INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600

THE ROLE OF GLYCOPROTEINS gE AND gI IN HERPES SIMPLEX VIRUS CELL-TO-CELL SPREAD

By
KEVIN S. DINGWELL, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

CELL-TO-CELL SPREAD OF HSV

DOCTOR OF PHILOSOPHY
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario, Canada

TITLE: The role of glycoproteins gE and gI in herpes simplex virus cell-to-cell spread

AUTHOR: Kevin S. Dingwell, B.Sc. (University of Ottawa)

SUPERVISOR: Dr. David C. Johnson

NUMBER OF PAGES: xvii, 229

ABSTRACT

Herpes simplex viruses (HSV) infect and spread through the mucosal and submucosal epithelium and can establish a latent infection in the peripheral nervous system. Under appropriate conditions, HSV may reactivate from the latent state, producing secondary disease near the site of the primary infection. A family of HSV encoded glycoproteins found within the virion envelope regulate and promote entry and spread of virus between susceptible cells. I have shown that a complex of the HSV glycoproteins E and I, were required for cell-to-cell spread of HSV across cellular junctions *in vivo* and *in vitro*, in a manner resistant to anti-HSV antibody neutralization. HSV gE^- and gI^- mutant viruses spread poorly in the corneal epithelium of mice and rabbits when compared to wild type HSV, and mutant viruses failed to spread to the brain and cause encephalitis. gE and gI were not required for entry of extracellular virus. Both gE^- and gI^- mutants produced similar yields of HSV compared to wild type HSV following a single round of viral replication, but when forced to spread to neighbouring cells, viral yields were 10-100 fold lower than wild type virus. These data suggest that HSV gE^- and gI^- mutant are unable to efficiently spread from cell-to-cell, and that the gE/gI complex facilitates transfer of HSV across cellular junctions. Since HSV infects neurons and apparently spreads across synaptic junctions, it was of interest to determine whether gE/gI were also

important for transneuronal transfer of HSV. gE^- and gI^- mutant viruses were markedly restricted in their ability to spread within the retina and to the major retinorecipient regions of the brain following injection into the vitreous body of the rat eye. In contrast, wild type virus spread rapidly and infected the majority of the neurons within the retina, and efficiently infected neurons within the brain. Together, these results demonstrated that gE/gI plays a significant role in promoting spread of HSV between cells.

To determine the molecular mechanism of how gE/gI facilitates cell-to-cell transmission of HSV, I examined the subcellular distribution of gE/gI in polarized human epithelial cells. Transport of gE to the cell surface required the coexpression of gI. At the surface of polarized cells, gE/gI accumulated along the lateral plasma membrane, with little or no gE/gI on the apical or basal plasma membrane domains. When gE/gI was expressed in subconfluent epithelial monolayers, gE/gI was found only at junctions between cells and not on those lateral surfaces not in contact with another cell. Together, these data support a model in which the gE/gI complex accumulates at cell junctions, perhaps by interacting with junctional components, and in so doing, mediates transfer of HSV across cell junctions.

ACKNOWLEDGEMENTS

"On fait la science avec des faits, comme on fait une maison avec des pierres; mais une accumulation de faits n'est pas plus une science qu'un tas de pierres n'est une maison."

Henri Poicare

I found this quote sometime during the last year of my undergraduate studies at the University of Ottawa. Six years have passed and I am beginning to understand what M. Poicare meant. I have my supervisor Dr. David Johnson and his colleagues at McMaster University and Oregon Health Sciences University to thank for helping me become a scientist. Special thanks to Linda Bonen and Jim Fenwick for getting me started in science. A special thanks to Mary Tang and Andrew Rainbow, who came to us with their observations that HSV gI mutants displayed a small plaque phenotype, and therefore got the ball rolling. I would also like to thank my lab mates and friends who have made my tenure as a graduate student a pleasure: Cindy Roop Beauchamp, Jessica Boname, Craig Brunetti, Peter Cheung, Chris Counter, Lloyd Hutchinson, Craig Smibert, Frank Jones, Pieter Jugovic, Steve Primorac, Roman Tomazin, Ian York , and all the members of the Natural Killers (a pathetic attempt at a softball team, but great fun nonetheless). In addition, I would like to thank Jay Nelson and the members of his lab at OHSU, especially Ken Fish and Andrew Townsend.

The completion of this thesis is due in part the help, guidance, support and

love of my family. Thanks to Mum and Pops, my big brother Graham, and my sister Hillary. Finally, I'd like to thank my favourite lab partner and my wife, Jessica.

Table of Contents

List of Figures

Figure

List of Tables

Tables

List of Abbreviations

α-MEM	alpha minimal essential medium
A	deoxyadenylate
Ad	Adenovirus
BHV	bovine herpes virus
BSA	bovine serum albumin
C	carbon
Ca	calcium
CAM	cell adhesion molecule
CEV	cell-associated enveloped virus
CHO	Chinese hamster ovary
CKII	casein kinase II
CNS	central nervous system
Cl	Cloride
CPE	cytopathic effect
DAB	3,3'-diaminobenzidine tetrahydrochloride dihydrate
DMEM	Dulbecco's modified minimal essential medium
DNA	Deoxyribonucleic acid
DOC	deoxyribonucleic acid
EBV	Epstein-Barr virus
EEV	extracellular enveloped virus
ER	endoplasmic reticulum
FBS	fetal bovine serum
FcR	Fc receptor
FHV	feline herpes virus

FITC	fluorescein isothiocyanate
G	deoxyguanylate
GAGs	glycosaminoglycans
hr	hour
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
hgg	human gamma globulin
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule 1
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IEV	intracellular enveloped virus
IMV	intracellular mature virus
HSV	herpes simplex virus
HVEM	herpes virus entry mediator
K	potassium
k_d	equilibrium dissociation constant
kbp	kilo base pairs
L	leucine
LATs	latency associated transcripts
LD_{50}	50% lethal dose
LFA	leucocyte function-associated antigen
LGN	lateral geniculate nucleus
M-6-P	mannose 6-phosphate
MPRs	mannose 6-phosphate receptors
MAb	monoclonal antibody

MDBK	Madin-Darby Bovine Kidney
MOI	multiplicity of infection
μCi	micro Curies
μg	micro gram
μl	micro litre
μM	micro molar
min	minutes
ml	mililitres
mM	milimolar
M	molar
N	asparagine
Na	sodium
nm	nanometers
NP40	Nonidet P-40
ORF	open reading frame
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PFU	plaque forming unit
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PrV	pseudorabies virus
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SC	superior colliculus
SCN	suprachiasmatic nucleus
TGN	trans Golgi network
TNG/NGF	tumor necrosis factor/nerve growth factor

Tris	tris(hydroxymethyl)aminomethane
TX100	Triton X-100
UL	unique long
US	unique short
w/v	weight/volume
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
W	tryptophane
X	any amino acid
Y	tyrosine

1 INTRODUCTION

1.1 *Herpesviridae*

Members of the *Herpesviridae* family are distributed throughout the animal kingdom, with over 100 viruses isolated to date (reviewed in Roizman 1996). There are currently 8 herpesviruses that infect humans; these include herpes simplex virus type 1 and 2 (HSV-1, -2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpes virus 6 and 7 (HHV6, 7), and the more recently identified HHV8 or KSHV (Kaposi's sarcoma (KS) associated herpesvirus) (Cesarman et al., 1995; Chang et al., 1994). In non-immunocomprimised individuals, herpesvirus infections are relatively benign (e.g., herpes labialis), yet in some instances can be life threatening (herpes encephalitis).

All herpesviruses have a common virion structure composed of an inner DNA core packaged within a proteinaceous capsid that, in turn, is surrounded by a tegument and lipid envelope. The double stranded linear DNA genome varies between 120 to 230 kbp (kilo base pairs) and can have a high % base composition of G + C 75 mole %. The icosahedral capsid exhibits a 5:3:2 symmetry constructed from equilateral triangles. Twelve pentavalent capsomers make up each of the vertices; 60 hexamers form the 20 faces, with 90 hexamers at the 30 edges for a total of 162 capsomers. Through the center of the long axis runs a 4 nm channel. The capsid is surrounded by an amorphous material called

the tegument that contains proteins required for the initiation of viral DNAtranscription and shut off of host cell transcription. Surrounding this complex is the virion envelope composed of host cell membranes and viral membrane glycoproteins. Together, herpesvirus virions vary in size from 120 nm which is typical for HSV-1 to over 300 nm as is the case for HCMV.

Herpesviruses are characterized by a number of features. First, they encode a large array of proteins, many of which are involved in viral transcription and DNA replication. Second, capsid assembly and viral DNA synthesis/packaging occurs in the nucleus. Third, virus replication is invariably a lytic event, resulting in the destruction of the host cell; and finally in those instances in which replication does not occur, the virus can initiate and maintain a latent state within the host. Variations within these properties have been used to differentiate the herpesviruses into three different classes: the *alphaherpesvirinae*, the *betaherpesvirinae*, and the *gammaherpesvirinae*.

All members of the *gammaherpesvirinae* class only replicate within their natural host range. These viruses replicate in lymphocytes, although some can infect fibroblast and/or epithelial cells. The representative human *gammaherpesvirinae* is EBV, the etiological agent of infectious mononucleosis and Burkitt's lymphoma.

The *betaherpesvirinae* are also characterized by a restricted host range. Their replication cycle is very slow and infected cells typically become enlarged (cytomegalia). HCMV, the largest of the herpesviruses is the leading cause of

congenital malformations in North America (Alford and Britt, 1990). In humans,latent HCMV DNA can be detected in a variety of tissues including secretory glands and kidneys but reactivated virus has only been derived from a dendritic cell lineage (Soderberg-Naucler et al., 1997).

Alphaherpesvirinae unlike the other classes of herpesviruses, exhibit a broad host range, which makes them particularly amenable for study in various animal models. Replication time is short and very efficient, with 50,000 to 200, 000 virions produced per infected cell (Corey and Spear, 1986), which inevitably results in the destruction of the host cell. Latent infections are established in sensory ganglia. The prototypical member of the family is herpes simplex virus (HSV).

1.2 Herpes simplex virus

HSV has had a long history with humans, and was first described by the ancient Greeks (Wildy, 1973). The word "herpes" is derived from the Greek word "herpo" meaning to creep, an apt description for the observed skin lesions. HSV remained solely as a description of a disease state until the early 20th century when its infectious nature was established by Lowenstein. Today, HSV is recognized as the etiological agent of diseases of the skin and mucous membranes. These include gingivostomatitis, herpes labialis, genital infections, keratoconjunctivitis as well as neonatal infections and HSV encephalitis (reviewed in Whitley, 1996).

Clinical symptoms of disease are frequently observed with primary HSV

infections and typically last longer and are more severe than those associated with reactivated virus (reviewed in Corey and Spear, 1986). The most frequent clinical manifestations of primary infections are gingivostomatitis and pharyngitis while herpes labialis is more frequently associated with recrudescences. Clinical manifestations, such as the severity and duration of the disease increase in immunocomprimised individuals (Whitley et al., 1984) In addition these individuals suffer from more frequent HSV infections (Whitley et al., 1984).

There are two serotypes HSV-1, and HSV-2, which are distinguished by restriction fragment length polymorphisms (Buchman et al., 1980). Approximately 50% of the sequences between HSV-1 and HSV-2 are highly conserved (Kieff et al., 1971). Both serotypes can cause clinically indistinguishable forms of either genital or oral/facial infections. However, HSV-1 typically is associated with disease above the waist while HSV-2 is below the waist. HSV-1 oral/facial recrudescences are more common than HSV-2 (Lafferty et al., 1985) and genital HSV-2 reactivation occurs 10 times more frequently than HSV-1 (Reeves et al., 1981).

1.2.1 Replication Cycle

HSV replication has been extensively studied for over 35 years following the development of methods for culturing the virus in vitro (Roizman and Roane, 1961). Although we still have yet to answer all the questions, the basic features of HSV lytic replication can be traced through a series of distinct stages. Infection is first initiated by the adsorption of the virus to the surface of a

susceptible cell followed by the fusion of the virion envelope with the plasma-membrane. The released nucleocapsid is transported from the periphery to the nucleus where it uncoats and the viral genome is injected into the cell nucleus. A cascade of viral gene expression ensues, resulting in the synthesis of viral proteins required for viral DNA synthesis and packaging. Following DNA replication, the viral DNA is encapsulated into capsids. These capsids are then enveloped by cellular membranes and released at the cell surface.

1.2.1.1 Adsorption and Entry.

The initial event in any viral infection is the binding of the virion to the cell surface. Viruses have been able to exploit a vast number of cell surface molecules as "viral receptors" that span a broad array of natural cellular activities (reviewed in Marsh and Helenius, 1989). They include cell adhesion molecules (Adenovirus), immunoregulatory molecules (e.g., CD4/HIV;(Lasky et al., 1987);Class I MHC/Semliki Forest virus, (Helenius et al., 1978)) and even carbohydrate moieties of cell surface glycoproteins (e.g., sialic acid/ Influenza virus; (Herrler et al., 1985)). Viral binding to the cell surface provides the close physical association required for subsequent penetration of the nucleocapsid into the cytoplasm. For enveloped viruses, binding proceeds a membrane fusion event between the viral envelope and cellular membranes. Fusion can occur either directly between the virion envelope and the plasma membrane or with endosomal membranes following virion endocytosis.

The process of HSV entry has been and continues to be a central focus of

investigation and to some extent consternation. HSV entry is a complicated issue, due to in part by the large number of glycoproteins that are required for penetration. At least four viral glycoproteins (gB, gD, gH/gL) are essential, however their cellular counterparts have been difficult to identify. Putative cellular receptor(s) for gD have been recently isolated (Brunetti et al., 1995; Brunetti et al., 1994; Montgomery et al., 1996). HSV has a broad host range and can infect a wide variety of cell types in culture albeit not always productively. This suggests that either the cellular receptor(s) for HSV are both widely distributed and highly conserved or alternatively, that HSV can use different receptors for different cells.

1.2.1.2 GLYCOPROTEINS

The events of HSV adsorption and entry into a susceptible cells are orchestrated by a number of viral encoded glycoproteins that decorate the surface of the virion lipid envelope. There are currently 16 known or putative membrane proteins, 11 of which are glycoproteins (reviewed in Roizman and Sears, 1996; Spear, 1993). The glycoproteins and their corresponding genes (gp/US#, or /UL#) are as follows: gB/UL27; gC/UL44; gD/US6; gE/US8; gG/US4; gH/UL22; gI/US7; gJ/US5; gK/UL53; gL/UL1; gM/UL10. Together they share a number of characteristics. i) They all have cleavable N-terminal signal sequences; ii) all with the exception of gL, have a membrane spanning domain near the N-terminus. In addition, gB has the potential to cross the membrane three times (Pellett et al., 1985), and gK is also suspected to span the membrane multiple times

although the exact topology is unknown (Debroy et al., 1985; Pogue-Geile and Spear, 1987; Ramaswamy and Holland, 1992). iii) Each has the potential for the addition of N-linked (Baines and Roizman, 1993; Spear, 1993) and in some cases O-linked oligosaccharides (Johnson and Spear, 1983). All glycoproteins with the exception of gK (Hutchinson et al., 1995) are found on the virion envelope (Spear, 1993). Only 5 of these glycoproteins including gB, gD, gH/gL, and gK are absolutely required for a productive infection in culture (Cai et al., 1988; Desai et al., 1988; Forrester et al., 1992; Hutchinson and Johnson, 1995; Ligas and Johnson, 1988; Roop et al., 1993). The remaining membrane associated proteins include UL11, UL20, UL34, UL43 and UL45. UL11 is myristylated (MacLean et al., 1992); UL34 and UL45 possess hydrophobic membrane regions that could be membrane spanning or membrane associated, although neither protein have sites for the addition of N-linked oligosaccharides (Spear, 1993). UL 20 and UL43 have multiple potential membrane spanning regions (MacLean et al., 1991). In fact, UL20 shares many characteristics of proteins that span the membrane multiple times: it's membrane associated, resistant to alkali extraction, and aggregates when boiled in SDS (Baines et al., 1991).

1.2.1.3 Adsorption

The initial interaction of HSV and a cell is with glucosaminoglycans (GAGs) moieties of cell surface proteoglycans (reviewed in Spear, 1993). GAGs are composed of repeating disaccharide units in which at least one of the two sugars contains an acidic group having a negative charge at pH 7 either a

carboxylate or sulfate group, and one must also be an amino sugar. GAGs can be covalently attached to a protein core forming proteoglycans in which GAGs make up to 95% of the weight of the molecule. HSV principally adsorbs to heperan sulfate (HS) moieties of cell surface proteoglycans (Shieh et al., 1992; WuDunn and Spear, 1989) although chondroitin sulfate (CS) may be used in certain cases (Banfield et al., 1995). The evidence which supports this conclusion is based on the following studies. First, treatment of cells with either heperinase or heparitinase which enzymatically removes HS from the cell surface reduced plaquing efficiency of wild type HSV by over 90%. Second soluble heparin (structurally similar to HS but more highly sulfated) added during infection also inhibited plaque formation (Shieh et al., 1992; WuDunn and Spear, 1989). Moreover, purified virions were able to bind to heparin affinity columns in physiological saline (WuDunn and Spear, 1989). Third, Chinese hamster ovary (CHO) cells that are deficient in HS synthesis bound HSV very poorly and were resistant to infection (Shieh et al., 1992).

In order to identify the virion component that was responsible for HS binding, Herold and colleagues identified HSV glycoproteins that showed affinity for heparin. They found that gC bound to a heparin-Sepharose affinity column in physiological saline (Herold et al., 1991). Together with the fact that HSV mutants unable to express gC (gC^-) were impaired in their ability to absorb to cells (Herold et al., 1991), these data strongly suggested that gC was the HSV component responsible for virion adsorption. Since gC^- mutants could still bind

to cells but at low levels, other viral glycoproteins also appeared to be involved. gB binds to heparin affinity columns (Herold et al., 1991) and the adsorption of gB^-/gC^- mutants is severely impaired compared to gC^- and wild type virions (Herold et al., 1994).

1.2.1.4 Secondary Receptors

Following adsorption to HS moieties, HSV appears to bind in a heparin-resistant fashion to the cell surface. This binding is mediated by gD on the virion envelope to a limited number of cell surface receptors. This is based on a series of studies using HSV mutants unable to express gD (gD^-) (Johnson and Ligas, 1988; Ligas and Johnson, 1988), a recombinant soluble form of gD (Johnson et al., 1990), and cell lines constitutively expressing gD (Campadelli-Fiume et al., 1988). gD was first shown to be essential for infectivity, as gD^- mutant viruses were unable to enter cells, yet the mutants displayed no defects in adsorption. Plaque production of these gD^- viruses could be enhanced following polyethylene glycol treatment suggesting that gD was involved in penetration. Evidence also suggested that gD was binding to a limited number of receptors on the cell surface. UV-inactivated HSV virions with and without gD when added to Vero cells, adsorbed onto a large number of sites ($> 4 \times 10^4$) and was not a saturable event (Herold et al., 1991; Johnson and Ligas, 1988). Vero cells pretreated with 5 $\times 10^3$ UV-inactivated (gD^+) particles at 4°C and then infected with wild type HSV-1, were resistant to infection by the challenge virus. In marked contrast cells

pretreated with UV-inactivated gD- particles, were infected by the challenge virus. This suggested that gD as part of the envelope of UV-inactivated particles was competing for a limited number of cellular receptors that were essential for viral entry (Johnson and Ligas, 1988). Transfected cell lines constitutively expressing gD were resistant to infection by HSV due to interference with viral penetration (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989), while cell lines expressing gB or gC were not (Arsenakis et al., 1986; Rosenthal et al., 1987). Further evidence that there are only a limited number specific HSV receptors came from experiments with soluble forms of HSV-1 and HSV-2 gD (Johnson et al., 1990). Both gD-1t and gD-2t could block plaque production by HSV-1 and HSV-2 while soluble forms of gB had no effect. Both forms of soluble gD bound in a saturable manner to a limited number of sites. It was estimated that soluble gD bound to approximately 4×10^5 to 5×10^5 sites per cell with a dissociation constant (k_d) within the range of $2.3\text{-}2.6 \times 10^{-7}$M. Specific binding of soluble gD to cell surface receptors also appears to be independent of heparin sulfate, as soluble gDt bound equally well to cells treated and untreated with either heperinase or heperitinase, enzymes that remove heparan sulfate glycosaminoglycans from the cell surface. In addition, heparin had no effect on soluble gD binding (Johnson et al., 1990). These results suggested that gD is essential for penetration and acts downstream of heparan sulfate mediated adsorption.

HSV adsorption to heparan sulfate glucosaminoglycans may be important

for subsequent gD-receptor mediated interaction. HSV infectivity was greatly diminished in cells unable to express heparan sulfate or in which heparan sulfate was enzymatically removed (Banfield et al., 1995; Shieh et al., 1992; WuDunn and Spear, 1989), in spite of the presence of cellular receptors. Therefore heparan sulfate may act to concentrate viral particles on the cell surface for subsequent low affinity binding of gD to its receptor (Johnson et al., 1990). gD affinity to its receptor(s) is appreciably lower than that observed for other viral receptors such as HIV gp120 for CD4 (k_d, 4 x10^{-9}M) (Lasky et al., 1987). However, the overall affinity or avidity of gD to its receptor may be significantly increased by mutliple interactions of gD molecules on the virion envelope with receptors on the cell surface.

