIBCO). Prior to infection, VS202 and gK-9 cells were passaged once in α-MEM containing 7% FCS.

The wild-type HSV-1 strains used in this study included KOS (Smith, 1964), obtained from J. Smiley (McMaster University, Hamilton, Ontario, Canada), and HSV-2 (333) provided by P.G. Spear (Northwestern University Medical School). Syncytial strains of HSV-1 included 804 (Little and Schaffer, 1981) which was obtained from P. Schaffer

(Dana-Farber Institute, Boston, Mass), Syn20 (Person et al., 1976; Read et al., 1980) and KTTS.1 (Cai et al., 1988b) which were obtained from T. Holland (Wayne State University, Detroit, Michigan), MP (Hoggan & Roizman, 1959), and tsB5 (Manservigi et al. 1977) obtained from P.G. Spear (Northwestern University Medical School) and syn082 (Cai et al., 1998b). HSV-1 strains MP and Syn20, were obtained independently and possess mutations affecting codon 40 (ala to thr or ala to val) in gK open reading frame (ORF) (Debroy et al. 1985, Pogue-Geile et al, 1987). MP has at least two other mutations; one which alters the size of the syncytial plaques and another which gives rise to the gC^- phenotype (Ruyechan et al., 1979; Bond & Person, 1984; Pogue-Geile et al., 1984). HSV-1 (HFEM) strain tsB5 contains three mutations which arise from amino acid substitutions in the gB ORF; a syn mutation which resides in the cytoplasmic tail of gB, and two mutations which alter residues in the gB ectodomain to produce a fast entry phenotype and a virus which is temperature sensitive for the accumulation of gB, the production of infectious virions and the capacity to induce cell fusion (Manservigi et al. 1977; Sarmiento et al., Haffey & Spear, 1980; Honess et al., 1980; Deluca et al., 1982; Bzik et al., 1984). HSV-1 (KTTS.1) was created by transferring a cloned DNA fragment including the gB^{syn} and fast-entry mutations in HFEM-tsb5 to an HSV-1 (KOS) genetic background (Cai et al., 1988b). HSV-1 syn082 is a virus derived by transferring a cloned DNA fragment containing the gK^{syn} mutation in HSV-1 (Syn20) to HSV-1 (KO82) which is unable to express gB^{wt} because a Hpa I linker was used to produce a stop mutation at codon 43 in the gB ORF (Cai et al., 1987). As mentioned previously, the syn mutation in 804 is now known to affect the UL53 gene (Roop et al., 1993).

Recombinant adenovirus expression vectors (Ad vectors) were grown and titrated on 293 cells (Ad5-transformed human embryo kidney cells) (Graham et al., 1977) were propagated in Joklik's modified medium supplemented with 10% horse serum or new born

calf serum and 1% P/S. For the large scale production of Ad vectors KB cell spinner cultures were employed.

The Ad vectors included AdE3$^-$ (AddlE3) (HAj-Ahmad & Graham, 1986), AdgC (Witmer et al., 1989), AdgD (Zheng et al., 1993), AdgE and AdgI (Hanke et al. 1990), AdgK (Hutchinson et al., 1992a), and AdgL (Hutchinson et al., 1992b). AdgB-8 (Bett et al., 1993) was constructed using the protocols described by Hanke et al. (1990) and is identical to its precursor AdgB-2 (Johnson et al., 1988) except that gB has been inserted into an Ad5 background rather than a hybrid Ad2/Ad5 background. Construction of AdgH, a recombinant Ad vector expressing HSV-1 gH, followed the strategy used to create AdgL (Hutchinson et al., 1992a). A 3.4-kb *Bgl*II fragment encompassing the gH coding sequences was excised from plasmid pUGHI (Gompels & Minson, 1989) and inserted into the *Bam*HI site of plasmid pAB26, which contains the rightward sequences of Ad 5 (Bett et al., 1993) yielding pABgH. AdgH was obtained after transfecting 293 cells with pABgH and pFG173 (Hanke et al., 1990) a plasmid containing the Ad5 genome except for a lethal deletion of viral sequences spanning the E3 region.

Plasmids. The plasmid pSG-28 contains the *Eco*RI EK(B) fragment of HSV-1 (KOS) (Goldin et al., 1981) inserted into pBR325 and was provided by M. Levine of the University of Michigan. pSV2HIS which contains the his D operon of *Salmonella typhimurium* encoding histidnol dehydrogenase under control of the simian virus 40 (SV40) early promoter and flanked by SV40 polyadenylation (poly-A) sequences (Hartman S.C. & R.C. Mulligan, 1988), was provided by R.A. Weinberg, (Whitehead Institute, Cambridge, Mass). The plasmid pSV2HISgD has the *Bam*HI J fragment of HSV-1 (KOS) inserted into the *Bam*H1 site of pSV2HIS (Ligas and Johnson, 1988). Plasmid pSV2HISsyn was constructed by excising a 3.4-kb *Eco*RI-*Bam*HI fragment containing the

UL53 gene (map units 0.723 to 0.745), from pSG-28 and ligating it to the unique *Bam*HI and *Eco*RI sites of pSV2HIS. A 1.3-kb *Kpn*I-*Bam*HI fragment derived from pSV2HISsyn and containing the UL53 open reading frame (ORF) was inserted into the multiple cloning site (MCS) of pUC19, to generate pUC19syn. A 1-kb *Nae*I fragment comprised of the UL53 ORF, was removed from pUC19syn and inserted between blunted *Bst*X1 sites within the MCS of pRc/CMV (Invitrogen Corporation, San Diego, California) yielding pCMVsyn. Plasmid pSV2HDK was created by removing a 1.9-kb *Hind*III-*Eco*RI fragment from pCMVsyn, and inserting it between the unique *Hind*III and *Eco*RI sites of pSV2HisgD (Ligas & Johnson, 1988), placing the UL53 ORF under control of the gD promoter and flanked by the bovine growth hormone poly-A site. Plasmid pKBXX (Cai et al., 1987) was produced by inserting a 4-kb *Bam*HI-*Xho*I fragment (map units 0.345 to 0.372) containing the wild-type gB gene and derived from pKBG-BS3 (Deluca et al., 1982), into the *Bam*HI-*Sal*I sites of pUC9. The *Bam*HI-*Xho*I fragment above contains the 2.7 kb gB-coding region, a 790 base-pair 5'-flanking sequence, and a 498 base-pair 3' flanking sequence. pK143 is one of many plasmids with a *Hpa*I linker (GTTAAC) inserted into *Alu*I, *Fnu*DII, *Hae*III, or *Rsa*I restriciton sites, within the gB coding sequences (Cai et al., 1988a). In pK143 the HpaI linker resides in an *Alu*I site between codons 816 and 817 of the gB ORF, where the addition of two residues (Val-Asn) produces the syncytial phenotype (Cai et al., 1988a). pKEB (Bond and Person, 1984) contains the HSV-1 (KOS) *Eco*RI B fragment (map units 0.724 to 0.86) inserted into pBR325. pKEB-S1 (Bond and Person, 1984) was created from pKEB and is composed of a 4.7 Kb *Eco*RI-*Sal*I fragment (map units 0.724 to 0.753 containing the UL53 gene), inserted into pBR325.

Synthetic peptides, antipeptide sera, and other antibodies. The synthetic peptide UL53-4

(residues 89 to 104 of gK, GRVVPFQVPPDATNRR) were synthesized and purified by Bachem Inc. (Torrance, Calif.) Rabbit antisera to this peptide was generated using the protocol described previously (Hutchinson et al 1992a and b). Monoclonal antibody (MAb) 15ßB2 (which recognizes HSV-1 or HSV-2 gB) (Johnson et al. 1988) was a gift from S. Bacchetti, McMAster University). LP2, which recognizes HSV-1 or HSV-2 gD (Minson et al. 1986) was a gift from T. Minson (Cambridge University). MAb II-481 specific for HSV-1 gE (Para et al., 1985) was a gift of P.G. Spear (Northwestern University Medical School). MAb 3114 and Mab 7520, specific for HSV-1 gE (Cross et al., 1987) were a generous gift of A. Cross (MRC Virology Unit, Glasgow) Pooled MAbs C1, C2, and C3, specific for gC (Holland et al., 1984) were gifts from J. Glorioso (University of Pittsburgh). Anti-tk is a polyclonal serum from rabbits injected with crystalline HSV-1 thymidine kinase produced in E.coli and was a generous gift of W. Summers (Yale University, New Haven, Conn.). Anti-ICP6 is rabbit antiserum directed to the large subunit of ribonucleotide reductase (ICP6) (Huszar et al. 1983) and was a gift from S. Bacchetti.

Transient expression of gB and/or gK in transfected cells. Vero cells were transfected with various DNA concentrations (ie. ranging from 0.0, 0.25, 0.5, 1.0, 2.0, to 4.0 µg/ml) of plasmids containing the gB^{wt} gene (pKBXX) or the gB^{syn} gene (pK143) according to the protocol outlined by Cai et al. (1988b). In addition, the concentration of gB plasmids remained constant in other experiments (0.25 or 0.5 µg/ml), and we varied the concentration of plasmid pKEB-S1 (ie. 0.0, 1.0, 2.0, 3.0 or 4.0 µg/ml), which contains the UL53-gK^{wt} gene. Transfected cells were incubated 24 hr and infected with HSV-1 syn082 using 1 PFU/cell. The number of fused and unfused cells were determined by microscopic examination 48 hr after transfection. Several fields of view were used and approximately

100 cells examined at each data point.

Construction of cell lines expressing gK. Subconfluent Vero cell monolayers (60 mm dishes) were transfected with 15 μg of pSV2HISsyn or pSV2HDK using the $CaPO^4$ technique (Graham and Van der Eb, 1973), and glycerol-shocked 4 hr later as outlined previously (Ligas and Johnson, 1988). After 2 days the confluent cell monolayers were trypsinized, and each 60mm dish was split 1:10, 1:15, or 1:20 into 150mm dishes containing DUL-his supplemented with 0.5mM histidnol and 7% FCS. After 10 days histidnol concentrations were increased to 1.2 mM and cells were incubated at 37°C for another 10 days. Next, isolated colonies of cells were trypsinized using cloning cylinders and placed in individual wells of a 6-well dish containing DUL-his supplemented with 0.5 mM histidnol and 7% FCS. Initially, transformants were screened by determining the UL53 gene copy number using a Southern dot blot assay. Later, when an HSV-1 type specific gK antisera became available, cell lines were tested for the production of gK protein by infecting the cells with HSV-2 (333), followed by immunoprecipitation of HSV-1 gK cell extracts labelled with both [^{35}S]methionine and [^{35}S]-cysteine ([^{35}S]Met-[^{35}S]Cys) (as described below). Based on these criteria the VS202 cell line was chosen from 210 pSV2HISsyn transformants and the gK-9 cell line was chosen from 302 pSV2HDK transformants.

Radiolabelling of cells, immunoprecipitation, and gel electrophoresis. Vero cells or Vero cells transformed with pSV2HISsyn or pSV2HDK were infected with HSV-1 or HSV-2 using 5 (or 30) PFU/cell. 4 hr (or 4.5 h) after infection, cells were washed three times with medium 199 lacking methionine and cysteine (labelling medium). The cells were then incubated for 5 hr with labelling medium containing 1% dialysed FCS and 200μCi

per ml of [^{35}S]Met-[^{35}S]Cys. Alternatively, Vero cell monolayers were infected with Adenovirus vectors using 30, 100, or 1000 PFU/cell and after 6 hr some monolayers were coinfected with HSV-1 using 5 PFU/cell. Cell monolayers infected with Ad vectors were radiolabelled from 2 to 6 hr (or 10 to 18 hr) post-infection using [^{35}S]Met-[^{35}S]Cys as described above. Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). Extracts derived from 5 x 10^4 to 5 x 10^5 cells were mixed with mouse ascites fluid (5 µl) or rabbit sera (5 or 20 µl), followed by protein A-Sepharose (100 µl of a 1:1 mixture with a binding capacity of ≈1 mg IgG, Pharmacia Chemicals, Dorval, Quebec, Canada) and washed using stringent conditions (Hutchinson et al., 1992a). The precipitated proteins were eluted with 50 mM Tris-HCl (pH 6.8) containing 2% SDS, 15% glycerol, bromophenol blue, and 2% ß-mercaptoethanol by heating at 37°C for 30 min. For electrophoretic we employed 10 or 12% SDS-polyacrylamide gels as described previously (Johnson and Feenstra, 1987). The gels were infused with Enhance (Dupont, Montreal, Quebec, Canada) and exposed at -70°C to XAR film (Eastman Kodak Co., Rochester, N.Y.). Densitometric quantification of viral proteins involved preflashed film which was scanned using a GS 300 Transmittance-Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco) in conjunction with Hoefer GS 350 Data System Software for IBM personal computers.

Cell-cell fusion assays. In order to analyze plaque morphology, wild-type HSV-1 (KOS), the gK^{syn} mutants Syn20, MP or 804 and the gB^{syn} mutants tsB5 or KTTS.1 plated on Vero, VS202 or gK-9 cell monolayers using a low multiplicity of infection. This was achieved by inoculating cell monolayers situated in 6-well plates with 0.5 ml of a 10 fold dilution series (10^{-2} to 10^{-8}) derived from each of the viral stocks. After a 2 hr incubation, the virus inoculum was removed and 2.0 ml of α-MEM containing 1% FCS and 0.2%

human τ-globulin (Connaught Laboratories, Mississauga, Ontario, Canada) was added. Vero cell monolayers were incubated for 24 hr and VS202 and gK-9 cells were incubated for 36 hr before the medium was removed and the cells were stained with crystal violet. For photography, cells were grown on sterilized glass coverslips.

To study fusion of cell monolayers, ≈4.2 x 10^5 Vero cells were seeded in 6-well plates incubated in growth medium at 37°C for 24 hr prior to infection. Some of the Vero cell monolayers were infected with Ad vectors using 30, 100, 300, and 1000 PFU/cell by diluting the virus in 0.7 ml α-MEM + 1% FCS, and allowing the virus to adsorb and penetrate for 2 hr at 37°C. Following Ad vector adsorption 1.3 ml α-MEM + 4% FCS was added to the monolayer. Six hr after the initial Ad vector infection, Vero cells were coinfected with HSV-1 (Syn20) or HSV-1 (KTTS.1) using 5 PFU/cell. To generate cell morphology controls, some of the uninfected Vero, VS202, and gK-9 cell monolayers were inoculated of wild-type HSV-1, or syncytial mutants HSV-1 (syn20) or HSV-1 (KTTS.1) at this time point using 5 pfu/cell, or left uninfected. The cells were incubated for an additional 12 hr at 37°C and then stained with crystal violet stain (ie. 18 hr after initiating the Ad vector infection). For photography, cells were grown on glass coverslips treated with poly-D-lysine (according to the manufacturers specifications Boehringer Mannheim, W. Germany) and stained with crystal violet.

Plaqueing efficiency, and production of infectious HSV-1 in Vero, VS202 and gK-9 cells. To assess plaqueing efficiency, 10-fold serial dilutions of the following HSV-1 strains: KOS, Syn20, or KTTS.1 were prepared in α-MEM containing 1% FCS. Vero, VS202 or gK-9 cell monolayers in 6-well dishes were infected (in triplicate) were incubated with 0.5 ml of each virus dilution incubated for 2 hr, after which the virus inoculum was removed and monolayers were overlaid with 2.0 ml of α-MEM containing 1% FCS and 0.1%

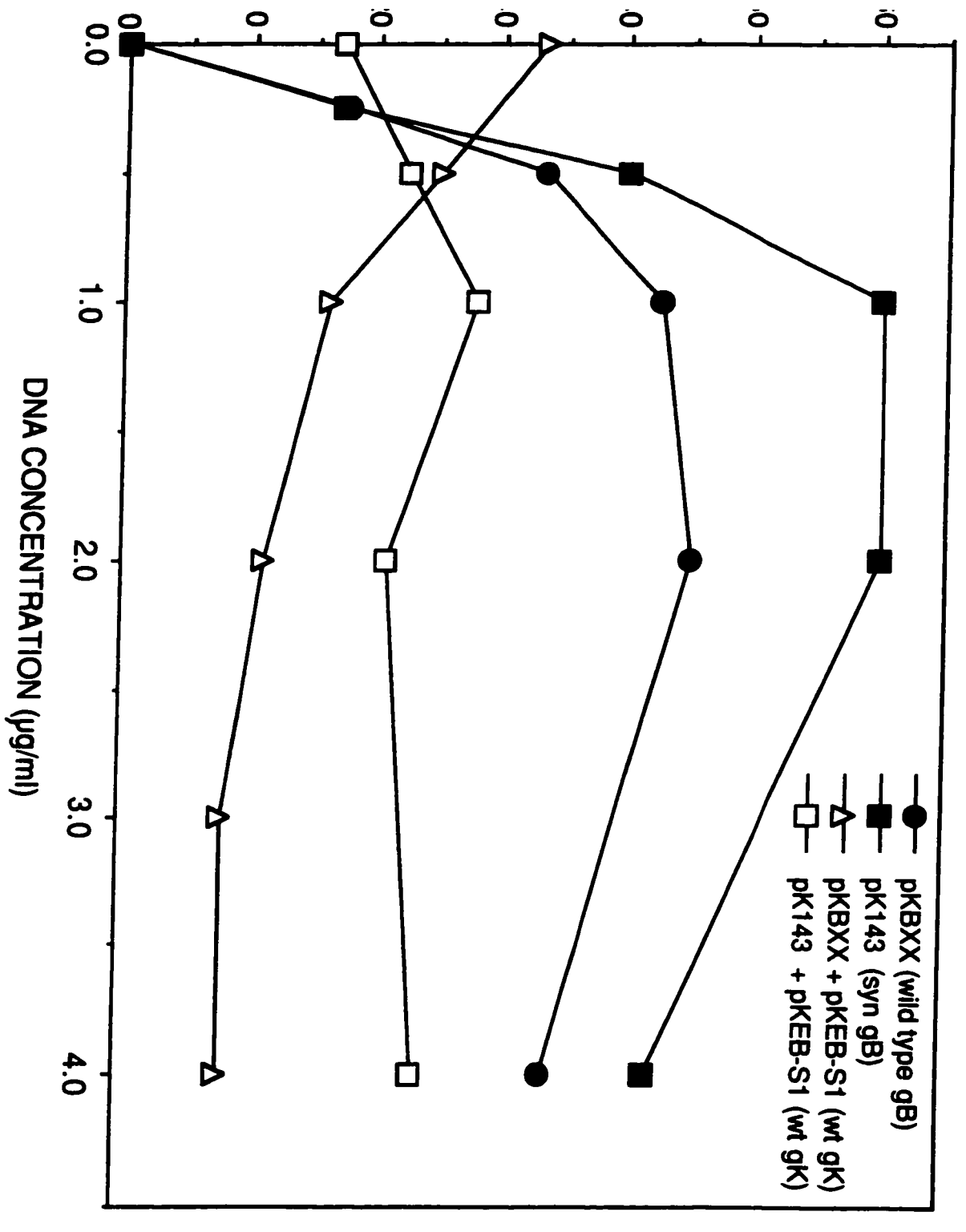
human τ-globulin (Connaught Laboratories, Mississauga, Ontario, Canada). Vero cells were incubated at 37°C for 24 h, whereas VS202 and gK-9 cells were incubated for 36 hr before the medium was removed and the cells were fixed with crystal violet.

HSV-1 replication in the three cell lines (Vero, VS202 or gK-9) was assessed by infecting cells with HSV-1 (KOS) using 5 PFU/cell or 0.2 PFU/cell in 0.5 ml α-MEM containing 1% FCS. After allowing virus to absorb and penetrate for 1.5 to 2 hr at 37°C, the virus inoculum was removed and 1.5 ml α-MEM containing 1% FCS was added. The cells were incubated at 37°C and 24 hr post-infection the cell monolayers and cell culture medium were harvested separately. The infected cells were disrupted by sonication, and plaque titrations were performed using Vero cell monolayers.

Results

Fusion of cells after transient expression of wild-type or syncytial forms of gB and gK followed by infection with HSV-1. A transient expression assay was devised to study the effect of expressing wild-type and mutant forms of gK and gB in cells. Vero cells were transfected with wild-type gB (gB^{wt}; pKBXX) or a syncytial variant of the gB protein (gB^{syn}; pK143), and then infected with HSV-1 (syn082) which expresses a syncytial form of gK (gK^{syn}) but does not express gB (Cai et al., 1988b). In the absence of gB (ie., when cells were not transfected with one of the gB plasmids) no fusion was observed (Fig. 1.3). However, when cells were transfected with plasmids encoding gB^{wt} (pKBXX) or gB^{syn} (pK143), fusion of cells infected with syn082 approached or exceeded 50% confirming previous results demonstrating that gB is required for membrane fusion (Cai et al., 1988b). Since the expression of gB in transfected cells did not approach 50% (Cai et al., 1988b), it appears likely that nonexpressing cells were recruited into fusion centres by

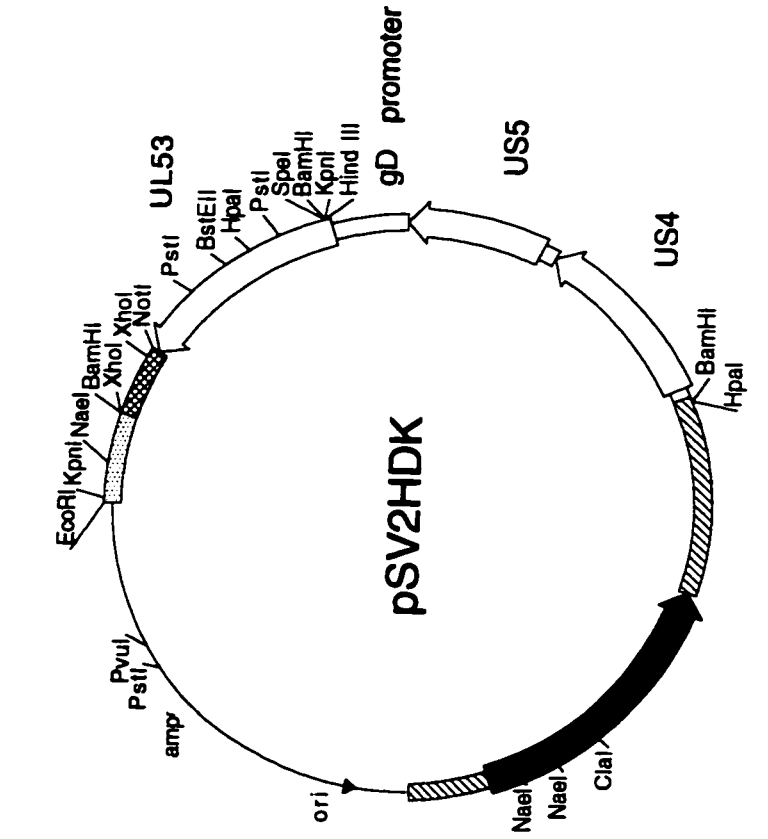
Figure 1.3 Wild-type gK inhibits fusion associated with gK^{syn}, but not gB^{syn}, in transfected cells infected with HSV-1 strain syn082. Vero cells seeded on coverslips in 60-mm dishes were transfected with various concentrations of plasmid DNA. Transient transfections with the following plasmids were performed: pKBXX (wild-type gB) alone (-●-), pK143 (syn-gB) alone (-■-), or cotransfections in which the concentration of pKBXX remained constant (0.5 μg/ml) and pKEB-S1 (wild-type gK) was varied from 0-4 μg/ml (-▲-) or where pK143 was kept constant (0.25 μg/ml) and the concentration of pKEB-S1 varied from 0-4 μg/ml (-□-). Twenty four hours after transfection, cells were infected using 1 PFU/cell with HSV-1 (syn082), a gB-negative virus which expresses syn-gK. Cell monolayers were incubated for another 24 hr, then fixed and stained, and measured microscopically. The extent of cell fusion was determined by measuring the fraction of cells remaining unfused. Each line represents the average of at least three experiments.


pKBXX (wild type gB)
pK143 (syn gB)
pKBXX + pKEB-S1 (wt gK)
pK143 + pKEB-S1 (wt gK)
DNA CONCENTRATION (µg/ml)
0.0
1.0
2.0
3.0
4.0

neighbouring gB^- cells. To evaluate the effects of expressing wild-type gK (gK^{wt}) has on HSV-induced membrane fusion, cells were transfected with a constant amount of the gB plasmids and varying amounts of pKEB-S1 (Bond & Person, 1984) which contains the wild-type UL53-gK gene, and then infected with the HSV-1 gB^-gK^{syn} mutant (syn082). In cells cotransfected with pKBXX (gB^{wt}), increasing the concentration of pKEB-S1 (gK^{wt}) caused a reduction in the amount of cell fusion (Fig. 1.3). In contrast, cell-cell fusion was not inhibited by increasing the quantity of pKEB-S1 (gK^{wt}) in cells cotransfected with pK143 (gB^{syn}). These data suggested that although gK^{wt} has the ability to suppress cell-cell fusion caused by gK^{syn} and gB^{wt}, wild-type gK could not reduce the fusion produced by cells expressing gB^{syn} plus gK^{syn}. Note that these results were considered semi-quantitative and were the basis for further experiments.

Construction of cell lines able to overexpress HSV-1 gK. To study the inhibition of fusion by wild-type gK further, stably transformed cell lines expressing high levels of gK were constructed. In addition cell lines which express gK in response to HSV infection were constructed because they have the potential to fulfil another objective; ie, complementation of an HSV-1 UL53-gK deletion mutant because preliminary data had suggested that gK is essential for virus replication (L. Hutchinson, unpublished results). Cell lines were constructed by transfecting Vero cells with one of two plasmids, pSV2HISsyn or pSV2HDK (Fig. 2.3). Both pSV2HISSyn and pSV2HDK plasmids utilize a pSV2HIS backbone and employ the simian virus 40 early promoter to drive expression of the selectable marker, histidnol dehydrogenase (Hartman and Mulligan, 1988) which permits the selection of cell lines featuring a high copy number of such plasmids. pSV2HISsyn contains an EcoRI-BamHI fragment (HSV-1 [KOS], map coordinates 0.723-

Figure 2.3. Plasmids used to express gK in Vero cell transformants. Plasmids, pSV2HDK and pSV2HISsyn were used to construct cell lines capable of expressing gK after HSV infection. pSV2HISsyn contains the UL53 promoter, open reading frame (ORF) and polyadenylation signal (poly A), as well as a fraction of the overlapping UL52 gene (open boxes). In pSV2HDK, the HSV-1 US4 gene, US5 gene and US6-gD promoter (open boxes) are upstream of the UL53 ORF and a bovine growth hormone polyadenylation signal (crosshatched box) and the M13 origin of replication (stippled box) are downstream of the UL53 ORF. Both pSV2HISsyn and pSV2HDK were derived from the pSV2HIS backbone (Hartman and Mulligan, 1988) and possess the selectable marker, histidnol dehydrogenase (black box) coupled to the SV40 early promoter and poly A signal (hatched boxes).


pSV2HDK
gD promoter
US5
US4
UL53
BamHI
HpaI
NaeI
NaeI
ClaI
ori
ampr
PvuI
PstI
EcoRI
KpnI
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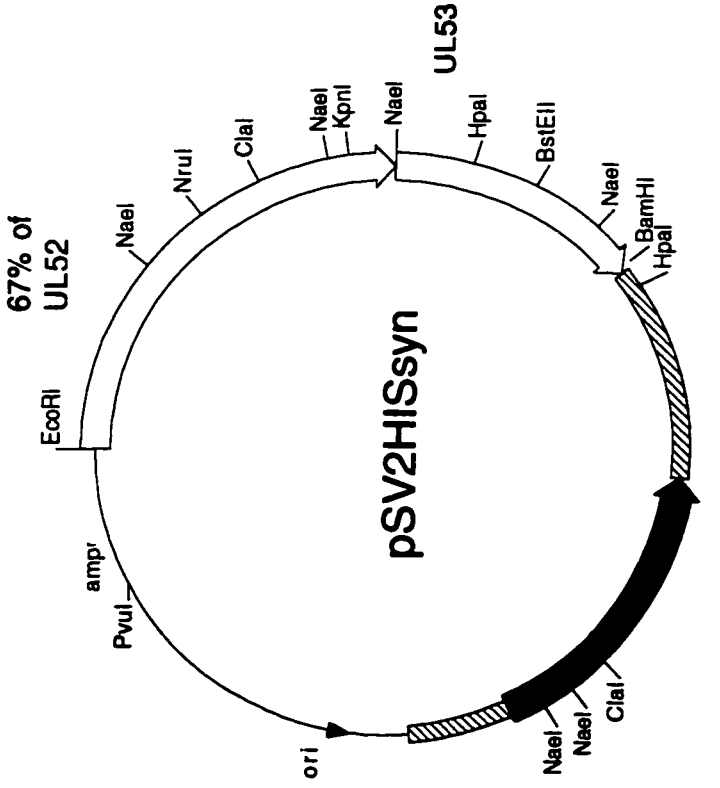
SpeI
BamHI
KpnI
Hind III


pSV2HISsyn
67% of
UL52
UL53
EcoRI
ampr
PvuI
ori
NaeI
NaeI
ClaI
NaeI
BamHI
HpaI
NaeI
BstEII
HpaI
NaeI
KpnI
NaeI
ClaI
NruI
NaeI

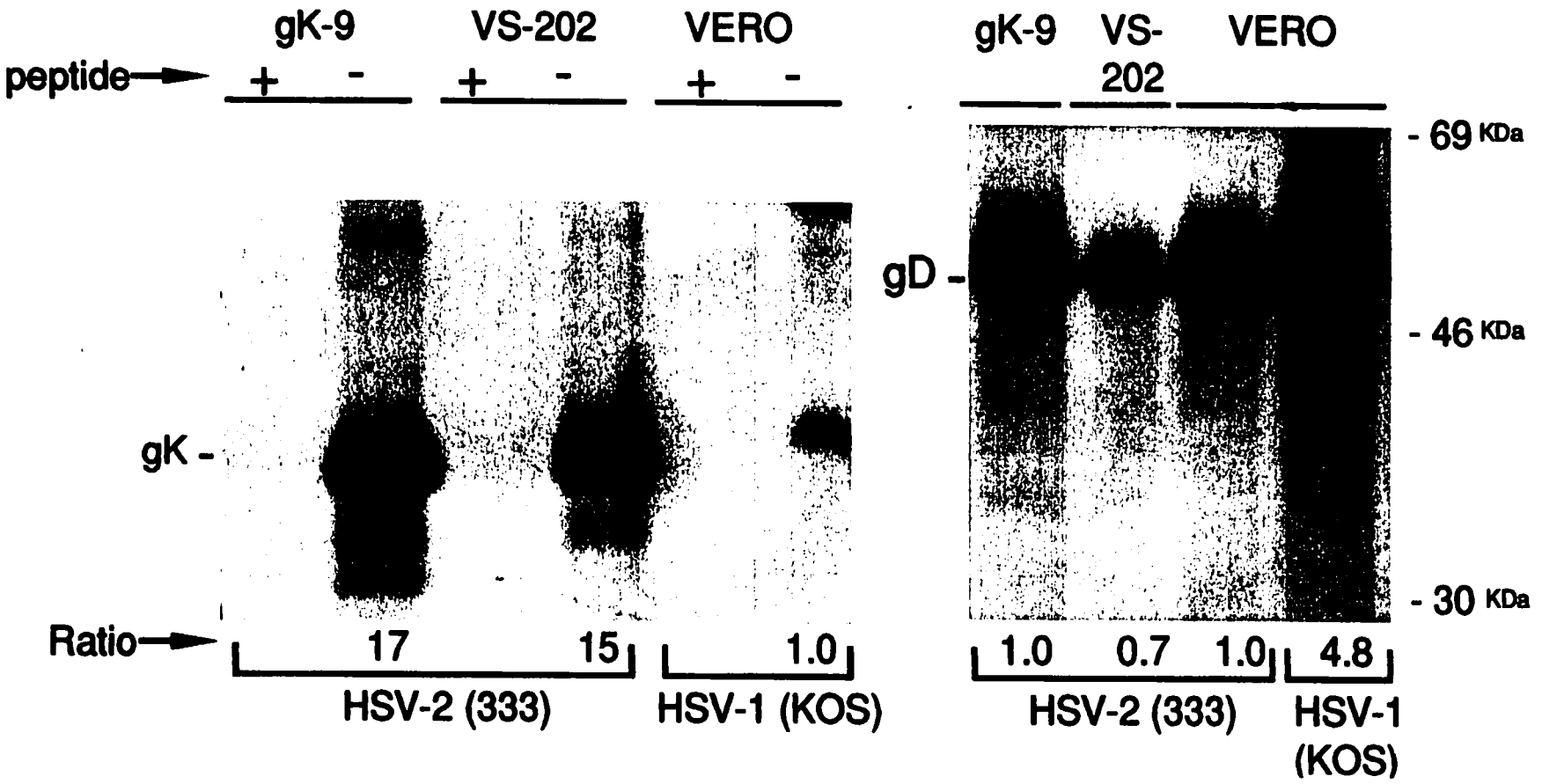
0.745) including the endogenous UL53 promoter and coding sequences and a fraction ofthe UL52 gene which lies upstream and overlaps the UL53 gene. pSV2HDK contains the UL53 open reading frame (ORF), but it has been placed under the control of an HSV-1 gD promoter. In previous studies, cell lines capable of expressing HSV-1 glycoproteins gB, gD, and gL have been constructed using plasmids which contain the endogenous gene (ie. the relevant ORF coupled to the native promoter) (Cai et al., 1987; Ligas & Johnson, 1988; Roop et al., 1993). These cells produce the glycoprotein in response to infection by HSV-1 or HSV-2 and the relevant protein can be detected with immunoassay that use type-specific antibodies. In a recent report describing the construction of an HSV-1 gH^- virus, the authors found it necessary to construct cell lines which accommodate the gH ORF coupled to the gD promoter (Forrester et al., 1992). Therefore, we included a similar strategy to increase the likelihood of obtaining the appropriate cell lines.

Cell lines expressing histidnol dehydrogenase are able to grow in $histidnol^-$:$histidine^-$ medium by catalyzing the oxidation of histidnol (toxic to cells) to histidine (an essential amino acid). Over 200 pSV2HISsyn transformants and over 300 pSV2HDK transformants were isolated and screened, initially for plasmid copy number by Southern blot analysis, and usually for expression of gK. To perform southern blot analysis, total cellular DNA was isolated, quantified by fluorometry, dot blotted onto nitrocellulose, and probed with nick-translated [^{32}P]-labelled pUC19syn or [^{32}P]-end labelled oligonucleotide AB924 (5' G CTG CAG GGA ACG GAC GGC GAG CAT 3') which maps to the first 30 base pairs downstream of the UL53 AUG initiation codon. UL53-gK expression was assessed by infecting cell lines with HSV-2 (333) and immunoprecipitating HSV-1 gK using the HSV-1 type specific antibody, anti-UL53-4 (Hutchinson et al., 1992b). Two cell lines VS202 and gK-9 were chosen for further study.

VS202 cells contain approximately 500 copies of pSV2HIS syn (endogenous UL53 gene), while gK-9 cells have approximately 200 copies of pSV2HDK (gD promoter:gK ORF) (data not shown). A third cell lines, BL-1, which contains approximately 20 copies of the overlapping UL52 and UL53 genes in an HSV-1 (KOS) BamHI L fragment, has been published elsewhere (Goldstein & Weller, 1988) and was included in these experiments as a control.

When gK-9 and VS202 cells were infected with HSV-2, large amounts of HSV-1 gK were synthesized (Fig 3.3). Laser densitometry analysis of autoradiographs revealed that HSV-2 infected gK-9 and VS202 cells produced at least 15 times, the levels of gK-1 expressed in HSV-1-infected Vero cells. Although the electrophoretic mobility of HSV-1 gK expressed by the cell lines appears to be different from that of HSV-1-infected Vero cells (Fig. 3.3); this condition is due to a variation in electrophoresis caused by loading of the Vero-derived gK in the outside lane of this gel. In other experiments, HSV-1 gK expressed the UL53 ORF in gK-9 or VS202 displayed an electrophoretic mobility identical to gK derived from HSV-1 infected Vero cells. HSV-1 and HSV-2 gD were also precipitated from cell extracts to establish the expression level for other HSV proteins in gK-9, VS202 and Vero cells. In the experiment shown in Fig. 3.3, HSV-1-infected Vero cells expressed approximately 5 times more gD than was observed in the HSV-2-infected gK-9 and VS202 cells, even though more gK was produced by the gK-9 and VS202 cells. Although BL-1 cells have been shown to complement HSV-1 UL52 null mutants (Goldstein & Weller, 1988), they did not express detectable levels of gK-1 after infection with HSV-2 (results not shown). In addition, uninfected VS202 and gK-9 cells did not express detectable amounts of gK (data not shown), suggesting that trans-acting viral factors are required to support gK synthesis.

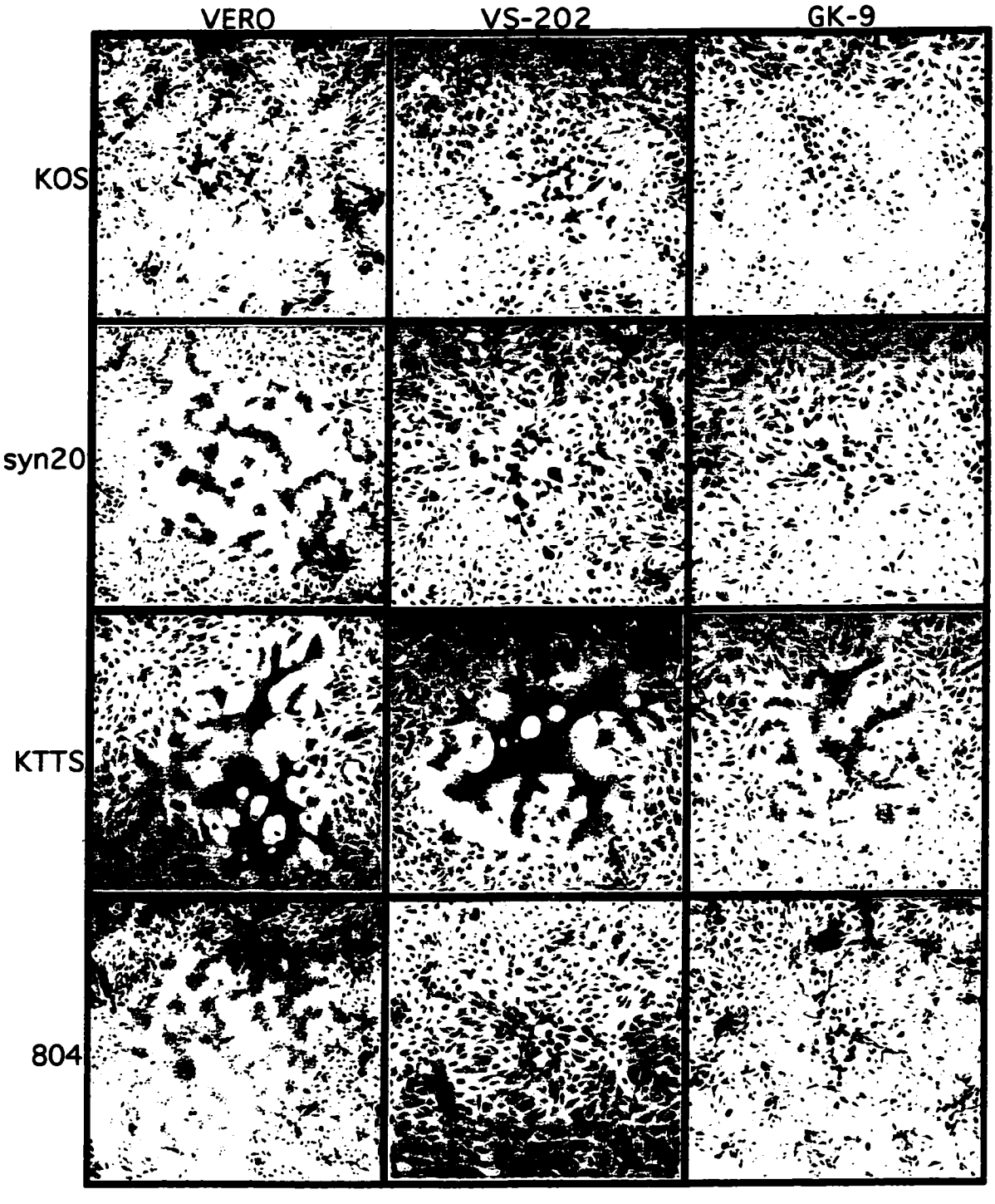
Figure 3.3 Expression of HSV-1 gK in Vero cell transformants after infection with HSV-2 (333). The gK-9 and VS202 cell lines are Vero cell transformants containing multiple copies of plasmids pSV2HDK or pSV2HisSyn, respectively. gK-9 and VS202 cells were infected with HSV-2 (333) and for comparison, Vero cells were infected with HSV-2 (333) or HSV-1 (KOS). 4.5 hr later, the infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 5 hours until 9.5 hr post-infection. (Left) Cell extracts were mixed with an excess (relative to the antigen available) of rabbit anti-UL53-4 peptide sera which had been preincubated with (+) or without (-) UL53-4 peptide. Anti-UL53-4 sera recognizes HSV-1 gK but not HSV-2 gK (Hutchinson et al., 1992b). (Right) gD was immunoprecipitated from cell extracts with MAb LP2 which recognizes both HSV-1 and HSV-2 gD. Antigen-antibody complexes were precipitated with an excess (relative to the Ab available) of protein A-Sepharose and washed using stringent conditions (see Material and Methods). Precipitated proteins were eluted with 2X sample buffer at 37°C for 30 min and separated using 12% polyacrylamide gels. Autoradiography was conducted with preflashed Xray film and laser densitometry used to quantify the relative amounts of gK and gD expressed by each cell line (Ratio). The positions of glycoproteins gK and gD and molecular mass markers of 69, 46, and 30 kDa are indicated.


gK-9
VS-202
VERO
peptide
+
-
+
-
+
-
gK -
Ratio
17
15
1.0
HSV-2 (333)
HSV-1 (KOS)
gK-9
VS-
202
VERO
gD -
- 69 KDa
- 46 KDa
- 30 KDa
1.0
0.7
1.0
4.8
HSV-2 (333)
HSV-1
(KOS)

Overexpression of gK^{wt} suppresses cell-cell fusion induced by HSV-1 syncytial mutants expressing gK^{syn}. In order to study the effects of gK^{wt} on HSV-induced cell fusion we chose to work with a number of well defined HSV-1 mutants with lesions in the UL53-gK or UL27-gB genes. HSV-1 MP, 804, and Syn20 have gK^{syn} mutations, whereas HSV-1 tsB5 and KTTS-1 possess gB^{syn} mutations (refer to Materials & Methods for details) (Read et al. 1980; Hoggan & Roizman 1959; Manservigi et al., 1977; Little and Schaffer, 1981; Cai et al. 1988; Roop et al., 1993). HSV-1 (KOS) was used as the wild-type control.

To examine plaque formation on Vero, gK-9 or VS202 cell monolayers, the cell lines were infected with wild-type HSV-1 (KOS) or one of the syncytial mutants using a low multiplicity of infection (MOI) and incubated in medium containing human τ-globulin. Vero cells were incubated at 37°C for 24 hr, whereas VS202 and gK-9 cells were incubated for 36 hr before they were fixed with crystal violet. Cell monolayers infected with HSV-1 (KOS) produced plaques with bordered by rounded cells aggregating together, a morphology typical of wild type HSV-1 viruses (Fig. 4.3). Vero cells infected with HSV-1 syncytial mutants Syn20, MP, tsB5, KTTS.1 or 804, fused with neighbouring infected and uninfected cells, forming plaques comprised of giant multinucleated cells (Fig. 4.3). Similarly, HSV-1 strains which express gB^{syn} (ie. tsB5 and KTTS.1), also exhibited syncytial plaque morphologies on VS202 and gK-9 cells, which were identical in appearance to those observed on Vero cells (Fig. 4.3, results not shown to avoid duplication). In contrast, HSV-1 strains with syn mutations in gK, ie. Syn20, 804 and MP, produce nonsyncytial plaques on VS202 and gK-9 cells, which exhibit characteristics reminiscent of cells infected with wild-type, nonsyncytial HSV strains (Fig.4.3, results not shown to avoid duplication). Both the wild type and syncytial plaques tended to be smaller and took longer to form, on VS202 and gK-9 cell monolayers relative to plaque

Figure 4.3 Morphology of plaques produced by HSV-1 syncytial mutants on gK-expressing cell lines. Vero, VS202, and gK-9 cells were grown on glass coverslips, and then infected with HSV-1 (KOS), or the syncytial mutants HSV-1 (syn20), HSV-1 (KTTS.1) and HSV-1 (804), using a dilution expected to produce individual plaques. HSV-1 infected cells were incubated for 24 h at 37°C, fixed and stained with crystal violet. Plaques were photographed at 100X microscope magnification using a Zeiss microscope.


VERO
VS-202
GK-9
KOS
syn20
KTTS
804

formation on the parental Vero cell monolayers. Other cell lines which express much lower levels of gK and can complement a gK$^-$ mutant virus did not suppress fusion of Syn20 (results not shown).

Overproduction of gK inhibits HSV-1 plaque formation and the production of infectious virions but not the synthesis of viral proteins. To define the process by which fusion is inhibited on VS202 and gK-9 cells, we examined the production of infectious virions and plaqueing efficiency on these cell lines. Stocks of HSV-1 KOS, Syn20, and KTTS.1 all produced fewer plaques on VS202 and gK-9 cell monolayers than was observed on Vero cells (Table 1.3). A 15 to 30 fold decrease in the number of plaques formed by wild-type and syncytial viruses was observed on VS202 cell monolayers and a 200 to 400 decline in plaque numbers was observed on gK-9 cells, relative to the amount of plaques detected on Vero cells. The more severe reduction in gK-9 plaqueing efficiency correlated with the smaller plaque size observed with these cells. Again, HSV-1 (KTTS.1) displayed a syncytial plaque morphology on all cell lines, despite the reduction in plaque numbers, but these plaques were smaller on VS202 and gK-9 cells than plaques found on Vero cell monolayers.

In an effort to understand the mechanism inhibiting plaque formation, we investigated the ability of VS202 and gK-9 cell lines to support virus replication. Vero, VS202 or gK-9 cells were infected with HSV-1 (KOS) using two MOIs, 5 PFU or 0.2 PFU per cell. Infected cells and medium were harvested after 24 hr and the production of infectious virions was determined. The yield of infectious virions obtained from the medium or cell extracts of VS202 and gK-9 monolayers, were lower than those obtained from Vero cells (Table 2.3). The decrease in virus production was greater when a low

TABLE 1.3 Plaqueing Efficiency of HSV-1 on Vero cells and gK-producing cells

	Relative number of virus plaques on each cell line[a]		
Virus[b]	Vero	VS202	gK-9
KOS	100 ± 5.6	6.0 ± 1.8	0.50 ± 0.040
Syn20	100 ± 5.8	6.6 ± 0.30	0.21 ± 0.030
KTTS.1	100 ± 5.6	3.4 ± 0.36	0.24 ± 0.070

[a] Vero, VS202, and gK-9 cell monolayers were infected in triplicate with various dilutions of virus stocks, the monolayers were overlaid with medium containing 0.1% human gamma-globulin, incubated for 36 hr, then stained with crystal violet and the plaques were counted.

[b] The HSV-1 (KOS), (Syn20), or (KTTS.1) titre on Vero cells was defined as 100 and the relative titres on cell lines VS202 and gK-9 are indicated.

TABLE 2.3 HSV-1 (KOS) Replication on Vero cells and gK-producing cells.

	Relative yields of infectious virus[c]			
	Initial m.o.i. = 5.0[b]		Initial m.o.i. = 0.2[b]	
Cell line[a]	Cells	Medium	Cells	Medium
Vero	100	100	100	100
VS202	21.1	20.8	9.43	3.82
gK-9	3.52	0.82	0.60	1.05

[a] Vero, VS202, and gK-9 cell monolayers were infected with HSV-1 (KOS) and incubated at 37°C for 24 hr.

[b] Initial multiplicities of infection (m.o.i.), 5 or 0.2 PFU/cell, were used.

[c] Infected cells were harvested and disrupted by sonication and cell culture supernatants were collected and viruses titrated using Vero cell monolayers. The titres of virus from VS-202 and gK-9 cells were given relative to their titre from Vero cells.

multiplicity of infection was used, and this change was comparable to the reduction observed in plaqueing efficiency, ie., about 11 fold for VS202 cells and 167 fold for gK-9 cells (Table 1.3; Table 2.3). In addition, the defect in virus replication caused by overexpressing gK had a greater impact on the release of infectious virus than the assembly of infectious intracellular virions. Together these results suggest that high levels of gK expression may interfere with the release and/or cell-to-cell spread of HSV-1 virions.

To further characterize virus replication in VS202 and gK-9 cells, we examined the expression of some typical viral proteins derived from the major regulatory classes. Vero, VS202, or gK-9 cells were infected with HSV-1 (KOS) using 5 PFU/cell and then cells were labelled with [^{35}S]methionine-[^{35}S]cysteine ([^{35}S]Met-[^{35}S]Cys). In the next step ICP6, which has been described as an immediate-early or α-protein and also a ß-protein, the early or ß-protein thymidine kinase (tk), and the leaky-late or τ_1 or proteins gD and gB, were immunoprecipitated from cell extracts. The expression of these HSV polypeptides in VS-202 and gK-9 cells was not markedly different from that observed in HSV-infected Vero cells (Fig. 5.3). Similar results were obtained when the cells were infected using 0.2 or 30 PFU/cell (results not shown). Therefore, inhibition of the gK^{syn}-induced cell-cell fusion by VS202 and gK-9 cells, does not result from a large reduction in viral protein synthesis. In support of this conclusion, HSV-1 strains expressing gB^{syn} were able to fuse cells (Fig. 4.3 & results not shown). Since the production of infectious virions as well as plaque formation were reduced in VS202 and gK-9 cells, it appears likely that other aspects of HSV replication are inhibited in cells expressing large amounts of UL53-gK, eg. envelopment and/or egress. However, further studies would be required to elucidate this point.

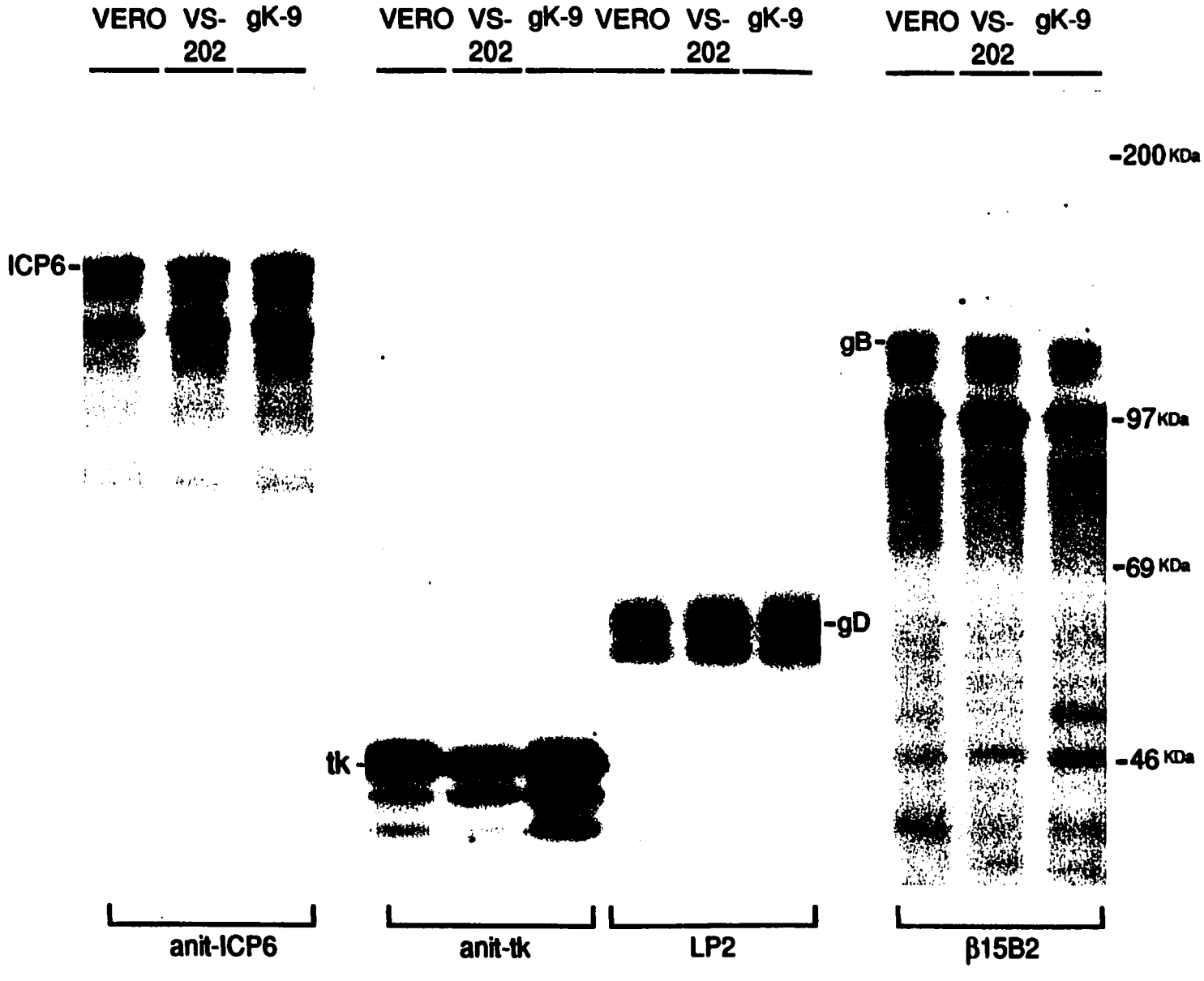

VERO
VS-202
gK-9
VERO
VS-202
gK-9
VERO
VS-202
gK-9
-200 KDa
ICP6-
gB-
-97 KDa
-69 KDa
-gD
tk-
-46 KDa
anit-ICP6
anit-tk
LP2
β15B2

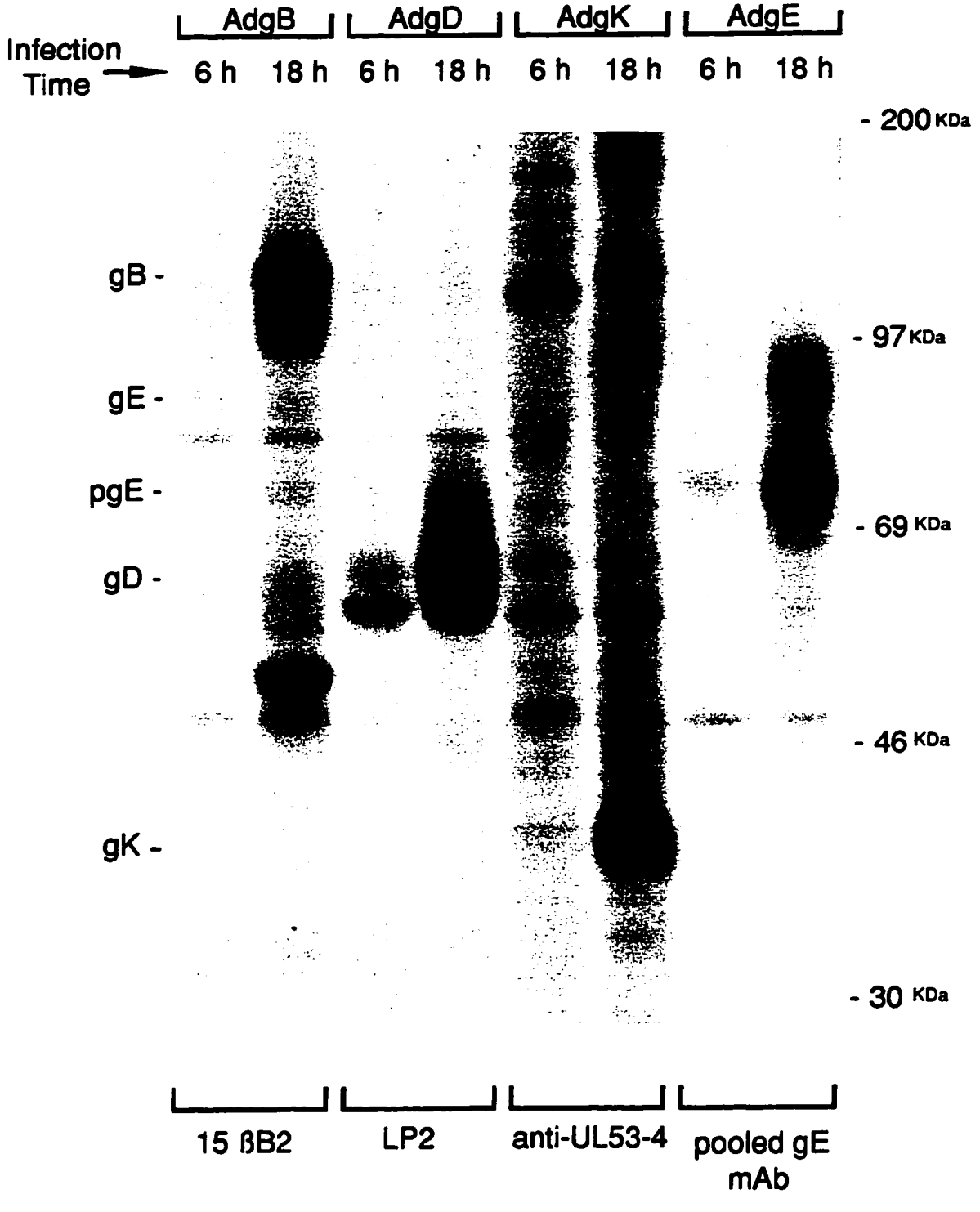
Figure 5.3 Expression of HSV proteins in gK expressing cells. Vero cell transformants gK-9 and VS202, or for comparison parental Vero cells, were infected with HSV-1 (KOS) at 5 PFU/cell and 3 hr later these cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 3.5 hours until 6.5 hr post-infection. Infected cell extracts (from approximately 4 x 10^5 cells) were mixed with an excess of the following antibodies: polyclonal rabbit serum specific for the large subunit of ribonucleotide reductase (ICP6), rabbit anti-tk serum, MAb 15ßB2 specific for gB, or MAb LP2 specific for gD. Antibody-antigen complexes were precipitated using protein A-sepharose (present in excess amounts) and washed three times with NP40-DOC. The precipitated proteins were eluted with 2X sample buffer at 100°C for 5 min and separated using 8.5% polyacrylamide gels. HSV-1 proteins ICP6, tk, gB, gD and molecular markers of 200, 97, 69 and 46 kDa are indicated.

Characterization of recombinant adenovirus vectors used to express HSV-1 glycoproteins. In the next set of experiments we used recombinant adenovirus (Ad) vectors to express gK and gB, as well as gC, gD, gE, gH:gL and gI, in order to assess the effect expressing these glycoproteins has on cell-cell fusion induced by HSV-1 strains carrying gB^{syn} or gK^{syn}. The methodology used to construct these Ad vectors has been published elsewhere (Johnson et al., 1988; Johnson, 1991; Graham & Prevec, 1991) and many of the Ad vectors have been described previously. Essentially, DNA sequences encoding HSV-1 glycoproteins were either coupled to an SV40 promoter (ie. gB, gC, gD, gE, gI, gK), or not (ie. gH, gL), and inserted into the E3 region of Ad5 (Hanke et al. 1990, Hutchinson et al. 1992a). AdgC (Witmer et al., 1990) expresses HSV-1 gC, AdgD (Zheng et al., 1993 expresses HSV-1 gD, AdgE (Hanke et al., 1990) expresses HSV-1 gE, AdgI (Hanke et al., 1990) expresses HSV-1 gI and AdgK expresses HSV-1 gK (Hutchinson et al., 1992b). AdgB8 has been designated AdgB in this paper, and is an Ad vector identical to AdgB2 (Johnson et al., 1988) except that the entire Ad genome is derived from Ad serotype 5 (ad5), whereas AdgB2 contains sequences from Ad serotype 2 (F.L. Graham, unpublished). AdgH was constructed using a strategy similar to the one used to create the HSV-1 gL expressing vector, AdgL (Hutchinson et al., 1992b), and contains the gH ORF situated in the E3 region of Ad5 without upstream SV40 promoter sequences. AddlE3 is an Ad5 mutant in lacking nonessential E3 sequences (Haj-Ahmad & Graham, 1986) and was used as a negative control in these experiments.

To ensure that the Ad vectors produce adequate quantities of each HSV-1 glycoprotein we examined the temporal expression and the relative abundance of these proteins in cells infected with individual Ad vectors (Fig. 6.3). This information also furnished the basis for subsequent experiments designed to evaluate the role of these

Figure 6.3 Expression of HSV-1 glycoproteins in cells infected with Adenovirus expression vectors, 6 h and 18 h after infection.

Vero cell monolayers were infected with AdgB, AdgD, AdgK or AdgE using 1000 PFU/cell and labelled with ^{35}S-methionine and ^{35}S-cysteine, from 2 h to 6 h (6 h) or from 10 h until 18 h (18 h) post-infection. Cell extracts were immunoprecipitated with MAb 15ßB2 (specific for gB) or LP2 (specific for gD), rabbit anti-UL53-4 peptide sera (specific for gK) or pooled anti-gE MAb's, II-481, 3114, and 7520. Antigen-Antibody complexes were eluted with 2X sample buffer at 37°C for 30 min and separated on 12% polyacrylamide gels. The HSV-1 glycoproteins gB, gE, pgE, gD, gK and molecular mass markers of 200, 97, 69, 46 and 30 KDa are indicated.


AdgB
AdgD
AdgK
AdgE
Infection Time
6 h
18 h
6 h
18 h
6 h
18 h
6 h
18 h
- 200 KDa
- 97 KDa
- 69 KDa
- 46 KDa
- 30 KDa
gB -
gE -
pgE -
gD -
gK -
15 ßB2
LP2
anti-UL53-4
pooled gE mAb

glycoproteins in HSV-induced cell-cell fusion. To assess the production of HSV-1 proteins after infection with Ad recombinants, Vero cells were infected with one of the following vectors: AdgB, AdgD, AdgE, AdgK (Fig. 6.3), AdgC, AdgI, or AdgH in conjunction with AdgL (not shown). Infected cells were labelled with [^{35}S]Met-[^{35}S]Cys from 2 to 6 hr, or 10 to 18 hr after infection, and the HSV-1 were immunoprecipitated, quantitatively. The gB and gK proteins could not be detected 6 hr after infection and only low levels gD and gE were observed, despite the relatively high MOI (1000 PFU/cell) which was used. By 18 hr post-infection the Ad vector-infected cells expressed gB, gD, gE, and gK in quantities approaching, or exceeding, those observed in HSV-infected cells (see Fig. 6.3 & 10.3). As expected, decreasing the Ad vector MOI caused a reduction in the accumulation of HSV-1 proteins. (Johnson et al., 1988; Table 3.3). Laser densitometry of preflashed autoradiographs derived from quantitative immunoprecipitation assays indicated that decreasing the Ad vector MOI from 1000 to 30 PFU/cell caused a 5 to 13 fold reduction in the expression of individual HSV-1 glycoproteins (Table 3.3). Infections involving intermediate MOIs yielded intermediate levels of protein synthesis.

In other experiments, Vero cells were infected with Ad vectors at an MOI of 30 PFU/cell and labelled from 1 to 6 hr, or 7 to 12 hr, or 11 to 18 hr, or 19 to 24 hr with [^{35}S]Met-[^{35}S]Cys. These conditions produced small amounts of gB after 6 hrs of infection, whereas gC, gD, gE, gH, gI, and gK, did not appear until 12 hr post-infection (data not shown). The quantity of gC, gD, gE, gI, and gK expressed by Ad vector-infected cells continued to increase until the final time point of 24 hr, but the rate of gB and gH synthesis levelled off by 12 hr or 18 hr, respectively (data not shown).

TABLE 3.3: Expression of HSV-1 Glycoproteins after infection with Ad vectors

Adenovirus[a] vector	Glycoprotein[b] measured	Relative expression[c] (arbitrary units)		Ratio
		MOI 30	MOI 1000	MOI 1000:MOI 30
AdgB	gB	11826	72141	6.1
AdgD	gD	5781	75160	13.0
AdgK	gK	9002	45012	5.0
AdgE	gE	8960	43008	4.8

[a] Vero cell monolayers were infected with AdgB, AdgD, AdgK, or AdgE using 30 or 1000 PFU/cell then labelled with [3^5S]-methionine and [^{35}S]-cysteine between 11.5 and 18 hr post-infection.

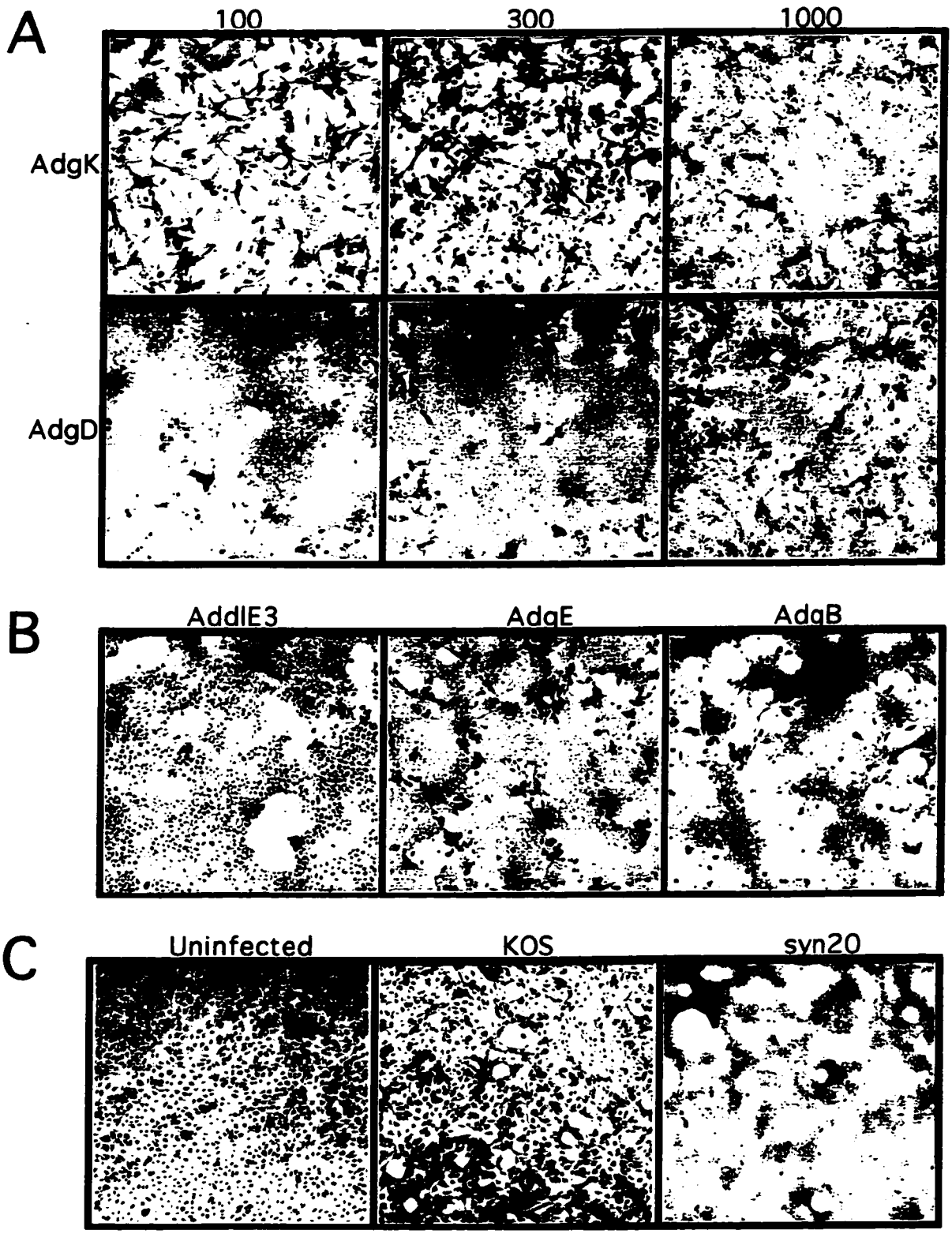
[b] Cell extracts were mixed with MAb 15ßB2 (specific for gB), MAb LP2 (specific for gD), anti-UL53-4 sera, or pooled anti-gE MAb's and protein A-Sepharose. Precipitated proteins were subjected to electrophoresis using 12% polyacrylamide gels.

[c] Densitometric analysis was performed to compare the levels of individual HSV-1 glycoproteins. Since different film exposure times were required for individual glycoproteins, comparisons between infections using different m.o.i.s are valid but comparisons of the relative levels of individual glycoproteins (e.g., gB vs gD) are not.

Expression of gK^{wt} or gB^{wt} inhibits fusion by HSV-1 syncytial mutants. Vero cell monolayers were infected with Ad vectors using 30, 100, 300, and 1000 PFU/cell (Time 0) and 6 hr later the cells were coinfected with HSV-1 (Syn20) at 5 PFU/cell. 18 hr after initiating the Ad vector infection, the cell monolayers were fixed and stained with crystal violet, prior to photography.

AdgK inhibited cell-cell fusion caused by the gK^{syn} mutant HSV-1 (Syn20), in a dose dependent manner, suppressing cell-cell fusion completely when the AdgK infection consisted of 1000 pfu/cell and to a lesser degree when an MOI of 300 or 100 pfu/cell was used (Fig. 7.3A). When Vero cells were subjected to AdgK (30 PFU/cell) prior to the Syn20 infection, all or most of the cells fused (results not shown). In contrast to the effect produced by AdgK, preinfection of Vero cells with 1000 pfu/cell of AddlE3, AdgE, AdgB (Fig. 7.3B), AdgC, AdgI, or AdgH in conjunction with AdgL, did not reduce the amount of Syn20-induced cell-cell fusion. In fact, the AdgH:AdgL coinfection seemed to enhance the syncytial phenotype of HSV mutants expressing gK^{syn} or gB^{syn}, (not shown) and this observation is consistent with the discovery that HSV-1 gH-deletion mutants form syncytial plaques on a complementing cell line which produces gH during the early phase of the replication cycle (Forrester et al., 1992). In control samples, Vero cells infected with wild-type HSV-1 (KOS) were rounded and in some cases displayed signs of cyto-aggregation but little or no fusion was observed (Fig. 7.3C). Infection of cells with AdgD also caused a reduction in Syn20-induced cell-cell fusion, but the effect was partial requiring higher MOIs, and appeared to arise from a block in virus entry known as gD-interference (Campadelli-Fiume et al., 1988; Johnson et al., 1989). Support for this hypothesis comes from the observation that gD was detected in AdgD infected cells when the HSV virions were introduced to cells (6 hr post-infection)(Fig. 6.3) and because gD

Figure 7.3 Effects of Adenovirus vectors on the morphology of Vero cells coinfected with HSV-1 (syn20). (A) Vero cell monolayers seeded on glass coverslips coated with poly-D-lysine, were first infected with Adenovirus vectors (AdgK, AdgD) using 100, 300, and 1000 PFU/cell or B) (AddlE3, AdgE, AdgB) using 1000 PFU/cell. After 6 h the Vero cell monolayers were coinfected with 5 PFU/cell of HSV-1 (Syn20) and incubated an additional 12 h. C) For comparison, Vero cells were infected with HSV-1 (KOS) or HSV-1 (syn20) using 5 PFU/cell and incubated for 12 h at 37°C or left uninfected (Uninfected). Cells were fixed and stained with crystal violet and photographed using a Zeiss microscope at 100X magnification.