Considerable effort has been made by a number of groups to identify a cellular receptor for HSV. Using soluble gD-1t and gD-2t, Brunetti and colleagues identified the the 275 kDa mannose-6-phosphate/insulin-like growth factor II receptor and the 46 kDa cation-independent mannose-6-phosphate receptor (M6R), as putative gD receptors (Brunetti et al., 1994). Soluble M6R, anti-M6R antibodies, and a synthetic ligand for the M6R could block plaque production in Vero and MDBK cells by up to 75% (Brunetti et al., 1995). Moreover, wild type HSV produced small plaques on mutant fibroblasts that were unable to add mannose-6-phosphate to glycoproteins, suggesting that the M6R played an important role in mediating entry of HSV into susceptible cells. However, there were no differences in the ability of wild type HSV to enter mouse cells deficient

in both the cation-dependent and independent M6Rs compared to normal mouse Balb/c cells (Brunetti et al., 1995). Although this latter result would imply that M6Rs are not important for HSV infections, it is more likely that HSV uses a number of receptors to mediate entry. This is illustrated by the fact that other HSV receptors have also been recently characterized. Huang and Campadelli-Fiume identified a 62 kDa protein using anti-idiotypic antibodies to the HSV neutralizing anti-gD MAb HD1, (Huang and Campadelli-Fiume, 1996). These anti-idiotypic antibodies could block the number and formation of plaques by HSV on monolayers of Vero and HEp-2 cells (Huang and Campadelli-Fiume, 1996). In additon, a previously uncharacterized member of the TNF/NGF receptor family called HVEM (herpes virus entry mediator) has been shown to mediate entry into HSV resistant CHO cells (Montgomery et al., 1996). Antibodies to HVEM could block entry of HSV into CHO cells and T-cell clones expressing HVEM, but not into HVEM expressing HeLa cells (Montgomery et al., 1996). Further, soluble forms of HVEM and gD could not block entry of HSV into HVEM expressing cells (Montgomery et al., 1996; Whitbeck et al., 1997). Together, these results suggest that the entry of HSV into cells is likely mediated by a number of different receptors, which is not surprising considering that HSV has such a broad host range, as well as the ability to enter a wide variety of cell types.

1.2.1.5 Penetration

Evidence from a number of studies suggest that penetration occurs

following fusion of the virion envelope with the plasma membrane and not by endocytosis. Early EM studies by Morgan et al., (1968), and later by Fuller and Lee (1992), demonstrated fusion of the virion envelope with the plasma membrane that can be inhibited by antibodies (Fuller and Lee, 1992; Fuller et al., 1989; Fuller and Spear, 1987; Morgan et al., 1968). In addition, HSV Fc receptor activity associated with virion particles can be transferred to the cell surface following infection, in the absence of viral gene expression (Para et al., 1980). Viral penetration is unlikely to be mediated by endocytosis, as conditions which prevent endocytosis such as potassium depletion and hypotonic shock have no effect (Wittels and Spear, 1991), nor does entry require the low pH-dependent endocytic pathway, (Koyama and Uchida, 1984; Koyama and Uchida, 1987; Wittels and Spear, 1991). If, however, endocytosis of virions does occur, it is likely a dead end pathway (Campadelli-Fiume et al., 1988; Koyama and Uchida, 1987).

Fusion of the virion envelope and the plasma membrane requires at least 4 glycoproteins and another poorly characterized tegument protein, and occurs fairly rapidly (Huang and Wagner, 1966; Koyama and Uchida, 1987). Together the glycoproteins gB, gD, the gH/gL oligomer, and UL25 are required for penetration. The best evidence that these glycoproteins are essential for penetration, is based on the generation of deletion mutants for the individual glycoproteins. HSV mutants unable to express either gB, gD, or gH/gL are able to bind to cells but do not enter and must be propagated on cell lines that supply the

glycoprotein in *trans* (Cai et al., 1988; Forrester et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993). Consistent with the mutational analysis, many monoclonal antibodies against gB,gD, and gH have strong neutralizing ability but have no affect on adsorption (Fuller et al., 1989; Fuller and Spear, 1987; Highlander et al., 1988; Highlander et al., 1987; Navarro et al., 1992). In addition, the tegument protein UL25 appears to be essential for penetration. A UL25 temperature sensitive (*ts*) mutant is unable to enter cells at the nonpermissive temperature and can block infection of wild type HSV (Addison et al., 1984; Preston, 1990).

Although many of the virion components required for fusion have been identified, how they actually mediate membrane fusion is unclear. It has been proposed that fusion of the virion envelope with the plasma membrane follows a number of distinct steps that require specific glycoprotein functions (Fuller and Lee, 1992). gD was thought to initiate a specific interaction with the cell surface which is followed by gH dependent activity (Fuller and Lee, 1992). This is similar to a previous proposal in as much that gH acted downstream of gD during cell fusion (Forrester et al., 1992). Unlike gD^- virions which are unable to inhibit infection by challenge virus (Johnson and Ligas, 1988), gH^- mutants block entry of wild type HSV (Forrester et al., 1992). Based on this assumption, gB can also be placed downstream of gD function, as gB^- mutants can also inhibit infection of wild type HSV (Lee and Fuller, 1993).

Individual glycoproteins have been observed by EM as a combination of

distinct morphological spike structures or patches projecting from the virion envelope (Stannard et al., 1987). In this case, the glycoproteins examined gB, gC and gD existed as distinct entities and did not appear to form complexes (Stannard et al., 1987). Hetero-oligomeric complexes of viral glycoproteins have not been observed other than gH/gL and gE/gI (Hutchinson et al., 1992; Johnson and Feenstra, 1987), suggesting that the glycoproteins may act independently but sequentially during fusion (Cooper, 1994). However, Handler and colleagues have identified hetero-oligomers of gB-gC, gC-gD and gD-gB following chemical crosslinking of purified virions (Handler et al., 1996). Using a modified entry assay in which crosslinking agents were added at various times during penetration, changes in the ability to isolate specific glycoprotein hetero-oligomers were observed, such that they were increasingly less accessible to cross-linking over time (Handler et al., 1996). Only the glycoproteins essential for penetration were refractory to crosslinking as the levels of oligomeric gC did not change during entry (Handler et al., 1996). The inability to crosslink the glycoproteins may reflect their role in penetration; as penetration proceeds, the glycoproteins may either bury themselves into the plasma membrane (Handler et al., 1996) as has been observed for other viruses (White, 1992), or alternatively, they may be dispersed within the plasma membrane and become too far apart to be crosslinked. In any case, it is still unknown whether the glycoproteins act either as a single unit (i.e., as a fusion machine) or individually and sequentially during fusion. Moreover, understanding virion-cell fusion still requires the

identification of the cellular components involved in the process.

1.2.1.6 SYNCYTIAL FORMATION (CELL-CELL FUSION)

If the events that regulate virion-cell fusion are unclear, even less is known about how HSV induces cell-cell fusion. Clinical HSV isolates normally do not cause fusion of cells in tissue culture (i.e., there is no recruitment of cells into polykaryocytes). However, in culture, HSV mutants (*syn*) arise that are able to elicit the formation of syncytia in a cell-type dependent manner (Bzik and Person, 1981; Ejercito et al., 1968; Spear, 1993). HSV induced cell-cell fusion depends for the most part on the expression of virion proteins by at least some of the cells that are involved (i.e., fusion from within; reviewed in Spear, 1993) , however there are some HSV mutants which can induce fusion from without (Falke D et al., 1985; Walev et al., 1991). In the latter case, the addition of high concentrations of HSV to cells leads to syncytial formation in the absence of viral protein expression (Walev et al., 1991). Fusion from without may be a multistep process in which normal virion entry occurs (virion-cell fusion), followed by a rapid unregulated fusion of the plasma membrane with its neighbour. Fusion from without can be inhibited by either heparin or prior treatment of cells with heperitinase (Walev et al., 1991).

Mutations in the HSV genome that result in HSV-induced cell-cell fusion map to 5 genes, 2 glycoproteins (gB,and gK), UL20, and UL24 (Baines et al., 1991; Jacobson et al., 1989; Sanders et al., 1982). Syncytial mutations for gB, and gK map to both the amino-(N-)terminal ectodomain and the C-terminus (Bzik

et al., 1984; Debroy et al., 1985; Dolter et al., 1994; Goodman and Engel, 1991; Pogue-Geile and Spear, 1987; Saharkhiz-Langroodi and Holland, 1997; Weise et al., 1987). Many of these *syn* mutations however, can be suppressed either by mutations in other genes such as UL45 (Haanes et al., 1994), and gH (Wilson et al., 1994), or by overexpression of the wild type gene (Hutchinson et al., 1993). In addition, HSV induced cell-cell fusion requires viral expression of gB, gD, gM as *syn* mutants that are unable to express gB, gD, or gM lose their *syn* phenotype (Cai et al., 1988; Davis-Poynter et al., 1994; Ligas and Johnson, 1988). It has been proposed that gE and gI are also important for expression of the syn phenotype (Balan et al., 1994; Davis-Poynter et al., 1994), however, HSV mutants lacking either gE or gI have been isolated that remain syn (Johnson et al., 1988; Neidhardt et al., 1987).

The role of gK and UL24 in cell-cell fusion appear to be more indirect. Both appear to have a more important role in viral egress (see section 1.2.2.11) rather than entry (Hutchinson and Johnson, 1995; Jayachandra et al., 1997). In HSV infected cell, gK is predominantly localized to nuclear and endoplasmic reticulum (ER) membranes and could not be detected in purified virions (Hutchinson and Johnson, 1995). Moreover, the UL24 *syn* phenotype is due to loss of expression (Jacobson et al., 1989; Sanders et al., 1982). Therefore, both proteins must elicit their effects on the plasma membrane by an indirect route, possibly by altering the functions of the cell's membrane fusion machinery.

Results from stable transformed cell lines as well as transient and viral

mediated expression of HSV glycoproteins have added to the apparent confusion about the process of cell-cell fusion. Cell-cell fusion of cell lines stably expressing gD and gH is cell-type dependent (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989) and may require an acidic environment (Butcher et al., 1990). Transient expression of single viral glycoproteins (gB, gD, and gH) by Adenovirus (Ad) vectors has resulted in little success in inducing fusion (Spear, 1993), and in some cases can actually suppress fusion by HSV *syn* mutants (Hutchinson et al., 1993). However, fusion may require the presence of all the necessary HSV glycoproteins as transient expression of gB, gD and gH/gL together induced spontaneous fusion to a limited extent in Cos cells (Turner et al., 1998). Cell-cell fusion and virion-cell fusion may occur through different mechanisms or be regulated in a different manner. This is illustrated by the fact that antibodies directed against gL can inhibit syncytial formation, yet cannot block entry of exogenous virus (Novotny et al., 1996). Therefore, results from either virion induced cell-cell fusion or from transient transfection experiments (Turner et al., 1998) must be interpreted with care.

1.2.2.7 Post penetration: viral gene expression and DNA replication

Fusion of the virion envelope with the plasma membrane releases the nucleocapsid into the cytoplasm where it may be transported rapidly via the microtubule (MT) network by dynein motors (Sodeik et al., 1997)to nuclear pores where the viral DNA is released into the nucleus (Batterson and Roizman, 1983;

Sodeik et al., 1997; Tognon et al., 1981). Concomitant with capsid entry, tegument proteins originally associated with the capsid, are released into the cytoplasm (Batterson and Roizman, 1983). Two of these tegument proteins are essential for the efficient initiation of the viral infection: UL41 (*vhs*, virion host shutoff) leads to immediate shut off of host protein synthesis (Fenwick and Everett, 1990; Read and Frenkel, 1983; Smibert et al., 1992) and UL48 (more commonly known as VP16 or α-TIF) is required for immediate early gene expression (Batterson and Roizman, 1983; Campbell et al., 1984).

1.2.2.8 Gene Expression

Within the nucleus, VP16 initiates a cascade of temporally controlled viral gene expression (Batterson and Roizman, 1983; Campbell et al., 1984; Honess and Roizman, 1974). The first genes to be expressed are the immediate early (IE) or α-genes (ICP0, ICP4, ICP22, ICP27, and ICP47) which all, except for ICP47, have transcriptional regulatory functions (Roizman and Sears, 1996). ICP47 down regulates surface expression of major histocompatibility class I (MHC I) and thus plays an important role in viral immune evasion (York and Johnson, 1994; York et al., 1994). IE gene expression peaks at 2-4 hours post infection (p.i.). The second wave of viral gene expression peaks between 5-7 hrs p.i., and involves early (E) or ß-genes, which are required for viral DNA synthesis (Honess and Roizman, 1974; Roizman and Sears, 1996). Viral gene expression concludes with the late (L) or γ-genes and requires prior viral DNA synthesis (Roizman and Roane, 1964). L genes code for structural proteins (i.e., capsid proteins), many of

the glycoproteins of the virion envelope, and proteins involved in viral egress (Roizman and Sears, 1996).

1.2.2.9 DNA replication

The linear double stranded HSV genome is of approximately 150 kbp, and is comprised of unique long (U_L) and a unique short (U_S) sequences that are covalently linked and bracketed by inverted repeats (reviewed in Roizman and Sears, 1996). Recombination between the reiterated repeats leads to isomerization of the U_L and U_S regions relative to each other (Chou and Roizman, 1985) yielding 4 isomers of equal molar concentrations (Delius and Clements, 1976; Hayward et al., 1975). Upon delivery into the infected cell nucleus, the viral DNA rapidly circularizes into a head-to-tail conformation that does not require de novo protein synthesis (Poffenberger and Roizman, 1985). This viral DNA structure then becomes the template for future viral DNA replication. The viral DNA replicates by a rolling circle mechanism forming large concatameric structures (Ben-Porat and Tokazewski, 1977; Jacob et al., 1979; Jacob and Roizman, 1977) that are first detected at 3 hrs. p.i., and can continue to accumulate for at least 12 hrs (Roizman et al., 1963). Packaging of the viral DNA into newly formed capsids is linked to sequence specific cleavage events within the a sequences of the repeats, a process that inevitably results in the packaging of genome length equivalents (Deiss et al., 1986; Deiss and Frenkel, 1986).

1.2.2.10 Capsid formation

HSV capsids are composed of the products of the following genes: UL18(VP23), UL19(VP5), UL26(VP21, VP22a, VP24), UL35(VP26), UL38(VP19c) (Braun et al., 1983; Costa et al., 1984; Davison et al., 1992; Gibson and Roizman, 1972; McGeoch et al., 1988; McNabb and Courtney, 1992; Rixon et al., 1990). Three types of capsids, A, B, and C, can be isolated by sucrose gradient centrifugation from HSV infected cell lysates (reviewed in Rixon, 1993). The B capsids are thought to be the precusor to A and C capsids; A capsids are abortive attempts of DNA packaging, while C capsids contain viral DNA and go on to form infectious virus. The pathway of HSV B capsid assembly has been partially defined using recombinant baculovirus expressed capsid proteins in either infected insect cells or in a cell free system using baculovirus infected cell lysates (Newcomb et al., 1994; Thomsen et al., 1994). The self assembly of the proteins UL26 and UL26.5 into a double core strucure acts a scaffold for the assembly of the outer icosahedral shell made up of UL18, UL15, UL35, and UL38 forming the B capsid (Newcomb et al., 1994; Thomsen et al., 1994).

1.2.2.11 Egress

Envelopment and egress of newly formed capsids to the cell surface is perhaps one of the most controversial issues within the HSV replication field. It is generally agreed upon that α-herpesvirus capsids bud into the perinuclear space, thereby acquiring an envelope from the inner nuclear membrane (Morgan et al., 1959; Schwartz and Roizman, 1969; Torrisi et al., 1992; Whealy et al., 1991).

Once enveloped within the perinuclear space, virions must make their way to the cell surface; it is at this point in which the disagreement develops. There are essentially two fields of thought. The first ER/Golgi model suggests that enveloped virions follow the secretory pathway and pass sequentially through the ER and Golgi compartments so that enveloped particles are in turn surrounded by cytoplasmic membrane visicles. Ultimately, vesicular traffic delivers the particles to the plasma membrane where they are released (Campadelli-Fiume et al., 1991; Johnson and Spear, 1982; Morgan et al., 1959; Schwartz and Roizman, 1969; Torrisi et al., 1992). In this case, immature glycoproteins acquired at the inner nuclear envelope are processed to mature forms while virions are in transit through the exocytic pathway (Johnson and Spear, 1982). Alternatively, the second model contends that egress relies on a deenvelopment/reenvelopment event (Gershon et al., 1994; Jones and Grose, 1988; Stackpole, 1969). Newly enveloped capsids within the ER lumen fuse with the ER membrane releasing naked nucleocapsids into cytoplasm. These capsids are subsequently reenveloped by budding into the Golgi apparatus or *trans* Golgi network (TGN), acquiring the mature glycoproteins, which have accumulated within the Golgi compartments via the exocytic pathway (Gershon et al., 1994; Jones and Grose, 1988).

Both models have relied heavily on static EM pictures of infected cells, and therefore it is difficult to determine whether virions are in the process of either envelopment or deenvelopment. However, recently the emphasis on EM

analysis has shifted to genetic and biochemical studies. The use of drugs such as Monensin and Brefeldin A have met with limitted success (Cheung et al., 1991; Johnson and Spear, 1982; Whealy et al., 1991). Both models require an active secretory system and any interruptions in this process have yielded results that have been difficult to interpret; data obtained from Brefeldin A treated cells have been used to support both models (Cheung et al., 1991; Whealy et al., 1991). Support for the ER/Golgi model has come from a study of a gD mutant in which nucleocapsids accumulated in the cytoplasm suggesting that this was a "dead end" pathway (Campadelli-Fiume et al., 1991). However, more recent biochemical evidence points toward the second model. The phospholipid content of membranes purified from extracellular virions was more similar to Golgi derived membranes than it was to the inner nuclear membrane (van Genderen et al., 1994). An HSV mutant in which the essential glycoprotein gH has been modified with a C-terminal ER retrieval signal KKXX (K, lysine; X, any amino acid), produced normal amounts of enveloped particles yet the infectivity was 100 fold lower, and the particles lacked gH (Browne et al., 1996). Since the ER retrieval signal within gH was at the C-terminus, the modified gH acquired by the virion at the inner nuclear envelope would be unaccessible to the ER/Golgi retrieval system and thus would have had no effect on infectious virion production. Together, these results are compatible with a deenvelopment/reenvelopment pathway, and argue against the ER/Golgi model. However, it was unclear in these studies whether immature enveloped particles

(i.e., those formed at the inner nuclear envelope) contained the mutant form of gH.

The membrane proteins UL20 and UL53 (gK), the myristylated protein UL11 and ICP34.5 appear to play important roles in viral egress (Baines and Roizman, 1992; Baines et al., 1991; Brown et al., 1994; Hutchinson and Johnson, 1995; MacLean et al., 1992). ICP34.5 function in egress is cell-type dependent; in BHK cells, enveloped particles become trapped between the inner and outer nuclear membranes while in mouse 3T6 cells, there appears to be a defect in initial envelopment as 90% of the nucleocapsids remain in the nucleus (Brown et al., 1994). Similarly, a UL11$^-$ mutant accumulated capsids in the nucleus (Baines and Roizman, 1992), suggesting that both proteins may function to facilitate envelopment of the capsid with the inner nuclear membrane.

In cells infected with gK$^-$ HSV mutants, enveloped particles did not reach the surface of cells, but accumulated as uneveloped capsids in the cytoplasm (Hutchinson and Johnson, 1995). Defects in viral egress were also observed in HSV infected cells that overexpress gK; enveloped virions accumulated between the inner and outer nuclear membranes or ER lumen. Together, the data suggested that gK may act to prevent fusion of the virion envelope and the ER membrane, and supports the ER/Golgi model of viral egress (Hutchinson and Johnson, 1995). However, this gK$^-$ mutant was constructed by the insertional inactivation of the gK gene with ICP6::ß-galactosidase gene cassette which resulted in the expression of the first 112 amino acids of the N-terminus of gK

(Hutchinson and Johnson, 1995). Another HSV gK$^-$ mutant, ΔgK, has recently been constructed and characterized (Jayachandra et al., 1997). In Vero cells infected with the ΔgK mutant, nucleocapsids accumulated within the nucleus, suggesting that gK is required for envelopment of the nucleocapsids at the inner nuclear membrane. ΔgK enveloped particles were also seen accumulating in the cytoplasm, where, in additon, single envelopes often contained numerous nucleocapsids (Jayachandra et al., 1997). Although there were differences between the two gK mutants, the observations support the hypothesis that gK as an inhibitor or regulator of membrane fusion. Loss of gK function would lead to uncontrolled fusion between virion envelopes producing multicapsid enveloped particles. How gK would act to prevent fusion between nucleocapsids is unclear as gK appears to be excluded from the virion envelope (Hutchinson et al., 1995). Since ΔgK infected cells resulted in the accumulation of capsids in the nucleus, it is more likely that loss of gK expression resulted in the aberrant envelopment of several capsids within a single envelope. gK may also play a role in egress of enveloped particles at the plasma membrane, although how gK could regulate fusion at the plasma membrane is unclear, as gK was predominantly found within ER membranes, and not in the Golgi apparatus or at the cell surface (Hutchinson and Johnson, 1995; Hutchinson et al., 1995). However, the alternative hypothesis is that gK is required for regulating the fusion of the cytoplasmic vesicles with the Golgi apparatus (Jayachandra et al., 1997).

Similar to gK$^-$ mutants, HSV mutants unable to express UL20 exhibit a defect in viral egress. In Vero cells infected with a UL20$^-$ HSV mutant, enveloped nucleocapsids accumulated in the perinuclear space/ER lumen, but there was also an increase in the number of naked capsids in the cytoplasm (Baines et al., 1991). UL20 may function in a cell type dependent manner since the phenotype was not as severe in 147TK$^-$ cells (Baines et al., 1991). This variation has been attributed to the differential disruption of the Golgi compartment: UL20 is required in Vero cells (where the Golgi compartment breaks down) for the efficient entry of virions into the exocytic pathway and for transport of viral glycoproteins from the TGN to the plasma membrane but UL20 is not required in 147TK- cells because the Golgi compartment remains intact (Avitabile et al., 1994). Due to the nature of the different phenotypes that have been observed with the various HSV egress mutants, the exact pathway that HSV uses is still unresolved. However, HSV may adopt different egress strategies based upon the cell type in which it is replicating. This should not be surprising considering the wide variety of cells that HSV infects (Roizman and Sears, 1996) and the concomitant variations in glycoprotein trafficking exhibited in different cell types (Keller and Simons, 1997).

1.3 HSV Infection of the Nervous System

Common to all herpesviruses is their ability to establish a latent state. For HSV, the site of latency is the neuron, particularly those that constitute sensory or autonomic ganglia (Corey and Spear, 1986) where the virus can remain latent for

the lifetime of the host (reviewed in Ho, 1992; Steiner, 1996; Stevens, 1989). Even before it had been established that HSV was the etiological agent for "fever blisters" both Cushing and Goodpasture proposed that the viral agent could establish a latent state (Cushing, 1905; Goodpasture, 1929). It was noted by Cushing that those patients undergoing treatment for trigeminal neuralgia developed herpetic lesions near regions of the trigeminal nerve branch that had been sectioned (Cushing, 1905). Goodpasture then postulated that "Following a primary infection the virus remains in a latent state with the ganglia after the local lesion has healed and that neural disturbances are frequently the basis of subsequent outbreaks" (Goodpasture, 1929). Goodpasture's postulate was then proven by Stevens and Cook (Stevens and Cook, 1971), as well as by others using ganglia from human cadavers and different animal model systems (Baringer and Swoveland, 1973; Bastian et al., 1972; Nesburn et al., 1972) . In these studies the presence of latent HSV was detected using an *in vitro* reactivation model in which infectious virus was recovered following the cocultivation of infected ganglion cells with permissive non-neuronal cells. Since then considerable effort has been made to towards understanding HSV's sophisticated host-virus relationship within the nervous system.

1.3.1 Replication cycle

Primary infection of the host by HSV is initiated within mucosal and submucosal tissues predominantly of the face, eye, and mouth. Replication within these tissues provides HSV access to axonal terminals of either sensory or

autonomic ganglion neurons. There is no requirement for extensive replication at the periphery to achieve neuronal infection, in fact many primary infections are asymptomatic (reviewed in Corey and Spear, 1986). However, the location of the primary infection specifies the eventual site of latency and the route of virus spread (Baringer and Swoveland, 1973). In humans, the trigeminal ganglion (TG) is the chief site for infected neurons followed closely by the sacral ganglion (Liesegang, 1992).

Infection of axons is thought to occur as with other cell types; the virion envelope fuses with the axonal membrane releasing the capsid into the axonoplasm. The capsid is transported along the axon (Cook and Stevens, 1973; Hill et al., 1972; Kristensson et al., 1971) by microtubule-associated fast axonal transport, approximately 5-10 mm/hr, to the neural soma (Kristensson et al., 1986; Penfold et al., 1994) where the viral DNA is released into the nucleus. While in humans the time for spread from the mucosa to the neuron is unknown, in mice and guinea pigs, it can happen in as little as 2 days (Klein et al., 1979; Stanberry et al., 1982). A productive infection of neurons and other cells in the ganglia can occur but this infection is usually limited in nature lasting for only 3-5 days (Simmons et al., 1992).

1.3.2 Latency

Following a limited replication, HSV infections develop into a state of latency in which the viral genome becomes quiescent (a non-replicating state). Formally, the term latency was defined operationally as the period of HSV

infection in which no infectious virus could be detected except by explant cocultivation (Stevens and Cook, 1971). Today, this definition has changed somewhat with the development of *in situ* hybridization and the polymerase chain reaction (PCR). Latent HSV referes to any virus that can be detected by these methods (i.e., PCR or in situ hybridization) in the absence of any infectious virus (reviewed in Steiner, 1996). Under appropriate conditions HSV reactivates, in which viral DNA replication is initated and progeny virus is transported back along the axon to the periphery, producing secondary recrudescences. For HSV-1, recrudescences commonly take the form of herpes labialis, but for many individuals reactivation is asymptomatic such that there is viral shedding yet no outward clinical disease (Corey and Spear, 1986). Reactivation of latent HSV within the nervous system (i.e, production of infectious virus) can be due to a number of factors such as physical damage to the nerve, UV exposure, menstruation and emotional stress.

The nature of the viral particle (i.e., whether it is either a naked capsid or enveloped particle) as it returns along the axon to the periphery is still under debate. EM studies have shown both enveloped and naked capsids within axons (Card et al., 1993; Hill et al., 1972; Lycke et al., 1988; Penfold et al., 1994). Using extensive EM studies of PrV (an α-herpesvirus member), infected vagal motor neurons, Card et al., suggest that viral progeny travel back to the periphery as enveloped capsids, and that envelopment follows the classical deenvelopment/reenvelopment pathway (Card et al., 1993). However, in recent

experiments based on an *in vitro* system of culturing human fetal dorsal root ganglia with autologous skin explants, Penfold and colleagues suggest otherwise (Penfold et al., 1994). They observed both enveloped and naked HSV capsids within infected neurons, but only naked capsids could be seen within axons. Further, they suggested that there exists a specialized form of HSV transport to the periphery such that capsids are transported via microtubules to the distal regions of the axon where the capsid buds into patches of HSV glycoproteins on the axon plasma membrane.

Reactivation of virus inevitably brings forward the question of the ultimate fate of the infected neurons. Replication of HSV in neurons in culture leads to certain death, yet many individuals suffer from hundreds of recrudescences and bear no discernable loss of sensation. Therefore, it would be hard to imagine that latently infected neurons die after HSV reactivation in these cells. In fact, analyses suggest that in the mouse model, of the 10% of neurons infected very few neurons die (Simmons and Tscharke, 1992).

1.3.2.1 Latent viral DNA

The physical form of the viral genome in latently infected neurons was first thought to be endless, as the viral DNA lacked detectable termini (Rock and Fraser, 1983; Rock and Fraser, 1985). These results were confirmed by Efstathiou et al., (1986), and together the data suggested that HSV DNA existed either integrated into the genome or was in an extrachromosomal state but one in which the viral DNA was circularized (Efstathiou et al., 1986; Rock and Fraser, 1983;

Rock and Fraser, 1985). Mellerick and Fraser were then able to separate viral from cellular DNA by buoyant density gradient centrifugation, indicating that the viral DNA was extrachromosomal, possibly as a circular episome (Mellerick and Fraser, 1987).

The number of neurons in a ganglia that harbour latent HSV is not well quantitated, principally due to the lack of success with in situ hybridization specific for HSV DNA (Ho, 1992; Steiner, 1996; Stevens, 1989). Some effort has been made to determine the copy number of HSV genomes per latently infected neurons. Based on several criteria including i) copy number of viral genomes per cell equivalent, ii) percent of neurons that yield virus following *in vitro* reactivation, and iii) the percent of neurons that make up a ganglion, several groups have estimated that there are between 10-1000 HSV genomes per latently infected neuron (Ho, 1992; Roizman and Sears, 1996; Stevens, 1989).

The mechanisms leading to the establishment and maintenance of a latent HSV infection are still unknown. HSV mutants unable to express either IE genes or VP16 were still capable of establishing latency and to reactivate (Katz et al., 1990; Leib et al., 1989; Steiner et al., 1990). Moreover, overexpression of VP16 (initiates expression of IE genes) did not inhibit the development or maintenance of latency (Sears et al., 1991), strongly suggesting that there are distinct mechanisms that set latency apart from a lytic infection.

Latently infected neurons do support a limited level of HSV specific transcription. These transcriptes are collectively referred to as LATs (latency

associated transcripts) (Deatly et al., 1987; Spivack and Fraser, 1987; Steiner et al., 1988; Stevens et al., 1987). LAT transcripts map exclusively to the long terminal repeat region of the HSV genome and are transcribed antisense to the ICP0 gene. LAT transcription initiates 3' to the ICP0 gene and extends approximately a third of the way into ICP0. There are two major LAT transcripts, one of 2.0 kb, and the other 1.5 kb in size; there are also transcripts present in smaller amounts referred to as minor LATs (mLATs). Together the major and minor LATs may be products of a larger transcript of 8.3 kb, itself commonly referred to as mLAT (Ho, 1992; Mitchell et al., 1990; Mitchell et al., 1990).

Since LATs appear to be the only transcripts detected, at least to date, it follows that they may have some function in either the establishment of, maintenance of, or reactivation of virus from the latent state. Studies by Javier et al, and Ho and Mocarski showed that LAT expression was not essential for either establishing or maintaining latency in a mouse model (Ho and Mocarski, 1989; Javier et al., 1988). However, if there is any evidence that LAT expression has a function, it may be in reactivation from the latent state (Hill et al., 1990; Hill et al., 1990; Leib et al., 1989; Steiner et al., 1989; Trousdale et al., 1991). Some evidence suggests that a cyclic AMP response element (CRE) within the LAT promoter may be required for efficient reactivation of HSV in the rabbit eye model, although it has no effect on the levels of the LAT transcripts (Bloom et al., 1997).

The LAT gene contains two open reading frames (ORFs), in which the larger of the two, ORF-2, is highly conserved among HSV-1 strains and could

code for a polypeptide of 305 amino acids (Wagner et al., 1988; Wechsler et al., 1989). Rabbit antibodies generated to a bacterial fusion protein containing the carboxy-terminus of ORF-2, recognized a protein by immunocytochemistry in HSV latently infected neurons *in vitro* but not in unifected or productively infected neurons (Doerig et al., 1991). In western blot analysis, a protein of approximately 80 kDa was detected and was designated latency-associated antigen (LAA) (Doerig et al., 1991). The expression of LAA only in HSV latently infected neurons suggested that LAA may have a role in the establishment, maintenance or reactivation of HSV from a latent state. To ascertain whether the LAA had any role during HSV latency, Farrell and colleagues (1993) generated a recombinant LAA-minus HSV mutant by inserting a frameshift 50 bp downstream of the the first ATG of the LAA ORF. Using the rabbit latency model, they could not detect any differences in the reactivation of the LAA- mutant compared to wild type HSV, suggesting that LAA does not play a role during HSV latency, or at least during reactivation (Farrell et al., 1993).

1.4 Viral Membrane Glycoproteins not Required For Replication in Cultured Cells

Of the 80 proteins made by HSV, over half of them are not required for efficient replication in tissue culture (reviewed in Roizman and Sears, 1996). Included among these nonessential proteins are 6 of the 11 HSV encoded glycoproteins. HSV mutants unable to express either glycoproteins gC, gE, gG, gI, gJ, or gM are capable of replicating in certain cultured cells (Ackermann et al.,

1986; Baines and Roizman, 1991; Baines and Roizman, 1993; Longnecker et al., 1987; Longnecker and Roizman, 1987; Longnecker and Roizman, 1986; Neidhardt et al., 1987; Weber et al., 1987). As it seem unlikely that HSV would conserve such a large number of proteins without a function, it seems reasonable to propose that they would have important functions *in vivo*. These glycoproteins could act either in the infection of diverse cell types, in promoting viral spread within tissues, or in protecting virus from the host immune system.

1.4.1 gC AS COMPLEMENT RECEPTOR:

In addition to playing a role in HSV adsorption to heparan sulfate GAGs, gC also functions as a receptor for the complement component C3b (Eisenberg et al., 1987; Friedman et al., 1984; Smiley et al., 1985; Tal-Singer et al., 1991). gC can protect HSV infected cells *in vitro* from complement-mediated immune lysis (Friedman et al., 1996; Fries et al., 1986; Kostavasili et al., 1997; McNearney et al., 1987). Complement component C3 is at the center of both the classical and alternative complement pathways (reviewed in Roitt, 1988). The alternative pathway is initiated by the activation of C3 to C3b. C3 has a naturally unstable thiodiester bond, which in combination with water or a plasma proteolytic enzyme, is split into the reactive intermediate C3b. C3b subsequently reacts with factors B and D forming the C3 convertase C3bBb. Under normal circumstances, C3bBb is inactivated by factor I, however C3bBb activity can be stabilized by binding properdin and to carbohydrate moities on the surface of microorganisms (bacteria and viruses). This creates a positive-feedback loop producing high

levels of activated C3bBb. The activated C3bBb converts C5 to C5b that together with a number of other complement factors form a pore complexes called the membrane attack complex (MAC). MACs result in a net influx of Na^+ and water into the cell, that inevitably leads to cell lysis. gC is thought to block the initiation of this cascade by inhibiting the binding of properdin and C5 to C3b (Kostavasili et al., 1997).

1.4.2 HSV Fc RECEPTOR:

Following infection with HSV types 1 and 2, many cell types in culture including cells of fibroblastic, epithelial and hematopoietic origin express a receptor for the Fc region of immunoglobulin G (IgG)(Costa et al., 1978; Dorval et al., 1979; Feorino et al., 1977; McTaggart et al., 1978; Nakamura et al., 1978; Watkins, 1964; Westmoreland and Watkins, 1974; Yasuda and Milgrom, 1968). Watkins in 1964, was the first to demonstate that HSV infected cells acquired IgG Fc receptors (Watkins, 1964). Watkins sensitized sheep erythrocytes with rabbit anti-sheep serum, and found that the only IgG coated erythrocytes adhered (hemadsorption) to HSV infected HeLa cells, whereas the erythrocytes did not adhere to uninfected cells. Moreover, Watkins could block hemadsorption by preincubating the HSV infected cells with rabbit anti-HSV serum while preimmune serum had no effect, suggesting that it was a viral- encoded or induced protein(s) that was responsible for hemadsorption. The specificity of HSV infected cells for the Fc region of IgG was then characterized by a number of studies. First, no hemadsorption was observed when the IgG fraction of the rabbit anti-sheep

erythrocyte serum was treated with pepsin prior to being added to the erythrocytes (Yasuda and Milgrom, 1968). Second, in experiments by Westmoreland and colleagues, whole IgG or Fc fractions of IgG labelled with ^{125}I bound to HSV infected cells while little of the labelled $F(ab')_2$ IgG fragments bound to infected cells (Westmoreland and Watkins, 1974). Together, these results suggested that a viral factor was expressed following infection and that it had affinity for the Fc domain of IgG.

At this time, however, one could have argued that HSV infection caused the induction of Fc receptor expression by the host cell. However, further characterization of the receptor activity strongly suggested that the Fc receptor was viral in nature. First, Fc receptor expression coincided with the replication cycle of HSV, first being detected 2 hrs after infection and reaching maximal activity by 8 hrs after infection (Nakamura et al., 1978; Watkins, 1965). In addition, the number of cells expressing receptor activity was dependent on the multiplicity of infection (MOI) (Costa et al., 1978). Second, Fc receptor activity could be blocked by inhibitors of either viral transcription or protein synthesis, however viral DNA synthesis was not required (Bourkas and Menezes, 1979; Costa et al., 1978; McTaggart et al., 1978; Watkins, 1965). The induced Fc receptor was also extremely sensitive to trypsin at concentrations as low as 0.1 mg/ml, compared with previously characterized Fc receptors of B and T cells which were resistant to concentrations greater than 10 mg/ml (Dickler, 1974; Warner et al., 1975). When HSV infected cells were treated with trypsin at 8 hrs

after infection, Fc receptor activity reappeared to maximal levels within 2 hrs. Thus, reappearance of the Fc receptor occurred well after host protein synthesis had been inhibited (Nakamura et al., 1978). Since it was known that rabbit anti-HSV F(ab')2 fragments could block either hemadsorption or binding of radiolabelled IgG to HSV infected cells (Adler et al., 1978; Yasuda and Milgrom, 1968), McTaggart and colleagues asked whether rabbit anti-cell $F(ab')_2$ fragments could block binding of labelled IgG to HSV infected HeLa cells. They found that only the anti-HSV $F(ab')_2$ fragments could block IgG binding to infected cells (McTaggart et al., 1978), strongly suggesting that a cellular component was not involved. Finally, experiments by Para et al., demonstrated the presence of Fc receptors in purified HSV virions and these receptors could be transferred to the cell surface following fusion of the virion envelope with the plasma membrane (Para et al., 1980). Taken together, these data indicated that the Fc receptor activity observed on the surface of HSV infected cells was due to a virus encoded protein(s).

1.4.2.1 Fc Receptor Specificity for IgG

The original observations of HSV Fc receptor activity involved binding of either rabbit or human sera (Nakamura et al., 1978; Watkins, 1964; Yasuda and Milgrom, 1968). Preliminary studies by Yasuda and Milgrom, showed that antibodies from human, rabbit, guinea pig and goat were able to facilitate hemadsorption to infected cells, while rat anti-sera could not (Yasuda and Milgrom, 1968). A more detailed study on Fc receptor species specificity was

undertaken by Johansson and colleagues. They found that the HSV Fc receptor was able to bind to human, swine, rabbit, goat, sheep and cow IgG, but not to mouse, guinea pig, hamster, rat, dog, cat or horse IgG (Johansson et al., 1985). Further analysis indicated that the Fc receptor exhibited a subclass specificity for the following IgGs: $IgG_4 > IgG_1 \geq IgG_2$. The HSV Fc receptor did not bind IgG_3 (Johansson et al., 1984; Wiger and Michaelsen, 1985). IgG class specific binding was independent of both the cell type infected and the HSV strain used (Johansson and Blomberg, 1987; Wiger and Michaelsen, 1985). Using a series of IgG fragments, Johansson et al were able to map the binding site of IgG to the Cγ2-Cγ-3 interface, the analogous site for protein A of *Staphylococcus aureus* (Johansson et al., 1986; Johansson et al., 1989; Schroder et al., 1987). The similarity in binding site on IgG for both the HSV Fc receptor and Protein A is supported by previous observations (Johansson et al., 1989). First, Protein A can inhibit binding of radiolabelled IgG to HSV infected cells in dose dependent manner; and second, Fc receptors expressed on the surface of HSV infected cells can block binding of FITC labelled Protein A to anti-cell IgG. Finally, Protein A exhibits the same binding specificity to human IgG subclasses

Protein A coated-sepharose beads are commonly used in immunoprecipitation experiments to isolate HSV IgG Fc receptor/non-immune IgG complexes from HSV infected cell lysates even though both the HSV Fc receptor and Protein A share the same IgG binding site. This observation can be explained by the the fact that much of the IgG is found aggregated and is not

monomeric. Thus within a single IgG aggregate, there would be Fc domains available for both HSV Fc receptor and Protein A binding.

1.4.2.2 Fc Receptor Components: Glycoproteins gE and gI

In an attempt to purify the viral Fc receptor, Baucke and Spear (1979) ran detergent extracts of HSV infected cells over rabbit IgG affinity columns (Baucke and Spear, 1979). They were able to purify three electrophoretic distinguishable Fc-binding species with apparent molecular weights ranging from 65,000 - 80,000 Da. Further analysis showed that these three species were different forms of a single processed glycoprotein termed gE. This was in agreement with previous studies suggesting that the HSV Fc receptor was a glycoprotein (Costa et al., 1978; McTaggart et al., 1978). These gE related proteins were a constituent of the cell surface and appeared concomitantly with the increase of Fc receptor activity on the surface of infected cells. Moreover, these gE related proteins could bind to IgG and binding was dependent upon the Fc region, as gE could not be purified using IgG columns treated with pepsin (i.e., $F(ab')_2$ columns).

Rabbit antibodies raised against the affinity purified gE related proeins could immunoprecipitate gE from a variety of HSV infected cell membrane extracts, and could also precipitate gE from various HSV-1 strains (Para et al., 1982). However, it was noted that the electrophoretic mobility of gE precipitated from HSV-1 was different from HSV-2 (Para et al., 1982). Using two sets of HSV-1 x HSV-2 intertypic recombinant viruses, Para and colleagues were able to exploit the differences in gE mobility to map gE between 0.85 to 0.97 map units,

corresponding to a position within the unique short region (Us) of the HSV genome (Para et al., 1982). This agreed with previous work by Hope and Marsden who had mapped gE to 0.832 to 0.95 map units using intertypic HSV recombinants (Hope and Marsden, 1983; Hope et al., 1982) Finally, using a selective mRNA hybridization strategy, Lee et al (1982) were able to map gE further to coordinates between 0.924 and 0.951 (Lee et al., 1982), which corresponded to the US8 gene (McGeoch et al., 1985). gE is 550 amino acids long and is the major sulfated HSV glycoprotein detected following 2D-electrophoresis of [^{35}S]inorganic sulfate labelled HSV infected cells (Hope and Marsden, 1983; Hope et al., 1982; McGeoch et al., 1985). Although the majority of sulfate is incorporated into N-linked oligosaccharides, some sulfate may be in other parts of gE, possibly the polypeptide backbone (Hope and Marsden, 1983). gE is also the only known HSV glycoprotein that incorporates [^{3}H] palmitate (Johnson and Spear, 1983). Fatty acid acylation occurs just prior to the shift in electrophoretic mobility of gE, suggesting that the fatty acid is incorporated into high mannose oligosaccharides before maturation in the Golgi (Johnson and Spear, 1983). No known function is associated with fatty acid acylation of gE.

gE was found to be complexed with another protein called gI (Johnson and Feenstra, 1987; Johnson et al., 1988). gI was first detected as a 70,000 Da species (referred to as g70) in ^{125}I labelled HSV-1 infected cell extracts immunoprecipitated with rabbit sera or IgG (Johnson and Feenstra, 1987). Most of the gI synthesized in virus infected cells appears to be complexed in a 1:1 molar

ratio with gE forming a complex that has for Fc receptor activity (Johnson and Feenstra, 1987) Further characterization showed that gI is a glycoprotein 390 amino acids long that maps to the US8 gene (Johnson and Feenstra, 1987; Johnson et al., 1988; Longnecker et al., 1987; McGeoch et al., 1985). Both gI and gE together as a complex are required for full Fc receptor activity (Bell et al., 1990; Hanke et al., 1990; Johnson and Feenstra, 1987; Johnson et al., 1988). Non immune rabbit serum was unable to precipitate gE or gI if cells were infected with either gE or gI null mutants (gE^- or gI^-). In addition cells infected with either gE^- or gI^- mutants were unable to bind labelled IgG whereas cells co-infected with the two mutants bound significant levels of the labelled IgG (Johnson et al., 1988). To confirm these results, Bell et al., and Hanke et al., independently used virus vectors to express gE and gI either alone or in combination in cells (Bell et al., 1990; Hanke et al., 1990). Using the labelled IgG assay, both groups found that both gE and gI were required for Fc receptor activity. However if a more sensitive hemadsorption assay was used, Fc receptor activity could be detected with gE alone, albeit at lower levels than with gE and gI together. Expression of gI alone gave no detectable hemadsorption. Therefore, gE and gI are both necessary and sufficient for full Fc receptor activity although gE along can bind IgG to a lesser extent (Bell et al., 1990; Hanke et al., 1990).