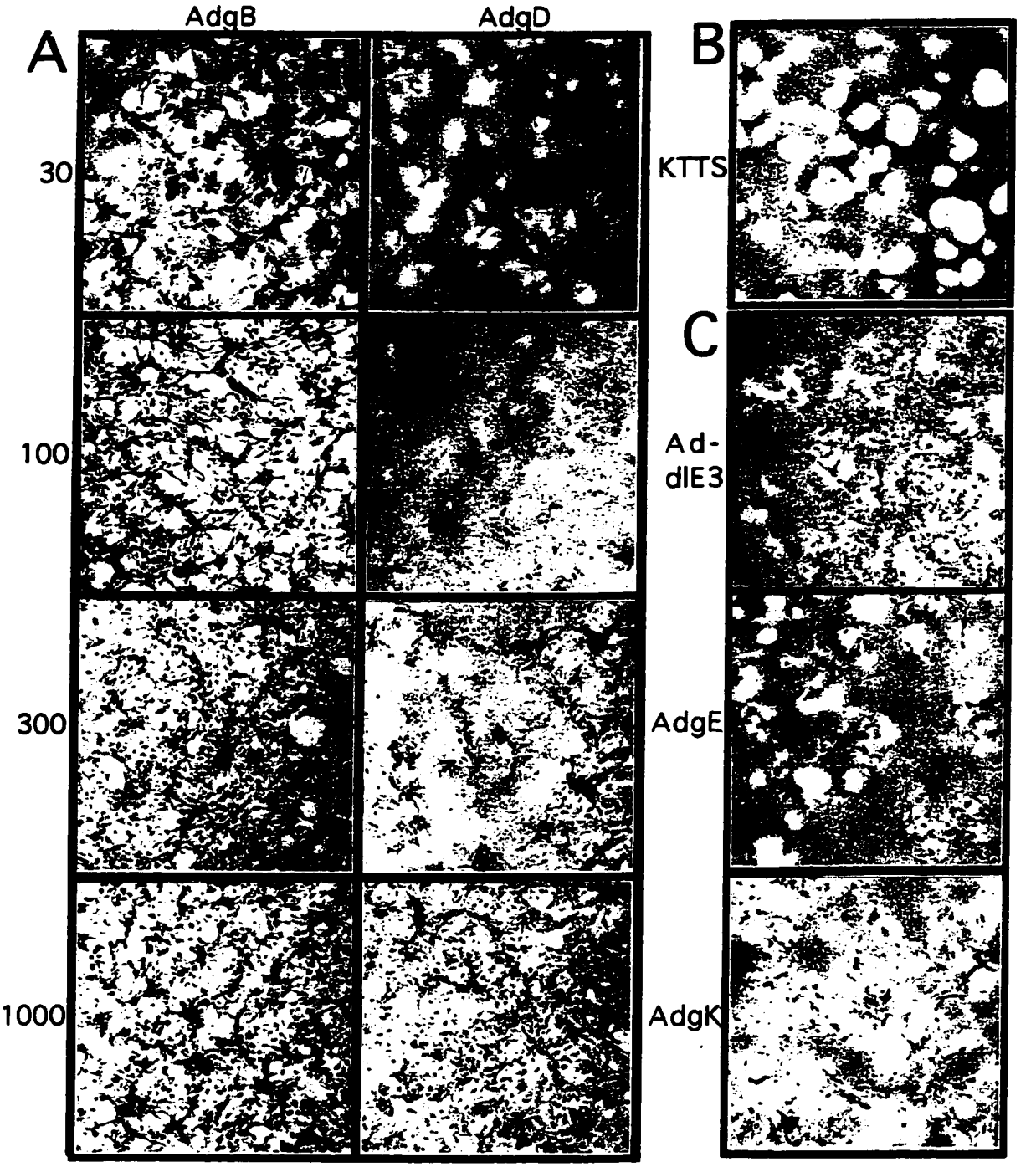

A
100
300
1000
AdgK
AdgD
B
AddlE3
AdgE
AdgB
C
Uninfected
KOS
syn20

prevented HSV-1 protein synthesis (see below).

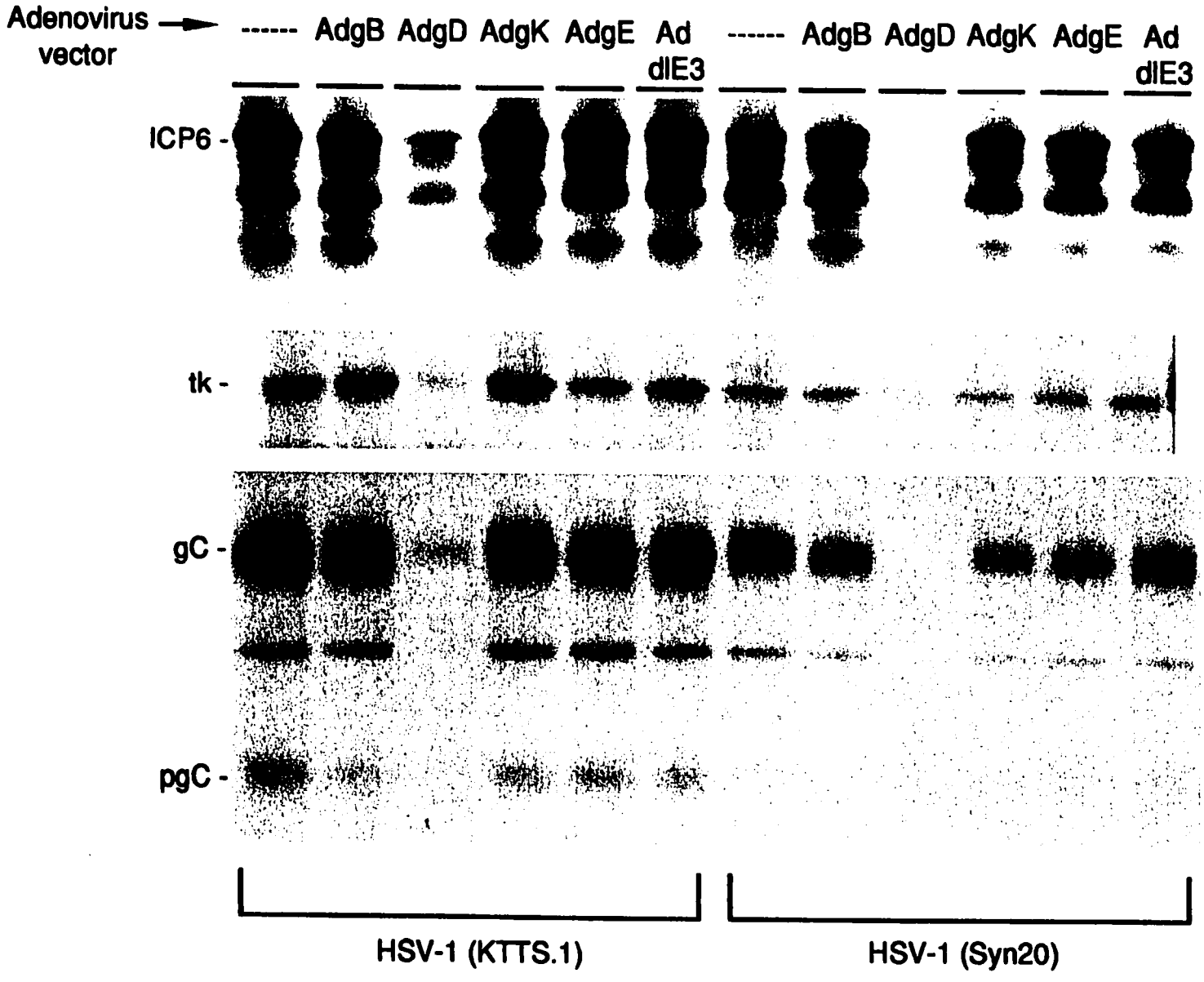
Although AdgB infection did not block fusion induced by the gK^{syn} mutant HSV-1 (Syn20), we were interested in the possibility that gB^{wt} expression might suppress fusion induced by KTTS.1, which encodes gB^{syn}. The entire Vero cell monolayer developed into a single giant multi-nucleated cell during the 12 hr period after infection with KTTS.1 (Fig. 8.3B), but the fusion induced by KTTS.1 was completely suppressed by preinfecting cells with 100 PFU/cell of AdgB (Fig 8.3A). Like the mixed infection containing AdgK & Syn20, coinfection of cells with AdgB & KTTS.1 resulted in a cell morphology reminiscent of wild-type HSV-1 infections (ie. cell rounding and clumping) (Fig. 7.3A, 7.3C, 8.3A). Again, preinfection of Vero cells with 300 or 1000 PFU/cell of AdgD reduced both cell fusion and cyto-aggregation suggesting that gD synthesis prior to HSV-infection can block virus infection (Fig. 8.3A). In contrast, Vero cells preinfected with 1000 PFU/cell of AddlE3, AdgE, AdgK (Fig 8.3C), AdgC, AdgI, AdgL, or AdgH plus AdgL (not shown) and coinfected with KTTS.1 fused extensively, indicating that these HSV-1 glycoproteins do not have the capacity to reduce KTTS.1-induced cell fusion.

Adenovirus vector replication does not affect the expression of HSV-1 proteins. It was formally possible that preinfection with Ad vectors, especially at high input MOIs might inhibit HSV replication or the production of viral proteins required for cell-cell fusion. In the early stages of the adenovirus life cycle, infections do not normally lead to marked host shut off or inhibition of HSV replication and the conditions employed in these experiments took advantage of this observation. For instance, no obvious differences in cell morphology were observed during the 18 hr Ad-vector infection, even though MOIs of 1000 were used. HSV-1 protein synthesis was also used as a criteria to determine

Figure 8.3. Effects of Adenovirus vectors on the morphology of Vero cells coinfected with HSV-1 (KTTS.1). (A) Vero cell monolayers, seeded on glass coverslips coated with poly-D-lysine, were first infected with Adenovirus vectors (AdgB, AdgD) using 30, 100, 300, and 1000 PFU/cell or (C) (AddlE3, AdgE, AdgK) using 1000 PFU/cell. After 6 hr the Vero cell monolayers were coinfected with 5 PFU/cell of HSV-1 (KTTS.1) (which expresses syn-gB) at 5 PFU/cell and incubated an additional 12 h. (B) For comparison, Vero cells were infected with HSV-1 (KTTS.1) using 5 PFU/cell and incubated for 12 h at 37°C. Cells were fixed and stained with crystal violet and photographed using a Zeiss microscope at 100X magnification.


AdgB
AdgD
A
30
100
300
1000
B
KTTS
C
Ad-dlE3
AdgE
AdgK

whether the decrease in HSV-induced membrane fusion produced by AdgB, AdgK, and AdgD involved repression of HSV-1 replication. We analyzed the synthesis of representative HSV-1 proteins derived from the major kinetic classes (α, ß and τ) expressed by HSV-1: ICP6, thymidine kinase (tk) and the true late τ_2-protein gC, to assess the effect of Ad vector replication on HSV-1 protein synthesis. Initially, Vero cells were infected with individual Ad vectors using an MOI of 1000 PFU/cell and to provide a standard base line for HSV-1 replication, the preinfection step was omitted from some dishes. After 6 hr the cells were infected with HSV-1 strain Syn20 or KTTS.1 (5 PFU/cell) and incubated another 4 hr before the cells were labelled metabolically with [^{35}S]Met-[^{35}S]Cys for 8 hr. Cell extracts were prepared and the HSV-1 proteins ICP6, thymidine kinase (tk), and gC were quantitatively immunoprecipitated. The overall levels of tK and gC were somewhat lower in Syn20-infected cells than in KTTS.1-infected cells and may reflect strain differences. With the exception of AdgD, preinfection with Ad vectors (ie. AdgB, AdgK, AdgE, or AddlE3) had little or no effect on the amount of ICP6, tk and gC produced by HSV-1 strains Syn20 or KTTS.1 (Fig. 9.3). In cells coinfected with AdgB, AdgK, AdgE, or AddlE3, laser densitometry analysis confirmed that the expression of HSV-1 proteins ICP6, and tk did not vary by more than 1.5 fold, above or below, the base line observed for cells containing only HSV-1 Syn20 or KTTS.1 (not shown). Mixed infections including syn20 and AdgB, or AdgK or AdgE or AddlE3, caused a small decrease in gC expression relative to syn-20 infected cells (Fig. 9.3). However, we do not expect that this small decrease in gC synthesis accounts for the substantial decline in gK^{syn} induced cell-cell fusion associated with the presence of AdgK, especially because AddLE3, AdgB, AdgE (Fig. 7.3B), AdgC, AdgI, and AdgH plus AdgL (not shown) did not reduce cell fusion. Introducing AdgD to cells prior to the HSV-1


Adenovirus vector

AdgB
AdgD
AdgK
AdgE
Ad dlE3

AdgB
AdgD
AdgK
AdgE
Ad dlE3
ICP6 -
tk -
gC -
pgC -
HSV-1 (KTTS.1)
HSV-1 (Syn20)

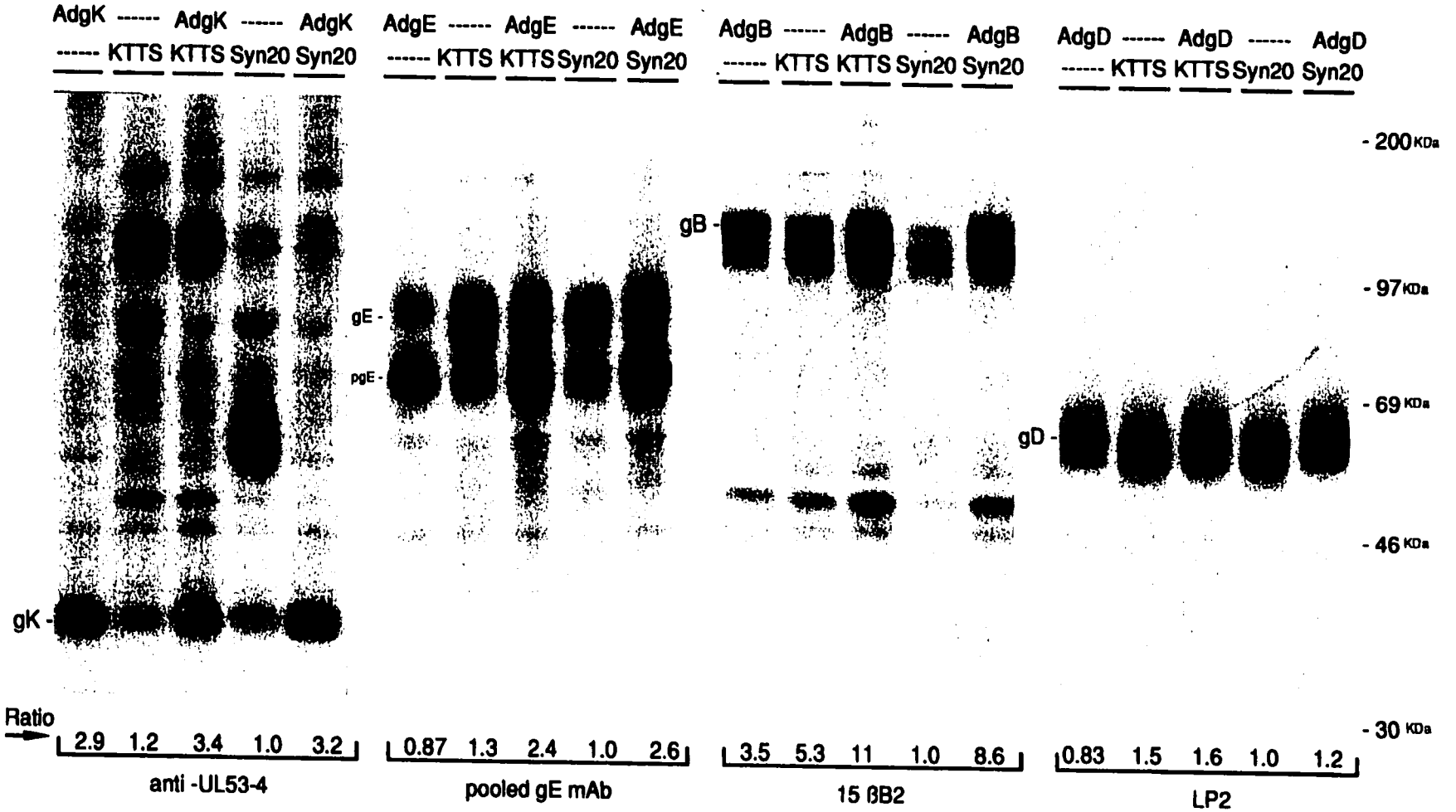
Figure 9.3 Expression of HSV-1 proteins when cells are preinfected with Adenovirus vectors. Vero cells were mock infected (---) or infected with the Adenovirus vectors AdgB, AdgD, AdgK, AdgE or AddlE3 using 1000 PFU/cell and 6 hours later, all cells were infected with HSV-1 (KTTS.1) or HSV-1 (Syn20) using 5 PFU/cell. After an additional 4 h incubation at 37°C, the cells were labelled for 8 h with ^{35}S-methionine and ^{35}S-cysteine. Extracts from infected cells were immunoprecipitated using a polyclonal rabbit serum specific for ICP6, rabbit anti-tk sera, or pooled anti-gC MAbs. Precipitated proteins were eluted with 2X sample buffer at 37°C for 30 min and analyzed using 12% polyacrylamide gels.

infection, resulted in low levels of ICP6, tk and gC protein synthesis, suggesting that the presence of gD in cellular membranes (Fig. 6.3) inhibited entry of HSV-1 into cells as has been previously reported (Campadelli-Fiume et al., 1988; Johnson & Spear, 1989).

In complementary experiments we monitored the level of the 72-kDa Ad5 DNA binding protein produced by AdgB, AdgD, AdgE, AdgK and AddlE3 in cells coinfected with HSV-1. Superinfection with Syn20 had little or no effect on the production of this protein whereas KTTS.1 caused a modest reduction in the levels of the 72-kDa protein observed in Ad vector-infected cells.

Production of HSV-1 glycoproteins in cells coinfected with recombinant Ad vectors and HSV-1 syncytial strains. Since Ad vector replication did not interfere with HSV-1 replication it seemed likely that it was the HSV-1 glycoproteins produced by Ad vectors which inhibited the cell-cell fusion associated with gB^{syn} or gK^{syn}. Furthermore, the specificity with which AdgK inhibited cell fusion induced by the gK^{syn} mutant, Syn20, AdgB inhibited cell fusion induced by the gB^{syn} mutant, KTTS.1, and AdgD universally reduced HSV-induced cell fusion and protein synthesis, is compatible with this hypothesis. To verify that Ad vectors produce HSV-1 glycoproteins in the quantities which would be expected to suppress HSV-induced cell fusion, we compared the quantity of gB, gK, gE and gD produced by Ad vectors alone, or in conjunction with HSV-1, to the levels observed in cells infected with HSV-1 alone. Vero cells were coinfected with HSV-1 and Ad vectors using the protocols used to inhibit cell fusion and the cells were labelled with [^{35}S]Met-[^{35}S]Cys for 8 hr. This experiment revealed a number of universal trends. In every case, cells infected with one of the Ad vectors and then with HSV-1 yielded more of the relevant glycoprotein than cells infected with HSV-1 alone (Fig. 10.3).

Figure 10.3 Expression of gK, gE, gB, and gD in cells infected with HSV-1 alone, Adenovirus vectors alone, or in coinfections. Vero cell monolayers were infected AdgK, AdgE, AdgB or AdgD at 1000 PFU/cell or left uninfected (-----). 6 hours later these cells were infected with HSV-1 (KTTS.1) or HSV-1 (Syn20) using 5 PFU/cell or left uninfected (-----). After an additional 4 hr, all cells were radiolabelled with ^{35}S-methionine and ^{35}S-cysteine for 8 h. Cell extracts were immunoprecipitated using an excess (relative to the antigen available) of the following antibodies; rabbit anti-UL53-4 peptide sera, pooled anti-gE MAbs (481-II, 3114, 7520), MAb 15ßB2 specific for gB or LP2 specific for gD, and an excess (relative to the Ab available) of protein A-sepharose. Antigen-antibody complexes were eluted at 37°C for 30 min and subjected to electrophoresis on 12% polyacrylamide gels. To quantify the relative amounts of HSV glycoproteins expressed in cells (Ratio), preflashed Xray film as used for autoradiography and laser densitometry as performed. Densitometry data for the precursor and mature forms of gE, gB, and gD was grouped together to formulate these ratios. HSV-1 glycoproteins gK, gE, gB, and gD and molecular mass markers of 200, 97, 69, 46 and 30 KDa are indicated.


AdgK ------ AdgK ------ AdgK
------ KTTS KTTS Syn20 Syn20
AdgE ------ AdgE ------ AdgE
------ KTTS KTTS Syn20 Syn20
AdgB ------ AdgB ------ AdgB
------ KTTS KTTS Syn20 Syn20
AdgD ------ AdgD ------ AdgD
------ KTTS KTTS Syn20 Syn20
- 200 KDa
- 97 KDa
- 69 KDa
- 46 KDa
- 30 KDa
gK -
gE -
pgE -
gB -
gD -
Ratio
2.9 1.2 3.4 1.0 3.2
anti -UL53-4
0.87 1.3 2.4 1.0 2.6
pooled gE mAb
3.5 5.3 11 1.0 8.6
15 ßB2
0.83 1.5 1.6 1.0 1.2
LP2

Coinfection of cells with AdgB and syn20 led to a dramatic increase in the gB^{wt} present in the syn20 infection, primarily because the cells infected with syn20 alone produce low levels of gB relative to other HSV-1 glycoproteins and compared to the level of gB synthesized by KTTS.1-infected cells (Fig. 10.3) and KOS-infected cells (data not shown). Nevertheless, overexpression of gB^{wt} did not suppress the cell-cell fusion associated with the gK^{syn} mutant HSV-1 (Syn20) and actually appeared to enhance syn20-induced cell fusion (data not shown). Although gB expression in cells coinfected with AdgB and KTTS.1 was only slightly higher (twofold) than the level produced in KTTS.1-infected cells, the gB^{wt} present in these co-infected cells suppressed the cell-cell fusion associated with the gB^{syn} mutant, KTTS.1 (Fig. 8.3). Since we have demonstrated that Ad vectors do not halt the expression of HSV-1 proteins, we suspect that equal amounts of gB^{wt} and gB^{syn} are expressed in cells coinfected with AdgB and KTTS.1. Further, we observed that cells coinfected with AdgK and syn20 contain approximately threefold more gK than the quantities present in cells infected with syn20 alone (Fig. 10.3) (and about two-thirds of this protein is expected to be gK^{wt}) and this was sufficient to suppress syn20-induced cell-cell fusion (Fig. 7.3). Comparable overexpression of gK^{wt} in cells coinfected with AdgK & KTTS.1 did not inhibit the cell fusion caused by the KTTS.1, a gB^{syn} mutant (Fig. 8.3). Note that the gD in Fig. 10.3, lane 4 (syn20, anti-UL53-4) is present because the immunoprecipitation was contaminated with anti-gD antibody and did not affect the level of gK present.

The electrophoretic mobility of HSV-1 glycoproteins B, D, and E produced by Ad vector-infected cells, differed from that of proteins synthesized in cells infected by HSV-1 alone. However, differences of this type have been noted previously, and these studies have demonstrated that the HSV-1 glycoproteins expressed using Ad vectors, retain

biological function (Johnson et al., 1988; Witmer et al., 1990; Hanke et al., 1991). In this study, only gK exhibited the same electrophoretic mobility regardless of whether gK was obtained from cells infected with the Ad vector or HSV-1. Furthermore, the oligosaccharide processing of glycoproteins B, D, and E differed in cells infected with HSV-1 or the relevant Ad vector. Immature gE was the predominant form detected in AdgE-infected cells, whereas mature gE was the principal form observed in HSV-infected cells. In contrast, the majority of gB produced by AdgB infected cells had been processed to the mature form, but similar amounts of immature and mature gB were found in HSV-infected cells and analogous differences were observed with gD. These observations may result from differences in the impact of HSV and Ad vectors on post-translational modification and transport of HSV glycoproteins. We also noticed an striking reduction in the quantity of gB expressed in syn20-infected cells compared to the synthesis of other HSV glycoproteins (Fig. 10.3). Person et al. (1982) previously noted that the gB processing and that of other HSV-1 glycoproteins was reduced in cells infected with syncytial HSV-1 viruses, but it is not clear whether these results are related.

Discussion

The fusion induced by three HSV-1 strains containing syn mutations in the UL53 (gK) gene was inhibited by expressing gK^{wt} in cells but not by expressing wild-type gB. Conversely, fusion induced by an HSV-1 mutant which expresses a syncytial form of gB was inhibited by expressing gB^{wt} but not by expressing wild-type gK. As such, the recessive phenotypes displayed by HSV-1 strains expressing gB^{syn} or gK^{syn} are characteristic of loss-of-function mutations. These results are consistent with previous

observations involving mixed infections with wild-type HSV-1 and HSV-1 strains expressing gK^{syn} (Read et al., 1980; Bond et al., 1982; Bzik and Person, 1981); however, here, the effects can be directly attributed to the gK protein and we have extended the analysis to include coexpression of gB^{wt} with gB^{syn}. Quantification of the levels of gK^{wt} and gB^{wt} required for the suppression of cell-cell fusion suggested that wild-type gK and gB were required at levels similar those observed in HSV-infected cells. For example, when cells were infected with AdgK, using 30 PFU/cell, expression of gK was 5-fold lower than when cells were infected with AdgK using 1000 PFU/cell and fusion induced by Syn20 (gK^{syn}) occurred at the lower MOI but not the higher AdgK MOI. Syn-20-induced fusion was inhibited on stable cell lines overexpressing gK. Furthermore we observed a dramatic reduction in HSV-1 plaqueing efficiency on these cells. This result might not have been unexpected because these cells overexpress gK by 15- to 17-fold relative to HSV-infected cells and the timing of expression of gK may be different, especially with gK-9 cells where the gD promoter was used. However, when plaques were produced on these cells by gK^{syn} mutants, the cells bordering plaques became rounded and clumped and were not fused. Overexpression of gB^{wt} by as much as 11-fold using AdgB had no effect on fusion induced by the gK mutant, Syn20, and overexpression of gK^{wt} by 15- to 17-fold in the transfected cell lines had no effect on fusion induced by the gB^{syn} mutant, KTTS.1. These observations argue that cell fusion is inhibited, not simply by overexpressing either of the wild-type glycoproteins but by correcting the deregulation of membrane fusion brought about by mutations in either protein. In addition, overexpression of any of the other HSV-1 glycoproteins, gC, gD, gE, gH/gL, or gI did not lead to suppression of membrane fusion, which lends credence to the hypothesis that gB and gK play direct roles in membrane fusion.

Previously, evidence has been presented that gB functions as a dimer or oligomer (Claesson-Welsh and Spear, 1986; Cai et al., 1988a; Highlander et al., 1991). Mutant forms of gB have been shown to interfere with gB^{wt} in complementation assays, indicating that these mutant forms were dominant (Cai et al., 1988a). Therefore, it was interesting that gB^{wt} could suppress fusion induced by gB^{syn} because the formation of oligomers containing mutant and wild-type gB molecules, would be expected. There are a number of possible explanations: (i) The gB^{syn}, altered in only a single residue was able to function in virus entry, unlike the mutant forms of gB described by Cai et al (1988a) which did not complement a gB^- virus. Thus, gB^{wt}/gB^{syn} hetero-oligomers might function normally and produce wild-type plaques. (ii) gB^{wt} may accumulate in AdgB-infected cells prior to infection with HSV-1, and by doing so, modify the cellular membranes to inhibit fusion before gB^{syn} is synthesized by the KTTS.1 infection.

It is by no means clear how gB and gK contribute to the fusion process. One model suggests that gB acts to induce fusion and gK suppresses or regulates the fusion process (see Introduction section.). This model is supported by evidence that gB participates directly in the fusion process including the observations that mutations in gB can affect the rate of entry of virus, produce the syn phenotype, or block virus entry into cells. If gB plays a direct role in HSV-induced membrane fusion as the findings to date suggest (see Introduction), and gB^{syn} is recessive to gB^{wt} as our data indicates, then it seems likely that the syncytial mutations which map to the cytosolic domain of gB (Bzik et al., 1984; Cai et al., 1988b, Weise et al., 1987; Baghian eet al., 1993; Gage et al., 1993), disrupt a regulatory domain which governs gB function (eg. innate fusogenic activity, or an ability to trigger the HSV-1 fusogenic machinery).

However, the vast majority of syn mutations, in at least some HSV-1 strains, map

to the UL53-gK gene (Read et al., 1980; Little & Schaffer, 1981; Bond et al., 1982; Bond & Person, 1984; Roop et al., 1993) supporting a central role for gK in fusion of infected cells. Person et al. (1976) reported that cell fusion begins 4-6 hr after infection and ends by 6-8 hr after infection in cells infected with wild-type HSV-1; whereas, fusion continues unabated until 10-12 hr in cells infected with gK^{syn} mutants. In addition, cells infected with gK^{syn} mutants fuse efficiently with uninfected or gK^{syn} mutant-infected cells, but not with wild-type HSV-infected cells (Keller, 1976; Lee & Spear, 1980). Therefore, one might hypothesize that gK^{syn} is unable to regulate the fusion process in these cells. Consistent with this hypothesis is the observation that gK appears to be expressed at low levels relative to other HSV-1 glycoproteins within infected cells (Hutchinson et al., 1992b). This finding suggests that gK may function in a catalytic or regulatory role to modulate membrane fusion, rather than acting as a structural protein directly involved in the fusion process, as is indicated for gB, gD and gH (Gompels & Minson, 1986; Cai et al., 1987; 1988; Ligas & Johnson, 1988; Forrester et al., 1992). Tight regulation of membrane fusion makes ample sense so that infected cells do not inappropriately fuse with uninfected cells and, by so doing, compromise virus replication. Since fusion begins relatively early in the replicative cycle, it appears likely that HSV transcriptional, translational, and packaging strategies would be negatively affected by the recruitment of uninfected cells into a polykaryocyte. Supporting this view, gK^{syn} mutants frequently produce lower titres of infectious virus and syncytial viruses are infrequently observed in clinical samples (Hoggan & Roizman, 1959; Ejercito et al., 1968; Read et al., 1980; Bzik et al., 1981).

There has been some speculation in the field (mainly unpublished) that the UL53 gene product, gK, might interact with other HSV-1 glycoproteins, eg., gB to reduce the

fusion capacity of these proteins. The data presented here is consistent with a model in which gK and gB interact, but also with models where the two proteins act independently in cell fusion. In certain instances we have observed gB among proteins which were immunoprecipitated in conjunction with gK using anti-peptide serum (see for example Fig. 10.3; left panel). However, these results should be viewed with some caution because gB forms oligomeric structures (Sarmiento and Spear, 1979; Claesson-Welsh and Spear, 1986; Cai et al., 1988a; Highlander et al., 1991) and frequently contaminates immunoprecipitated material.

Recently, we constructed HSV-1 mutant viruses unable to express gK by using gK expressing Vero cell lines to complement the mutant viruses (Hutchinson and Johnson, 1995). Studies of these mutants have shown that UL53-gK is required for efficient virus replication in cultured cells and gK-mutants show defects in virus egress. These observations are similar to those of Baines and Roizman (1991) who found that a UL20 mutant had a syn phenotype and was defective in viral egress. Thus, a second model explaining cell fusion induced by syn viruses would suggest that imbalances in the subcellular distributions of various HSV glycoproteins can be elicited by transport defects involving the gK or UL20 protein. By this model, fusion might be induced because, for example, the plasma membrane is enriched with gB or some fusion protein and not with other viral proteins or structures which suppress fusion.

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CHAPTER 4

PUBLISHED MANUSCRIPT

Hutchinson, Lloyd, Cindy Roop-Beauchamp, and David C. Johnson. (1995) Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. J. of Virology. 69:4556-4563.

PREAMBLE

The observation that UL53-gK exists as a single 40-kDa protein, whereas other HSV-1 glycoproteins are found as two protein species differing in N-linked and O-linked oligosaccharides, raised the possibility that gK is not processed in the Golgi apparatus. Since UL53-gK has been implicated in HSV-induced cell-cell fusion, we wished to discover if gK ever reaches the Golgi apparatus or the cell surface because subcellular distribution will determine if gK plays a direct or indirect role in the membrane fusion process. Immunofluorescence microscopy was used to examine the subcellular localization of gK and an endoglycosidase H digestion was used to evaluate the processing of gK N-linked oligosaccharides. The low levels of gK expressed by HSV-infected cells represented the greatest technical difficulty encountered in these studies.

Herpes Simplex Virus Glycoprotein K Is Known To Influence Fusion of Infected Cells, yet Is Not on the Cell Surface

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Syncytial mutants of herpes simplex virus (HSV) cause extensive fusion of cultured cells, whereas wild-type HSV primarily causes cell rounding and aggregation. A large fraction of syncytial viruses contain mutations in the UL53 gene, which encodes glycoprotein K (gK). Previously, we demonstrated that wild-type and syncytial forms of gK are expressed at similar levels and possess identical electrophoretic mobilities. Using immunofluorescence, we show that gK is not transported to the surfaces of cells infected with either wild-type or syncytial HSV. Instead, gK accumulates in the perinuclear and nuclear membranes of cells. This finding is in contrast to the behavior of all other HSV glycoproteins described to date, which reach the cell surface. When gK was expressed in the absence of other HSV proteins, using a recombinant adenovirus vector, a similar perinuclear and nuclear pattern was observed. In addition, gK remained sensitive to endoglycosidase H, consistent with the hypothesis that gK does not reach the Golgi apparatus and is retained in the endoplasmic reticulum and nuclear envelope. Therefore, although gK mutations promote fusion between the surface membranes of HSV-infected cells, the glycoprotein does not reach the plasma membrane and, thus, must influence fusion indirectly.

Entry of herpes simplex virus (HSV) into host cells is thought to involve fusion of the virion envelope with the plasma membrane (reviewed in reference 52). By what is thought to be an analogous process, newly synthesized viral glycoproteins appearing in the plasma membranes of infected cells can cause infected cells to fuse. Wild-type HSV type 1 (HSV-1) fuses cells rarely, whereas syncytial (syn) mutants of HSV-1 cause extensive cell fusion (14, 22, 40). In at least some virus strains, the majority of syn mutations map to the UL53 gene (3, 41, 44, 46, 48), which encodes the hydrophobic, multiple membrane-spanning glycoprotein gK (24, 43). Of the mutant UL53 genes that have been sequenced, all contained amino acid substitutions at residue 40 within the N-terminal extracellular domain of gK (12, 42), and we refer to these proteins as syncytial forms of gK. Apart from a few observations, little is known about how gK functions during virus replication or in membrane fusion. HSV-1-infected cells express relatively low levels of gK (24), and transfected cells or cells infected with recombinant adenovirus vectors expressing wild-type gK resist fusion induced by infection with syncytial mutants of HSV gK (25). The importance of gK in the replication of other herpesviruses is underscored by the observation that other members of the alphaherpesvirus family all share homologs of the protein (10, 11, 33, 39, 54, 57). HSV-1 expresses at least 10 other membrane glycoproteins, as well as additional membrane proteins which are not glycosylated (1, 2, 23, 24, 37, 51), and a subset of these proteins, gB, gD, and the gH-gL complex, are known to be essential for virus entry into cells and for cell fusion (6, 7, 15, 18, 36, 46).

The other HSV-1 glycoproteins described to date all appear in infected cells as two protein species, differing in N-linked and O-linked oligosaccharides, so that the immature form of the protein is processed to the mature form during transit through the Golgi apparatus (23, 32, 51, 52). In contrast, gK exists as a single 40-kDa protein species, which is modified with N-linked oligosaccharides, in cells infected with both wild-type and syncytial HSV-1 (24). Given this difference and the apparent role of gK in cell fusion, it was of interest to determine whether gK reaches the Golgi apparatus and the cell surface. In this report, we demonstrate that gK remains in an endoglycosidase H (endo H)-sensitive form and does not reach the surfaces of infected cells.

gK is retained in the perinuclear and nuclear membranes of HSV-1-infected cells. Immunofluorescence microscopy was used to evaluate the subcellular localization of gK and to compare this with that of gD, which is found in the plasma membrane and Golgi apparatus as well as the endoplasmic reticulum (ER) and nuclear membranes (8, 9, 29, 50). Immunofluorescence experiments were performed as previously described (21, 56), with the following modifications. Vero cells were cultured on glass coverslips and infected with HSV-1 (20 or 30 PFU per cell) for 9 to 14 h or left uninfected. All cells were washed with phosphate-buffered saline (PBS) (pH 7.2) containing 1 mM $MgCl_2$ (PBS-Mg), fixed with 2 or 4% paraformaldehyde for 10 min, and rinsed with PBS-Mg. To examine internal distribution of the proteins, the cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min (56), or for surface immunofluorescence, the cells were not permeabilized. Excess aldehyde groups were inactivated with 0.2 M ethanolamine, pH 7.5, for 1.5 h; the cells were washed with PBS-Mg containing 2% bovine serum albumin (BSA) (PBS-BSA) and incubated with PBS-BSA containing 2% goat serum for 1 to 3 h. Rabbit anti-gD serum (4) was diluted 1:500 or 1:1,000, or individual or pooled gK-specific antipeptide sera were diluted 1:200 in PBS-BSA and added to cells for 1 to 2 h. The gK-specific antipeptide antibodies are directed to hydrophilic domains in gK and were characterized in an earlier study (24). Anti-UL53-1 reacts with residues 31 to 46 of gK, anti-UL53-3 recognizes residues 273 to 289, and anti-UL53-4 recognizes residues 89 to 104. All primary antisera were extensively preadsorbed against fixed, permeabilized, uninfected

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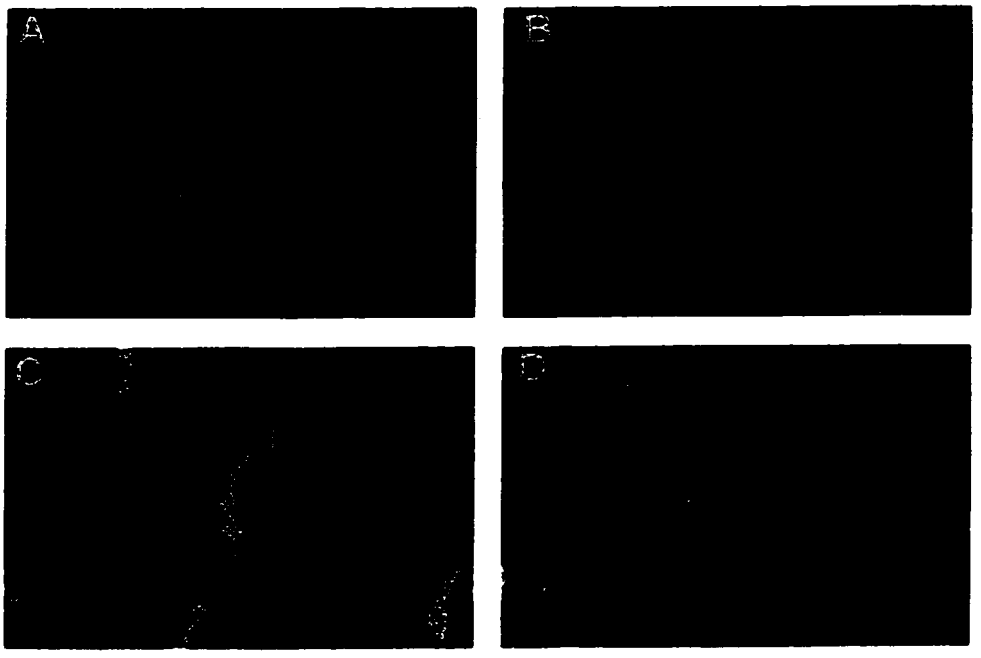

FIG. 1. gK is not transported to the surfaces of HSV-1-infected cells. Vero cell monolayers growing on glass coverslips were infected with HSV-1 (KOS) at 20 PFU per cell. After 12 h, all cells were fixed with 4% paraformaldehyde for 10 min and then either permeabilized with 0.2% Triton X-100 (A and C) or not permeabilized (B and D). The cells were incubated with a mixture of anti-UL53-1 and anti-UL53-4 antibodies (A and B) or rabbit anti-gD antibody (C and D) and subsequently washed with PBS-BSA before incubation with fluorescein-conjugated goat anti-rabbit IgG antibody. The coverslips were washed with PBS-BSA and mounted on glass slides. Photography was done using a Reichert fluorescence microscope.

Vero cells and then centrifuged at 18,000 × *g* for 1 h and filtered through a 0.2-μm-pore-size filter before use. After incubation with these primary antibodies, the cells were washed with PBS-BSA and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) serum (immunoaffinity purified; Jackson Immuno Research Laboratories, West Grove, Pa.) that had been diluted 1:60 in PBS-BSA. Coverslips were mounted on microscope slides, using 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) (21) or Vecta Shield fluorescence mounting medium (Vector Laboratories Inc., Burlingame, Calif.).

Both the cellular distribution (this study) and the relative abundance (24) of gK differed from those of gD. In permeabilized, HSV-1-infected cells, gD was found in nuclear and perinuclear regions of the cells, as well as distributed throughout cytoplasmic and surface membranes, and was associated with cellular processes (Fig. 1C). When the cells were not permeabilized there was also strong, granular, surface fluorescence (Fig. 1D). By contrast, gK was relatively localized almost exclusively to the perinuclear and nuclear membranes, and low levels of gK-specific fluorescence were detected in permeabilized cells (Fig. 1A); fluorescence was not detected on the cell surface when the cells were not permeabilized (Fig. 1B). The low levels of gK-specific fluorescence associated with permeabilized cells were consistent with previous observations that gK is expressed at low levels in HSV-infected cells (24). A small fraction of nonpermeabilized cells exhibited weak fluorescence after staining with anti-gK antibodies; however, the pattern of fluorescence was similar to that of permeabilized cells, i.e., a perinuclear pattern was observed, indicating that membrane damage had been caused, probably by HSV infection (not shown).

The results shown in Fig. 1 were obtained with a mixture of two antipeptide sera, anti-UL53-1 and anti-UL53-4. It was formally possible that only a single antipeptide antibody actually reacted with gK in these experiments and that the epitope for this antibody was masked as the protein matured. This might cause the antibodies to react with only the immature form of gK present in the ER and nuclear envelope but not with gK on the plasma membrane. Therefore, we examined whether each of the individual anti-gK antibodies could react individually with gK in HSV-infected cells. In addition, we considered the possibility that the fluorescence pattern obtained with the anti-gK antibodies was affected by binding of the Fc domain of rabbit antibodies to the HSV-1 Fc receptor. Therefore, we used the HSV-1 gE-negative mutant, F-gEβ, which does not have detectable Fc receptor activity (13). When HSV-1-infected Vero cells were fixed, permeabilized, and then stained with either anti-UL53-1, anti-UL53-3, or anti-UL53-4 serum, a pattern of fluorescence similar to that observed in Fig. 1 was obtained (not shown). However, the fluorescence produced by individual antisera was lower than that with pooled sera, making photography difficult. Increasing the concentration of individual antisera had little effect, suggesting that each antibody contributed to the fluorescent signal in an additive fashion.

To provide evidence that all three antipeptide antibodies individually reacted with gK and to rule out the possibility that a small fraction of gK was on the cell surface, we used a Vero cell transformant (gK-9) which contains ~200 copies of the UL53-gK gene and expresses 10-fold more gK after infection with HSV-1 than is observed in comparably infected Vero cells (25). gK-9 cells were infected with F-gEβ, fixed, and then permeabilized and stained with either anti-UL53-1, anti-

UL53-3, or anti-UL53-4 antibody. All three anti-gK antibodies produced a pattern characterized by a perinuclear ring extending into the cytoplasm from the nuclear envelope, and there was an absence of staining of the plasma membrane and cell processes (Fig. 2A, C, and E). There was little or no fluorescence when nonpermeabilized, F-gEβ-infected gK-9 cells were stained with individual anti-gK antibodies (Fig. 2B, D, and F), and the low level of fluorescence that could occasionally be detected was not significantly different than that observed with preimmune sera (Fig. 2J). By contrast, gD was associated with the plasma membrane and cellular processes as well as being localized to perinuclear and nuclear membranes of HSV-infected gK-9 cells (Fig. 2G and H).

Since the anti-UL53-1 and anti-UL53-4 peptide sera are directed toward sequences in gK flanking the N-linked glycosylation signals (24, 43), we would expect that the antibodies would detect gK on the cell surface. Moreover, glycosylation does not interfere with the ability of these antisera to react with gK (24). Similar results were obtained when cells were stained with anti-gK antibodies prior to fixation (results not shown). Therefore it appears highly unlikely that the epitopes for all three antipeptide sera are lost during maturation of gK; this was supported by pulse-chase experiments in which anti-UL53-4 antibody (see Fig. 5) and anti-UL53-1 and anti-UL53-3 antibodies (not shown) reacted with gK produced after a relatively long chase period. With permeabilized gK-9 cells, the intensity of fluorescence obtained with individual and pooled anti-gK antipeptide sera was similar to that observed with the anti-gD antibodies: both antibodies produced intense fluorescence. Therefore, we would have expected to easily detect gK on the cell surface, if a significant fraction of the protein was present there.

Syncytial forms of gK are not expressed on the surfaces of infected cells. Two independently isolated HSV-1 syncytial strains, MP (22) and syn-20 (44), possess mutations affecting a single amino acid in the UL53 open reading frame (12, 42) and express gK proteins which exhibit electrophoretic mobilities similar to that of wild-type gK (24). To determine if syncytial mutations alter the subcellular distribution of gK, Vero cells were infected with MP and immunofluorescence analysis was performed as described above. The subcellular distribution of gK was more difficult to discern in these experiments; however, gK-specific fluorescence was observed in a region surrounding the clumped nuclei of MP-infected cells when the cells were permeabilized (Fig. 3A). No fluorescence, above the background observed with preimmune serum (Fig. 3F), was observed when nonpermeabilized, MP-infected cells were stained with anti-gK antibodies (Fig. 3B). Again, gD was associated with both perinuclear and surface membranes (Fig. 3C), and nonpermeabilized cells displayed strong gD-specific fluorescence (Fig. 3D). Similar results to those shown for HSV-1 MP were obtained with HSV-1 syn-20 (not shown). Therefore, it appears that the mutations in MP and syn-20 gK do not grossly affect intracellular distribution of the protein, at least not sufficiently so as to promote their transport to the cell surface.

gK expressed in the absence of other HSV-1 proteins is perinuclear. Earlier studies with another HSV glycoprotein, gH, demonstrated that this protein must form a complex with a second glycoprotein, gL, in order to be transported to the cell surface (16, 23, 45). Therefore, it was of interest to determine whether the retention of gK in HSV-infected cells was related to the expression of other HSV polypeptides. HSV glycoproteins expressed by adenovirus vectors exhibit targeting and functional properties similar to glycoproteins expressed by HSV (20, 23, 25, 29). We used an adenovirus vector, AdgK (24, 25), to express gK in the absence of other HSV proteins. Vero cell monolayers were infected with AdgK (1,000 PFU per cell) for 27 h or with AddlE3, a control adenovirus lacking E3 sequences (19). Previously, we demonstrated that under these conditions of infection, AdgK produces approximately three times the quantity of gK normally expressed in Vero cells infected with HSV for 12 h (25). Twenty-seven hours after infection is relatively early in the replicative cycle of adenovirus, and cytopathic effects were not noted, though a relatively high multiplicity of infection (1,000 PFU per cell) of AdgK was used. Anti-gK antibodies stained the perinuclear and nuclear membranes of permeabilized, AdgK-infected cells (Fig. 4A); this was not observed in AddlE3-infected cells (Fig. 4C). There was no gK on the surfaces of AdgK-infected cells (Fig. 4B). Therefore, it appears that gK is localized to perinuclear and nuclear membranes without a requirement for expression of other HSV polypeptides.

N-linked oligosaccharides on gK are not processed. To investigate the intracellular transport of gK further, we characterized posttranslational processing of gK oligosaccharides by using endo H, which removes immature, high-mannose oligosaccharides but not mature, complex oligosaccharides produced after glycoproteins move into the Golgi apparatus (34). Radiolabelling of cells, immunoprecipitations, and gel electrophoresis have been described previously (23–25) and were performed with modifications described in the legend to Fig. 5. After cells were pulse-labelled for 20 min with [^{35}S]methionine and [^{35}S]cysteine, a major species of 40 kDa as well as a minor protein species of 38 kDa was immunoprecipitated from HSV-infected cells and these proteins were not observed when the anti-gK serum was preincubated with peptide (Fig. 5). A single form of gK with an electrophoretic mobility of 29 kDa was observed after extracts were digested with endo H, supporting the conclusion that both the 40- and 38-kDa proteins contained immature, high-mannose oligosaccharides. Of two potential glycosylation signals in gK, one includes an aspartic acid residue (12, 42) which often reduces the use of a site (34) and therefore, the 38-kDa protein is likely a partially modified form of gK. This conclusion is supported by in vitro translation experiments (24) and when gK was expressed using insect cells (17). The disappearance of the 38-kDa protein during the 90-min chase (Fig. 5) may be due to breakdown/proteolysis, which has perhaps been accelerated because of the underglycosylation. Nevertheless, the 40-kDa protein appears to be the predominant form of gK in infected cells (24–26).

Similarly, the 40-kDa protein was the predominant form observed in the chase samples (Fig. 5). Moreover, the 40-kDa protein remained sensitive to endo H in both chase samples (Fig. 5). In contrast, other HSV glycoproteins gD, gE, and gI became predominantly resistant to endo H after the 235-min chase (not shown). The upper part of the gel was not included because this region included intense bands derived from the HSV Fc receptor proteins gE and gI, which are commonly observed in such immunoprecipitations (27). However, a more complete study using these antibodies demonstrated that there are no gK species with electrophoretic mobilities slower than 40 kDa (24). The intensity of the gK band dropped moderately during the chase periods; however, this reduction is probably the result of protein turnover, since the intensity of the gE and gI bands declined in a proportionate fashion during the chase in this experiment. It is possible that a fraction of gK is converted to an endo H-resistant form and that this fraction is not recognized by the anti-UL53-4 antibody; however, this is unlikely because similar results were obtained with the anti-UL53-1 and anti-UL53-3 antibodies and we and others have found that these antibodies do not detect proteins other than 38 and 40 kDa on Western immunoblots (results not shown;

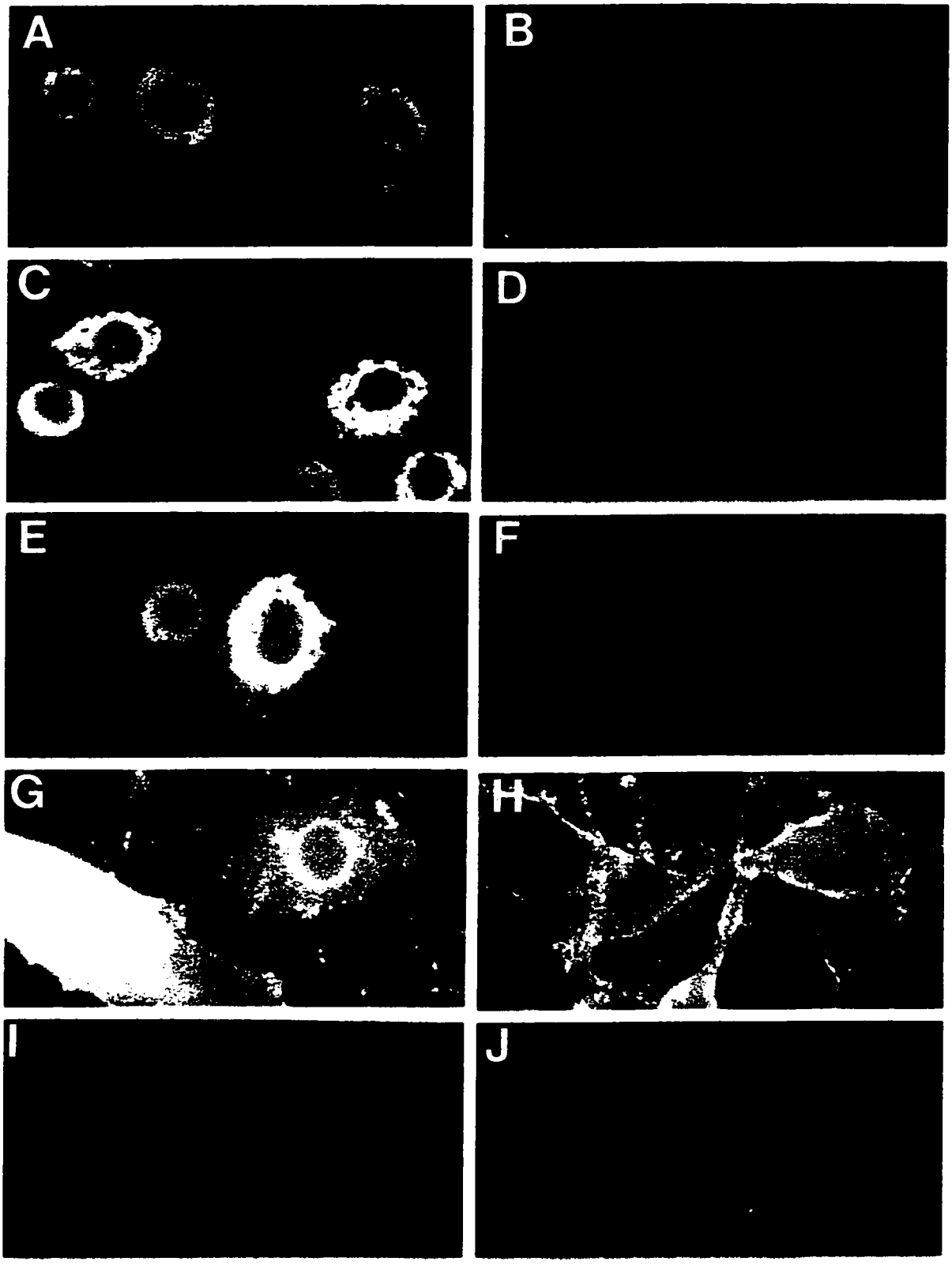

FIG. 2. Immunofluorescence staining of gK in HSV-1-infected gK-9 cells. gK-9 cells were infected with HSV-1 (F-gEβ) at 30 PFU per cell, and after 12 h the cells were fixed with 2% paraformaldehyde and then some monolayers were permeabilized with 0.2% Triton X-100 for 5 min (A, C, E, G, and I), while others were not permeabilized (B, D, F, H, and J). The cells were incubated with individual antisera directed against the UL53-1 peptide (A and B), the UL53-3 peptide (C and D), or the UL53-4 peptide (E and F) or with anti-gD antiserum (G and H) or preimmune serum (I and J). The cells were washed, incubated with fluorescein-conjugated goat anti-rabbit IgG antibodies, washed again, and then mounted on glass slides.

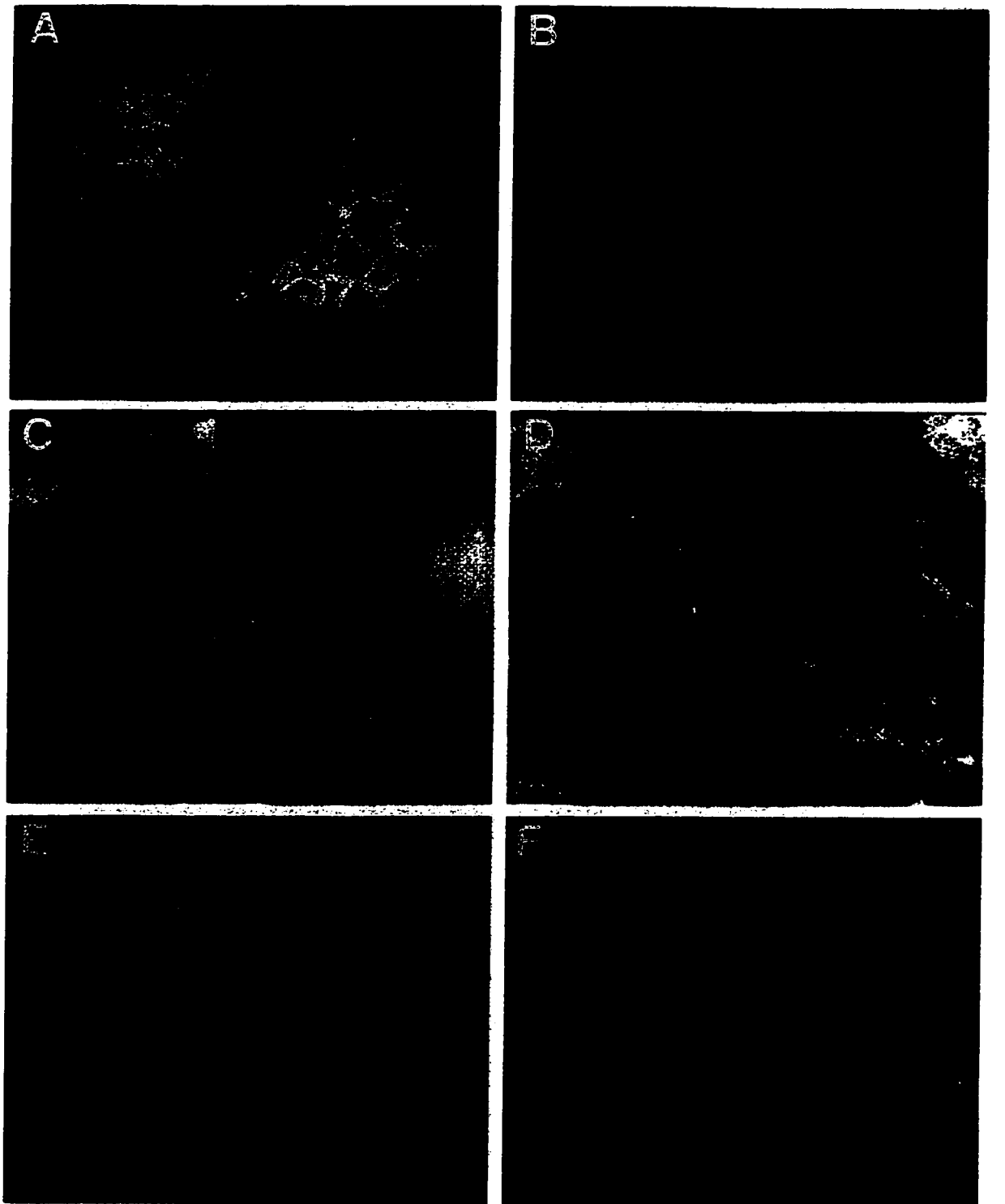

FIG. 3. gK is restricted to the perinuclear region of cells infected with HSV-1 syncytial mutants. Vero cell monolayers growing on glass coverslips were infected with HSV-1 strain MP at 30 PFU per cell, and 13 h later the cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.2% Triton X-100 (A, C, and E) or were not permeabilized (B, D, and F). The cells were incubated with a mixture of rabbit anti-UL53-1 and anti-UL53-4 sera (A and B), rabbit anti-gD serum (C and D), or preimmune sera (E and F) and then washed and incubated with fluorescein-conjugated goat anti-rabbit IgG. Coverslips were washed, mounted on slides, and fluorescence photographed.

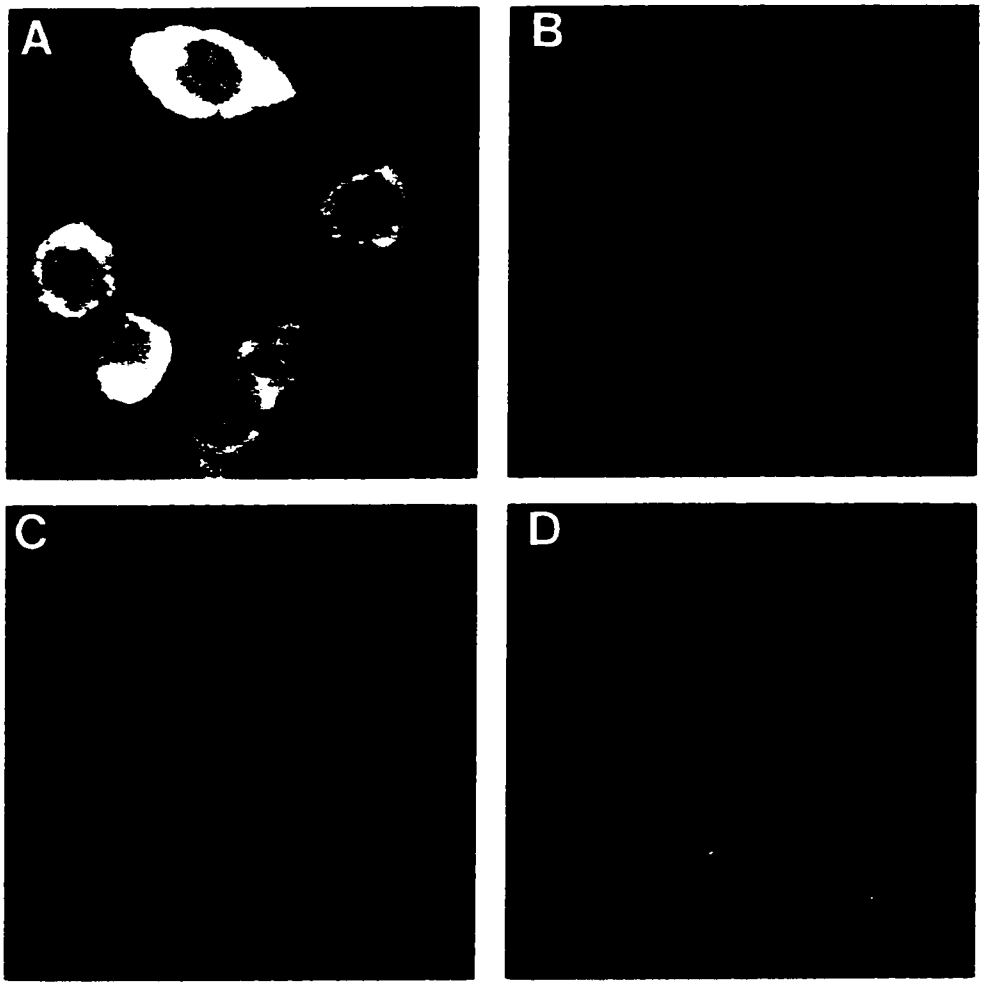

FIG. 4. Immunofluorescence staining of cells infected with AdgK, an adenovirus vector expressing gK. Vero cells growing on glass coverslips were infected with AdgK (A and B) or AddlE3 (C and D) at 1,000 PFU per cell. At 27 h after infection, the cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.2% Triton X-100 (A and C) or not permeabilized (B and D); then they were incubated with a mixture of anti-UL53-1 and anti-UL53-4 sera. The cells were washed and incubated with fluorescein-conjugated goat anti-rabbit IgG serum. The coverslips were washed and mounted on glass slides.

17). Similarly, gK expressed at much higher levels in gK-9 cells did not become endo H resistant and syncytial forms of gK remained endo H sensitive (not shown).

Conclusions. The results presented here support the conclusion that HSV-1 gK is retained in the nuclear envelope and ER in HSV-infected cells. Immunofluorescence staining with three different gK-specific antipeptide sera demonstrated that gK accumulates in perinuclear and nuclear membranes of HSV-infected Vero cells. Moreover, gK could not be detected on the surfaces of HSV-infected cells, a result which distinguishes gK from all other HSV glycoproteins studied to date (1, 16, 18, 20, 23, 27–31, 45, 53). gK expressed by using a recombinant adenovirus vector was restricted to perinuclear and nuclear membranes, supporting the conclusion that other HSV proteins are not required for intracellular retention of gK. In addition, gK produced by syncytial HSV-1 did not reach the cell surface.

Supporting the hypothesis that gK does not leave the perinuclear compartment of cells, gK oligosaccharides remained entirely endo H sensitive. This result supports the immunofluorescence data and suggests that gK does not reach the medial Golgi. It should be noted that the mature form of HSV-1 gB also contains a fraction of N-linked oligosaccharides which remains sensitive to endo H, though other gB oligosaccharides acquire endo H resistance (32, 55) and the other HSV glycoproteins characterized to date become predominantly endo H resistant (1, 32, 45, 51). We know of no example of a herpesvirus glycoprotein which reaches the cell surface without oligosaccharide processing in the Golgi apparatus. gK does not possess obvious ER retention motifs previously identified in type I and type II membrane proteins or the KDEL/HDEL retention motif of ER luminal proteins (reviewed in reference 49).

There is ample evidence that mutations in gK produce striking effects in cells, causing extensive cell fusion within 4 to 6 h after HSV infection (40). These observations have suggested that gK plays a central role in regulating fusion of infected cells and, perhaps, fusion of the virion envelope with cellular membranes during the process of virus entry into cells. Several models have been put forward to explain the effects of gK mutations on infected cells, though none of these models have been confirmed experimentally. It has been proposed that gK (i) possesses an innate fusion-inducing activity which is triggered by mutations in the protein, (ii) catalyzes or indirectly influences a process which controls or regulates the functions of viral fusion proteins, (iii) interacts with components of the fusion complex in the plasma membrane and virion envelope

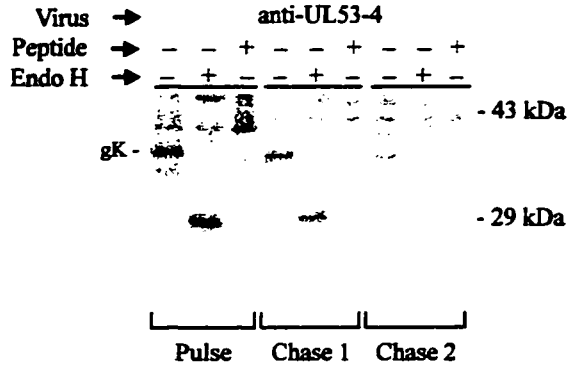

FIG. 5. gK oligosaccharides are not processed in HSV-1-infected cells. Vero cells were infected with HSV-1(F) at 20 PFU per cell. After 4 h, the cells were labelled with [^{35}S]methionine and [^{35}S]cysteine (100 μCi of each per ml) for 20 min (Pulse) and cell extracts were made immediately or the label was chased for 90 (Chase 1) or 235 (Chase 2) min before cell extracts were made. Cell extracts (from approximately 10^6 cells) were clarified by centrifugation and then mixed with 10 μl of anti-UL53-4 serum (−) or with 10 μl of anti-UL53-4 serum preincubated with 100 μg of UL53-4 peptide (+). Antigen-antibody complexes were washed and incubated in denaturing buffer (0.5% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol) at 37°C for 30 min, and then the mixture was diluted and proteins were digested with endo H (+) or mock digested (−) for 3 h at 37°C. Precipitated proteins were diluted in sample buffer, placed at 37°C for 30 min, and subjected to electrophoresis on SDS-polyacrylamide (14%) gels. The gels were dried and analyzed using a Molecular Dynamics Image Quant phosphoimager. The positions of gK and molecular mass markers of 43 and 29 kDa are indicated.

to regulate fusion, or (iv) influences cell surface transport of the HSV or host cell proteins which affect cell-cell fusion (5, 25, 26, 35, 38, 44, 47, 51, 52). The results presented here rule out a number of these possibilities because gK is not present in the plasma membrane and, therefore, it is unlikely that it plays a direct role in fusion of cells. Moreover, the small quantities of gK expressed in HSV-infected cells relative to other glycoproteins involved in cell-cell fusion is more consistent with a regulatory role for gK. It is also unlikely that gK is associated with mature virus particles at the cell surface, because we could not detect gK at the cell surface by immunofluorescence (Fig. 1). In addition, we have been unable to detect gK labelled with either [^{35}S]methionine and [^{35}S]cysteine or ^{125}I in virus particles purified from the medium of infected cells (not shown), although a previous communication (24) indicated incorrectly that we had done so. If gK is not part of the virus particle, it appears unlikely that it participates directly in fusion of the virion envelope during entry.

Therefore, gK is an unusual HSV glycoprotein that accumulates in cytoplasmic membranes, the ER, and the nuclear envelope and does not reach the cell surface. We have recently constructed an HSV-1 mutant unable to express gK, and this mutant is unable to produce infectious viruses; instead, viruses accumulate within intracellular membranes (26). The phenotype of the mutant is consistent with the internal localization of gK observed here, and the results demonstrate that gK is essential for virus replication.

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REFERENCES

1. **Baines, J. D., and B. Roizman.** 1993. The U_L10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J. Virol. **67**:1441–1452.
2. **Baines, J. D., P. L. Ward, G. Campadelli-Fiume, and B. Roizman.** 1991. The U_L20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J. Virol. **65**:6414–6424.
3. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132**:368–376.
4. **Brunetti, C. R., R. L. Burke, B. Hoflack, T. Ludwig, K. S. Dingwell, and D. C. Johnson.** 1995. Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. J. Virol. **69**:3517–3528.
5. **Bzik, D. J., and S. Person.** 1981. Dependence of herpes simplex virus type 1-induced cell fusion on cell type. Virology **110**:35–42.
6. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in entry and cell fusion. J. Virol. **62**:2596–2604.
7. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
8. **Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg.** 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. J. Virol. **27**:172–181.
9. **Compton, T., and R. J. Courtney.** 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1. J. Virol. **36**:429–439.
10. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759–1816.
11. **Debroy, C.** 1990. Nucleotide sequence of the herpes simplex virus type-2 syn gene that causes cell fusion. Gene **88**:257–277.
12. **Debroy, C., N. Pederson, and S. Person.** 1985. Nucleotide sequence of herpes simplex virus type 1 gene that causes cell fusion. Virology **145**:36–48.
13. **Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson.** 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J. Virol. **68**:834–845.
14. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2**:357–364.
15. **Forrester, A. J., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and A. C. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J. Virol. **66**:341–348.
16. **Forrester, A. J., V. Sullivan, A. Simmons, B. A. Blacklaws, G. L. Smith, A. A. Nash, and A. C. Minson.** 1991. Induction of protective immunity with antibody to herpes simplex virus type 1 glycoprotein H (gH) and analysis of the immune response to gH expressed in recombinant vaccinia virus. J. Gen. Virol. **72**:369–375.
17. **Ghiasi, H., S. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1994. Characterization of baculovirus-expressed herpes simplex virus type 1 glycoprotein K. J. Virol. **68**:2347–2354.
18. **Gompels U.A., and A. C. Minson.** 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology **153**:230–247.
19. **Haj-Ahmad, Y., and F. L. Graham.** 1986. Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J. Virol. **57**:267–274.
20. **Hanke, T., F. L. Graham, V. Lulitanond, and D. C. Johnson.** 1990. Herpes simplex virus IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology **177**:437–444.
21. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 411. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
23. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66**:2240–2250.
24. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66**:5603–5609.
25. **Hutchinson, L., F. L. Graham, W. Cai, C. Debroy, S. Person, and D. C. Johnson.** 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology **196**:514–531.
26. **Hutchinson, L., and D. C. Johnson.** 1995. Herpes simplex virus glycoprotein K promotes egress of virus particles. Submitted for publication.
27. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61**:2208–2216.
28. **Johnson, D. C., M. C. Frame, N. W. Ligas, A. M. Cross, and N. D. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62**:1347–1354.