It has also been proposed that gE alone acts as low affinity receptor for complexed IgG whereas the gE/gI complex acts as a high affinity receptor for monomeric IgG (Bell et al., 1990; Dubin et al., 1990). However, the sensitivity of

the assays used to compare Fc receptor activity, IgG-coated red blood cells versus monomeric radiolabelled IgG, are very different (Bell et al., 1990; Dubin et al., 1990; Hanke et al., 1990). Therefore, it is not clear what are the affinities of gE versus gE/gI for IgG. The affinity of gE/gI for monomeric IgG has been estimated at 2×10^{-7} M (Johansson and Blomberg, 1990) which is relatively low compared to the range of $2\text{-}5 \times 10^{-9}$ M reported for the human FcγRI receptor (Allen and Seed, 1989; Shopes et al., 1990)

1.4.2.2 Function of HSV Fc Receptor Activity in vitro

Both gE and gI are dispensable for HSV replication in certain cultured cells (Longnecker and Roizman, 1987; Longnecker and Roizman, 1986; Neidhardt et al., 1987), however their ability to act as a Fc receptor suggested that gE/gI may play a role in modulating the host immune response. It had been previously proposed that Fc receptors on tumour cells or viruses may protect them from immune lysis (Kerbel and Davies, 1974; Sjogren et al., 1971). Aggregates of IgG bound to the surface of the cells through Fc receptor may mask or block access to tumour-specific antigens. Using an *in vitro* complement-mediated cytolysis assay, Adler et al., demonstrated that heat aggregated rabbit IgG could block antibody dependent complement mediated lysis of HSV infected cells by up to 90%. The addition of aggregated nonimmune $F(ab')_2$ fragments had no effect on killing. Similarly, preincubation of HSV-2 infected cells with preimmune rabbit antiserum or purified Fc fragments protected cells from antibody dependent neutralization (Dowler and Veltri, 1984). Moreover, in the presence of aggregated nonimmune

IgG HSV Fc receptor activity could block cell mediated killing of HSV infected cells by as much as 67% (Adler et al., 1978). Together, these experiments demonstrated that the HSV Fc receptor could protect HSV infected cells *in vitro* from complement-dependent and -independent immune cytolysis.

The mechanism by which the aggregated nonimmune IgG provided protection to the infected cells is unclear. It was suggested that the aggregated nonimmune IgG, bound to the cell surface Fc receptor, masked the presence of HSV specific antigens by sterically blocking the binding of immune IgG (Adler et al., 1978). In the classical complement pathway, binding of antibody to antigen leads the association with complement component C1q. Together with C1r and C1s, a trimolecular complex is formed C1qrs, that is capable of cleaving the next component in the pathway C4. The product, C4b interacts with C2 forming the C3 convertase C4b2b (i.e, equivalent to activated C3bBb). From then on the cascade is essentially as the alternative pathway in which C5 is recruited to form the MAC (see above), and cell lysis ensues. Since antigen specific antibodies could not bind to the cell surface there would not be a concomitant binding of C1q to the immune IgG and thus inhibition of the complement cascade. In addition, this blocking effect would also limit the ability for cell mediated lysis (also referred to as antibody-dependent cell-mediated cytotoxicity (ADCC)). Leukocyte activation requires binding of the Fc domain of the IgG-antigen complex to the leukocyte's Fc receptor. gE/gI expressed on the surface of the infected cell inhibit leukocyte activation by blocking (via binding to aggregated

non-immune IgG) the immune cell's Fc receptors access to immune IgG bound at the infected cell surface.

An alternative proposal for HSV Fc receptor protection from the immune system is the so called "double binding" or bipolar bridging (Lehner et al., 1975). In this case immune IgG is bound by its Fab end to its antigen while the Fc domain is bound to a Fc receptor, and thus forms a "bridge" on the cell surface. In this form, IgG is unable to fix complement component C1q or bind to Fc receptors of immune cells. Purified HSV gE$^-$ virions were 10-100 fold more susceptible to complement-mediated neutralization in the presence of rabbit immune serum compared to wild type virus (i.e., express Fc receptors) (Frank and Friedman, 1989). However, no protection from neutralization was evident for wild type virus in the presence of mouse antibodies which do not bind the HSV Fc receptor (Frank and Friedman, 1989). Moreover, bipolar bridging by gE/gI protected wild type infected cells from ADCC. HSV gE/gI could also inhibit binding of C1q to immune IgG thereby inhibiting the classical complement pathway (Dubin et al., 1991). Similarly, bipolar bridging was shown by van Vleit and colleagues who observed reduced binding of FITC-coupled protein A to HSV infected cells that were treated with anti-cellular IgG, compared to cell infected with an HSV gE$^-$ mutant. In this case, bipolar bridging occurred between the IgG bound to its cellular antigen and to the HSV Fc receptor. Thus bipolar bridging effectively eliminated the ability for the labelled protein A to bind to the Fc domain of the bound IgG (Van Vliet et al., 1992). In addition, the HSV Fc

receptor was able reduce by two fold the Fc-dependent attachment of human polymorphonuclear leukocytes (PMN) to HSV infected cells compared to uninfected cells (Van Vliet et al., 1992).

1.4.2.3 ROLE FOR gE/gI IN VIVO:

It is not known at this time whether gE/gI can act *in vivo* as a Fc receptor and protect HSV infected cells from complement-dependent and -independent immune cytolysis. However, there is some evidence that HSV gE^- mutants are severely reduced in pathogenicity (Kudelova et al., 1991; Neidhardt et al., 1987; Rajcani et al., 1990; Rajcani et al., 1990; Schranz et al., 1989). The first gE^- mutant described grew to titres of only 25-30% of that of the parent virus, and produced smaller plaques on Vero cell monolayers (Neidhardt et al., 1987). However the striking observation was the disparity in neurovirulence (ie., capacity of the virus to cause death) of the gE^- mutant compared to the wild type parent. Following intracerebral inoculation of mice, the LD_{50} (50% lethal dose) of the wild type virus was 1 PFU (plaque forming unit) per mouse. In marked contrast, the gE^- mutant LD_{50} was 1.4×10^5 PFU per animal (Neidhardt et al., 1987).

HSV-1 ANGpath is a highly pathogenic strain of virus which was derived by repeated intracerebral passaging of the HSV-1 stain ANG in mice (Kaerner et al., 1983; Kumel et al., 1986). When injected intraperitoneally (i.p.), into DBA-2 mice, ANGpath spread efficiently to the spleen and other visceral organs, followed quickly by the infection of the nervous system (spinal cord and brain

stem) (Rajcani et al., 1990). In over 70% of the animals infected, the mice died due to encephalitis. The less pathogenic ANG strain could spread within the peritoneal cavity to a limited extent and could infect the peripheral nervous system, yet the infection did not lead to encephalitis. However, a gE^- mutant derived from ANGpath was exceptionally nonpathogenic; there was only a limited replication within the spleen and the mutant failed to spread to other organs and the nervous system. Moreover, the gE^- mutant failed to spread to the trigeminal ganglion following corneal inoculation in mice and rabbits (Kudelova et al., 1991; Rajcani et al., 1990; Rajcani et al., 1990). Thus the loss of gE expression substantially decreased the virulence of HSV-1, to the point where the pathogenesis of highly virulent strain is reduced to below that of a normally nonpathogenic virus. These observation suggest that gE is an important determinant of HSV pathogenicity (including neurovirulence) and that gE and likely gI, are required for the efficient spread of virus *in vivo*. However, it is unclear whether the differences in pathogenicity between the wild type virus and the gE^- mutant virus were related to the loss of Fc receptor activity.

Chapter 2 Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells

2.0 Preface

In the following publication, I carried out the experiments in Figures 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 2.10. Figure 2.3 and Table 2.1 were carried out by Q.Tang and R. Hendricks at the University of Illinois at Chicago. The original observation that HSV gI$^-$ mutants produced small plaques on monolayers of normal human fibroblasts was made by M. Tang and A. Rainbow. C. Brunetti constructed the plasmid pgEßgal that I later used to derive the recombinant gE$^-$ HSV mutant F-gEß.

JOURNAL OF VIROLOGY, Feb. 1994, p. 834–845
0022-538X/94/$04.00+0

Vol. 68, No. 2

Herpes Simplex Virus Glycoproteins E and I Facilitate Cell-to-Cell Spread In Vivo and across Junctions of Cultured Cells

KEVIN S. DINGWELL,[1,2] CRAIG R. BRUNETTI,[1] ROBERT L. HENDRICKS,[3] QIZHI TANG,[4] MARY TANG,[2] ANDREW J. RAINBOW,[2] AND DAVID C. JOHNSON[1]*

Molecular Virology and Immunology Program, Department of Pathology,[1] and Department of Biology,[2] McMaster University, Hamilton, Ontario, Canada L8N 3Z5, and Departments of Ophthalmology and Visual Sciences[3] and Microbiology and Immunology,[4] The University of Illinois, Chicago, Illinois 60612

Received 11 October 1993/Accepted 17 October 1993

Herpes simplex virus (HSV) glycoproteins E and I (gE and gI) can act as a receptor for the Fc domain of immunoglobulin G (IgG). To examine the role of HSV IgG Fc receptor in viral pathogenesis, rabbits and mice were infected by the corneal route with HSV gE$^-$ or gI$^-$ mutants. Wild-type HSV-1 produced large dendritic lesions in the corneal epithelium and subsequent stromal disease leading to viral encephalitis, whereas gE$^-$ and gI$^-$ mutant viruses produced microscopic punctate or small dendritic lesions in the epithelium and no corneal disease or encephalitis. These differences were not related to the ability of the gE-gI oligomer to bind IgG because the differences were observed before the appearance of anti-HSV IgG and in mice, in which IgG binds to the Fc receptor poorly or not at all. Mutant viruses produced small plaques on monolayers of normal human fibroblasts and epithelial cells. Replication of gE$^-$ and gI$^-$ mutant viruses in human fibroblasts were normal, and the rates of entry of mutant and wild-type viruses into fibroblasts were similar; however, spread of gE$^-$ and gI$^-$ mutant viruses from cell to cell was significantly slower than that of wild-type HSV-1. In experiments in which fibroblast monolayers were infected with low multiplicities of virus and multiple rounds of infection occurred, the presence of neutralizing antibodies in the culture medium caused the yields of mutant viruses to drop dramatically, whereas there was a lesser effect on the production of wild-type HSV. It appears that cell-to-cell transmission of wild-type HSV-1 occurs by at least two mechanisms: (i) release of virus from cells and entry of extracellular virus into a neighboring cell and (ii) transfer of virus across cell junctions in a manner resistant to neutralizing antibodies. Our results suggest that gE$^-$ and gI$^-$ mutants are defective in the latter mechanism of spread, suggesting the possibility that the gE-gI complex facilitates virus transfer across cell junctions, a mode of spread which may predominate in some tissues. It is ironic that the gE-gI complex, usually considered an IgG Fc receptor, may, through its ability to mediate cell-to-cell spread, actually protect HSV from IgG in a manner different than previously thought.

Herpes simplex viruses (HSVs) can initiate infection of cultured cells by adsorbing onto the cell surface and then penetrating across cellular membranes so that the nucleocapsid enters the cytoplasm. However, spread of HSV in tissues involves additional features because viruses frequently move across cellular junctions where cells are closely cemented or through extracellular matrix where cells are more distantly separated. Cell-to-cell spread of HSV in monolayers of certain cultured cells can also involve infection of adjacent cells across cell junctions because plaques can be produced in the presence of neutralizing antibodies. Major participants in these processes include the HSV glycoproteins which comprise the protein component of the virion envelope. HSV type 1 (HSV-1) encodes at least 11 glycoproteins, of which gB, gD, gH, gK, and gL are essential for productive infections in cultured cells (10, 18, 40, 46, 60). Virus mutants lacking gB, gD, and either gH or gL, which form a hetero-oligomer (39), are unable to enter cells (10, 18, 44, 60). There is evidence suggesting that gB and gH/gL function directly in the fusion of viral and cellular membranes during virus penetration (8, 9, 10, 21, 23, 34, 56) and that gD binds to cellular proteins (7, 11, 42, 44, 45). In addition, gB and gC probably mediate interactions with heparan sulfate proteoglycans during virus adsorption onto the cell surface (33, 61). The remaining five glycoproteins; gE, gI, gG, gJ, and gM, are considered nonessential in that they are dispensable for virus infection and replication in cultured cells (2, 3, 43, 48, 49, 65).

Since it seems unlikely that HSV conserves unnecessary genes, it is probable that these so-called nonessential glycoproteins play important roles in vivo in the infection of diverse cell types, movement of virus through tissue, or protection from host immune responses. gC functions as a receptor for the complement component C3b (17, 20), and a complex of gE and gI acts as a receptor for immunoglobulin G (IgG) (4, 25, 43). It appears likely that by binding complement or the Fc domain of IgG, these viral proteins may protect HSV-infected cells from complement-mediated immune lysis, in the case of the Fc receptor by causing IgG aggregation or by reducing the ability of complement components to bind to virus- or cell-associated IgG (1, 15, 19, 25).

HSV-1 gE mutants exhibit decreased neurovirulence. Neidhardt et al. (57) reported that an HSV-1 gE mutant was much less neurovirulent than parental strain HSV-1(ANG). Rajcnai et al. (59) found that a gE mutant spread poorly following intraperitoneal inoculation and failed to spread axonally following corneal inoculation. A pseudorabies virus (PrV) mutant deficient in the PrV gE homolog, gI, displayed reduced ability to spread in the rat central nervous system (12). These observations suggested that HSV gE and the PrV homolog function in vivo by facilitating virus spread; however, it has been difficult to interpret some of the in vivo data involving

* Corresponding author. Mailing address: Room 4H30/HSC, McMaster University, 1200 Main St. West, Hamilton, Ontario, Canada L8N 3Z5. Phone: 905-529-7070, ext. 22359. Fax: 905-546-9940.

gE-negative mutants because it is not known whether IgG Fc receptor activity participates in virus spread, perhaps by reducing immune surveillance or cytolysis of virus-infected cells.

We examined the pathogenicity of HSV-1 gE$^-$ and gI$^-$ mutants after infecting the eyes of rabbits and mice. Mutants unable to express gE or gI produced very small punctate or dendritic lesions in the corneal epithelium, in comparison with the large dendritic lesions produced by wild-type HSV-1. This difference was unrelated to anti-HSV IgG because it was observed before IgG appeared and in mice, in which IgG does not bind to the Fc receptor. Furthermore, we showed that cell-to-cell spread of these mutant viruses was markedly reduced in monolayers of human fibroblasts and epithelial cells. The decreased cell-to-cell spread was not related to differences in the rate or extent of virus entry into these cells. Thus, gE and gI are important in spread of HSV in epithelial tissues and in monolayers of certain cultured human cell types which form cellular junctions.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human R970-5 (here called R970) cells were grown in alpha minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS). Normal human fibroblasts were grown in α-MEM supplemented with 10% FBS. HSV-1 strain F (obtained from P. G. Spear, Northwestern University Medical School), F-US7kan (43), 17syn+ (here called 17) (6), *in*1404 (43), and F-gEβ were propagated and titered on Vero cells.

Antibodies. Monoclonal antibody (MAb) 3104, specific for gI (43), and 3114, specific for gE, were gifts of Anne Cross and Nigel Stow (Institute of Virology, Glasgow, United Kingdom). MAbs LP11, which is specific for gH (18), and LP2, specific for gD (55), were gifts from A. C. Minson (University of Cambridge, Cambridge, United Kingdom). MAb DL11, specific for gD (16), was a gift G. Cohen (University of Pennsylvania). Anti-thymidine kinase (anti-TK) polyclonal rabbit serum was generously provided by W. Summers (Yale University). MAb 17βA1, specific for the large subunit of ICP6 (38), was a gift of S. Bacchetti (McMaster University, Ontario, Canada). Rabbit serum against HSV-1 (MacIntyre strain) was purchased from Dako (Dimension Laboratories, Mississauga, Ontario, Canada). Peroxidase-coupled goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Polyclonal sera specific for gE and gI (R gE/gI) was raised in rabbits inoculated with recombinant adenoviruses AdgE and AdgI (25) by intraperitoneal injection of animals with 10^9 PFU of both AdgE and AdgI.

Labelling of cells with [^{35}S]methionine, immunoprecipitations, and gel electrophoresis. Human R970 cells or normal fibroblasts grown on 35-mm dishes were infected with 5 or 10 PFU of either HSV-1 strain F or 17 or mutant F-gEβ, *in*1404, or F-US7kan per cell. After 2 or 5 h, medium was removed and the monolayer was washed twice with medium 199 lacking methionine and then radiolabeled with [^{35}S]methionine (50 μCi/ml; Amersham, Oakville, Ontario, Canada) in medium 199 lacking methionine for 2 or 3 h. Cell extracts were made by using Nonidet P-40 (NP-40)–deoxycholate (DOC) extraction buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 0.5% sodium DOC, 1% NP-40) containing 2 mg of bovine serum albumin per ml and 1 mM phenylmethylsulfonyl fluoride and stored at −70°C. Cell extracts were thawed at room temperature, subjected to a high-speed centrifugation, and mixed with sera or MAbs for 2 h at 4°C, and then protein A-Sepharose (Pharmacia, Dorval, Quebec, Canada) was added for an additional 2 h at 4°C on a rotating wheel. Protein A-Sepharose beads were washed three times with NP-40–DOC buffer, and precipitated proteins were eluted with twofold-concentrated sample buffer (100 mM Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 20% glycerol, bromophenol blue) and boiled for 5 min. Samples were electrophoresed in a 8.5% *N,N'*-diallyltartardiamide cross-linked polyacrylamide gel as described by Heine et al. (26) at 50 V for 14 to 16 h. Gels were fixed for 1 h, washed twice in distilled water for 2 min, enlightened (Enlightening; Dupont, Mississauga, Ontario, Canada) for 30 min, dried, and placed in contact with Kodak XAR film.

Construction of plasmid pgEβgal and HSV-1 gE$^-$ mutant F-gEβ. All enzymes used to construct plasmids were purchased from Bethesda Research Laboratories (Burlington, Ontario, Canada) or New England Biolabs (Mississauga, Ontario, Canada). Plasmid DNA was prepared by using Qiagen 500 columns (Qiagen Inc., Chatsworth, Calif.). Plasmid pgEβgal was constructed from pSV2XXXgEN (25) by replacing a *Sma*I fragment encompassing the 5' coding sequences of the gE gene with a 4.3-kb *Bam*HI fragment containing the ICP6::*lacZ* cassette from pD6p (22). Vero cell monolayers (60% confluent 100-mm dishes) were transfected with pgEβgal and viral DNA derived from Vero cells infected with HSV-1 strain F, using the $CaPO_4$ technique (24) as previously described (46). Recombinant viruses which had the gE gene replaced with the β-galactosidase gene were identified as blue plaques following screening with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim Canada, Ltd., Dorval, Quebec, Canada) as previously described (46). Recombinant viruses were plaque purified three times. Viral DNAs were extracted from infected cells (62), and recombination was confirmed by Southern blot analysis (50).

Ocular HSV-1 infections. Six-week-old female A-strain mice (Frederick Cancer Research and Development Center, Frederick, Md.) were anesthetized with 2.0 mg of ketamine hydrochloride (Vetalar; Park-Davis, Morris Plains, N.J.) and 0.04 mg of acepromazine maleate (Aveco Co., Inc., Fort Dodge, Iowa) in 0.1 ml of RPMI 1640 injected intramuscularly. Corneas of anesthetized mice were scarified in a crisscross fashion with a sterile 30-gauge needle, the eyes were infected topically with 3 μl (10^5 PFU) of HSV-1 F, F-US7kan, or F-gEβ, and then the eyelids were rubbed. Two days postinfection (p.i.), the eyes of infected mice were instilled with 3 μl of fluorescein solution and photographed by using a vertically mounted slit lamp with a 27× objective and a cobalt blue light source. After 7 to 14 days, herpes stromal keratitis was observed in animals inoculated with wild-type HSV-1 F as previously described (32).

Single and multistep growth curves of HSV-1 replication. Normal human fibroblasts were seeded in 35-mm dishes and grown to confluency. Cell monolayers were infected with F, F-gEβ, or F-US7kan, using 5 (single-step growth curves) or 0.001 (multistep growth curves) PFU per cell for 2 h, the media were aspirated, the cells were washed once with phosphate-buffered saline (PBS), and fresh media were added. In some instances, the media were supplemented with 0.1% human gamma globulin (HGG) to neutralize the spread of extracellular virus. At various times p.i., cells and media were collected separately and frozen at −70°C. Samples were later thawed, sonicated, and plaqued on Vero cells under standard plaque assay conditions, using 0.1% HGG. After 48 h, the cell monolayers were fixed and then stained with crystal violet and plaques were counted.