29. **Johnson, D. C., G. Ghosh-Choudhury, J. R. Smiley, L. Fallis, and F. L. Graham.** 1988. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology **164**:1–14.
30. **Johnson, D. C., and J. R. Smiley.** 1985. Intracellular transport of herpes simplex virus gD occurs more rapidly in uninfected cells than in infected cells. J. Virol. **54**:682–689.
31. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43**:1102–1112.
32. **Johnson, D. C., and P. G. Spear.** 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell **32**:987–997.
33. **Klupp, B. G., J. Baumeister, N. Visser, and T. C. Mettenleiter.** 1993. Identification and characterization of glycoproteins gK and gL of pseudorabies virus, abstr. C-94, p. C-94. *In* Abstracts of the XVIII International Herpes Virus Workshop. University of Pittsburgh, Pittsburgh, Pa.
34. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54**:631–664.
35. **Lee, G. T.-Y., and P. G. Spear.** 1980. Viral and cellular factors that influence cell fusion induced by herpes simplex virus. Virology **107**:402–414.
36. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
37. **Maclean, C. A., S. Efstathiou, M. L. Elliot, F. E. Jamieson, and D. J. McGeoch.** 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J. Gen. Virol. **72**:897–906.
38. **Manservigi, R., P. G. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different glycoproteins. Proc. Natl. Acad. Sci. USA **74**:3913–3917.
39. **McGeoch, D. J., C. Cunningham, G. McIntyre, and A. Dolan.** 1991. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. J. Gen. Virol. **72**:3057–3075.
40. **Person, S., R. W. Knowles, G. S. Read, S. C. Warner, and V. C. Bond.** 1976. Kinetics of cell fusion induced by a syncytia-producing mutant of herpes simplex virus type 1. J. Virol. **17**:183–190.
41. **Pogue-Geile, K. L., G. T.-Y. Lee, S. K. Shapira, and P. G. Spear.** 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology **136**:100–109.
42. **Pogue-Geile, K. L., and P. G. Spear.** 1987. The single base pair substitution responsible for the syn phenotype of herpes simplex virus type 1, strain MP. Virology **157**:67–74.
43. **Ramaswamy, R., and T. C. Holland.** 1992. In vitro characterization of the HSV-1 UL53 gene product. Virology **186**:579–587.
44. **Read, G. S., S. Person, and P. M. Keller.** 1980. Genetic studies of cell fusion induced by herpes simplex virus type 1. J. Virol. **35**:105–113.
45. **Roberts, S. R., M. Ponce deLeon, G. H. Cohen, and R. J. Eisenberg.** 1991. Analysis of the intracellular maturation of the herpes simplex virus type 1 glycoprotein gH in infected and transfected cells. Virology **184**:609–624.
46. **Roop, C., L. Hutchinson, and D. C. Johnson.** 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J. Virol. **67**:2285–2297.
47. **Ruhlig, M. A., and S. Person.** 1977. Alterations of neutral glycolipids in cells infected with syncytium-producing mutants of herpes simplex virus type 1. J. Gen. Virol. **63**:277–287.
48. **Ruyechen, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–687.
49. **Schutze, M. P., A. Peterson, and M. R. Jackson.** 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. EMBO J. **13**:1696–1705.
50. **Sodora, D. L., G. H. Cohen, and R. J. Eisenberg.** 1989. Influence of asparagine-linked oligosaccharides on antigenicity, processing, and cell surface expression of herpes simplex virus type 1 glycoprotein D. J. Virol. **63**:5184–5193.
51. **Spear, P. G.** 1984. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Publishing Corp., New York.
52. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Boca Raton, Fla.
53. **Sullivan, V., and G. L. Smith.** 1987. Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: neutralization of HSV-1 infectivity with anti-gG antibody. J. Gen. Virol. **68**:2587–2598.
54. **Telford, E. A., M. S. Watson, K. McBride, and A. J. Davison.** 1992. The DNA sequence of equine herpesvirus-1. Virology **189**:304–316.
55. **Wenske, E. A., M. W. Bratton, and R. J. Courtney.** 1982. Endo-β-*N*-acetylglucosaminidase H sensitivity of precursors to herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. **44**:241–248.
56. **York, I., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to $CD8^+$ T lymphocytes. Cell **77**:525–535.
57. **Zhao, Y., R. Holden, R. N. Harty, and D. J. O'Callaghan.** 1992. Identification and transcriptional analyses of the UL3 and UL4 genes of equine herpesvirus 1, homologs of the ICP27 and glycoprotein K genes of herpes simplex virus. J. Virol. **66**:5363–5372.

CHAPTER 5

PUBLISHED MANUSCRIPT

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PREAMBLE

Membrane fusion events induced by HSV-1 are essential for virus penetration into host cells, nucleocapsid envelopment, virus egress and intercellular transmission of virus from infected to uninfected cells (Baines et al., 1991; Campadelli-Fiume et al., 1991; Baines & Roizman, 1992; Balan et al., 1994; reviewed by Spear, 1993). Since mutations in UL53-gK confer a syncytial phenotype to HSV-1 characterized by massive amounts of cell-cell fusion (reviewed in Dolter et al., 1994), it seems likely that gK encodes an activity which regulates the fusion of cell surface membranes. Nevertheless, gK does not reach or the cell surface and cannot be detected in virus particles (see Chapter 4), suggesting that gK is not directly involved in membrane fusion. In order to investigate the role gK plays in governing HSV-induced membrane fusion and in HSV replication, an HSV-1 gK-negative mutant (F-gKß) was constructed and characterized.

Although there had been extensive efforts in at least four laboratories to delete the UL53 gene from the HSV-1 genome, none to date had been successful (MacLean et al., 1991; S. Person, personal communication, T. Holland, personal communication, G. Kousoulas, personal communication). To achieve this goal I had to overcome a number of technical difficulties which arose in part because UL53-gK is essential for HSV-1

replication and because alterations in the temporal expression and/or over-abundance of gK are toxic to HSV-1 replication. Therefore, cell lines which produce gK in response to an HSV-infection, and display expression kinetics that mimic a wild-type HSV-1 infection, were required for this study. In addition, the UL52 gene overlaps the UL53 open reading frame (ORF) and is essential for HSV-1 replication, a point which was taken into consideration and increased the difficulty of constructing the gK^- mutant. Since little was known about the regulatory domains controlling UL53 mRNA synthesis, it was necessary to create cell lines which have the UL53-gK ORF downstream of the endogenous UL53 promoter as well as cell lines which placed the UL53-gK downstream of the heterologous HSV-1 US6-gD promoter. Type specific antisera which recognizes HSV-1 gK (but not HSV-2 gK) were developed for this study, and used to screen ≈600 cell lines for the production of HSV-1 gK after an infection with HSV-2. A few cell lines expressed gK at levels similar to those observed in cells infected with wild-type HSV-1, and were used to isolate the gK-negative mutant F-gKß.

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Herpes Simplex Virus Glycoprotein K Promotes Egress of Virus Particles

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Herpes simplex virus (HSV) glycoprotein K (gK) is thought to be intimately involved in the process by which infected cells fuse because HSV syncytial mutations frequently alter the gK (UL53) gene. Previously, we characterized gK produced in cells infected with wild-type HSV or syncytial HSV mutants and found that the glycoprotein was localized to nuclear and endoplasmic reticulum membranes and did not reach the cell surface (L. Hutchinson, C. Roop, and D. C. Johnson, J. Virol. 69:4556–4563, 1995). In this study, we have characterized a mutant HSV type 1, denoted F-gKβ, in which a *lacZ* gene cassette was inserted into the gK coding sequences. Since gK was found to be essential for virus replication, F-gKβ was propagated on complementing cells which can express gK. F-gKβ produced normal plaques bounded by nonfused cells when plated on complementing cells, although syncytia were observed when the cells produced smaller amounts of gK. In contrast, F-gKβ produced only microscopic plaques on Vero cells and normal human fibroblasts (which do not express gK) and these plaques were reduced by 10^2 to 10^6 in number. Further, large numbers of nonenveloped capsids accumulated in the cytoplasm of F-gKβ-infected Vero cells, virus particles did not reach the cell surface, and the few enveloped particles that were produced exhibited a reduced capacity to enter cells and initiate an infection of complementing cells. Overexpression of gK in HSV-infected cells also caused defects in virus egress, although particles accumulated in the perinuclear space and large multilamellar membranous structures juxtaposed with the nuclear envelope were observed. Together, these results demonstrate that gK regulates or facilitates egress of HSV from cells. How this property is connected to cell fusion is not clear. In this regard, gK may alter cell surface transport of viral particles or other viral components directly involved in the fusion process.

The fusion of cellular and viral membranes induced by herpes simplex virus (HSV) is essential for virus penetration into host cells, nucleocapsid envelopment, virus egress, and transfer of virus from infected to uninfected cells (10, 16, 40, 46, 57). These processes are complex and not well understood, primarily because a large number of HSV proteins (many of unknown function) have been implicated in membrane fusion and because cellular factors involved in the processes have largely not been described.

During production of plaques on monolayers of cultured cells, HSV can spread by direct cell-to-cell transmission, presumably across cell contacts or junctions (reviewed in reference 19). By a similar, but perhaps distinct, process, HSV can cause cultured cells to fuse at neutral pH. Wild-type strains of HSV usually promote aggregation of cultured cells, although fusion of cultured cells is observed to a limited degree and polykaryocytes have been observed in herpetic lesions of infected individuals (51, 65). By contrast, syncytial mutants of HSV, which can arise on passage of virus in cultured cells, cause cells to fuse with uninfected cells, producing massive syncytia. It is thought that the syncytial mutations affect viral proteins which play a direct role in the membrane fusion events, e.g., glycoprotein B (gB), or which regulate the fusion process in some unknown manner (reviewed in reference 65).

Syncytial mutations can arise in at least four different viral genes: the UL20 gene, which encodes a membrane protein necessary for viral egress (4, 43); the UL24 gene, which encodes a cytosolic protein of unknown function (35, 60); the UL27 (gB) gene (8, 59); and the UL53 gene (7, 53, 59), which encodes glycoprotein K (gK) (32). Deletion mutants lacking either the UL20 or UL24 coding sequences form syncytial plaques, implying that these proteins are not required for fusion and that loss of either protein induces fusion by deregulating some aspect of virus replication. By contrast, syncytial mutations in gB primarily involve amino acid substitutions or truncations (2, 10, 23, 68). Other point mutations in gB alter the rate of HSV entry (8, 23), and gB null mutants cannot enter cells and are blocked in virus penetration and cell fusion (10, 11, 46). Therefore, it appears likely that gB plays a direct role in membrane fusion events. Mutations in the UL20, UL24, and UL27 (gB) genes are infrequently observed, at least in some strains of HSV type 1 (HSV-1), and mutations in the UL53 (gK) gene predominate (6, 7, 41, 56, 58). The mutations in gK described to date have been restricted to amino acid substitutions (17, 20, 54), and it has been suggested that gK somehow regulates the fusion of cell surface membranes (reviewed in reference 34). Nonetheless, recent observations indicate that gK does not reach the cell surface and cannot be detected in virus particles (34), suggesting that gK is not directly involved in membrane fusion.

There is also evidence, primarily from studies of virus mutants, demonstrating that gB, gD, and gH/gL are required for cell fusion as well as for virus entry into cells (10, 11, 16, 22, 40, 58). In addition, HSV membrane proteins, not absolutely required for virus penetration, have also been implicated in cell fusion. Cell fusion was not observed with HSV-1 mutants carrying a syncytial form of gB and unable to express gE, gI, or gM or a membrane protein encoded by the UL45 gene (5, 16, 28). The role of the gE/gI heterooligomer in cell fusion is consistent

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with the observation that gE/gI fosters efficient cell-to-cell transmission of HSV across cell junctions (5, 19). However, others have reported that syncytial mutants lacking gE (47) or gI (19, 36) retain the syncytial phenotype, implying that these deletion mutants possess syncytial mutations in a gene other than UL27 (gB), e.g., that encoding gK. In addition, a variety of cell factors, including glycosaminoglycans, can influence fusion of infected cells (9, 62, 63).

In order to characterize the role of gK in membrane fusion and in HSV replication, we constructed and characterized an HSV-1 mutant, F-gKβ, unable to express gK. F-gKβ formed extremely rare microscopic plaques in the absence of gK, indicating that gK was required for HSV replication. Enveloped and unenveloped virus particles accumulated in the cytoplasm of cells in the absence of gK, and virus particles were not observed on the cell surface. When gK was overexpressed in the cells, there were similar disruptions in virus egress, although in this case the virus accumulated in the perinuclear space.

MATERIALS AND METHODS

Cells and viruses. Vero cells and normal human fibroblasts were grown in alpha-minimal essential medium (alpha-MEM) supplemented with 7 to 10% fetal calf serum (FCS), 1% penicillin-streptomycin (P/S; GIBCO Laboratories, Burlington, Ontario, Canada), and 0.3% L-glutamine (GIBCO). VK243, VK295, VK302, VK308, and gK-9 cells (33) were maintained in Dulbecco's modified Eagle medium lacking histidine (DUL-his) supplemented with 0.5 mM histidinol (Sigma Chemical Co., St. Louis, Mo.), 7% FCS, 1% P/S, and 0.3% L-glutamine. Prior to infection, VK243, VK295, VK302, VK308, and gK-9 cells were passaged once in alpha-MEM containing 7% FCS. HSV-1 wild-type strains F (obtained from P. G. Spear, University of Chicago, Chicago, Ill.) and KOS (obtained from J. Smiley, McMaster University, Hamilton, Ontario, Canada) and HSV-2 strain 333 (obtained from P. G. Spear, University of Chicago) were propagated on and had their titers determined on Vero cells. The gK-defective mutant strain, F-gKβ-308, was grown and had its titers determined on VK302 cells.

Plasmids. Plasmid pSV2HISsyn (33) includes a 3.4-kb *Eco*RI-*Bam*HI fragment from pSG28 (25) encoding the UL53 gene as well as a selectable marker, histidinol dehydrogenase from pSV2HIS (30). Plasmid pUC19syn (33) contains the UL53 open reading frame (ORF) within a 1.3-kb *Kpn*I-*Bam*HI fragment derived from pSV2HISsyn (33) inserted into the *Kpn*I and *Bam*HI sites of pUC19. Plasmid pD6p (a generous gift of S. Weller, University of Connecticut) contains a 4.3-kb ICP6::*lacZ* gene cassette bounded by *Bam*HI sites (26), and this fragment was excised, end repaired with T4 polymerase, and ligated into the unique *Hpa*I site (within the UL53 ORF) of pUC19syn so that the *lacZ* and UL53 genes were in the same direction, creating plasmid p19SZ. A 1.0-kb *Nae*I fragment, containing the UL53 ORF, was removed from pUC19syn and inserted in the *Sma*I site of pRIT2T (Pharmacia Chemicals, Dorval, Quebec, Canada), yielding pRIT2Tsyn, a protein A-gK fusion expression vector. Plasmid pCMVsyn (33) was constructed by blunt-end ligation of the 1.0-kb *Nae*I fragment from pUC19syn (33) into end-repaired *Bst*XI sites of pRc/CMV (Invitrogen Corporation, San Diego, Calif.), placing the UL53 ORF under control of the cytomegalovirus (CMV) immediate-early (IE) promoter and flanked by the bovine growth hormone polyadenylation site. Plasmid pSV2HDK (33) carries a 1.9-kb *Hin*dIII-*Eco*RI fragment comprising the UL53 ORF and the bovine growth hormone polyadenylation site from pCMVsyn (33) downstream of the HSV gD promoter in pSV2HISgD (40). A 2-kb *Bgl*II-*Xba*I fragment derived from pCMVsyn and containing a CMV IE promoter::UL53 gene cassette was inserted into the multiple cloning site within the US7-gI gene of pSS17L (21, 40) to create pSS17syn. Oligonucleotide AB924 (5'-GCT GCA GGG AAC GGA CGG CGA GCA T-3'), which hybridizes at the 5' end of the UL53 ORF (purchased from The Institute for Molecular Biology and Biotechnology, McMaster University), was used for Southern blot analysis.

Antibodies. Synthetic peptide UL53-4 and rabbit anti-UL53-4 peptide sera have been described previously (32). Monoclonal antibody (MAb) 15βB2, which recognizes HSV-1 and HSV-2 gB (37), was a gift of S. Bacchetti (McMaster University). MAb LP2, which reacts with HSV-1 and HSV-2 gD (44), was a gift of T. Minson (Cambridge University). MAb 53S directed towards HSV gH was obtained as a hybridoma from the American Type Culture Collection. Rabbit anti-thymidine kinase (TK) is directed to HSV TK produced in *Escherichia coli* and was a generous gift from W. Summers (Yale University).

Construction of Vero cells expressing gK and the gK-negative HSV-1, F-gKβ. Vero cells were transfected with plasmid pSV2HDK or pSV2HISsyn, and transformants were selected by using medium lacking histidine and containing 0.15 to 1 mM histidinol, as previously described (33). Isolated colonies of cells were cloned by using cloning cylinders, and the cells were screened initially by dot blot Southern analysis with oligonucleotide AB924. Cells found to contain more than five copies of the UL53 (gK) gene were characterized further for the inducible expression of gK after infection with HSV-2, and then cells were labelled with [^{35}S]methionine and immunoprecipitated with the anti-UL53-4 serum, which does not recognize HSV-2 gK (32). Three cell lines capable of expressing gK, VK243, VK295, and VK308, were cotransfected with plasmid p19SZ DNA and infectious HSV-1 (F) genomic DNA extracted from cytoplasmic HSV nucleocapsids (64) by the $CaPO_4$ method (27) followed by a glycerol shock (40). Transfected cells were incubated for 2 to 4 days at 37°C and then harvested and sonicated after extensive virus-induced cytopathic effect was observed. Virus preparations were diluted and plated on the progenitor cell line, and recombinant viruses which expressed β-galactosidase were detected by using an overlay of alpha-MEM containing 2% FCS, 0.5% low-melting-temperature agarose, and 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim Canada Ltd., Dorval, Quebec, Canada) per ml. Blue plaques were picked, subjected to three additional rounds of plaque purification on the appropriate cell line, and finally cloned by limiting dilution.

Production of infectious virus, plaque-forming efficiency, and plaque morphology of F-gKβ on different cell types. The replication and plaque-forming efficiencies of wild-type F and F-gKβ were assessed by infecting Vero or VK302 cell monolayers with 2 PFU per cell and harvesting the cells in medium (the cells were resuspended in 1/10 the volume of cell culture supernatant) after 24 h at 37°C. The cells were disrupted by one round of freeze-thawing followed by sonication, and then titers of viruses were determined on monolayers of normal human fibroblasts or Vero or VK302 cells. After 1.5 to 2 h at 37°C, the virus inoculum was removed and cells were incubated in alpha-MEM containing 1% FCS and 0.1% human gamma globulin for 96 h and then stained with crystal violet. Plaques were photographed with a Zeiss inverted microscope.

Radiolabelling of proteins, immunoprecipitation, and gel electrophoresis. Vero cells or Vero cell transformants were infected with HSV-1 or HSV-2 by using 2.5, 5, or 30 PFU per cell. Two to 4 h after infection, the cells were washed three times with medium 199 lacking methionine and cysteine and containing 1% dialyzed FCS (labelling medium) and then incubated for 2 to 5 h with labelling medium containing [^{35}S]methionine and [^{35}S]cysteine (50 to 200 μCi of each per ml). Pulse-chase experiments were performed by washing cells three times with labelling medium 3 h after infection and incubating cells for 20 min with [^{35}S]methionine and [^{35}S]cysteine (100 μCi of each per ml) in labelling medium. Cell extracts were made, by using a solution of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate (NP-40/DOC buffer) containing 2 mg of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5 mM phenanthroline, and 10 μg of aprotinin (Sigma) per ml, immediately (pulse) or after the cells had been washed with alpha-MEM containing 1% FCS and incubated an additional 90 or 210 min (chases). Insoluble material was removed by centrifugation at high speed (86,000 × *g*) for 60 min, and in some instances the extracts were precleared by incubation with *Staphylococcus aureus* Cowan and rabbit anti-gE/gI serum, as previously described (29, 31). Extracts derived from 3.5×10^5 to 5.5×10^5 cells were incubated with mouse or rabbit antibodies (1 to 10 μl of serum or ascites fluid) at 4°C for 90 min and then with protein A-Sepharose (50 to 100 μl; Pharmacia Chemicals). In some experiments, anti-UL53-4 peptide serum (5 or 10 μl) was preincubated with 50 or 100 μg of synthetic UL53-4 peptide for 60 min at 4°C. Antigen-antibody complexes were washed three times with NP-40/DOC buffer or under more stringent conditions, as described previously (31), and then precipitated proteins were eluted by adding 50 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, bromophenol blue, and 2% β-mercaptoethanol and the samples were heated at 37°C for 30 min (gK samples) or at 100°C for 5 min. Proteins were subjected to electrophoresis as described previously (31), and the gels were dried and analyzed by using a Molecular Dynamics Image Quant phosphoimager or, alternatively, infused with Enhance (Dupont, Montreal, Quebec, Canada), dried, and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.).

Electron microscopy. Vero or VK302 cells were infected with wild-type HSV-1 (F) or F-gKβ by using 5 PFU per cell, and then the cells were incubated at 37°C for 20 to 24 h. The cells were washed with phosphate-buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 15 min. The cells were scraped from the dishes, collected by centrifugation at 800 × *g* for 5 min, resuspended in cacodylate buffer, and processed for electron microscopy as described elsewhere (38).

RESULTS

Construction of a mutant HSV-1 unable to express gK. The UL52 and UL53 genes partially overlap, and the UL52 gene is essential for viral DNA synthesis (26). Therefore, to construct an HSV-1 UL53 mutant, it was important not to interrupt the overlapping UL52 gene. On the basis of this consideration, we constructed a plasmid, p19SZ, in which an ICP6 promoter::*lacZ* cassette (26) was inserted into an *Hpa*I site 336 nucleotides downstream of the UL53 start codon (Fig. 1), allowing expression of about one-third of the gK polypeptide (17, 54).

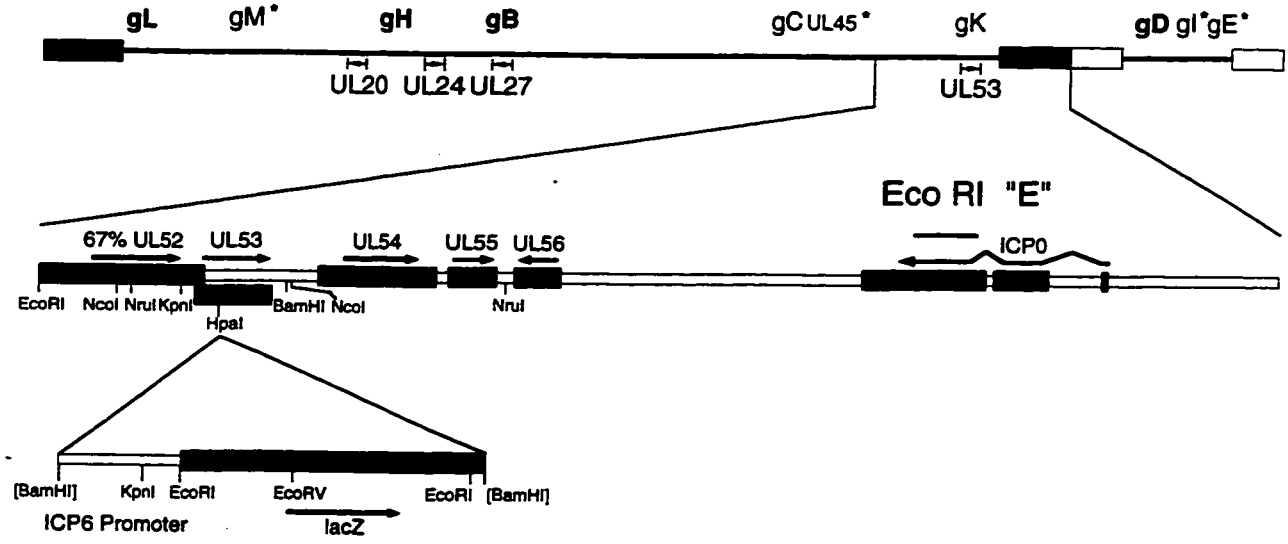

FIG. 1. Schematic representations of the HSV-1 genome, genes involved in cell fusion, and the genomic structure of the gK mutant virus. The HSV-1 genome is represented as unique long (U_L) and short (U_S) segments flanked by inverted repeats (filled and open boxes). The positions of genes encoding proteins which govern HSV-induced cell fusion are indicated along the genomic map. Mutations in the UL20, UL24, UL27-gB, and UL53-gK genes (indicated below the map) can produce the syncytial phenotype. The UL1-gL, UL22-gH, UL27-gB, and US6-gD glycoprotein genes (boldface type) are essential for HSV-1 replication in cultured cells, cell fusion, and virus entry. Virus-induced cell fusion associated with a syncytial mutation in gB requires HSV genes encoding UL10-gM, US7-gI, US8-gE, and UL45-membrane protein (asterisks). The *Eco*RI fragment of HSV-1 genomic DNA, containing 67% of the UL52 gene and the UL53, UL54, UL55, UL56, and ICP0 genes, is shown magnified to indicate the insertion of an ICP6::*lacZ* gene cassette into the UL53 gene at an *Hpa*I site (located 336 nucleotides into the UL53 ORF). Brackets around the *Bam*HI sites indicate that these sites were lost when the ICP6::*lacZ* cassette was cloned into the *Hpa*I site in UL53.

Cotransfection of Vero cells with HSV-1 (F) DNA and p19SZ yielded recombinant viruses which produced blue plaques under overlays containing X-Gal. However, Southern blot analysis revealed that the entire p19SZ plasmid had been introduced into the HSV-1 genome, regenerating the wild-type UL53 gene, probably through a single recombination crossover event (data not shown). MacLean et al. (43) previously reported unsuccessful efforts to mutate the UL53 gene; thus, it appeared that gK was essential for replication in these cultured cells.

To complement gK mutants, we constructed cell lines which could express gK. Previous attempts to increase gK synthesis by introducing a human CMV promoter-driven UL53 gene cassette into the HSV-1 genome (unpublished data) suggested that altering the temporal expression or abundance of gK interfered with virus replication. Efforts to produce cell lines which overexpress gK confirmed this hypothesis and also suggested that gK was toxic to cells when expressed even at low levels (33). Consequently, we endeavored to establish cell lines which produced gK in response to HSV infection yet expressed little or no gK before infection. Vero cells were transfected with either of two plasmids: pSV2HISsyn, which has the UL53 gene coupled to its own promoter, or pSV2HDK, which contains the UL53 ORF under control of the HSV-1 gD promoter (33). Both plasmids carry the selectable marker histidinol dehydrogenase (30, 40).

Histidinol-resistant transformants were screened initially for plasmid copy number by Southern dot blot analysis. Those cells which had over five copies of the plasmid were subsequently screened for gK expression by infecting the cells with HSV-2 and immunoprecipitating HSV-1 gK with an antipeptide serum, anti-UL53-4, which does not recognize HSV-2 gK (32). Among the 302 pSV2HDK transformants that were characterized, cell lines VK243, VK295, and VK308 represent 3 of 19 clones that produced detectable amounts of gK and a fourth cell transformant, VK302, produced substantially higher levels of gK (Fig. 2). Certain of the 210 cell lines derived from pSV2HISsyn expressed low but detectable levels of gK (data not shown) and were not characterized further. As with other cell lines carrying gD promoter constructs (22, 40), VK243, VK295, VK308, and VK302 cells did not express detectable amounts of gK prior to infection (data not shown). The prominent bands ranging from 60 to 90 kDa in HSV-1 (KOS)-infected cells were derived from HSV immunoglobulin G Fc receptor proteins gE and gI (32, 36).

To obtain an HSV-1 gK mutant, VK243, VK295, and VK308 cells were cotransfected with infectious HSV-1 (F) DNA and plasmid p19SZ. Viruses derived from the tranfections of individual cell lines were screened for blue plaques and subjected to four additional rounds of plaque purification with the same cells. Three mutants, F-gKβ-243, F-gKβ-295, and F-gKβ-308, derived independently from cell lines VK243, VK295, and VK308, respectively, were examined by Southern blot analysis using oligonucleotide AB924, which hybridizes to the first 25 nucleotides of the UL53 ORF (Fig. 1). The presence of 6.1-kb *Nco*I and 9.2-kb *Nru*I fragments and the absence of the wild-type 2.1-kb *Nco*I and 5.0-kb *Nru*I fragments indicated that F-gKβ-243, F-gKβ-295, and F-gKβ-308 contained only the disrupted form of the UL53 gene (Fig. 3). Other Southern blot analyses verified that plasmid sequences were not introduced into the mutant viruses (data not shown). All three gK-negative viruses exhibited a syncytial phenotype when plated on VK243, VK295, and VK308 cells and other cell lines that expressed detectable quantities of gK (data not shown), probably because all of these cells produce lower levels of gK than are observed in cells infected with wild-type HSV-1 (Fig. 2). In contrast, all three viruses produced nonsyncytial plaques when plated on VK302 cells (F-gKβ-308 is shown in Fig. 5), which express gK at levels more similar to those in HSV-infected cells. Therefore, subsequent experiments employed the VK302

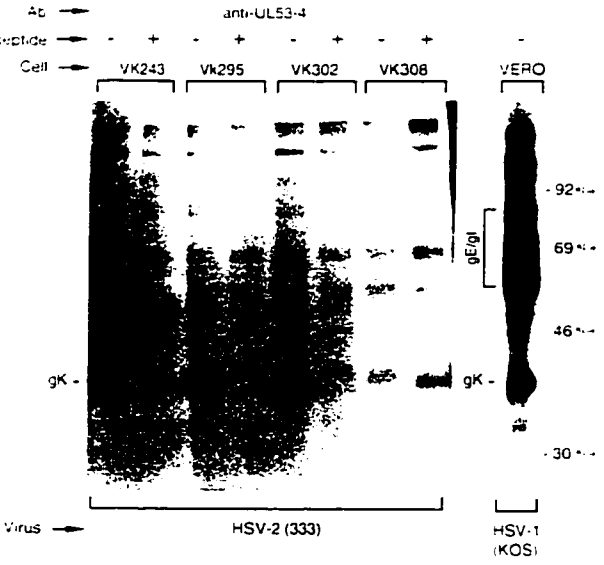

FIG. 2. Expression of HSV-1 gK in Vero cell transformants after infection with HSV-2. Transfected cell lines VK243, VK295, VK302, and VK308 were infected with HSV-2, and for comparison, Vero cells were infected with HSV-1 (KOS). Infected cells were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine for 5 h beginning 4.5 h after infection. Extracts of the cells were made and then incubated with rabbit anti-UL53-4 peptide sera, which had been preincubated with (+) or without (−) UL53-4 peptide. Anti-UL53-4 recognizes HSV-1 gK but not HSV-2 gK (32). Antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions (see Materials and Methods). Precipitated proteins were eluted at 37°C for 30 min, and electrophoresis was performed with 12% polyacrylamide gels. The positions of gK, HSV-1 immunoglobulin G Fc receptor proteins gE and gI, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated. Ab, antibody.

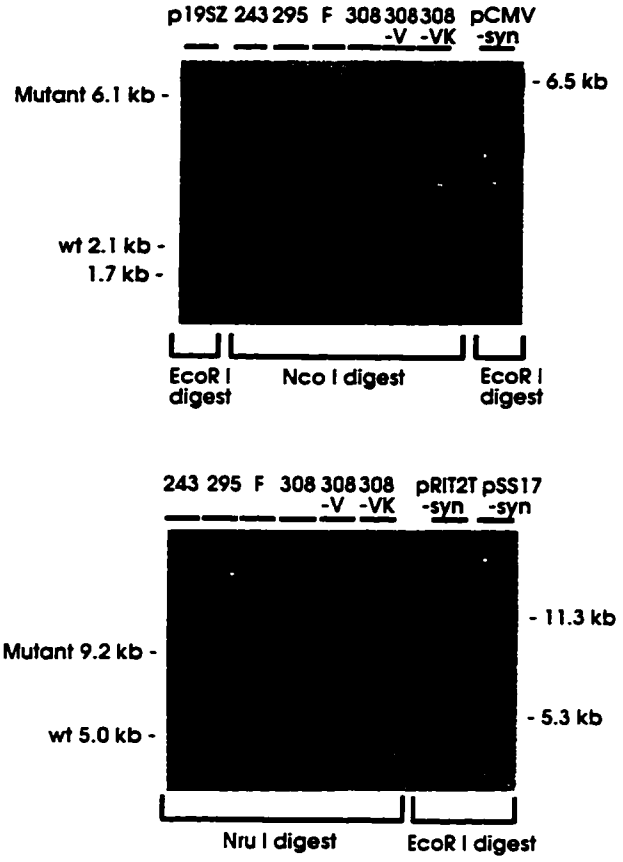

FIG. 3. Southern blot analysis of mutant (F-gKβ) viral DNA. Viral DNA was extracted from Vero cells infected with mutant viruses or wild-type HSV-1 (F) and then digested with *Nco*I (top panel) or *Nru*I (bottom panel). As markers, plasmids p19SZ, pCMVsyn, pRIT2Tsyn, and pSS17syn were digested with *Eco*RI yielding 1.7-, 6.5-, 5.3-, and 11.3-kb fragments. DNA fragments were separated on a 1% agarose gel, transferred to nylon membranes, and probed with a ^{32}P-labelled oligonucleotide, AB924, which hybridizes with the first 25 nucleotides of the UL53 ORF. Lanes: 243 (F-gKβ-243), a mutant isolated after transfection and plaque purification on VK243 cells; 295 (F-gKβ-295), a mutant isolated after transfection and plaque purification on VK295 cells; F, the wild-type HSV-1 strain; 308 (F-gKβ-308), a mutant isolated after transfection and plaque purification on VK308 cells. Lanes 308-V (F-gKβ-308V) and 308-VK (F-gKβ-308-VK) indicate virus preparations after additional rounds of plaque purification.

cell line and the F-gKβ-308 isolate, which will henceforth be designated F-gKβ. All three of the initial isolates failed to produce plaques on Vero cells and exhibited defects similar to those of F-gKβ in other experiments.

Virus mutant F-gKβ does not express gK. We next examined the ability of mutant viruses to express gK. Vero or VK302 cells were infected with F-gKβ or wild-type HSV-1 (F) labelled with [^{35}S]methionine and [^{35}S]cysteine, and cell extracts were mixed with anti-UL53-4 peptide serum, which recognizes gK residues 89 to 104 (32). The anti-UL53-4 antibodies precipitated the 40-kDa gK protein from extracts of wild-type-strain F-infected Vero cells and also from F-gKβ-infected VK302 cells (Fig. 4). The full-length 40-kDa gK protein was not detected in extracts of F-gKβ-infected Vero cells; instead, a protein of 28 kDa was detected. This 28-kDa protein was also expressed in F-gKβ-infected VK302 cells, in addition to the 40-kDa gK polypeptide (Fig. 4). The 40-kDa gK protein and the novel 28-kDa protein were not observed when anti-UL53-4 serum was preincubated with the UL53-4 peptide (Fig. 4). Identical results were obtained with the anti-UL53-1 peptide serum (data not shown), which reacts with gK residues 31 to 46 (32). Examination of DNA sequences at the insertion site of the ICP6::*lacZ* cassette into the UL53 gene indicated that the first 112 residues of gK (including the signal peptide and N-linked glycosylation sites but none of the transmembrane domains) were fused to 26 residues derived from the ICP6::*lacZ* cassette. The addition of N-linked oligosaccharides would be expected to alter the electrophoretic mobility of the F-gKβ fusion protein from the predicted value of 15 kDa to the apparent size of approximately 28 kDa. This is based on the observation that the apparent molecular mass of wild-type gK increases from 29 kDa to approximately 40 kDa on glycosylation (32). The finding that this protein is recognized by two gK-specific antipeptide sera strongly supports the view that the 28-kDa protein is derived from the N terminus of gK.

Plaque production by F-gKβ and marker rescue experiments. F-gKβ formed plaques on VK302 cells, although these plaques were reduced in size compared with those produced by wild-type HSV-1 (F) (Fig. 5), suggesting that complementation was incomplete. Wild-type HSV-1 produced normal plaques on VK-302 cells, and equal quantities of infectious viruses were produced by wild-type HSV-1 on VK-302 and Vero cells (data not shown). By contrast, F-gKβ failed to form normal plaques on Vero cells (Fig. 5) and on human R970 cells (data not shown). At very low frequencies ($<10^{-5}$ relative to the number of plaques formed on VK302 cells), microscopic plaques (consisting of three to six infected cells) were observed on Vero cell

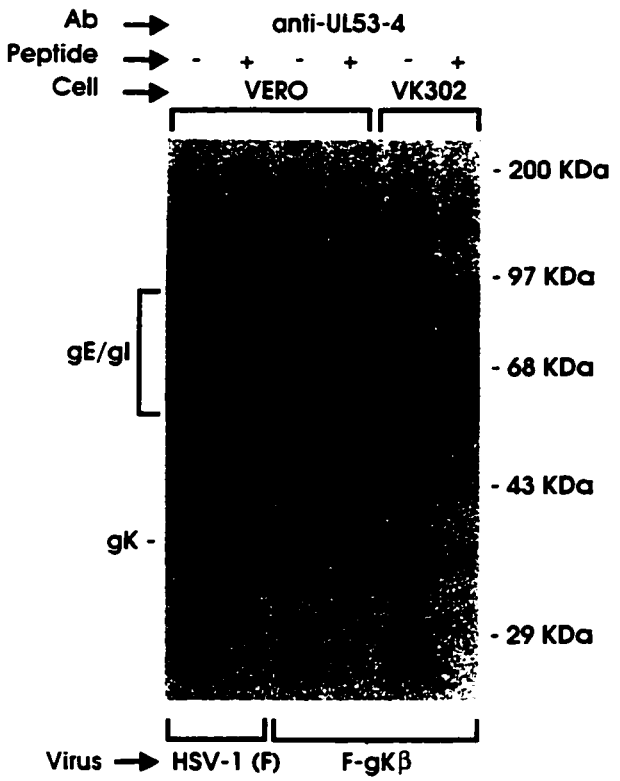

FIG. 4. Expression of gK in Vero and VK302 cells infected with F-gKβ. Vero cells and VK302 cells were infected with F-gKβ, and for comparison, Vero cells were infected with wild-type HSV-1 (F). Infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine, and extracts were mixed with anti-UL53-4 serum (-) or with anti-UL53-4 serum preincubated with peptide UL53-4 (+). Antigen-antibody complexes were eluted from protein A-Sepharose at 37°C for 30 min, subjected to electrophoresis on 12% polyacrylamide gels, dried, and analyzed with a Molecular Dynamics Image Quant phosphoimager. The positions of the 40-kDa form of HSV-1 gK, the HSV immunoglobulin G Fc receptor proteins gE and gI, and marker proteins of 200, 97, 68, 43, and 29 kDa are indicated. Ab, antibody.

monolayers infected with approximately 1 PFU of F-gKβ per cell, and the plaques were detected only after extended periods (72 to 96 h) (Fig. 5; Table 1). F-gKβ also formed microscopic plaques on normal human fibroblasts; however, the reduction in plaque-forming efficiency was less striking than with Vero cells (Fig. 5; Table 1), possibly because small HSV-1 plaques are more easily detected on fibroblast monolayers. It is highly unlikely that these microscopic F-gKβ plaques were caused by wild-type revertants, which can contaminate HSV-1 preparations derived from complementing cells. These revertants apparently derive from recombination events between viral and cellular copies of the gene (11, 58); however, this type of recombination is unlikely because VK302 cells lack the HSV-1 sequences flanking the UL53 ORF. In addition, we have not detected wild-type HSV-1, even at low levels, in the F-gKβ stocks.

To verify that the defects in virus replication were related to the mutation in the UL53 gene and not defects in other genes, a marker rescue experiment was performed. Vero cells were cotransfected with F-gKβ and either plasmid pUC19syn, which contains the wild-type UL53 gene, or pUC19. No wild-type plaques were observed with pUC19, whereas hundreds of plaques were observed on monolayers transfected with pUC19 syn and F-gKβ DNA. Several recombinants derived from the pUC19syn transfection were plaque purified and found to replicate normally on Vero cells (data not shown). Therefore, we conclude that gK is required for production of HSV plaques on monkey Vero cells and human fibroblasts and R970 cells.

Markedly less infectious virus is produced in the absence of gK. To evaluate the effects the gK mutation on production of infectious viruses, we infected Vero cells or VK302 cells with F-gKβ or wild-type F, using 2 PFU per cell, and harvested the cells after 24 h. Under these conditions, over 95% of the cells expressed viral antigens and were thus infected by the input virus (data not shown), so that this can be considered a single-step growth analysis. Infectious viruses were assayed by plaque titration with VK302 cells to determine if virions produced in the absence of gK were capable of initiating an infection of complementing cells. The titers of wild type HSV-1 (F) derived from Vero cells and F-gKβ derived from complementing VK302 cells were virtually identical, demonstrating good complementation in this single-step infection (Table 1). F-gKβ virions produced on Vero cells (lacking gK) formed plaques on VK302 cells; however, these were reduced in number by about 100-fold relative to numbers obtained with F-gKβ preparations derived from VK302 cells. Further, Vero cells infected with F-gKβ at 2 PFU per cell yielded only approximately 0.5 PFU per cell when this virus was plated on VK302 cells, consistent with the notion that gK is required for HSV replication. The data shown in Table 1 involve infectious virus derived from cells which were suspended in the culture media and then disrupted by sonication. However, we also analyzed infectious virus present in the culture media from F-gKβ-infected Vero cells and found a 300- to 1,000-fold reduction in the amount of virus produced relative to the amount produced by F-gKβ-infected VK-302 cells. Therefore, in the absence of gK, markedly less infectious virus was produced.

To further evaluate the infectivity of virus particles lacking gK, we examined expression of early viral proteins in cells infected with F-gKβ. VK302 or Vero cells were infected with Vero-derived HSV-1 (F), Vero-derived F-gKβ, or VK302-derived F-gKβ, and then the cells were labeled with [^{35}S]methionine and [^{35}S]cysteine and HSV-1 early proteins, TK, or gD was immunoprecipitated. Since approximately equal amounts of infectious HSV-1 (F) and F-gKβ were produced on Vero and VK302, respectively (Table 1), cells were infected with equal quantities of all three virus stocks, which corresponded to approximately 2 PFU per cell for Vero-derived F and VK302-derived F-gKβ. Under these conditions, Vero-derived F-gKβ (lacking gK) expressed 50-fold less TK and gD in both Vero and VK302 than was observed in cells infected with wild-type HSV-1 or with VK302-derived F-gKβ (Fig. 6). Similar results were observed when two HSV-1 immediate-early proteins, ICP4 and ICP6, were immunoprecipitated (data not shown). When the input of Vero-derived F-gKβ was increased about 100-fold, so that cells were infected with 2.5 PFU per cell (PFU here refers to plaques formed on VK302 cells), the expression of TK increased to 20% of that observed with HSV-1 (F) (data not shown). These results show clearly that HSV-1 virions derived from cells in which gK is not expressed display a reduced capacity to enter cells and initiate early stages of virus replication.

Subcellular distribution of the virus particles in cells lacking gK. F-gKβ-infected Vero or VK302 cells and HSV-1 (F)-infected Vero cells were examined by electron microscopy to determine if there were defects in virus assembly or egress in the absence of gK. Similar quantities of virus particles were observed in Vero cells infected with F and with F-gKβ and in F-gKβ-infected VK302 cells; however, the distribution of the

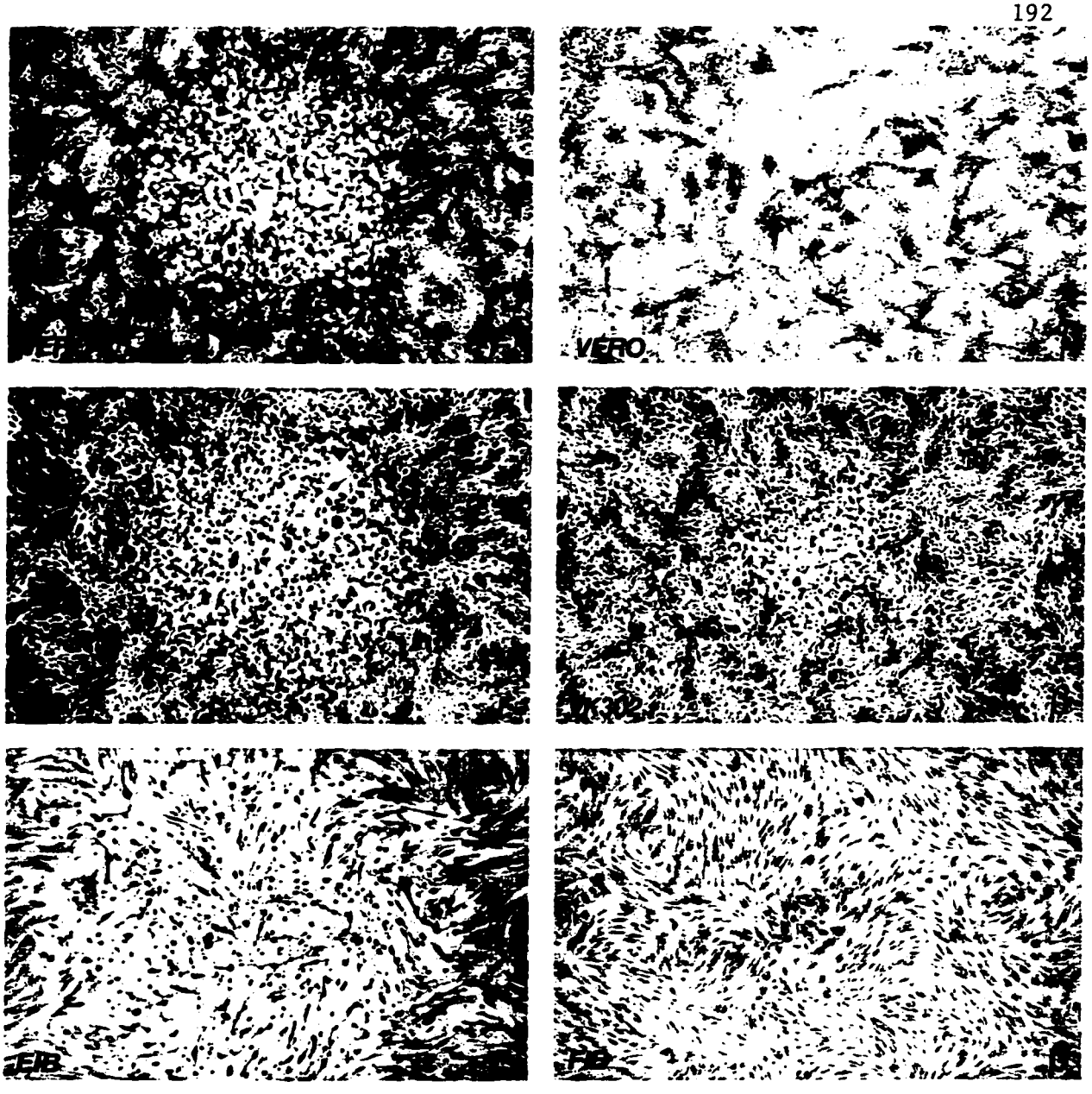

FIG. 5. Morphology of F-gKβ plaques produced on normal human fibroblasts and Vero and VK302 cells. Monolayers of Vero (A and B) or VK302 cells (C and D) or human fibroblasts (FIB) (E and F) were infected with wild-type HSV-1 (F) (A, C, and E) or the gK-negative mutant, F-gKβ (B, D, and F), overlaid with medium containing 0.1% human gamma globulin, and incubated at 37°C. After 74 h, the cells were fixed and stained with crystal violet. Plaques were photographed with a Zeiss inverted microscope. Magnification, ×92.

particles and the ratio of enveloped to nonenveloped virus particles differed quite dramatically (Fig. 7; Table 2). Virus particles were rarely observed on the surfaces of F-gKβ-infected Vero cells, whereas enveloped virions were frequent and numerous components of the surfaces of F-infected Vero cells and of F-gKβ-infected VK-302 cells (Fig. 7). The few virions which were occasionally observed near the surface of F-gKβ-infected Vero cells were mostly unenveloped particles and were often surrounded by cell debris, indicating that the particles originated from damaged cells. The lack of virus particles on the surfaces of F-gKβ-infected Vero cells was consistent with our observation that there was a 300- to 1,000-fold decrease in the number of infectious virus particles which accumulated in the cell culture supernatant (see above). Large numbers of unenveloped capsids accumulated in the cytoplasm of F-gKβ-infected Vero cells and, to a lesser extent, enveloped virions were found within membrane vesicles (Fig. 7; Table 2). Moreover, enveloped virions in the cytoplasm of F-gKβ-infected Vero cells were frequently morphologically aberrant, e.g., there were several capsids within a single envelope or

TABLE 1. Production of infectious virus and plaque formation by F-gKβ on noncomplementing cells

Virus	Cell type grown on[a]	Genotype	Phenotype	Cell type on which titers determined[b]	Titer (PFU/ml)	Titer relative to VK302[c]
F	Vero	gK⁺	gK⁺	Vero	8.6×10^8	1.02
				VK302	8.4×10^8	1.00
				Fibroblasts	6.0×10^8	0.71
F-gKβ	Vero	gK⁻	gK⁻	Vero	$<10^{2}$[d]	$<10^{-6}$
				VK302	8.8×10^8	1.00
				Fibroblasts	4.8×10^{5}[d]	0.054
F-gKβ	VK302	gK⁻	gK⁻	Vero	$<10^{2}$[d]	$<10^{-6}$
				VK302	8.2×10^8	1.00
				Fibroblasts	5.5×10^{7}[d]	0.067

[a] The indicated cells were infected with 2 PFU per cell and incubated at 37°C for 24 h. Infected cells were harvested in cell culture media and disrupted by sonication.
[b] Serial dilutions were plated on the indicated cells and then incubated at 37°C for 96 h.
[c] The virus titer on VK302 cells was defined as 1.00, and the relative titers on fibroblasts and Vero cells are indicated.
[d] Microscopic plaques were composed of 3 to 6 cells, whereas plaques caused by wild-type HSV-1 were composed of 100 to 200 cells.

partially enveloped structures (Fig. 7, panel 3). By contrast, in wild-type-F-infected Vero cells and F-gKβ-infected VK-302 cells, there were fewer unenveloped particles in the cytoplasm and the majority of enveloped particles that accumulated inside cells were found in the space between the inner and outer nuclear membranes (perinuclear space). In the absence of gK, there were fivefold fewer enveloped particles present in the perinuclear space (Table 2). For all three samples, F-gKβ-infected Vero cells, F-infected Vero cells, and F-gKβ-infected VK302 cells, over 3,500 virus particles in association with at least 15 randomly selected cells were counted; thus, the observations should be considered highly significant. These results demonstrated that virus particles produced in the absence of gK seldom reach the cell surface and extracellular space and accumulate predominantly in the cytoplasm as unenveloped particles and morphologically aberrant particles. It should also be noted that although there were four- to eightfold fewer enveloped particles produced in F-gKβ-infected Vero cells (Table 2), these enveloped particles were of very low specific infectivity because infectious viruses were reduced 100-fold (Table 1).

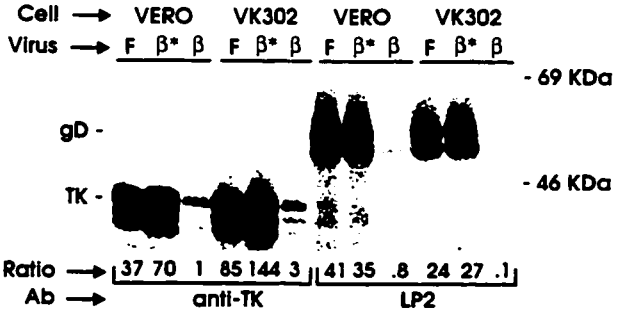

FIG. 6. Expression of early viral proteins in cells infected with wild-type HSV-1 (F) or the gK-negative mutant F-gKβ. Preparations of wild-type HSV-1 (F) or F-gKβ were produced with Vero cells (F and β) or with complementing VK302 cells for F-gKβ (β*) by infecting the cells with 2.0 PFU per cell. The virus preparations were made in parallel, and equal amounts of the two preparations were used to infect Vero or VK302 cells [2.5 PFU per cell for HSV-1 (F) and F-gKβ (β*)]. After 2 h, the cells were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine for 2 h. Cell extracts were mixed with MAb LP2, specific for gD, or rabbit serum specific for TK (anti-TK) and subsequently with protein A-Sepharose. The precipitated proteins were separated by electrophoresis in 8.5% polyacrylamide gels. The gels were dried, and the relative amount of each HSV-1 protein was quantified with a phosphoimager (Ratio). HSV-1 proteins gD and TK and molecular mass markers of 69 and 46 kDa are indicated. Ab, antibody.

Processing and transport of viral glycoproteins in F-gKβ-infected Vero cells. Previous studies have suggested that inhibition of HSV glycoprotein glycosylation or processing can result in the intracytoplasmic accumulation of virus particles and inhibition of virus egress (13, 28, 49, 52, 59). Since egress of HSV-1 appears to be impaired in viruses lacking gK, we investigated whether the maturation of HSV-1 glycoproteins was altered in F-gKβ-infected Vero cells. Vero and VK302 cells were infected with wild-type F or F-gKβ and radiolabelled by using a pulse-chase protocol, and gB, gD, gH, or TK was immunoprecipitated from cell extracts. Since F-gKβ was derived from complementing cells, similar quantities of gB, gD, and gH were observed in wild-type-F- and F-gKβ-infected cells, but in the F-gKβ-infected Vero cells we would not expect expression of gK. On the basis of their reduced electrophoretic mobilities after a chase period, the HSV glycoproteins were converted to mature glycoproteins with similar kinetics in cells infected with mutant and wild-type viruses (Fig. 8). Proteolytic degradation of a fraction of gB and TK was observed, a common phenomenon in extracts from Vero cells (50). These results indicated that, although virus egress appears to be inhibited in the absence of gK, the bulk of the HSV-1 glycoproteins were processed to the mature forms in the medial Golgi and *trans*-Golgi apparatus and probably reach the cell surface. Because the enveloped virus particles which accumulated in F-gKβ-infected cells were distributed throughout the cytoplasm as well as in the perinuclear space, it is likely that the particles contained a mixture of processed and unprocessed forms of the glycoproteins, though analysis of the total glycoprotein expressed in cells does not necessarily address this point (4).

In studies employing a UL20⁻ HSV-1, which also exhibits an egress defect in Vero cells, evidence was presented that HSV gD and gC were present in reduced amounts on the cell surface (1). Consequently, we used confocal immunofluorescence microscopy to compare subcellular localization and cell surface expression of gD in Vero cells infected with wild-type HSV-1 (F) and F-gKβ. Although these results were not absolutely quantitative, the comparison showed clearly that there were no large differences in the cellular distribution or abundance of gD in F-gKβ-infected Vero cells relative to wild-type-F-infected Vero cells (data not shown). Therefore, the transport of

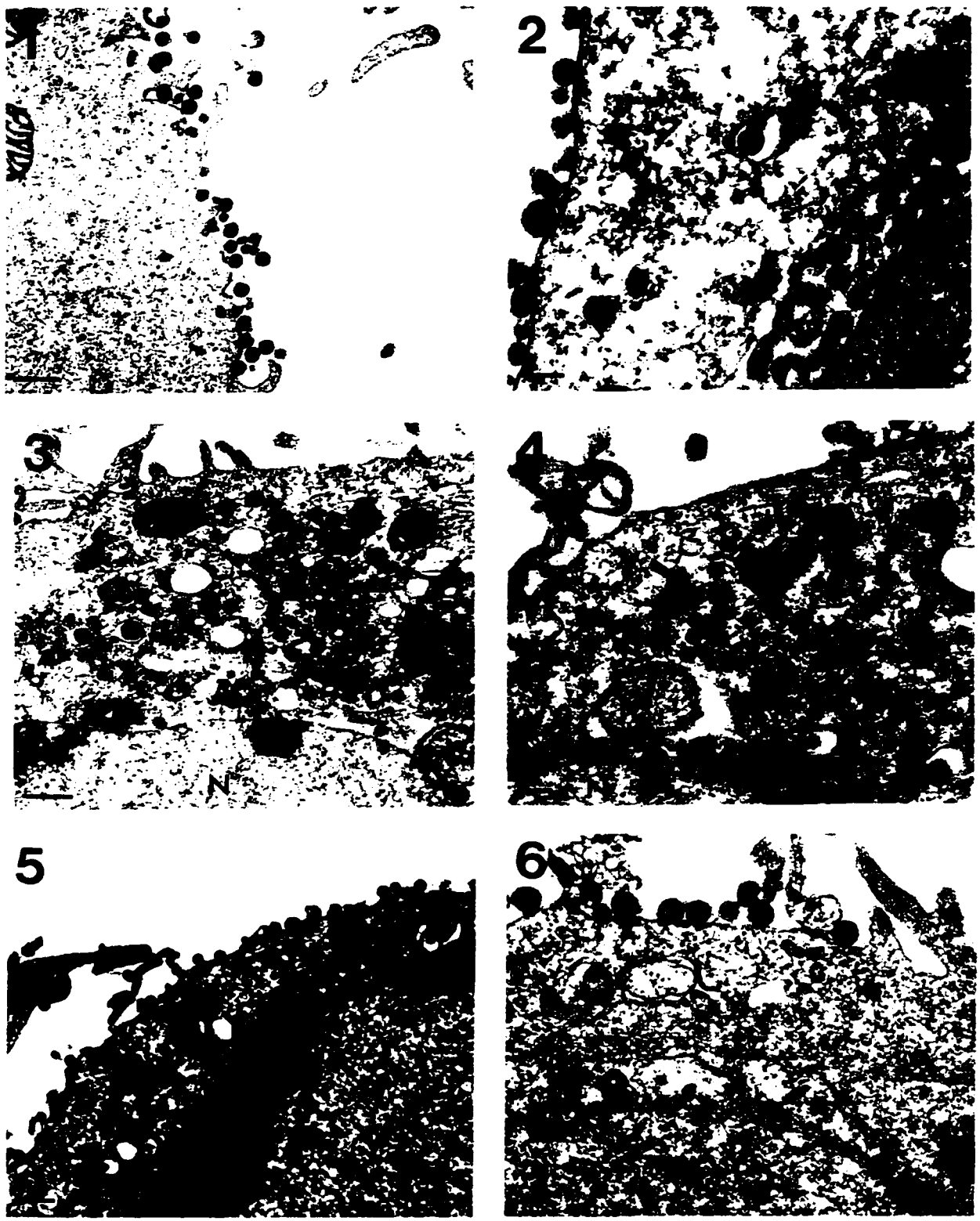

FIG. 7. Electron micrographs of cells infected with HSV-1 (F) or mutant F-gKβ. Vero cells were infected with wild-type HSV-1 (F) (1 and 2) or F-gKβ (3 and 4), and VK302 cells were infected with F-gKβ (5 and 6) by using 5 PFU/cell and then the cells were incubated at 37°C for 20 to 24 h. The cells were fixed with 2% glutaraldehyde and prepared for electron microscopy. The arrow indicates cytoplasmic unenveloped nucleocapsids which accumulate in F-gKβ-infected Vero cells. N, nucleus. Bars, 500 nm in panels 1, 3, and 5 and 200 nm in panels 2, 4, and 6.

TABLE 2. Distribution of virions in Vero cells infected with HSV-1 (F) or F-gKβ

Subcellular locale	Virion structure	Avg no. (%) of particles/cell[a]	
		HSV-1 (F)	F-gKβ
Nucleus	Nonenveloped	34 (14)	36 (15)
Perinuclear space	Enveloped	99 (39)	18 (7.3)
Cytoplasm	Enveloped	22 (8.8)	38 (15)
	Nonenveloped	13 (5.2)	120 (49)
Cell Surface	Enveloped	80 (32)	8 (3.2)[b]
	Nonenveloped	3 (1.2)	27 (11)[b]
Total (all locations)		251 (100)	247 (100)

[a] Virus particles present in the nucleus, perinuclear space, and cytoplasm and on the cell surface were counted in electron micrographs of at least 15 randomly sampled Vero cells infected with HSV-1 (F) or F-gKβ. The numbers represent the average number of particles per cell, and the numbers in parentheses indicate the percentage of particles per cell.

[b] The virus particles were loosely associated with the cell surface and appeared to originate from cells that were damaged.

the bulk of the HSV glycoproteins was not dramatically affected in the absence of gK.

Overexpression of gK inhibits HSV egress. Previously, we described and characterized a Vero cell transformant, gK-9, which contains several hundred copies of the UL53 (gK) gene and expresses approximately 15-fold more gK after infection with HSV-1 than is observed to be present in comparably infected Vero cells (33). HSV-1 forms plaques inefficiently on gK-9 cells, such that the numbers of plaques were reduced by approximately 200-fold relative to the number produced on Vero cells (33). It appeared possible that overexpression of the gK protein might also inhibit virus egress. gK-9 cells were infected with wild-type HSV-1 (F), and then after 20 h the cells were prepared for electron microscopy. As with F-gKβ-infected Vero cells, virus particles were rarely observed on the surfaces of infected gK-9 cells (Fig. 9). However, in contrast to the observations with F-gKβ-infected Vero cells, virtually all of the enveloped virus particles found in infected gK-9 cells accumulated in the perinuclear space and cytoplasmic nucleocapsids were not detected. Moreover, we frequently observed large multilamellar membranous structures juxtaposed with the nuclear membranes of infected gK-9 cells (Fig. 9, panel 4, arrow). Since gK-9 cells do not exhibit defects in HSV protein synthesis, glycosylation, or protein transport (33, 34), it would appear that overexpression of gK causes defects in virus egress, just as there are defects in this process when gK is not expressed.

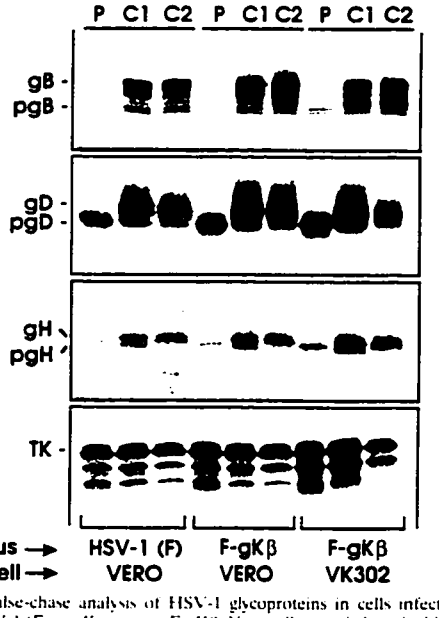

FIG. 8. Pulse-chase analysis of HSV-1 glycoproteins in cells infected with wild-type HSV-1 (F) or gK-negative F-gKβ. Vero cells were infected with HSV-1 (F) or F-gKβ, and VK302 cells were infected with F-gKβ (derived from VK302 cells). After 3 h, infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min and then cell extracts were made immediately (P) or the cells were washed with medium containing methionine and cysteine and incubated in this medium for 90 min (C1) or 210 min (C2) before cell extracts were made. Cell extracts were mixed with MAb 15βB2, specific for gB; LP2, specific for gD; 53S, specific for gH, or rabbit anti-TK serum, and precipitated proteins were eluted from protein-A Sepharose and analyzed on 12% acrylamide gels. The positions of HSV-1 proteins pgB, gB, pgD, gD, pgH, gH, and TK are indicated.

DISCUSSION

In this report, we describe an HSV-1 mutant, F-gKβ, which is unable to express gK and demonstrate that gK is essential for virus replication in cultured monkey Vero cells, human R970 cells, and normal human fibroblasts. The primary defect in replication of this gK-negative mutant appears to lie in virus egress from the nucleus of infected cells to the cell surface. In Vero cells infected with F-gKβ, virus particles were rarely observed on the cell surface, whereas in cells infected with wild-type HSV-1, the surface membranes were frequently encrusted with virus particles late in the infection. Moreover, large numbers of unenveloped cytoplasmic particles accumulated in the cytoplasm of F-gKβ-infected Vero cells. Those enveloped virus particles that were present in the cytoplasm of F-gKβ-infected Vero cells were of low specific infectivity, and the particles entered host cells or initiated expression of early proteins poorly. For example, expression of TK was reduced by 70-fold when virus particles were produced in cells lacking gK. gK$^-$ viruses, produced in Vero cells, displayed a 100-fold reduction in plaque formation on complementing cells, even though the number of enveloped capsids observed in F-gKβ-infected Vero cells was only 2- to 4-fold lower than was observed in F-infected Vero or F-gKβ-infected VK-302 cells. Therefore, only a small fraction of the enveloped particles produced in the absence of gK were actually able to initiate an infection. The low infectivity of these viruses was amplified during production of virus plaques, so that plaques were extremely rare, microscopic, and detected only after extended periods.

In order to avoid disrupting the UL52 gene, which is required for HSV DNA replication (26), we inserted a *lacZ* gene cassette into a region of the UL53 ORF which allowed expression of 112 residues of gK (approximately one-third of the protein) fused to 26 residues derived from the ICP6::*lacZ* gene cassette. Because this protein contains the gK signal peptide, it is probable that the protein passes into the endoplasmic reticulum (ER), where the signal peptide is cleaved, since the signal peptide is removed from a truncated gK protein translated in vitro (55). Without the membrane-spanning domains of gK or a means of localizing itself to membranes, it is highly doubtful that the truncated protein could function like gK in any fashion. It is conceivable that the truncated protein was somehow

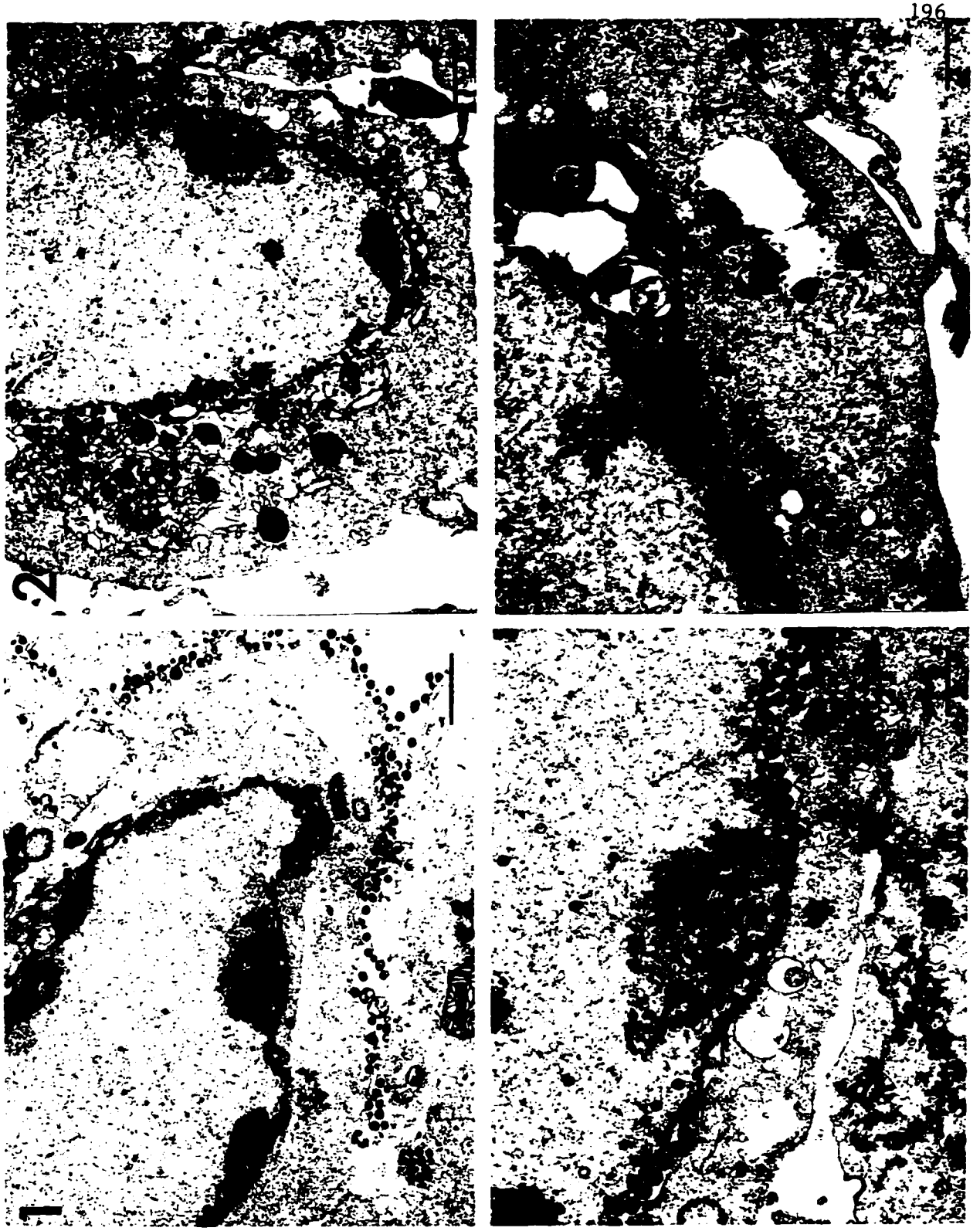

FIG. 9. Electron micrographs of HSV-infected Vero or gK-9 cells, which overexpress gK. Monolayers of Vero cells (1) or gK-9 cells (2, 3, and 4) were infected with HSV-1 (F) by using 5 PFU per cell and then the cells were incubated at 37°C for 22 h, fixed with 2% glutaraldehyde, and processed for electron micrographs. The arrow indicates a multilamellar vesicle juxtaposed with the nuclear envelope. Bars, 1 μm in panels 1 and 2 and 500 nm in panels 3 and 4.

poisonous to HSV replication; however, this did not occur, because the truncated protein was expressed in F-gKβ-infected VK-302 cells, where replication was normal. In addition, we would argue that the phenotype of F-gKβ, which shows defects in virus egress from the nuclear envelope to cytoplasmic membranes, is consistent with previous observations that gK is localized to nuclear and ER membranes (34). Moreover, to our knowledge there are no precedents for negative effects stemming from such truncated proteins.

Several models have been proposed to account for egress of herpesviruses from cells. There is consensus that viral nucleocapsids, formed in the nucleus, acquire a lipid envelope containing immature forms of the HSV glycoproteins at the inner nuclear envelope and that these particles transiently accumulate in the perinuclear space i.e., the region between the inner and outer nuclear membranes (15, 45, 61, 66, 69). However, there is controversy over the process by which these perinuclear particles reach the cell surface. One camp suggests that enveloped virus particles are transported to the cell surface in the interior of a series of membranous vesicles or tubules derived from the ER and Golgi apparatus (12, 38, 45, 61, 66). Others have argued that enveloped particles present in the perinuclear space can fuse with cellular membranes (e.g., the outer nuclear envelope) producing unenveloped cytoplasmic capsids. These unenveloped capsids are thought to subsequently acquire an envelope derived from cytoplasmic, e.g., Golgi-apparatus-derived, membranes modified with viral glycoproteins (24, 39, 48, 69). Much of the debate as to how herpesviruses exit cells surrounds observations of nucleocapsids juxtaposed with cytoplasmic membrane patches, presumably modified with viral glycoproteins. From these static electron microscopic images, it is by no means clear whether particles associated with cytosolic membranes are in the process of envelopment or deenvelopment. Similarly, studies using inhibitors of membrane transport processes do not discriminate between these models of virus egress (14, 38). Cytoplasmic nucleocapsids can be observed in HSV-infected cells; however, these capsids have been attributed to a dead-end pathway in which enveloped particles fuse with cytoplasmic vesicles (12). It is possible that the egress pathway used by HSV differs from that of other herpesviruses, especially in different cell types. Until further genetic and biochemical analyses of this problem are carried out, the question of how herpesviruses exit cells will remain open.

Virus mutants such as that characterized here, and others that are described below, offer some of the best opportunities for understanding herpesvirus egress. It appears unlikely that gK alters the initial envelopment of capsids at the inner nuclear envelope because normal or larger than normal amounts of virus particles reach the cytoplasm of F-gKβ-infected Vero cells. However, in the absence of gK, reduced numbers of enveloped particles were observed in the perinuclear space. Moreover, dramatically increased numbers of unenveloped capsids were observed in the cytoplasm of F-gKβ-infected Vero cells. These results demonstrate that gK facilitates transport of virus particles through the cytoplasm to the cell surface and plays a role in the earliest transport processes involving movement of enveloped particles from the perinuclear space.

On the basis of the two models for herpesvirus egress, gK could function in either of two ways. If enveloped virus particles transit through the cytoplasmic vesicular network, as described, e.g., by Johnson and Spear (38), then gK may prevent fusion between cellular and viral membranes, so that the particles do not lose their envelope during transport. By this model, cytosolic capsids would be considered a dead-end product (12). Further, gK may inhibit fusion between the virion envelope and the outer nuclear envelope, because in the absence of gK fewer enveloped virions accumulated in the perinuclear space. However, if the second model, described by Jones and Grose (39) and Whealy et al. (69), involving a series of envelopments and deenvelopments is correct, one could hypothesize that gK promotes envelopment of capsids on cytoplasmic membranes. gK is primarily localized to a perinuclear region as determined by immunofluorescence experiments, and gK oligosaccharides are not processed in the medial Golgi and *trans*-Golgi apparatus, consistent with localization in the nuclear envelope, the ER, and the *cis*-Golgi apparatus (34). Thus, if indeed gK functions to promote envelopment of cytosolic capsids, it should do so in the ER or *cis*-Golgi apparatus. This might also be considered unlikely because most of the electron microscopic studies propose reenvelopment in the *trans*-Golgi apparatus; in addition, gK has not been detected in the virus envelope (34). Also, a role for gK in promoting reenvelopment in the cytoplasm is difficult to rationalize with observations of decreased numbers of enveloped particles in the perinuclear space.

Consistent with the hypothesis that gK functions during the early stages of virus egress, i.e., in nuclear and ER membranes, overexpression of gK in HSV-infected gK-9 cells caused enveloped particles to accumulate in the perinuclear space. It is interesting that the absence of gK has the opposite effect, causing reduced numbers of enveloped particles to accumulate in the perinuclear space. Moreover, gK overexpression was associated with accumulation of large multilamellar vesicles, apparently derived from the outer nuclear envelope. If one hypothesizes that gK functions to inhibit membrane fusion events, overexpression of wild-type gK might also inhibit vesicular transport of viruses from the perinuclear space, since membrane fusion occurs as vesicles pinch off the ER and nuclear envelope. This could cause accumulation of virus particles in the perinuclear space and multilamellar vesicles next to the nuclear envelope. In the absence of gK, unrestricted fusion of enveloped particles with the outer nuclear envelope might reduce the numbers of particles present in the perinuclear space. In the deenvelopment-reenvelopment model of egress, by which it could be argued that gK facilitates envelopment of capsids at cytoplasmic sites, it is more difficult to understand how gK overexpression would promote accumulation of perinuclear particles or multilamellar vesicles. By this model, if one argues that gK possesses a property which promotes envelopment, overexpression of the glycoprotein might not be expected to cause particles to accumulate in the perinuclear space.

Considering all of this, we favor a model of HSV egress in which enveloped virus particles transit from the perinuclear space through the Golgi network in membrane vesicles in order to gain access to the cell surface. We propose that in this model, gK prevents fusion between virion and cellular membranes. In the absence of gK, there is unrestricted fusion between viral and cellular membranes (the outer nuclear envelope, the ER, and perhaps the Golgi network), so that capsids accumulate in the cytoplasm. Overexpression of gK apparently has the opposite effect, obstructing vesicular transport of virus particles out of the perinuclear space, a process which requires membrane fusion. However, it should be noted that we have not observed inhibition of HSV glycoprotein transport in HSV-infected gK-9 cells (33, 34).

A number of other HSV-1 mutants with defects in virus egress have been described. An HSV-1 mutant expressing a temperature-sensitive form of gH retains infectious particles containing gH in cells and secretes noninfectious virus particles lacking gH (18). It is not clear whether these extracellular

particles lose gH, e.g., by proteolysis during egress, or whether mutant gH is not incorporated into the envelope of those particles which ultimately escape cells. An HSV-1 mutant with a defect in the UL11 gene product, a myristylated membrane protein, displays reduced levels of cell surface and extracellular virus and increased quantities of unenveloped capsids in the cytoplasm (3, 42). The ratio of unenveloped/enveloped cytoplasmic nucleocapsids produced by the UL11 mutant was approximately 3:1, similar to that observed with our gK mutant. The UL11 mutant can produce plaques, although these are reduced in number. However, the UL11 defect appears much less severe than the defect with the gK$^-$ mutant described here. Notwithstanding this difference, the UL11 and gK mutations produce similar phenotypes, suggesting that the proteins have similar functions and may, perhaps, interact in some manner to promote passage of enveloped particles through the cytoplasm. The UL20 gene product has some properties similar to those of gK, since both proteins are multimembrane-spanning proteins and are predominately localized to the nuclear and perinuclear membranes, although UL20 is also localized to the Golgi apparatus (4, 17, 34, 43, 54, 67). However, in contrast to the gK mutation, which caused accumulation of capsids and enveloped particles in the cytoplasm, the UL20 mutation caused accumulation of enveloped particles in the perinuclear space of Vero cells (4). The phenotype of the UL20$^-$ mutant was more similar to that we observed when gK was overexpressed. This result again points to the complexity of the viral egress process.

It is not entirely clear how the results reported here relate to previous observations that point mutations in gK lead to fusion of infected cells. Since gK cannot be detected on the surfaces of infected cells or in virions (34), it is unlikely that gK is present in the fusion complex used during cell fusion or as virus enters cells. The most likely explanation for the syncytial phenotype of gK mutants, given the observations reported here, is that these point mutations alter transport of the fusion complex or some other viral component, e.g., the regulator of fusion to the cell surface. Consistent with this hypothesis are two observations. When gK was expressed at less than wild-type levels, e.g., in F-gKβ-infected VK243, VK295, or VK308 cells, syncytial plaques were produced; thus, underexpression of gK, an internal protein, promotes fusion between surface membranes. Secondly, we recently observed that infection of cells with syncytial HSV mutants leads to the accumulation of larger than normal quantities of cytoplasmic virus particles (33a). Furthermore, the UL20$^-$ mutant formed small, syncytial plaques on 143 cells, similar to those we observed when gK was expressed at reduced levels. These results suggest that production of syncytia by HSV mutants is linked to defects in egress of virus particles and add additional support to the hypothesis that gK regulates fusion at intracellular sites. One explanation of cell fusion would be that fewer virus particles or other viral components reach the surfaces of cells infected with syncytial mutants, yet viral fusion complexes e.g., gB, gD, and gH/gL, reach the surface and function in an unregulated manner. Clearly, the relationship between HSV egress and cell fusion is complex, and further characterization of gK and gK mutants will be necessary before this protein's role in virus egress can be adequately understood.

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REFERENCES

1. **Avitabile, E., P. L. Ward, C. Di Lassaro, M. R. Torrisi, B. Roizman, and G. Campadelli-Fiume.** 1994. The herpes simplex virus U_L20 protein compensates for the differential disruption of exocytosis of virions and viral membrane glycoproteins associated with fragmentation of the Golgi apparatus. J. Virol. **68**:7397–7405.
2. **Baghian, A., L. Huang, S. Newman, S. Jayachandra, and K. G. Kousoulas.** 1993. Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant *amb*1511-7 causes extensive cell fusion. J. Virol. **67**:2396–2401.
3. **Baines, J. D., and B. Roizman.** 1992. The U_L11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J. Virol. **66**:5168–5174.
4. **Baines, J. D., P. L. Ward, G. Campadelli-Fiume, and B. Roizman.** 1991. The U_L20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J. Virol. **65**:6414–6424.
5. **Balan, P., N. Davis-Poynter, S. Bell, H. Atkinson, H. Browne, and T. Minson.** 1994. An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI, or the putative gJ. J. Gen. Virol. **75**:1245–1258.
6. **Bond, V. C., and S. Person.** 1982. The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. J. Gen. Virol. **61**:245–254.
7. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132**:368–376.
8. **Bzik, D. J., B. A. Fox, N. A. Deluca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137**:185–190.
9. **Bzik, D. J., and S. Person.** 1981. Dependence of herpes simplex virus type 1-induced cell fusion on cell type. Virology **110**:35–42.
10. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in entry and cell fusion. J. Virol. **62**:2596–2604.
11. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
12. **Campadelli-Fiume, G., F. Farabegoli, S. Di Gaeta, and B. Roizman.** 1991. Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. **65**:1589–1595.
13. **Campadelli-Fiume, G., L. Poletti, F. Dall'Olio, and F. Serafini-Cessi.** 1982. Infectivity and glycoprotein processing of herpes simplex virus type 1 grown in a ricin-resistant cell line deficient in *N*-acetylglucosaminyl transferase I. J. Virol. **43**:1061–1071.
14. **Cheung, P., B. W. Banfield, and F. Tufaro.** 1991. Brefeldin A arrests the maturation and egress of herpes simplex virus particles during infection. J. Virol. **65**:1893–1904.
15. **Compton, T., and R. J. Courtney.** 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus-infected cells. J. Virol. **49**:594–597.
16. **Davis-Poynter, N., S. Bell, T. Minson, and H. Browne.** 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J. Virol. **68**:7586–7590.
17. **Debroy, C., N. Pederson, and S. Person.** 1985. Nucleotide sequence of herpes simplex virus type 1 gene that causes cell fusion. Virology **145**:36–48.
18. **Desai, P. J., P. A. Schaffer, and A. C. Minson.** 1988. Excretion of non-infectious virus particles lacking glycoprotein H by a temperature sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J. Gen. Virol. **69**:1147–1156.
19. **Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson.** 1993. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J. Virol. **68**:834–845.
20. **Dolter, K. E., R. Ramaswamy, and T. C. Holland.** 1994. Syncytial mutations in the herpes simplex virus type 1 gK (UL53) gene occur in two distinct domains. J. Virol. **68**:8277–8281.
21. **Feenstra, V., M. Jodaie, and D. C. Johnson.** 1990. Deletions in herpes simplex virus glycoprotein D define nonessential and essential domains. J. Virol. **64**:2096–2102.
22. **Forrester, A. J., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and A. C. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 deleted for glycoprotein H sequences. J. Virol. **66**:341–348.
23. **Gage, P. J., M. Levine, and J. C. Glorioso.** 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. J. Virol. **67**:2191–2201.
24. **Gershon, A. A., D. L. Sherman, Z. Zhu, C. A. Gabel, R. T. Ambron, and**

M. D. Gershon. 1994. Intracellular transport on newly synthesized varicella-zoster virus: envelopment in the *trans*-Golgi network. J. Virol. **68**:6372–6390.
25. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38**:50–58.
26. **Goldstein, D. J., and S. K. Weller.** 1988. An ICP6::*lacZ* insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. **62**:2970–2977.
27. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of adenovirus 5 DNA. Virology **52**:456–467.
28. **Haanes, E. J., C. M. Nelson, C. L. Soule, and J. L. Goodman.** 1994. The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. J. Virol. **68**:5824–5834.
29. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 411–463. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Hartman, S. C., and R. C. Mulligan.** 1988. Two dominant-acting selectable markers for gene transfer studies in mammalian cells. Proc. Natl. Acad. Sci. USA **85**:8047–8051.
31. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66**:2240–2250.
32. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66**:5603–5609.
33. **Hutchinson, L., F. L. Graham, W. Cai, C. Debroy, S. Person, and D. C. Johnson.** 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology **196**:514–531.
33a. **Hutchinson, L., and D. C. Johnson.** Unpublished data.
34. **Hutchinson, L., C. Roop, and D. C. Johnson.** 1995. Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. J. Virol. **69**:4556–4563.
35. **Jacobson, J. G., S. L. Martin, and D. M. Coen.** 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. **63**:1839–1843.
36. **Johnson, D. C., M. C. Frame, N. W. Ligas, A. M. Cross, and N. D. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62**:1347–1354.
37. **Johnson, D. C., G. Ghosh-Choudhury, J. R. Smiley, L. Fallis, and F. L. Graham.** 1988. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology **164**:1–14.
38. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43**:1102–1112.
39. **Jones, F., and C. Grose.** 1988. Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. J. Virol. **62**: 2701–2711.
40. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
41. **Little, S. P., and P. A. Schaffer.** 1981. Expression of the syncytial (syn) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two syn loci. Virology **112**:686–697.
42. **MacLean, C. A., B. Clark, and D. J. McGeoch.** 1989. Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristilated. J. Gen. Virol. **70**:3147–3157.
43. **MacLean, C. A., S. Efstathiou, M. L. Elliot, F. E. Jamieson, and D. J. McGeoch.** 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J. Gen. Virol. **72**:897–906.
44. **Minson, A. C., T. C. Hodgman, P. Digard, D. C. Hancock, S. E. Bell, and E. A. Buckmaster.** 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. J. Gen. Virol. **67**:1001–1013.
45. **Morgan, C., H. M. Rose, M. Holden, and E. P. Jones.** 1959. Electron microscope observations on the development of herpes simplex virus. J. Exp. Med. **110**:643–656.
46. **Navarro, D., P. Paz, and L. Pereira.** 1992. Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology **186**:99–112.
47. **Neidhardt, H., C. H. Schroder, and H. C. Kaerner.** 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. J. Virol. **61**:600–603.
48. **Nii, S., C. Morgan, and H. M. Rose.** 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. J. Virol. **2**:517–536.
49. **Olofsson, S., M. Milla, C. Hirschberg, C. E. De, and R. Datema.** 1988. Inhibition of terminal N- and O-glycosylation specific for herpesvirus-infected cells: mechanism of an inhibitor of sugar nucleotide transport across Golgi membranes. Virology **166**:440–450.
50. **Pereira, L., D. Dondero, and B. Roizman.** 1982. Herpes simplex virus glycoprotein gA/B: evidence that the infected Vero cell products comap and arise by proteolysis. J. Virol. **44**:88–97.
51. **Person, S., R. W. Knowles, G. S. Read, S. C. Warner, and V. C. Bond.** 1976. Kinetics of cell fusion induced by a syncytia-producing mutant of herpes simplex virus type 1. J. Virol. **17**:183–190.
52. **Pizer, L. I., G. H. Cohen, and R. J. Eisenberg.** 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. J. Virol. **34**:142–153.
53. **Pogue-Geile, K. L., G. T.-Y. Lee, S. K. Shapira, and P. G. Spear.** 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology **136**:100–109.
54. **Pogue-Geile, K. L., and P. G. Spear.** 1987. The single base pair substitution responsible for the syn phenotype of herpes simplex virus type 1, strain MP. Virology **157**:67–74.
55. **Ramaswamy, R., and T. C. Holland.** 1992. In vitro characterization of the HSV-1 UL53 gene product. Virology **186**:579–587.
56. **Read, G. S., S. Person, and P. M. Keller.** 1980. Genetic studies of cell fusion induced by herpes simplex virus type 1. J. Virol. **35**:105–113.
57. **Rodriguez, J. E., T. Moninger, and C. Grose.** 1993. Entry and egress of varicella virus blocked by same anti-gH monoclonal antibody. Virology **196**: 840–844.
58. **Roop, C., L. Hutchinson, and D. C. Johnson.** 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells and its particles lack glycoprotein H. J. Virol. **67**:2285–2297.
59. **Ruyechen, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–687.
60. **Sanders, P. G., N. M. Wilkie, and A. J. Davison.** 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. J. Gen. Virol. **63**:277–287.
61. **Schwartz, J., and B. Roizman.** 1969. Concerning the egress of herpes simplex virus from infected cells. Electron microscope observations. Virology **38**:42–49.
62. **Serafini-Cessi, F., F. Dall'Olio, M. Scanavini, and G. Campadelli-Fiume.** 1983. Processing of herpes simplex virus type 1 glycans in cells defective in glycosyl transferase of the Golgi system: relationship to cell fusion and virion egress. Virology **131**:59–70.
63. **Shieh, M.-T., and P. G. Spear.** Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. J. Virol. **68**:1224–1228.
64. **Smiley, J., B. Fong, and W.-C. Leung.** 1981. Construction of a double jointed herpes simplex virus DNA molecule: inverted repeats are required for segment inversion and direct repeats promote deletions. Virology **113**:345–362.
65. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Boca Raton, Fla.
66. **Torrisi, M. R., C. Di Lazzara, A. Pavan, L. Pereira, and G. Campadelli-Fiume.** 1992. Herpes simplex virus envelopment and maturation studied by fracture label. J. Virol. **66**:554–561.
67. **Ward, P. L., G. Campadelli-Fiume, E. Avitabile, and B. Roizman.** 1994. Localization and putative function of the U_L20 membrane protein in cells infected with herpes simplex virus 1. J. Virol. **68**:7406–7417.
68. **Weise, K., H. C. Kaerner, J. Glorioso, and C. H. Schroder.** 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J. Gen. Virol. **68**:1909–1919.
69. **Whealy, M. E., J. P. Card, R. P. Meade, A. K. Robbins, and L. W. Enquist.** 1991. Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. **65**:1066–1081.

CHAPTER 6

Discussion

1.0 Historical Perspective

When this project began our knowledge of the UL53 gene was limited to the discovery that the vast majority of syncytial variants, in at least some strains of HSV-1, carry syn-1 mutations mapping to this open reading frame (Keller, 1976a; Little & Schaffer, 1981; Bond et al., 1982; Bond & Person, 1984; Read et al., 1980; Debroy et al., 1985; Pogue-Geile et al., 1987; Roop et al., 1993; Dolter et al., 1994). Complementation experiments (ie. mixed infection of cells with wild-type and syn-1 mutants of HSV-1) designed to clarify the role of UL53 in HSV-induced membrane fusion, had yielded conflicting results. Although initial inquiries had suggested that the syn-1 phenotype is recessive to the wild-type (wt) morphology (Roizman, 1962; Keller, 1976a; Manservigi et al., 1977), subsequent analysis demonstrated that dominance of the wild-type morphology is dependent both on, cell-type and on the syn-1 variant used (Lee & Spear, 1980; Read et al., 1980; Bzik & Person, 1981; Bond et al., 1982). For instance, syn-1 variants have been described which exhibit a plaque morphology that is recessive to, or dominant over wild-type HSV-1 in a variety of cell types (Read et al., 1980; Little & Schaffer, 1981; Bond et al., 1982), whereas other syn-1 mutants exhibit a phenotype which is dominant in mixed infections of HEL cells, co-dominant in CV-1 cells, and recessive to wild-type virus in HEp-2 cells (Roizman, 1962; Keller, 1976a; Manservigi et al., 1977; Bzik & Person, 1981).

At least four interpretations can be put forward to explain the results of

complementation experiments. (1) Recessive syn-1 mutations could represent a loss-of-function suggesting that wt-UL53 is a regulatory protein which prevents uncontrolled cell-cell fusion. (2) Loss-of function mutations could also produce the dominant and co-dominant phenotype if wt-UL53 is assembled into inactive homo- or hetero-oligomers. (3) In addition, dominant and codominant syn-1 mutations could denote a gain of function indicating that mutant UL53 induces fusion. (4) Finally, the genetic backgrounds of wt and syn-1 strains are likely to vary and the presence of secondary loci may determine if the syn-1 phenotype is dominant or recessive in mixed infections with "wild-type" virus (eg. mutations in US6-gD, US8-gE, US7-gI, UL22-gH, UL10-gM or UL45 can reduce cell-cell fusion without diminishing virus infectivity [Feenstra et al., 1990; Balan et al., 1992; Davis-Poynter et al., 1994; Haanes et al., 1994; Wilson et al., 1994], and there are mutations in UL27-gB, US6-gD or UL10-gM which may enhance cell-cell fusion but do not confer a syncytial phenotype [Izumi & Stevens, 1990; Yuhasz & Stevens, 1993]).

Consequently, the function of wt-UL53 remained unclear. UL53 could act directly or indirectly, to inhibit cell-cell fusion and syn-1 mutations may abolish this function. Alternatively, UL53 may participate in cell-cell fusion directly, and syn-1 mutations may remove the regulatory controls governing this process. For example, UL53 could activate the HSV-1 fusogenic machinery, or exercise a fusogenic activity intrinsic to UL53 function. Nevertheless, the expectation that UL53 would exert a regulatory influence on the membrane fusion events required for virus penetration eventually prevailed, largely because the product of the syn-3 locus, UL27-gB, had been characterized and was thought to be a logical candidate for the HSV-1 "fusogenic factor" (see Introduction: section 3.2.1; Manservigi et al., 1977; Sarmiento et al., 1979; Haffey & Spear, 1980; Cai et al., 1987, 1988).

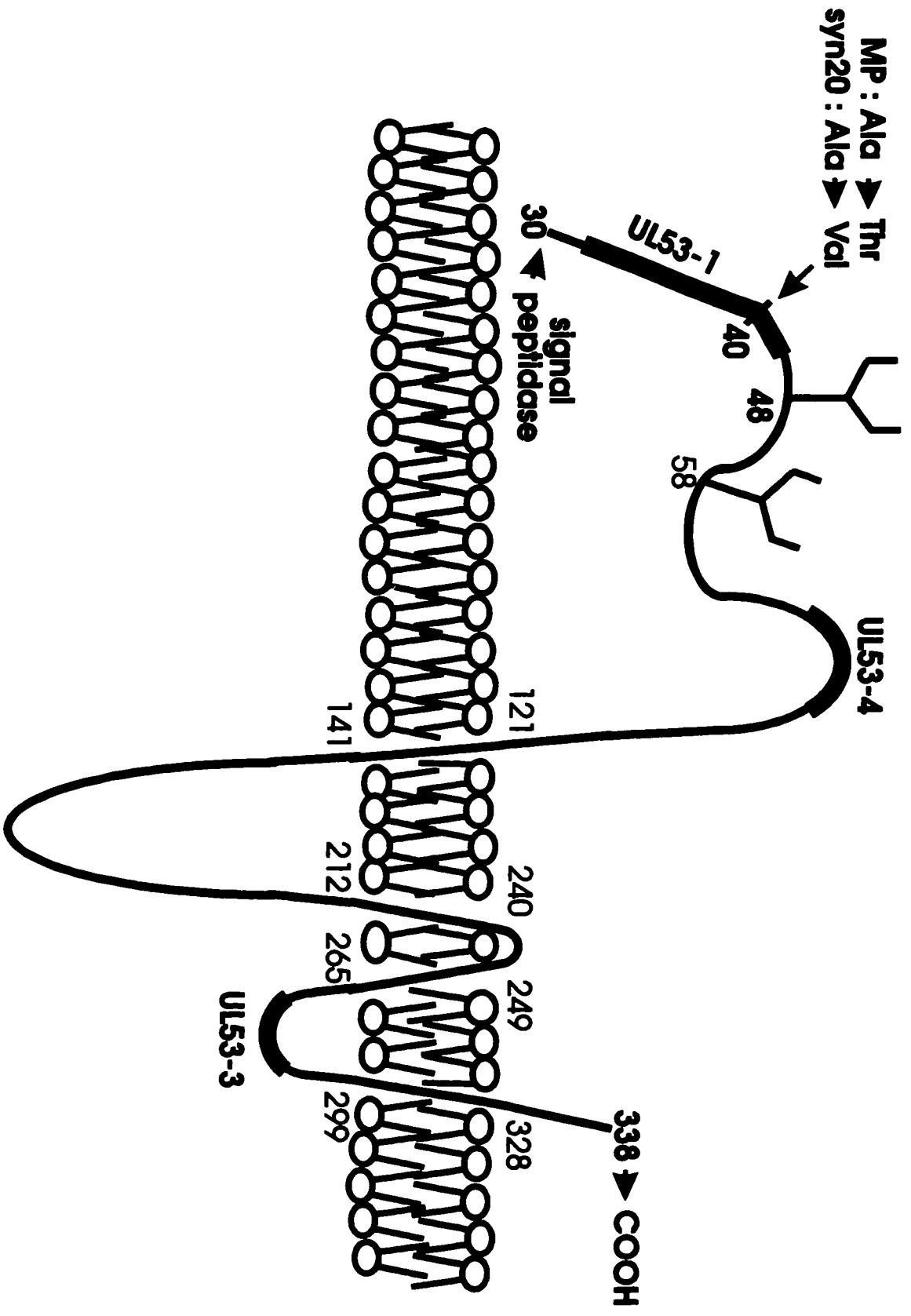
1.1 Attributes of the HSV-1 UL53 open reading frame and UL53 homologs from other Alphaherpesvirinae.

Prior to commencement of this thesis, UL53 genes from HSV-1 and VZV had been cloned and sequenced (Debroy et al., 1985; Davison & Scott, 1986; Pogue-Geile et al., 1987), and in the following years the UL53 homologs of other alphaherpesviruses (eg. BHV-1, EHV-1, ILTV, MDV, PRV & VZV) have been sequenced (Zhao et al., 1992; Telford et al., 1992; Ren et al., 1994; Baumeister et al., 1995; Johnson et al., 1995; Khadr et al., 1996). Furthermore, homologs of UL53 have been identified without exception, in all of the alphaherpesviruses studied to date, highlighting the importance of UL53 in alphaherpesvirus replication.

The amino acid sequence specified by the HSV-1 UL53 gene is indicative of a very hydrophobic protein likely to have multiple membrane spanning domains, and the UL53 homologs of other alphaherpesviruses share these characteristics. The UL53 homologs are predicted to be type 1 or III membrane glycoproteins and share the following attributes: (1) a similar size (312 to 354 residues), (2) similar hydrophobicity profiles, (3) an N-terminal signal sequence, (4) a hydrophillic region, downstream of the signal peptide which includes at least two consensus sites for N-linked glycosylation, and (5) four hydrophobic, potentially membrane spanning domains, which occupy a similar position in each of the UL53 homologs (see Figures 1.6 and 2.6) (Debroy et al., 1985; Pogue-Geile et al., 1987; Zhao et al., 1992; Ren et al., 1994; Baumeister et al., 1995; Johnson et al., 1995; Khadr et al., 1996; Nakai and Kanehisa, 1992). A comparison of the amino acid sequences from the UL53 homologs of six mammalian and two avian alpha-herpesviruses revealed a high degree of conservation, ranging from 21% amino acid identity between HSV-1 and ILTV, to 84% identity between HSV-1 and HSV-2 (Gene Works program, 1992). The average sequence identity between any given UL53

Figure 1.6 Model of UL53 protein.

This model is based on several publications (Debroy et al., 1985; Pogue-Geile & Spear, 1987; Ramaswamy & Holland, 1992) and computer data generated by the PSORT server (psort@nibb.ac.jp, Nakai & Kanehisa, 1992). The N-linked glycosylation signals (residues 48 & 58), and the approximate location of the peptides (UL53-1, UL53-3, UL53-4) used to generate the anti-UL53 antibodies, are indicated. In addition, the amino acid substitutions at residue 40 responsible for the syncytial phenotype of HSV-1 strains MP and syn20 are noted.


MP : Ala → Thr
syn20 : Ala → Val
UL53-1
30
signal peptidase
40
48
58
UL53-4
121
141
212
240
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UL53-3
299
328
338 → COOH

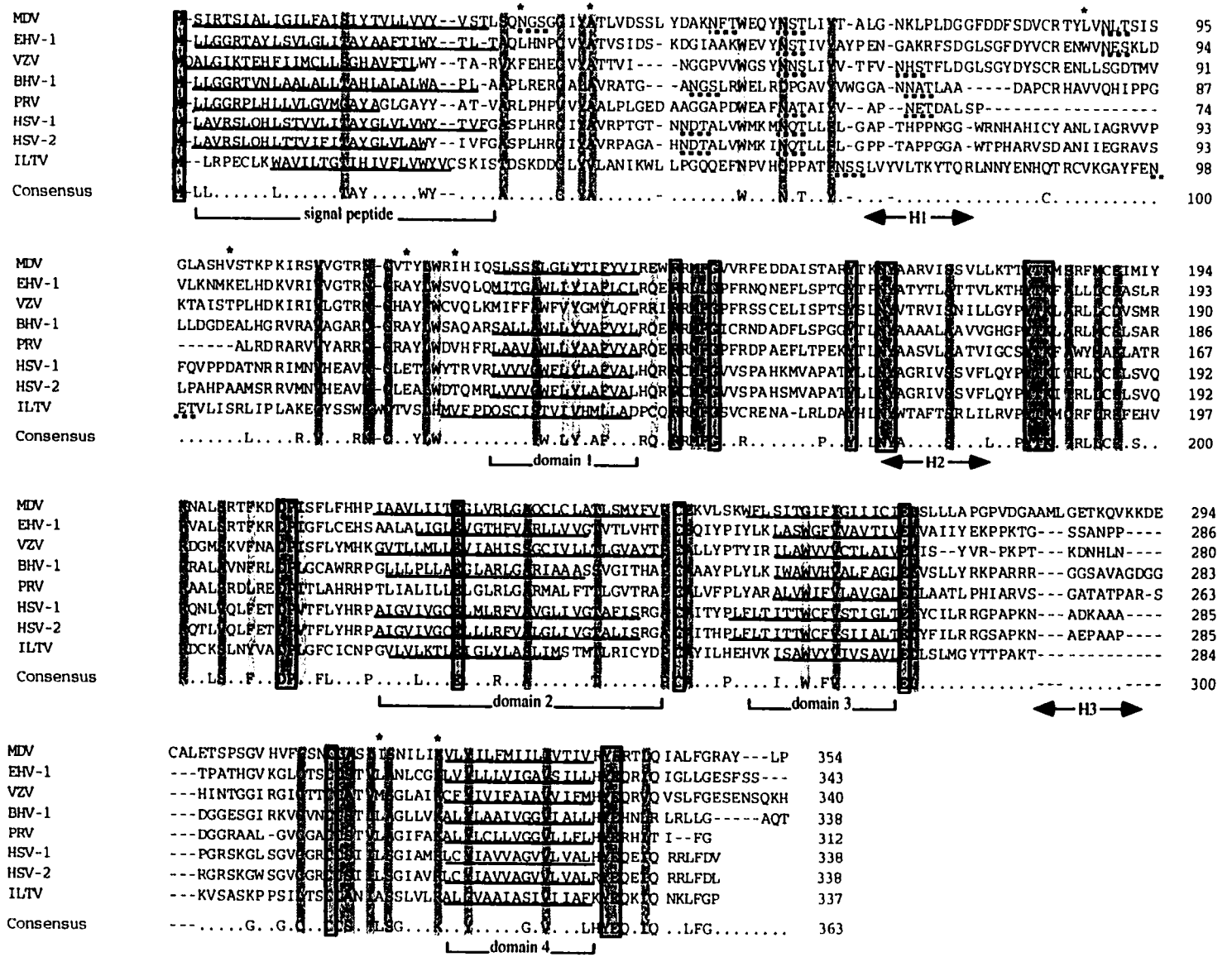

MDV
EHV-1
VZV
BHV-1
PRV
HSV-1
HSV-2
ILTV
Consensus
signal peptide
H1
H2
H3
domain 1
domain 2
domain 3
domain 4

Figure 2.6 Consensus Alignment of eight UL53 homologs

The Gene Works (version 3.3) homology program was used to align the amino acids sequences of MDV ORF53 (Ren et al., 1994), EHV-1 UL4 (Zhao et al., 1992), VZV ORF5 (Davison & Scott, 1986), BHV-1 UL53 (Khadr et al., 1996), PRV UL53 (Baumeister et al., 1995), HSV-1 UL53 (Debroy et al., 1985; McGeoch et al., 1988), HSV-2 UL53 (Debroy, 1990; McGeoch et al., 1991) and ILTV gK (Johnson et al., 1995). Numbers at the right represent the number of amino acids from the N-terminus of each protein. Potential signal peptides and hydrophobic domains (domains 1, 2, 3, 4) are underlined. Hydrophillic domains (H1, H2, H3), N-linked glycosylation signals (dashed lines under NX[T/S]), and the location of amino acid substitutions (*) detected in HSV-1 syn-1 mutants with lesions in the UL53 open reading frame (Debroy et al., 1985; Pogue-Geile & Spear, 1987; Dolter et al., 1994), are indicated. Residues conserved by all gK homologs are boxed and shaded in grey, whereas conservative substitutions or residues present in seven homologs are simply shaded in grey. Letter symbols for residues conserved by at least 5 homologs are listed in the consensus below the ILTV sequence.

counterpart is about 30 to 40%. This level of conservation exceeded that of the UL22-gH and UL10-gM homologs and among glycoproteins of the alphaherpesvirus family these values are second only to UL27-gB, an observation consistent with an essential role for UL53 in alphaherpesvirus replication (McGeoch et al., 1988; Klupp & Mettenleiter, 1991; Telford et al., 1992; Spear, 1993; Gompels et al., 1995).

A region of average hydrophobicity (hydrophillic region 2) flanked by hydrophobic domains one and two is the most highly conserved among UL53 homologs. Surprisingly, none of the syn-1 mutations uncovered to date are located in hydrophillic region 2 (see Figure 2.6). Two regions, one located downstream of the signal peptide and another between hydrophobic domains three and four, contain the greatest number of hydrophillic amino acids (hydrophillic regions 1 & 3) and constitute the least conserved areas in UL53. However, hydrophillic regions 1 and 3 have retained several motifs adjacent to hydrophobic domains, which are preserved in at least seven of the eight UL53 homologs. Hydrophillic region 1 includes two motifs, C(ILV)YA and C..YLW, situated at opposite ends of this domain, whereas hydrophillic region 3 accommodates the motif C..CCS.(ILV)LSG juxtaposed to hydrophobic domain 4 (The '.' represents X or any amino acid). The presence of cysteines in all of these motifs may indicate that they are involved in the formation of disulphide bonds required for UL53-gK structure. Interestingly, all of the recognized syn-1 mutations map inside or nearby one of the three motifs (see Figure 2.6), and seven of ten HSV-1 syn-1 variants have amino acid substitutions in the residues maintained by a majority of UL53 homologs (Debroy et al., 1985; Pogue-Geile et al., 1987; Dolter et al., 1994). Five of these isolates contain a mutation which converts the conserved residue, Ala-40, within the C(IVL)YA motif, to Val or Thr, suggesting that this motif might comprise a functional domain common to all UL53 homologs (Debroy et al.,

1985; Pogue-Geile et al., 1987; Dolter et al., 1994). In contrast to the conservative change (Ala-40 ->Val or Thr) which might be expected to have minor consequences with respect to overall protein structure, the other syn-1 mutations are more drastic and replace charged amino acids with hydrophobic residues (eg. Arg-310 -> Leu), or alter structure by manipulating proline residues (eg. Pro-33 ->Ser or Leu-304 ->Pro) or introducing N-linked glycosylation sites (eg. Asp-99 ->Asn) (Dolter et al., 1994).

In addition, the hydrophobic domains of UL53 homologs also share some common attributes. For example, the position of many residues within each of the hydrophobic domains has been retained by the majority of UL53 homologs (eg. domain #1 - W.LY.AF; #2 - L...E...R..A.....T; #3 - I..W.FV ; #4 - Y.....G.V; where `.' represents those residues which are not conserved). All of these hydrophobic domains may be membrane spanning. However, the presence of conserved residues in each of the hydrophobic domains is inconsistent with the premise that they perform a single function (eg. bridging the membrane), although it is impossible to predict what the other function(s) might be (eg. oligomerization, ion channel?).

Even though the two most hydrophillic regions in UL53 are the least conserved it is a proline, seven polar (4 Tyr, 1 Gly, 1 Asn & 1 Thr) and six charged (3 Glu, 1 Asp, 1 Lys, & 1 Arg) amino acids, situated in areas outside the transmembrane domains, which are maintained in all UL53 counterparts. Most of these amino acids are arranged in conserved motifs located in hydrophillic region 2 (eg. RRMFG, Y.LNYA, YTK(FILM).RLLCE, and F..DP..FL) or the carboxyl-tail of UL53 (eg. YEQ.IQ..LFG) (underlined residues conserved in all homologs and the `.' represents X or any amino acid). The prevalence of tyrosine residues in many of these domains is suggestive, and consequently there is a chance that tyrosine phosphorylation plays a role in UL53 function.

In addition, the position of two cysteines are retained in all UL53 homologs, and the location of another four cysteines are preserved in seven of the eight alphaherpesviruses. As such, these cysteines may contribute to the overall conformation of the UL53 protein.

2.0 Identification, characterization and cellular transport of the UL53 gene product, glycoprotein K.

As the first step in defining the role of UL53 in HSV-induced membrane fusion and virus replication I endeavoured to demonstrate that a UL53 gene product is present in HSV-infected cells during the lytic replication cycle. With availability of the UL53 coding sequence it was possible to prepare antibodies to synthetic peptides from specific regions of the UL53 open reading frame. Using a panel of anti-peptide sera which recognize two of three hydrophillic regions in UL53 (hydrophillic regions 1 & 3; see Figure 1.6), I established that the HSV-1 UL53 gene encodes a single 40-kDa glycoprotein modified by N-linked oligosaccharides (Hutchinson et al., 1992). The 40-kDa protein has been designated gK, the ninth HSV glycoprotein to be described. As such, I am the first and at present, the only person to show that a protein is expressed from the UL53 gene in HSV-1 or HSV-2 infected cells. To date, none of the proteins specified by the UL53 homologs of other alphaherpesviruses (eg. BHV-1, EHV-1, ILTV, MDV, PRV & VZV) have been identified (Davison & Scott, 1986; Zhao et al., 1992;Ren et al., 1994; Baumeister et al., 1995; Johnson et al., 1995; Khadr et al., 1996).

Of the other HSV-1 glycoproteins characterized to date, all have appeared as two glycosylated species, an immature form with high-mannose N-linked oligosaccharides and a mature form which contains processed N-linked oligosaccharides and O-linked oligo-saccharides (Johnson & Spear, 1983; Hutchinson et al., 1992; Baines & Roizman, 1993; Spear, 1993). In contrast, a single electrophoretic form of UL53-gK was detected in HSV-

infected cells, and thus UL53-gK was unusual in this respect (Hutchinson et al., 1992). A trivial explanation for this finding is the suggestion that additional UL53-gK species are hidden by the presence of HSV immunoglobulin G (IgG) Fc receptors, which are also precipitated by anti-peptide sera (Johnson & Feenstra, 1987; Johnson et al., 1988b). Western blot analysis and follow up experiments which employed HSV-1 mutants lacking the Fc receptor proteins, US8-gE and US7-gI, or a recombinant adenovirus vector which expresses UL53-gK, ruled out this possibility (Hutchinson et al., 1992, 1995a; Ghiasi et al., 1994).

Endoglycosidase H digestion revealed that the N-linked oligosaccharides exhibited by UL53-gK were of the high-mannose type (Hutchinson et al., 1995). Under normal circumstances, N-linked oligosaccharides become resistant to endoglycosidase H digestion after processing in the cis-Golgi (CG) where high mannose residues are removed by mannosidase I (Kornfeld & Kornfeld, 1985). Since there are other examples of mature HSV-1 glycoproteins which contain a small proportion of unprocessed oligosaccharides (eg. UL27-gB & UL22-gH [Johnson & Spear, 1983; Wenske, 1982; Roberts et al., 1991]), this result opened the door to two possibilities: 1) UL53-gK is transported to the Golgi apparatus but not processed or 2) UL53-gK is retained in the endoplasmic reticulum (ER) or intermediate compartment (IC) (for review see Pelham, 1995; Rabouille & Nilsson, 1995) of HSV-infected cells.

The latter possibility appears to reflect the true situation, because immunofluorescence analysis did not detect UL53-gK at the cell surface. Instead, UL53-gK accumulated in the perinuclear and nuclear membranes of HSV-infected cells (Hutchinson et al., 1993a,b, 1995). In addition, Ward et al. (1994) later confirmed that UL53-gK is an intracellular protein because it was not among the viral proteins present in

the plasma membranes of biotinylated cells. This result was unexpected, considering that UL53-gK has been implicated in cell-cell fusion, and to the best of my knowledge represented a unique discovery because all of the HSV-1 membrane proteins examined to that point were expressed on the cell surface. (Johnson & Spear, 1983; Johnson & Smiley, 1985; Gompels & Minson, 1986; Johnson & Feenstra, 1987; Sullivan & Smith, 1987; Johnson et al., 1988ab; Hanke et al., 1990; Forrester et al., 1991; Hutchinson et al., 1992; Roberts et al., 1991; Purves et al., 1992; Baines & Roizman, 1993 Liang et al., 1996).

Interestingly, UL20, another hydrophobic membrane protein with four potential membrane-spanning domains and the site of a syncytial mutation (McGeoch et al., 1988; MacLean et al., 1991; Baines et al., 1991), is now known to reside in nuclear membranes but is not expressed on the cell surface (Ward et al., 1994). In addition, a substantial fraction of UL20 colocalized with ß-COP, a vesicle coat protein associated with the Golgi-derived vesicles that mediate intra-Golgi transport and also with retrograde traffic from the Golgi to ER and IC (Orci et al., 1993, 1994; for review see Harter, 1995; Pelham, 1995).

Although UL53-gK and UL20 seem to occupy a different subset of intracellular compartments (ie. UL20 is found in the Golgi whereas UL53-gK is not), it will be necessary to pinpoint their exact position within the exocytic pathway, since the location of UL20 and UL53-gK should provide additional clues regarding their role in HSV replication and cell-cell fusion. Colocalization studies which correlate the presence of UL20 and UL53-gK with the resident proteins of specific exocytic compartments (eg. ER, IC, cis-Golgi, medial-Golgi, trans-Golgi and TGN) should resolve this issue. Moreover, verification that UL53-gK lacks the O-linked oligosaccharides common to many HSV-1 glycoproteins (Johnson & Spear, 1983), would provide further conformation that UL53-gK is not transported beyond the ER and IC.

2.1 ER retrieval and retention signals.

Two mechanisms exist to ensure that resident proteins of the exocytic pathway remain in the appropriate compartments (reviewed by Nilsson & Warren, 1994; Rabouille & Nilsson, 1995). The first type depends on a retention signal which allows forward movement along the bulk-flow pathway and when the correct location has been reached, keeps the resident protein at this site by blocking access to anterograde transport vesicles (Nilsson et al., 1993; Bretscher & Munro, 1993). The second mechanism, termed a retrieval signal, returns the proteins from downstream compartments after they have escaped. This process relies on specific recognition of escaped protein retrieval motifs by cellular components involved in retrograde transport (Pelham & Munro, 1993).

ER retrieval signals include the KDEL motif of luminal ER proteins, the C-terminal K(X)KXX motif of type I ER membrane proteins, and the N-terminal RR motif of type II ER membrane proteins (Pelham, 1989, 1990; Jackson et al., 1990; Schutze et al., 1994). Although retrieval motifs are sufficient for ER localization, they permit reporter molecules to acquire Golgi modifications in compartments including the trans-Golgi (TG) cisternae, before the escaped proteins are returned to the ER (Jackson et al., 1993; Schutze et al., 1994; see Luzio & Banting, 1994 for review). In contrast, endogenous ER proteins possess retention signals in addition to retrieval signals, and the N-linked oligosaccharides on these proteins do not acquire Golgi modifications (Brands et al., 1985; Jackson et al., 1993; Sato et al., 1996; Szczesna-Skorupa et al., 1995).

2.1.1 UL53-gK contains an ER retention signal.

The attributes of UL53-gK (eg. endoH sensitivity) are reminiscent of endogenous ER proteins, indicating that UL53-gK may accommodate an ER retention signal although

it does not preclude the possibility that localization of UL53-gK also involves ER retrieval. Since membrane-spanning domains are known to act as retention signals (Smith & Blobel, 1993; Machamer, 1993; Szczesna-Skorupa et al., 1995) it is possible that one or more of the four potential transmembrane domains featured by UL53-gK perform this function.

In addition, it has been suggested that the retention events associated with membrane spanning domains of resident proteins of the secretory pathway are not receptor-mediated since they can not be saturated by overexpression (Nilsson & Warren, 1994). For example, overproduction of the trans-Golgi (TG) enzymes, ß1,4-galactosyltransferase (GalT) or α-2,6-sialyltransferase (SialyT), could not overcome the block in cell-surface transport, but did cause these proteins to accumulate in pre-TG compartments (eg. ER, CG, & MG) (Nilsson et al., 1991; Munro, 1991). Similarly, UL53-gK is not expressed on the cell surface even when the protein is produced at much higher levels than those observed in wild-type HSV-1 infections (Hutchinson et al., 1995). Instead, the perinuclear membranes appear to proliferate and UL53-gK is retained in the ER without Golgi modifications, a result which is consistent with the hypothesis that UL53-gK contains an ER retention signal.

2.1.2 ER retention of UL53-gK: Retention mediated by membrane thickness?

Two models have been described which explain how membrane-spanning domains are apt to mediate retention and there is evidence to support both. These models have been referred to as `retention through oligomerization' (Nilsson et al., 1993) and `retention caused by a change in membrane thickness' (Bretscher & Munro, 1993).

The first, "retention through membrane thickness", is based on several observations:

an increasing cholesterol gradient in the anterograde exocytic pathway ensures that the plasma membrane is thicker than membranes of the ER (Orci et al, 1981), the transmembrane domains of intracellular residents are often shorter than those of plasma membrane proteins (Masibay et al., 1993), and the length of the membrane-spanning domain (not the primary sequence) is an important factor in determining the position of resident proteins in the exocytic pathway (Munro, 1991; Masibay et al., 1993; Dahdal & Colley, 1993). At least three of the four potential trans-membrane domains in UL53-gK contain 14 or 15 consecutive uncharged, largely hydrophobic amino acids located between positively charged residues (Pogue-Geile & Spear, 1987). Since a hydrophobic region of 14-15 residues is considered short for a membrane-spanning domain (Adams & Rose, 1985), membrane thickness may be a determinant for UL53-gK retention in the ER.

2.1.3 ER retention of UL53-gK: Retention through oligomerization?

In the second model, "retention through oligomerization", interactions between the membrane-spanning domains of resident proteins contribute to the formation of oligomeric structures, which are then excluded from anterograde transport vesicles due to size constraints (Nilsson et al., 1991, 1993; Swift & Machamer, 1991).

Enzymes of the medial-Golgi can be isolated as hetero-oligomers (Nilsson et al., 1994) and form a matrix which is resistant to detergent extraction, yet they can be dismantled reversibly when salt is added (Slusarewicz et al., 1994). Resident proteins of the ER, are also arranged in detergent-resistant aggregates (Hortsch et al., 1987). In addition, viral membrane proteins have been described which employ oligomerization to maintain their intracellular status. For instance, the membrane spanning domains of Coronaviridae M proteins (formerly called E1 proteins) mediate oligomer assembly in the

Golgi apparatus, and this process has been associated with retention (Swift & Machamer, 1991; Machamer et al., 1993; Weisz et al., 1993; Krijnse et al., 1995)

I have demonstrated that UL53-gK has the capacity to form oligomeric structures, although the significance of this finding is unclear (see Chapter 2 and Discussion Figures 3.6A, 3.6B, 3.6C). If one assumes that protein aggregation is analogous to oligomer assembly, then the possibility that membrane spanning domains in UL53-gK are involved in oligomer assembly is suggested by the observation that UL53-gK made *in vitro* aggregates when heated. Moreover, the degree of aggregation is influenced by the number of potential membrane spanning domains (Hutchinson et al., 1992; Ramaswamy & Holland, 1992). Furthermore, the hydrophobic domains in UL53-gK contain residues which are preserved by the majority of UL53 homologs, an observation which is consistent with the hypothesis above. There should be no selective pressure to retain any of these residues if these hydrophobic regions are simply membrane spanning domains, since any one of the hydrophobic amino acids could potentially preform the same function. In addition, hydrophobic domain 3 appears to be the major aggregation determinant (Ramaswamy et al., 1992). Interestingly, polar amino acids make up thirty-three percent of all residues in hydrophobic domain 3 (Figure 2.6), an unusual property for a membrane spanning domain (Pogue-Geile et al., 1987; Ramaswamy et al., 1992). Polar residues lining one face of a predicted α-helix in a transmembrane domain from coronavirus M protein, make an important contribution to Golgi retention and oligomerization of that molecule (Machamer et al., 1993). Therefore, polar residues in hydrophobic domain 3 of UL53-gK may have a similar function.

ER retention of UL53-gK may be related to an ability to form oligomeric assemblies. Two observations are pertinent to this hypothesis: (1) small amounts of

Figure 3.6A. Oligomers precipitated with anti-UL53 peptide sera from AdgK infected cells expressing high levels of UL53-gK.

R970 cells were infected with AdgK at 300 or 1000 PFU/cell or infected with HSV-1 (KOS) using a multiplicity of infection (MOI) of 20 PFU/cell. Ad vector-infected cells were incubated with labelling medium (Hutchinson et al., 1993) containing 200 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine from 24 to 29 hr post-infection and the HSV-infected cells were labelled [^{35}S]Met-[^{35}S]Cys from 6 to 11 hr post-infection. Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). Extracts derived from ≈4.7 x 10^5 cells were mixed with 10 μl of anti-UL53-1 serum preincubated in the presence (+) or absence (-) of peptide UL53-1 or 10 μl of anti-UL53-4 serum preincubated with (+) or without (-) peptide UL53-4. Antigen-antibody complexes were precipitated with protein-A sepharose using the methodology outlined in Hutchinson et al. (1993), subjected to stringent washes (Hutchinson et al., 1992a), eluted at 37°C for 30 min and separated on 13% SDS-polyacrylamide gels as described (Johnson & Feenstra, 1987). The HSV-1 Fc receptor proteins (gE:gI), glycoprotein K (gK), potential gK dimer and gK trimer, and molecular mass markers of 200, 92, 69, 46, and 30 kDa are indicated.

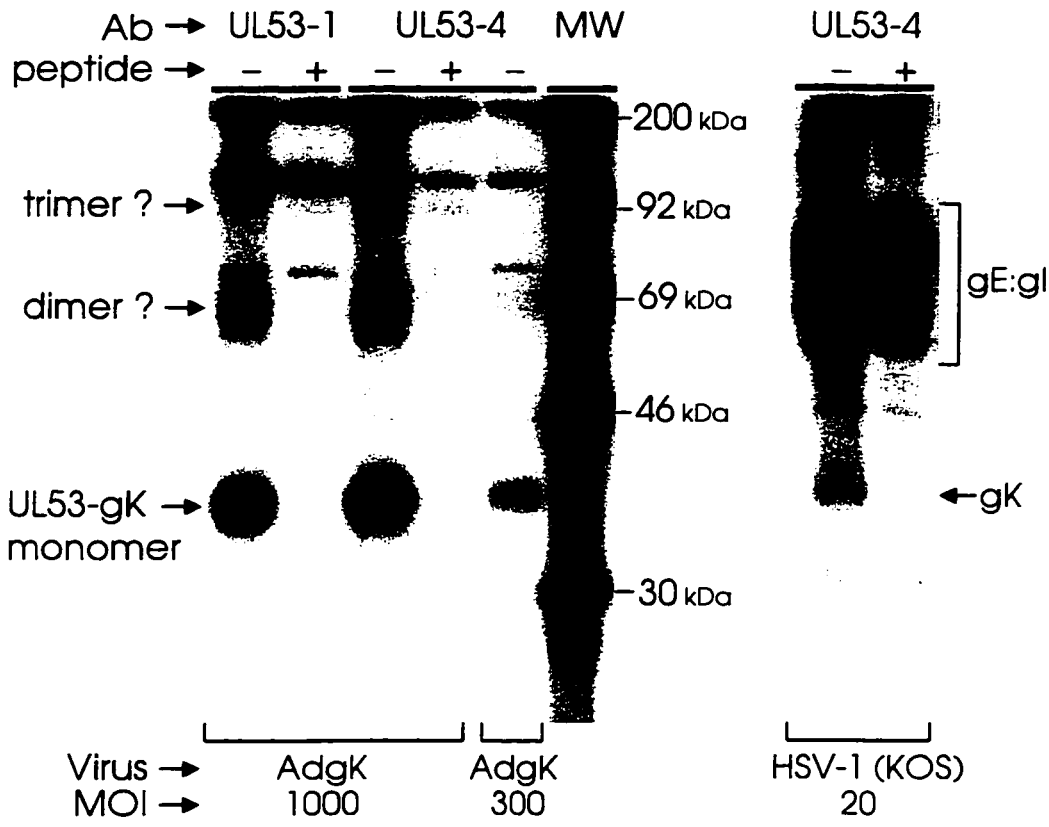
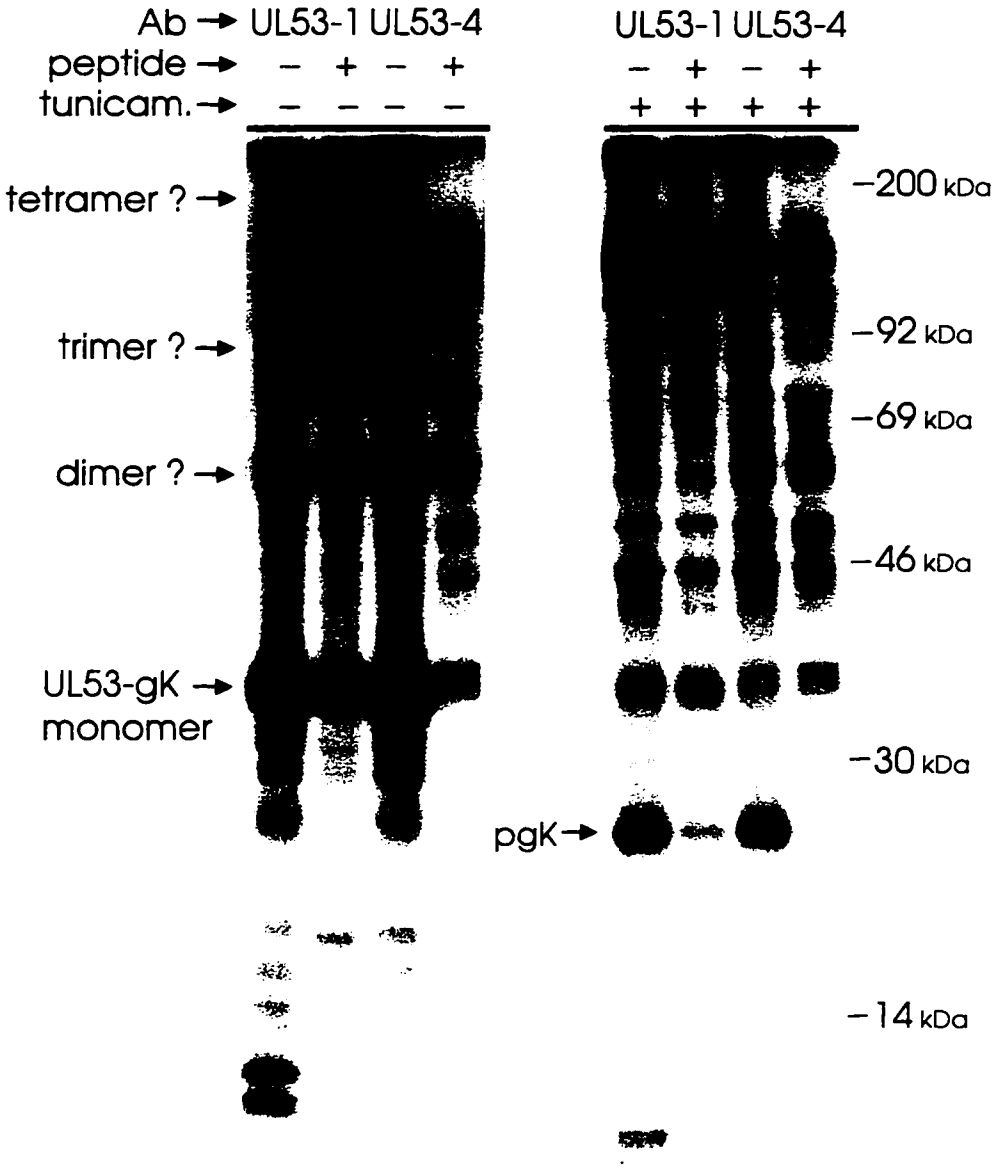

Ab →
UL53-1
UL53-4
MW
UL53-4
peptide →
– + – + –
– +
200 kDa
92 kDa
69 kDa
46 kDa
30 kDa
trimer ? →
dimer ? →
UL53-gK →
monomer
gE:gI
←gK
Virus →
MOI →
AdgK
1000
AdgK
300
HSV-1 (KOS)
20

Figure 3.6B. Oligomers precipitated with anti-UL53 peptide sera from HSV-2-infected gK-9 cells expressing high levels of UL53-gK.

gK-9 cells which contain ≈200 copies of the UL53 gene downstream of the US6-gD promoter were infected with HSV-2 using 20 PFU/cell in the absence (-) or presence of 1.0 μg/ml of tunicamycin (tunicam) per ml (+) or in its absence (-). 4.5 hr after infection cells were incubated with labelling medium (Hutchinson et al., 1993) containing 200 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine until 9 hr post-infection. Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). Extracts derived from ≈1 x 10^6 cells were mixed with 10 μl of anti-UL53-1 serum preincubated in the presence (+) or absence (-) of peptide UL53-1 or 10 μl of anti-UL53-4 serum preincubated with (+) or without (-) peptide UL53-4. Antigen-antibody complexes were precipitated with protein-A sepharose using the methodology outlined in Hutchinson et al. (1993), eluted at 37°C for 30 min and separated on 12% SDS-polyacrylamide gels as described (Johnson & Feenstra, 1987). Glycoprotein K (UL53-gK), potential gK dimers, trimers and tetramers as well as, molecular mass markers of 200, 92, 69, 46, 30 and 14 kDa are indicated.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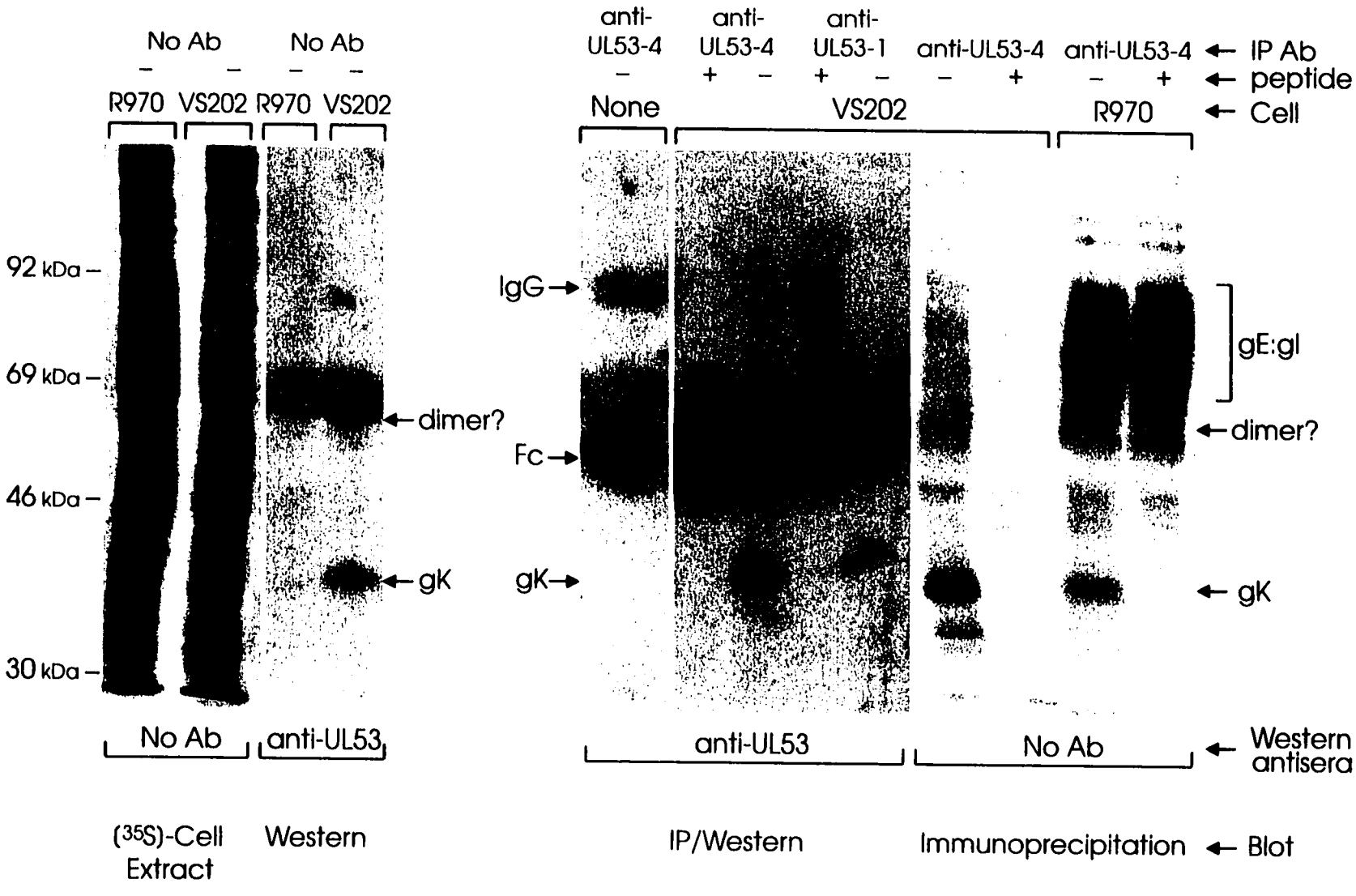
peptide → – + – +
tunicam. → – – – –
UL53-1 UL53-4
– + – +
+ + + +
tetramer ? →
trimer ? →
dimer ? →
UL53-gK →
monomer
pgK →
–200 kDa
–92 kDa
–69 kDa
–46 kDa
–30 kDa
–14 kDa

Figure 3.6C. Western blot analysis of oligomers produced by VS202 cells. VS202 cells which contain ≈500 copies of the UL53 gene with endogenous promoter sequences, were infected with HSV-2 (333) at 30 PFU/cell, and R970 cells (which lack gK) were infected with HSV-1 (KOS) using 30 PFU/cell. 4.75 hr after infection some dishes of cells ([^{35}S]-Cell extract, & Immunoprecipitation) were incubated with labelling medium (Hutchinson et al., 1993) containing 200 µCi/ml of both [^{35}S]Met and [^{35}S]Cys until 9 hr postinfection, and other dishes were not labelled (Western, & IP/Western). Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). For the immunoprecipitation assay and IP/Western, extracts derived from ≈1 x 10^6 cells were mixed with 10 µl of anti-UL53-1 serum preincubated in the presence (+) or absence (-) of peptide UL53-1 or 10 µl of anti-UL53-4 serum pre-incubated with (+) or without (-) peptide UL53-4 and precipitated with protein-A sepharose using the methodology outlined in Hutchinson et al. (1993). Antigen-antibody complexes (Immunoprecipitation & IP/Western) and cell lysates ([^{35}S]-Cell extract & Western) were incubated in sample buffer (50 mM Tris-HCl containing 2% SDS, 2% ß-mercaptoethanol, and 15% glycerol) at 37°C for 30 min. Proteins eluted from the antigen-antibody complexes and cell lysates comprising ≈3 x 10^4 cells ([^{35}S]-Cell extract) or ≈1 x 10^5 cells (Western) were separated on 8.5% SDS-polyacrylamide gels as described (Johnson & Feenstra, 1987). All proteins were transferred to nitrocellulose membranes and the gK-related proteins present in Western and IP/Western samples were detected with anti-UL53-1 sera, followed by [^{125}I]-labelled protein A. Western blot analysis of the anti-UL53-4 sera (5 µl) in the absence of cell extracts (None) indicates the major protein band present in the central portion of IP/Western blots is derived from the IgG heavy chain (Fc) which can bind to protein A, and larger bands may be comprised of IgG molecules which were not fully denatured by the elution conditions (ie. 37° for 30 min in sample buffer). The gK monomer and potential dimer, HSV-1 gE:gI, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated.


No Ab
No Ab
R970
VS202
R970
VS202
92 kDa
69 kDa
46 kDa
30 kDa
dimer?
gK
No Ab
anti-UL53
(35S)-Cell Extract
Western
anti-UL53-4
anti-UL53-4
anti-UL53-1
anti-UL53-4
anti-UL53-4
IP Ab
peptide
Cell
None
VS202
R970
IgG
Fc
gK
gE:gI
dimer?
gK
anti-UL53
No Ab
Western antisera
IP/Western
Immunoprecipitation
Blot

monomeric UL53-gK were detected in the detergent extracts of HSV-1 infected cells, (2) oligomeric forms of UL53-gK were detected only when UL53-gK is expressed at levels exceeding those observed in cells infected with wild-type HSV-1 (Figure 3.6A, 3.6B, 3.6C). Thus, it is conceivable that a substantial fraction of UL53-gK is situated in a proteinaceous complex, resistant to detergent extraction. This possibility is amenable to experimental evaluation. A combination of western blot analysis and the use of conditions which solubilize the detergent-resistant pellets obtained from HSV-1 infected cells, should be sufficient to confirm or reject this theory. The use of gel filtration techniques and cross-linking reagents which often stabilize oligomeric complexes may provide additional information regarding the true nature of the UL53-gK oligomers.

In addition, there is evidence to suggest that oligomeric forms of UL53-gK constitute a functional unit, and may even be necessary for UL53-gK activity. Support for this idea stems from the studies of Little & Shaffer (1981) who used the morphology of single plaques, arising from infectious centres generated by co-infecting cells with two syncytial strains, to assign each mutant to a different complementation group. These experiments were based on the assumption that syn mutations are recessive, and the idea that the HSV-1 strains which carry different syncytial loci will complement each other by providing the missing function in *trans*. However, this assay cannot distinguish between a complementation event produced by two different proteins and the possibility that a single protein has two domains that function independently, or alternatively a single protein which participates in a multimer and requires the presence of two distinct domains in order to form a functional unit. Of particular interest, two syncytial mutants (78R and 804), both of which are thought to contain syn-1 mutations, produce wild-type plaques in a mixed infection (Little & Schaffer, 1981; Roop et al., 1993) and other examples of this phenomenon have been reported (Read et al., 1980; Bond et al., 1982). Furthermore,

Dolter et al. (1994) have revealed that the syncytial mutations in UL53-gK arise in two distinct regions suggesting that UL53-gK may possess two functional domains which control cell-cell fusion. Therefore, UL53-gK may operate as an oligomer which can be assembled from a pool of mutant subunits and still retain activity, provided that the monomers contain mutations which reside in different functional domains.

In other experiments Little & Schaffer coinfected cells with different syn-1 mutants (eg. MP & 804) or the combination of a syn-1 mutant (MP) and syn-3 mutant (HFEM), but failed to detect any complementation (ie. observed a syncytial plaque morphology in the mixed infection). However, these results are best explained by the discovery that HSV-1 strain MP, the predominant syn-1 mutant used in complementation assays, exhibited a co-dominant phenotype in mixed infections with wild-type HSV-1 on the cell line employed by Little & Schaffer (1981) (Read et al., 1980; Bond et al., 1982). Other HSV-1 protiens which have the capacity to form oligomeric structures and accommodate more than one functional domain share similar properties. When protein subunits containing mutations in different domains are co-expressed they may form a functional oligomer, or the mutant protein may exhibit the ability to trans-inhibit the wild-type counterpart (Godowski & Knipe, 1985; Cai et al., 1988; Gao & Knipe, 1991; Cavalcoli et al., 1993; Chowdury & Batterson, 1994).

On the other hand, one must consider the possibility that UL53-gK oligomerization may simply be a by-product of the envelopment process, because a large number of HSV-1 membrane proteins form homo- and hetero-oligomers (see Introduction Section 2.5; Sarmiento & Spear, 1979; Eberle & Courtney, 1982; Eisenberg et al., 1982; Gibson & Spear, 1983; Zhu & Courtney, 1988; 1994; Johnson & Feenstra, 1987; Johnson et al., 1988b; Hutchinson et al., 1992; Roop et al., 1993; Handler et al., 1996).

2.1.4 Does UL53-gK contain an ER retrieval signal?

Although UL53-gK does not possess any of the well characterized ER retrieval signals (eg. the C-terminal K(X)KXX motif of type I ER membrane proteins, or the N-terminal RR motif of type II ER membrane proteins), it would not be surprising if this sorting mechanism is used by UL53-gK, since many cellular proteins employ both retention and retrieval to maintain their position in the exocytic pathway (Luizo & Banting, 1993; Nilsson & Warren, 1994; Hammon & Helenius, 1995; Rabouille & Nilsson, 1995; Szczesna-Skorupa et al., 1995; Sato et al., 1996).

In a recent study, Mallabiabarrena et al. (1995) identified a novel tyrosine-containing motif YXXLXXR which acts as an ER retrieval signal. Tyrosine-based signals which resemble this ER retrieval motif also play an important role in sorting proteins to the basolateral domains of polarized epithelial cells (eg. polymeric IgG receptor [YSAF]), membrane compartments including the TGN (eg. TGN38, [YQRL]; furin, [YKGL]) and lysosomes (LAMPI, [YQTI]) or both (eg. M6PR^{cd} [AYRGV & YQRL]), as well as the rapid internalization of plasma membrane proteins via clathrin coated pits (eg. transferrin receptor, [YTRF]) (Johnson et al., 1990; Bos et al., 1993; Luzio & Banting, 1993; Wong & Hong, 1993; Nilsson & Warren, 1994). The tyrosine-based motifs of different proteins are sometimes interchangeable and will mediate endocytosis and/or retrieval of proteins when placed in the proper context (Luzio & Banting, 1993; Sato et al., 1996). However, the targeting function of these tetrapeptide sequences can be separated from their ability to act as internalization signals. Surrounding residues often determine the targeting specificity of the tyrosine-containing sequence, either as direct participants in the routing function, by introducing post-translational modifications (eg. phosphorylation or proteolytic cleavage), or by modifying the position and conformation of the targeting motif (Luzio & Banting, 1993; Guamieri et al. 1993; Humphrey et al., 1993; Wong & Hong, 1993;

Meresse & Hoflack, 1993). In other instances, the internalization-retrieval signal appears to work independently of the targeting motif (Luzio & Banting, 1993).