Assay for rate of HSV penetration into cells. The low-pH citric acid entry assay, used to measure the rate of viral penetration, was adapted from methods of Huang and Wagner

(37) and Highlander et al. (35). Normal human fibroblasts grown on 12-well dishes were placed at 4°C for 30 min prior to a 2-h adsorption period at 4°C with 100 PFU of HSV-1 F, F-US7kan, or F-gEβ. Viruses were removed, the cells were washed three times with PBS, fresh medium was added, and the dishes were shifted to a 37°C incubator to allow viruses to penetrate. At various times following the temperature shift, the media were replaced with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) or PBS for 1 min. These buffers were aspirated, the cells were washed three times with PBS, and fresh medium supplemented with 0.1% HGG and 1% FBS was added. After 48 h, the cell monolayers were fixed and stained with crystal violet and plaques were counted.

Immunoperoxidase staining of fibroblasts. Normal human fibroblasts were seeded on glass coverslips in 35-mm dishes and infected with HSV-1 F, F-US7kan, or F-gEβ at a multiplicity of infection of 0.002. At 2 h p.i., the inoculum was replaced with fresh medium supplemented with 0.1% HGG or with other antibodies. At 12-h intervals, medium was removed and coverslips were washed once with PBS, washed once with water, and air dried. Cells were fixed with 100% acetone (−20°C) for 10 min, washed briefly in water, air dried, and stored at −20°C. Coverslips were incubated with Dako rabbit anti-HSV-1 serum diluted 1:100 in PBST (PBS supplemented with 0.05% Tween 20) and incubated at 37°C for 1 h. Cells were washed three times with PBST and incubated with a 1:100 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) at 37°C for 30 min. Cells were washed three times in PBST, and peroxidase substrate (equal volumes of 0.02% hydrogen peroxide and 1 mg of DAB [3,3'-diaminobenzidine tetrahydrochloride dihydrate; Bio-Rad] per ml in 100 mM Tris [pH 7.2]) was added for 20 min. Coverslips were washed three times in water, air dried, and mounted on glass slides.

RESULTS

Construction of an HSV-1 mutant, F-gEβ, unable to express gE. HSV-1 mutant *in*1404, which was used in previous studies, was derived from strain 17 and does not express gE because an oligonucleotide was inserted into a *Hpa*I site near the 5' terminus of the gE coding sequences, producing a stop codon (43). In certain animal experiments, there appeared to be selective pressure for *in*1404 to revert to wild type, perhaps related to the fact that the coding sequences for gE are intact (19a). Therefore, we constructed a second gE-negative mutant in which a large fraction of the gE coding sequence was deleted from HSV-1 strain F so as to simplify comparisons with mutant F-US7kan (43), a gI⁻ mutant derived from F. Plasmid pgEβgal (Fig. 1) was constructed from pSV2XXXgEN (25) by replacing a *Sma*I fragment with a 4.3-kb *Bam*HI fragment containing the ICP6::*lacZ* cassette of pD6p (22). The *lacZ* insertion begins 4 bp upstream of the ATG initiation codon for gE and deletes approximately 60% of the coding sequences. Vero cells were cotransfected with HSV-1 F viral DNA and pgEβgal, and virus progeny were screened by using agarose overlays containing X-Gal. A recombinant virus, F-gEβ, was isolated, and Southern blot analysis was used to confirm the structure of the viral DNA (data not shown).

To test for expression of gE, human R970 cells were infected with wild-type HSV-1 F or mutant F-gEβ or *in*1404, the cells were labelled with [^{35}S]methionine, and gE was immunoprecipitated. A MAb specific for gE (3114) and a polyclonal serum produced in rabbits inoculated with adenovirus vectors expressing gE and gI (R gE/gI) (25) both precipitated a complex of gE and gI from extracts of cells infected with wild-type HSV-1 F, but no gE was detected in extracts from cells infected with F-gEβ or *in*1404 (Fig. 2). Similarly, the rabbit IgG present in rabbit preimmune serum precipitated the gE-gI complex from extracts of cells infected with wild-type HSV-1 as previously reported (43), but the gE-gI complex was not detected in extracts from F-gEβ- or *in*1404-infected cells. Rabbit polyclonal anti-gE/gI antibodies and MAb 3104, specific for gI, precipitated gI but not gE from extracts of cells infected with F-gEβ and *in*1404. Therefore, F-gEβ does not express gE but expresses gI.

F-US7kan and F-gEβ display a reduced pathogenicity in the mouse eye. Rabbits and mice were infected by the corneal route with gE⁻ or gI⁻ mutant or wild-type HSV, and disease was evaluated as previously described (28). Only the results for mice will be described, although very similar results were also obtained for rabbits. In two identical experiments, three groups of four mice (6 weeks old; A strain) were topically infected with each virus by corneal scarification. Two days later, epithelial lesions were examined and photographed by using a slit lamp following instillation of a fluorescein dye. A marked difference between the ability of wild-type HSV-1 F and the mutants F-gEβ and F-US7kan to produce epithelial lesions was observed (Fig. 3 and Table 1). Wild-type HSV-1 produced very large lesions with an amorphous center and dendritic projections, the gE⁻ mutant produced barely perceptible, punctate keratopathies, and the gI⁻ mutant, F-US7kan, produced smaller dendritic lesions, but these were clearly distinct from those produced by wild-type F (Fig. 3). At 7 to 14 days p.i., herpes stromal keratitis was observed in mice infected with wild-type F but not in mice inoculated with either mutant (Table 1). All mice infected with F succumbed to encephalitis and were sacrificed, whereas no signs of viral encephalitis were observed in mice infected with F-US7kan or F-gEβ. Patterns of disease illustrated in Table 1 and Fig. 3 were uniformly observed within each group of mice. Large differences in epithelial disease were observed by 2 days postinoculation and would, therefore, not be related to the IgG Fc receptor activity of the gE-gI complex, since it appears highly unlikely that anti-HSV IgG could appear in this short period of time. In the rabbit eye, anti-HSV IgG or IgA cannot be detected until 5 to 13 days p.i. (13, 27). In addition, mouse IgG binds poorly or not at all to the HSV Fc receptor (41). In other experiments (not shown), rabbits or mice infected with wild-type HSV-1 strain 17 displayed large dendritic lesions, whereas the lesions produced by the gE-negative mutant *in*1404 (43) were small, barely detected, punctate lesions. In these experiments performed for 2 to 5 days, there was insufficient time for *in*1404 to revert to wild type. Therefore, these differences in pathogenicity were observed with two independently isolated gE mutants and a gI mutant, supporting the premise that this phenotype was related to the absence of the gE-gI hetero-oligomer rather than to mutations in other genes.

Mutants unable to express gE or gI produce small plaques on monolayers of human fibroblasts. To investigate the basis for the reduced pathogenicity of HSV-1 gE or gI mutants, we characterized the replication of mutant viruses in a number of different cultured cells. On several nontransformed fibroblasts derived from human skin biopsies, we observed a dramatic decrease in the size of plaques produced by F-gEβ and F-US7kan relative to those produced by wild-type HSV-1 F (Fig. 4). Similarly, *in*1404 produced much smaller plaques than parental wild-type strain 17 on fibroblast monolayers, and several other gI-negative mutants displayed the small-plaque phenotype (not shown). Since these other mutant viruses were

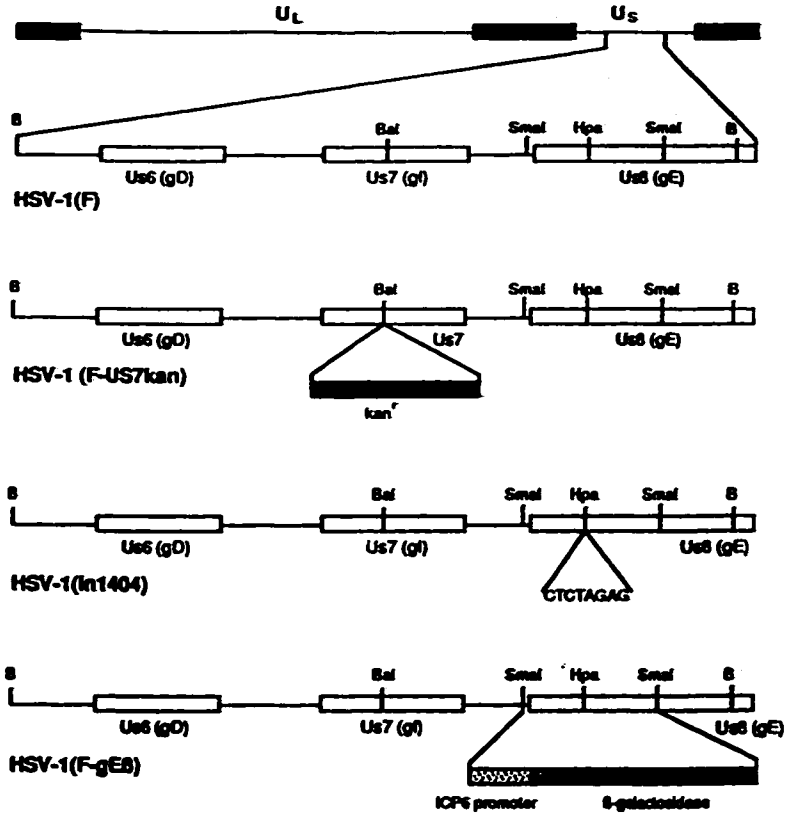


FIG. 1. Schematic representations of DNA sequence rearrangements in HSV-1 gE⁻ and gI⁻ mutant viruses. The region of HSV-1 DNA which includes the *Bam*HI J fragment containing the US6 (gD), US7 (gI), and US8 (gE) genes (51) is depicted for the HSV-1 recombinants F-US7kan, *in*1404, and F-gEβ. F-US7kan was derived from F-gDβ after gI coding sequences were disrupted by the insertion of a kanamycin resistance gene cassette into a unique *Bal*I site (43). *in*1404 was derived from HSV-1 strain 17 by insertion of an 8-bp *Xba*I linker into an *Hpa*I site near the 5′ end of the US8 coding sequences (43). In F-gEβ, an ICP6::β-galactosidase cassette (22) replaces 60% of gE coding sequences. B, *Bam*HI; Bal, *Bal*I; Hpa, *Hpa*I; UL, unique long region of HSV genome; US, unique short region.

independent isolates, it is unlikely that mutations other than those in gE or gI contributed substantially to the small-plaque phenotype. There was a small but significant difference in the size of plaques produced on Vero cells by F-gEβ versus those produced by F on Vero cells; however, this difference was not normally observed until the plaques were relatively large (Fig. 4). The plaque phenotype of F-US7kan was partially syncytial on Vero cells, perhaps because F-US7kan was derived from F-gDβ, which produces syncytial plaques on complementing VD60 cells (46). However, we did not observe fusion of human fibroblasts infected with F-US7kan, and thus the syncytial mutation is not likely to contribute to the differences in plaque sizes on fibroblasts. In addition, a wild-type revertant of F-gDβ, wt 61, which expresses gD and gI (62a), produced normal large plaques on fibroblasts, whereas other gI⁻ mutants derived from F-gDβ produced small plaques (not shown), suggesting that any secondary mutations in F-US7kan which produce a syncytial phenotype on Vero cells did not affect plaque morphology on fibroblasts and confirming a role for gI in the small-plaque phenotype. In other experiments, we detected a similar small-plaque phenotype when F-US7kan, *in*1404, and F-gEβ were plated on monolayers of human fibroblasts which had been transformed with plasmids contain-

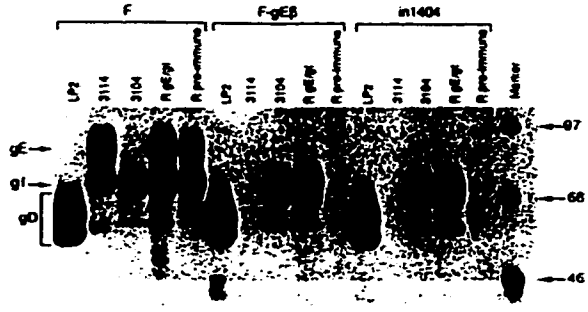


FIG. 2. Expression of gD, gI, and gE in cells infected with gE mutants F-gEβ and *in*1404. Human R970 cells were infected with F, F-gEβ, or *in*1404, and the cells were radiolabelled with [^{35}S]methionine from 5 h until 7 h p.i. Cell extracts were immunoprecipitated with anti-gD MAb LP2, anti-gE MAb 3114, anti-gI MAb 3104, rabbit polyclonal anti-gE/gI serum (R gE/gI), or a rabbit preimmune serum (R pre-immune). Immunoprecipitated proteins were subjected to electrophoresis through 8.5% polyacrylamide gels. The positions of gD, gE, and gI as well as molecular size markers (Marker) of 97, 68, and 46 kDa are indicated.

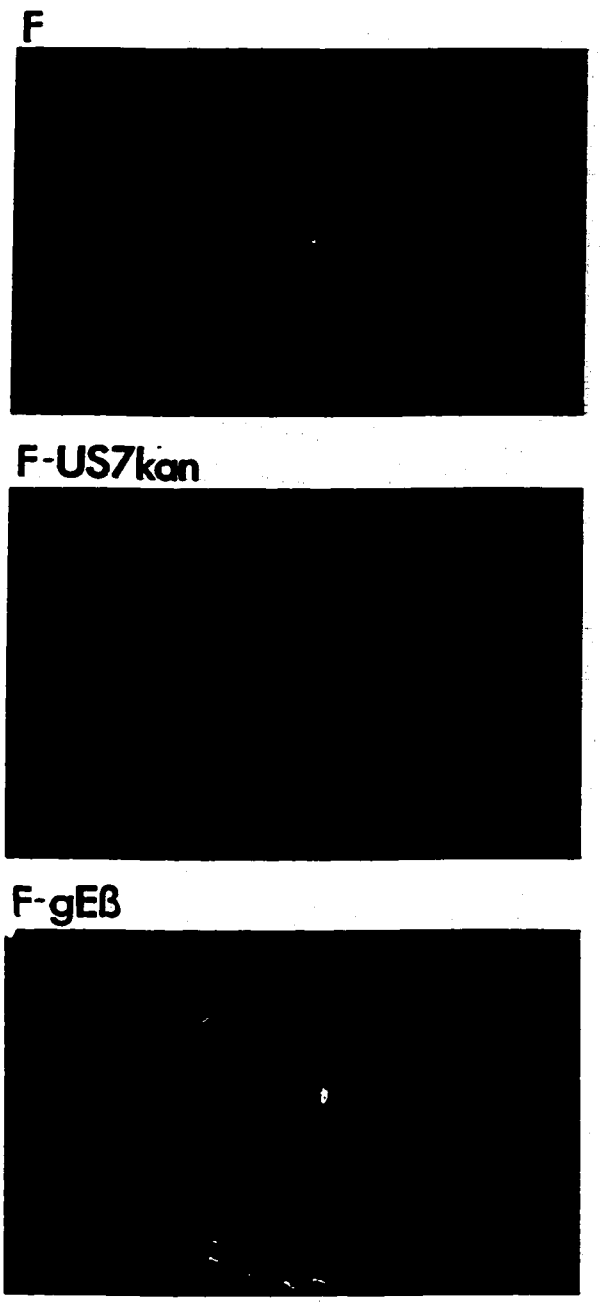


FIG. 3. Epithelial lesions caused by gE⁻ and gI⁻ mutant viruses in the cornea of mice. The corneas of anesthetized mice were infected with either wild-type HSV-1 strain F, the gI⁻ mutant F-US7kan, or the gE⁻ mutant F-gEß. The corneas were examined and photographed 2 days after infection by instilling fluorescein and using a slit lamp with a cobalt blue light source.

ing the simian virus 40 large T antigen and with some but not all epithelial cells (not shown). Since both the gE⁻ and gI⁻ mutant viruses produced small plaques on cells displaying a flattened, well-spread morphology with extensive cell contacts

TABLE 1. Pathogenicity of HSV-1 gE⁻ and gI⁻ mutants in mice infected by the corneal route

HSV strain	Presence of disease			
	Epithelium[a]	Skin[b]	HSK[c]	Encephalitis[d]
F	+++	Severe	++	+
F-US7kan	++	None	None	−
F-gEß	+	None	None	−

[a] Epithelial lesions were observed 2 days after infection by instilling fluorescein and using a slit lamp. Lesions were graded as mild (+), moderate (++), or severe (+++).

[b] Periocular skin disease was measured 14 days after infection.

[c] Herpes stromal keratitis (HSK) was measured 14 days after infection and was graded as mild (+), moderate (++), severe (+++), or perforated cornea (++++).

[d] Mice began to die as a result of encephalitis by 14 days p.i. and were sacrificed. Mice infected with F-US7kan and F-gEß showed no signs of encephalitis.

and there were often no differences between wild-type and mutant viruses with more rounded cells possessing fewer cell junctions, we suspected that these differences were related to cell morphology or intercellular junctions.

To more accurately quantify the spread of virus infection in monolayers of human fibroblasts, we stained cell monolayers infected with wild-type and mutant viruses, using anti-HSV antibodies and an anti-IgG peroxidase conjugate (Fig. 5). At 36 h p.i., plaques produced by wild-type F contained over 200 stained cells per plaque whereas plaques produced by F-gEß and F-US7kan contained an average of 56 cells per plaque, and these differences were statistically significant (Student unpaired t test, $P < 0.005$). After 60 h of infection, all cells in dishes infected with wild-type F were stained by immunoperoxidase, whereas defined regions of infection were observed with F-gEß and F-US7kan. In these experiments, the numbers of plaques produced on fibroblast monolayers by mutant and wild-type viruses were not different, and similar results were obtained when *in*1404 and 17 were compared (results not shown). Therefore, it appears that cell-to-cell spread of wild-type HSV-1 is more efficient than that of the gE⁻ or gI⁻ mutant viruses in human fibroblasts.

HSV-1 mutants unable to express gE or gI replicate normally on human fibroblasts but spread poorly from cell to cell. The most likely explanation for reduced plaque sizes by gE⁻ and gI⁻ mutant viruses was that certain aspects of virus replication were inhibited in human fibroblasts so that progeny viruses were produced more slowly or less efficiently. To test this hypothesis, a single round of HSV replication was followed by infecting fibroblasts at a multiplicity of infection of 5 PFU per cell and harvesting cells and culture media independently. No differences were detected in the yields of infectious virus either in the media (Fig. 6A) or associated with cells (Fig. 6B) in three independent experiments. Similarly, no differences in yields of infectious viruses were obtained following infection of fibroblasts with wild-type strain 17 and gE mutant *in*1404 (not shown).

Since we observed marked differences in the plaque sizes produced by mutant and wild-type viruses on human fibroblasts, we expected that the yields of infectious viruses produced after multiple rounds of infection (initiated by using 0.001 PFU per cell) would also be different. In this assay, F-gEß and F-US7kan produced approximately 10- to 20-fold less infectious virus in the cell culture supernatant than was produced by F after 48 and 60 h of infection (Fig. 7A). The amount of cell-associated virus produced in fibroblasts was reduced by 5- and 10-fold in cells infected with the gE⁻ and

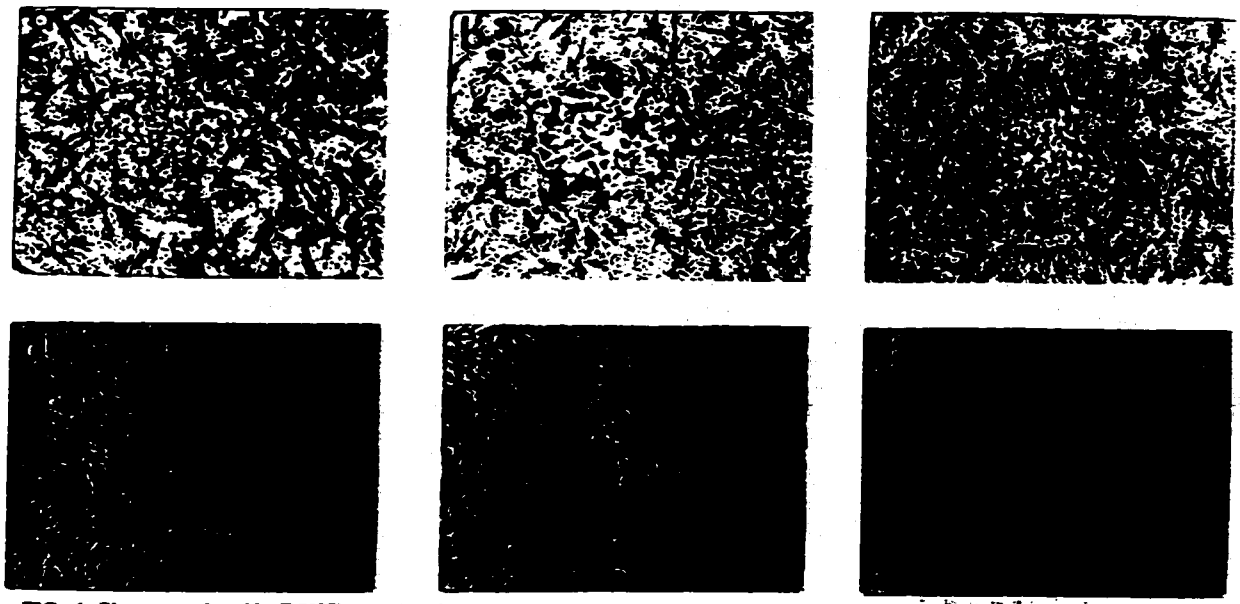


FIG. 4. Plaques produced by F-US7kan and F-gEβ on monolayers of Vero cells and normal human fibroblasts. Monolayers of Vero cells (a to c) or normal human fibroblasts (d to f) were infected with wild-type F (a and d), F-US7kan (b and e), or F-gEβ (c and f) at 0.001 PFU per cell. At 2 h p.i., cell monolayers were washed once with PBS and α-MEM supplemented with 1% FBS, and 0.1% HGG was added for a further 48 h. The cells were fixed and then stained with crystal violet.

gI⁻ mutants relative to wild-type HSV-1 (Fig. 7B). However, there was a much greater difference (100- to 200-fold) in the yields of infectious virus produced by wild-type versus mutant virus when HGG, a source of neutralizing antibodies, was kept present in the culture medium (Fig. 7C). Moreover, there was little difference in the titers of virus from wild-type HSV-1-infected cells incubated with or without HGG, whereas there were dramatic (20- to 100-fold) differences in yields of infectious virus produced by gE⁻ and gI⁻ mutant viruses with and without HGG (compare Fig. 7B and C). Again, similar results were obtained when *in*1404 and wild-type 17 were compared (not shown). These results suggest that a primary mode of spread of wild-type HSV-1 in these cells is from cell to cell, by mechanisms which do not place the virus in contact with neutralizing antibodies. Apparently, gE⁻ and gI⁻ mutants function poorly in this form of direct cell-to-cell transmission.