The majority of these tyrosine-containing motifs share some common features which include; (1) a common sequence, meaning the tyrosine residue can be separated from a bulky hydrophobic amino acid by any two other amino acids and (2) a common structure which places the critical tyrosine residue at the end of a tight beta-turn structure within the cytoplasmic domains of type 1 and type II membrane proteins (Trowbridge, 1991; Vaux, 1992; Pelham & Munro, 1993). However, there are some exceptions to these general rules. In particular, VZV glycoprotein E contains a TGN targeting motif (AYRV) and the tyrosine in this sequence is separated from valine by a single residue (Zhu et al., 1996). In addition, the novel ER retrieval motif, YXXLXXR, forms an elongated α-helix:ß-turn instead of the traditional ß-turn structure more frequently observed (Mallabiabarrena et al., 1995). However, Mallabiabarrena et al. (1995), note that the arrangement of tyrosine and leucine residues within the α-helix segment of the ER retrieval motif, is reminiscent of the beta-turn structure adopted by tyrosine-containing endocytosis signals.

Interestingly, the conservation of tyrosine residues among homologs of UL53-gK (ie. 73%, 53% or 27% of tyrosines are conserved in five, seven or all eight homologs, respectively; see Table 1.6) is on par with the preservation of cysteine residues, which often play an essential role in the tertiary structure of proteins (ie. 67%, 50%, or 17% of cysteines are conserved in five, seven or all eight homologs, respectively) (Debroy et al., 1985; Pogue-Geile et al., 1987; Davision & Scott, 1986; Debroy, 1990; Zhao et al., 1992; Telford et al., 1992; Ren et al., 1994; Baumeister et al., 1995; Johnson et al., 1996; Khadr et al., 1996). Moreover, two of the tyrosine-containing motifs conserved by all of homologs of UL53-gK, appear to meet the criteria introduced above for tyrosine-based

Table 1.6 Conserved amino acids among UL53 homologs.

Amino acid residue	Number in consensus*	Total number in gK	% conserved
A	7	31	22%
C	8	12	**67%**
D	1	5	20%
E	4	8	**50%**
F	6	14	43%
G	6	23	26%
H	1	10	10%
I	2	21	10%
K	2	6	33%
L	17	40	43%
M	2	7	29%
N	3	12	25%
P	6	17	35%
Q	3	10	30%
R	9	24	38%
S	5	14	36%
T	4	23	17%
Y	11	15	**73%**
V	3	40	8%
W	5	6	**83%**

- * consensus obtained from Figure 2.6
- residues in bold are highly conserved between gK homologs

retrieval signals.

The probable internal cytosolic loop of UL53-gK (see Figures 1.6 & 2.6) contains a tyrosine within the highly conserved sequence "LXXP **YTK(FILM)** XRLLCEXS", and this tyrosine is predicted to occupy the end of a tight ß-turn (Debroy et al., 1985). Of specific interest, the sequence YTK(FILM) is almost identical to the internalization-retrieval sequences of the transferrin receptor (TfR; eg. YTRF) and the cation-independent mannose-6-phosphate receptor ($M6PR^{ci}$; eg. YKYSKV). TfR is a cell surface receptor which is rapidly recycled and $M6PR^{ci}$ is involved in lysosomal enzyme sorting from the TGN, with additional stops that include cycling between the plasma membrane and the TGN (Collawn et al., 1990). Consequently, the conserved sequences which surround the YTK(FILM) motif, or an independent signalling domain maintained by the UL53-gK homologs, is likely to determine the targeting specificity of this four-amino-acid peptide, provided that the YTL(FILM) motif is indeed an ER retrieval sequence.

The putative cytoplasmic tail of UL53-gK homologs also include a tyrosine within the highly conserved sequence LHYEQXIQXXLFG, and this tyrosine is predicted to reside in one of the six potential α-helices accommodated by UL53-gK (Debroy et al., 1985) (underlined residues are conserved in all UL53 homologs). As such, the YEQXI sequence resembles the ER retrieval signal (YXXLXXR) more closely than the internalization motif of plasma membrane receptors (Mallabiabarrena et al., 1995). However, the spacing of the tyrosine and isoleucine residues does not conform to that of the other tyrosine-based targeting signals described above (ie. a spacing of 1 or 2 but not 3 residues). Consequently, the YEQXI motif may be involved in protein-protein interactions which are unrelated to ER retention or serve another function entirely (eg. phosphorylation of the tyrosine residue could induce a conformational change in UL53-gK). Alternatively,

the YEQXI motif may constitute a novel adaptation of the traditional ER retrieval sequence, or even a novel tyrosine-containing targeting signal.

It is interesting to note that UL53-gK was observed in the plasma membranes of insect cells infected with a baculovirus expression vector (Bac-gK) (Ghiasi et al., 1994). It is tempting to speculate that insect cells lack one or more of the potential ER retention mechanisms which influence UL53-gK transport in mammalian cells (eg. the tyrosine-based retrieval system). In support of this hypothesis, HSV-1 glycoprotein UL22-gH expressed in the absence of other HSV-1 proteins is retained in the ER of mammalian cells, but a substantial fraction of UL22-gH is transported to the cell surface when the same conditions are applied to insect cells. On the other hand, cell surface expression of UL53-gK could be related to the cytopathic effects caused by baculovirus infection or overexpression of UL53-gK may overwhelm the insect cell ER retrieval machinery.

To verify that UL53-gK contains an ER retention and/or retrieval signal and determine the mechanism which mediates ER localization of UL53-gK (eg. retention through oligomerization vs. membrane thickness), additional experiments will be required. Transfer experiments which exchange the membrane spanning domains, probable cytosolic loop and the carboxyl-terminus of UL53-gK with analogous regions in a reporter molecule (eg. G protein of VSV), can be used to identify the domains in UL53-gK which mediate retention, retrieval, or protein oligomerization. Confirmation that UL53-gK homologs from other alphaherpesviruses are retained in the ER compartment would strengthen the evidence suggesting that all of these proteins perform similar functions during alphaherpesvirus replication, and provide a convincing rationale for site-directed mutagenesis of the functional domains identified in transfer experiments. When the ER retention-retrievals signals in UL53-gK have been identified, these sequences can be

removed or replaced with targeting domains for different exocytic compartments in order to provide further insight into UL53-gK function during HSV-1 replication.

Since tyrosine-containing motifs must be located in cytoplasmic domains to act as targeting signals, an understanding of UL53-gK membrane topology is crucial. To date, the extent of our knowledge about gK topology is restricted to the discovery that hydrophillic region 1 is exposed to the lumen of the endoplasmic reticulum, indicating UL53-gK has a type 1 topology (Hutchinson et al., 1992; Ramaswamy & Holland, 1992). Furthermore, the primary sequence of hydrophillic regions 1 and 3 are poorly conserved relative to hydrophillic region 2 (probable internal cytosolic loop), and consequently the orientation of each domain is likely to have important implications regarding UL53-gK function (eg. binding to cytosolic constituents rather than interactions with cellular and/or viral components in the ER lumen).

Because experimental procedures which employ *in vitro* protein synthesis are amenable to assays including protease sensitivity, oligosaccharide analysis, and the accessability of antibody epitopes, they are commonly used to investigate the topology of membrane proteins. Inquiries of this nature may provide useful data regarding the orientation of the hydrophillic regions in UL53-gK. However, the physical properties of UL53-gK synthesized *in vitro* deviate from UL53-gK manufactured in mammalian cells (eg. heat sensitivity & electrophoretic mobility), indicating that there may be differences in the folding or processing of UL53-gK made in these two systems (Hutchinson et al., 1992). Therefore, any studies which employ an *in vitro* expression system to investigate UL53-gK structure, should recognize the potential for irregularities, and demonstrate caution when interpreting this data.

The feasibility of many of the prospective experiments described in this discussion

(eg. UL53-gK intracellular localization and membrane topology) will depend on the availability of reliable anti-sera directed against different domains of UL53-gK, or the ability to epitope tag regions of the UL53-gK without inducing radical changes to protein conformation or function. Studies to date have employed rabbit antisera directed towards peptides from hydrophillic region 1 (UL53-1, residues 31-46 & UL53-4, residues 89-104) and hydrophillic region 3 (UL53-3, residues 89-104). Of these peptides, only the peptide UL53-4 consistently produced a strong antibody response in rabbits (MacLean et al., 1991; Hutchinson et al., 1992, 1995). Other peptides, including one that encompasses the cytoplasmic tail of UL53-gK (residues 327-338) did not produce an antibody response in rabbits (MacLean et al., 1991; H. Ghosh, personal communication). Nevertheless, the development of peptide anti-sera specific for hydrophillic region 2 (residues 140-212) has not been attempted, and would provide a valuable tool since this domain represents one of the most conserved regions between homologs of UL53-gK (see Figure 2.6). For instance, anti-sera directed toward this domain might be used to identify functional domains in UL53-gK, in addition to providing information in the experiments described above.

The major drawback of peptide antisera revolves around their inability to recognize complex, discontinuous epitopes and consequently, their usefulness may be limited if these regions of UL53-gK are involved in the organization of protein oligomers. Therefore, it would be desirable to produce antibodies which recognize the native UL53-gK protein. Over the last several years, I and others in this laboratory have employed a variety of techniques designed to generate additional UL53-gK antibodies, largely without success. These strategies have included: (1) efforts to produce MAbs against UL53-gK peptides conjugated to bovine serum albumin (S. Primorac, personal communication), (2) injection of rabbits with a live, replicating adenovirus vector (AdgK) which expresses native UL53-

gK, (Hutchinson, Ghosh & Johnson, unpublished results), (3) the use of adenovirus and baculovirus expression systems (AdgK or vAc-gK1) in an attempt to purify UL53-gK from polyacrylamide gels, in quantities that are sufficient to raise antibodies in rabbits or mice (Hutchinson et al., 1992; Ghiasi et al., 1994; Hutchinson, unpublished results). In addition, the UL53-gK ORF was cloned into a bacterial protein A-expression vector (pRIT2Tsyn) which generates a fusion protein amenable to purification from bacterial extracts (Hutchinson et al., 1993). However, this approach was abandoned over concerns regarding the potential for misfolding in bacterial cells, since they lack an endoplasmic reticulum, and also the tendency of UL53-gK to aggregate. I did consider expressing the first hydrophillic region from UL53-gK in bacteria (residues 31-122), insect or mammalian cells (residues 1-122) which would reduce the possibility of misfolding, and expedite purification since this segment has the characteristics of a secreted protein. However, antibodies directed to this polypeptide would provide only a marginal improvement to the repertoire of UL53-gK antibodies already available, given that two of the peptide anti-sera bind to this domain. Since few options remained, I tagged the carboxyl-terminus of UL53-gK with a MAb epitope derived from influenza haemagglutinin and rescued the chimeric protein into HSV-1 (Kolodziej & R.A. Young, 1991; Hutchinson & Johnson, unpublished results). Although the HA epitope did not appear to interfere significantly with HSV-1 replication, MAbs to this epitope could not recognize the chimeric protein in immuno-assays (eg. western blot, immunofluorescence, immuno-precipitation).

In my view there is one remaining approach which could yield an anti-sera certain to recognize UL53-gK in its native form. Extracts from sonicated rabbit or mouse fibroblasts, infected with an adenovirus vector expressing UL53-gK, could be used as the immunogen needed to produce polyclonal and/or monoclonal antibodies. Replication

competent adenovirus vectors (eg. AdgK) could be used to augment the immune response, or alternatively, replication incompetent adenovirus vectors could be constructed to avoid complications which may arise from the creation of antibodies which recognize adenovirus proteins. Subsequently, antisera obtained from these animals could be screened by using cell extracts of HSV-1 infected gK-9 cells, which overexpress UL53-gK, as the antigen source. A similar approach, which employed extracts of baculovirus (vAc-gK1) infected cells, has been used successfully to produce UL53-gK specific anti-sera in mice (Ghiasi et al., 1994). These antibodies would be particularly useful in supplementary experiments designed to confirm that UL53-gK is not transported to the surface of HSV-infected cells (eg. cell fractionation, or an [^{125}I] surface label).

3.0 UL53-gK is required of HSV-1 egress.

All of the membrane proteins located in the virus envelope have also been detected in the plasma membrane (see Introduction Section 2.5). Consequently, the discovery that UL53-gK is an intracellular protein was unexpected, and called into question the prevailing belief that subsequent studies would show that UL53-gK is an envelope protein which regulates the membrane fusion events required for virus penetration. Furthermore, retention of viral membrane proteins in specific compartments of the exocytic pathway is not an uncommon occurrence, and is often linked to the site of capsid envelopment for those enveloped viruses which replicate in the cytoplasm (for review see Pettersson, 1991). For instance, the envelope proteins of Punta Toro virus (Bunyavirus) and rubella (Togavirus) are transported to Golgi compartments, where the virus is thought to acquire an envelope (Matsuoka et al., 1988; Chen et al., 1991; Hobman et al., 1995), whereas glycoproteins of Hepatitis B virus and Human foamy virus (retrovirus) collect in

membranes of the endoplasmic reticulum, the putative site of capsid envelopment for all of these viruses (Bruss & Ganem, 1991; Nemeckova et al., 1994; Goepfert et al., 1994; for review see Pettersson, 1991).

To differentiate between both of these possibilities it was necessary to inactivate the UL53 gene, and observe the effect on HSV-1 replication. To achieve this objective, I tried to disrupt UL53-gK function by inserting an ICP6 promoter::*lacZ* cassette into the UL53 gene. Since my initial attempts were unsuccessful, and the results of other herpesvirus researchers mirrored my own (MacLean et al., 1991), I expected UL53-gK to supply a function which is absolutely essential for HSV-1 replication in cultured cells. To complement UL53-gK mutants, I endeavoured to construct cell lines which expressed UL53-gK in response to HSV-1 infection. This goal was more difficult to achieve than I had anticipated, largely because the temporal expression and/or the abundance of UL53-gK must fall within a strict range to support HSV-1 replication (Hutchinson et al., 1993, 1995b). However, intensive screening efforts produced cell lines which supported HSV-1 replication, and expressed quantities of UL53-gK comparable to the levels observed in cells infected with wild-type HSV-1. Using these cell lines I generated an HSV-1 mutant, F-gKß, which is unable to express UL53-gK, and demonstrated for the first time that UL53-gK is essential for virus replication in cultured monkey Vero cells, human R970 cells, and normal human fibroblasts.

This phenotype results, primarily from a fundamental defect in virus egress, which affects the transport of enveloped virions from the perinuclear space to the plasma membrane. Subsequent studies demonstrated that Vero cells infected with F-gKß assemble viral nucleocapsids in quantities typical of a wild-type infection. Nevertheless, very few enveloped F-gKß particles were conveyed to the cell surface, in contrast to wild-type

particles which reach the plasma membrane in large numbers. Instead, a large number of unenveloped capsids accumulated in the cytoplasm of F-gKß-infected cells. In addition, the physical traits of those enveloped virions which were observed, often differed from the attributes of enveloped virions present in cells infected with wild-type HSV-1 (eg. two or more capsids in a single envelope; unusually large quantities of tegument) (see Fig. 7.6 of Chapter 5). Furthermore, these differences may have translated into the 25-50 fold decrease in the infectivity exhibited by enveloped virions made in the absence of UL53-gK.

3.1 HSV-1 egress is a directed process.

Two models have been described which endeavour to explain the egress mechanism used by herpesviruses (see Introduction: section 3.7). In the first, enveloped virions employ vacuoles derived from the outer nuclear membrane to vacate the perinuclear space, and migrate to the plasma membrane in membrane bound compartments derived from membranes of the exocytic pathway (Johnson & Spear, 1982; Torrisi et al., 1992). Advocates of the second model assert that fusion events, between the envelope of perinuclear virions and the outer nuclear membrane, release naked capsids into the cytoplasm and invoke a subsequent re-envelopment step (eg. at the trans-Golgi) to ensure that capsids acquire an infection-competent membrane (Stackpole, 1969; Jones & Grose, 1988; Whealy et al., 1991; Ni, 1992; Browne et al., 1996).

Although there is evidence that the virion and its components use elements of the host cell exocytic pathway during virus egress, the results presented here and those of other studies, clearly show that HSV-1 egress is a directed process involving specific viral proteins (Baines et al., 1991; Baines & Roizman, 1992; Avitabile et al., 1994; Brown et al., 1994; Ward et al., 1994). Therefore, the egress pathway used by HSV-1 virions is

distinct from the default pathway used by cells to export a macromolecule destined for secretion. Ward et al. (1994), have also suggested that transport vesicles containing HSV virions may have an origin which differs from the transport vesicles accommodating viral glycoproteins. This is because viral glycoproteins acquire post-translational modifications in cells infected with a UL20-negative HSV-1 mutant, yet virion exocytosis is blocked in a pre-Golgi compartment (Baines et al., 1991; Avitabile et al., 1994). My results (Chapter 5) appear to support this hypothesis. The processing and transport of the bulk of HSV glycoproteins remained largely unaffected in the absence of gK, yet virus egress was reduced dramatically.

3.2 UL53-gK expression, transport and function during HSV-1 replication: impact on UL53-gK's role in virus egress.

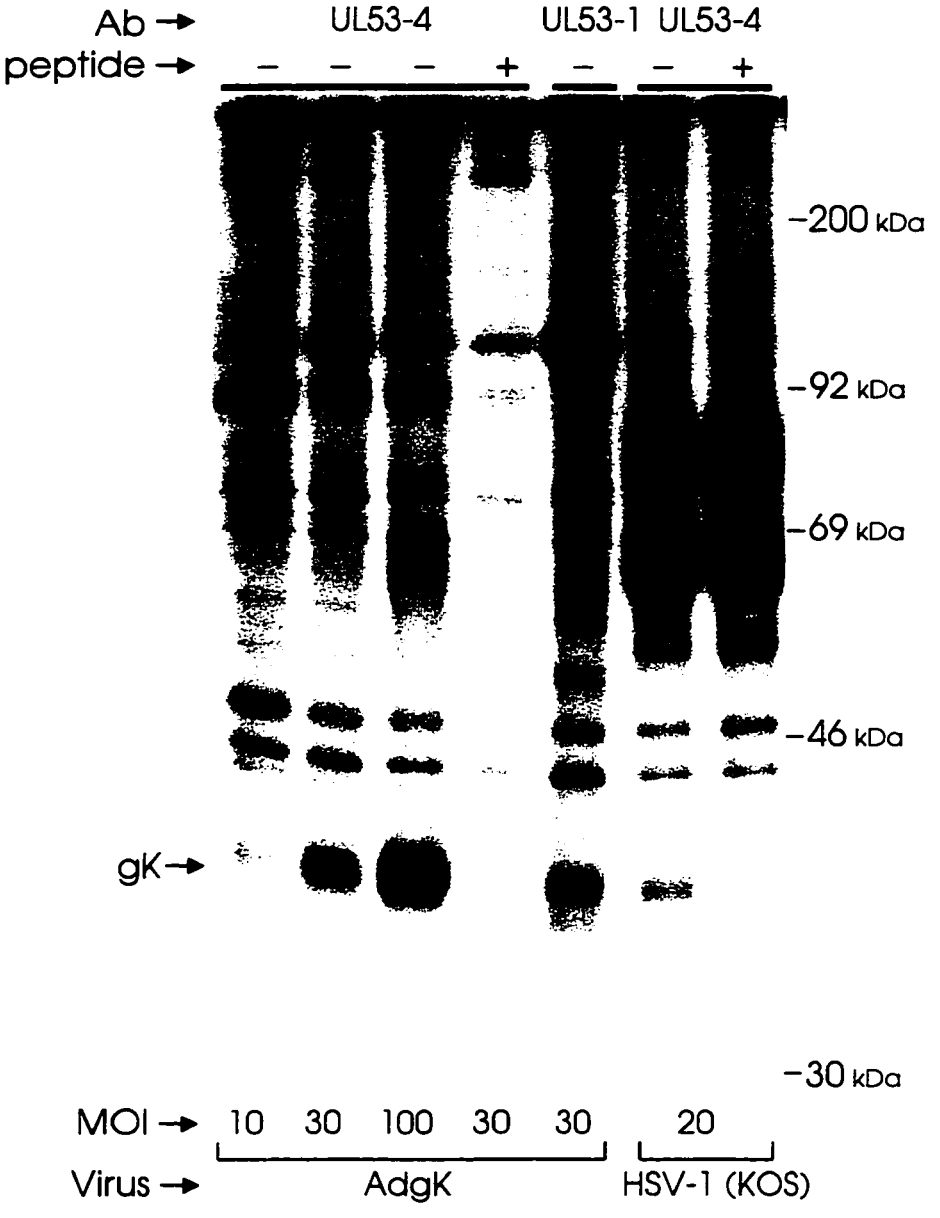
Since the exact events which govern virion transport between the site of envelopment and the extracellular space have yet to be defined, it is impossible to state unequivocally what UL53-gK's role in this process might be. Any attempt to assign UL53-gK to a specific step in an egress pathway, must take several observations into consideration: (1) small amounts of UL53-gK were detected inside HSV-1 infected cells, (2) UL53-gK is retained in the ER and/or the intermediate compartment (IC) (3) UL53-gK was not detected in the virion envelope, (4) naked capsids accumulated in the cytoplasm of cells infected with F-gKß, (5) enveloped capsids in the cytoplasm of F-gKß-infected cells exhibit abnormalities and this morphology was accompanied by a 10-100 fold reduction in specific infectivity, and (6) in cells overexpressing UL53-gK, HSV-1 particles accumulated in the perinuclear space.

3.2.1 Low levels of UL53-gK are maintained in HSV-infected cells

The small quantity of UL53-gK present in HSV-1 infected cells is indicative of a regulatory role for UL53-gK in HSV-1 replication, rather than a structural role which has been associated with other HSV-1 glycoproteins. This conclusion is based on the observation that components of the virion envelope, namely the HSV-1 IgG Fc receptor proteins (US8-gE:US7-gI), are precipitated by anti-UL53 peptide sera (see Chapter 2). Increasing the volume of UL53-gK peptide antisera used in immunoprecipitation assays, enhanced the recovery of Fc receptors from HSV-infected cells, but had little or no effect on the levels of UL53-gK precipitated from HSV-infected cells. Corollary experiments which employed a constant volume of UL53-gK peptide antisera and varied the quantity of UL53-gK antigen, also support the conclusion that UL53-gK is the limiting factor in immunoprecipitation assays (see Figure 4.6). In addition, the intensity of the HSV-1 Fc receptor proteins (US8-gE:US7-gI) observed following autoradiography exceeded that of UL53-gK, even though each of these proteins contain a comparable number of cysteine and methionine residues (gE = 10 Met, 13 Cys; gI = 6 Met, 10 Cys; gK = 7 Met, 12 Cys) (see Fig. 4.6 of Chapter 2). Since, US8-gE:US7-gI are minor components of cellular membranes relative to other HSV-1 glycoproteins (eg. gB, gC, gD), these results serve to accentuate the scarcity of UL53-gK in HSV-1 infected cells (based on [^{3}H]mannose label and the # of N-linked glycosylation signals in each glycoprotein: gB = 7, gC = 7, gD = 3, gE = 2, gI = 3, gH = 7) (Heine et al., 1974; Kousoulas et al., 1983; Spear, 1985). It could be argued that the anti-UL53 peptide sera used in these studies recognized only a fraction of the UL53-gK inside HSV-1 infected cells. However, data from northern blot experiments would seem to support the conclusion that HSV-1 infected cells contain small amounts of UL53-gK (Uprichard & Knipe, 1996).

Figure 4.6 Small quantities of UL53-gK are produced in HSV-infected cells.

R970 cells were infected with AdgK at 10, 30 or 100 PFU/cell or infected with HSV-1 (KOS) using a multiplicity of infection (MOI) of 20 PFU/cell. Ad vector-infected cells were incubated with labelling medium (Hutchinson et al., 1993) containing 200 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine from 22 to 27 hr post-infection and the HSV-infected cells were labelled [^{35}S]Met-[^{35}S]Cys from 4 to 9 hr post-infection. Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). Extracts derived from ≈1 x 10^6 cells were mixed with 10 μl of anti-UL53-1 serum preincubated in the presence (+) or absence (-) of peptide UL53-1 or 10 μl of anti-UL53-4 serum preincubated with (+) or without (-) peptide UL53-4. Antigen-antibody complexes were precipitated with protein-A sepharose using the methodology outlined in Hutchinson et al. (1993), eluted at 37°C for 30 min and separated on 12% SDS-polyacrylamide gels as described (Johnson & Feenstra, 1987). The HSV-1 Fc receptor proteins (gE:gI), glycoprotein K (gK) and molecular mass markers of 200, 92, 69, 46, and 30 kDa are indicated.


Ab →
UL53-4
UL53-1
UL53-4
peptide →
– – – + – – +
–200 kDa
–92 kDa
–69 kDa
–46 kDa
gK →
–30 kDa
MOI → 10 30 100 30 30 20
Virus → AdgK HSV-1 (KOS)

For instance, mRNA expression from the UL52 and UL53 genes is governed by similar transcriptional controls (ie. as a beta or gamma-1 gene), a result which is consistent with the effect PAA had on UL53-gK protein expression, and both of these genes produce comparable quantities of mRNA (Chapter 2, Uprichard & Knipe, 1996). Since low levels of UL52 protein are maintained within virus-infected cells (Olivo et al., 1989) the same may be true for UL53-gK, if UL53 mRNA transcripts are translated with the same efficiency as UL52 mRNA.

3.2.2 UL53-gK is not an envelope protein.

The failure to detect UL53-gK in the virion envelope also strengthens the premise that UL53-gK makes an indirect contribution to virion structure, presumably as a regulatory protein which is essential for HSV-1 egress and therefore viral replication (Chapter 4). Nevertheless, it is possible that unforeseen factors have prevented UL53-gK from being detected in the virion. UL53-gK may be present within HSV-1 virions in such minute quantities that the signal intensity fell below the level of detection. Although it is conceivable that UL53-gK protein labelled with either [^{35}S]methionine and [^{35}S]cysteine or ^{125}I might escape detection (Chapter 4), it is less likely that the virus particles purified from the medium of infected cells would resist antibody-mediated complement lysis, if UL53-gK was present in the virion envelope. However, Steve Primorac, a member of our laboratory was unable to demonstrate UL53-gK is a component of the virion envelope using this technique (S. Primorac, personal communication). Likewise, cell extracts containing baculovirus-expressed UL53-gK were unable to elicit neutralizing-antibodies from vaccinated mice (Ghiasi et al., 1994). In contrast, glycoproteins B, C, D, E, I and H:L, induced the production of neutralizing antibodies in rabbits and in mice (Browne et

al., 1993; for review see Para et al., 1985; Ghiasi et al., 1995). Since immunization with US4-gG fails to produce neutralizing antibodies in mice (Ghiasi et al., 1992), and individual MAbs which recognize HSV-1 glycoproteins including UL27-gB, UL44-gC, US6-gD and US8-gE frequently lack neutralizing activity even in the presence of complement (for review see Para et al., 1983), there is still a chance that UL53-gK specific antisera binds to the virion but has a minimal impact on virion infectivity. Immunoelectron microscopy could be used to resolve this issue. Alternatively, the epitopes in hydrophillic region 1 recognized by the peptide antisera used in these experiments (α-UL53-1 & α-UL53-4) may be hidden (eg. in an oligomeric structure), or removed by proteolysis if/when UL53-gK is incorporated into the virion envelope. If hydrophillic region 1 is masked by a conformational change triggered if/when UL53-gK is assembled into the virion envelope, then the denaturing conditions provided by western blot analysis may be sufficient to expose the UL53-1 and UL53-4 epitopes. Conversely, if hydrophillic region 1 is removed by proteolysis, then it would be difficult, if not impossible, to detect UL53-gK in virions with the antisera that is currently available.

3.3 Potential role for UL53-gK in the exocytic and the re-envelopment egress models.

Based on the characteristics of the gK deletion mutant (F-gKß), the observations described above, and the models for herpesvirus egress presented in the literature, UL53-gK could function in either of two ways. HSV-1 virions may retain the envelope they acquire at the inner nuclear membrane, and exit cells in vacuoles derived from the membranes of the exocytic pathway (ie. outer nuclear membrane, ER, IC, Golgi, TGN, etc), as proposed in the exocytic model. Thus, UL53-gK may suppress intracellular fusion events between viral and cellular membranes to prevent the premature loss of virion

envelopes (see Introduction: section 3.7.1). According to this model, the release of naked capsids into the cytoplasm is a dead-end process (Campadelli-Fiume et al., 1991). Conversely, UL53-gK may facilitate re-envelopment if perinuclear virions penetrate the outer nuclear membrane, shed the primary envelope acquired from the inner nuclear membrane, and bud through the cytoplasmic face of membranes derived from the exocytic network (eg. Golgi) to obtain a secondary (final) envelope.

3.3.1 Role for UL53-gK in the exocytic egress model.

If one accepts the premise that UL53-gK prevents fusion between the virion envelope and cellular membranes (eg. Golgi membranes), then one must ask how UL53-gK can accomplish this task, given that gK does not reside in the virion envelope and remains in the ER and/or the intermediate compartment (IC). The salient points are as follows: (1) small amounts of UL53-gK were detected inside HSV-1 infected cells, (2) gK is retained in the ER and/or IC, (3) UL53-gK was not detected in the virion envelope, (4) naked capsids accumulated in the cytoplasm of cells infected with F-gKß, (5) enveloped capsids in the cytoplasm of F-gKß-infected cells were abnormal and displayed a 10-100 fold reduction in specific infectivity, (6) HSV-1 particles accumulated in the perinuclear space of cells expressing high levels of UL53-gK).

To explain this data one could propose that UL53-gK binds to a cellular receptor required for HSV-1 induced membrane fusion and prevent undesirable fusion events by retaining the putative receptor in the ER, or by inducing the rapid degradation of this molecule. By this model, UL53-gK does not need to be present in subsequent compartments of exocytic pathway, or the virion envelope to prevent fusion between virion and cellular membranes.

In cells infected with F-gKß, the absence of UL53-gK and resulting availability of cellular "fusion" receptors would permit the membrane fusion events which release naked capsids into the cytoplasm. The reduction in the number of enveloped virions within the perinuclear space, and the concomitant increase in the number of naked capsids observed in the cytoplasm of cells infected with F-gKß, is also consistent with this hypothesis (see Chapter 5). Enveloped particles containing two or more capsids may be the result of virion-virion fusion events, if for example, the putative HSV "fusion" receptor is transported to the inner nuclear membrane and incorporated into the virion envelope when UL53-gK is absent. A variety of cellular receptors have been detected in the envelopes of herpesvirions providing additional support for this idea (eg. CD4 in HSV-1; MHCII in EBV) (Dolter et al., 1993; Knox & Young, 1995).

The suggestion that UL53-gK binds to and/or induces rapid degradation of a cellular "fusion" receptor for HSV-1 is not without precedence. Like UL53-gK, the Vpu gene product of human immunodeficiency virus type 1 (HIV-1) is a small hydrophobic protein that enables efficient virus maturation and release (Klimkait et al., 1990). To mediate this function the Vpu protein interacts with the cytoplasmic domain of the HIV-1 receptor CD4, to induce the rapid degradation of CD4 molecules in the ER (Vincent et al., 1993). In addition, other viral proteins have been identified which retain plasma membrane proteins in the ER. For example, the adenovirus E19 glycoprotein contains a dilysine-ER-retention motif and acts to suppress the Ad-specific cytotoxic T lymphocyte immune response by holding MHC class 1 molecules in the ER (Jackson et al., 1990; Cox et al., 1991). If UL53-gK performs a function comparable to HIV-1 Vpu and interferes with the transport of a cellular "fusion" receptor, then constitutive expression of UL53-gK should block HSV-1 entry. To confirm or reject this hypothesis, one could infect cells

with an adenovirus expression vectors and measure HSV-1 entry by comparing HSV-1 IE protein synthesis in the cells expressing UL53-gK, with cells that do not.

Overexpression of UL53-gK caused enveloped virions to accumulate in the perinuclear space, again suggesting that UL53-gK functions during the early stages of virus egress, (ie. in nuclear and ER membranes). However, it is difficult to reconcile this observation with the hypothesis that UL53-gK sequesters a cellular "fusion" receptor in the ER and/or IC, to inhibit membrane fusion events. If the primary role of UL53-gK is in fact to sequester a cellular "fusion" receptor, then overexpression of UL53-gK should have little or no effect on virus egress. To resolve the apparent contradiction, one must suggest that overexpression of UL53-gK leads to nonspecific effects which do not occur during wild-type HSV-1 infections. Hepatitis B virus (HBV) provides an example of such a phenomenon. Overexpression of the HBV envelope protein L inhibits the release of HBV virions, yet mutant L proteins which no longer possess this activity still allow virion formation, indicating that the function mediating ER retention of virus particles is not required for virus egress (Bruss & Ganem, 1991).

It is doubtful that a change in the temporal control of UL53-gK synthesis, caused by introducing the UL53 gene to the cellular genome, could produce this phenotype. UL53-gK is normally expressed as an early or leaky late protein from the HSV genome during virus replication (see Chapter 2, Uprichard & Knipe, 1996) and many studies report that virus genes placed in the context of the cellular genome are regulated as early genes (Arsenakis et al., 1986; for review see Roizman & Sears, 1996).

Instead, overexpression of UL53-gK may have forced the protein into membrane domains from which it is normally excluded (eg. specialized budding domains in the outer nuclear membrane?). In these circumstances, UL53-gK may interfere with the function of

other viral proteins (eg. UL20) involved in HSV-1 egress, or alter the structure of membranes (eg. through oligomerization) to prevent the membrane fusion events required for vesicular transport of HSV-1 virions from the perinuclear space.

Alternatively, retention of enveloped virions between the nuclear membranes in cells expressing high levels of UL53-gK may be indicative of normal UL53-gK function. For instance, large multilamellar vesicles apparently derived from the nuclear envelope were frequently observed in those cell lines which overexpress UL53-gK (see Chapter 5). Similarly, the proliferation and reduplication of intracellular membranes has been observed in cells infected with wild-type HSV-1, albeit at a much reduced frequency (Rodriguez & Dubois-Dalcq, 1978; for review see Nii, 1992). Therefore, the physical presence of UL53-gK in membranes may be enough to suppress fusion between virion and cellular membranes, and overexpression UL53-gK may be sufficient to inhibit vesicular transport of viruses from the perinuclear space, since membrane fusion must occur for vesicles to pinch off the ER and nuclear envelope. This mechanism could lead to the build up of enveloped virions in the perinuclear space, and provide the conditions required for the formation of multilamellar vesicles next to the nuclear envelope. Based on this model, a shortage of UL53-gK could result in unrestricted fusion of enveloped virions with the outer nuclear membrane, causing a reduction in perinuclear virions and concomitant increase in naked cytoplasmic capsids similar to that observed in F-gKß-infected cells. However, a block in vesicular transport from the ER might be expected to affect the intracellular movement and cell surface transport of membrane glycoproteins, yet the cell lines expressing large amounts of UL53-gK did not exhibit any obvious defects in HSV protein synthesis, glycosylation or protein transport (see Chapter 3 & 4). Nevertheless these studies focused primarily on HSV-1 glycoprotein D and were not absolutely

quantitative, so it is possible that a fraction of US6-gD or another HSV membrane protein(s) is retained inside cells overexpressing UL53-gK. To determine if/how gK affects protein transport in HSV-infected cells techniques including fluorescence-activated cell sorting (FACS) could be used both to quantify and compare the cell surface transport of HSV-1 membrane proteins (eg. gB, gD, gE:gI, gH:gL, gM, UL45) in HSV-infected cells which overexpress UL53-gK, express wild-type levels of UL53-gK or lack UL53-gK altogether.

3.3.2 Role for UL53-gK in the de-envelopment-re-envelopment egress model

In the event that HSV-1 employs the de-envelopment-re-envelopment route first described by Stackpole (1969) to exit cells, one could hypothesize that UL53-gK promotes envelopment of capsids at the cytoplasmic face of the endoplasmic reticulum or intermediate compartment. By comparison, envelope proteins from a variety of virus families (eg. Bunyaviridae [MG], Hepatitis B virus [ER], Paramyxoviridae [PM], Poxviridae [orthopoxviruses, IC & TG], Retroviridae [spumaviruses, ER; lentiviruses, PM], Rhabdoviridae [PM], Togaviridae [rubiviruses, MG; alphaviruses, PM] contain retention signals which target these proteins to specific membrane compartments where they mediate budding of infectious particles through cytoplasmic membranes, including those derived from the ER, IC, elements of the Golgi apparatus, and the plasma membrane (Matsuoka et al., 1988; Chen et al., 1991; Bruss & Ganem, 1991; Nemeckova et al., 1994; Roberts et al., 1995; Rodriguez & Smith, 1992; Engelstad & Smith, 1993; Sodeik et al., 1993; Schmelz et al., 1994; Goepfert et al., 1994; Owens et al., 1991; Lodge et al., 1994; Fuller et al., 1984; Thomas et al., 1993; Luan & Glaser, 1994; Luan et al., 1995; Hobman et al., 1995; for reviews see Pettersson, 1991; Kingsbury, 1996; Moss, 1996; Schlesinger &

Schlesinger, 1996; Schmaljohn, 1996; Wagner & Rose, 1996).

To direct capsid envelopment at cytoplasmic membranes, envelope proteins must possess certain attributes: a targeting signal which sorts the envelope protein to a specific cellular membrane (eg. ER, IC, Golgi apparatus or PM), and the ability to interact with the matrix or capsid proteins of the virus. In addition, envelope proteins in conjunction with matrix proteins may be involved in fission, the process by which virion particles pinch off from the enveloping membrane (Lenard, 1996). Transport and retention of UL53-gK in the ER and/or IC membranes fulfils the first of these requirements, but as yet there has been no indication that UL53-gK interacts with HSV-1 tegument and/or capsid proteins. Furthermore, the syncytial phenotype associated with UL53-gK could represent a loss of regulatory controls governing the envelope fission process. In support of a role for UL53-gK in re-envelopment, the appearance of aberrant virions in F-gKß-infected cells (ie. 2 capsids in a single envelope) which exhibit a 10-100 fold reduction in specific infectivity, could be interpreted as abnormal re-envelopment, triggered by the absence of UL53-gK. Nevertheless, intracellular targeting by itself, does not establish that UL53-gK is involved in capsid re-envelopment. For example, targeting signals specify accumulation of coronavirus M proteins in the Golgi complex, but capsid envelopment is thought to take place on membranes of the intermediate compartment (Tooze et al., 1984, 1988; Machamer et al., 1990, 1993; Klumperman et al., 1994; Locker et al., 1994, 1995; Opstelten et al., 1995; for review see Holmes & Lai, 1996).

If indeed UL53-gK acts to facilitate envelopment of cytosolic capsids, the distribution of UL53-gK should determine the site of HSV-1 re-envelopment, given that enveloped viruses usually bud through the cellular membrane to which their membrane proteins are targeted. In accordance with the observation that UL53-gK is retained inside

cells, membrane proteins which support capsid envelopment do not need to be transported to the cell surface to fulfil this function (for reviews see Pettersson, 1991; Kingsbury, 1996; Moss, 1996; Schlesinger & Schlesinger, 1996; Schmaljohn, 1996; Wagner & Rose, 1996). To date, most electron microscopy studies have proposed re-envelopment in the trans-Golgi apparatus (eg. Whealy et al., 1991; Gershon et al., 1994), although immunoelectron microscopy - lectin binding studies indicate that re-envelopment at the ER, IC, or cis-Golgi remains a possibility (Di Lassaro et al., 1995). However, the intracellular location of UL53-gK and the presence of unprocessed oligosaccharides, would seem to suggest that re-envelopment should occur in the ER or intermediate compartment. Nevertheless, the perinuclear signal observed in the immunofluorescence experiments (see Chapter 4) is also consistent with the accumulation of UL53-gK in a Golgi compartment (Klumperman et al., 1994). Interestingly, glycoprotein G1 of Uukuniemi virus (of the Bunyaviridae) is transported to the Golgi apparatus, yet remains endo H sensitive (Melin et al., 1995). Consequently, the possibility that UL53-gK is transported to the Golgi apparatus where it contributes to re-envelopment, can not be excluded, until the intracellular location of UL53-gK has been confirmed by a co-localization study.

The majority of enveloped viruses replicate in the cytoplasm (eg. Bunyaviridae, Paramyxoviridae, Poxviridae, Retroviridae, Rhabdoviridae, and Togaviridae, but not the Orthomyxoviridae) and thus, must bud through cytoplasmic membranes to produce infectious particles (for reviews see Pettersson, 1991; Holmes & Lai, 1996; Kingsbury, 1996; Lamb & Krug, 1996; Moss, 1996; Schlesinger & Schlesinger, 1996; Schmaljohn, 1996; Wagner & Rose, 1996). In addition, all of the viral membrane proteins implicated in capsid envelopment are incorporated into the virion envelope during the budding process. In contrast, HSV-1 replicates in the nucleus and acquires an envelope at the inner

nuclear membrane.

Hence the absolute requirement for envelopment at cytoplasmic membranes has been removed. Moreover, the idea that UL53-gK takes part in capsid re-envelopment might be considered unlikely, because UL53-gK has not been detected in the virion envelope (Chapter 4). A role for gK in promoting re-envelopment in the cytoplasm is also difficult to rationalize with the reduced number of enveloped virions located between the inner and outer nuclear membranes, in cells infected with F-gKß relative to wild-type HSV (Chapter 5).

However, I can not rule out the possibility that HSV-1 sheds the envelope obtained from the inner nuclear membrane and secures a second envelope from cytoplasmic membranes through a process involving UL53-gK. If this event in fact occurs, UL53-gK may have been situated in the virion envelope, yet escaped detection for one of the reasons discussed earlier (see Discussion: section 3.2.2: UL53-gK is not an envelope protein). Alternatively, UL53-gK may participate in a re-envelopment mechanism which excludes UL53-gK from the virion envelope, for example as a prerequisite of the fission process. Since the HSV-1 envelope contains more than 14 different membrane proteins, but the majority of virus families possess less than four envelope proteins, it is conceivable that the envelopment proteins of other virus families reside in the virus envelope because they supply additional functions (eg. during virus entry). By contrast, the availability of 14 HSV-1 envelope proteins to supply viral functions, could eliminate the need for UL53-gK in the virion envelope.

Another discovery which must be considered when debating the merits of UL53-gK participation in the de-envelopment-re-envelopment model, concerns the observation that enveloped virions accumulate in the perinuclear space of cells expressing large

amounts of UL53-gK, and the recognition that this condition is accompanied by the development of multilamellar vesicles. Again it is difficult to reconcile these results with the view that UL53-gK contains a property that promotes re-envelopment. In the de-envelopment-re-envelopment model of virus egress, it could be argued that UL53-gK facilitates envelopment of capsids at the cytoplasmic face of intracellular membranes and therefore, overexpression of the glycoprotein might not be expected to cause particles to accumulate in the perinuclear space. However, these results are not sufficient to dismiss the possibility that UL53-gK plays a role in re-envelopment. Analogous reports have demonstrated that envelope protein L of hepatitis B virus (HBV) is absolutely required for HBV capsid envelopment at the cytoplasmic face of ER membranes, yet overexpression of L protein caused enveloped HBV particles to accumulate in the ER lumen (Bruss & Ganem, 1991). Consequently, retention of the HSV-1 particles in cells expressing large amounts of UL53-gK may be related to nonspecific effects which do not occur during wild-type HSV-1 infections, as discussed previously (see Discussion: section 3.3.1: Role for UL53-gK in the exocytic egress model).

3.4 **Defining the HSV-1 Egress Pathway.**

A clear understanding of HSV-1 egress will be necessary before UL53-gK can be assigned to a specific role in the egress process with any certainty. At present, there is evidence to support both models of virus egress and it is conceivable that both are correct, introducing the potential for one pathway to be favoured over the other, in different cell types. Consequently, specific experiments are needed to investigate this possibility. Two strategies offer the greatest of chance success: 1) define the intracellular membrane which provides the envelope to extracellular virions (eg. membranes derived from the inner

nuclear envelope vs. the cis-Golgi), 2) compare the viral proteins obtained from perinuclear virions, extracellular virions and the plasma membrane to identify differences which can only be attributed to the envelopment process (eg. a change in the virion-protein composition or the presence of post-translational alterations).

To identify the origin of extracellular envelopes one could demonstrate that the resident proteins of a specific intracellular compartment (eg. the inner nuclear envelope or the cis-Golgi) are incorporated into extracellular virions. Since the cellular proteins CD4 and MHCII have been detected in the envelope of herpesviruses, this approach could entail cellular proteins (eg. cis-Golgi mannosidase II) (Dolter et al., 1993; Knox & Young, 1995).

Alternatively, the retention signals from other type 1 membrane proteins could be introduced to an HSV-1 glycoprotein (eg. US6-gD) in order to localize this chimera to specific organelles (eg. inner nuclear membrane, ER, cis-, medial-, or trans-Golgi) (Smith & Blobel, 1993; Machamer et al., 1990; Swift & Machamer, 1991; Chen et al., 1991a; Matsuoka et al., 1994; Hobman et al., 1995; Locker et al., 1994; 1995; for review see Machamer, 1993). The retention signals, which are typically transmembrane domains, can be used to replace the membrane anchor in US6-gD. This should reduce the possibility that their presence will exclude US6-gD from the virion envelope, given that GPI-linked forms of gD (which lack a transmembrane domain altogether) are incorporated into the envelope of bovine herpes virus (Liang et al., 1995b). Moreover, it is important to distinguish retention signals, which do not allow proteins to move beyond a given compartment and produce unambiguous results, from retrieval signals (eg. K(X)KXX used by Browne et al., 1996), which permit membrane proteins to move between exocytic compartments and produce results that are difficult to interpret.

Finally, immunoelectron microscopy could be used to determine the intracellular location of cellular proteins CD4 and MHCII as well as the GPI-linked forms of gD. Since these proteins are incorporated into the envelope of extracellular virions, and are not indigenous to the viral genome, knowledge of their intracellular distribution may allow one of the egress models to be discarded (eg. transport to the inner nuclear membrane is a prerequisite of the exocytic model) (Dolter et al., 1993; Knox & Young, 1995; Liang et al., 1995b).

The second strategy (described above) is based on the concept that extracellular virions which undergo re-envelopment may contain a different subset (or ratio) of viral proteins than the perinuclear virions, since the distribution of HSV-1 structural components (eg. tegument & envelope proteins) may differ between the cytoplasm and the nucleus. The availability of a UL20-negative virus which assembles perinuclear and extracellular particles on one cell type, and perinuclear particles on another, should facilitate these experiments (Avitabile et al., 1994). On the other hand, HSV virions may obtain their final envelope from the inner nuclear membrane. In that case, post-translational modifications made to the transmembrane domain or cytosolic tail of the HSV-1 glycoproteins transported to the inner nuclear membrane and incorporated into the virion envelope, may differ from viral glycoproteins which reside in the plasma membrane, since these proteins are exposed to different environments (eg. the cytoplasmic tail of viral gE:gI may exhibit a different phosphorylation pattern than cellular gE:gI). It is important to note that all of these experiments are based on the protein composition of the viral envelope rather than phospholipid analysis. Since the envelope proteins of several virus families (eg. Rhabdoviridae) induce the formation of domains enriched in specific phospholipids prior to capsid envelopment, the same may be true of HSV-1 membrane proteins and

consequently, phospholipid analysis is apt to produce unreliable results (Pessin & Glaser, 1980; Marquardt et al., 1993; Luan & Glaser, 1994; Luan et al., 1995).

3.4.1 Defining the role of UL53-gK in HSV-1 replication.

Although the route that HSV-1 virions use to exit cells remains uncertain, several avenues of research may provide additional insight into the role of UL53-gK in HSV-1 replication. For instance, the polypeptide profile of the enveloped virions produced in F-gKß-infected Vero cells could be compared to the profile of intracellular virions generated by wild-type HSV-1. If the aberrant virions observed inside F-gKß-infected cells (eg. 2 capsids in one envelope) stem from an abnormal re-envelopment process, the protein content (or ratio) is likely to differ from wild-type virions. In contrast, the composition of aberrant F-gKß virions which arise from virion-virion fusion events should be similar to wild-type HSV.

As an adjunct to these experiments, the production and release of L-particles from F-gKß infected cells could be investigated. Since noninfectious L-particles often contain proteins which H-particles lack (eg. ICP4), they may arise by a different mechanism (eg. envelopment at cytoplasmic membranes) (Szilagyi & Cunningham, 1991; McLauchlan & Rixon, 1992; Dargan et al., 1995; Yang & Courtney, 1995). In addition L-particles may exit cells by a different route, since the intracellular distribution of H- and L-particles has been shown to differ (Rixon et al., 1992). Therefore it would be of interest to determine if UL53-gK is required for the assembly and/or the egress of HSV-1 L-particles. A combination of F-gKß (gK-negative virus) and BHK21 cells should be used in an investigation of this type, since the ratio of L-particles:H-particles produced by BHK21 cells is 1:1 and less than 1:1300 on Vero cells (Yang & Courtney, 1995).

Furthermore, studies designed to analyze F-gKß H- and L- particles could be used to verify that non-infectious H-particles (enveloped virions) do not escape from F-gKß-infected cells into the extracellular medium. Although enveloped virions were rarely observed on the surface of F-gKß infected cells in electron micrographs, there is a remote possibility that non-infectious H-particles lacking UL44-gC, UL27-gB, and US6-gD (the principal receptor binding proteins), may have been washed away when cells were prepared for electron microscopy. This possibility is underscored by reports that the egress of infectious particles from cells treated with glycosylation inhibitors is affected more severely than the release of noninfectious particles (Ghosh-Choudhury et al., 1987; Kuhn et al., 1988; Koyama & Uchida, 1989; Cheung et al., 1991; Chatterjee & Sarkar, 1992).

Other experiments may also provide useful information concerning UL53-gK function. For example, the membrane topology of UL53-gK is likely to be significant. If UL53-gK is involved in capsid re-envelopment, then one might predict that the most highly conserved regions in UL53-gK face the cytoplasm. Depending on the outcome of this analysis, one could alter the membrane topology of UL53-gK by modifying the gK-signal peptide, as a way to confirm this hypothesis (Bruss & Vieluf, 1995; Spiess, 1995). Further confirmation that conserved regions in UL53-gK are sufficient for gK function could be furnished through the use of substitution experiments which place the gK homologs from other alphaherpesviruses into the TK locus of F-gKß.

In the distant future, it may be possible to replace the ER retention signals in UL53-gK with domains that target gK to other membrane compartments (eg. Golgi), and thereby assess the importance of UL53-gK ER retention for HSV-1 replication. One could foresee a situation where retention of UL53-gK within the medial-Golgi could be sufficient

to support HSV-1 replication, assuming that UL53-gK blocks intracellular fusion events by sequestering a cellular receptor, or insufficient if UL53-gK mediates re-envelopment of cytoplasmic capsids.

4.0 Syn-1 mutations in UL53-gK influence cell-cell fusion through an indirect mechanism

For many years, a desire to reveal the role UL53-gK plays in cell-cell fusion has been a major ambition of researchers dedicated to understanding HSV-1-induced membrane fusion, and has constituted one of the most challenging aspects of this thesis. In an attempt to define the contribution syncytial forms of UL53-gK make to cell-cell fusion, I compared the traits of UL53-gK made by syn-1 HSV mutants (eg. abundance, turnover, electrophoretic mobility, glycosylation, & cellular distribution) with UL53-gK produced by wild-type strains of HSV-1 (Chapter 2, 3, & 4).

Given that cell-cell fusion has been observed in HSV-1 infected cells expressing low levels of UL53-gK (Chapter 5), one might predict that syn-1 mutations destabilize UL53-gK leading to rapid degradation of the protein. Another conjecture with merit is the potential for syn-1 mutations to alter the cellular distribution and possibly the post-translational processing of UL53-gK oligosaccharides. Surprisingly, neither of these phenomena materialized. In fact, the attributes of UL53-gK produced by syncytial variants (syn-gK) and wild-type HSV-1 (wt-gK) appear to be identical (Chapters 2, 3, & 4). However, it is conceivable that syn-1 mutations produce subtle alterations in UL53-gK structure, and MAbs which recognize discontinuous epitopes may be able to detect a change of this nature sometime in the future.

There is overwhelming evidence to suggest that point mutations in UL53-gK produce striking effects in HSV-infected cells, which manifest as the massive amounts of

cell-cell fusion observed 4 to 6 h post-infection. These observations led to the suggestion that UL53-gK occupies a pivotal role in the regulatory mechanism controlling cell-cell fusion, and perhaps fusion of the virion envelope with the plasma membrane during virus entry. Several models have been put forward to explain the effect syn-1 (UL53-gK) mutations have on HSV-infected cells, although none of these models have been confirmed experimentally (see Figure 5.6). It has been proposed that UL53-gK contains an innate fusion-inducing activity which is triggered by mutations in the protein, as well as the opposite view that UL53-gK acts to suppress or regulate the fusion process (Keller, 1976; Rhulig & Person, 1977; Manservigi et al., 1977; Spear, 1984; Read et al., 1980; Hutchinson et al., 1993, 1995; Dolter et al., 1994). Among the potential mechanisms that have been suggested is the prospect that UL53-gK inhibits cell-cell fusion by (1) interacting with components of the fusion complex in the plasma membrane and virion envelope to regulate fusion, (2) influencing the cell surface transport of the HSV-1 or cellular proteins which affect cell-cell fusion, (3) transforming the cell surface, directly or indirectly, to create membranes resistant to HSV-induced cell-cell fusion, or (4) acting to catalyze or indirectly influence a process which governs the function of molecules involved in cell-cell fusion (eg. post-translational modifications or ion flux) (Keller, 1976; Rhulig & Person, 1977; Lee & Spear, 1980; Read et al., 1980; Bzik & Person, 1981; McKenzie et al., 1987; Hutchinson et al., 1993, 1995; Spear, 1984, 1993; Baghian & Kousoulas, 1994). Since UL53-gK has not been detected in the plasma membrane of HSV-infected cells or the HSV-1 virion (Chapter, 4), it is unlikely that UL53-gK plays a direct role in membrane fusion, either as a component of the fusion complex used during cell-cell fusion or as virus enters cells. Moreover, the small quantities of UL53-gK expressed by HSV-infected cells, relative to other membrane proteins involved in cell-cell

Figure 5.6 Mutations in UL53-gK induce cell-cell fusion: Description of the mechanisms proposed to date.

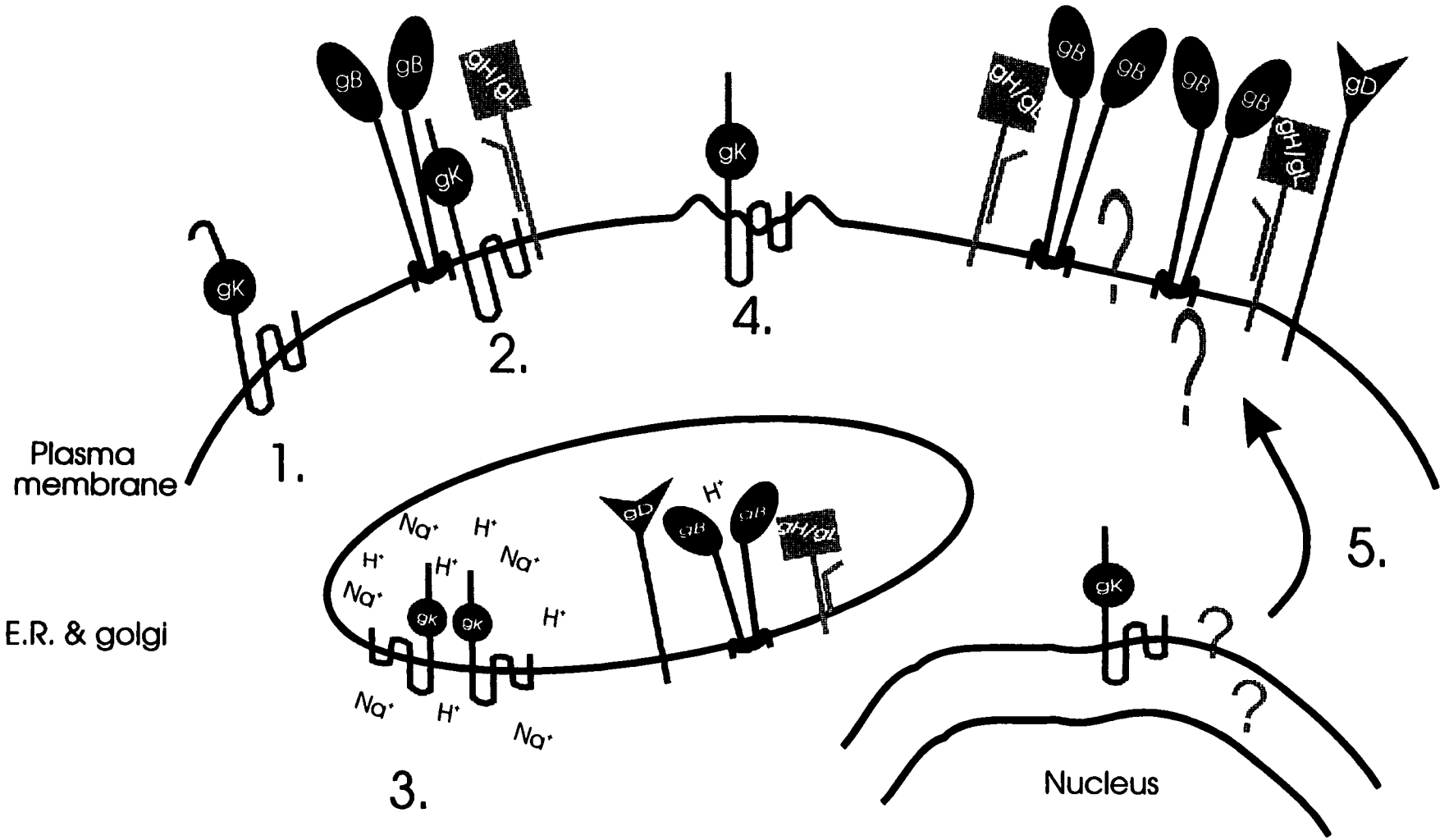
1. UL53-gK contains an innate fusion-inducing activity triggered by syncytial mutations.

2. gK acts to suppress or regulate fusion by interacting with plasma membrane proteins (eg. gB, gH:gL, gD) which participate in cell fusion.

3. gK may act to catalyze or indirectly influence a process which governs the function of molecules involved in cell-cell fusion. For example, post-translational modifications may alter the folding or structure of HSV fusion protein(s) placing these molecules in an "off" conformation, whereas mutations in gK may disrupt HSV-1 replication in a manner that impedes this process. Alternatively, gK could influence, or even act as ion channel which alters the environment of intracellular compartments to inhibit internal membrane fusion events, perhaps by placing the HSV fusion protein(s) in an inactive state?

4. gK controls cell-cell fusion by transforming the cell surface, directly or indirectly (not shown), to create membranes resistant to HSV-induced cell-cell fusion.

5. gK influences the cell surface transport of the viral proteins which mediate and/or regulate cell-cell fusion and mutations in gK create an imbalance in the subcellular distribution of these proteins. It is also conceivable that mutations in gK alter the cell surface transport of a cellular receptor protein(s) required for cell-cell fusion, a prospect which has been proposed in the Discussion below. In addition, the previous models did not consider the possibility that HSV particles mediate cell-cell fusion, rather than viral proteins in the plasma membrane, and because gK is required for virus egress gK^{syn} mutations may affect the transit of HSV virions to influence cell-cell fusion (see Chapter 5, and Discussion below).


gB
gB
gH/gL
gK
gK
gB
gB
gB
gB
gH/gL
gD
gH/gL
?
?
?
Plasma membrane
1.
2.
4.
5.
E.R. & golgi
Na+
H+
H+
H+
Na+
Na+
gK
gK
H+
Na+
H+
Na+
gD
gB
gB
gH/gL
H+
gK
?
?
Nucleus
3.

fusion, is more consistent with a regulatory role for UL53-gK. In addition, complementation experiments performed in Chapter 3 confirmed that wild-type UL53-gK has the capacity to suppress cell-cell fusion associated with syn-1 mutants (eg. syn20, MP, 804), including strains which exhibit co-dominant traits in Vero cells infected with a 1:1 mixture of wild-type HSV-1 and a syn-1 mutant (Hoggan & Roizman, 1959; Person et al., 1976; Bzik & Person, 1981; Little & Schaffer, 1981; Debroy et al., 1985; Pogue-Geile et al., 1987; Hutchinson et al., 1993; Roop et al., 1993). These results are consistent with previous studies which employed mixed infections to assess UL53-gK function (Lee & Spear, 1980; Read et al., 1980; Bzik & Person, 1981), but are the first to demonstrate that UL53-gK is directly responsible for the reduction in cell-cell fusion. One or more of the wild-type strains used in mixed infection studies may harbour secondary mutations in nonessential loci required for cell-cell fusion (eg. US8-gE, US7-gI, UL10-gM, UL45), whereas complementation experiments performed in Chapter 3 employed UL53-gK expression vectors (Hutchinson et al., 1993; Baanes et al., 1994; Davis-Poynter et al., 1994). As such, the recessive phenotype exhibited by syn-1 mutants is indicative of a loss-of-function mutation. Still, the underlying basis for the syn-1 phenotype remains unclear, and in all probability cell-cell fusion induced by mutations in UL53-gK is related to the role UL53-gK plays in virus egress.

4.1 The syncytial phenotype may be linked to defects in virus egress.

Evidence supporting the position that the egress defects and the syn-1 phenotype caused by mutations in UL53-gK may have a common basis, stems in part, from the discovery that other HSV proteins involved in virus egress have also been implicated in cell-cell fusion. A number of HSV-1 mutants with defects in virus egress have been

described which fall into this category.

Cells infected with an HSV-1 mutant expressing a temperature-sensitive form of gH produce infectious particles which are retained inside cells, whereas the particles released from cells are noninfectious and lack gH (Desai et al., 1988). It is not clear whether these particles lose UL22-gH, e.g., by proteolysis during egress, or whether mutant gH is not incorporated into the envelope of those particles which ultimately escape cells. Interestingly, the presence of UL22-gH is absolutely required for HSV-induced cell-cell fusion, and MAbs specific for the gH homolog of a closely related alphaherpesvirus, VZV, block the entry and egress of that herpesvirus (Rodriguez et al., 1993; Davis-Poynter et al., 1994). Similarly, US6-gD is absolutely required for HSV-induced cell-cell fusion, and mutations in this protein have been described which cause unenveloped capsids to congregate in the cytoplasm of infected cells (Ligas & Johnson, 1988; Davis-Poynter et al., 1994; Campadelli-Fiume et al., 1991). Surprisingly, UL22-gH or US6-gD can be deleted from the virus genome without affecting capsid envelopment or virus egress. However, the possibility that these proteins make an essential contribution to the assembly of infectious virions (apart from their role in virus entry), has not been ruled out (Ligas & Johnson, 1988; Feenstra et al., 1990; Forrester et al., 1992; Roop et al., 1993; Wilson et al., 1994).

In addition, syncytial mutations have been identified in many of the HSV proteins which have a direct role in virus egress. Like UL53-gK, the UL20 gene product is an intracellular membrane protein with four potential membrane spanning domains, and deletion of the UL20 gene produces a host-range defect in virus egress, or the syncytial phenotype in those cell lines which support the replication of $UL20^-$ mutants (Baines et al., 1991; MacLean et al., 1991). However, UL20 is transported to the Golgi apparatus and

enveloped virions accumulate in the perinuclear space of Vero cells infected with a UL20-deletion mutant, whereas UL53-gK is retained in the ER and defective particles (naked and enveloped capsids) accumulate in the cytoplasm of Vero cells infected with the gK-negative mutant, F-gKß (Debroy et al., 1985; Pogue-Geile et al., 1987; Baines et al., 1991; Ward et al., 1994; Hutchinson et al., 1995a,b; see Chapter 4 & 5). Deletion of ICP34.5 also manifests in a host range defect characterized by the accumulation of perinuclear virions in some cell lines, but not in others. As such, the attributes of ICP34.5$^-$ and UL20$^-$ mutants are reminiscent of the properties displayed by wild-type HSV-1 when UL53-gK is overexpressed. Furthermore, a syncytial mutation (syn-6) has been mapped to a region of the genome flanked by genes encoding ICP0 and ICP34.5 (Romanelli et al., 1991). Although the syncytial mutation has not been assigned to a specific open reading frame, this outcome raises the possibility that ICP34.5 is the affected gene. Another indication that syncytium formation is linked to defects in virus egress, is provided by the observation that the number of cytoplasmic virus particles is elevated in cells infected with syn-1 (UL53-gK) or syn-3 (UL27-gB) mutants relative to wild-type HSV-1 (Chapter 5). Taken together these results suggest that defects in virus egress may contribute to the syncytial phenotype and add credence to the hypothesis that UL53-gK regulates cell-cell fusion by acting at intracellular sites. Consequently, any attempt to explain the syncytial phenotype produced by point mutations in UL53-gK, should be in keeping with the properties displayed by the gK-negative mutant, F-gKß.

4.1.1 The syn-1 phenotype: Relationship to the de-envelopment-re-envelopment model of virus egress

If one subscribes to the de-envelopment-re-envelopment model of virus egress, and the premise that UL53-gK facilitates capsid envelopment at the cytoplasmic face of ER

and/or IC membranes, then the proposal that syn-1 mutations affect the cell surface transport of HSV proteins with activities that trigger cell-cell fusion, or serve to regulate the fusion process, remains appealing (Lee & Spear, 1980; Hutchinson et al., 1993, 1995a,b; Spear, 1993). According to this hypothesis, fusion might be induced by an imbalance in the subcellular distribution of HSV gene products which govern cell-cell fusion, if for example, the plasma membrane is enriched with elements of the viral fusion complex (eg. gB, gD, & gH:gL) or deficient in viral components which suppress fusion.

This theory also recognizes the potential for a viral fusion complex within the virion envelope, as well as the plasma membrane, to function in an unregulated manner and thereby induce cell-cell fusion. For instance, syn-1 mutations could disrupt capsid re-envelopment, reducing the abundance and/or interactions of regulatory proteins with the viral fusion complex in mature HSV-1 virions. Nevertheless, no one has demonstrated that the HSV-1 virions produced by syn-1 mutants have the capacity to bridge cells and induce cell-cell fusion in the absence of viral protein synthesis (ie. fusion-from-without) (Nii & Kamahora, 1961a; Person et al., 1976; Walev et al., 1991a,b). Instead, all of the studies which have examined this question, report that viral protein synthesis is essential for HSV-induced fusion among cells exposed to syn-1 mutants (ie. fusion-from-within) (see Keller, 1976b for review; Kousoulas et al., 1978; Falke et al., 1985). Moreover, the production of infectious virions is not a prerequisite of cell-cell fusion (Keller, 1976b; Kousoulas et al., 1978; Lee & Spear, 1980). As such, these results favour the idea that it is viral fusion proteins located in the plasma membrane which mediate cell-cell fusion.

Therefore, it is somewhat surprising that Vero cells infected with the gK-negative mutant, F-gKß, do not form syncytia. To explain these results, one could suggest that syn mutations in UL53-gK have a greater effect on the cell surface transport of regulatory

proteins than that of viral fusion proteins (eg. gB, gH:gL), and when UL53-gK is removed entirely, these effects may extend to one or more of the HSV proteins which make an essential contribution to cell-cell fusion. In support of this suggestion, the movement of at least some HSV glycoproteins to the plasma membrane, is reduced in cells infected with HSV syncytial mutants (eg. gK^{syn}, gB^{syn}, UL20$^-$) (Person et al., 1982; Avitabile et al., 1994). Alternatively, UL53-gK may possess additional functions required for cell-cell fusion, that have yet to be identified. Studies which compare the cellular distribution of the viral proteins promoting and/or controlling syncytium formation, in cells infected with wild-type HSV-1, F-gKß, and syn-1 mutants may resolve these issues (eg. gB, gC, gD, gE:gI, gH:gL, gK, gM, UL20, UL24, UL45 and possibly ICP34.5 & UL25, among others which have yet to be discovered) (Hutchinson et al., 1993; Davis-Poynter et al., 1994; Baanes et al., 1994; Spear, 1993).

4.1.2 The syn-1 phenotype: Relationship to the exocytic model of virus egress

Within the context of an exocytic egress model, the concept that UL53-gK sequesters a cellular "fusion" receptor to block intracellular fusion events, also has the capacity to explain how mutations in UL53-gK could produce the syncytial phenotype. If syn-1 mutations in UL53-gK permit an HSV-1 "fusion" receptor to escape from the ER, the availability of these receptors at the cell surface should greatly enhance the likelihood of cell-cell fusion, at least in high MOI infections. Consistent with this hypothesis, syn-1 mutant infected cells fuse more readily with uninfected cells or syn-1 mutant infected cells, than with cells infected with wild-type HSV-1 (Keller, 1976a; Read et al., 1980; Lee & Spear, 1980).

In addition, certain predictions arise from this theory. For example, naked capsids

should accumulate in the cytoplasm of cells infected with syn-1 mutants and a corresponding reduction in virus egress should be observed, if the syn-1 defects in UL53-gK truly increase the availability of a cellular "fusion" receptor. Interestingly, syn-1 mutants often produce a lower titre (eg. 5-10 fold decline) then wild-type HSV-1 in one step growth experiments (Read et al., 1980; Bzik & Person, 1981; Little & Schaffer, 1981). Furthermore, the replication of wild-type HSV-1 is invariably reduced in mixed infections that include syn-1 variants, whereas growth of the syn-1 variant is generally enhanced by the presence of wild-type HSV-1. Whether the reduced yields observed in both of these experiments stem from a decline in virus production, or a decrease in specific infectivity caused by defects in particle assembly is not known, but each of these possibilities could be examined. However, it should be noted that the reduction in virus yield cannot be attributed to syn-1 mutations with absolute certainty, because secondary mutations which have the potential to disrupt HSV replication, may have been generated by the mutagenesis strategy used in these studies (eg. Person et al., 1976; Little & Schaffer, 1981).

According to current dogma, it is US6-gD rather than UL53-gK which is the logical candidate to sequester an HSV-1 "fusion" receptor and thereby prevent fusion events between intracellular membranes and the virion envelope. This view is based on the observation that cells transfected with US6-gD are resistant to infection with HSV-1 (Campadelli et al., 1988), and the discovery that naked capsids accumulate in the cytoplasm of cells infected with HSV-1 variants containing mutations in US6-gD (Campadelli et al., 1991). Nevertheless, it is possible that HSV-1 has evolved more than one mechanism to combat intracellular fusion events.

By comparison, the HIV-1 genome contains the receptor binding-fusion protein

gp120/gp41, as well as Vpu a molecule which retains the HIV-1 receptor, CD4, in the ER. As such, cells containing gp120/gp41 in their plasma membranes do not produce syncytia when mixed with $CD4^-$ Hela cells expressing Vpu (Vincent et al., 1993). In addition, Raja et al. (1993) have circumvented the Vpu function of HIV-1 by incorporating ER retention signals into CD4 and producing molecules that are either partially or fully retained in the ER. When Raja et al. (1993) performed membrane fusion (syncytium) assays in cells co-transfected with CD4 molecules and gp120/gp41, they found that the HIV-1 gp120/gp41 induced membrane fusion was appreciably reduced in the presence of wild-type CD4 or the mutant CD4 molecules described above. Saturation of the CD4 binding site on gp120/gp41 appeared to be the predominant reason for the reduction in cell-cell fusion observed in cells expressing CD4 and gp120/gp41, although partial or complete retention of the CD4:gp120/gp41 complex in the ER further reduced syncytia formation in co-transfected cells (Raja et al., 1993). Therefore, HSV-1 may use a similar mechanism to prevent membrane fusion events which have the potential to interfere with virus egress, or contribute to syncytium formation.

Accordingly, UL53-gK and US6-gD may interact with separate regions of the same cellular "fusion" receptor. Based on this model, the mutations in US6-gD which induce naked capsids to accumulate in the cytoplasm of infected cells may place US6-gD in an active conformation, thereby reducing the need for receptor interactions which promote membrane fusion. To test this premise, cell lines which express high levels of UL53-gK (eg. gK-9 & VS202) could be infected with the gD mutants and examined using electron microscopy. Under normal circumstances enveloped virions are retained in the perinuclear space of gK-9 cells (see Chapters 3 & 5), but one would still expect naked capsids to accumulate in the cytoplasm of gK-9 cells infected with US6-gD mutants, if the mutations

described above alter gD-structure and diminish the requirement for receptor binding prior to membrane fusion. Alternatively, UL53-gK and US6-gD may suppress membrane fusion events by blocking interactions between envelope proteins and distinct "fusion" receptors, given the evidence that HSV-1 binds to a number of different cell surface receptors (Sears et al., 1991; Shieh et al., 1993; Spear, 1993; Subramanian et al., 1994; Brunetti et al., 1994; 1995; Montgomery et al., 1996).

There are however, some potential drawbacks to the theory that syn-1 mutations in UL53-gK free up a cellular receptor, and by doing so, permit cell-cell fusion to occur. For instance, cells infected with F-gKß do not form syncytia, even though UL53-gK is absent from these cells. Nevertheless, there may be an explanation for this apparent contradiction (ie. virus particles may be responsible for cell-cell fusion).

At present, our understanding of the process surrounding syncytium formation is extremely limited. A single fusion event may be sufficient to induce cell-cell fusion between two cells (ie. through expansion of the fusion pore). Conversely, multiple fusion events may be required to induce cell-cell fusion. In addition, we do not know if cell-cell fusion is mediated by HSV-1 membrane proteins in the plasma membrane of infected cells, or by the membrane proteins within the envelopes of HSV-1 heavy (H-) and/or light (L-) particles located on the surface of HSV-infected cells.

In light of the points introduced earlier (see Discussion: section 4.1.1) one might predict that viral glycoproteins in the plasma membrane mediate cell-cell fusion. On the other hand, none of the studies discussed above have ruled out the possibility that noninfectious L-particles (or pre-viral DNA replication enveloped particles [PREPs]) facilitate syncytium formation (Keller, 1976b; Kousoulas et al., 1978; Lee & Spear, 1980; Szilagyi & Cunningham, 1991; Dargan et al., 1995). Since HSV-1 virions produced by

syn-3 (gB) mutants have the capacity to induce cell-cell fusion in the absence of viral protein synthesis, this idea deserves consideration (Falke et al., 1985; Walev et al., 1991a,b). Moreover, L-particles which are defined by the absence of a capsid structure, retain the ability to enter cells and supply functions which promote HSV-1 replication, indicating that they are also fusion competent (McLauchlan et al., 1992).

If HSV-1 H- and/or L-particles must be present at the cell surface for cell-cell fusion to occur, then HSV-infected cells lacking UL53-gK could not be expected to form syncytia since very few, if any, intact virions reached the surface of cells infected with F-gKß (Chapter 5). Several observations are compatible with this theory. First, HSV-1 mutants lacking UL20 produce syncytial plaques only on those cell lines which also support virus egress and the transport of UL20-negative virions to the cell surface (Baines et al., 1991). Additional support for this viewpoint stems from the observation that syn-3 (UL27-gB) mutants form plaques with a syncytial phenotype on cell lines expressing high levels of UL53-gK (eg. VS202 & gK-9 cells), yet the plaque numbers decreased 30-400 fold on these cell lines, and this reduction is equivalent to the decline in plaque numbers produced by wild-type HSV-1 and syn-1 mutants (see Chapter 3). Since HSV-1 egress is obstructed in cell lines which overexpress UL53-gK, but protein synthesis, glycosylation and protein transport appear to be unaffected, it is conceivable that H- and/or L-particles play a role in cell-cell fusion (see Chapter 3, 4, & 5). Finally, independent isolates of F-gKß, the gK-negative virus, exhibit a syncytial phenotype when plated on cell lines which express small quantities of UL53-gK relative to the levels expressed by wild-type HSV-1 in Vero cells (eg. VK243, VK295, and VK308 cells) (see Chapter 5). In contrast, all three viruses produced nonsyncytial plaques when plated on VK302 cells, which produce UL53-gK at levels comparable to those detected in cells infected with wild-type HSV (see

Chapter 5). Since VK243, VK295, and VK308 cell lines support F-gKß replication and produce an abundant number of infectious extracellular virions, but Vero cells lacking gK do not, this data serves to reinforce the idea that HSV-1 H- and/or L-particles play a role in cell-cell fusion. These results may indicate that the small amount of UL53-gK expressed by each of these cell lines is enough to sustain moderate levels of virus egress, yet falls below the levels required to sequester the putative cellular "fusion" receptor.

Taken together, these findings also have important implications regarding the cell-to-cell spread of HSV-1. At least two models have been put forward to explain this phenomena. For instance, enveloped particles transported to the surface of HSV-infected cells, may be responsible for all aspects of cell-to-cell spread (Dingwell et al., 1994). Alternatively, viral glycoproteins situated in the plasma membrane may trigger fusion events between opposing membranes, and create intercellular junctions which contribute to the cell-to-cell spread of HSV-1 virions (Desai et al., 1988; Davis-Poynter et al., 1994). Since egress is also required for plaque formation it seems likely that enveloped virions must be transported to the cell surface before cell-to-cell spread can occur (Baines et al., 1991; Baines & Roizman, 1992; Brown et al., 1994; Chapters 3 & 5). As such, this requirement is compatible with the premise that it is enveloped particles located on the cell surface which mediate cell-to-cell spread, rather than viral glycoproteins situated in the plasma membrane.

In spite of the fact that cell-to-cell spread correlates with virus egress, there is still a chance however unlikely, that protein(s) required for cell-to-cell spread never reach the plasma membranes of cells infected with HSV-1 egress mutants. For example, a reduction in the cell surface transport of HSV glycoproteins (eg. gB, gC, gD) has been observed in cells infected with a variety of HSV syncytial mutants (eg. syn-1:gK, syn-3:gB, UL20)

(Person et al., 1982; Avitabile et al., 1994). It is also possible that viral proteins situated in the plasma membrane create intercellular junctions, but the virus particles assembled by HSV-1 egress mutants contain unidentified defects which prevent any capsids that are transported to adjacent cells from initiating a productive infection. Fortunately, both of these possibilities are amenable to experimental analysis. Techniques including fluorescence-activated cell sorting (FACS) could be used to track the appearance of HSV-1 membrane proteins (eg. gB, gD, gE:gI, gH:gL, gM, UL45) in the plasma membrane of cells infected with HSV-1 egress mutants. In addition, cells infected with HSV-1 egress mutants could be used to seed an uninfected monolayer, and immunoassays or PCR techniques could be employed to detect the spread of noninfectious capsids to adjacent "uninfected" cells.

The theory that point mutations in UL53-gK liberate a cellular "fusion" receptor to produce the syncytial phenotype may have additional limitations. If this mechanism is responsible for the syn-1 phenotype, then cells infected with wild-type HSV-1 might be expected to form syncytia with uninfected cells, boasting a full complement of cell surface receptor proteins. Although low levels of cell-cell fusion can be detected in wild-type infections, this phenomena differs from the syncytial phenotype in several ways. Cell-cell fusion induced by wild-type HSV-1 occurs during a two hour interval (ie. 5-7 hrs post-infection), proceeds at a slower rate, and is much less extensive than the cell-cell fusion observed when cells are infected with syn-1 mutants (Person et al., 1976; Read et al., 1980; Bond et al., 1982). Similar results were obtained in experiments that mixed cells accommodating wild-type HSV and the syn-1 mutant (MP), with uninfected cells (Lee & Spear, 1981). Specifically, co-infected cells exhibit a reduced capacity to recruit uninfected cells into polykaryocytes, compared to cells infected with HSV-1 (MP) alone

(Lee & Spear, 1981). Perhaps the cellular "fusion" receptor must be present on the surface of both cells for cell-cell fusion to occur, if the syn-1 phenotype is actually caused by defects in the ability of UL53-gK to sequester a cellular "fusion" receptor. For example, cellular "fusion" receptors on opposing cells might strengthen cell-cell contacts required for membrane fusion, and delay the cytopathic effects normally observed in wild-type HSV-1 infections (eg. cell rounding). A mechanism of this type might enable syn-1 mutants to induce cell-cell fusion beyond the two hour interval observed in wild-type infections.

Alternatively, the syn-1 mutations in UL53-gK may influence cell-cell fusion through a completely different mechanism. For example, mutations in UL53-gK may alter the structural conformation of a HSV-1 fusion complex, if gK acts as an ion channel, influences post-translational modifications or facilitates another process which has yet to be identified. To explore this possibility, a panel of MAbs specific for discontinous epitopes in HSV-1 fusion proteins (eg. gB, gD, gH:gL), could be used to compare the folding of HSV-1 glycoproteins located in the plasma membranes of cells infected with wild-type HSV-1, with those of a syn-1 (UL53-gK) mutant or gK$^-$ virus.

4.2 Defining the role UL53-gK plays in cell-cell fusion.

A better understanding of the function UL53-gK performs during virus egress is likely to provide important clues about the mechanism by which point mutations in UL53-gK produce the syncytial phenotype. Nevertheless, it will still be necessary to identify the HSV-1 proteins which mediate cell-cell fusion induced by syn-1 mutants, and recreate this phenomena outside the context of an HSV-1 infection, before the syn-1 phenotype can be assigned to a specific process.

The experimental approach used by Person and colleagues (Cai et al., 1988a), and later adopted by Davis-Poynter et al. (1994) in studies designed to catalogue the HSV-1 proteins which mediate cell-cell fusion induced by syn-3 (UL27-gB) mutants, could also be used to identify HSV-1 proteins required for the syn-1 phenotype. To date, UL27-gB is the only HSV-1 protein known to play a fundamental role in the cell-cell fusion induced by syn-1 mutants (Cai et al., 1988a), whereas functions supplied by gD, gE:gI, gH:gL gM and UL45 are necessary for the syn-3 phenotype of UL27-gB mutants (Davis-Poynter et al., 1994; Baanes et al., 1994). Therefore, it is likely that one or more of these proteins (eg. gD, gH:gL), in addition to other HSV-1 proteins synthesized prior to DNA replication, promote cell-cell fusion in cells infected with syn-1 mutants (Keller, 1976b; Kousoulas et al., 1978; Ligas & Johnson, 1988; Desai et al., 1988; Forrester et al., 1992). Of specific interest, is the possibility that viral proteins which have been implicated in the assembly of L- and H-particles are also required for cell-cell fusion, in cells treated with pharmacological agents that block HSV-1 DNA replication (eg. elements of the tegument) (Rixon et al., 1992; Weinheimer et al., 1992; Dargan et al., 1995). Since viral proteins which have been implicated in the assembly of L-particles and H-particles may be required for syncytium formation in cells treated with pharmacological agents that block HSV-1 DNA replication, deletion of these proteins should be a priority (eg. elements of the tegument) (Rixon et al., 1992; Weinheimer et al., 1992; Dargan et al., 1995). If the assembly of L-particles (or pre-viral DNA replication enveloped particles [PREPs]) proves to be a prerequisite of cell-cell fusion, this result would lend credence to the suggestion that it is L-particles situated on the surface of HSV-infected cells which are responsible for syncytium formation.

The gK-negative virus, F-gKß, is an ideal tool for these studies given that plaque

formation is dependent on the presence of UL53-gK. Syn-gK could be used to repair the lethal defect in F-gKß, confer the syncytial phenotype, and serve as an insertional mutagen to knock out genes encoding proteins which have the potential to contribute directly or indirectly to the syn-1 phenotype. Furthermore, the HSV envelope proteins required for cell-cell fusion, but not for penetration or replication in cultured cells (eg. gE:gI, gM, UL45), could be tagged with Golgi retention signals (Machamer, 1993; Spear, 1993; Davis-Poynter et al., 1994; Haanes et al., 1994). This method could be used to determine if any of these proteins must be situated in the plasma membrane to sustain the syn-1 phenotype, given that one or more of them may be involved in cell surface interactions which promote cell-cell fusion. In addition, this experiment may provide insight into the mechanisms controlling cell-to-cell spread and virus egress. Nevertheless, this approach can not establish that non-essential membrane proteins facilitate an intracellular process required for cell-cell fusion (eg. virion assembly), since it can not separate the action of viral proteins in the plasma membrane from those of envelope proteins contained in extracellular virions. However, the discovery that HSV glycoproteins (eg. gB, gC, gD, gE, gG) are targeted to the basolateral domains of polarized epithelial cells might be used to rule out the possibility that extracellular virions induce cell-cell fusion, provided that the release of HSV virions from polarized epithelial cells follows a different pattern (Srinivas et al., 1986). For example, antibodies to gD, gB or gH may block cell fusion and intercellular spread when added to the basolateral side, but not the apical side of polarized epithelial cells (Tashiro et al., 1992). In addition, recombinant glycoproteins tagged with an apical targeting signal may permit virus egress without supporting cell-cell fusion or cell-to-cell spread, if HSV glycoproteins must be present in the plasma membrane to mediate these events (Lisanti et al., 1989; Liang et al., 1993, 1995b; Zurzolo et al., 1993;

Matter & Mellman, 1994).

Several strategies have been used in an attempt to reproduce cell-cell fusion outside the framework of an HSV-1 infection. However, all of the investigations launched to date have been largely unsuccessful. Transfected cells expressing individual glycoproteins (eg. gB, gC, gD, gE) have been examined (Johnson & Smiley, 1985; Ali et al., 1987; Campadelli-Fiume et al., 1988a; Butcher et al., 1990; Spear, 1993). Davis-Poynter et al. (1994) used recombinant vaccinia viruses to co-express different combinations of the HSV-1 glycoproteins gB, gD, gE:gI, gH:gL, gK, and syncytial forms of gB and gK (gB^{ANG}, gK^{syn20} & gK^{MP} mutations) (Debroy et al., 1985; Pogue-Geile et al., 1987; Weise et al., 1987; Balan et al., 1994). In addition, adenovirus (Ad) expression vectors which have been used extensively by the laboratories of Dr. D.C. Johnson and Dr. P.G. Spear, were employed in similar studies (Johnson et al., 1988; Witmer et al., 1990; Zheng et al., 1993; Hanke et al., 1990; Hutchinson et al., 1992a,b, 1993, Hutchinson, Primorac, Graham & Johnson, unpublished results; Spear, 1993; T. Terry-Allison, M.J. Novotny, and P.G. Spear, unpublished results).

In an effort to define the viral proteins which mediate cell-cell fusion, I and other colleagues, constructed adenovirus expression vectors containing the HSV-1 glycoproteins gB, gC, gD, gE:gI, gH:gL, gK (Johnson et al., 1988; Witmer et al., 1990; Zheng et al., 1993; Hanke et al., 1990; Hutchinson et al., 1992a,b, 1993) and syncytial forms of gK and gB (gK^{syn20} & gB^{pK143} mutations) (Debroy et al., 1985; Cai et al., 1988, Hutchinson, Roop, Graham & Johnson, unpublished results). Using protocols which have been described elsewhere, sequences from the Ad5 E3 region were replaced with the open reading frames from each of the HSV glycoproteins, with the exception of the gB^{pK143} gene which supplanted the E1 region of Ad5 (Johnson et al., 1988a; Johnson, 1991; Graham & Prevec,

1991; Bett et al., 1993; Hitt et al., 1994, 1995). Confirmation that HSV glycoproteins were expressed in cells coinfected with combinations of up to eight Ad vectors (eg. gB, gC, gD, gE:gI, gH:gL, syn-gK), was achieved with quantitative immunoprecipitation assays, and the appropriate antibodies (see Figure 6.6). A variety of cell lines (eg. Vero, Hela, R970, Human fibroblasts) were infected with different combinations of the Ad vectors using 2, or 10 pfu per cell for each recombinant. Moreover, we also employed a multiplicity of Ad-infection designed to produce HSV-1 glycoproteins in the proportions observed in cells infected with the HSV-1 syn-1 mutant, Syn20 (see Table 2.6 , Figure 6.6) (Person et al., 1976; Debroy et al., 1985). Ad vector-infected cells were inspected at 12 hr intervals until 96 hr post-infection, but no evidence of cell-cell fusion was detected, an observation in keeping with the results of other laboratories (Spear, 1993; Davis-Poynter et al., 1994; T. Terry-Allison, M.J. Novotny, and P.G. Spear, unpublished results).

These experiments were designed with the aim of removing any impediments which might influence the capacity of HSV-1 glycoproteins to induce cell-cell fusion. For example, the composition and the abundance of viral glycoproteins in the plasma membrane could be a critical determinant of cell-cell fusion. This possibility was addressed by adjusting the MOI of each Ad vector to mimic the expression characteristics of HSV-1 (syn20), according to projections based on the level of HSV-1 glycoproteins synthesized 24 hrs after Vero cells had been infected with a mixture of eight Ad vectors at an MOI of 10 pfu/cell for each recombinant. Furthermore, the cytopathic effects caused by viral expression vectors are apt to influence the ability of HSV-1 glycoproteins to induce cell-cell fusion. In this respect adenovirus vectors have several advantages over vaccinia virus recombinants. Ad vectors have a much longer replication cycle reflecting the moderate cytotoxicity of this system, and unlike vaccinia recombinants, contain very

Table 2.6 Expression of HSV-1 glycoproteins by different Ad vector combinations

HSV-1 glycoprotein[b]	Advector cocktails[a] B	C	D	E	I	H:L	K	K^{syn}	B^{syn}
1[c]	x	x	x	x	x	x	x		
2	x	x	x	x		x	x		
3	x	x	x			x	x		
4	x		x			x	x		
5	x	x	x			x			
6	x		x			x			
7	x					x			
8		x				x			
9			x			x			
10						x	x		
11				x		x			
12			x			x	x		
13	x					x	x		
14		x				x	x		
15						x			
16	x		x						
17	x						x		
18			x				x		
19	x		x				x		
20	x	x	x						
21		x	x			x			
22	x	x	x	x	x	x			
23	x	x				x			
24		x		x		x			
25	x	x	x	x	x	x	x		
26	x		x			x			
27	x		x			x	x		
28	x		x				x		
29			x			x			
30	x		x						
31	x						x		
32	x	x	x	x	x	x		x	
33	x		x	x	x	x		x	
33									x

[a] Cell lines were infected with Ad vector combinations and following a 2 hr adsorption, the virus inoculum was removed and monolayers were overlaid with α-MEM containing 4% FCS, monitored up to 5 days and stained with crystal violet.

[b] DNA sequences encoding HSV-1 glycoproteins were inserted into the E3 region of Ad5 except gBsyn which is situated in the E1 region of Ad5.

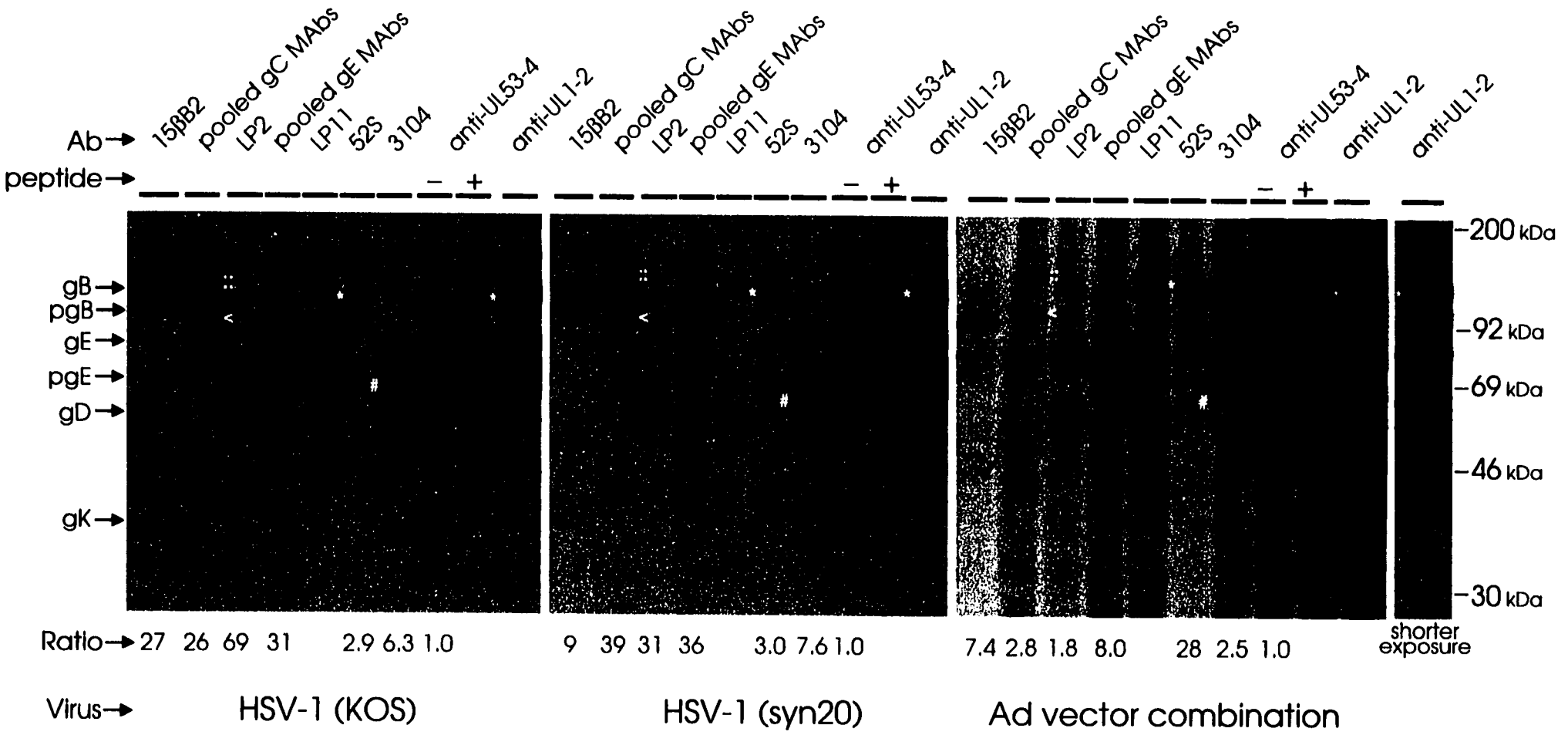
[c] In samples 1 to 24 Vero cells were infected with combinations of Ad vectors at 1, 4, or 20 pfu/cell for each recombinant.

In samples 25 to 31, Fibroblasts, R970, or Hela cells were infected with Ad vector combinations at 2 or 10 pfu/cell for each recombinant or a multiplicity of infection (MOI) designed to produce HSV-1 glycoproteins in quantities similar to the amounts observed in HSV-infected cells (see Figure 6.6)

In samples 32 and 33, Vero, R970, and 293 cells were infected with combinations of Ad vectors using MOIs of 10 or 30 pfu/cell.

In sample 33, Vero, R970, and 293 cells were infected with $AdgB^{syn}$ using an MOI of 30, 100, or 300 pfu/cell.

Figure 6.6 Quantification of HSV-1 glycoproteins B, C, D, E, H, I, and K expressed by Ad vectors, wild-type HSV-1 (KOS) or the gK^{syn} mutant HSV-1 (syn20). Vero cells were infected with a combination of AdgB, AdgC, AdgD, AdgE, AdgH, AdgI, AdgK & AdgL at 20 PFU/cell or infected with HSV-1 strains (KOS or syn20) using 5 PFU/cell. Ad vector-infected cells were incubated with labelling medium (Hutchinson et al., 1993) containing 125 µCi/ml of both [^{35}S]methionine and [^{35}S]cysteine from 19 to 24 hr post-infection and the HSV-infected cells were labelled [^{35}S]Met-[^{35}S]Cys from 2 to 7 hr post-infection. Note that ≈100% of syn20-infected cells had fused 7 hr after infection. Cell extracts were made and prepared as previously described (Hutchinson et al., 1992a). Extracts derived from 4.9, 9.8 or 19.6 x 10^4 cells were mixed with MAb 15ßB2, specific for gB, MAb LP2, specific for gD, pooled anti-gC MAbs C1, C2, and C3, pooled anti-gE MAbs, II481, 3114, and 7520, MAb LP11, specific for gH, MAb 52S, specific for gH, MAb 3104, specific for gI, anti-UL53-4 peptide sera, specific for gK, or rabbit anti-UL1-2 peptide sera, specific for gL (for description of Abs see Johnson et al., 1988b, Hutchinson et al., 1992, 1993). In addition, the anti-UL53-4 serum was preincubated with (+) or without (-) UL53-4 peptide. Antigen-antibody complexes were precipitated with protein-A sepharose using the methodology outlined in Hutchinson et al. (1993), eluted at 37°C for 30 min and separated on 12% SDS-polyacrylamide gels as described (Johnson & Feenstra, 1987). The relative levels of glycoproteins expressed in cells were quantified by densitometry (ratio) according to published protocols (Hutchinson et al., 1993). In addition, a direct comparison of the quantity of gH expressed by Ad vectors, KOS and syn20 produced a KOS:syn20:Ad ratio of 4.3 :1 : 3.7. The HSV-1 glycoproteins pgB, gB, pgC (<), gC (::), gD, pgE, gE, gH (*), gI (#), gK and molecular mass markers of 200, 97, 69, 46, and 30 kDa are indicated.


Ab→
15βB2
pooled gC MAbs
LP2
pooled gE MAbs
LP11
52S
3104
anti-UL53-4
anti-UL1-2
peptide→
−
+
gB→
pgB→
gE→
pgE→
gD→
gK→
200 kDa
92 kDa
69 kDa
46 kDa
30 kDa
Ratio→ 27 26 69 31 2.9 6.3 1.0
9 39 31 36 3.0 7.6 1.0
7.4 2.8 1.8 8.0 28 2.5 1.0
shorter exposure
Virus→
HSV-1 (KOS)
HSV-1 (syn20)
Ad vector combination

few endogenous membrane proteins, which have the potential to complicate the interpretation of these experiments. In addition, Ad vectors produce infectious particles which are nonenveloped, reducing the likelihood that the host cell exocytic pathway will be disrupted, and therefore glycoprotein transport to the plasma membrane should be relatively normal.

In spite of these precautions, the Ad vectors failed to produce syncytia indicating that additional HSV-1 proteins may be required for cell-cell fusion (eg. UL10-gM, UL45 or tegument proteins such as UL25, UL48-VP16 & UL49) (Addison et al., 1984; Weinheimer et al., 1992; Davis-Poynter et al., 1994, Haanes et al., 1994; Elliot et al., 1995; Ali et al., 1996). These proteins have not been included in coexpression experiments because the appropriate expression vectors are not yet available. The recent improvements in vector technology should be considered when creating additional expression vectors, since the new generation of E1$^-$ Ad vectors have a cytopathic impact which is minimal to nonexistent (Bett et al., 1993; Hitt et al., 1995). It is conceivable that the cytopathic effects of adenovirus replication inhibited syncytium formation in coexpression experiments which used the E3$^-$ Ad vectors described above. With this in mind, members of the Johnson laboratory including myself have re-constructed E1$^-$ Ad vectors containing the HSV-1 glycoproteins gB^{pK143}, gD, gE, gH, gI and gL and when other vectors (eg. syn-gK, gM, UL45, UL25, UL48-VP16, & UL49) become available it may be possible to produce syncytia using E1$^-$ Ad recombinants (C. Brunetti, K. Dingwell, L. Hutchinson, F.L. Grahmam & D.C. Johnson, unpublished results).

Although our understanding of HSV-induced cell-cell fusion is far from complete, some avenues for future research are suggested by data presented in this thesis. Among the glycoproteins retained by Alphaherpesvirinae, the level of conservation displayed by

UL53-gK homologs is second only to UL27-gB (McGeoch et al., 1988; Klupp & Mettenleiter, 1991; Telford et al., 1992; Spear, 1993; Gompels et al., 1995). In contrast, UL10-gM is the most highly conserved glycoprotein among members of the Beta- and Gammaherpesvirinae, and these herpesviruses lack a UL53-gK homolog altogether (Gompels, 1995; Telford, 1995; Nicolas, 1996). Since members of the Alpha-, Beta-, and Gammaherpesvirinae tend to reside within different tissues in the infected host (eg. α = epidermo-neurotropism vs. τ = lymphotropic), deletion of UL53-gK may produce a defect in virus egress which is tissue specific, and analogous to the host range effects caused by deleting the UL20 or gamma1-34.5 genes (Baines et al., 1991, Browne et al., 1994).

Interestingly, syn-1 mutants induce cell-cell fusion on some cell lines but not on others, and this phenotype may indicate that host cell functions have the ability to compensate for defects in UL53-gK (Lee & Spear, 1980; Bzik & Person, 1981). Therefore, F-gKß (a gK^- mutant) should be plated on a wide variety of cell types (eg. neuronal, epithelial cells) to determine if UL53-gK is a universal requirement of virus egress and cell-to-cell spread. Moreover, this methodology can also be used to explore the possibility that UL53-gK, like UL20, is a regulatory protein which is <u>not required</u> for cell-cell fusion. For instance, gK^- strains, like the $UL20^-$ mutant, may form syncytial plaques on cell lines which support F-gKß replication, and permit enveloped virions to reach the cell surface (Baines et al., 1991). As an adjunct to these experiments, syncytial mutations (eg. syn-3:UL27-gB) could be introduced to F-gKß, in order to clarify whether UL53-gK is required for syncytia formation in cells which do not support F-gKß replication.

Another avenue of research is suggested by the observation that many of the HSV-1 proteins (eg. US6-gD, UL22-gH, UL53-gK, UL20) which govern cell-cell fusion can influence virus egress (Debroy et al., 1985; Desai et al., 1988; Campadelli-Fiume et al.,

1991; Baines et al., 1991; Hutchinson et al., 1995). This raises the possibility that other HSV-1 proteins (eg. syn-5:UL24, UL10-gM, UL45) involved in cell-cell fusion play a role in HSV egress or virion assembly, a prospect which has not been investigated to date (Jacobson et al., 1989b; Baanes et al., 1994; Davis-Poynter et al., 1994). Furthermore, the small plaque phenotype displayed by UL24$^-$, UL10$^-$, & UL45$^-$ mutants is compatible with this hypothesis (Jacobson et al., 1989b; Baines & Roizman, 1991; 1993; MacLean et al., 1993; Visalli & Brandt, 1991, 1993).

In addition, many of the HSV-1 membrane proteins required for the syn-3 (UL27-gB) phenotype feature cytoplasmic domains which are greater than 100 residues in length (eg. gB, gE, gI, gM & UL45 but not gD & gH:gL), whereas other HSV-1 membrane proteins have small cytoplasmic tails less than 30 residues in length (Davis-Poynter et al., 1994; Baanes et al., 1994; Spear, 1993). Although UL27-gB is the only one of these proteins (ie. gE: gI, gM, UL45) which is essential for HSV-1 replication in tissue cultured cells, the cytoplasmic domains of nonessential membrane proteins may enhance virion assembly by interacting with elements of the virion tegument (Spear, 1993). This hypothesis could be tested by examining the protein composition and specific infectivity of extracellular virions produced by HSV deletion mutants, since this information is not yet available (Spear, 1993; Baines & Roizman, 1993; Visalli & Brandt, 1993; Balan et al., 1994; Dingwell et al., 1994).

There is evidence to suggest that gB homologs of Alphaherpesvirinae (eg. PRV), and the Gammaherpesvirinae (eg. EBV, MHV) participate in the production or egress of infectious virions (Peeters et al., 1992a; Stewart et al., 1994; Herrold et al., 1996). At present, there has been no indication that HSV-1 UL27-gB (syn-3 locus) contributes to the assembly, envelopment, or release of HSV-1 virions (Cai et al., 1987; Desai et al., 1994).

Extracellular virions are produced by UL27-gB deletion mutants and appear to contain a full complement of HSV-1 capsid, tegument and envelope proteins (Desai et al., 1994). However, careful studies quantifying the release of gB^- virions (or for that matter gD^-, gH^-, or gL^- virions) from HSV-infected cells have not been published. For example, no one has used electron microscopy, western blot analysis or radiolabelling techniques to compare the number of extracellular virions produced by cells infected with gB^- mutants and wild-type HSV-1. Since HSV-1 gB is absolutely required for HSV-1 entry, gB participation in other facets of HSV-1 replication may have escaped detection (eg. tegument disassembly) (Sarmiento et al., 1979; Little & Schaffer, 1981; Cai et al., 1987, 1988). To test this hypothesis, the UL27-gB entry function could be replaced with a protein chimera composed of two elements: (1) a functional ectodomain derived from a viral fusion protein which functions at neutral pH (eg. HIV gP160), and (2) the transmembrane and cytoplasmic tail of an HSV-1 membrane protein (eg. gB & gD). This technique could also be used to determine if other HSV-1 proteins with essential roles in HSV-1 entry (eg. gD, gH:gL), are involved in other facets of HSV-1 replication, since many viral fusion proteins (eg. HIV-1 gp160) act as receptor binding proteins in addition to facilitating membrane fusion.

5.0 Conclusions.

In conclusion I have identified the UL53-gene product, gK, and established that this protein exists as a single 40-kDa species containing high mannose, unprocessed N-linked oligosaccharides. These studies also revealed that gK is <u>not</u> expressed on the surface of infected cells, and suggested that gK is maintained in ER membranes by retention signals. These features are unique to gK, since the other HSV glycoproteins

reach the cell surface, and appear as two forms differing in N-linked and O-linked oligosaccharides. Glycoprotein K displayed the ability to form oligomeric structures, and because oligomerization has been implicated in retention, it is conceivable that this process mediates gK ER-retention. In addition, the tyrosine-based motif (YTK[FILM]) was observed in all of the gK homologs described to date, and it is possible that this sequence acts as an ER retention signal.

I have also demonstrated that gK is essential for HSV-1 replication and that gK plays a crucial role in virus egress. F-gKß, an HSV-1 mutant is unable to express gK, formed microscopic plaques (3-6 infected cells) on gK$^-$ cell lines and these plaques were reduced by 10^2 to 10^6 in number. In virus infections lacking gK, large quantities of unenveloped capsids accumulated in the cytoplasm and virus particles did not reach the cell surface. The few enveloped particles that were assembled in gK$^-$ cells exhibited a reduced capacity to enter cells and initiate an infection of complementing gK$^-$ cells. In addition, overexpression of gK in HSV-infected cells also caused defects in virus egress, although particles accumulated in the perinuclear space and large multilamellar membranous structures juxtaposed with the nuclear envelope were frequently observed.

Other HSV-1 egress mutants have been described (eg. UL11, UL20, US6-gD, ICP34.5), and some of these mutants possess a phenotype reminiscent of F-gKß (eg. UL11, US6-gD mutants). However, HSV mutants lacking these proteins display a marginal to moderate decrease in the production of infectious virions (eg. 3 to 50 fold), and the conditions which block plaque formation are confined to specific cell types (ie. host range defect) (Baines et al., 1991; Baines & Roizman, 1992; MacLean et al., 1992; Brown et al., 1994). In contrast, gK was required for HSV-1 replication in all cell types tested to date, suggesting that gK may play a fundamental role in virus egress which

cannot be replaced by a host cell function. This hypothesis is also consistent with the observation that gK homologs constitute the second most highly conserved group of alphaherpesvirus glycoproteins. Since gK homologs are restricted to the Alpha-herpesvirinae the egress functions supplied by gK may contribute to the epidermo-neurotropism displayed by members of this subfamily.

Small quantities of gK were expressed in HSV-infected cells compared to other HSV-1 glycoproteins with a direct role in cell-cell fusion, and the syncytial phenotype produced by mutations in gK was recessive to the actions of a wild-type protein. Based on these findings, and the observation that gK is <u>not</u> transported to the plasma membrane or incorporated into the virion envelope, I have proposed that gK is a regulatory protein which influences membrane fusion through an indirect mechanism. In the absence of gK expression, defects in virus egress were observed and large numbers of nonenveloped capsids accumulated in the cytoplasm of HSV-infected cells. This result raised the possibility that syn mutations in gK induce cell-cell fusion by altering the cell surface transport, or protein composition of HSV-1 virions. Furthermore, transport defects involving gK^{syn} could also produce an imbalance in the subcellular distribution of the viral components (eg. "fusion complex" or "fusion inhibitor") and/or cellular proteins (eg. "fusion receptor") which govern the fusion process. By this model, fusion might be induced by a decrease in the abundance of fusion inhibitors at the cell surface, or alternatively by an increase in the concentration of fusion factors within the plasma membrane.

In summary, this work represents an important advance in the fields of HSV-induced cell-cell fusion and virus egress. Moreover, these studies should provide a foundation for future discoveries in both of these areas. In addition, gK^{-} viruses could

provide the basis for a rationally attenuated HSV-1 vaccine, since HSV-1 requires gK for multi-cycle growth and gK^- viruses have the ability to complete at least one replication cycle in noncomplementing cells. For instance, prophylactic vaccination of animals with a gH^- mutant (also a single cycle virus), granted animals with a high degree of protection against subsequent high dose challenges with wild-type virus (Farrell et al., 1994; McLean et al., 1994). However, gK^- viruses may have an advantage over the other single cycle mutants (eg. gB^-, gD^-, gH^-), since the missing proteins are major constituents of the virion (Handler et al., 1996) and induce a protective immune response in animal models (McDermott et al., 1989; Forrester et al., 1991; Browne et al., 1993; Ghiasi et al., 1995), whereas the protection provided by gK immunization is marginal (Ghiasi et al., 1994).

REFERENCES

Ace, C.I., M.A. Dalrymples, F.H. Ramsay, V.G. Preston, and C.M. Preston. 1988. Mutational analysis of the herpes simplex virus type 1 trans-inducing factor, Vmw65. J. Gen. Virol. 69:2595-2605.

Ace, C.I., T.A. McKee, J.M. Ryan, J.M. Cameron and C.M. Preston. 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J. Virol. 63:2260-2269.

Adams, G.A., and J.K. Rose. 1985. Strucutral requirements of a membrane-spanning domain for protein anchoring and cell surface transport. Cell. 41:1007-1015.

Addison, C., F.J. Rixon, J.W. Palfreyman, M. OHara, and V.G. Preston. 1984. Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology 138:246-259.

Addison, C., F.J. Rixon, and V.G. Preston. 1990. Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. 71:2377-2384.

Ackermann, M., J. Chou, M. Sarmiento, R.A. Lerner, and B. Roizman. 1986. Identification by antibody to a synthetic peptide of a protein specified by a diploid gene located in the terminal repeats of the L component of herpes simplex virus. J. Virol. 58:843-850.

Ackermann, M., R. Longnecker, B. Roizman, and L. Pereira. 1986. Identification, properties, and gene location of a novel glycoprotein specified by herpes simplex virus 1. Virology 50:207-220.

Adlish, J.D., R.S. Lahijani, and St. Jeor, S.C. 1990. Identification of a putative cell receptor for human cytomegalovirus. Virology. 176:337-345.

Albrecht, J.-C., J. Nicholas, D. Biller, K.R. Cameron, B. Biesinger, C. Newman, S. Wittmann, M.A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess. 1992. Primary structure of the herpesvirus saimiri genome.
J. Virol. 66:5047-5048.

Albright, A.G., and F.J. Jenkins. 1993. The herpes simplex virus UL37 protein is phosphorylated in infected cells. J. Virol. 67:4842-4847.

Ali, M.A., M. Butcher, and H.P. Ghosh. 1987. Expression and nuclear envelope localization of biologically active fusion glycoprotein gB of herpes simplex virus in mammalian cells using cloned DNA. Proc. Natl. Acad. Sci. USA. 84:5675-5679.

Ali, M.A., B. Forghani, and E.M. Cantin. 1996. Characterization of an essential HSV-1 protein encoded by the UL25 gene reported to be involved in virus penetration and capsid assembly. Virology. 216:278-283.

Al-Kobaisi, M.F., F.J. Rixon, I. McDougall, and V.G. Preston. 1991. The herpes simplex virus UL33 gene product is required for the assembly of full capsids. Virology. 180:380-388.

Andrews, D. 1989. Examining protein translocation in cell-free systems and microinjected *Xenopus* oocytes. Biotechniques 7:960-967.

Arsenakis, M., L. Foa-Tomasi, V. Speziali, B. Roizman, and G. Campadelli-Fiume. 1986. Expression and regulation of glycoprotein C gene of herpes simplex virus 1 resident in a clonal L cell line. J. Virol. 58:367-376.

Arvin, A.M. 1996. Varicella-Zoster virus. p. 2547-2586. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Avitabile, E., S. Di Gaeta, M. R. Torrisi, P.W. Ward, B. Roizman, and B. Campadelli-Fiume. 1995. Redistribution of microtubules and golgi apparatus in herpes simplex virus-infected cells and their role in viral exocytosis. J. Virol. 69:7472-7482.

Avitabile, E., P.L. Ward, C. Di Lassaro, M.R. Torrisi, B. Roizman and G. Campadelli-Fiume. 1994. The herpes simplex virus UL20 protein compensates for the differential disruption of exocytosis of virions and viral membrane glycoproteins associated with fragmentation of the golgi apparatus. J. Virol. 68:7397-7405.

Babic, N., T.C. Mettenleiter, A. Flamand, and G. Ugolini. 1993. Role of essential glycoproteins gII and gp50 in transneruonal transfer of pseudorabies virus from the hypoglossal nerves of mice. J. Virol. 67:4421-4426.

Baer, R., A.T. Bankier, M.D. Biggin, P.L. Deninger, P.J. Farrell, T.J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffnell, and B.G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) 310:207-211.

Baghian, A., M.A. Dietrich and K.G. Kousoulas. 1992. Cell fusion caused by herpes simplex virus-1 (HSV-1) strains tsB5 and MP is inhibited at pH 6.7 and pH 7.0. Arch. Virol. 122:119-131.

Baghian, A., L. Huang, S. Newman, S. Jayachandra, and K.G. Kousoulas. 1993. Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant amb1511-7 causes extensive cell fusion. 67:2396-2401.

Baines, J.D., R.J. Jacob, L. Simmerman, and B. Roizman. 1995. The herpes simplex

viurs 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. J. Virol. 69:825-833.

Baines, J.D., A.P. Poon, J. Rovnak and B. Roizman. 1994. The herpes simplex virus 1 UL15 gene encodes two protein and is reuqired for cleavage of genomic viral DNA. J. Virol. 68:8118-8124. Jim says that this protein is probably the endonuclease that cleaves replicated DNA into genome unit lengths.

Baines, J.D., and B. Roizman. 1991. The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. 65: 938-944.

Baines, J.D., and B. Roizman. 1993. The UL10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J. Virol. 67:1441-1452.

Baines, J.D., and B. Roizman. 1992. The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J. Virol. 66:5168-5174.

Baines, J.D., P.L. Ward, G. Campadelli-Fiume and B. Roizman. 1991. The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J. Virol. 65:6414-6424.

Balachandran, N. and L.M. Hutt-Fletcher. 1985. Synthesis and processing of glycoprotein gG of herpes simplex virus type 2. J. Virol. 54:825-832.

Balan, P., N. Davis-Poynter, S. Bell, H. Atkinson, H. Browne, and T. Minson. 1994. An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI, or the putative gJ. J. Gen. Virol. 75:1245-1258.

Baldwin, B.R., M. Kleinberg and S. Keay. 1996. Molecular cloning and expression of receptor peptides that block human cytomegalovirus/cell fusion. Biochem. Biophys. Res. Commun. 219:668-673.

Banfield, B.W., Y. Leduc, L. Esford, K. Schubert, and F. Tufaro. 1995b. Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: Evidence for a proteoglycan-independent virus entry pathway. J. Virol. 69:3290-3298.

Banfield, B.W., Y. Leduc, L. Esford, R.J. Visalli, C.R. Brandt, and F. Tufaro. 1995a. Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. Virology. 208:531-539.

Banfield, B.W., and F. Tufaro. 1990. Herpes simplex virus particles are unable to transverse the secretory pathway in the mouse L-cell mutant gro29. J. Virol. 64:5716-

5729.

Baradaran, K., C.E. Dabrowski and P.A. Schaffer. 1994. Transcriptional analysis of the region of the herpes simplex virus type 1 genome containing the UL8, UL9, and UL10 genes and identification of a novel delayed-early gene product, OBPC. J. Virol. 68:4251-4261.

Baranowski, E., J. Dubuisson, P.-P. Pastoret, and E. Thirty. 1993. Idenfification of 198K, 93K, and 42K glycoproteins of bovine herpesvirus-1 by monoclonal antibodies. Arch. Virol. 133:97-111.

Baranowski, E., J. Dubuisson, S. van Drunen Littel-van den Hurk, L.A. Babiuk, A. Michel, P.-P. Pastoret, and E. Thirty. 1995. Synthesis and processing of bovine herpesvirus-1 glycoprotein H. Virology. 206:651-654.

Barker, D.E., and B. Roizman. 1990. Identification of three genes nonessential for growth in cell culture near the right terminus of the unique sequences of long component of herpes simplex virus 1. Virology. 177:684-691.

Barker , D.E., and B. Roizman. 1992. The unique sequence of the herpes simplex virus 1 L component contains an additional open reading frame designated UL49.5 J. Virol. 66:562-566.

Barnett, B.C., A. Dolan, E.A. Telford, A.J. Davison, and D.J. McGeoch. 1992. A novel herpes simplex virus gene (UL49a) encodes a putative membrane protein with counterparts in other herpesviruses. J. Gen. Virol. 73:2167-2171.

Basu, S., G. Dubin, M. Basu, V. Nguyen, and H.M. Friedman. 1995. Characterization of regions of herpes simplex virus type 1 glycoprotein E involved in binding the Fc domain of monomeric IgG and in forming a complex with glycoprotein I.

Batterson, W., and B. Roizman. 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J. Virol. 46:371-377.

Batterson, W., D. Furlong, and B. Roizman. 1983. Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. 45:397-407.

Baumeister, J., B. G. Klupp, and T.C. Mettenleiter. 1995. Pseudorabies virus and equine herpesvirus 1 share a nonessential gene which is absent in other herpesviruses and located adjacent to a highly conserved gene cluster. J. Virol. 69:5560-5567.

Becker, Y. 1995. HSV-1 brain infection by the olfactory nerve route and virus latency and reactivation may cause learning and behavioral deficiencies and violence in children and adults: a point of view. Virus Genes. 10:217-226.

Bell, S., M. Cranage, L. Borysiewicz, and T. Minson. 1990. Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoprotein E and I of herps simplex virus type 1. J. Virol. 64:2181.

Ben-Porat, T., A.S. Kaplan, B. Stehn, and A.S. Rubenstein. 1976. Concatermeric forms of intracellular herpesvirus DNA. Virology. 69:547-560.

Bett, A.J., W. Haddara, L. Prevec, and F.L. Graham. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. USA. 91:8802-8806.

Bett, A.J., L. Prevec, and F.L. Graham. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. J. Virol. 67:5911-5921.

Blacklaws, B.A., S. Krishna, A.C. Minson, and A.A. Nash. 1990. Immunogenicity of herpes simplex virus type 1 glycoproteins expressed in vaccinia virus recombinants. Virology 177:727-736.

Blewett, E. and V. Misra. 1991. Cleavage of the bovine herpesvirus glycoprotein B is not essential for its function. J. Gen. Virol. 72:2083-2090.

Boehmer, P.E., M.C. Craigie, N.D. Stow and I.R. Lehman. 1994. Association of origin binding protein and single strand DNA-binding protein, ICP8 during herpes simplex virus type 1 DNA replication *in vivo*. J. Biol. Chem. 269:29329-29334.

Boehmer, P.E., M.S. Dodson and I.R. Lehman. 1993. The herpes simplex virus type-1 origin binding protein. DNA helicase activity. J. Biol. Chem. 268:1220-1225.
Boehmer, P.E. and I.R. Lehman. 1993a. Herpes simplex virus type 1 ICP8: helix-destabilizing properties. J. Virol. 67:711-715.

Boehmer, P.E., and I.R. Lehman. 1993b. Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8. Proc. Natl. Acad. Sci. USA. 90:8444-8448.

Bond V.C., S. Person and S.C. Warner. 1982. The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. J. Gen. Virol. 61:245-254.

Bond, V.C., and S. Person. 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology 132:368-376.

Booy, F.P., B.L. Trus, A.J. Davison and A.C. Steven. 1996. The capsid architecture of channel catfish virus, an evolutionarily distant herpesvirus, is largely conserved in the absence of discernable sequence homolgy with herpes simplex virus. Virology. 215:134-141.

Booy, F.P., B.L. Trus, W.W. Newcomb, J.C. Brown, J.F. Conway and A.C. Steven. 1994.

Finding a needle in a haystack: detection of a small protein (the 12 kDa VP26) in a large complex (the 200 MDa capsid of herpes simplex virus). Proc. Natl. Acad. Sci. USA 91:5652-5656.

Borchers, K., M. Ozel, G. Pauli, H.R. Gelderbolm, and H. Ludwig. 1990. Conserved epitopes of simianvirus SA 8 and bovine herpesvirus type 2. Arch. Virol. 111:1-14.

Bos, K., C. Wraight, and K.K. Stanley. 1993. TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. EMBO J. 12:2219-2228.

Bosch, V., and M. Pawlita. 1990. Mutational analysis of the human immunodeficiency virus type 1 env gene product proteolytic cleavage site. J. Virol. 64:2337-2344.

Brandimarti, R., T. Huang, B. Roizman, and G. Campadelli-Fiume. 1994. Mapping of herpes simplex virus 1 genes with mutations which overcome host restrictions to infection. Proc. Natl. Acad. Sci. USA. 91:5406-5410.

Brandli, A.W. 1991. Mammalian glycosylation mutants as tools for the analysis and reconstitution of protein transport. Biochem. J. 276:1-12.

Brands, R. M.D. Snider, Y. Hino, S.S. Park, H.V. Gelboin, and J.E. Rothman. 1985. Retention of membrane proteins by the endoplasmic reticulum. J. Cell Biol. 101:1724-1732.

Bretscher, M.S., and S. Munro. 1993. Cholesteraol and the Golgi apparatus. Science. 261:1280-1281.

Britt, W.J., and C.A. Alford. 1996. Cytomegalovirus. p. 2493-2523. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Britt, W.J., and L.G. Vugler. 1989. Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J. Virol. 63:403-410.

Britt, W.J., and L.G. Vulger. 1992. Oligomerization of human cytomegalovirus major envelope glycoprotein complex gB (gp55-116). J. Virol. 66:6747-6754.

Brown, C.R., M.S. Nakamura, J.D. Mosca, G.S. Hayward, S.E. Straus, and L.P. Perera. 1995. Herpes simplex virus trans-regulatory protein ICP27 stabilizes and binds to 3' ends of labile mRNA. J. Virol. 69:7187-7195.

Brown, S.M. J. Harland, A.R. MacLean, J. Podlech, and J.B. Clements. 1994. Cell type and cell state determine differentiatial *in vitro* growth and nonveurovirulent phenotype of ICP34.5-negative herpes simplex virus types 1 and 2. J. Gen. Virol. 75:2367-2377.

Brown, S.M., A.R. MacLean, J.D. Aitken and J. Harland. 1994. ICP34.5 infuences herpes simplex virus type 1 maturation and egress from infected cells *in vitro*. J. Gen. Virol. 75:3679-3686.

Brown, S.M., D.A. Ritchie, and J.H. Subak-Sharpe. 1973. Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. 18:329-346.

Browne, H., V. Baxter, and T. Minson. 1993. Analysis of protective immune responses to the glycoptorein H-glycoprotein L complex of herpes simplex virus type 1. J. Gen. Virol. 74:2813-2817.

Browne, H., S. Bell, T. Minson, and D.W. Wilson. 1996. An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. J. Virol. 70:4311-4316.

Brucher, K.H., W. Garten, H.D. Klenk, E. Shaw, and K. Radsak. 1990. Inhibition of endoproteolytic cleavage of cytomegalovirus (HCMV) glycoprotein B by palmitoyl-peptidyl-chloromethyl ketone. Virology. 178:617-620.

Bruckner, R.C., J.J. Crute, M.S. Dodson, and I.R. Lehman. 1991. The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. 266:2669-2674.

Brunetti, C.R., R.L. Burke, S. Kornfeld, W. Gregory, F.R. Masiarz, K.S. Dingwell, and D.C. Johnson. 1994. Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. J. Biol. Chem. 269:17067-17074.

Brunetti, C.R., R.L. Burke, B. Hoffack, T. Ludwig, K. Dingwell, and D.C. Johnson. 1995. Role of mannose 6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. J. Virol. 69:3517-3528.

Bruss, V., and D. Ganem. 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Natl. Acad. Sci. USA. 88:1059-1063.

Bruss, V., and K. Vieluf. 1995. Functions of the internal pre-S domain of the large surface protein in hepatitis B virus particle morphogenesis. J. Virol. 69:6652-6657.

Buckmaster, E.A., U. Gompels and A.C. Minson. 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately 115 x 103 molecular weight. Virology 139:408-413.

Buckmaster, E.A., M.P. Cranage, C.S. McLean, R.R.A. Coombs, and A.C. Minson. 1984. the use of monoclonal antibodies to differentiate isolates of herpes simplex types 1 and 2 by neutralization and reverse passive haemagglutination tests. J. Med. Virol. 13:193-202.

Bush, M., D.R. Yager, M. Gao, K. Weisshart, A.I. Marcy, D.M. Coen, and D.M. Knipe. 1991. Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA binding protein. J. Virol. 65:1082-1089.

Butcher, M., K. Raviprakash, and H.P. Ghosh. 1990. Acid pH-induced fusion of cells by herpes simplex virus glycoproteins gB and gD. J. Biol. Chem. 265:5862-5868.

Byrne, K.M., D. Horohov, and K. Kousoulas. 1995. Glycoprotein B of bovine herpesvirus-1 binds heparin. Virology. 209:230-235.

Bzik, D.J., and S. Person. 1981. Dependence of herpes simplex virus type 1-induced cell fusion on cell type. Virology 110:35-42.

Bzik, D.J., B.A. Fox, N.A. Deluca, and S. Person. 1984a. Nucleotide sequence specifying the glycoprotein gB of herpes simplex virus type 1. Virology 133:301-314.

Bzik, D.J., B.A. Fox, N.A. Deluca, and S. Person. 1984b. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: Mutations affecting rate of virus entry and cell fusion. Virology 137:185-190.

Cai, W., B. Gu, and S. Person. 1988a. Role of glycoprotein B of herpes simplex virus type 1 in entry and cell fusion. J. Virol. 62:2596-2604.

Cai, W., S. Person, C. DeBroy and B. Gu. 1988b. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. J. Mol. Biol. 201:575-588.

Cai, W., S. Person, S.C. Warner, J. Zhou, and N.A. DeLuca. 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. 61:714-721.

Cai, W., and P.A. Schaffer. 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viarl DNA. J. Virol. 63:4579-4589.

Cai, W., and P.A. Schaffer. 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol. 66:2904-2915.

Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman. 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation for the virus. J. Virol. 62:159-167.

Campadelli-Fiume, G., E. Avitabile, S. Fini, D. Stirpe, M. Arsenakis, and B. Roizman. 1988a. Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH-independent fusion in a cell line that constitutively expresses the glycoprotein. Virology. 166:598-602.

Campadelli, G., R. Brandimarti, C. Di Lazzaro, P.L. Warc, B. Roizman, and M.R. Torrisi. 1993. Fragmentation and dispersal of golgi proteins and redistribution of glycoproteins and glycoplipids processed through the golgi apparatus after infection with herpes simplex virus 1. Proc. Natl. Acad. Sci. USA. 90:2798-2802.

Campadelli-Fiume, G., F. Farabegoli, S. Di Gaeta and B. Roizman. 1991. Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. 65:1589-1595.

Campadelli-Fiume, G., L. Poletti, F. Dall'Olio, and F. Serafini-Cessi. 1982. Infectivity and glycoprotein processing of herpes simplex virus type 1 grown in a ricin-resistant cell line deficient in N-acetylglucosaminyl transferase I. J. Virol. 43: 1061-1071.

Campadelli-Fiume, G., S. Qi, E. Avitable, L. Foa-Tomasi, R. Brandimarte, and B. Roizman. 1990a. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. 64: 6070-6079.

Campadelli-Fiume, G., D. Stripe, A. Boscaro, E. Avitabile, L. Foa-Tomasi, D. Barker, and B. Roizman. 1990b. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. Virology 178:213-222.

Campbell, M.E.M., J.W. Palfreyman, and C.M. Preston. 1984. Identification of the herpes simplex virus DNA sequences which encodes a trans-acting polypeptide responsible for the stimulation of the immediate early transcription. J. Mol. Biol. 180:1-19.

Caras, I.W., G.N. Weddell, M.A. Davitz, V. Nussenzweig, and D.W.J. Martin. 1987. Signal for attachment of phospholipid membrane anchor in decay accelerating factor. Science. 238:1280-1283.

Card, J.P., M.E. Whealy, A.K. Robbins, and L.W. Enquist. 1992. Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. 66:3032-3041.

Card, J.P., M.E. Whealy, A.K. Robbins, R.Y. Moore, and L.W. Enquist. 1991. Two herpesvirus strains are transported differentially in the rodent visual system. Neuron. 6:957-969.

Carrigan, D.R. W.R. Drobyski, S.K., Russler, M.A. Tapper, K.K. Knox, and R.C. Ash. 1991. Interstitial pneumonitis associated with human herpesvirus-6 infection after marrow transplantation. Lancet 338:147-149.

Carrozza, M.J., and N.A. DeLuca. 1996. Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol Cell. Biol. 16:3085-3093.

Carter, K.L., and B. Roizman. 1996. The promoter and trnascriptional unit of a novel

herpes simplex virus 1 alpha gene are contained in, and encode a protein in frame with, the open reading frame of the alpha 22 gene. J. Virol. 70:172-178.

Cassai, E.N., M. Sarmiento and P.G. Spear. 1975. Comparison of the virion proteins specified by herpes simplex virus types 1 and 2. J. Virol. 16:1327-1331.

Cassai, E. R. Manservigi, A. Corallini, and M. Terni. 1976. Plaque dissociation of herpes simplex viruses: biochemical and biological characters of the viral variants. Intervirology. 6:212-223.

Cavalcoli, J.D., A. Baghian, F.L. Homa, and K.G. Kousoulas. 1993. Resolution of genotypic and phenotypic properties of herpes simplex virus type 1 temperature-sensitive mutant (KOS) tsZ47: evidence for alellic complementation in the UL28 gene. Virology. 197:23-34.

Challberg, M.D. 1986. A method for identifying the viral genes required for herpes DNA replication. Proc. Natl. Acad. Sci. USA. 83:9094-9098.

Challberg, M.D., and T.J. Kelly. 1989. Animal virus DNA replication. Annu. Rev. Bichem. 58:671-717.

Chang, Y.E., A.P.W. Poon and B. Roizman. 1996. Properties of the protein encoded by the UL32 open reading frame of herpes simplex virus 1. J. Virol. 70:3938-3946.

Chapsal, J.M., and L. Pereira. 1988. Characterization of epitopes on native and denatured forms of herpes simplex virus glycoprotein B. Virology 164:427-434.

Chase, C.C., K. Carter-Allen, C. Lohff, and G.J. Letchworth. 1990. Bovine cells expressing bovine herpes virus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. J. Virol. 64:4866-4872.

Chatterjee, S., and S. Sarkar. 1992. Studies on endoplamic reticulum-golgi complex cycling pathway in herpes simplex virus-infected and brefeldin A-treated human fibroblast cells. Virology. 191:327-337.

Chee, A.S., T.A. Bankier, S. Beck, R. Bohni, C. Brown, R. Cerny, T. Horsnell, C. Hutchison, T. Kouzarides, J. Martignetti, E. Preddie, S. Satchwell, P. Tomlinson, K. Westen, and B. Barrell. 1990. Analysis of the protein content of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. 154:125-185.

Chen, S.Y., Y. Matsuoka, and R.W. Compans. 1991. Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. Virology. 183:351-365.

Chen, S.Y., Y. Matsuoka, and R.W. Compans. 1991a. Assembly and polarized release of Punta Toro virus and effects of brefeldin A. J. Virol. 65:1427-1439.

Chernomordik, L., M.M. Kozlov, and J. Zimmerberg. 1995. Lipids in biological membrane fusion. J. Membrane Biol. 146:1-14.

Cheung, P., B.W. Banfield, and F. Tufaro. 1991. Brefeldin A arrests the maturation and egress of herpes simplex virus particles during infection. J. Virol. 65:1893-1904.

Chiang, H.-Y., G.H. Cohen, and R.J. Eisenberg. 1994. Identification of functional regions of herpes simplex virus glycoproteins gD by using linker insertion mutagenesis. J. Virol. 68:2529-2543.

Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P.D. Ponath, L. Wu, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The ß-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell. 85:1135-1148.

Chou, J., J.J. Chen, M. Gross, and B. Roizman. 1995. Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma1(34.5) mutants of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA. 92:10516-10520.

Chou, J., E.R. Kern, R.J. Whitley and B. Roizman. 1990. Mapping of herpes simplex virus-1 neurovirulence to gamma-1(34.5), a gene non-essential for growth in culture. Science 250:1262-1265

Chou, J. and B. Roizman. 1986. The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequece of the L component. J. Virol. 57:629-637.

Chou, J., and B. Roizman. 1990. The herpes simplex virus 1 gene for ICP34.5, which maps in inverted repeats, is conserved in several limited-passage isolates but not in strain 17syn+. J. Virol. 64:1014-1020.

Chou, J., and B. Roizman. 1992. The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA. 89:3266-3270.

Chowdhury, S.I. and W. Batterson. 1994. Transinhibition of herpes simplex virus replication by an inducible cell-resident gene encoding a dysfunctional UL38-VP19c capsid protein. Virus Res. 33:67-87.

Claesson-Welsh, L., and P.G. Spear. 1986. Oligomerization of herpes simplex virus glycoprotein B. J. Virol. 60:803-806.

Clapham, P.R., J.N. Wever, D. Whitby, K. McIntosh, A.C. Dalgleish, P.J. Maddon, K.C.

Deen, R.W. Sweet, and R.A. Weiss. 1989. Soluble CD4 blocks the infectiviety of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. Nature. 337:368-370.

Clements, J.B., R.J. Watson, and N.M. Wilkie. 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell. 12:275-285.

Cohen, G.H., M. Katze, C. Hydrean-Stern, and R.J. Eisenberg. 1978. Type-Common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular weight envelope glycoprotein. J. Virol. 27:172-181.

Cohen, G.H., D. Long, and R.J. Eisenberg. 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. J. Virol. 36:429-439

Cohen, G.H., D. Long, J.T. Matthews, M. May, and R. Eisenberg. 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. 46:679-689.

Cohen, G.H., M. Ponce de Leon, H. Diggelmann, W.C. Lawrence, S. Vernon, and F. H. Eisenberg. 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. J. Virol. 16:1327-1331.

Cohen, J.I., and K.E. Seidel. 1994. Absence of varicella-zoster virus (VZV) glycoprotein V does not alter growth of VZV *in vitro* or sensitivity to heparin. J. Gen. Virol. 75:3087-3093.

Cohen, J.I., and S.E. Straus. 1996. Varicella-Zoster virus and its replication. p. 2525-2546. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Compton, T. 1995. Towards a definition of the HCMV entry pathway. Scand. J. Infect Dis. Suppl. 99:30-32.

Compton, T., and R.J. Courtney. 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus-infected cells. J. Virol. 49:594-597.

Conley, A.J., D.M. Knipe, P.C. Jones, and B. Roizman. 1981. Molecular genetics of herps simplex virus. VII. Characterization of a temperature-sensitive mutant produced by *in vitro* mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J. Virol. 37:191-206.

Cook, W.J., B. Gu, N.A. DeLuca, E.B. Moynihan and D.M. Coen. 1995. Induction of transcription by a viral regulatory protein depends on the relative strengths of functional TATA boxes. Mol. Cell. Biol. 15:4998-5006.

Copeland, C.S., R.W. Doms, E.M. Bolzau, R.G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J. Cell. Biol. 103:1179-1191.

Corey, L., and P.G. Spear. 1986. Infections with herpes simplex viruses. N. Engl. J. Med. 314:686-691.

Coulter, L.J., H.W.M. Moss, J. Lang and D.J. mcGeoch. 1993. A mutant of herpes simplex virus type 1 in which the UL13 protein kinase gene is disrupted. J. Gen. Virol. 74:387-395.

Courtney, R.J. 1979. Herpes simplex virus protein synthesis in the presence of 2-deoxy-D-glucose. Virology. 73:286-294.

Courtney, R.J., S.M. Steiner, and M. Benyesh-Melnick. 1973. Effects of 2-deoxy-D-glucose on herpes simplex virus replication. Virology. 52:447-455.

Cox, J.H., J.R. Bennink, and J.W. Yewdell. 1991. Retention of adenovirus E19 glycoprotein in the endoplasmic reticulum is essential to its ability to block antigen presentation. J. Exp. Med. 174:1629-1637.

Crabb, B.S., M.J. Studdert. 1995. Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus). Adv. Virus Res. 45:153-190.

Cranage, M.P., T. Kouzarides, A.T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S.E. Bell, A.C. Minson and G.L. Smith. 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralising antibodies via its expression in a recombinant vaccinia virus. EMBO J. 5:3057-3063.

Cranage, M.P., G.L. Smith, S.E. Bell, H. Hart, C. Brown, A.T. Bankier, P. Tomlinson, B.G. Barrell, and A.C. Minson. 1988. Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXLF2 product, varicella zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. J. Virol. 62:1416-1422.

Cross, A.M., R.G. Hope, and H.S. Marsden. 1987. Generation and properties of the glycoprotein E-related 32K/34K/35K amd 55/57K polypeptides encoded by herpes simplex virus type 1. J. Gen. Virol. 68:2093-2104.

Crute, J.J. and I.R. Lehman. 1991. Herpes simplex virus-1 helicase-primase. Physical and catalytic properties. J. Biol. Chem. 266:4484-4488.

Crute, J.J., T. Tsurumi, L. Zhu, S.K. Weller, P.D. Olivo, M.D. Challberg, E.S. Mocarski, and I.R. Lehman. 1989. Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. Proc. Natl. Acad. Sci. USA. 86:2186-2189.

Cunningham, C. A.J. Davison, A. Dolan, M.C. Frame, D.J. McGeoch, D.M. Meredith, H.W.M. Moss, and A.C. Orr. 1992. The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase. J. Gen. Virol. 73:303-311.

Curtin, K.D., and D.M. Knipe. 1993. Altered properties of the herpes simplex virus ICP8 DNA-binding protein in cells infected with ICP27 mutant viruses. Virology. 196:1-14

Dahdal, R.Y., and K.J. Colley. Specific sequences in the signal anchor of the ß-galactoside α-2,6-sialyltransferase are not essential for golgi localization. J. Biol Chem. 268:9908-9916.

Dalgleish, A.G., P.C.L. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, and R.A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature. 312:763-768.

Dargan, D.J. 1986. The structure and assembly of herpesviruses. In Electron Microscopy of Proteins, vol. 5, Virus Structure, pp. 359-437. Edited by J.R. Harris and R.W. Horne. London: Academic Press.

Dargan, D.J., A.H. Patel, and J.H. Subak-Sharpe. 1995. PREPs: Herpes simplex virus type 1-specific particles produced by infected cells when viral DNA replication is blocked. J. Virol. 69:4924-4932.

Davison, A.J. 1992. Channel catfish virus: a new type of herpesvirus. Virology. 186:9-14.

Davison, A.J., and J.E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. 67:1759-1816.

Davison, A.J. and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. 68:1067-1079.

Davison, A.J., and N.M. Wilkie. 1981. Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. J. Gen. Virol. 55:315-331.

Davison, M.D., F.J. Rixon, and A.J. Davison. 1992. Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. J. Gen. Virol. 73:2709-2713.

Davis-Poynter, N., S. Bell, T. Minson, and H. Browne. 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J. Virol. 68:7586-7590.

Dean, H.J., and A.K. Cheung. 1993. A 3' coterminal gene cluster in pseudorabies virus contains herpes simplex virus UL1, UL2, and UL3 gene homologs and a unique UL3.5

open reading frame. J. Virol. 67:5955-5961.

Dean, H.J., S.S. Terhune, M.T. Shieh, N. Susmarski, and P.G. Spear. 1994. Single amino acid substitutions in gD of herpes ismplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity. Virology. 199:67-80.

Dean, J.J., M.S. Warner, S.S. Terhune, R.M. Johnson, and P.G. Spear. 1995. Viral determinants of the variable sensitivity of herpes simplex virus strains to gD-mediated interference. J. Virol. 69:5171-5176.

Debroy, C. 1990. Nucleotide sequence of the Herpes simplex virus type-2 syn gene that causes cell fusion. Gene. 88:257-277.

Debroy, C., N. Pederson, and S. Person. 1985. Nucleotide sequence of herpes simplex virus type 1 gene that causes cell fusion. Virology 145:36-48.

DeBruyn Kops, A. and D. Knipe. 1988. Formation of DNA replication structures in herpes virus infected cells requires a viral DNA binding protein. Cell. 55:857-868.

Dechman, I.C., M. Hagen, and P.J. McCann III. 1992. Herpes simplex virus type 1 protease expressed in *Esherichia coli* exhibits autoprocessing and specific cleavage of the ICP35 assembly protein. J. Virol. 66:7362-7367.

Deluca, N., D.J. Bzik, V.C. Bond, S. Person, and W. Snipes. 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology 122:411-423.

Deluca, N. S. Person, D.J. Bzik, and W. Snipes. 1984. Genome locations of temperature-sensitive mutants in glycoprotein gB of herpes simplex virus type 1. Virology. 137:382-389.