F-US7kan, F-gEβ, and wild-type F enter cells with similar kinetics. To examine the kinetics of virus entry, we used a low-pH inactivation assay previously described by Huang and Wagner (37) and adapted for HSV by Highlander et al. (35). Cells were incubated with virus at 4°C, allowing virus adsorption onto cells; then the cells were moved to 37°C to allow virus penetration into cells and at various times briefly treated with a low-pH citrate buffer to cause unpenetrated viruses to be released or inactivated. Rates of entry were determined by quantifying plaques produced on monolayers incubated for various times, using untreated cells as controls. F and F-gEβ entered fibroblasts with very similar kinetics (Fig. 8); in other experiments, a second gE⁻ mutant, *in*1404, entered fibroblasts with kinetics identical to those of wild-type strain 17 (not shown). F-US7kan entry reproducibly lagged behind that of F and F-gEβ at early time points (Fig. 8), while at later times, e.g. 60 and 90 min, entry into cells had been completed and the final extents of entry for all three viruses were similar or identical. The slower rate of entry observed with F-US7kan appears not to explain the observed differences in cell-to-cell spread of gI⁻ and gE⁻ viruses because the final extent of entry measured at 60 min was similar for all three viruses and because the two gE⁻ mutants, which also formed small plaques, entered with kinetics similar to those of wild-type strains F and 17.

To further study entry of the mutant viruses into cells, we infected fibroblasts with 5 PFU per cell and quantified an HSV-1 immediate-early protein, ICP6, and an early protein, TK, although only the results for TK are shown. Similar quantities of TK were immunoprecipitated from extracts of cells infected with F-gEβ, F-US7kan, and wild-type F (Fig. 9). Similar results were observed when ICP6 was quantitated and when *in*1404 and wild-type 17 were compared (not shown). Thus, gE⁻ and gI⁻ mutant viruses and wild-type HSV-1 caused equal quantities of early viral proteins to be produced, and the lag in F-US7kan penetration (Fig. 8) does not affect the production of immediate-early and early HSV-1 proteins in these cells. In other experiments, fibroblasts were infected with wild-type and mutant viruses at 1 PFU per cell, and again equal quantities of TK were produced by mutant and wild-type viruses (not shown).

The small-plaque phenotype of gE⁻ and gI⁻ mutants is not explained by loss of Fc receptor activity. In the plaque assays described above, cell monolayers were incubated with virus for 2 h and then overlaid with medium supplemented with pooled HGG, which contains anti-HSV antibodies, to inhibit formation of satellite plaques. Since gE⁻ and gI⁻ viruses are deficient in Fc receptor activity, it was formally possible that the differences in plaque size might be related to an inability to bind human IgG. To test this possibility and to extend these analyses, fibroblasts were infected with HSV-1 and then incubated without antibodies or with a panel of mouse and rabbit

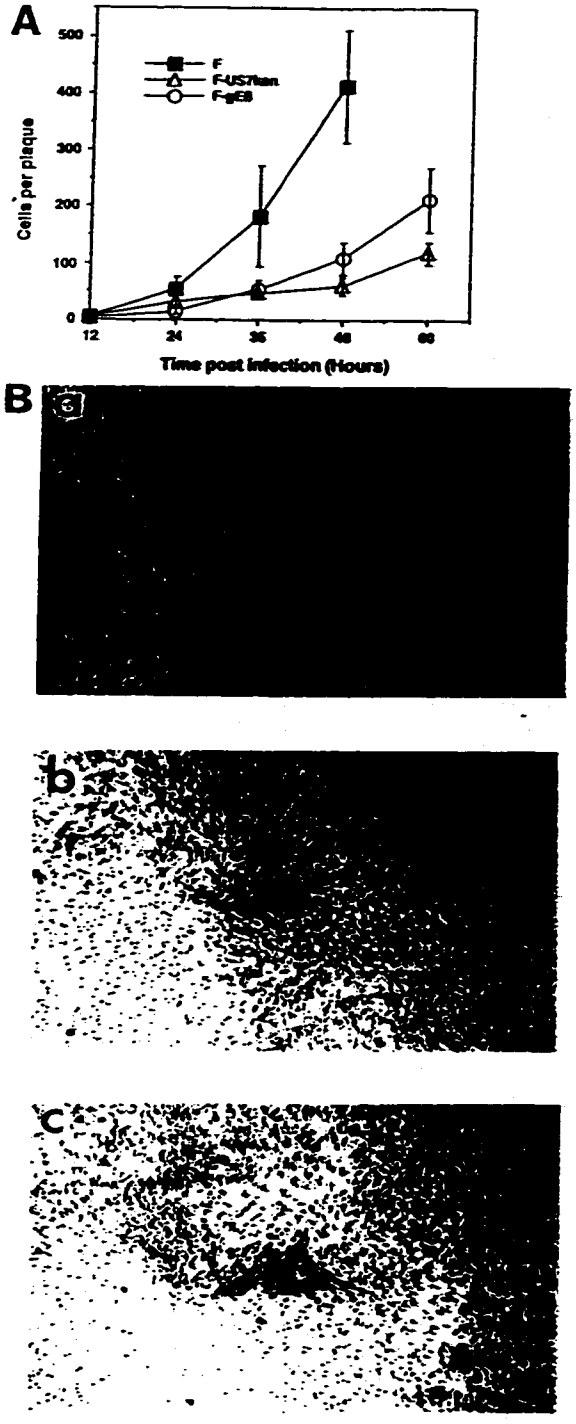


FIG. 5. Immunoperoxidase staining of infected cells in plaques produced by gE⁻ and gI⁻ mutant viruses. (A) Monolayers of normal fibroblasts seeded on glass coverslips were infected with 0.001 PFU per cell and maintained in α-MEM supplemented with 1% FBS and 0.1% HGG. At 12-h intervals, the cells were fixed with acetone and stained with rabbit anti-HSV antibodies, peroxidase-conjugated anti-rabbit antibodies, and peroxidase substrate. The numbers of infected cells in 10 representative plaques at each time point were counted and averaged. At 36 h p.i., the number of cells infected by wild-type F is significantly different from the number of cells infected by F-gEβ or F-US7kan (Student unpaired t test, $P < 0.005$). (B) Representative immunoperoxidase-stained plaques produced 48 h p.i. with wild-type F (a), the gI⁻ mutant F-US7kan (b), or the gE⁻ mutant F-gEβ (c).

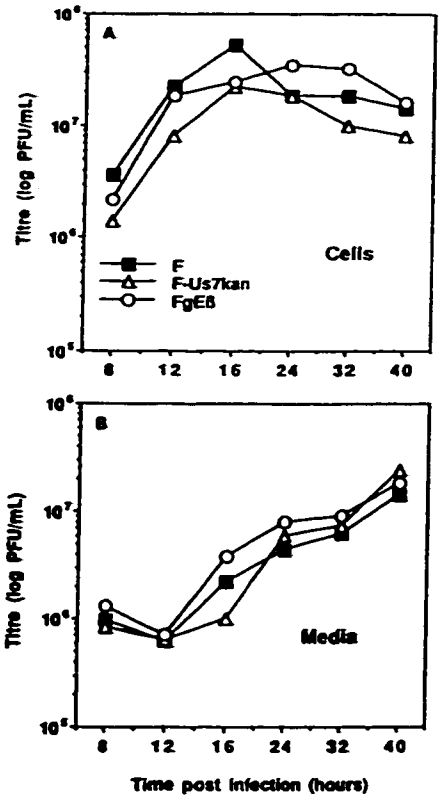


FIG. 6. Production of infectious viruses by HSV-1 gE⁻ and gI⁻ mutants infecting human fibroblasts. Normal fibroblasts growing in six-well dishes were infected with F, F-US7kan, or F-gEβ at 5 PFU per cell. At the indicated times after infection, cells (A) and media (B) were collected and frozen at −70°C. Samples were thawed, sonicated, and titered under standard plaque assay conditions on Vero cell monolayers. The results shown are representative of three separate experiments, but individual points shown are the results of a single determination because of the relatively large number of samples.

HSV-specific antibodies, both neutralizing and nonneutralizing. When no antibodies were present, plaques produced by wild-type F were large; in many cases, by 48 h of infection, it was difficult to count the numbers of cells infected, whereas plaques produced by F-gEβ or F-US7kan were much smaller (Fig. 10, no serum). Two strongly neutralizing mouse anti-gD MAbs, LP2 and DL11, reduced the size of plaques produced by both wild-type and mutant viruses in a fashion similar to HGG; however, again there were significant differences between the sizes of plaques produced by the mutant viruses and those produced by wild-type viruses. It has been difficult or impossible to detect binding of mouse IgG to the HSV Fc

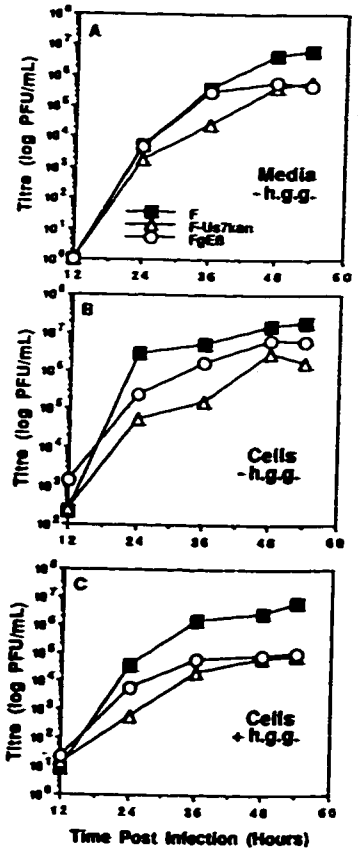


FIG. 7. Spread of gE$^-$ and gI$^-$ mutant viruses through fibroblast monolayers. Normal human fibroblasts growing in 35-mm dishes were infected with F, F-US7kan, or F-gEß at 0.001 PFU per cell; after 2 h, the monolayers were washed with PBS, and α-MEM–1% FBS (A and B) or α-MEM–1% FBS supplemented with 0.1% HGG (C) was added. At the indicated times, media (A) or cells (B and C) were collected and frozen at −70°C. In panel C, cells were washed twice with medium twice to remove HGG before being collected. Samples were later thawed, sonicated, and titered by plaque assay on Vero cell monolayers. The results shown are representative of three separate experiments, but individual points shown are the results of a single determination because of the relatively large number of samples.

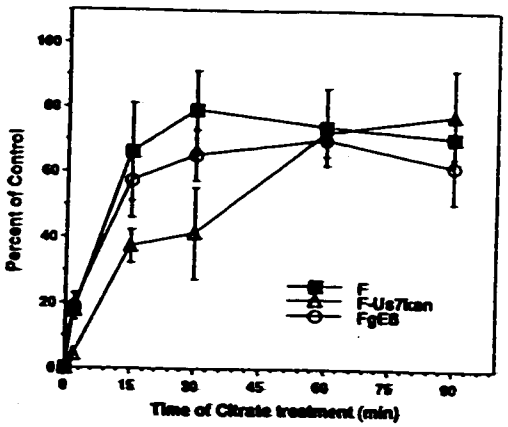


FIG. 8. Kinetics of penetration of gE$^-$ and gI$^-$ mutant viruses into human fibroblasts. Confluent monolayers of human fibroblasts growing in 12-well dishes were incubated with approximately 100 PFU of either F, F-US7kan, or F-gEß for 2 h at 4°C. Following removal of the viruses, cells were washed three times to remove unbound virus, and the temperature was shifted to 37°C. At various times, cell monolayers in triplicate were treated for 1 min with citrate buffer (pH 3.0) or with PBS. The cell monolayers were then washed three times with PBS and incubated for 2 days in α-MEM–1% FBS supplemented with 0.1% HGG. Cells were fixed and then stained with crystal violet, and plaques were counted. At each time point, the average number of plaques produced on citrate-treated monolayers was divided by the average number of plaques produced on PBS-treated monolayers and expressed as a percentage. Error bars represent standard deviations.

receptor (41, 43); thus, these mouse MAbs would not be expected to alter spread of virus by interacting with the Fc receptor. Other mouse MAbs, e.g., LP11, which is specific for gH, decreased wild-type and mutant HSV-1 plaque sizes to a lesser extent; however, again there were marked differences in the sizes of mutant and wild-type plaques. Rabbit antibodies, which can bind to the HSV Fc receptor, either neutralizing (e.g., rabbit anti-gD serum) or nonneutralizing (e.g. anti-gE/gI), gave similar results. In other experiments, these differences between mutant and wild-type HSV-1 were also observed when fibroblasts were overlaid with methylcellulose (not shown). The results showed clearly that gE$^-$ and gI$^-$ mutants spread poorly through fibroblast monolayers whether antibodies capable of neutralizing HSV or binding to the Fc receptor were present or not; thus, this effect was unrelated to the Fc receptor activity of the gE-gI hetero-oligomer.

DISCUSSION

These experiments were initiated to investigate the protective effects of the HSV IgG Fc receptor in rodent corneal models of HSV pathogenesis. Our premise was that the gE-gI hetero-oligomer acting as an Fc receptor might protect HSV-1 from immune surveillance or cytolysis. Mutants lacking either gE or gI were used because full Fc receptor activity requires both gE and gI (4, 25, 43). Marked differences in the epithelial lesions caused by wild-type and mutant viruses were observed in both rabbits and mice; however, to our surprise, these

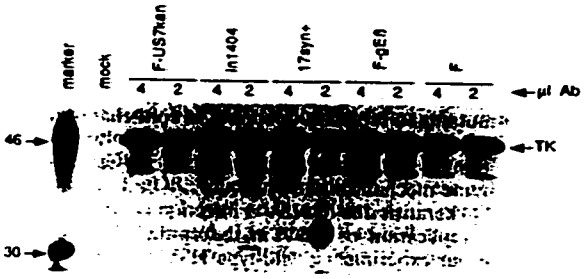


FIG. 9. Expression of HSV-1 TK in fibroblasts infected with gE$^-$ and gI$^-$ mutants. Human fibroblasts were infected with F, F-US7kan, F-gEß, 17, or *in*1404 at 5 PFU per cell and labelled with [^{35}S]methionine from 5 h until 7 h p.i. Cell extracts were made, and TK was immunoprecipitated with either 2 or 4 µl of rabbit anti-TK serum. Note that in one of the 17syn+ lanes, the anomaly is due to the presence of an air bubble in the gel. Positions of TK and molecular mass markers of 46 and 30 kDa are indicated.

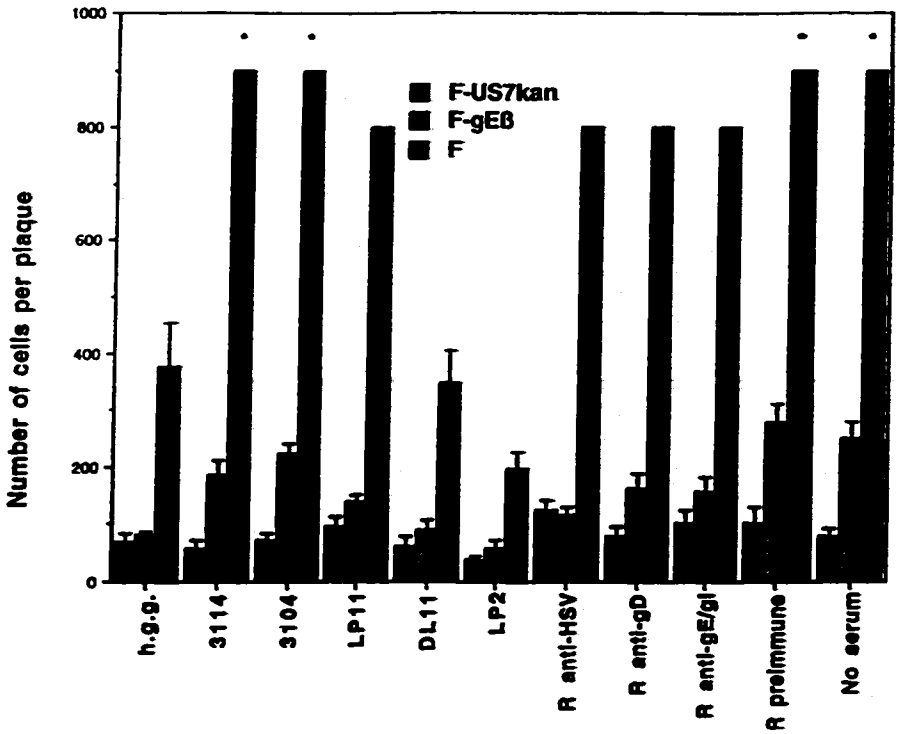


FIG. 10. Effects of murine and rabbit IgG on plaque sizes. Normal human fibroblasts were seeded on glass coverslips and infected with F, F-US7kan, or F-gEß at 0.001 PFU per cell. At 2 h p.i., cell monolayers were washed with PBS and incubated with α-MEM supplemented with 1% FBS and either 0.1% HGG or one of the murine or rabbit antibodies; mouse ascites fluids and rabbit serum were diluted 1:1,000. The cells were fixed with acetone 48 h p.i. and stained with anti-HSV-immunoperoxidase. The total number of infected cells per plaque was counted for 10 representative plaques and expressed as an average. Error bars represent standard deviations. Bars representing monolayers infected with F and treated with MAb LP11, R anti-HSV serum, R anti-gD serum, or R anti-gE/gI serum represent estimates of approximately 800 cells per plaque, although these were difficult to count. The asterisks over bars representing F-infected monolayers treated with no serum, R preimmune serum, and MAbs 3114 and 3104 refer to instances when HSV-1 F infected all cells in the monolayer (we estimate >1,500 cells per plaque); thus, it was impossible to definitively determine the numbers of cells per plaque.

differences were observed well before IgG appeared in the eye, i.e., 2 or 3 days after inoculation. Detectable levels of anti-HSV IgG or IgA were not observed in the rabbit eye until 5 to 13 days after infection of nonimmunized animals (13, 27). Although we know of no quantitative studies of murine IgG in the eye, murine IgG binds poorly or not at all to the HSV Fc receptor (41, 43); thus, it is unlikely that the differences observed in mice were related to the absence of a functional IgG Fc receptor. Together, these results suggest that the loss of virulence of HSV gE$^-$ and gI$^-$ mutants is due to the inability of these mutants to replicate or spread in epithelial tissue and not due to loss of IgG binding activity, although it is formally possible that the Fc receptor complex affects resistance to other immune mechanisms.

Epithelial keratitis due to HSV-1 infection is associated with the active replication of HSV in the epithelial cells of the cornea. In our experiments, wild-type HSV-1 strain F caused severe epithelial keratitis characterized by large dendritic lesions and extensive stromal disease, and all of the infected mice developed encephalitis. By contrast, gE$^-$ and gI$^-$ mutant viruses produced small punctate epithelial lesions and no stromal disease, and the animals displayed no encephalitis. It appears likely that the restricted stromal disease caused by mutant viruses is an outcome of reduced virus spread in the epithelium so that less virus reaches the stroma. However, there is also evidence that certain virus isolates can cause extensive stromal disease while producing little epithelial disease (14, 63). Stromal disease is thought to be largely due to immunopathological responses to viral antigen and does not occur in T-cell-deficient mice (28, 31, 53), and virus and host factors contribute to progression from epithelium to stroma (14, 29, 30, 54, 63, 64). The relationship between epithelial and stromal disease in the cornea is not clear. For instance, some HSV-1 strains produce large epithelial lesions, establish latent infections in the neurons of the trigeminal ganglia, but fail to reactivate from latency and also fail to induce stromal disease. In contrast, other strains produce very little epithelial disease but cause severe stromal keratitis (27). Until stromal inflammation is better understood, we cannot speculate on the role of the gE-gI hetero-oligomer in this process. We also note that these results are not limited to ocular models of HSV infection because wild-type HSV-1 F produced easily detected lesions in female guinea pig vaginal epithelium, yet no lesions could be detected in F-US7kan-infected animals (5).

To study further the role of the gE-gI complex in HSV infection, replication of the mutant viruses was examined in a number of cultured rodent and human cell types. HSV is normally propagated in transformed monkey or human cells which are often morphologically and metabolically different from the cells normally infected in vivo (epithelial cells,

fibroblasts, keratinocytes, neurons, etc.). Mutants unable to express gE or gI produced small plaques on monolayers of normal human fibroblasts and certain epithelial cells. The interpretation of these results was initially complicated by the fact that our standard plaque assays included human antibodies which can neutralize HSV and prevent production of satellite plaques. However, in the absence of antibodies, wild-type HSV-1 also spread through these monolayers much more efficiently than did gE$^-$ and gI$^-$ mutant viruses. Similar results were obtained when monolayers were overlaid with mouse antibodies, nonneutralizing antibodies, or methylcellulose. In considering the relationship between the observations that gE$^-$ and gI$^-$ mutants produce small plaques in human fibroblast monolayers and small lesions in the eye, we note that Stulting et al. (63) found a correlation between the ability of an HSV-1 strain to replicate in mouse primary fibroblasts and keratinocytes and its ability to induce stromal keratitis.