DeLuca, N.A., and P.A. Schaffer. 1985. Isolation and characterization of deletion mutants of herpes simplex virus tyep1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. 56:558-570.

DeLuca, N.A., and P.A. Schaffer. 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 proteins ICP4. Mol. Cell Biol. 8:1997-1208.

DeLuca, N.A., and P.A Schaffer. 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. 62:732-743.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. DiMarzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman, and N.R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature. 381:661-666.

Desai, P., N.A. DeLuca, J.C. Glorioso and S. Person. Mutations in herpes simplex virus type 1 gene encoding VP5 (UL19) and VP23 (UL18) abrogate capsid formation and cleavage of replicated DNA. J. Virol. 67:1357-1364.

Desai, P., F.L. Homa, S. Person, and J.C. Glorioso. 1994. A genetic selection method for the transfer of HSV-1 glycoprotein B mutations from plasmid to the viral genome: preliminary characterization of transdominance and entry kinetics of mutant viruses. Virology. 204:312-322.

Desai, P.R., Ramakrishnan, Z.W. Lin, B. Osak, J.C. Glorioso, and M. Levine. 1993. The RR1 gene of herpes simplex virus type 1 is uniquely transactivated by ICP0 during infection. J. Virol. 67:6125-6135.

Desai, P.J., P.A. Schaffer, and A.C. Minson. 1988. Excretion of non-infectious virus particles lacking glycoprotein H by a temperature sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J. Gen. Virol. 69:1147-1156.

Desai, P., S.C. Watkins, and S. Person. 1994. The size and symmetry of B capsids of herpes simplex virus type 1 are determined by the products of the UL26 open reading frame. J. Virol. 68:5365-5374.

DiIanni, C.L., D.A. Drier, I.C. Deckman, P.J. McCann III, F. Liu, B. Roizman, R.J. Colonno, and M.G. Cordingley. 1993. Identification of the herpes simplex virus-1 protease cleavage sites by direct sequence analysis of autoproteolytic cleavage products. J. Biol. Chem. 268:2048-2051.

Dingwell, K.S., C.R. Brunetti, R.L. Hendricks, Q. Tang, M. Tang, A.J. Rainbow, and D.C. Johnson. 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread *in vivo* and across junctions of cultured cells. J. Virol. 68:834-845.

Dingwell, K.S., L.C. Doering, and D.C. Johnson. 1995. Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J. Virol. 69: 7087-7098.

Dolan, A. E. McKie, A.R. MacLean, and D.J. McGeoch. 1992. Status of the ICP34.5 gene in hereps simplex virus type 1 strain 17. J. Gen. Virol. 73:971-973.

Dolter, K.E., R. Ramaswamy, and T.C. Holland. 1994. Syncytial mutations in the herpes simplex virus type 1 gK (UL53) gene occur in two distinct domains. J. Virol. 68:8277-8281.

Dolter, K.E., S. R. King, and T.C. Holland. 1993. Incorporation of CD4 into virions by a recombinant herpes simplex virus. J. Virol. 67:189-195.

Doms, R., R. Lamb, J. Rose, and A. Heleniu. 1993. Folding and assembly of viral membrane proteins. Virology. 193:545-562.

Doranz, B.J., J. Rucker, U. Yi, R.J. Smyth, M. Samson, S.C. Peiper, M. Parmentier, R.G. Collman, and R.W. Doms. 1996. A dual-tropic primary HIV-1 isolate the uses fusin and the ß-chemokine receptors CKR-5, CKR-3, and CDR-2b as fusion cofactors. Cell. 85:1149-1158.

Dorsky, D. and C.S. Crumpacker. 1988. Expression of herpes simplex type 1 DNA polymerase gene by in virto translation and effects of gene deletions on acitivity. J. Virol. 62:3224-3232.

Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, X.Y. Huang, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P. Moore, and W.A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature. 381:667-673.

Dubbs, D.R., and S. Kit. 1964. Mutant strains of herpes simplex deficient in thymidine kinase-induced activity. Virology. 22:493-502.

Drobyski, W.R., W.M. Dunne, E.M. Burd, K.K. Knox, R.C. Ash, M.M. Horowitz, N. Flemenbery, and D.R. Carrigan. 1993. Human herpesvirus-6 (HHV-6) infection in allogeneic bone marrow transplant recipients. I. Evidence of a marrow suppressive role for HHV-6 *in vivo*. J. Infect. Dis. 167:735-739.

Dubin, G., S. Basu, D.L.P. Mallory, M. Basu, R. Tal-singer, and H.M. Friedman. 1994. Characterization of domains of herpes simplex virus type 1 glycoprotein E involved in Fc binding activity for immunoglobulin G aggregates. J. Virol. 68:2478-2486.

Dubin ,G., I. Frank, and H.M. Friedman. 1990. Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. J. Virol. 64:2725-2731.

Dubin, G., N.O. Fishman, R.J. Eisenberg, G.H. Cohen, and H.M. Friedman. 1992. The role of herpes simplex virus glycoproteins in immune evasion. Current Topics Microbiol. Immunol. 179:111-120.

Dubin, G., and H. Jiang. 1995. Expression of herpes simplex virus type 1 glycoprotein L (gL) in transfected mammalian cells: Evidence that gL is not independently anchored to cell membranes. J. Virol. 69:4564-4568.

Duus, K.M., C. Hatfield, and C. Grose. 1995. Cell surface expression and fusion by the varicella-zoster virus gH:gL glycoprotein complex: analysis by laser scanning confocal microscopy. Virology. 210:429-440.

Duus, K.M., and C. Grose. 1996. Multiple regulatory effects of Varicella-Zoster virus (VZV) gL on trafficking patterns and fusogenic properties of VZV gH. J. Virology. J. Virology. 70:8961-8971.

Earl, P.L., R.W. Doms, and B. Moss. 1990. Oligomeric structure of the human

immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA. 87:648-652.

Earl, P.L., R.W. Doms, and B. Moss. 1992. Multimeric CD4 binding exhibited by human and simian immunodeficiency virus envelope protein dimers. J. Virol. 66:5610-5614.

Eberle, R., and R. Courtney. 1982. Multimeric forms of herpes simplex virus type 2 glycoproteins. J. Virol. 41:348-351.

Eberle, R., and R.J. Courtney. 1980. gA and gB glycoproteins of herpes simplex virus type 1: two forms of a single polypeptide. J. Virol. 36:665-675.

Ebert, S.N., D. Subramanian, S.S. Shtrom, I.K. Chumg, D.S. Parris and M.T. Muller. 1994. Association of the p170 form of human topoisomerase II and progeny viral DNA in cells infected with herpes simlex virus type 1. J. Virol. 68:1010-1020.

Einfeld, D., and E. Hunter. 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc. Natl. Acad. Sci. USA. 85:8688-8692.

Eisenberg, R.J., C. Hydrean-Stern, and G.H. Cohen. 1979. Structural analysis of precursor and procduct forms of type-common envelope glycoprotein D (CP-1 antigen) of herpes simplex virus type 1. J. Virol. 31:608-620.

Eisenberg, R.J., M. Ponce de Leon, L. Pereira, D. Long, and G.H. Cohen. 1982. Purification of glycoprotein gD of herpes simplex viruses types 1 and 2 by use of monoclonal antibody. J. Virol. 41:1099-1104.

Ejercito, P.M., E.D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. 2:357-364.

Elias, C.M. Gustafsson and O. Hammarsten. 1990. The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication oriS. J. Biol. Chem. 265: 17167-17173.

Ellinger, K., F. Neipel, L. Foa-Tomasi, G. Campadelli-Fiume, and B. Fleckstein. 1993. The glycoprotien B homologue of human herpesvirus 6. J. Gen. Virol. 74:495-500.

Elliott, G., G. Mouzakitis and P. O'Hare. 1995. VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. J. Virol. 69:7932-7941.

Eloit, M., D. Fargeaud, F. L'Haridon, and B. Toma. 1988. Identification of the pseudorabies virus glycoprotein gp50 as a major target of neutralizing antibodies. Arch. Virol. 99:45-56.

Engel, J.P., E.P. Boyer, and J.L. Goodman. 1993. Two novel single amino acid syncytial mutations in the carboxy terminus of glycoprotein B of herpes simplex virus type 1 confer a unique pathogenic phenotype. Virology. 192:112-120.

Engelstad, M., and G.L. Smith. 1993. The vaccinia virus 42-kDa envelope proteins is required for the envelopment and egress of extracellular virus and for virus virulence. Virology. 194:627-637.

Everett, R.D. 1984. Trans-activation of transcription by herpes virus products: Requirements for two HSV-1 immediate early polypeptides for maximum activity. EMBO J. 3:3135-3141.

Fairfax, M.R., T. Schacker, R.W. Cone, A.C. Collier, and L. Corey. 1994. Human herpesvirus 6 DNA in blood cells of human immunodeficiency virus-infected men: Correlation of high levels with high CD4 cell counts. J. Infect Dis. 169:1342-1345.

Falke , D., A. Knoblich, and S. Muller. 1985. Fusion from without induced by herpes simplex virus type 1. Intervirology 24:211-219.

Farnham, A.E. 1958. The formation of microscopic plaques by herpes simplex virus in Hela cells. Virology. 6:317-327.

Feenstra, V., M. Jodaie, and D.C. Johnson. 1990. Deletions in herpes simplex virus glycoprotein D define nonessential and essential domains. J. Virol. 64:2096-2102.

Fehler, F., J.M. Herrmann, A. Saalmuller, T.C. Mettenleiter, and G.M. Keil. 1992. Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. J. Virol. 66:831-839.

Felser, J., S. Strauss, and J. Ostrove. 1987. Varicella-zoster virus complements herpes simplex virus type 1 temperature -sensitive mutants. J. Virol. 61:225-228.

Fenwick, M.L. and R.D. Everett. 1990a. Transfer of UL41, the gene controlling virion-associated host cell shutoff, between different strains of herpes simplex virus. J. Gen. virol. 61:1225-1228.

Fenwick, M.L. and R.D. Everett. 1990b. Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. J. Gen. Virol. 71:2961-2967.

Fenwick, M.L., and M.M. McMenamin. 1984. Early virion-associated suppression of cellular protien synthesis by herpes simplex virus is accompanied by inactivation of mRNA. J. Gen. Virol. 65:1225-1228.

Fenwick, M.L., and S.A. Owen. 1988. On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. J. Gen. Virol. 69:2869-2877.

Fierer, D.S., and M.D. Challberg. 1992. Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. J. Virol. 66:3986-3986.

Fitzpatrick, D. T. Zamb, and L. Babiuk. 1990. Expression of bovine herpes virus type 1 glycoprotein gI intransfected bovine cells induces spontaneous fusion. J. Gen. Virol. 71:1215-1219.

Fenwick M.L. and R.D. Everett, R.D. 1990. Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. J. Gen. Virol. 71:2961-2967.

Fenwick, M.L. and M.J. Walker. 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. 41:37-51.

Foa-Tomasi, L., E. Avitabile, A. Boscaro, R. Brandimarti, R. Gualandri, R. Manservigi, F. Dall'Olio, F. Serfini-Cessi, and G. Campadelli-Fuime. 1991a. Herpes simplex virus (HSV) glycoprotein H is partially processed in a cell line that expresses the glycoprotein and fully processed in cells infected with deletion or ts mutants in the known HSV glycopoteins. Virology 180:474-482.

Foa-Tomasi, L., A. Boscara, G.S. Di, and G. Campadelli-Fiume. 1991b. Monoclonal antibodies to gp100 (gH) inhibit penetration of human herpesvirus 6 and polykaryocyte formation in susceptible cells. J. Virol. 65:4124-4129.

Forghani, B., L. Ni and C. Grose. 1994. Neutralization epitope of the varicella-zoster virus gH:gL glycoprotein complex. Virology. 199:458-462.

Forrester, A.J., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter and A.C. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 deleted for glycoprotein H sequences. J. Virol. 66:341-348.

Forrester, A.J., V. Sullivan, A. Simmons, B.A. Blacklaws, G.L. Smith, A.A. Nash and A.C. Minson. 1991. Induction of protective immunity with antibody to herpes simplex virus type 1 glycoprotein H (gH) and analysis of the immune reponse to gH expressed in recombinant vaccinia virus. J. Gen. Virol. 72:369-375.

Frame, M.C., H.S. Marsden, and D.J. McGeoch. 1986a. Novel herpes simplex virus type 1 glycoproteins identified by antiserum against a synthetic oligopeptide from the predicted product of gene US4. J. Gen. Virol. 67:745-751.

Frame, M.C., D.J. McGeoch, F.J. Rixon, A.C. Orr and H.S. Marsden. 1986b. The 10K virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. Virology 150:321-322.

Frenkel N., and E. Roffman. 1996. Human Herpesvirus 7. p. 2609-2622. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Frenkel, N., E.C., Schirmer, L.S., Wyatt, G. Katsafanas, E. Roffman, R.M. Danovich, and C.H. June. 1990. Isolation of a new herpesvirus from human CD4+ T cells. Proc. Natl. Acad. Sci. USA. 87:748-752.

Fuchs, W., B.G. Klupp, H. Granzow, H.-J. Rziha, and T.C. Mettenleiter. 1996. Identification and characterization of the Pseudorabies virus UL3.5 protein, which is involved in virus egress. J. Virol. 70:3517-3527.

Fuller A.O. and W.-C. Lee. 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. 66:5002-5012.

Fuller, A.O., R.E. Santos, and P.G. Spear. 1989. Potent neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. 63:3435-3443.

Fuller, A.O., and P.G. Spear. 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. J. Virol. 55:475-482.

Fuller, A.O., and P.G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus type 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA 84:5454-5458.

Fuller, S.D., J.A. Berriman, S.J. Butcher, and B.E. Gowen. 1995. Low pH induces swiveling of the glycoprotein heterodimers in the Semiliki forset virus spike complex. Cell. 81:715-725.

Fuller, S., C.H. von Bonsdorff, and K. Simons. Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line, MDCK. Cell. 38:65-67.

Gage, P.J., M. Levine and J.C. Glorioso. 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. J. Virol. 67:2191-2201.

Gallo, M.L., D.I. Dorsky, C.S. Crumpacker and D.S. Parris. 1989. The essential 65-kilodalton DNA-binding protein of herpes simplex sitmulates the virus-encoded DNA polymerase. J. Virol. 63:5023-5029.

Gao, M., and D.M. Knipe. 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. J. Virol. 65:2666-2675.

Gao, M., L. Matusick-Kumar, W. Hurlburt, S.F. DiTusa, W.W. Newcomb, J.C. Brown, P.J. McCann III, I. Deckman, and R.J. Colonno. 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. J. Virol.

68:3702-3712.

Garten, W., A. Stieneke, E. Shaw, P. Wikstrom, and H.-D. Klenk. 1989. Inhibition of proteolytic activation of influenza virus hemagglutinin by specific peptidyl chloroalkyl ketones. Virology. 172:25-31.

Ge, H., and R.G. Roeder. 1994. Purification, cloning and characterization of a human coactivator, PC4, that meidates transcriptional activation of class II genes. Cell. 78:513-523.

Gelman, I.H., and S. Silverstein. 1986. Co-ordinate regulation of herpes simplex virus gene expresion is mediated by the functional interaction of two immediated early gene products. J. Mol. Biol. 191:395-409.

Georgopoulou, U., A. Michaelidou, B. Roizman, and P. Mavromara-Nazos. 1993. Identification of a new transcriptional unit that yields a gene product within the unique sequences of the short component of the herpes simplex virus 1 genome. J. Virol. 67:3961-3968.

Gerber, S.I., B.J. Belval, and B.C. Herold. 1995. Differences in the role of glycoprotein C of HSV-1 and HSV-2 in viral binding may contribute to serotype diferences in cell tropism. Virology. 214:29-39.

Gershon, A., L. Cosio, and P.A. Brunell. 1973. Observations on the growth of varicella-zoster virus in human diploid cells. J. Gen. Virol. 18:21-31.

Gershon, A.A., D.L. Sherman, Z. Zhu, C.A. Gabel, R.T. Ambron and M.D. Gershon. 1994. Intracellular transport on newly synthesized varicella-zoster virus: Final envelopment in the trans-Golgi network. J. Virol. 68:6372-6390.

Gerster, T., and R.G. Roeder. 1988. A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA. 85:6347-6351.

Gething M., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. Cell. 46:939-950.

Ghiasi, H., S. Bahri, A.B. Nesfurn, and S.L. Wechsler. 1995. Protection against herpes simplex virus-induced eye disease after vaccination with seven individually expressed herpes simplex virus 1 glycoproteins. Investigative Ophthalmology & Visual Science. 36:1352-1360.

Ghiasi, H., A.B. Nesburn, and S.L. Wechsler. 1991. Cell surface expression of herpes simplex virus type 1 glycoprotein H in recombinant baculovirus-infected cells. Virology 185:187-194.

Ghiasi, H., S. Slanina, A.B. Nesburn and S.L. Wechsler. 1994. Characterization of baculovirus-expressed herpes simplex virus type 1 glycoprotein K. J. Virol. 68:2347-2354.

Ghosh-Choudhury, N., A. Graham, and H.P. Ghosh. 1987. Herpes simplex virus type 2 glycoprotein biogenesis: effect of monensin on glycoprotein maturation, intracellular transport and virus infectivity. J. Gen. Virol. 68:1939-1949.

Ghosh-Choudhury, N., M. Butcher, E. Reid. and H.P. Ghosh. 1994. Effect of tunicamycin and monensin on the transport to the cell surface and secretion of a viral membrane glycoprotein containing both N- and O-linked sugars. Biochem. Cell Biol. 72:20-25.

Gibson, W. and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. 10:1044-1052.

Gibson, M.G., and P.G. Spear. 1985. Insertion mutants of herpes simplex viruses have a duplication of the glycoprotein D gene and espress two different forms of glycoprotein D. J. Virol. 48:396-404.

Gilbert, R., and H.P. Ghosh. 1993. Immunoelectron microscopic localization of herpes simplex virus glycoprotein gB in the nuclear envelope of infected cells. Virus Research 28:217-231.

Gilbert, R., K. Ghosh, L. Rasile, and H.P. Ghosh. 1994. Membrane anchoring domain of herpes simplex viurs glycoprotein gB is sufficient for nuclear envelope localization. J. Virol. 68:2272-2285.

Glorioso, J., M. S. Szczesiul, S.D. Marlin, and M. Levine. 1983. Inhibition of glycosylation of herpes simplex virus glycoproteins: Identification of antigenic and immunogenic partially glycosylated glycopeptides on the cell surface membrane. Virology. 126:1-18.

Godowski, P.J., and D.M. Knipe. 1985. Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. J. Virol. 55:357-365.

Goepfert, P.A., G. Wang, and M.J. Mulligan. 1994. Identification of an ER retrieval signal in a retroviral glycoprotein. Cell 82:543-544.

Goldin, A.L., R.M. Sandri-Goldin, M. Levine, and J.C. Glorioso. 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. 38:50-58.

Goldstein, D.J., and S.K. Weller. 1988a. Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: Characterization of an ICP6 deletion mutant. Virology. 166:41-51.

Goldstein, D.J., and S.K. Weller. 1988b. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: Isolation and characterization of an ICP6 lac Z insertion mutant. J. Virol. 62:196-205.

Goldstein, D.J., and S.K. Weller. 1988. An ICP6:lacZ insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. 62:2970-2977.

Goltz, M., H. Broll, A. Mankertz, W. Weigelt, H. Ludwig, H.J. Buhk, and K. Borchers. 1994. Glycoprotein B of bovine herpesvirus type 4: its phylogenetic relationship to gB equivalents of the herpesviruses. Virus Genes. 9:53-59.

Gompels, U.A., A.L. Carss, C. Saxby, D.C. Hancock, A. Forrester, and A.C. Minson. 1991. Characterization and sequence analyses of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. J. Virol. 65:2393-2401.

Gompels, U., M.A. Craxton, and R.W. Honess. 1988. Conservation of the gH gene in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri. J. Gen. Virol. 69:2819-2829.

Gompels, U.A. J. Luxton, K.K. Kehl, and D.R. Carrigan. 1994. Chronic bone marrow suppression in immunocompetent adult by human herpesvirus 6. Lancet. 343:735-736.

Gompels, U., and A.C. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology 153:230-247.

Gompels, U.A., and A.C. Minson. 1989. Antigenic properties and cellular localization of herpes simplex virus glycoprotein H synthesized in a mammalian cell experimental system. J. Virol. 63:4744-4755.

Gompels, U.A., J. Nicholas, G. Lawrence, M. Jones, B.J. Thomson, M.E.D. Martin, S. Efstathiou, M. Craxton, and H.A. Macaulay. 1995. The DNA Sequence of human herpesvirus-6: Structure, coding content, and genome evolution. Virology 209:29-51.

Gong, M., and E. Kieff. 1990. Intracellular trafficking of two major Epstein-Barr virus glycoproteins, gp350/220 and gp110. J. Virol. 64:1507-1516.

Goodman, J.L., and J.P. Engel. 1991. Altered pathogenesis in herpes simplex virus type 1 infection due to a syncytial mutation mapping to the carboxy terminus of glycoprotein B. J. Virol. 65:1770-1778.

Goodrich, J.A., R. Hoey, C.J. Thut, A. Admon, and R. Tjian. 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell. 75:519-530.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36:59-72.

Graham, F.L., and Prevec, L. 1991. Manipulation of adenovirus vectors, Vol. 7, p. 109-128. In E.J. Murray and J.M. Walker (Eds.), Methods in Molecular Biology: Gene Transfer and Expression Techniques. Humana press, Clifton, NJ.

Graham, F.L., and A.J. van der Eb. 1973. A new technique for the assay of infectivity of adenovirus 5 DNA. Virology 52:456-467.

Greaves, R., and P. O'Hare. 1989. Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J. Virol. 63:1641-1650.

Grose, C., D.M. Perrotta, P.A. Brunell and G.C. Smith. 1979. Cell-free varicella-zoster virus in cultured human melanoma cells. J. Gen. Virol. 43:15-27.

Gruenheid, S., L. Gatzke, H. Meadows, and F. Tufaro. 1993. Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate protoglycans. J. Virol. 67:93-100.

Gu, B., R. Kuddus, and N.A. DeLuca. 1995. Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechamism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. Mol Cell Biol. 15:3618-3626.

Gurevich, I. 1992. Varicella zoster and herpes simplex virus infections. Heart & Lung. 21:85-91.

Gustafsson, C.M., O. Hammarsten, M. Falkenberg, and P. Elias. 1994. Herpes simplex virus DNA replication: a spacer sequence directs the ATP-dependent formation of a nucleoprotein complex at oriS. Proc. Natl. Acad. Sci. USA. 91:4629-4633.

Haanes, E.J., C.M. Nelson, C.L. Soule, and J.L. Goodman. 1994. The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. J. Virol. 68:5824-5834.

Haddad, R.S., and L.M. Hutt-Fletcher. 1989. Depletion of glycoprotein gp85 from virosomes made with Epstein-Barr virus proteins abolishes their ability to fuse with virus receptor-bearing cells. J. Virol. 63:4998-5005.

Haffey, M.L., and P.G. Spear. 1980. Alteratins in glycoprotein gB specified by mutants and their partial revertants in herpes simplex virus 1 and relationship to other mutant phenotypes. J. Virol. 35:114-128.

Haj-Ahmad, Y., and F.L. Graham (1986). Development of a helper-independent human

adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J. Virol. 57:267-274.

Hall, C.B., C.E. Long, K.C. Schnabel, M.T. Caserta, K.M. McIntyre, M.A. Costanzo, A. Knott, S. Dewhurst, R.A. Insel, and L.G. Epstein. 1994. Human herpesvirus-6 infection in children - A prospective sutdy of complications and reactivation New Engl. J. Med. 331:432-438.

Hall, J.D., D.M. Coen, B.L. Fisher, M. Weisslitz, S. Randall, R.E., Almy, P.T. Gelep, and P.A. Schaffer. 1984. Generation of genetic diversity in herpes simplex virus: an antimutator phenotype maps to the DNA polymerase locus. Virology. 132:26-37.

Hallenberger, S., V. Bosh, H. Angliker, E. Shaw, H.D. Klenk, and W. Garten. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature. 360:358-361.

Hammarsten, O., X. Yao, and P. Elias. 1996. Inhibition of topoisomerase II by ICRF-193 prevents efficient replication of herpes simplex virus type 1. J. Virol. 70:4523-4529.

Hammond, C., and A. Helenius. 1995. Quality control in the secretory pathway. Curr. Opin. Cell Biol. 7:523-529.

Handler, C.G., R.J. Eisenberg, and G.H. Cohen. 1996a. Oligomeric structure of glycoproteins in herpes simplex virus type 1. J. Virol. 70:6067-6075.

Handler, C.G., G.H. Cohen, and R.J. Eisenberg. 1996b. Cross-Linking of glycoprotein oligomers during herpes simplex virus type 1 entry. J. Virol. 70:6076-6082.

Hanke, T., F.L. Graham, V. Lulitanond, and D.C. Johnson. 1990. Herpes simplex virus IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology 177:437-444.

Hardy, W.R., and R.M. Sandri-Goldin. 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J. Virol. 68:7790-7799.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual, p. 411. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Harson, R., and C. Grose. 1995. Egress of varicella-zoster virus from the melanoma cells: a tropism for the melanoyte. J. Virol. 69:4994-5010.

Harter, C. 1995. COP-coated vesicles in intracellular protein transport. FEBS Lett. 369:89-92.

Hartman, S.C., and R.C. Mulligan. 1988. Two dominant-acting selectable markers for gene transfer studies in mammalian cells. Proc. Natl. Acad. Sci. USA 85:8047-8051.

Hay, B., S.B. Prusiner, and V.R. Lingappa. 1987. Evidence for a secretory form of the cellular prion protein. Biochemistry. 26:8110-8115.

Hayward, G.S., R.J. Jacob, S.C. Wadsworth and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short segments. Proc. Natl. Acad. Sci. USA 72:4243-4247.

Haywood, A.M., 1994. Virus receptors: binding, adhesion strengthening, and changes in viral structure. J. Virol. 68:1-5.

He, B., J. Chou, D.A. Liebermann, B. Hoffman and B. Roizman. 1996. The carboxyl terminus of the murin MyD116 gene substitutes for the corresponding domain of the gamma(1)34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. J. Virol. 70:84-90.

Heffner, S., F. Kovacs, B.G. Klupp, and T.C. Mettenleiter. 1993. Glycoprotein gp50-negative pseudorabies virus: a novel approach toward a nonspreading live herpesvirus vaccine. J. Virol. 1529-1537.

Heifetz, A., R.W. Keenan, and A.D. Elbein. 1979. Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichylphospgate Glc NAc-1-phosphate transferase. Biochemistry. 18:2186-2192.

Heine, J.W., R.W. Honess, E.Cassai, and B. Roizman. 1974. Proteins specified by herpes simplex virus. XIII. The virion polypeptides of type 1 strains. J. Virol. 14:640-651.

Heinman, T., M. Gong, J. Sample, and E. Kieff. 1988. Identification of the Epstein-Barr virus gp85 gene. J. Virol. 62:1101-1107.

Hernandez, T.R., and I.R. Lehman. 1990. Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. J. Biol. Chem. 265:11227-11232.

Herold, B.C., S.I. Gerber, T. Polonsky, B.J. Belval, P.N. Shakleee, and K. Holme. 1995. Identification of structural features of heparin required for inhibition of herpes simplex virus type 1 binding. Virology. 206:1108-1116.

Herold, B.C., R.J. Visalli, N. Susmarski, C.R. Brandt, and P.G. Spear. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J. Gen. Virol. 75:1211-1222.

Herold, B.C., D. Wudunn, N. Soltys, and P.G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. 65:1090-1098.

Herrold, R.E., A. Marchini, S. Fruehling, and R. Longnecker. 1996. Glycoprotein 110, the Epstein-Barr virus homolog of herpes simplex virus glycoprotein B is essential for

Epstein-Barr virus replication *in vivo*. J. Virol. 70:2049-2054.

Hidaka, Y., T. Sakai, Y. Toh, and R. Mori. 1991. Glycoprotein C of herpes simpelx virus type 1 is essential for the virus to evade antibody-independent complement-mediated virus inactivation and lysis of virus-infected cells. J. Gen. Virol. 72:915-921.

Hidaka, Y., S. Sakuma, Y. Kumano, H. Minagawa, and R. Mori. 1990. Characterization of glycoprotein C-negative mutants of herpes simplex virus type 1 isolated from a patient with keratitis. Arch. Virol. 113:195-207.

Highlander, S.L., W. Cai, S.Person, M. Levine, and J.C. Glorioso. 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. 62:1881-1888.

Highlander, S.L., D.J. Dorney, P.J. Gage, T.C. Holland, W. Cai, S. Person, M. Levine, and J.C. Glorioso. 1989. Identification of mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. 61:3356-3364.

Highlander, S.L., W.F. Goins, S. Person, T.C. Holland, M. Levine, and J.C. Glorioso. 1991. Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. J. Virol. 65:4275-4283.

Highlander, S.L., S.L. Sutherland, P.J. Gage, D.C. Johnson, M. Levine, and J.C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotien D inhibit virus penetration. J. Virol. 61:3356-3364.

Hitt, M., A.J. Bett, C. Addison, L. Prevec and F.L. Graham. 1995. Techniques for human adenovirus vectors construction and characterization. In: "Methods in Molecular Genetics" Ed. K.W. Adolph, Academic Press 7B. Orlando, Florida. p. 13-30.

Hitt, M., A.J. Bett, L. Prevec and F.L. Graham. Construction and propogation of human adenovirus vectors. In: "Cell Biology: A Laboratory Handbook" Ed. J.E. Celis. Academic Press. p. 479-490.

Hobman, T.C., L. Woodward, and M.G. Farquhar. 1995. Targeting of a heterodimeric membrane protein complex to the Golgi: rubella virus E2 glycoprotein contains a transmembrane Golgi retention signal. Mol. Biol. Cell. 6:7-20.

Hoggan, M.D., and B. Roizman. 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. 70:208-219.

Hoggan, M.D., B. Roizman, and T.B. Turner. 1960. The effect of temperature of incubation on the spread of herpes simplex virus in an immune environment in cell culture. Amer. J. Hyg. 70:208-219.

Holland, L.E., K.P. Anderson, C. Shipman, Jr., and E.K. Wagner. 1980. Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology. 101:10-24.

Holland L.E., K.P. Anderson, J.R. Stringer, and E.K. Wagner. 1979. Isolation and localization of hepes simplex virus type 1 mRNA abundant before viral DNA synthesis. J. Virol. 31:447-462.

Holland, T.C., F.L. Homa, S.D. Marlin, M. Levine, and J. Glorioso. 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. 34:716-742.

Holland, T.C., and S. Person. 1977. Ammonium chloride inhibits cell fusion induced by syn mutants of herpes simplex virus type 1. J. Virol. 23:213-215.

Holmes, K.V., and M.M.C. Lai. Coronaviridae: The viruses and their replication. p. 1075-1096. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Homa, F.L., D.J.M. Purifoy, J.C. Glorioso, and M. Levine. 1986. Molecular basis of the glycoprotein C-negative phenotypes of herpes simplex virus type 1 mutants selected with a virus-neutralizing monoclonal antobody. J. Virol. 58:281-289.

Honess, R.W., A. Buchan, I.W., Halliburton, and D.H. Watson. 1980. Recombination and linkage between structural and regulatory genes of herpes simplex virus type 1: Study of the functional organization of the genome. J. Virol. 34:716-742.

Honess, R.W., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. 1. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14:8-19.

Honess, R.W., and B. Roizman. 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA. 72:1276-1280.

Huff, V., W. Cai, J.C. Glorioso, and M. Levine. 1988. The carboxy-terminal 41 amino acids of herpes simplex virus type 1 glycoprotein B are not essential for production of infectious virus particles. J. Virol. 62:4403-4406.

Humphrey, J.S., P.J. Peters, L.C. Yuan, and J.S. Bonifacino. 1993. Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. J. Cell Biol. 120:1123-1135.

Huszar, D., and S. Bacchetti. 1983. Is ribonucleotide reductase the transforming function of herpes simplex virus 2? Nature 302:76-79.

Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A.C. Minson, and D.C. Johnson. 1992a. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. 66:2240-2250.

Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F.L. Graham, and D.C. Johnson. 1992b. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. 66:5603-5609.

Hutchinson, L., F.L. Graham, W. Cai, C. Debroy, S. Person, and D.C. Johnson. 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology 196:514-531.

Hutchinson, L., C. Roop and D.C. Johnson. 1993a. Glycoprotein K of HSV-1 is important for Viral Egress, Production of Infectious Progeny and Does Not Reach the Cell Surface. Abstract # C93 of the XVIII International Herpesvirus Workshop.

Hutchinson, L.M., C. Roop and D.C. Johnson. 1993b. The Role of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K, In Virus Induced Membrane Fusion. Abstract 1197, p306a of the American Society for Cell Biology, Thirty-third Annual Meeting (supplement to Molecular Biology of the Cell, Vol. 4, 1993).

Hutchinson, L., C. Roop and D.C. Johnson. 1995a. Herpes simplex virus glycoprotein K which is known to influence fusion of infected cells, yet is not transported to the cell surface. J. Virol. 69: 4556-4563.

Hutchinson, L., and D.C. Johnson. 1995b. Herpes simplex virus glycoprotien K promotes egress of virus particles. J. Virol. 69:5401-5413.

Irmiere, A. and W. Gibson. 1983. Isolation and characterization of noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. Virology 130:118-133.

Izumim K.M., and J.G. Stevens. 1990. Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. J. Exp. Med. 172:487-497\6.

Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 9:3153-3162.

Jackson, M.R., T. Nilsson, P.A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. J. Cell Biol. 121:317-333.

Jacob, R.J., L.S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA XII. Accumulation of head-to tail concatemers in nuclei of infected cells and their role in

the generation of four isomeric arrangements of viral DNA. J. Virol. 29:448-457.

Jacobs, L. 1994. Glycoprotein E of pseudorabies virus and homologous proteins in other alphaherpesvirinae. Arch. Virol. 137:209-228.

Jacobs, L., H.J. Rziha, T.G. Kimman, A.L. Gielkens and J.T. Van Oirschot. 1993a. Deleting valine-125 and cysteine-126 in glycoprotein gI of pseudorabies virus strain NIA-3 decreases plaque size and reduces virulence in mice. Arch. Virol. 131:251-264.

Jacobs, L., W.A.M. Mulder, J.T., Van Oirschot, A.L. J. Gielkens, and T.G. Kimman. 1993b. Deleting two amino acids in glycoprotein gI of pseudrabies virus decreases virulence and neurotropism for pigs, but does not affect immunogenicity. J. Gen. Virol. 74:2201-2206.

Jacobson, J.G., D.A. Leib, D.J. Goldstein, C.L. Bogard, P.A. Schaffer, S.K. Weller, and D.M. Coen. 1989a. A herpes simplex virus ribonucleotide reductase deletion muant is defective for productive acute and reactivatable latent infection of mice and for replication in mouse cells. Virology. 173:276-283.

Jacobson,J.G., S.L. Martin and D.M. Coen. 1989b. A Conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. 63:1839-1843.

Jamieson, A.T., G.A. Gentry. and J.H. Subak-Sharpe. 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes simplex virus. J. Gen. Virol. 24:465-480.

Jensen, H.L, J. Rygaard, and B. Norrild. 1995. A time-related study of brefeldin A effects in HSV-1 infected cultured human fibroblasts. APMIS 193:530-539.

Johnson, C.F., W. Chan, and S. Kornfeld. 1992. Cation-dependent mannose-6-phosphate receptor contains two internalization signals in its cytoplasmic domain. Proc. Natl. Acad. Sci. USA. 87:10010-10014.

Johnson, D.C. 1991. Adenovirus vectors as potential vaccines against herpes simplex virus. Rev. Inf. Dis. 13 (Suppl. 11), S912-916.

Johnson, D.C., R.L. Burke and T. Gregory. 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. of Virol. 64:2569-2576.

Johnson, D.C., and V. Feenstra. 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. 61:2208-2216.

Johnson, D.C., M.C. Frame, N.W. Ligas, A.M. Cross, and N.D. Stow. 1988b. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral

glycoproteins, gE and gI. J. Virol. 62:1347-1354.

Johnson, D.C., G. Ghosh-Choudhury, J.R. Smiley, L. Fallis, and F.L. Graham. 1988a. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164:1-14.

Johnson, D.C. and M.W. Ligas. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus specific cell surface receptors. J. Virol. 62:1486-1494.

Johnson, D.C., and J.R. Smiley. 1985. Intracellular transport of herpes simplex virus gD occurs more rapidly in uninfected cells than in infected cells. J. Virol. 54:682-689.

Johnson, D.C. and P.G. Spear. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. 43:1102-1112. (gB, gC, gD on cell surface -iodination)

Johnson, D.C., and P.G. Spear. 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell 32:987-997.

Johnson, D.C., M. Wittels, and P.G. Spear. 1984. Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. 52:238-247.

Johnson, M.A., C.T. Prideaux, K. Kongsuwan, S.G. Tyack, and M. Sheppard. 1995. ICP27 immediated early gene, glycoprotein K (gK) and DNA helicase homologues of infectious laryngotracheitis virus (gallid herpesvirus 1) SA-2 strain. Arch. Virol. 140:623-634.

Johnson, R.M., and P.G. Spear. 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. 63:819-827.

Jones, F.E., C.A. Smibert, and J.R. Smiley. 1995. Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. J. Virol. 69:4863-4871.

Jones, F., and C. Grose. 1988. Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. J. Virol. 62:2701-2711.

Jons, A., H. Granzow, R. Kuchling and T.C. Mettenleiter. 1996. The UL49.5 gene of Pseudorabies virus codes for an O-glycosylated structural protein of the viral envelope. J. Virol. 70:1237-1241.

Karger, A., and T.C. Mettenleiter. 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology. 194:654-664.

Karger, A., and T.C. Mettenleiter. 1996. Identification of cell surface molecules that interact with pseudorabies virus. J. Virol. 70:2138-2145.

Karger, A., A. Saalmuller, F. Tufaro, B.W. Banfield, and T.C. Mettenleiter. 1995. Cell surface proteoglycans are not essential for infection by pseudorabies virus. J. Virol. 69:3482-3489.

Katan, M., A. Haigh, C.P. Verrijzer, P.C. van der Vliet, and P. O'Hare. 1990. Characteization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. Nucleic Acids Res. 18:6871-6880.

Katz, F.N., J.E., Rothman, V.R. Lingappa, G. Blobel, and H.R. Lodish. 1977. Membrane assembly *in vitro*: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. Proc. Natl. Acad. Sci. USA. 74:3278-3282.

Kaye, J.F., U.A. Gompels and A.C. Minson. 1992. Glycoprotein gH of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. J. Gen. Virol. 73:2693-2698.

Keay, S. and B. Baldwin. 1991. Anti-idiotype antibodies that mimic gp86 (gH) of human cytomegalovirus inhibit viral fusion but not attachment. J. Virol. 65:5124-5128.

Keay, S., and B. Baldwin. 1992. The human fibroblast receptor for gp86 (gH) of human cytomegalovirus is a phophorylated glycoprotein. J. Virol. 66:4834-4838.

Keay, S. T.C. Merigan, and L. Rasmussen. 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc. Natl. Acad. Sci. USA. 86:10100-10103.

Kehm, R., E. Lorentzen, A. Rosen-Wolff and G. Darai. 1994. *In vitro* expression of UL56 gene of herpes simplex virus type 1; detection of UL56 gene product in infected cells and in virions. Virus Res. 33:55-66.

Keller, J.M. 1976a. The expression of the syn$^-$ gene of herpes simplex virus type 1. I. Morphology of Infected cells. 69:490-499.

Keller, J.M. 1976b. The expression of the syn$^-$ gene of herpes simplex virus type 1. II. Requirements for macromoleucular synthesis. 72:402-409.

Keller, P.M., A.J. Davison, R.S. Lowe, M.W. Rieman, and R.W. Elis. 1987. Identification and sequence of the gene encoding gpIII (gH), a major glycoprotein of varicella zoster virus. Virology 157:526-533.

Keller, P.M., B.J, Neff, and R.W. Ellis. 1984. Three major glycoprotein genes of varicella-zoster virus whose products have neutralizaiton epitopes. J. Virol. 52:293-297.

Kembel, G.W., T. Daniell, and J.M. White. 1994. Lipid-anchored influenza hemaggutinin promotes hemifusion, not complete fusion. Cell. 76:383-391.

Kerr, C.L., and T.H. Pennington. 1984. The effect of monensin on virion production and protein secretion in pseudorabies virus infected cells. J. Gen. Virol. 65:1033-1041.

Khadr, A., S.K. Tikoo, L.A. Babiuk and S. van Drunen Littel-van den Hurk. 1996. Sequence and expression of a bovine herpesvirus-1 gene homologous to the glycoprotein K-encoding gene of herpes simplex virus -1. Gene. 168:189-193

Khattar, S.K., S. van Drunen Littel-van den Harke, S.K. Attah-Poku, L.A. Babiuk and S. K. Tikoo. 1996. Identification and characterization of a bovineherpesvirus-1 (BHV-1) glycoprotein gL which is required for proper antigenicity, processing, and transport of BHV-1 glycoprotein gH. Virology 219:66-76.

Khattar, S.K., S. van Drunen Littel0vanden Hurk, L.A. Babiuk, and S.K. Tikoo. 1995. Identification and transcriptional analysis of a 3'coterminal gene cluster containing UL1, UL2, UL3, and UL3.5 open reading frames of bovine herpesvirus-1. Virology. 213:28-37.

Kieff, E.D., S.L. Bachenheimer, and B. Roizman. 1971. Size, composition and structure of the DNA of subtypes 1 and 2 of herpes simplex virus. J. Virol. 8:125-129.

Kieff, E. 1996. Epstein-Barr virus and its replication. p. 2343-2396. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Kikuchi, G.E., J.C. Glorioso, and R. Nairn. 1990. Cross-linking studies show that herpes simplex virus type 1 glycoprotein C molecules are clustered in the membrane of infected cells. J. Gen. Virol. 71:455-462.

Kingsbury, D.W. 1996. Paramyxoviridae and their replication. p. 945-962. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Klenk, H.D., and W. Garten. 1994. Host cell proteases controlling virus pathogenicity. Trends Microbiol. 2:39-43.

Klimkait, T., K. Strebel, M.D. Hoggan, M.A. Martin, and J.M. Orenstein. 1990. The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. J. Virol. 64:621-629.

Klinedinst, D.K., and M.D. Challberg. 1994. Helicase-primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. J. Virol. 68:3693-3701.

Klumperman, J., J.K. Locker, A. Meijer, M.C. Horzinek, H.J. Geuze, and P.J. Rottier. 1994. Coronavirus M proteins accumulate in the Golgi complex beyond the site of virion budding. J. Virol. 68:6523-6534.

Klupp, B.G., J. Baumeister, N. Visser and T.C. Mettenleiter. 1993. Identification and characterization of glycoproteins gK and gL of pseudorabies virus, abstr. C-94, p. C-94 In Abstracts of the XVIII International Herpes Virus Workshop. University of Pittsburgh, Pittsburgh, Pa.

Klupp, B.G., J. Baumeister, A. Karger, N. Visser, and T.C. Mettenleiter. 1994 Identification and characterization of a novel structural glycoprotein in pseudorabies virus, gL. J. Virol. 68:3868-3878.

Klupp, B.G., and T.C. Mettenleiter. 1991. Sequence and expression of the glycoprotein gH gene of pseudorabies virus. Virology 157:732-741.

Knox, P.G., and L.S. Young. 1995. Epstein-Barr virus infection of CR2-transfected epithelial cells reveals the presence of MHC class II on the virion. Virology. 213:147-157.

Kohn, A., J. Gitelman, and M. Inbar. 1980. Interaction of poyunsaturated fatty acids with animal cells and enveloped viruses. Antimicrob. Agents Chemother. 18:962-968.

Kolodziej, P.A., and R.A. Young. 1991. Epitope Tagging and protein surveillance. Methods in Enzymology. 194:508-519.

Komuro, M., M. Tajima and K. Kato. 1989. Transformation of Golgi membrane into the envelope of herpes simplex virus in rat anterior pituitary cells. Eur. J. Cell Biol. 50:398-406.

Koop, K.e., J. Duncan, and J.R. Smiley. 1993. Binding sites for the herpes simplex virus immediate early protein ICP4 impose an increased dependence on viral DNA replication on simple model promoters located in the viral genome. J. Virol. 67:7254-7263.

Kopp, A., and T.C. Mettenleiter. 1992. Stable rescue of a glycoprotein gII deletion mutant of pseudorabies virus by glycoprotien gI of bovine herpesvirus 1. J. Virol. 66:2754-2764.

Kopp, E. Blewett, V. Misra, and T.C. Mettenleiter. 1994. Proteolytic cleavage of bovine herpesvirus 1 (BHV-1) glycoprotein gB is not necessary for its function in BHV-1 or pseudorabies virus. J. Virol. 68:1667-1674.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharies. Annu. Rev. Biochem. 54:631-664.

Kousoulas, K.G., D.J. Bzik, N. DeLuca, and S. Person. 1983a. The effect of ammonium

chloride and tunicamycin on the glycoprotein content and infectivity of herpes simplex virus type 1. Virology 125:468-474.

Kousoulas, K.G., D.J. Bzik, and S. Person. 1983b. Effect of ionophore monensin on herpes simplex virus type 1-induced cell fusion, glycoprotein synthesis, and virion infectivity. Intervirology. 20:56-60.

Kousoulas, K.G., S. Person, and T.C. Holland. 1978. Timing of some of the molecular events required for cell fusion induced by herpes simplex virus type 1. J. Virol. 27:505-512.

Kousoulas, K.G., S. Person, and T.C. Holland. 1982. Herpes simplex virus type 1 cell fusion occurs in the presence of ammonium chloride-inhibited glycoproteins. Virology. 123:257-263.

Koyama, A.H., and T. Uchida. 1984. Inhibition of multiplication of herpes simplex virus type 1 by ammonium chloride and chloroquine. Virology. 138:332-335.

Koyama, A.H., and T. Uchida. 1987. The mode of entry of herpes simpelx virus type 1 into Vero cells. Microbiol. Immunol. 31:123-30.

Koyama, A.H., and T. Uchida. 1989. The effect of ammonium chloride on the multiplication of herpes simplex virus type 1 in Vero cells. Virus Research. 13:271-282.

Knowles, R.W., and S. Person. 1976. Effects of 2-deoxyglucose, glucosamine, and mannose on cell fusion and the glycoproteins of herpes simplex virus. J. Virol. 18:644-651.

Kristensson, K., E. Lycke, M. Roytta, B. Svennerholm, and A. Vahlne. 1986. Neuritic transport of herpes simplex virus in rat sensory neurons *in vitro*. Effects of substances interacting with microtubular function and axonal flow [nocodazole, taxol, and erythro-9-3(2-hydroxy-nonyl)adenine]. J. Gen. Virol. 67:2023-2038.

Kristie, T.M., J.H. LeBowitz, and P.A. Sharp. 1989. The octamer-binding proteins form multi-protein-DNA complexes with the HSV alpha-TIF regulatory protein. EMBO J. 8:4229-4238.

Kristie, T.M., J.L. Pomerantz, T.C. Twomey, S.A. Parent, and P.A. Sharp. 1995. The cellular C1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. J. Biol. chem. 270:4387-4394.

Kristie, T.M., and B. Roizman. 1988. Differentiation and DNA contact points of the host proteins binding at the cis-site for the virion-mediated induction of alpha genes of herpes simplex virus 1. J. Virol. 62:1145-1157.

Kristie, T.M. and P.A. Sharp. 1990. Interactions of the Oct-1 POU subdomains with

specific DNA sequences and with the HSV alpha-trans-activator protein. Genes Dev. 4:2383-2396.

Kristie, T.M. and P.A. Sharp. 1993. Purification of the C1 factor required for the stable recognition of the Oct-1 homeodomain by the herpes simplex virus alph-transinducing factor (VP16). J. Biol. Chem. 268:6525-6534.

Kuhn, J.E., B.R. Eing, R. Brossmer, K. Munk, and R.W. Braun. 1988. Removal of N-linked carbohydrates decreases the infectivity of herpes simplex virus type 1. J. Gen. Virol. 69:2847-2858.

Kuhn, J.E., M.D. Kramer, W. Willenbacher, U. Wieland, E.U. Lorentzen, and R.W. Braun. 1990. Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. J. Virol. 64:2491-2497.

Kwong, A.D., and N. Frenkel. 1987. Herpes simplex virus infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA. 84:1926-1930.

Ladin, B.F., M.I. Blankenship, and T. Ben-Porat. 1980. Replication of herpesvirus DNA. V. Maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. J. virol. 33:1151-1164.

Lagunoff, M. and B. Roizman. 1994. Expression of a herpes simplex virus 1 open reading frame antisense to the gamma(1)34.5 gene and transcribed by an RNA 3' coterminal with the unspliced latency associated transcript. J. Virol. 68:6021-6028.

Lagunoff, M., and B. Roizman. 1995. The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus 1 genome. J. Virol. 69:3615-3623.

Lagunoff, G. Randall, and B. Roizman. 1996. Phenotypic properties of herpes simplex virus 1 containing a derepressed open reading frame P gene. J. Virol. 70:1810-1817.

Lam, A., C.A. Smibert, K.E. Koop, C. Lavery, J.P. Capone, S.P. Weinheimer, and J.R. Smiley. 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J. 15:2575-2581.

Lamb, R.A. 1993. Paramyxovirus fusion: A hypothesis for change. Virology. 197:1-11.

Lamb, R.A., and R.M. Krug. 1996. Orthomyxoviridae: The viruses and their replication, p. 1353-1393. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London). 227:680-685.

Langeland, N., A.M. Oyan, H.S. Marsden, A. Cross, J.C. Glorioso, L.J. Moore, and L. Haar. 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. J. Virol. 64:1271-1277.

Laquerre, S., S. Person, and J.C. Glorioso. 1996. Glycoprotein B of herpes simplex virus type 1 oligomerizes through the intermolecular interaction of a 28-amino-acid domain. J. Virol. 70:1640-1650.

Lawrence, G.L., J. Nicholas, and B.G. Barrell. 1995. Human herpes virus 6 (strain U1102) encodes homologues of the conserved herpesvirus glycoprotein gM and the alphaherpesvirus origin-binding protein. J. Gen. Virol. 76:147-152.

Lazzaro, C.D., G. Campadelli-Fiume, and M.R. Torrisi. 1995. Intermediate forms of glycoconjugates are present in the envelope of herpes simplex virions during their transport along the exocytic pathway. Virology. 214:619-623.

Lee, G. T.-Y., and P.G. Spear. 1980. Viral and cellular factors that influence cell fusion induced by herpes simplex virus. Virology 107:402-414

Lee, G. T.-Y., M.F. Para, and P.G. Spear. 1982. Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. 43:41-49.

Lee, G. T.-Y., K.L. Pogue-Geile, L. Pereira, and P.G. Spear. 1992. Expression of herpes simplex virus glycoprotein C from a DNA fragment inserted into the thymidine kinase gene of this virus. Proc. Natl. Acad. Sci. USA 79:6612-6616.

Lee, S.S.-K., Q. Dong, T.S.-F. Wang, and I.R. Lehman. 1995. Interaction of herpes simplex virus 1 origin-binding protein with DNA polymerase alpha. Proc. Natl. Acad. Sci. USA. 92:7882-7886.

Lee, W.C., and A.O. Fuller. 1993. Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not heparan sulfate. J. Virol. 67:5088-5097.

Lehner, R., H. Meyer, and M. Mach. 1989. Identification and characterization of a human cytomegalovirus gene coding for a membrane protein that is conserved among the human herpesviruses. J. Virol. 63:3792-3800.

Lenard, J., 1996. Negative-strand virus M and retrovirus MA proteins: all in the family? Virology. 216:289-298.

Levitan, D.B., and H.A. Blough. 1976. Preliminary biochemical characterization of the factor(s) responsible for herpesvirus-induced exogenous fusion. J. Virol. 18:1081-1087.

Li, Q., S.M. Turk, and L.M. Hutt-Fletcher. 1995A. The Epstein-Barr virus (EBV) BZLF2

gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. J. Virol. 69:3987-3994.

Li, Y., S. van Drunen Littel-van den Hurk, L.A. Babiuk, and X. Liang. 1995. Characterization of cell-binding properties of bovine herpesvirus 1 glycoproteins B, C, and D: Identification of a dual cell-binding function of gB. J. Virol. 69:4758-4768.

Liang, X., L.A. Babiuk, S. van Drunen Littel-van den Hurk, D.R. Fitzpatrick, and T.J. Zamb. 1991. Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. J. Virol. 65:1124-1132.

Liang, X., L.A. Babiuk, and T.J. Zamb. 1991a. Pseudorabies virus gIII and bovine herpesvirus I gIII share complementary functions. J. Virol. 65:5553-5557.

Liang, X., L.A. Babiuk, and T.J. Zamb. 1993. Mapping of heparin-binding structures on bovine herpesvirus 1 and pseudorabies virus gIII glycoproteins. Virology. 194:233-243.

Liang, X., B. Chow, C. Raggo, and L.A. Babiuk. 1996. Bovine herpesvirus 1 UL49.5 homolog gene encodes a novel viral envelope protein that forms a disulfide-linked complex with a second virion structural protein. J. Virol. 70:1448-1454.

Liang, X., B. Chow, U. Li, C. Raggo, D. Yoo, S. Attah-Poku and L.A. Babiuk. 1995a. Characterization of bovine herpesvirus 1 UL49 homolog gene and product: Bovine herpesvirus 1 UL49 homolog is dispensable for virus growth. J. Virol. 69:3863-3867.

Liang, X, C. Pyne, U. Li, L.A. Babiuk and J. Kowalski. 1995b. Delineation of the essential funciton of bovine herpesvirus 1 gD: and indication for the modulatory role of gD in virus entry. Virology. 207:429-441.

Liang, X., M. Tang, T.J. Zamb, L.A. Babiuk, J., Kowalski and M. L. Tykocinski. 1993. Expression of glycoprotein gIII-human decay-accelerating factor chimera on the bovine herpesvirus 1 virion via a glycosyl phosphatidylinositol-based membrane anchor. J. Virol. 67:4896-4904.

Liesegang, T.J. 1991. Biology and molecular aspects of herpes simplex and varicella zoster virus infections. Opthamology. 99:781-797.

Ligas, M.W., and D.C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by ß-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. 62:1486-1494.

Lin, R.-S., I. Ha, E. Maldonado, D. Reinberg, and M. Green. 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature. 353:569-571.

Lingappa, V.R., A. Devellers-Thiery, and G. Blobel. 1977. Nascent prehormones are intermediates in the biosynthesis of authentic pituitary growth hormone and prolactin.

Proc. natl. Acad. Sci. USA. 74:2432-2436.

Lisanti, M.P., I. W. Caras, M.A. Davitz, and E. Rodriguez-Boulan. A glycophopholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. J. Cell Biol. 109:2145-2156.

Little, S.P. and P.A. Schaffer. 1981. Expression of the syncytial (syn) phenotype in HSV-1, strain KOS: Genetic and phenotypic studies of mutants in two syn loci. Virology 112:686-697.

Little, S.P., J.T. Jofre, R.J. Courtney, and P.A. Schaffer. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology 115:149-158.

Liu, D.X., U.A., Gompels, L. Foa-Tomasi, and G. Campadelli-Fiume. 1993b. Human herpesvirus-6 glycoprotein H and L homologs are ycomponents of the gp100 complex and the gH external domain is the target for neutralizing monoclonal antibodis. Virology 197:12-22.

Liu, D.X., U.A. Gompels, J. Nicholas, and C. Lelliott. 1993a. Identification and expression of the human herpesvirus 6 glycoprotein H and interaction with an accessory 40 K glycoprotein. J. Gen. Virol. 74:1847-1857.

Liu, F., and B. Roizman. 1991a. The promoters, transcriptional unit, and coding sequences of herpes simplex virus 1 family 35 proteins are contained within and in frame with the UL26 open reading frame. J. Virol. 65:206-212.

Liu, F., and B. Roizman. 1991b. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. 65:5149-5156.

Liu, F., and B. Roizman. 1992. Differenctiation of multiple domains in the hereps simplex virus 1 protease encoded by the UL26 gene. Proc. Natl. Acad. Sci. USA. 89:2076-2080.

Liu, F. and B. Roizman. 1993. Characterizaiton of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus 1 UL26 protein. J. Virol. 67:1300-1309.

Locker, J.K., J. Klumperman, V. Oorschot, M.C. Horzinek, H.J. Geuze, and P.J. Rottier. 1994. The cytoplasmic tail of mouse hepatitis virus M protein is essential but not sufficient for its retention in the Golgi complex. J. biol. Chem. 269:28263-28269.

Locker, J.K., D.J. Opstelten, M. Ericsson, M.C. Horzinek, and P.J. Rottier. 1995. Oligomerization of a trans-Golgi/trans-Golgi network retained protein occurs in the Golgi complex and may be part of its retention. J. Biol. Chem. 270:8815-8821.

Lockshon, D., and D.A. Galloway. 1988. Sequence and structural requirements of a herpes simplex viral DNA replication origin. Mol. Cell. Biol. 8:4018-4027.

Lodge, R., H. Gottlinger, D. Gabuzda, E.A. Cohen, and G. Lemay. 1994. The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells.

Loh, L. 1991. Synthesis and processing of the major envelope glycoprotein of murine cytomegalovirus. Virology. 180:239-250.

Longnecker, R., S. Chatterjee, R.J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Natl. Acad. Sci. USA 84:4303-4307.

Longnecker, R., and B. Roizman. 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene.

Longnecker, R., and B. Roizman. 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 gene. Science 236:573-576.

Luan, P., and M. Glaser. 1994. Formation of membrane domains by the envelope proteins of vesicular stomatitis virus. Biochemistry. 33:4483-4489.

Luan, P., L. Yang, and M. Glaser. 1995. Formation of membrane domains created during the budding of viesicular stomatitis virus. A model for selective lipid and protein sorting in biological membranes. Biochemistry. 34:9874-9883.

Luciw, P.A. 1996. Human Immunodeficiency viruses and their replication, p. 1881-1953. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Ludwig, H., H. Becht, and R. Rott. 1974. Inhibition of herpes virus-induced cell fusion by concanavalin A, antisera, and 2-deoxy-D-glucose. J. Virol. 14:307-314.

Luzio, J.P., and G. Banting. 1993. Eukaryotic membrane traffic: retrieval and retention mechanisms to achieve organelle residence. TIBS. 18:395-398.

Lukacs, N., H.-J. Thiel, T.C. Mettenleiter, and H.-J. Rziha. 1985. Demonstration of three major species of pseudorabies virus glycoproteins and identification of a disulfide-linked glycoprotein complex. J. Virol. 53:166-174.

Lusso, P., M.A. De, M. Malnati, F. Lori, S.E. De Rocco, M. Baseler, and R.C. Gallo. 1991. Induction of CD4 and susceptibility to HIV-1 infection in human CD8+ T lymphocytes by human herpesvirus 6. Nature. 349:533-535.

Lusso, P., M.S. Malnati, A. Garzino-Demo, R.W. Crowley, E.O. Long, and R.C. Gallow. 1993. Infection of natural killer cells by human herpesvirus 6. Nature. 362:458-462.

Machamer, C.E. 1993. Targeting and retention of golgi membrane proteins. Curr. Opin. Cell Biol. 5:606-612.

Machamer, C.E, M.G. Grim, A. Esquela, S.W. Chung, M. Rols, K. Ryan, and A.M. Swift. 1993. Retention of a cis Golgi protein requires polar residues on one face of a predicted alpha-helix in the transmembrane domain. Mol Biol. Cell. 4:695-704.

Machamer, C.E., S.A. Mentone, J.K. Rose, and M.G. Farquhar. 1990. The E1 glycoprotein of an avian coronavirus is targeted to the cis-Golgi complex. Proc. Natl. Acad. Sci. USA. 87:6944-6948.

Mackem, S., and B. Roizman. 1982. Diffentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. Proc. Natl. Acad. Sci. 79:4917-4912.

Mackem, S., and B. Roizman. 1982. Structural features of the herpes simplex virus alpha genes 4, 0, and 27 promoter-regulatory sequences which confer alpna regulation on chimeric thymidine kinase genes. J. Virol. 44:939-949.

MacLean, C.A., B. Clark, and D.J. McGeoch. 1989. Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristilated. J. Gen. Virol. 70:3147-3157.

MacLean, CA., A. Dolan, F.E., Jamieson, D.J. McGeoch. 1992. The myristylated virion proteins of herpes simplex virus type 1: investigation of their role in the virus life cycle. J. Gen. Virol. 73:539-547.

MacLean, C.A., S. Efstathiou, M.L. Elliot, F.E. Jamieson, and D.J. McGeoch. 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J. Gen. Virol. 72:897-906.

MacLean, C.A., L.M. Robertson, And F. E. Jamieson. 1993. Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role *in vivo*. J. Gen. Virol. 74:975-983.

Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 47:333-348.

Mallabiabarrena, A., M.A. Jimenez, M. Rico, and B. Alarcon. 1995. A tyrosine-containing motif mediates ER retention of CD3-epsilon and adopts a helix-turn structure.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Manservigi, R., P.G. spear and A. Buchan. 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different glycoproteins. Proc. Natl. Acad. Sci. USA 74:3913-3917.

Mao, J.C.-H., E.E. Robishaw, and L.R. Overby. 1975. Inhibition of DNA polymerase from herpes simplex virus-infected WI-38 cells by phosphonoacetic acid. J. Virol. 15:1281-1283.

Marquardt, M.T., T. Phalen, and M. Kielian. 1993. Cholesterol is required for the exit pathway of Semiliki Forest virus J. Cell Biol. 123:57-65.

Marlin, S.D., S.L. Highlander, T.C. Holland, M. levine, and J.C. Glorioso. 1986. Antigenic variation (mar mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. 59:142-153.

Marsden, H.S., A. Buckmaster, J.W. Palfreyman, R.G. Hope, and A.C. Minson. 1984. Characterization of the 92,000-dalton glycoprotein induced by herpes simplex virus type 2. J. Virol. 50:547-554.

Marsden, H.S., I.K. Crombie, and J.H. Subak-Sharpe. 1976. Control of proteins synthesis in herpesvirus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. J. Gen. Vriol. 31:347-372.

Masibay, A.S., P.V. Balaji, E.E. Boeggeman, and P.K. Qasba. 1993. Mutational analysis of the Golgi retention signal of bovine ß-1,4-galactosyltransferase. J. Biol. Chem. 268:9908-9916.

Matsuoka, Y., T. Ihara, D.H. Bishop, and R.W. Compans. 1988. Intracellular accumulation of Punta Toro virus glycoproteins expressed from cloned cDNA. Virology. 167:251-260.

Matsuoka, S.Y. Chen, and R.W. Compans. 1994. A signal for Golgi retention in the bunyavirus G1 glycoprotein. J. Biol. Chem. 269:22565-22573.

Matter, K., and I. Mellman. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. Curr. Opin. Cell Biol. 6:545-554.

Matusick-Kumar, L., W. Hurlburt, S.P. Weinheimer, W.W. Newcomb, J.C. Brown and M. Gao. 1994. Phenotype of the herpes simplex virus type 1 protease substrate ICP35 mutant virus. J. Virol. 68:5384-5394.

Matusick-Kumar, L., P.J. McCann III, B.J. Robertson, W.W. Newcomb, J.C. Brown, and M. Gao. 1995a. Release of the catalytic domain No from the herpes simplex virus type 1 protease is required for viral growth. J. Virol. 69:7113-7121.

Matusick-Kumar, L., W.W. Newcomb, J.C. Brown, P.J. McCann III, W. Hurlburt, S.P. Weinheimer, and M. Gao. 1995b. The C-terminal 25 amino acids of the protease and its substrate ICP35 of herpes simplex virus type 1 are involved in the formation of sealed capsids. J. Virol. 69:4347-4356.

Maul, G.G., A.M. Ishov, and R.D. Everett. 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology. 217:67-75.

McCann, P.J., III, D.R. O'Boyle II, and I.C. Deckman. 1994. Investigaion of the specificity of the herpes simplex virus type 1 protease by point mutagenesis of the autoproteolysis sites. J. Virol. 68:526-529.

McCarthy, A.M., L. McMahan, and P.A. Schaffer. 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. 63:18-27.

McClain, S.S., and A.O. Fuller. 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology. 198:690-702.

McCombs, R.M., J.P. Brunschwig, R. Mirkovic and M. Benyesh-Melnick. 1971. Electron microscopic characterization of a herpeslike virus isolated from tree shrews. Virology. 45:816-820.

McCune, J., L. Rabin, M. Feiberg, M. Lieberman, J. Kosek, G. Reyes, and I. Weissman. 1988. Endoproteolytic cleavage of gp 160 is required for the activation of human immunodeficiency virus. Cell 53:55-67.

McFarlane, M., J.I. Dakis, and C.M. Preston. 1992. Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65. J. Gen. Virol. 73:285-292.

McGeoch, D.J., and S. Cook. 1994. Molecular phylogeny of the alphaherpesvirinae subfamily and a proposed evolutionary timescale. J. Mol. Biol. 238:9-22.

McGeoch, D.J., C. Cunningham, G. McIntyre, and A. Dolan. 1991. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. J. Gen Virol. 72:3057-3075.

McGeoch, D.J., M.A. Dalrymple, A.J. Davison, A. Donlan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69:1531-1574.

McGeoch, D.J., and A.J. Davison. 1986. DNA sequence of the herpes simplex virus type 1 gene encoding glycoprotein H and identification of homologues in the genomes of varicella zoster virus and Epstein-Barr virus. Nucleic Acids Res. 14:111-114.

McGeoch, D.J., A. Dolan, S. Donald and B.H. K. Brauer. 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex type 1. Nucleic Acids Res. 14:1727-1745.

McGrath, B.J., and C.L. Newman. 1994. Genital herpes simplex infections in patients with the acquired immunodeficiency syndrome. Rev. Therapeutics. 14:529-542.

McGregor, F., A. Phelan, J. Dunlop, and J.B. Clements. 1996. Regulation of herpes simplex virus poly (A) usage and the action of immediate-early protein IE63 in the early-late switch. J. Virol. 70:1931-1940.

McGrory, W.J., D.S. Bautista, and F.L. Graham. 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology 163:614-617.

McKenzie, R.C. R.M. Epand, and D.C. Johnson. 1987. Cyclosporin A inhibits herpes simplex virus-induced cell fusion but not virus penetration into cells. Virology. 159:1-9.

McKay, E.M., B. McVey, H.S. Marsden, S.M. Brown, and A.R. Maclean. 1993. The herpes simplex virus type 1 strain 17 open reading frame RL1 encodes a polypeptide of apparent Mr 37K equivalent to ICP34.5 of herpes simplex virus type 1 strain F. J. Gen. Virol. 74:2493-2497.

McKie, E.A., R. G. Hope, S.M. Brown, and A.R. MacLean. 1994. Characterization of the herpes simplex virus type 1 strain 17+ neurovirulence gene RL1 and its expression in a bacterial system. J. Gen. Virol. 75:733-741.

McLauchlan, J., and F.J. Rixon. 1992. Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: Integrity of the tegument does not depend on the presence of capsid or envelope. J. Gen. Virol. 73:269-276.

McLauchlan, J., C. Addison, M.C. Craigie and F.J. Rixon. 1992. Noninfectious L-particles supply functions which can facilitate infection by HSV-1. Virology. 190:682-688.

McLauchlan, K. Liefkens and N.D. Stow. 1994. The herpes simplex virus type 1 UL37 gene product is a component of virus particles. J. Gen. Virol. 75:2047-2052.

McNabb, D.S. and R.J. Courtney. 1992. Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. Virology. 190:221-232.

McKnight, J.L.C., T.M. Kristie, and B. Roizman. 1987. Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. Proc. Natl. Acad. Sci. USA. 84:7061-7065.

Meignier, B., R. Longnecker, P. Mavromara-Nazos, A.E. Sears, and B. Roizmain. 1988.

Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus type 1. Virology. 162:251-254.

Meier J.L., and S.E. Straus. 1992. Comparative biology of latent varicella-zoster virus and herpes simplex virus infections. J. Infect. Dis. 166 (Suppl 1):S13-23.

Melin, L, R. Persson, A. Andersson, A. Berstrom, R. Ronnholm, and R.F. Pettersson. 1995. The membrane glycoprotein G1 of Uukuniemi virus contains a signal for localization to the Golgi complex. Virus Res. 36:49-66.

Meredith, D.M., J.M. Stocks, G.R. Whittaker, I.W. Halliburton, B.W. Snowden, and R.A. Killington. 1989. Identification of the gB homologues of equine herpesvirus type 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J. Gen. Virol. 70:1161-1172.

Mettenleiter, T.C. 1993. Pseudorabies (Aujeszky's disease) virus: state of the art. Acta Vet. Hung. 42:153-177.

Mettenleiter, T.C. 1994. Initiation and spread of alpha-herpesvirus infections. Trends Microbiol. 2:2-4.

Mettenleiter, T.C., C. Schreurs, F. Zuckermann, T. Ben-Porat, and A.S. Daplan. 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. 62:2712-2717.

Mettenleiter, T.C., and P.G. Spear. 1993. Glycoprotein gB (gII) of pseudorabies virus can functionally substitute for glycoprotein gB in herpes simplex virus type 1. J. Virol. 68:500-504.

Mettenleiter, T.C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat. 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. 64:278-286.

Meyer, A.L., E.A. Petrovskis, W.P. Duffus, D.R. Thomsen, and L.E. Post. 1991. Cloning and sequence of an infectious bovine rhinotracheitis virus (BHV-1) gene homologous to glycoprotein H of herpes simplex virus. Biochim. Biophys. Acta 1090:267-269.

Michaelis, C., B.W. Banfield, S. Gruenheid, Y. Tsang, R. Lippe, W.A. Jefferies, B.W. Wattenberg, and F. Tufaro. 1992. Toxin resistance and reduced secretion in a mouse L-cell mutant defective in herpes virus propagation. Biochem Cell Biol. 70:1209-1217.

Miller, N. and L.M. Hutt-Fletcher. 1988. A monoclonal antibody to glycoprotein gp85 inhibits fusion but not attachment of Epstein-Barr virus. J. Virol. 62:2366-2372.

Miller, N., and L.M. Hutt-Fletcher. 1992. Epstein-Barr virus enters B cells and epithelial cells by different routes. J. Virol. 66:3409-3414.

Minson, A.C., T.C. Hodgman, P. Digard, D.C. Hancock, S.E. Bell, and E.A. Buckmaster. 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. J. Gen. Virol. 67:1001-1013.

Misra, V., and E.L., Blewett. 1991. Construction of herpes simplex viruses that are pseudodiploid for the glycoprotein B gene: a strategy for studying the function of an essential herpes virus gene. J. Gen. Virol. 72:385-392.

Mocarski, E.S. 1996. Cytomegaloviruses and their replication. p. 2447-2492. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Mocarski, E.S. and B. Roizman. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell. 31:89-97.

Montalvo, E.A., and C. Grose. 1986. Neutralization epitope of varicella zoster virus on native viral glycoprotin gp118 (VZV glycoprotein gpIII). Virology 149:230-241.