The small-plaque phenotype and decreased virulence of gE$^-$ and gI$^-$ mutants might have resulted from defects in a number of aspects of virus replication, including adsorption, penetration, production or egress of infectious particles, or cell-to-cell spread of the viruses. Viral mutants and wild-type HSV-1 applied to human fibroblasts, using either 1 or 5 PFU per cell, entered the cells normally and produced equivalent quantities of viral proteins and infectious viruses which were shed normally into the cell culture supernatant. Other studies (not shown) involving electron microscopy indicated that mutant and wild-type virus particles were distributed in an identical fashion in infected cells and reached the cell surface. However, when fibroblast monolayers were infected with gE$^-$ and gI$^-$ mutants under conditions in which only a fraction of the cells were infected and neutralizing antibodies were kept present in the overlay medium, the production of infectious viruses over several rounds of replication was 100- to 200-fold lower than that produced by wild-type virus. Moreover, the yields of gE$^-$ and gI$^-$ mutant viruses were 20- to 100-fold lower when neutralizing antibodies were present than observed in the absence of antibodies; by contrast, neutralizing antibodies had little or no effect on the production of wild-type HSV-1. From these data, we concluded that viruses lacking gE or gI spread poorly from cell to cell across cellular junctions; furthermore, the results support the notion that direct cell-to-cell transmission across cell junctions can be a primary mechanism for spread of HSV-1 in some cell types. In transformed monkey Vero cells, the effect of these mutations was much less evident, although on careful examination, gE$^-$ mutants F-gEβ and *in*1404 produced slightly smaller plaques than their respective wild-type parents did, especially when infected cell monolayers were incubated for additional hours.

One important concern with HSV mutants engineered by transfection of viral DNA is that secondary mutations can arise which may cause unwanted phenotypic changes. This concern is particularly acute when HSV mutants are propagated in cultured cells and tested in animal models. To confirm that the observed phenotype is related to mutations in a particular gene, virus revertants are often characterized or, alternatively, multiple mutants can be characterized. In these studies, we found that two independently isolated gE mutants, F-gEβ and *in*1404, and a gI mutant, F-US7kan, produced smaller epithelial lesions than did their wild-type parents and produced microscopic plaques on fibroblasts. In all subsequent experiments designed to elucidate differences between mutant and wild-type viruses, *in*1404, F-gEβ, and F-US7kan behaved identically or similarly. Furthermore, a number of other independently isolated gI-negative mutants also produced small plaques on human fibroblasts (not shown). Since gE and gI are extensively complexed and dependent on one another for function (43), it appears highly likely that these phenotypes, i.e., inability to spread in vivo and in vitro, are caused by the absence of gE or gI and not by secondary mutations; however, it is formally possible that other mutations were acquired during the construction of these virus mutants. Our observations with these HSV-1 gE$^-$ and gI$^-$ mutants further strengthen the hypothesis that gE and gI function as a complex, whether to bind IgG or to facilitate cell-to-cell spread by an unknown mechanism.

During the course of these studies, a report describing effects of a mutation in the PrV virus gI protein, a homolog of HSV-1 gE, appeared; in this study, it was found that the mutant virus was deficient in direct cell-to-cell transmission (66). The mutant virus replicated normally in cells but was less able to form syncytia and spread primarily by adsorption of released virus to uninfected cells, whereas wild-type virus spread primarily by direct cell-to-cell transfer. Another PrV gI$^-$ mutant virus was restricted in its ability to spread in the rat nervous system (12). Together, these results for PrV, previous results for HSV-1 gE mutant viruses (52, 57, 59), and the observations described here involving both gE and gI strongly support a general role for the gE-gI complex in cell-to-cell spread in a variety of human and rodent tissues.

The spread of HSV through tissue is likely to be a complicated series of events mediated by the large repertoire of viral membrane glycoproteins. It is quite apparent that HSV can spread from an infected cell to an uninfected neighbor by either of two distinct pathways. Viruses can detach from infected cells and then adsorb onto and enter uninfected cells, or virus particles can be transferred across junctions between cells so that the viruses do not come in contact with neutralizing antibodies. It appears that HSV is infrequently released from infected cells because electron micrographs of HSV-infected cells frequently show large numbers of virus particles encrusting the cell surface and the quantities of infectious HSV released by cultured cells are often low. HSV-1 gE and gI appear to be unimportant in entry of extracellular virus into cells, but both proteins play important roles in direct cell-to-cell spread across cell junctions. This latter mode of virus spread apparently leads to infection of neighboring cells through a set of receptors different from those used for entry of exogenous virus, and it appears that the gE-gI heterooligomer engages these receptors. Another subset of herpesvirus glycoproteins are apparently required for entry of extracellular virus but not for cell-to-cell transmission. For example, PrV mutants unable to express gp50, a homolog of HSV gD, were found to be severely compromised in their ability to enter cells but after entering a host cell can spread from cell to cell and produce plaques without gp50 (58). Similarly, we have found that HSV-1 gD-negative mutants can produce small plaques on human fibroblasts when the infections are initiated with complemented virus (14a), supporting the notion that gD is not absolutely required for cell-to-cell spread. In these experiments, F-gDβ, which is unable to express gD and gI, could not produce plaques on fibroblasts, again confirming the requirement for gE and gI in this cell-to-cell spread.

The propensity to spread directly from cell-to-cell across cell junctions appears to be very much dependent on the cell type. Cell contacts are frequently formed between fibroblasts and epithelial cells, and both of these cell types form diverse junctional complexes, including adherens junctions, desmosomes, and tight junctions. However, other cells, e.g., Vero and MDBK cells, also possess cell junctions and can spread in monolayers of these cells (in the presence of neutralizing HSV antibodies), and yet the phenotypes of gE$^-$ and gI$^-$ mutants

were much less obvious with these cells. A second type of direct cell-to-cell transmission of HSV involves fusion of infected cells with uninfected cells which can occur with wild-type HSV but is more conspicuous with syncytial mutants. Again, HSV-induced cell fusion is very much dependent on cell type and appears to involve mechanisms similar to those involved in cell-to-cell transmission of virus whereby cells do not fuse. To date, few efforts have been made to characterize interactions between HSV and components of cellular junctions; however, these studies have been initiated and should provide valuable information as to how herpesviruses spread in tissue.

At the present time, we have only hints as to how gE and gI might function to mediate cell-to-cell spread of HSV across cellular junctions. HSV mutants lacking gE or gI can spread in the presence of neutralizing antibodies and in rodent eyes, although much less efficiently than wild-type viruses, suggesting that alternate pathways for cell-to-cell spread exist. An important caveat here is that a double mutant lacking both gE and gI was not tested, and thus it is possible that such a mutant would display a more absolute phenotype. However, we suspect that the gE-gI hetero-oligomer functions to facilitate cell-to-cell transmission of HSV by interacting with cellular receptor molecules localized to cell junctions. In this regard, it is interesting to note that HSV gE-gI binds only weakly to the Fc domain of IgG, and it has been difficult or impossible to demonstrate IgG binding to the gE-gI homologs in PrV (67) and varicella-zoster virus (47). Therefore, it is tempting to speculate that the HSV gE-gI heterooligomer functions not by binding to IgG but by binding to other cell surface proteins which are members of the IgG supergene family. It is particularly interesting that the gE polypeptide contains a 14-amino-acid region which is homologous with other cell adhesion proteins known to bind sulfated glycoconjugates, e.g., thrombospondin (36).

ACKNOWLEDGMENTS

We thank the following colleagues for gifts of MAbs and antiserum: Anne Cross, Nigel Stow, Silvia Bacchetti, Gary Cohen, Roselyn Eisenberg, Tony Minson, and Bill Summers. We also recognize the excellent technical contributions of Cindy Roop and Steven Primorac. Once again we thank Ian York, Craig Smibert, and Jim Smiley for helpful discussions.

This work was funded by the Medical Research Council of Canada and the National Cancer Institute of Canada (NCIC). D.C.J. is a senior scientist of the NCIC.

REFERENCES

1. Adler, R., J. C. Glorioso, J. Cossman, and M. Levine. 1978. Possible role of Fc receptors on cells infected and transformed by herpes virus: escape from immune cytolysis. Infect. Immun. 21: 442–447.
2. 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.
3. 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.
4. Bell, S., M. Cranage, L. Borysiewicz, and T. Minson. 1990. Induction of immunoglobulin G by Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J. Virol. 64:2181–2186.
5. Bourne, N., and L. Stanberry. Unpublished data.
6. 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, and recombination analysis leading to a linkage map. J. Gen. Virol. 18:329–346.
7. Brunetti, C. R., K. S. Dingwell, R. L. Burke, T. Gregory, and D. C. Johnson. 1992. A putative receptor protein for herpes simplex virus which is bound by glycoprotein D, abstr. 124. Abstr. Am. Soc. Virol.
8. Buckmaster, E. A., U. Gompels, and A. C. Minson. 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately 115 × 10^3 molecular weight. Virology 139:4408–4413.
9. Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence specifying the glycoprotein gB of herpes simplex virus type 1. Virology 133:301–314.
10. Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62:2596–2604.
11. Campadelli-Fiume, C., 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 of the virus. J. Virol. 62:159–167.
12. Card, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist. 1992. Pseudorabies virus envelope glycoproteins gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. 66:3032–3041.
13. Carter, C., and D. L. Easty. 1981. Experimental ulcerative herpetic keratitis. I. Systemic immune responses and resistance to corneal infection. Br. J. Ophthalmol. 65:77–81.
14. Centifanto-Fitzgerald, Y. M., T. Yamaguchi, H. E. Kaufmann, M. Tegnon, and B. Roizman. 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. J. Exp. Med. 155:475–489.
14a. Dingwell, K. S., and D. C. Johnson. Unpublished data.
15. Dowler, K. W., and R. W. Veltri. 1984. In vitro neutralization of HSV-2: inhibition by binding of normal IgG and purified Fc to virion Fc receptor (FcR). J. Med. Virol. 13:251–259.
16. Eisenberg, R. J., D. Long, M. Ponce de Leon, J. T. Matthews, P. G. Spear, M. G. Gibson, L. A. Laskey, P. Berman, E. Golub, and G. H. Cohen. 1985. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. J. Virol. 53:634–644.
17. Eisenberg, R. J., M. Ponce de Leon, H. M. Friedman, L. F. Fries, M. M. Frank, J. C. Hastings, and G. H. Cohen. 1987. Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. Microb. Pathog. 3:423–435.
18. Forrester, A. J., G. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and A. C. Minson. 1992. Construction and properties of a mutant herpes simplex virus type 1 deleted for glycoprotein H sequences. J. Virol. 66:341–348.
19. Frank, I., and H. M. Friedman. 1989. A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J. Virol. 63:4479–4488.
19a. Frazer, N. Personal communication.
20. Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines. 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the complement component C3b on infected cells. Nature (London) 309:633–635.
21. Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. 63:3435–3443.
22. 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 viral growth and DNA synthesis. J. Virol. 62:2970–2977.
23. 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.
24. 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.
25. Hanke, T., F. L. Graham, V. Lulitanond, and D. C. Johnson. 1990. Herpes simplex virus IgG receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology 177:437–444.
26. Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman. 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptide of type 1 strains. J. Virol. 14:640–651.
27. Hendricks, R. L. Unpublished data.
28. Hendricks, R. L., R. J. Epstein, and T. M. Tumpey. 1989. The

effect of cellular immune tolerance to HSV-1 antigens on the immunopathology of HSV-1 keratitis. Invest. Ophthalmol. Visual Sci. 30:105–115.
29. Hendricks, R. L., M. Janowicz, and T. M. Tumpey. 1992. Critical role of corneal Langerhans cells in the CD4 but not CD8 mediated immunopathology in herpes simplex virus-1-infected mouse corneas. J. Immunol. 148:2522–2529.
30. Hendricks, R. L., and T. M. Tumpey. 1990. Contribution of virus and immune factors to herpes simplex virus type 1 induced corneal pathology. Invest. Ophthamol. Visual Sci. 31:1929–1939.
31. Hendricks, R. L., and T. M. Tumpey. 1991. Concurrent regeneration of T lymphocytes and susceptibility to HSV-1 corneal stromal disease. Curr. Eye Res. 10:47–53.
32. Hendricks, R. L., T. M. Tumpey, and A. Finnegan. 1992. IFN-gamma and IL-2 are protective in the skin but pathologic in the corneas of HSV-1 infected mice. J. Immunol. 149:3023–3028.
33. 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.
34. 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 *mar* mutation in herpes simplex type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. 63:730–738.
35. Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes virus glycoprotein D inhibits virus penetration. J. Virol. 61:3356–3364.
36. Holt, G. D., H. C. Krivan, G. J. Gasic, and V. Ginsburg. 1989. Antistasin, an inhibitor of coagulation and metastasis, binds to sulfatide (Gal (3-S04) β1-1Cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates. J. Biol. Chem. 264:12138–12140.
37. Huang, A., and R. Wagner. 1964. Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Med. 116:863–869.
38. Huszar, D., S. Beharry, and S. Bacchetti. 1983. Herpes simplex virus induced ribonucleotide: development of antibodies specific for the enzyme. J. Gen. Virol. 64:1327–1355.
39. Hutchinson, L., H. Browne, U. 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.
40. Hutchinson, L., and D. C. Johnson. Unpublished data.
41. Johansson, P. J. H., E. B. Myhre, and J. Blomberg. 1985. Specificity of Fc receptors induced by herpes simplex virus type 1: comparison of immunoglobulin G from different animal species. J. Virol. 56:489–494.
42. Johnson, D. C., R. 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. Virol. 64:2569–2576.
43. Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. G. 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.
44. Johnson, D. C., and M. W. Ligas. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: qualitative evidence for virus-specific cell surface receptors. J. Virol. 62:4605–4612.
45. 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.
46. 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.
47. Litwin, V., W. Jackson, and C. Grose. 1992. Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. J. Virol. 66:3643–3651.
48. Longnecker, R., and B. Roizman. 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S function α sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. 58:583–591.
49. Longnecker, R., and B. Roizman. 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science 236:573–576.
50. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex type 1. J. Mol. Biol. 181:1–13.
52. Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman. 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology 162:251–254.
53. Metcalf, J. F., D. S. Hamilton, and R. W. Reichert. 1979. Herpetic keratitis in athymic (nude) mice. Infect. Immunol. 26:1164–1171.
54. Metcalf, J. F., and B. A. Michaelis. 1984. Herpetic keratitis in inbred mice. Invest. Ophthamol. Visual Sci. 25:1222–1225.
55. Minson, A. C., T. C. Hodgman, P. Digard, D. C. Hancock, S. E. Bell, and E. A. Buckmaster. 1986. An analysis of the 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.
56. Navarro, D., P. Paz, and L. Pereira. 1992. Domains of herpes simplex virus 1 glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology 186:99–112.
57. Neidhardt, H., C. H. Schröder, and H. C. Kaerner. 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infection. J. Virol. 61:600–603.
58. Peeters, B., J. Pol, A. Gielkens, and R. Moormann. 1993. Envelope glycoprotein gp50 of pseudorabies virus is essential for virus entry but it not required for viral spread in mice. J. Virol. 67:170–177.
59. 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. Acta Virol. 34:305–320.
60. 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.
61. Sears, A. E., B. S. McGuire, 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.
62. Smiley, J. R., 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 deletion. Virology 113:345–362.
62a. Smiley, J. R., D. C. Johnson, L. I. Pizer, and R. D. Everett. 1992. The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein D (gD) promoter are not essential for efficient gD transcription during virus infection. J. Virol. 66:623–631.
63. Stulting, R. D., J. C. Kindle, and A. J. Nahmias. 1985. Patterns of herpes simplex keratitis in inbred mice. Invest. Ophthalmol. Visual Sci. 26:1360–1367.
64. Wander, A. H., Y. M. Centifanto, and H. E. Kaufman. 1980. Strain specificity of clinical isolates of herpes simplex virus. Arch. Ophthamol. 98:1458–1461.
65. 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.
66. 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.
67. Zuckermann, F. A., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat. 1988. Complex between glycoprotein gI and gp63 of pseudorabies virus: its effects on virus replication. J. Virol. 62: 4622–4626.

Chapter 3 Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus

3.0 Preface

In the following publication, I carried out all the experiments (Figures 3.1 to 3.9). The initiation of this work was made possible by the collaboration with L. Doering who is a neuroanatomist at McMaster University. Dr. Doering showed me how to do the intraocular injections, crysectioning of retinal and brain sections, as well as the immunohitochemistry of these sections. The majority of the work was done in Dr. Doering's lab.

JOURNAL OF VIROLOGY, Nov. 1995, p. 7087–7098
0022-538X/95/$04.00+0

Vol. 69, No. 11

Glycoproteins E and I Facilitate Neuron-to-Neuron Spread of Herpes Simplex Virus

KEVIN S. DINGWELL,[1,2] LAURIE C. DOERING,[3] AND DAVID C. JOHNSON[1]*

Cancer Research Group, Institute of Molecular Biology and Biotechnology,[1] Department of Biology,[2] and Department of Anatomy,[3] McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Received 22 May 1995/Accepted 28 July 1995

Two herpes simplex virus (HSV) glycoproteins E and I (gE and gI) form a heterooligomer which acts as an Fc receptor and also facilitates cell-to-cell spread of virus in epithelial tissues and between certain cultured cells. By contrast, gE-gI is not required for infection of cells by extracellular virus. HSV glycoproteins gD and gJ are encoded by neighboring genes, and gD is required for both virus entry into cells and cell-to-cell spread, whereas gJ has not been shown to influence these processes. Since HSV infects neurons and apparently spreads across synaptic junctions, it was of interest to determine whether gD, gE, gI, and gJ are also important for interneuronal transfer of virus. We tested the roles of these glycoproteins in neuron-to-neuron transmission of HSV type 1 (HSV-1) by injecting mutant viruses unable to express these glycoproteins into the vitreous body of the rat eye. The spread of virus infection was measured in neuron-rich layers of the retina and in the major retinorecipient areas of the brain. Wild-type HSV-1 and a gJ$^-$ mutant spread rapidly between synaptically linked retinal neurons and efficiently infected major retinorecipient areas of the brain. gD mutants, derived from complementing cells, infected only a few neurons and did not spread in the retina or brain. Mutants unable to express gE or gI were markedly restricted in their ability to spread within the retina, produced 10-fold-less virus in the retina, and spread inefficiently to the brain. Furthermore, when compared with wild-type HSV-1, gE$^-$ and gI$^-$ mutants spread inefficiently from cell to cell in cultures of neurons derived from rat trigeminal ganglia. Together, our results suggest that the gE-gI heterooligomer is required for efficient neuron-to-neuron transmission through synaptically linked neuronal pathways.

Herpes simplex viruses (HSV) have developed a sophisticated relationship with the human nervous system. Following primary infections of mucosal and submucosal tissues, HSV infects sensory neurons and is transported in axons (11, 23, 31) by microtubule-associated fast axonal transport (30, 46) to sensory ganglia. The virus may transiently replicate and spread to other neurons and glial cells, although, in time, a latent infection is established and there is no virus replication (51). Later, under appropriate conditions, HSV may reactivate from latently infected neurons, and newly replicated virus is transported back to the periphery, producing secondary mucosal infections. Although virus may spread past the sensory ganglia to the central nervous system (CNS), damage to either the peripheral or central nervous system in humans is rare.

The spread of HSV and other alphaherpesviruses through the CNSs of experimental animals follows synaptically linked pathways, suggesting a mechanism of direct transneuronal transport across the synapse (8, 32, 41, 42, 53, 54). Supporting this hypothesis, virus particles in the synaptic cleft have been observed (8, 46). Additionally, the rate of virus spread in the nervous system suggests direct neuron-to-neuron transfer of virus, rather than spread via infected glial cells (39, 53). The spread of HSV between cells, either across cell junctions, synapses, or other structures, is apparently a complex process involving a subset of the viral envelope glycoproteins that are also necessary for entry of virus into cells.

HSV type 1 (HSV-1) encodes at least 11 glycoproteins, and 5 of these glycoproteins, gB, gD, gH, gK, and gL, are essential for productive infections in cultured cells and entry of extracellular virus particles into cells (7, 19, 24, 33, 49). The remaining glycoproteins, gC, gE, gI, gG, gJ, and gM, are not essential for virus infection and replication in cultured cells (2, 3, 35–37, 40, 50, 56). Since it seems unlikely that HSV would conserve nonessential genes, it is probable that these glycoproteins play important roles in vivo in infection of diverse cell types, movement of virus through tissues, or protection from the host immune responses. gE and gI form a complex which acts as a receptor for the Fc domain of immunoglobulin G (IgG) (5, 21, 26). The gE-gI Fc receptor may protect HSV-infected cells from complement-mediated immune lysis by causing IgG aggregation or by reducing the ability of complement components to bind to virus- or cell-associated IgG (1, 16, 20, 21). However, protective effects of this type have not been demonstrated in vivo in an animal model, and there is mounting evidence that the gE-gI heterooligomer is important to facilitate virus spread in vivo, a property that is apparently unrelated to the IgG Fc receptor activity (for a recent review, see reference 58).

HSV-1 mutants lacking either gE or gI are inhibited in their capacity to spread between certain types of cultured cells by the direct cell contact route and the mutants spread poorly in epithelial tissues (4, 13). However, gE$^-$ and gI$^-$ mutants do not display defects in entry of extracellular virus particles into the same cells. Thus, cell-to-cell spread of viruses, apparently across cell junctions, has features that are distinct from entry of extracellular virus. There is also some evidence that the gE-gI heterooligomer is important for cell-to-cell transfer in the nervous system. HSV-1 mutants lacking gE exhibited decreased neurovirulence following intracerebral infections (40), and spread into the nervous system was reduced after infection at the periphery (4, 47). Pseudorabies virus (PrV), another mem-

* Corresponding author. Mailing address: Room 4H30 HSC, McMaster University, 1200 Main St. West, Hamilton, Ontario, Canada L8N 3Z5. Phone: (905) 525-9140, ext. 22395. Fax: (905) 546-9940.