Montalvo, E.A., and C. Grose. 1987. Assembly and processing of the disulfide-linked varicellar-zoster virus glycoprotein gpII(140). J. Virol. 61:2877-2884.

Montalvo, E.A., R.T. Parmley and C. Grose. 1986. Varicella-zoster viral glycoprotein envelopment: ultrastructural cytochemical localization. J. Histochem. Cytochem. 34:281-284.

Montgomery, R.I., M.S. Warner, B.J. Lum, and P.G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell. 87:427-436.

Moore, P.S., S.-J. Gao, G. Dominguez, E. Cesarman, O. Lungu, D.M. Knowles, R. Garber, P.E. Pellett, D.J. McGeoch, and Y. Chang. 1996. Primary Characterization of a herpesvirus agent associated with Kaposi's Sarcoma. J. Virol. 70:549-558.

Morgan, C., S. A. Ellison, H.M. Rose, And D.H. Moore. 1954. Structure and development of viruses as observed in the electron microscope. 1. Herpes simplex virus. J. Exp. Med. 100:195-202.

Morgan, C., H.M. Rose, M. Holden and E.P. Jones. 1959. Electron microscope observations on the development of herpes simplex virus. J. Exp. Med. 110:643-656.

Morgan, C. H.M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2:507-516.

Morrison, T.G., and A. Portner. 1991. Structure, function and intracellular processing of the glycoproteins of paramxyoviridae, p. 347-382. in D. W. Kingsbury (ed.), The

paramyxoviruses. Plenum Press, New York.

Moscona, A., and R.W. Peluso. 1991. Fusion properties of cells persistently infected with human parainfluencza virus type 3: Participation of hemagglutinin-neuraminidase in membrane fusion. J. Virol. 65:2773-2777.

Moscona, A., and R.W. Peluso. 1992. Fusion properties of cells infected with human parainfluenza virus type 3: Receptor requirements for viral spread and virus-mediated membrane fusion. J. Virol. 66:6280-6287.

Moss, B. 1996. Poxviridae: The viruses and their replication. p. 2637-2671. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Muggeridge, M.L., W.C. Wilcox, G.H. Cohen and R.J. Eisenberg. 1990. Identification of a site on herpes simplex virus type 1 glycoprotein D that is essential for infectivity. J. Virol. 64:3617-3626.

Mulder, W.A., L. Jacobs, J. Priem, G.L. Kok, F. Wagenaar, T.G. Kimman, and J.M. Pol. 1994. Glycoprotein gE-negative pseudorabies virus has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the porcine central nervous system. J. Gen. Virol. 75:3095-3106.

Mulder, W., J. Pol, T. Kimman, G. Kok, J. Priem, and B. Peeters. Glycoprotein D-negative pseudorabies virus can spread transneuronally via direct neuron-to-neuron transmission in its natural host, the pig, but not after additional inactivation of gE or gI. J. Virol. 70:2191-2200.

Mullen, M.A., S. Gerstberger, D.M. Ciufo, J.D. Mosca, and G.S. Hayward. 1995. Evaluation of colocalization interactions between the IE110, IE175, and IE63 transactivator proteins of herpes simplex virus within subcellular punctate structures. J. Virol. 69:476-491.

Munro, S. 1991. Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify golgi retention. EMBO J. 10:3577-3588.

Nahmias, A.J., and S. Kibrick. 1964. Inhibitory effect of heparin on herpes simplex virus. J. Bacteriol. 87:1060-1066.

Nakai, K. and M. Kanehisa. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14:897-911. PSORT server: psort@nibb.ac.jp

Nash, T.C., and J.G. Spivack. 1994. The UL55 and UL56 genes of herpes simplex virus type 1 are not required for viral replication, intraperitoneal virulence, or establishment of latency in mice. Virology. 204:794-798.

Navarro, D., P. Paz, and L. Pereira. 1992. Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology 186:99-112.

Navarro, D., P. Paz, S. Tugizov, K. Topp, J. LaVail, and L. Pereira. 1993. Glycoprotein B of human cytomegalovirus promotes virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells. Virology. 197:143-158.u

Nazerian, K and R.L. Witter. 1970. Cell-free transmission and *in vivo* replication of Marek's disease virus. J. Virol. 5:388-397.

Neidhardt, H., C.H. Schroder, and H.C. Kaerner. 1987. Herpes simplex virus type 1 glycoprotein E is not indispensible for viral infectivity. J. Virol. 61:600-603.

Nemeckova, S., D. Kunke, M. Press, V. Nemecek, and L. Kutinova. 1994. A carboxy-terminal portion of the preS1 domain of hepatitis B virus (HBV) occasioned retention in endoplasmic reticulum of HBV envelope proteins expressed by recombinant vaccinia viruses. Virology. 202:1024-1027.

Newcomb, W.W., and J.C. Brown, 1989. Use of Ar+ plasma etching to localize structural proteins in the capsid of herpes simplex virus type 1. J. Virol. 63:4697-4702.

Newcomb, W.W., and J.C. Brown. 1991. Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. J. Virol. 65:613-620.

Newcomb, W.W., and J.C. Brown. 1994. Incuced extrusion of DNA from the capsid of herpes simplex virus type-1. J. Virol. 68:433-440.

Newcomb, W.W., B.L. Trus, F.P. Booy, A.C. Steven, J.S. Wall and J.C. Brown. 1993. Structure of the herpes simplex virus capsid: molecular composition of the pentons and triplexes. J. Mol. Biol. 232:499-511.

Nicola, A.Y., S.H. Willis, N.N. Naidoo, R.J. Eisenberg, and G.H. Cohen. 1996. Structure-Function analysis of soluble forms of herpes simplex viurs glycprotein D. J. Virol. 70:3815-3822.

Nicolas, J. 1996. Determination and analysis of the complete nucleotide sequence of human herpesvirus 7. J. Virol. 70:5975-5989.

Nicholson, P., C. Addison, A.M. Cross, J. Kennard, V.G. Preston, and F.J. Rixon. 1994. Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires abundant scaffolding protein VP22a. J. Gen. Virol. 75:1091-1099.

Nicolson, L., A. Cullinane, and D. Onions. 1990. The nucleotide sequence of an equine herpesvirus 4 gene homologue of the herpes simplex virus type 1 glycoprotein H gene. J.

Gen. Virol. 71:1793-1800.

Nii, S. 1971. Electron microscopic observations on FL cells infected with herpes simpelx virus. I. Viral forms. Biken J. 14:177-190.

Nii, S. 1992. Electron microscopic study on the development of herpesviruses. J. Electron. Microsc. 41:414-423.

Nii, S., and J. Kamahora. 1961. Studies on the growth of a newly isolated herpes simplex virus *in vitro*. Biken's J. 4:75-96.

Nii, S., and J. Kamahora. 1961a. Cytopathic cahanges induced by herpes simplex virus. Biken's J. 4:255-270.

Nii, S., C. Morgan, and H.M. Rose. 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. J. Virol. 2:517-536.

Nii, S., M. Yoshida, F. Uno, T. Kurata, K. Ikuta, and K. Tamanishi. 1990. Replication of human herpesvirus 6 (HHV-6): morphological aspects. Advances in Exp. Med. Biol. 278:19-28.

Nilsson, T. M.H. Hoe, P. Slusarewicz, C. Rabouille, R. Watson, F. Hunte, G. Watzele, E.G. Berger, and G. Warren. 1994. Kin Recognition between medial Golgi enzymes in HeLa cells. EMBO J. 13:562-574.

Nilsson, T., J.M. Lucocq, D. Mackay, G. Warren. 1991. The membrane spanning domain of ß-1,4-Galactoslytransferase specifies trans-Golgi localization. EMBO J. 10:3567-3575.

Nilsson, T., P. Slusarewicz, M.H. Hoe, and G. Warren. 1993. Kin Recognition: A model for the retention of Golgi enzymes. FEBS Lett. 330:1-4

Nilsson T., and G. Warren. 1994. Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. Curr. Opin. Cell Biol. 6:517-521.

Noble, A.G., G.T.-Y. Lee, R. Sprague, M.L. Parish, and P.G. Spear. 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology 129:218-224.

Norrild, B. and B. Pedersen. 1982. Effect of tuncamycin on the synthesis of herpes simplex virus type 1 glycoproteins and their expression on the cell surface. J. Virol. 395-402.

Novotny, M., M.L. Parish, and P.G. Spear. 1996. Variability of herpes simplex virus 1 gL and anti-gL antibodies that inhibit cell fusion but not viral infectivity. Virology. 221:1-13.

Nowlin D.M., N.R. Cooper, and T. Compton. 1991. Expression of a human cytomegalovirus receptor correlates with infectibility of cells. J. Virol. 65:3114-3121.

O'Donnell, M.E., P. Elias, B.E. Funnell and I.R. Lehman. 1987. Interaction between the DNA polymerase and single-stranded DNA-binding protein (infected cell protein 8) of herpes simplex virus 1. J. Biol. Chem. 262:4260-4266.

O'Hare, P., and C.R. Goding. 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell. 52:435-445.

O'Hare, P., C.R. Goding, and A. Haigh. 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J. 7:4231-4238.

O'Hare, P., and G.S. Haywood. 1985. Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. 56:723-733.

O'Hare, P., and G.S. Hayward. 1985. Evidence for a direct role for both the 175,000 and 110,000-molecular weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. 53:751-760.

Olivio, P.D., N.J. Nelson, and M.D. Challberg. 1991. Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. USA. 85:5414-5418.

Olivio, P.D., N.J. Nelson, and M.D. Challberg. 1989. Herpes simplex virus type 1 gene products required for DNA replication: identification and overexpression. J. Virol. 63:196-204.

Olofsson, S. 1992. Carbohydrates in herpesvirus infections. APMIS. 100:84-95.

Olofsson, S., J. Blomberg, and E. Lycke. 1981. O-glycosidic carbohydrate-peptide linkages of herpes simplex virus glycoproteins. Arch. Virol. 70:321-329.

Olofsson, S., M. Lundstrom, H. Marsden, S. Jeansson, and A. Vahlne. 1986. Characterization of a herpes simplex virus type 2-specified glycoprotein with affinity for N-acetylgalactosamine-specific lectins and its identification as g92K or gG. J. Gen. Virol. 67:737-744.

Olofsson, S., M. Milla, C. Hirschberg, C.E. De, and R. Datema. 1988. Inhibition of terminal N- and O-glycosylation specific for herpesvirus-infected cells: mechanism of an inhibitor of sugar nucleotide transport across Golgi membranes. Virology 166:440-450.

Oloffson, S., I. Sjoblam, M. Lundstrom, S. Jeannson, and E. Lycke. 1983. Glycoprotein

C of herpes simplex virus type 1: Characterization of O-linked oligosaccharides. J. gen. Virol. 64:2735-

Okazaki, K., E. Honda, T. Minetoma, and T. Kumagai. 1986. Mechanisms of neutralization by monoclonal antibodies to different antigenic sites on the bovine herpesvirus type 1 glycoproteins. Virology. 150:260-264.

Okasaki, K., T. Matsuzaki, Y. Sugahara, J. Okada, M. Hasebe, Y. Iwamura, M. Ohnishi, T. Kanno, M. Shimizu, E. Honda, et al., 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. Virology 181:666-670.

Opstelten, D.J., M.J. Raamsman, K. Wolfs, M.C. Horzinek, and P.J. Rottier. 1995. Envelope glycoproteins interactions in coronavirus assembly. J. Cell Biol. 131:339-349.

Orci, L, R. Montesano, P. Meda, F. Malaisse-Lagae, D. Brown, A. Perrelet, and P. Vassalli. 1981. Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. Proc. Natl. Acad. Sci. USA. 78:293-297.

Oroskar, A.A., and G.S. Read. 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. J. Virol. 61:604-606.

Oroskar, A.A., and G.S. Read. 1989. Control of mRNA stability by the virion host-shutoff function of herpes simplex virus. J. Virol. 63:1897-1906.

Osterrieder., N., A. Neubauer, C. Brandmuller, B. Braun, O.-R. Kaaden and J.D. Baines. 1996. The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus thpe 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. J. Virol. 70:4110-4115.

Otsuka, H. and X. Xuan. 1996. Construction of bovine herpesvirus-1 (BHV-1) recombinants which express pseudorabies virus (PRV) glycoproteins gB, gC, gD, and gE. Arch. Virol. 141:57-71.

Owens, R.J., J.W. Dubay, E. Hunter, and R.W. Compans. 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. Proc. Natl. Acad. Sci. USA. 88:3987-3991.

Pak, A.S., D.N. Everly, K. Knight, and G.S. Read. 1995. The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral proteins. Virology. 211:491-506.

Para, M.F., M.L. Parish, A.G. Noble, and P.G. Spear. 1985. Potent neutralizing antibodies associated with glycoprotein D specific monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. 55:483-488.

Para, M.F., R.B. Bauke, and P.G. Spear. 1980. Immunoglobin G (Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. 34:512-520.

Patel, A.H., and J.B. Maclean. 1995. The product of the UL6 gene of herpes simplex virus type 1 is associated with virus capsids. Virology. 206:465-478.

Patel, A.H., F.J. Rixon, C. Cunningham, and A.J. Davison. 1996. Isolation and characterization of herpes simplex virus type 1 mutant defective in the UL6 gene. Virology. 217: 111.123.

Paterson, R.G., and R.A. Lamb. 1987. Ability of the hydrophobic fusion related external domain of a paramyxovirus F protein to act as a membrane anchor. Cell. 48:441-452.

Peake, M.L., P. Nystrom and L.I. Pizer. 1982. Herpesvirus glycoprotein synthesis and insertion into plasma membranes. J. Virol. 42:678-690.

Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann. 1992a. Pseudorabies virus envelope glycoprtoeins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. 66:894-905.

Peeters, B., N. de Wind, R. Broer, A. Gielkens, and R. Moormann. 1992b. Glycoprotein H of pseudorabies virus is essential for entry and cell-to-cell spread of the virus. J. Virol. 66:3888-3892.

Peeters, B., J. Pol, A. Gielkens, and R. Moormann. 1993. Envelope glycoprotein gp50 of pseudorabies virus is essential for virus entry but is not required for viral spread in mice. J. Virol. 67:170-177.

Pelham, H. 1989. Control of protein exit from the endoplasmic reticulum. Annu. Rev. Cell Biol. 5:1-23.

Pelham, H.R.B. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. TIBS. 15:483-486.

Pelham, H.R.B. 1995. Sorting and retrieval between the endoplasmic reticulum and Golgi apparatus. Curr. Opin. Cell Biol. 7:530-535.

Pelham, H.R.B., and S. Munro. 1993. Sorting of membrane proteins in the secretory pathway. Cell. 75:603-605

Pellet, P.E., M.D. Biggin, B. Barrell, and B. Roizman. 1985. Epstein-Barr virus may encode a protein showing significant amino acid and predicted secondary-structure homology with glycoprotein B of herpes simplex virus type 1. J. Virol. 56:807-813.

Pellet, P.E., and J.B. Black. 1996. Human Herpesvirus 6. p. 2587-2608. In B.N. Fields,

D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Pellet, P.E., J.L.C. McKnight, F.J. Jenkins, and B. Roizman. 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing alpha genes. Proc. Natl. Acad. Sci. USA. 82:5870-5874.

Penfold, M.E.T., P. Armati, and A.L. Cunningham. 1994. Axonal transport of herpes simplex virions to epidermal cells: Evidence for a specialized mode of virus transport and assembly. Proc. Natl. Acad. Sci. 91:6529-6533.

Pepinsky, R.B., R. Tizard, R.J. Mattaliano, L.K. Sinclair, G.T. Miller, J.L. Browning, E.P. Chaw, C. Burne, K.-S. Huang, D.

Pereira, L., D. Dondero, B. Norrild, and B. Roizman. 1981. Differential immunologic and electrophoretic properties of glycoproteins gA and gB of HSV-2 produced in HEp-2 and Vero cells. Proc. Natl. Acad. Sci. USA. 78:5202-5206.

Pereira, L., D.V. Dondero, and B. Roizman. 1982. Herpes simplex virus glycoprtoein gA/B: Evidence that the infected Vero cell products co-map and arise by proteolysis. J. Virol. 44:88-97.

Perez, L., and L. Carrasco. 1994. Involvement of the vacuolar H(+)-ATPase in animal virus entry. J. Gen. Virol. 75:2595-2606.

Person, S., S. Laquerre, D. Prashant, and J. Hempel. 1993. Herpes simplex virus type 1 capsid protein, VP21, originates within the UL26 open reading frames. J. Gen. Virol. 74:2269-2273.

Person, S., R.W. Knowles, G.S. Read, S.C. Warner, and V.C. Bond. 1976. Kinetics of cell fusion induced by a syncytia-producing mutant of herpes simplex virus type 1. J. Virol. 17:183-190.

Person, S., K.G. Kousoulas, R.W. Knowles, G.S. Read, T.C. Holland, P.M. Keller, and S.C. Warner. 1982. Glycoprotein processing in mutants of HSV-1 that induced cell fusion. Virology 117:293-306.

Pertuiset, B., M. Boccara, J. Cebrian, N. Berthelot, S. Chousterman, F. Puvion-Dutilleul, J. Sisman, and P. Sheldric. 1989. Physical mapping and nucleotide sequence of a herpes simplex virus type 1 gene required for capsid assembly. J. Virol. 63:2169-2179.

Pessin, J.E., and M. Glaser. 1980. Budding of Rous sarcoma virus and vesicular stomatitis virus from localized lipid regions in the plasma membrane of chicken embryo fibroblasts. J. Biol. Chem. 255:9044-9050.

Petrovskis, E.A., A.L. Meyer, and L.E. Post. 1988. Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producin viral glycoprotein gp50. J. Virol. 62:2196-2199.

Petrovskis, E.A., J.G. Timmins, M.A. Armentrout, C.C. Marchioli, R.J. Yancey, and L.E. Post. 1986. DNA sequence of the gene from pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. 59:216-223.

Pettersson, R.F. 1991. Protein localization and virus assembly at intracellular membranes. Curr. Top. Microbiol. Immunol. 170:67-106.

Pfeffer, S., and J.E. Rothman. 1987. Biosynthetic protein transport and sorting by the encoplasmic reticulum and Golgi. Annu. Rev. Biochem. 56:829-852.

Pilling, A., A.J. davison, E.A.R. Telford and D.M. Meredith. 1994. The equine herpesvirus type 1 glycoprotein homologous to herps simplex virus type 1 glycoprotein M is a major constituent of the virus particle. J. Gen. Virol. 75:439-442.

Pizer, L.I., G.H. Cohen, and R.J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. J. Virol. 34:142-153.

Pogue-Geile, K.L., G.T.-Y. Lee, S.K. Shapira, and P.G. Spear. 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology 136:100-109.

Pogue-Geile, K.L., and P.G. Spear. 1987. The single base pair substitution responsible for the syn phenotype of herpes simplex virus type 1, strain MP. Virology 157:67-74.

Poliquin, L., G. Levine, and G.C. Shore. 1985. Involvement of the Golgi apparatus and a restructured nuclear envelope during biogenesis and transport of herpes simplex virus glycoproteins. J. Histochem. Cytochem. 33:875-883

Polvino-Bodnar, M., P.K. Orberg, and P.A. Schaffer. 1987. Herpes simplex virus 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. J. Virol. 61:3528-3535.

Poon, A.P. and B. Roizman. 1995. The phenotype *in vitro* and infected cells of herpes simplex virus 1 alpha trans-inducing factor (VP16) carrying temperature-sensitive mutations introduced by substitution of cysteines. J. Virol. 69:7658-7667.

Post, L.E., S. Mackem, and B. Roizman. 1981. Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell. 24:555-565.

Powell, K.L., and Watson D.H. 1975. Some structural antigens of herpes simplex virus type 1. J. Gen. Virol. 29:167-177.

Powell, K.L., and E. Littler, and D.J.M. Purifoy. 1981. Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. 39:894-902.

Pratt, L. Lachter, C. Hession, A.Z. Frey, and B.P. Wallner. 1988. Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. J. Biol. Chem. 263:10799-10811.

Preston, V.G., J.A. Coates, and F.J. Rixon. 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. 45:1056-1064.

Preston, C.M., M.C. Frame, and M.E.M. Campbell. 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell. 52:425-434.

Preston, V.G., F.J. Rixon, I.M. McDougall, M. McGregor, and M.F. al Kobaisi. 1992. Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. Virology. 186:87-98.

Purves, F.C., D. Spector, and B. Roizman. 1992. UL34 the target of the herpes simplex virus US3 protein kinase, is a membrane protein which in its unphosphorylated state associates with novel phosphoproteins. J. Virol. 66:4295-4303.

Purves, F.C., W.O. Ogle, and B. Roizman. 1993. Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. Proc. Natl. Acad. Sci. USA. 90:6701-6705.

Pyles, B.R., N.M. Sawtell, and R.L. Thompson. 1992. Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. J. Virol. 66:6706-6713.

Qingxue, L., S.M. Turk, and L.M. Hutt-Fletcher. 1995. The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells.
J. Virol. 69:3987-3994.

Rabouille, C., and T. Nilsson. 1995. Redrawing compartmental boundaries in the exocytic pathway. FEBS Lett. 369:97-100.

Radsak, K., K.H. Brucher, W. Britt, H. Shion, D. Schneider, and A. Kolbert. 1990. Nuclear compartmentalization of glycoprotein B of human cytomegalovirus. Virology 177:515-522.

Raja, N.U., M.J. Vincent, and M.A. Jabbar. 1993. Analysis of endoproteolytic cleavage and intracellular transport of human immunodeficiency virus type 1 envelope glycoproteins

using mutant CD4 molecules bearing the transmembrane endoplasmic reticulum retention signal. J. Gen. Virol. 74:2085-2097.

Rajcani, J., U. Herget, and H.C. Kaerner. 1990. Spread of herpes simplex virus (HSV) strains SC16, ANG, ANGpath and its glyC minus and glyE minus mutants in DBA-2 mice. Act Virol. 34:305-320.

Ramaswamy, R., and T.C. Holland. 1992. *In vitro* characterization of the HSV-1 UL53 Gene Product. Virology 186:579-587.

Rasile, L., K. Ghosh, K. Raviprakash, and H.P. Ghosh. 1993. Effects of deletions in the carboxy-terminal hydrophobic region of herpes simplex virus glycoprotein gB on intracellular transport and membrane anchoring. J. Virol. 67:2191-2201.

Rasmussen, L., J. Mullenax, M. Nelson, and T.C. Merigan. 1985. Human cytomegalovirus polypeptides stimulate neutralizing antibody *in vivo*. Virology. 145:186-190.

Rasmussen, L., M. Nelson, D.C. Kelsall, and T.C. Merigan. 1984. Murine monoclonal antibody to a single protein neutralizes the infectivity of human cytomegalovirus. Proc. Natl. Acad. Sci. USA. 81:876-880.

Rauh, I., F. Weiland, F. Fehler, G. Keil, and T.C. Mettenleiter. 1991. Pseudorabies virus mutants lacking the essential glycoprotein gII can be complemented by glycoprotein gI of bovine herpesvirus 1. J. Virol. 65:621-631.

Rauh I., and T.C. Mettenleiter. 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. 65:5348-5356.

Read, G.S., and N. Frenkel. 1983. Herpes simplex virus mutants defective in the virion shut-off of host polypeptide synthesis and exhibiting abnormal synthesis of alpha(immediate early) viral polypeptides. J. Virol. 46:498-512.

Read, G.S., S. Person, and P.M. Keller. 1980. Genetic studies of cell fusion induced by herpes simplex virus type 1. J. Virol. 35:105-113.

Ren, D., L.F. Lee, and P.M. Coussens. 1994. Identification and characterization of marek's disease virus genes homologous to ICP27 and glycoprotein K of herpes simplex virus-1. Virology. 204:242-260.

Reschke, M., B. Reis, K. Noding, D. Rohsiepe, A. Richter, T. Mockenhaupt, W. Garten, and K. Radsak. 1996. J. Gen. Virol. in press.

Richman, D.D., A. Buckmaster, S. Bell, C. Hodgman, and A.C. Minson. 1986. Identification of a new glycoprotein of herpes simplex virus type 1 and genetic mapping of the gene that codes for it. J. Virol. 57:647-655.

Rhim, J.S., H.Y. Cho, and R.J. Huebner. 1975. Non-producer human cells induced by murine sarcoma cells. Int. J. Cancer 15:23-29.

Rice, S.A., and D.M. Knipe. 1990. Genetic evidence for two distinct functions of the herpes simplex viurs alpha protein ICP27. J. Virol. 64:1704-1715.

Rice, S.A., M.C. Long, V. Lam, P.A. Schaffer, and C.A. Spencer. 1995. Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. J. Virol. 69:5550-5559.

Rickinson, A.B., and E. Kieff. 1996. Epstein-Barr Virus. p. 2397-2246. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Riggio, M.P., and D.E. Onions. 1993. DNA sequence of a gene cluster in the equine herpesvirus-4 genome which contains a newly identified herpsvirus gene encoding a membrane protein. Arch. Virol. 133:171-178.

Rixon, F.J. 1993. Strucutre and assembly of herpesviruses. Semin. Virol. 4:135-144.

Rixon, F.J., C. Addison and J. McLauchlan. 1992. Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. J. Gen. Virol. 73:277-284.

Rixon, F.J., A.M. Cross, C. Addison, and V.G. Preston. 1988. The products of herpes simplex virus type 1 gene UL26 which are involved in DNA packaging are strongly associated with empty by not with full capsids. J. Gen. Virol. 69:2879-2891.

Roberts, S.R., R.W. Compans, and G.W. Wertz. 1995. Respiratory syncytial virus matures at the apical surface of polarized epithelial cells. J. Virol. 69:2667-2673.

Roberts, S.R., M. Ponce deLeon, G.H. Cohen, and R.J. Eisenberg. 1991. Analysis of the intracellular maturation of the herpes simplex virus type 1 glycoprotein gH in infected and transfected cells. Virology 184:609-624.

Robertson, B.J., P.J. McCann III, L. Matusick-Kumar, W.W. Newcomb, J.C. Brown, R.J. Colonno, and M. Gao. 1996. Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids. J. Virol. 70:4317-4328.

Robertson, G.R., N.A. Scott, J.M. Miller, M. Sabine, M. Zheng, C.W. Bell, and J.M. Whalley. 1991. Sequence characteristics of a gene in equine herpesvirus 1 homologous to glycoprotein gH of herpes simplex virus. DNA Sequence 1:241-249.

Rodriguez, J.E. T. Moninger and C. Grose. 1993. Entry and Egress of varicella virus blocked by same anti-gH monoclonal antibody. Virology. 196:840-844.

Rodriguez, J.F., and G.L. Smith. 1990. IPTG-dependent vaccinia virus: identification of a virus protein (p14) enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. 18:5347-5351.

Rodriguez, M., and M. Dubois-Dalq. 1978. Intramembrane changes occurring during maturation of herpes simplex virus type 1: freeze-fracture study. J. Virol. 26:435-447.

Roizman, B. 1962. Polykaryocytosis induced by virus. Proc. Natl. Acad. Sci. USA. 48:228-234.

Roizman, B. 1963. The programming of herpes virus multiplication in doubly-infected and in puromycin-treated cells. Proc. Nat. Acad. Sci. USA. 49:165-171.

Roizman, B. 1990. Herpesviridae: a brief introduction, In Virology, 2nd Edition. p. 1787-1793. B.N. Fields (ed.), Raven Press, New York.

Roizman, B. 1996. Herpesviridae. p. 2221-2230. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Roizman, B., L.E. Carmichael, F. Deinhardt, G. de The, A.J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, K. Wolf. 1981. Herpesviriadae: definition, provisional nomenclature and taxonomy. Intervirology. 16:201-217.

Roizman, B., R.C. Desrosiers, B. Fleckenstein, C. Lopez, A.C. Minson, and M.J. Studdert. 1992. The family Herpesviridae: an update. Arch. Virol. 123:425-449.

Roizman, B. and D. Furlong. 1974. The replication of herpesviruses. p. 229-403. In Comprehensive Virology, Vol. 3. p. 229-403. H. Fraenkel-Conrat and R.R. Wagner, (eds). Plenum, New York.

Roizman, B., and P.G. Sears. 1990. Herpes simplex viruses and their replication. In Fields Virology, 2nd Edition. p. 1795-1841. B.N. Fields and D.M. Knipe, (eds.), Raven Press, New York.

Roizman, B., and A.E. Sears. 1996. Herpes simplex viruses and their replication. p. 2231-2295. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Roller, R.J., and B. Roizman. 1994. A herpes simplex virus 1 US11-expressing cell lines is resistant to herpes simplex virus infection at a step in viral entry mediated by glycoprotein D. J. Virol. 68:2196-2199.

Romanelli, M.G., E.M. Cattozzo, L. Faggioli, and M. Toggon. 1991. Fine mapping and characterization of the Syn6 locus in the herpes simplex virus type 1 genome. J. Gen. Virol. 72:1991-1995.

Romanelli, M.G., P. Mavromara-Nazos, D. Spector and B. Roizman. 1992. Mutational analysis of the ICP4 binding sites in the 5' transcrived noncoding domains of the herpes simplex virus 1 UL49.5 gamma-2 gene. J. Virol. 66:4855-4863.

Roop, C., L. Hutchinson, and D.C. Johnson. 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells and its particles lack glycoprotein H. J. Virol. 67:2285-2297.

Rose, J.D., and R.W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. Annu. Rev. Cell Biol. 4:257-288.

Rosenthal, K.S., J. Killius, C.M. Hodnichak, T.M. Venetta, L. Gyurgyik, and K. Janiga. 1989. Mild acidic pH inhibition of the major pathway of herpes simplex virus entry into HEp-2 cells. J. Gen. Virol. 70:857-867.

Rosenthal, K.L, J.R. Smiley, S. South, and D.C. Johnson. 1987. Cells expressing herpes simplex virus glycoprotein C, but not gB, gD, or gE, are recognized by murine virus-specific cytotoxic T lymphocytes. J. Virol. 61:2438-2447.

Rosen-Wolff, A., W. Lamade, C. Berkowitz, Y. Becker, and G. Darai. 1991. Elimination of UL56 gene by insertion of lacZ cassette between nucleotide position 116030 to 121753 of the herpes simplex virus type 1 genome abrogates intraperitoneal pathogenicity in tree shrews and mice. Virus Res. 20:205-221.

Ross, L., M. Sanderson, S. Scott, M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70:1789-1804.

Rothman, E. J.P. Albert, J.P. Geoff, and N. Frenkel. 1990. Putative site for the acquisition of hmuan herpesvirus 6 virion tegument. J. Virol. 64:6308-6313.

Ruhlig, M.A., and S. Person. 1977. Alterations of neutral glycolipids in cells infected with syncytium-producing mutants of herpes simplex virus type 1. J. Gen. Virol. 63:277-287.

Ruyechen, W.T., L.S. Morse, D.M. Knipe, and B. Roizman. 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. 29:677-687.

Sacks, W.R., C.C. Greene, D.P. Aschman, and P.A. Schaffer. 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. 55:796-805.

Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne. 1988. Bal4-VP16 is an unusually potent transcriptional activator. Nature. 335:563-564.

Samaniego, L.A., A.L. Webb, and N.A. DeLuca. 1995. Functional interactions between

herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. J. Virol. 69:5705-5715.

Sanbrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Ed. C. Nolan, Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Sanders, P.G., N.M. Wilkie, and A.J. Davison. 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. J. Gen. Virol. 63:277-287.

Sanders, V.J., A.E. Waddell, S.L. Felisan, X. Li, A.J. Conrad, and W.W. Tourtellotte. 1996. Herpes simplex virus in postmortem multiple sclerosis brain tissue. Arch. Neurol. 53:125-133.

Sarmiento, M., M. Haffey, and P.G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. 29:1149-1158.

Sarmiento, M., and P.G. Spear. 1979. Membrane proteins specified by herpes simplex viruses IV. Conformation of the virion glycoprotein designated VP7 (B2). J. Virol. 29:1159-1167

Sato, M., K. Sato, and A. Nakano. 1996. Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. J. Cell Biol. 134:279-293.

Sawitzky, D., H Hampl, and K.O. Habermehl. 1990. Comparison of herparin-sensitive attachment of pseudorabies virus (PRV) and herpes simplex virus type 1 and identification of heparin-binding PRV glycoproteins. J. Gen. Virol. 71:1221-1225.

Schaffer, P.A., J.P. Brunschwig, R.M. McComb and M. Benyesh-Melnick. 1974. Electron microscopic studies of temperature-sensitive mutants of herpes simplex virus type 1. Virology 62:444-457.

Schek, N., and S.L. Bachenheimer. 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. 55:601-610.

Schlesinger S., and M.J. Schlesinger. 1996. Togaviridae: The viruses and their replication. p. 825-840. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Schmaljohn, C.S. 1996. Bunyaviridae: The viruses and their replication. p. 1447-1472. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Schmelter, J., J. Knez, J.R. Smiley, and J.P. Capone. 1996. Identification and characterization of a small modular domain in the herpes simplex virus host shutoff protein sufficient for interaction with VP16. J. Virol. 70:2124-2131.

Schmelz, B. Sodeik, M. Ericsson, E.J. Wolffe, H. Shida, G. Hiller, and G. Griffiths. 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J. Virol. 68:130-147.

Schreurs, C. T.C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat. 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J. of Virol. 62:2251-2257.

Schutze, M.P., A. Peterson, and M.R. Jackson. 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. EMBO J. 13:1696-1705.

Schwartz, J., and B. Roizman. 1969. Concerning the egress of herpes simplex virus from infected cells. Electron microscope observations. Virology 38:42-49.

Sears, A.E., I.E. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus 1 mutant deleted in the alpha22 gene: Growth and gene expression, in permissive and restrictive cells and establishment of latency in mice. J. Virol. 55:338-346.

Sears, A.E., B.S. McGwire, and B. Roizman. 1991. Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. Proc. Natl. Acad. Sci. USA. 88:5087-5091.

Sechoy, O., J.R. Philippot, and A. Bienvenue. 1987. F protein-F protein interaction within the Sendai virus identified by native bonding or chemical cross-linking. J. Biol. Chem. 262:11519-11523.

Sekulovich, R.E., K. Leary, and R.M. Sandri-Goldin. 1988. The herpes simplex virus type 1 alpha protein ICP27 can act as a trans-repressor or a trans-activator in combination with ICP4 and ICP0. J. Virol. 62:4510-4522.

Semenza, J.C., K.G. Hardwick, N. Dean, and H.R.B. Pelham. 1990. ERD2 a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. Cell 61:1349-1357.

Serafini-Cessi, F., F. Dall'Olio, M. Scanavini, and G. Campadelli-Fiume. 1983. Processing of herpes simplex virus 1 glycans in cells defective in glycosyl transferase of the Golgi system: relationship to cell fusion and virion egress. Virology 131:59-70.

Serafini-Cessi, F., F. Dall'Olio, N. Malagolini, L. Pereira, and G. Campadelli-Fiume. 1988. Comparative study on O-linked oligosaccharides of glycoprotein D of herpes simplex virus types 1 and 2. J. Gen. Virol. 69:869-877.

Severi, B., M.P. Landini, M. Musiani, and M. Zerbini. 1979. A study of the passage of human cytomegalovirus from the nucleus to the cytoplasm. Microbiologica. 2:265-273.

Severi B., Severi B., M.P. Landini, and E. Govoni. 1988. Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases. Arch Virol. 98:51-64.

Shao, L., L.M. Rapp, and S.K. Weller. 1993. Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology. 196:146-162.

Sheldrick, P., and N. Berthelot. 1975. Inverted repititions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. 39:667-678.

Shepard, A.A. A.N. Imbalzano and N.A. DeLuca. 1989. Separation of primary structural components conferring autoregulation transactivation, and DNA-bindng properties to the herpes simplex virus transcriptional regulatory protein ICP4.

Sherman, G., and S.L. Bachenheimer. 1987. DNA processing in temperature-sensitive morpogenic mutants of HSV-1. Virology 163:471-480.

Shieh, M.-T. and P.G. Spear. 1994. Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. J. Virol. 68:1224-1228.

Shieh, M.-T., D. WuDunn, R.I. Montgomery, J.D. Esko, and P.G. Spear. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. 116:1273-1281.

Showalter, S.D., M. Zwieg, and B. Hampar. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. Infect. Immun. 34:684-692.

Silver, S., and B. Roizman. 1985. gamma 2-thymidine kinase chimeras are identically transcribed but regulated as gamma 2 genes in herpes simplex virus genomes and as beta genes in cell genomes. Mol. Cell Biol. 5:518-528.

Siminoff, P., and M.G. Menefee. 1966. Normal and 5-bromo-deoxyuridine-inhibited development of herpes simplex virus. An electron microscope study. Exp. Cell. Res. 44:241-255.

Slusarewicz, P., T. Nilsson, N. Hui, R. Watson, and G. Warren. 1994. Isolation of a matrix that binds medial Golgi enzymes. J. Cell. Biol. 124:405-413.

Smibert, C.A., B. Popova, P. Xiao, J.P. Capone, and J.R. Smiley. 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J. Virol. 68:2239-2246.

Smiley, J., B. Fong, and W.-C. Leung. 1981. Construction of a double jointed herpes simplex virus DNA molecule: inverted repeats are required for segment inversion and direct repeats promote deletions. Virology 113:345-362.

Smith, K.O. 1964. Relationships between the envelope and the infectivity of herpes simplex virus. Proc. Soc. Exp. Biol. Med. 115:814-816.

Smith S., and G. Blobel. 1993. The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. J. Cell Biol. 120:631-637.

Snoddy, D., D.C. Johnson, and H.P. Ghosh. 1990. *In vitro* expression and characterization of the HSV-1 syn gene product, abstr. 213. Abstr. 15th Int. Herpesvirus Workshop, Washington, D.C.

Snowden, B.W., and I.W. Halliburton. 1985. Identification of cross-reacting glycoproteins of four herpesviruses by western blotting. J. Gen. Virol. 66:2039-2044.

Sodeik, B., R.W. Doms, M. Ericsson, G. Hiller, C.E. Machamer, W. van 't Hof, G. van Meer, B. Moss, and G. Griffiths. 1993. Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. J. Cell Biol. 121:521-541.

Sodora, D.L., G.H. Cohen, and R.J. Eisenberg. 1989. Influence of asparagine-linked oligosaccharides on antigenicity, processing, and cell surface expression of herpes simplex virus type 1 glycoprotein D. J. Virol. 63:5184-5193.

Sodora, D.L., R. J. Eisenberg, and G.H. Cohen. 1991. Characterization of a recombinant herpes simplex virus which expresses a glycoprotein D lacking asparagine-linked oligosaccharides. J. Virol. 65:4432-4441.

Solheim, J.C., N.A. Johnson, B.M. Carreno, W.R. Lie, and T.H. Hansen. 1995. ß-2-microglobulin with an endoplasmic reticulum retention signal increases the surface expression of folded class I major histocompatibility complex molecules. Eur. J. Immunol. 25:3011-3016.

Solomon, K.A., A.K. Robbins, and L.W. Enquist. 1991. Mutations in the C-terminal hydrophobic domain of pseudorabies virus gIII affect both membrane anchoring and protein export. J. Virol. 65:5952-5960.

Solomon, K.A., A.K. Robbins, M.E. Whealy and L.W. Enquist. 1990. The putative cytoplasmic domain of the pseudorabies virus envelope glycoprotein gIII, the herpes simplex virus type 1 gC homolog, is not required for normal export and localization. J. Virol. 64:3516-3521.

Spaete, R.R. and E.S. Mocarski. 1985. The a sequence of the cytomegalovirus genome

functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. 54:817-824.

Spaete, R.R., K. Perot, P.I. Scott, J.A. Nelson, M.F. Stinski, and C. Pachl. 1993. Coexpression of truncated human cytomegalovirus gH with the Ul115 gene product or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. Virology. 193:853-861.

Spaete, R., R. Thayer, W. Probert, F. Masiarz, S. Chamberlain, L. Rasmussen, T. Merigan, and C. Pachl. 1988. Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage. Virology. 167:207-225.

Spear, P.G. 1976. Membrane proteins specified by herpes simplex virus type 1. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. 17:991-1008.

Spear, P.G. 1985. Glycoproteins specified by herpes simplex viruses, p. 315-356. In B. Roizman (ed.), The herpesviruses., Vol. 3. Plenum Publishing Corp., New York.

Spear, P.G. 1993. Membrane fusion induced by herpes simplex virus. p. 201-232. In J. Bentz (ed.), Viral Fusion Mechanisms. CRC Press, Boca Raton, Fla

Spear, P.G. 1993. Entry of alphaherpesviruses into cells. Semin. Virol. 4:167-180.

Spear, P.G., M.T. Shieh, B.C. Herold, D. WuDunn, and T.I. Koshy. 1992. Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. Adv. Exp. Med. Biol. 313:341-353.

Spear, P.G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9:143-159.

Spiess, M., Heads or tails - what determines the orientation of proteins in the membrane. FEBS Lett. 369:76-79.

Spivack, J.G., W.H. Prusoff, and T.R. Tritton. 1982. A study of the antiviral mechanism of action of 2-deoxy-D-glucose: normally glycosylated proteins are not strictly required for herpes simplex virus attachment but increase viral penetration and infectivity.

Srinivas, R.V., N. Balachandran, F.V. Alonso-Caplen, and R.W. Compans. 1986. Expression of herpes simplex virus glycoproteins in polarized epithelial cells. J. Virol. 58:689-693.

Stackpole, C.W. 1969. Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperature. J. Virol. 4:75-93.

Stannard, L.M., A.O. Fuller and P.G. Spear. 1987. Herpes simplex virus glycoproteins

associated with different morphological entities projecting from the virion envelope. J. Gen. Virol. 68:715-725.

Steineke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. 11:2407-2414.

Steinhart, W.L., C.M. Nicolet, and J.L. Howland. 1981. Incorporation of [32P]-phosphate into membrane phospholipids during infection of cultured human fibroblasts by herpes simpelx virus type 1. Intervirology. 16:80-85.

Stern, S., M. Tanaka, and W. Herr. 1989. The Oct-1 homeo-domain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature. 341:783-786.

Steven, A.C., C.R. Roberts, J. Hay, M.E., Bisher, T. Pun, and B. Trus. 1986. Hexavalent capsomers of herpes simplex virus type 2: symmetry, shape, dimensions, and oligomeric status. J. Virol. 57:578-584.

Stow, N.D. 1982. Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. EMBO J. 1:863-867.

Stow, N.D., E.C. McMonagle and A.J. Davison. 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. 11:8205-8220.

Stringer, K.F., C.J. Ingles, and J. Greenblatt. 1990. Direct and selective binding of an acidic transcriptional activation domain to the TAT-box factor TFIID. Nature. 345:783-786.

Strnad, B.C., T. Schuster, R. Klein, R.F. Hopkins III., T. Witmer, R.H. Neubauer, and H. Rabin. 1982. Production and characterization of monoclonal antibodies against the Epstein-Barr virus membrane antigen. J. Virol. 41:4683-4690.

Stuart, D. 1994. Docking mission accomplished. Nature. 371:19-20.

Subramanian, G., D.S. McClain, A. Perez, and A.O. Fuller. 1994. Swine testis cells contain functional heparan sulfate but are defective in entry of herpes simplex virus. J. Virol. 68:5667-5676.

Sullivan, D., G. Allen, and D. O'Callaghan. 1989. Synthesis and processing of equine herpesvirus type 1 glycoprotein 14. Virology. 173:638-646.

Sullivan, V., and G.L. Smith. 1987. Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: Neutralization of HSV-1 infectivity with anti-gG antibody. J. Gen. Virol. 68:2587-2598.

Swift, A.M., and C.E. Machamer. 1991. A golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. J. Cell Biol. 115:19-30.

Svennerholm, B., S. Jeansson, A. Vahlne, and E. Lycke. 1991. Involvement of glycoprotein C (gC) in adsorption of herpes simplex virus type 1 (HSV-1) to the cell. Arch Virol. 120:273-279.

Svennerholm, B., S. Olofsson, R. Lunden, A. Vahline, and E. Lycke. 1982. Adsorption and penetration of enveloped herpes simplex virus particles modified by tunicamycin or 2-deoxy-D-glucose. J. Gen. Virol. 63:343-349.

Szilagyi, J.F., and C. Cunningham. 1991. Identification and characterization of a novel noninfectious herpes simplex virus-related particle. J. Gen. Virol. 72:661-668.

Szczesna-Skorupa, E., K. Ahn, C.D. Chen, B. Doray, and B. Kemper. 1995. The cytoplasmic and N-terminal transmembrane domains of cytochrome P450 contain independent signals for retention in the endoplasmic reticulum. J. Biol. Chem. 270:24327-24333.

Talbot, P and J.D. Almeida. 1977. Human cytomegalovirus: purification of enveloped virions and dense bodies. J. Gen. Virol. 36:345-349.

Tal-Singer, R. R.J. Eixenberg, T. Valyi-Nagy, N.W. Fraser, and G.H. Cohen. 1994. N-linked oligosaccharides on herpes simplex virus glycoprotein gD are not essential for establishment of viral latency or reactivation in the mouse eye model. Virology. 202:1050-1053.

Tal-Singer, R., C. Peng, M. Ponce de Leon, W.R. Abrams, B.W. Banfield, F. Tufaro, G.H. Cohen, and R.J. Eisenberg. 1995. Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. J. Virol. 69:4471-4483.

Tang, I.T.-L., and D.H. Shepp. 1992. Herpes simplex virus infection in cancer patients: Prevention and Treatment.

Tashiro, M., J.T. Seto, S. Choosakul, H. Hegemann, H.D. Klenk, and R. Rott. 1992. Changes in specific cleavability of the Sendai virus fusion protein: implication for pathogenicity in mice. J. Gen. Virol. 73:1575-1579.

Tashiro, M., M. Yamakawa, K. Tobita, H.-D. Klenk, R. Rott, and J. Seto. 1990. Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. J. Virol. 64:3627-3634.

Tashiro, M., M. Yamakawa, K. Tobita, H.-D. Klenk, J. Seto. and R. Rott. 1992. Significance of basolateral domain of polarized MDCK cells for Sendai virus-induced cell fusion. Arch. Virol. 125:129-139.

Tatman, J.D., V.G. Preston, P. Nicholson, R.M. Elliot, and F.J. Rixon. 1994. Assembly of herpes simplex virus type 1 caspids using a panel of recombinant baculoviruses. J. Gen. Virol. 75:1101-1113.

Taylor, H.P. and N.R. Cooper. 1990. The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. J. Virol. 64:2484-2490.

Telford, E.A.R., M.S. Watson, H.C. Aird, J. Perry and A.J. Davison. 1995. The DNA sequence of Equine herpesvirus 2. J. Mol. Biol. 249:520-528.

Telford, E.A., M.S. Watson, K. McBride and A.J. Davison. 1992. The DNA sequence of equine herpesvirus-1. Virology 189:304-316

Tengelsen, N.E. Pederson, P.R. Shaver, M.W. Wathen and F.L. Homa. 1993. Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J. Virol. 67:3470-3480.

Tenney, D.J., A.K. Sheaffer, W.W. Hurlburt, M. Bifano and R.K. Hamatake. 1995. Sequence-dependent primer synthesis by the herpes simplex virus helicase-primase complex. J. Biol. Chem. 270:9129-9136.

Thaker, S.R., D.L. Stine, T.J. Zamb and S. Srikumaran. 1994. Identification of a putative cellular receptor for bovine herpesvirus 1. J. Gen. Virol. 75:2303-2309.

Thomas, D.C., C.B. Brewer, and M.G. Roth. 1993. Vesicular stomatitis virus glycoprotein contains a dominant cytoplasmic basolateral sorting signal critically dependent upon a tyrosine. J. Biol. Chem. 268:3313-3320.

Thompson, C.C., and S.L. McKnight. 1992. Anatomy of an enhancer. Trends Genet. 8:232-236.

Thomsen, D.R., W.W. Newcomb, J.C. Brown, and F.L. Homa. 1995. Assembly of the herpes simplex virus capsid: requirement for the carboxy-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. J. Virol. 69:3690-3703.

Thomsen, D.R., L.L. Roof, and F.L. Homa. 1994. Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. J. Virol. 68:2442-2457.

Tikoo, S.K. D.R. Fitzpatrick, L.A. Babiuk, and T.J. Zamb. 1990. Molecular cloning, sequenceing, and expression of functional bovine herpesvirus 1 glycoprotein gIV (gD) in transfected bovine cells. J. Virol. 64:5132-5142.

Tikoo, S.K. M. Campos, and L.A. Babiuk. 1995. Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. Adv. Virus Res. 45:191-223.

Tognon, M. D. Furlong, A.J. Conley. and B. Roizman. 1981. Molecular genetics of herpes simplex virus. V. Characterization of a mutant defective in ability to form plaques at low temperatures and ina viral function which prevents accumulation of coreless capsids at nuclear pores late in infection. J. Virol. 40:870-880.

Tognon, M., R. Guandalini, M.G. Romanelli, R. Manservigi, and B. Trevisani. 1990. Phenotypic and genotypic characterization of locus syn 5 in herpes simplex virus 1. Virus Research. 18:135-150.

Toh, Y., S. Tanaka, Y. Liu, Y. Hidaka, and R. Mori. 1993. Molecular characterization of naturally occuring glycoprotein C-negative herpes simplex virus type 1. Arch. Virol. 129:119-130.

Tomazin, R., A.B. Hill, P. Jugovic, I. York, P. van Endert, H.L. Ploegh, D.W. Andrews, and D.C. Johnson. 1996. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. EMBO. 15:3256-3266.

Tooze, J., S.A. Tooze, and G. Warren. 1984. Replication of coronavirus MHV-A59 in sac cells: determination of the first site of budding of progeny virions. Eur. J. Cell Biol. 33:281-293.

Tooze, J., S.A. Tooze, and G. Warren. 1988. Site of addition of N-acetyl-galactosamine to the E1 glycoprotein of mouse hepatitis virus-A59. J. Cell Biol. 106:1475-1487.

Torrisi, M.R., C. Di Lazzara, A. Pavan, L. Pereira, and G. Campadelli-Fiume. 1992. Herpes simplex virus envelopment and maturation studied by fracture label. J. Virol. 66:554-561.

Torrisi, M.R., M. Cirone, A. Pavan, C. Zompetta, G. Barile, L. Frati and A. Faggioni. 1989. Localization of Epstein-Barr virus envelope glycoproteins on the inner nuclear membrane of virus-producing cells. J. Virol. 63:828-832.

Triezenberg, S.J., K.L. LaMarco, and S.L. McKnight. 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. 2:730-742.

Trus, B.L., F.L. Homa, F.P. Booy, W.W. Newcomb, D.R. Thomsen, N. Cheng, J.C. Brown and A.C. Steven. 1995. Herpes simplex virus capsids assembled in insect cells infected with recombinant baculoviruses: structural authenticity and localization of VP26.

Tugizov, S., E. Maidji, and L. Pereira. 1996. Role of apical and basolateral membranes in replication of human cytomegalovirus in polarized retinal pigment epithelial cells. J. Gen. Virol. 77:61-74.

Tugizov, S., D. Navarro, P. Paz, Y. Wang, I. Qadri, and L. Pereira. 1994. Function of human cytomegalovirus glycoprotein B: syncytium formation in cells constitutively

expressing gB is blocked by virus-neutralizing antibodies. Virology. 201:263-276.

Uprichard, S.L., and D.M. Knipe. 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J. Virol. 70:1969-1980.

Van Drunen Littel-van den Hurk, S., T. Zamb, and L.A. Babiuk. 1989. Synthesis, cellular location, and immunogenicity of bovine herpesvirus 1 glycoproteins gI and gIII expressed by recombinant vaccinia virus. J. Virol. 63:2159-2168.

Van Genderen, I.L., R. Brandimarti, M.R. Torrisi, G. Campadelli and G. van Meer. 1994. The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. Virology. 200:831-836.

Varmuza, S.L., and J.R. Smiley. 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell. 41:793-802.

Vey, W. Schafer, B. Reis, R. Ohuchi, W. Britt, W. Garten, H.D. Klenk, and K. Radsak. 1995. Proteolytic processing of human cytomegalovirus glycoprotein B (gp UL55) is mediated by the human endoprotease furin. Virology. 206:746-749.

Vincent, M.J., N.U. Raja, and M.A. Jabbar. 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of chimeric envelope glycoproteins bearing the cytoplasmic and anchor domains of CD4: role of the cytoplasmic domain in Vpu-induced degradation in the endoplasmic reticulum. J. Virol. 67:5538-5549.

Visalli, R.J., and C.R. Brandt. 1991. The HSV-1 UL45 gene product is not required for growth in Vero cells. Virology 185:419-423.

Visalli, R.J., and C.R. Brandt. 1993. The HSV-1 UL45 18-kDa gene product is a true late protein and a component of the virion. Virus Res. 29:167-178.

Vlazny, D.A., and N. Frenkel. 1981. Replication of herpes simplex virus DNA: localization of replication recognition signals within defective virus genomes. Proc. Natl. Acad. Sci. USA. 78:742-746.

Vlanzy, D.A., A. Kwong, and N. Frenkel. 1982. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. Proc. Natl. Acad. Sci. USA. 79:1423-1427.

Wadsworth, S., R.J. Jacob, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA II. Size, composition, and arrangement or inverted terminal repititions. J. Virol. 15:1487-1497.

Wagner, R.R., and J.K. Rose. 1996. Rhabdoviridae: The viruses and their replication. p. 1121-1135. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd

ed. Lippincott-Raven Publishers, Philadelphia.

Wahlberg, J.M., and H. Garoff. 1992. Membrane fusion process of Semilki Forest virus. I. Low pH-induced rearrangement in spike protein quaternary structure preceds virus penetration into cells. J. Cell Biol. 116:339-348.

Walev, I., K. Weise and D. Falke. 1991a. Differentiation of herpes simplex virus-induced fusion from without and fusion from within by cyclosporin A and compound 48/80. J. Gen. Virol. 72:1377-1382.

Walev, I., C. Wollert, K. Weise, and D. Falke. 1991b. Characterization of fusion from without induced by herpes simplex virus. Arch. Virol. 117:29-44.

Walter, P. and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. Methods in Enzymology 96:84-93.

Ward, P.L., D.E. Barker and B. Roizman. 1996. A novel herpes simplex virus 1 gene, UL43.5, maps antisense to the UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. J. Virol. 2674-2690.

Ward, P.L., G. Campadelli-Fiume, E. Avitabile, and B. Roizman. 1994. Localization and putative function of the UL20 membrane protein in cells infected with herpes simplex virus 1. J. Virol. 68:7406-7417.

Ward P.L. and B. Roizman. 1994. Herpes simplex genes: the blueprint of a successful human pathogen. Trends in Genetics. 10:267-274.

Wathen, M.W., and L.M.K. Wathen. 1984. Isolation, characterization, and physical mapping of a pseudorabies virus mutant containing antigenically altered gp50. J. Virol. 51:57-62.

Watson, R.J., and J.B. Clements. 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature. 285:329-330.

Weber, P.C., M. Levine, and J.C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science 236:576-579.

Weinheimer, S.P., B.A. Boyd, S.K. Durham, J.L. Resnick and D.R. O'Boyle II. 1992. Deletion of the VP16 open reading frame of herpes simplex virus type 1. J. Virol. 66:258-269.

Weinheimer, S.P., P.J. McCann III, D.R. O'Boyle II, J.T. stevens, B.A. Boyd, D.A. Drier, G.A. Yamanaka, C.L. DiIanni, I.C. Deckman, and M.G. Cordingley. 1993. Autoproteolysis of herpes simplex virus type 1 portease releases an active catalytic domain found in intermediate capsid particles. J. Virol. 67:5813-5822.

Weise, K., H.C. Kaerner, J. Glorioso, and C.H. Schroder. 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J. Gen. Virol. 68:1909-1919.

Weiss, C.P., J.A. Levy, and J.M. White. 1990. Oligomeric organization of gp120 on infectious immunodeficiency virus type 1 particles. J. Virol. 64:5674-5677.

Weiss, C.D., and J.M. White. 1993. Characterization of stable chinese hamster ovary cells expressing wild-type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. 67:7076-7066.

Weisz, OA., A.M. Swift, C.E. Machamer. 1993. Oligomerization of a membrane protein correlates with its retention in the Golgi complex. J. Cell Biol. 122:1185-1196.

Weller, S.K., K.J. Lee, D.J. Sabourin, and P.A. Schaffer. 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. 45:354-366.

Weller, S.K., A. Spadaro, J.E. Schaffer, A.W. Murray, A.M. Maxam, and P.A. Schaffer. 1985. Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. 5:930-942.

Wenske, E.A., M.W. Bratton and R.J. Courtney. 1982. Endo-B-N-acetylglucosaminidase H sensitivity of precursors to herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. 44:241-248.

Whalley, J.M., G. Robertson, N. Scott, G. Hudson, C. Bell, and L. Woodworth. 1989. Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. J. Gen. Virol. 70:383-394.

Whealy, M.E., K. Baumeister, A.K. Robbins and L.W. Enquist. 1988. A herpesvirus vector for expression of glycosylated membrane antigens: fusion proteins of pseudorabies virus gIII and human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. 62:4185-4194.

Whealy, M.E., J.P. Card, R.P. Meade, A.K. Robbins and L.W. Enquist. 1991. Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. 65:1066-1081.

Whealy, M.E., J.P. Card, A.K. Robbins, J.R. Dubin, H.-J. Rzihz, and L.W. Enquist. 1993. Specific psuedorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. 67:3786-3797.

Whealy, M.E., A.K. Robbins and L.W. Enquist. 1989. Replacement of the pseudorabies

virus glycoprotein gIII gene with its postulated homolog, the glycoprotein gC gene of herpes simplex virus type 1. J. Virol. 63:4055-4059.

Whealy, M., A. Robbins and L.W. Enquist. 1990. The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. J. Virol. 64:1946-1955.

Whealy, M.E., A.K. Robbins, F. Tufaro, and L.W. Enquist. 1992. A cellular function is required for pseudorabies virus envelope glycoprotein processing and virus egress. J. Virol. 66:3803-3810.

White, J.M. 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. 52:675-697.

Whitley, R.J. 1996. Herpes Simplex Viruses. p. 2297-2342. In B.N. Fields, D.M. Knipe, P.M. Howley et al. (ed.), Fields Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Whitley, R.J., and F. Lakeman. 1995. Herpes simplex virus infections of the central nervous system: Therapeutic and diagnostic considerations. Clin. Infect. Diseases. 20:414-420.

Wildy, P., W.C. Russell and R.W. Horne. 1960. The morphology of herpes virus. Virology. 12:204-222.

Wiley, D.C., and J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotien of influenza virus. Annu. Rev. Biochem. 56:365-373.

Wilson, A.C., K. LaMarco, M.G. Peterson, and W. Herr. 1993. The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein. Cell. 74:115-125.

Wilson, D.W., N. Davis-Poynter and A.C. Minson. 1994. Mutations in theh cytoplasmic tail of herpes simplex virus glycoprotein H suppress cell fusion by a syncytial strain. J. Virol. 68:6985-6993.

Witmer, L.A., K.L. Rosenthal, F.L. Graham, H.M. Friedman, A.Yee, and D.C. Johnson. 1990. Cytotoxic T lymphocytes specific for herpes simplex virus (HSV) studied using adenovirus vectors expressing HSV glycoproteins. J. Gen. Virol. 71:387-396.

Wittels, M., and P.G. Spear. 1991. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res. 18:271-290.

White, J.M. 1992. Membrane fusion. Science. 258:917-924.

Wolfer, U., V. Kruft, D. Sawitzky, H. Hampl, B. Wittmann-liebold, and K.-O. Habermehl.

1990. Processing of pseudorabies virus glycoprotein gII. J. Virol. 64:3122-3125.

Wong, S.H., and W. Hong. The SXYQRL sequence in the cytoplasmic domain of TGN38 plays a major role in trans-Golgi network localization. J. Cell Biol. 120:1123-1135.

Wright, J.F., A. kurosky, and S. Wasi. 1994. An endothelial cell-surface form of annexin II binds human cytomegalovirus. Biochem. Biophys. Res. Commun. 198:983-989.

Wu, C.A., N. Nelson, D. McGeoch and M.D. Chalberg. 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. 62:435-443.

WuDunn, D. and P.G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63:52-58.

Xiao, H., A. Pearson, B. Coulombe, R. Truat, S. Zhang, J.L. Regier, S.L Triezenberg, D. Reinberg, O. Flores, C.J. Ingles, and J. Greenblatt. 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol. Cell. Biol. 14:7013-7024.

Xiao, P., and J.P. Capone. 1990. A cellular factor binds to the herpes simplex virus type transactivator Vmw65 and is required for Vmw65-dependent protein-DNA comples assembly with Oct-1. Mol. Cell. Biol. 10:4974-4977.

Yamamoto, N. Hamada, and M. Shingu. 1993. Two novel genes of herpes simplex virus type 1 involved in cell fusion. Kurume Med. J. 40:169-175.

Yamanishi, K., T. Okuno, K. Shiraki, M. Takahashi, T. Kondo, T. Asano, and T. Kurata. 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1:1065-1067.

Yang, Y.-Y., and R.J. Courtney. 1995. Influence of the host cell on the association of ICP4 and ICP0 with herpes simplex virus type 1. Virology. 211:209-217.

Yao F., and P.A. Schaffer. 1994. Physical interaction between the herpes simplex virus type 1 immediate-early regulatory proteins ICP0 and ICP4. J. Virol. 68:8158-8168.

Yawsen, L. R., E.B. Stephens, L.C. Davenport, and L.M. Hutt-Fletcher. 1993. epstein-Barr virus glycoprotein gp85 associates with the BKRF2 gene product and is incompletely processed as a recombinant protein. Virology 195:387-396.

Yoshida, L.F. Lee, N. Yanagida, and K. Nazerian. 1994. Identification and characterization of a Marek's disease virus gene homologous to glycoprotein L of herpes simplex virus. Virology. 204:414-419.

Yoshida, M., F. Uno, Z.L. Bai, M. Yamada, S. Nii, T. Sata, T. Kurata, K. Yamanishi, and

M. Takahashi. 1989. Electron microscopic study of a herpes-type virus isolated from an infant with exanthem subitum. Microbiol. Immunol. 33:147-154.

Yoshida, S., L.F. lee, N. Yanagida, and K. Nazerian. 1994. Mutational analysis of the proteolytic cleavage site of glycoprotein B (gB) of Marek's disease virus. Gene. 150:3030-306.

Yuhasz, S.A., and J.G. Stevens. 1993. Glycoprotein B is a specific determinant of herpes simplex virus type 1 neuroinvasiveness. J. Virol. 67:5948-5954.

Zelus, B.D., R.s. Stewart, and J. Ross. 1996. The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity *in vitro*. J. Virol. 70:2411-2419.

Zelnik, V. 1995. Marek's disease and new approaches to its control. Acta Virol. 39:53-63.

Zhang, Y. and J.L.C. McKnight. 1993. Herpes simplex virus type 1 UL46 and UL47 deletion mutants lack VP11 and VP12 or VP13 and VP14, respectively, and exhibit altered thymidine kinase expression. J. Virol. 67:1482-1492.

Zhao, Y., R. Holden, R.N. Harty and D.J. O'Callaghan. 1992. Identification and transcriptional analyses of the UL3 and UL4 genes of equine herpesvirus 1, homologs of the ICP27 and glycoprotein K genes of herpes simplex virus. J. Virol. 66:5363-5372.

Zheng, B., F.L. Graham, D.C. Johnson, T. Hanke, M.R. McDermott, L. Prevec. 1993. Immunogenicity of tandem repeats of a linear neutralizing epitope of the herpes simplex protein which expressed in recombinant Adenovirus vectors. Submitted for publication.

Zhou, Z.H., J. He, J. Jakana, J.D. Tatman, F.J. Rixon, and W. Chiu. 1995. Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. Nature Struct. Biol. 2:1026-30.

Zhou, Z.H., B.V.V. Prasad., J. Jakana, F. Rixon, and W. Chiu. 1994. Protein subunit structures in the herpes simplex virus A-capsid determined from 400 kV spot scan electron cryomicroscopy. J. Molec. Biol. 242:458-469.

Zhu, Q. and R.J. Courtney. 1988. Chemical cross-linking of glycoproteins on the envelope of herpes simplex virus. Virology 167:377-384.

Zhu, Q. and R.J. Courtney. 1994. Chemical cross-linking of virion envelope and tegument proteins of herpes simplex virus type 1. Virology 204:590-599.

Zhu, Z., W. Cai, and P.A. Schaffer. 1994. Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0. J. Virol. 68:3027-3040.

Zhu, Z., M. Gershon, R. Ambron, C. Gabel, and A. Gershon. 1995a. Infection of cells by varicella zoster virus: inhibition of viral entry by mannose 6-phosphate and heparin. Proc. Natl. Acad. Sci. USA. 92:3546-3550.

Zhu, Z., M.D. Gershon, U. Hao, R.T. Ambron, C.A. Gabel, and A.A. Gershon. 1995. Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. J. Virol. 69:7951-7959.

Zhu, Z., U. Hao, M.D. Gershon, R.T. Ambron, and A.A. Gershon. 1996. Targeting of glycoprotein I (gE) of varicella-zoster virus to the trans-Golgi network by an AYRV sequence and an acidic amino acid-rich patch in the cytoplasmic domain of the molecule. J. Virol. 70:6563-6575.

Zhu, Z, and P.A. Schaffer. 1995. Intracellular localization of the herpes simplex virus type 1 major transcriptional regulatory protein, ICP4, is affected by ICP27. J. Virol. 69:49-59.

Zsak, L., N. Sugg, T. Ben-Porat, A.K. Robbins, M.E. Whealy, and L.W. Enquist. 1991. The gIII glycoprotein of pseudorabies virus is involved in two distinct steps of virus attachment. J. Virol. 65:4317-4324.

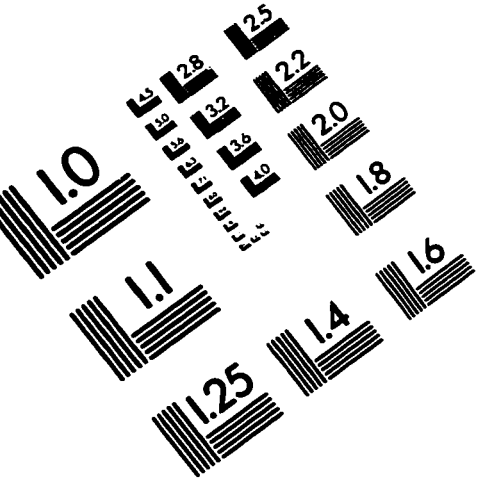
Zsak, L.F., Zuckermann, N. Sugg, and T. Ben-Porat. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. 66:2316-2325.

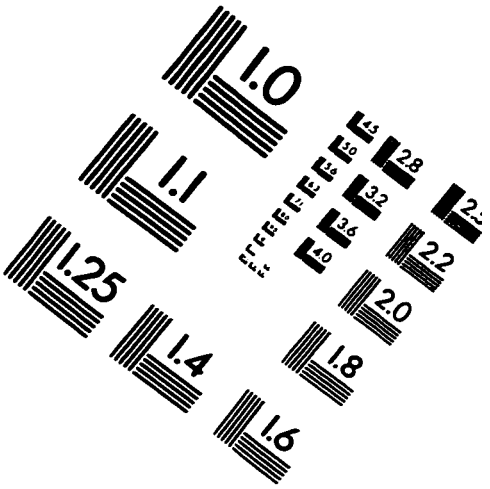
Zuckermann F.A., T.C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat. 1988. Complex between glycoproteins gI and gp63 of pseudrabies virus: its effect on virus replication. J. Virol. 62:4622-4626.

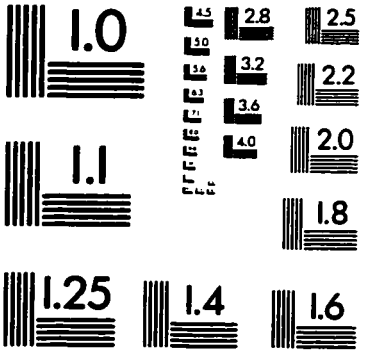
Zezulak, K.M., and P.G. Spear. 1984. Mapping of the structural gene of the herpes simplex virus type 2 counterpart of herpes simplex virus type 1 glycoprotein C and identification of a type 2 mutant which does not express this glycoprotein. J. Virol. 49:741-747.

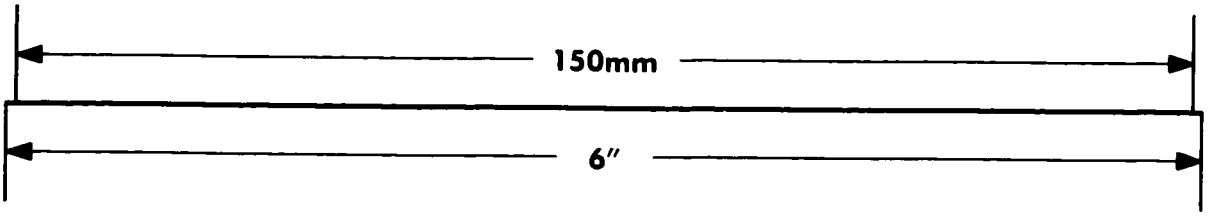
Zurzolo, C., M.P. Lisanti, I.W. Caras, L. Nitsch, and E. Rodriguez-Boulan. 1993. Glycosylphosphatidylinositol-anchored proteins are preferentially targeted to the basolateral surface in Fischer rat thyroid epithelial cells. J. Cell Biol. 121:1031-1039.

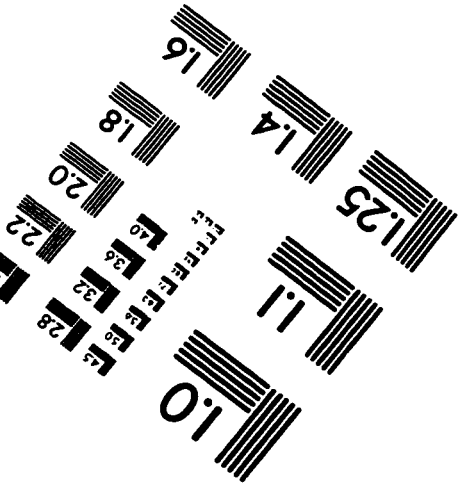
IMAGE EVALUATION TEST TARGET (QA-3)

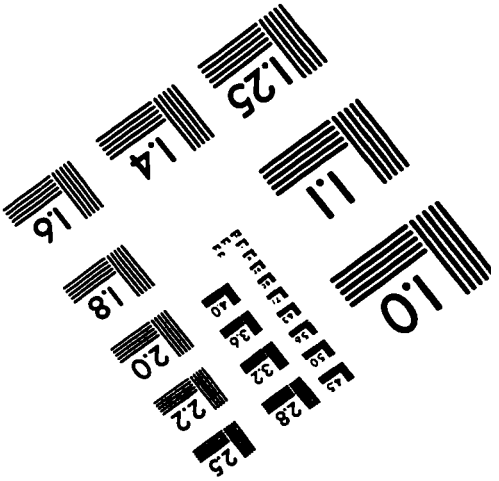












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