TABLE 1. HSV-1 mutants unable to express glycoprotein D, E, I, or J and a wild-type recombinant derived from F-gEβ

Virus	Phenotype and genotype	Reference
F	$gD^+ gE^+ gI^+ gJ^+$; wild-type HSV-1	17
F-US7kan	$gD^+ gE^+ gI^- gJ^+$; kanamycin resistance gene inserted into the US7 (gI) gene	26
F-gEβ	$gD^+ gE^- gI^+ gJ^+$; β-galactosidase gene replaces the US8 (gE) coding sequences	13
F-US5β	$gD^+ gE^+ gI^+ gJ^-$; insertion of the β-galactosidase gene in the US5 gene (gJ)	59
F-US6kan	$gD^- gE^+ gI^+ gJ^+$; kanamycin resistance gene inserted into the US6 gene (gD)	27
F-gDβ	$gD^- gE^+ gI^- gJ^+$; β-galactosidase replaces all gD coding sequences and part of the gI gene	27
wt61	$gD^+ gE^+ gI^+ gJ^+$; derived from F-gDβ by rescue with plasmid containing wild-type gD and gI genes	52

ber of the *Alphaherpesvirinae* subfamily, similarly depends on gE and gI for cell-to-cell spread in cultured cells and experimental animals (9, 57, 60).

Many previous studies on the spread of HSV and PrV gE^- and gI^- mutants into the nervous system have involved infection protocols in which experimental animals were infected at the periphery. In these investigations, the mutant viruses replicated poorly or spread poorly at the site of primary infection and less virus was produced. Therefore, it was frequently difficult to determine whether the reduced levels of virus produced less infection in the nervous system or whether the gE-gI heterooligomer was required for transmission of virus into the nervous system and transneuronal spread. In this study, we characterized the roles of gE and gI and two other glycoproteins, gD and gJ, in neuron-to-neuron spread of HSV-1. Mutants unable to express gD, gE, gI, or gJ were injected into the vitreous body of the rat eye, and the spread of virus in the neuron-rich tissue of the retina and retinorecipient regions of the brain was characterized. Neuron-to-neuron spread of the viruses was also studied by using cultured rat trigeminal ganglion neurons. The results support the hypothesis that gE and gI promote efficient transneuronal transport of HSV.

MATERIALS AND METHODS

Cells and viruses. Vero (African green monkey kidney) cells were passaged in α-minimal essential medium (α-MEM) containing 5% fetal bovine serum (FBS). VD60 cells (33) were passaged in Dulbecco's MEM lacking histidine supplemented with 5 mM histidinol and 5% FBS. Wild-type HSV-1 strain F (17), the gE deletion mutant F-gEβ (13), the gI^- mutant F-US7kan (26), the gJ^- mutant F-US5β (59), and wt61 (52), were all propagated and titered on Vero cells. The gD^- HSV-1 mutant F-US6kan (27) and the $gD^- gI^-$ mutant F-gDβ (33) were propagated on VD60 cells. For a description of mutant viruses, see Table 1.

Rat neuronal cultures. Trigeminal ganglia from 5- to 7-day-old Wistar rat pups were collected aseptically in Leibovitz's L-15 medium. Pooled ganglia were treated with 0.1% trypsin for 45 min, α-MEM containing 10% FBS was added, and the resulting mixture was repeatedly mechanically triturated through a Pasteur pipette. Connective tissues were allowed to settle, and the supernatant containing dissociated neurons was collected. Neurons were spun down at 300 × *g* for 5 min, resuspended in α-MEM supplemented with 50 ng of 2.5S nerve growth factor (MEMN) (Gibco BRL, Burlington, Ontario, Canada)–10% FBS, and plated on glass coverslips or tissue culture dishes previously treated with 0.1 mg of poly-o-lysine (Boehringer Mannheim) per ml and 10 μg of laminin (Boehringer Mannheim) per ml. The nonneuronal cell population was eliminated by repeated treatments with 10 μM cytosine arabinoside (Sigma) at 2-day intervals.

Ocular HSV infections. Six-week-old female Wistar rats (two animals per virus) were anesthetized with chloral hydrate and injected with HSV-1 F or HSV-1 mutants (5×10^6 PFU) into the vitreous body of one eye, using a 10-μl Hamilton syringe. At the indicated times, animals were overdosed with chloral hydrate and perfused through the heart with normal saline and then with 4% paraformaldehyde. The brain and injected eye were removed, postfixed overnight in paraformaldehyde, and then equilibrated in 15 or 30% sucrose, respectively. The brain was frozen in 2-methylbutanol cooled to −60°C, and 12-μm-thick serial sections were taken through the coronal plane of the retinorecipient regions. Floating sections were incubated overnight at 4°C with rabbit anti-HSV-1 antibodies (Dako, Dimension Laboratories, Mississauga, Ontario, Canada) diluted 1:100 in phosphate-buffered saline (PBS). Sections were washed three times in PBS for 5 min each and incubated with a 1:100 dilution of fluorescein-conjugated goat anti-rabbit IgG (Terochem Labs, Markham, Ontario, Canada) for 4 h at room temperature, washed three times in PBS for 5 min each, and then mounted on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.). The cornea and lens were removed from the eye cup, the vitreous fluid was removed with a gentle PBS wash, and the retina was imbedded in OCT compound (Tissue-Tek [Miles Inc., Elkart, Ind.]) and 12-μm-thick serial cross-sections were taken and collected onto gelatin-coated glass slides. Sections were immunostained as described above. Immunofluorescence was detected with a Reichert microscope, and photographs were taken with a Konica camera, Reichert Photostar optic system, and Kodak T-max 400 black-and-white film. Control sections from animals not infected with HSV-1 or processed without primary antibodies or with nonimmune rabbit serum showed no specific immunofluorescence. Immunoreactive cells were observed only in regions of the brain contralateral to the injected eye.

Production of infectious HSV in the retina. Replication of HSV mutants in the eye was tested by anesthetizing rats with chloral hydrate and injecting wild-type F or mutant viruses (5×10^6 PFU) into the vitreous body of both eyes. After 1, 2, or 5 days, rats were overdosed with chloral hydrate and the eyes were removed. The cornea and lens were removed from the eye cup, and the retina was washed with PBS and then frozen at −70°C. Retinas were thawed, suspended in 1-ml portions of α-MEM containing 1% FBS, and homogenized with a small (2-ml) Dounce homogenizer. Homogenates were then titered on Vero cell monolayers.

Production of infectious HSV in neuronal cultures. Cultures of dissociated rat neurons were infected with wild-type HSV-1 (F) or HSV-1 mutants at 1 or 10 PFU/cell (the titers given here were derived with Vero cells; under these conditions, approximately 1 in 10 to 1 in 20 neurons were infected when 1 PFU/cell was used) in α-MEM containing 1% FBS for 2 h. Inoculum was removed, and fresh MEMN–1% FBS was added. For experiments designed to follow virus spread through the cultures, MEMN–1% FBS supplemented with 0.1% human gamma globulin, a source of anti-HSV neutralizing antibodies, was added to the cultures after infection. At appropriate times, cells and media were collected and frozen at −70°C. Samples were thawed and sonicated, and HSV-1 was titered on Vero cells.

Immunofluorescence of neuronal cultures. Neurons were infected with wild-type HSV-1 or HSV-1 mutants at 1 PFU/cell in α-MEM containing 1% FBS for 2 h, and as noted above, these conditions of infection caused only approximately 1 in 10 or 1 in 20 of the neurons to be infected. The virus was removed, and cells were washed and then incubated in MEMN–1% FBS supplemented with 0.1% human gamma globulin, a source of anti-HSV neutralizing antibodies. After 48 h, the medium was removed, and the cells were washed gently with PBS, fixed in 4% paraformaldehyde for 10 min, washed extensively in PBS, and then permeabilized with 0.2% Triton X-100 for 5 min each. Cells were washed with PBS and then incubated in PBS containing 1% normal goat serum, 1% normal horse serum, and 0.2% Tween 20 (GHTPBS) for 30 min. The cells were then incubated simultaneously with mouse anti-160-kDa neurofilament antibodies (Boehringer Mannheim) diluted 1:50 in GHTPBS and rabbit anti-HSV-1 antibodies (Dako) diluted 1:5,000 for 90 min at 37°C. The cells were washed three times with GHTPBS for 5 min each and then incubated with fluorescein-conjugated goat anti-rabbit IgG antibodies and Texas red-conjugated horse anti-mouse IgG antibodies (1:100 diluted; Vector Laboratories) for 90 min at 37°C. Cells were washed three times with GHTPBS for 5 min each and then mounted on microscope slides with Vectashield mounting medium.

RESULTS

HSV gE^-, gI^-, and gD^- mutants spread inefficiently or not at all in the rat retina. Since gE and gI are important for efficient spread of HSV from cell-to-cell and gD is required for virus entry and spread in epithelial cells and fibroblasts, it was of interest to determine whether these glycoproteins were also important for neuron-to-neuron transmission of virus. Initially, we chose to study the spread of HSV-1 mutants after injection into the eye, where virus infection can spread through the neuron-rich layers of the retina and to the retinorecipient regions of the brain.

In all vertebrates, the retina is an inverted structure in which the photoreceptors are located in the outer layers of the eye (most distant from impinging light entering through the lens). Thus, light must pass through most of the retina before it can be detected by photoreceptors, namely, the rods and cones that are present in the bacillary, outer nuclear and outer plexiform

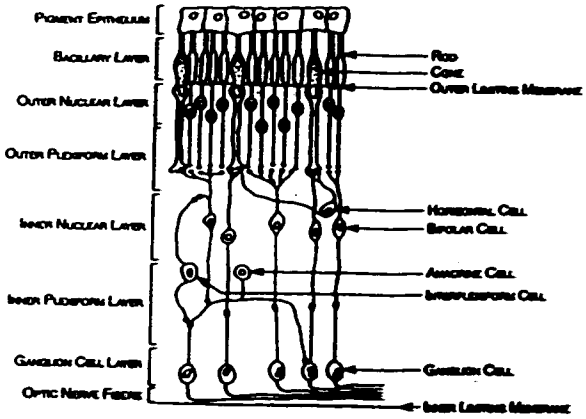


FIG. 1. Cellular organization of the vertebrate retina. The vertebrate retina is composed of eight parallel layers of cells. Light (from the bottom of this figure) impinges on the retina and traverses most of these layers, stimulating photoreceptors (near the top of this figure). The outer layer of the retina, the pigment epithelium, supports and insulates the photoreceptors. The photoreceptors (rods and cones) synapse with bipolar cells located in the inner nuclear layer. Bipolar cells in turn synapse with ganglion cells, located in the ganglion cell layer, which give rise to axons forming the optic nerve. Signals from the photoreceptors in this central pathway are modulated by several different associated neurons which constitute the lateral pathway. Horizontal cells, located in the inner nuclear layer, have synaptic connections with the photoreceptors. Other associated neurons modulating the signal are amacrine cells, located in the inner nuclear and ganglion cell layers, and form synaptic connections with ganglion cells, bipolar cells, and other associated neurons including interplexiform cells. In our experiments, HSV was injected into the vitreous body of the eye, the region adjacent to the inner limiting membrane (at the bottom of the figure).

layers (Fig. 1). Visual signals are conveyed from the photoreceptors through connections linking three distinct types of neurons, making up the central visual pathway of the retina. Hypopolarization of the photoreceptors leads to activation of bipolar cells present in the inner nuclear and inner plexiform layers of the retina. Bipolar cells are in turn connected to ganglion cells, the third type of neuron in the central pathway. Axons from ganglion cells give rise to optic nerve fibers which synapse with neurons located in the visual centers of the brain. Signals conveyed in the central pathway (vertically) from photoreceptors to bipolar cells to ganglion cells are modulated by other neurons, namely, horizontal cells, amacrine cells, and interplexiform cells, which synapse with neurons of the vertical pathway and constitute the lateral signal pathway (Fig. 1). The retina, although very thin, is an intricate system of neuronal connections in which neurons make up a large fraction of the cells. Therefore, the retina provides an excellent model in which to study transneuronal transmission of HSV.

Wild-type HSV-1 and mutant HSV-1 unable to express gD, gE, gI, or gJ (for description of mutant viruses, see Table 1) were injected into the vitreous body of the rat eye, and the spread of virus in the retina was assessed after 2 and 5 days by immunofluorescence. In retinas from animals infected with wild-type HSV-1, distinct foci of infection which extended from the ganglion cell layer to the outer nuclear layer were evident after 2 days. This result suggested that virus had infected ganglion cells and then spread through the central visual pathway connecting the ganglion cells to the photoreceptors (Fig. 2 and 3). Foci of infection were observed 2 days after infection with wild-type HSV-1 and with mutants unable to express gE (F-gEβ), gI (F-US7kan), or gJ (F-US5β), and the numbers of foci produced by the mutants did not appear to differ from those produced by the wild type, although no careful statistical analysis was performed. These observations suggest that the primary infection of retinal ganglion cells was not affected by the loss of gE, gI, or gJ. Confirming this, gE$^-$, gI$^-$, or gJ$^-$ HSV-1 mutants produced as much infectious virus after 1 or 2 days as was produced by wild-type HSV-1 (Fig. 4). Therefore, gE$^-$, gI$^-$, and gJ$^-$ mutants were able to initiate infection and replicate in retinal neurons as efficiently as wild-type HSV-1. No immunostaining was observed when sections were incubated with rabbit nonimmune sera, and thus, the immunostaining was not altered by HSV Fc receptor activity.

When retinas were infected with the gD$^-$ mutant (F-US6kan) or the gD$^-$ gI$^-$ mutant (F-gDβ), infected foci were observed and the number of these foci appeared to be similar to those produced by wild-type HSV-1 (Fig. 3). However, with both the gD$^-$ mutants, the spread of virus infection to the outer layers of the retina containing the photoreceptors was reduced. Infection of neurons other than the ganglion cells was observed with gD$^-$ mutants but was more restricted than with wild-type HSV-1 and could be related to initial infection of neurons other than ganglion cells or to a limited amount of virus spread in the absence of gD. Since gD is essential for entry of HSV into all the cultured cells tested to date, infections with these two gD$^-$ viruses involved virus stocks produced on complementing VD60 cells, which express gD (33). A recombinant virus, wt61, which was derived from F-gDβ by rescuing the gD and gI coding sequences, also produced equal numbers of foci of infection and replicated as well as wild-type F (Fig. 3 and 4). Therefore, gD$^-$ viruses, when supplied with gD *in trans*, were able to initiate infections of retina neurons, but in the absence of gD, the viruses were severely restricted in their ability to spread between neurons.

After 5 days, retinas infected with the gE$^-$ mutant (F-gEβ) or the gI$^-$ mutant (F-US7kan) displayed much less HSV-specific staining than retinas infected with wild-type HSV-1 (Fig. 2). The wild-type HSV-1 (F) spread throughout the various neuronal cell layers of the retina, infecting the majority of the retinal neurons. By contrast, the infection with F-US7kan or F-gEβ was markedly restricted in this spread. Lateral spread of these two mutants within the retina appeared reduced, so that columns of neurons in the central visual pathway were infected, but there was not broad infection throughout the retina, as was observed with wild-type virus. In addition, the yields of infectious virus produced by the gE$^-$ and gI$^-$ mutants after 5 days were 10-fold lower than that produced by wild-type HSV-1, yet the yields of virus produced after 1 and 2 days were not different (Fig. 4). There was not a substantial increase in the amount of infectious wild-type HSV-1 produced between 2 and 5 days in the retina, even though higher levels of viral antigens were observed by immunofluorescence after 5 days. These results may be related to inefficient production of infectious HSV-1 in retinal neurons. HSV-1 replicates poorly in rodent cells, and this effect may be more pronounced in retinal neurons. However, it is clear from these results that the gE$^-$ and gI$^-$ viruses replicate less efficiently in the retina than wild-type HSV-1, and this result appears to be related to decreased cell-to-cell spread and not to an inability to produce an initial infection of the retina.

In contrast to the gE$^-$ and gI$^-$ mutants, the gD$^-$ mutant (F-US6kan) and the gD$^-$ gI$^-$ mutant (F-gDβ) failed to spread in a detectable fashion from the initial point of infection. After 5 days, expression of viral antigens was difficult to detect and it appeared that these two mutant viruses were largely cleared (Fig. 3). We did not attempt to detect infectious viruses in the retina 5 days after infection with F-US5kan or F-gDβ, since these viruses, lacking gD, would not enter and infect most cultured cells (33), and because there is persuasive evidence

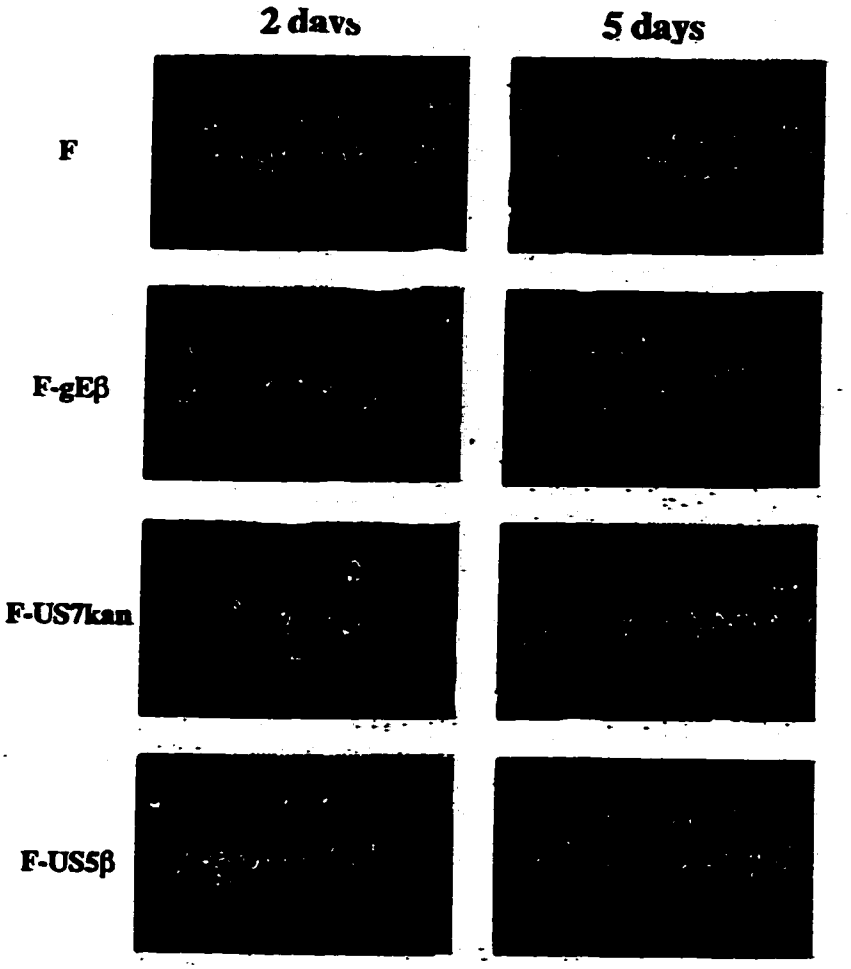


FIG. 2. Infections of the rat retina by gE⁻, gI⁻, and gJ⁻ mutant viruses. The wild-type HSV-1 strain F, the gE⁻ mutant (F-gEβ), the gI⁻ mutant (F-US7kan), or the gJ⁻ mutant (F-US5β) was injected into the vitreous body of one eye of each anesthetized rat, using 5×10^6 PFU of each virus. Two or five days after infection, anesthetized animals were perfused with 4% paraformaldehyde, and then the retinas were postfixed overnight in 4% paraformaldehyde and equilibrated in 30% sucrose. Retinas were imbedded in OCT compound and sectioned, and sections mounted on gelatin-coated glass slides were stained with rabbit anti-HSV antibodies and then with FITC-conjugated anti-rabbit antibodies. No detectable staining was observed with rabbit nonimmune serum. Sections were photographed with a Reichert fluorescence microscope and camera and Kodak T-max 400 film.

that the viruses were not being transmitted from neuron to neuron in the retina. wt61, which was rescued from F-gDβ, spread throughout the retina after 5 days and produced levels of infectious virus similar to those observed with wild-type F at both early and late times (Fig. 3 and 4). The gJ⁻ mutant, F-US5β, also behaved like wild-type HSV-1 (F). Thus, gD is essential for the spread of HSV-1 in the retina, but gJ is not.

Spread of HSV-1 mutants from retinal ganglion cells to the major visual centers in the brain. Axons of retinal ganglion cells project to six regions in the brain (for reviews, see references 43 and 48). Following an intravitreal inoculation of the rat eye and infection of the retina with HSV, several of these retinorecipient nuclei become infected. This is thought to involve anterograde intra-axonal transport of virus from retinal ganglion cells whose axons project to recipient neurons in the CNS (42). We followed the spread of the HSV-1 mutants from retinal ganglion cells to three retinorecipient nuclei in the brain: (i) the suprachiasmatic nuclei (SCN), located in the hypothalamus, which is involved in controlling circadian rhythms; (ii) the lateral geniculate nucleus (LGN), subdivided into dorsal and ventral lateral nuclei (dLGN and vLGN, respectively), which relays information to the visual cortex and to several subcortical areas; and (iii) the superior colliculus (SC), also known as the optic tectum, which facilitates shifts in gaze (for a review, see reference 43) (Fig. 5). Two or five days after retinal infection, animals were anesthetized and then perfused with 4% paraformaldehyde, and the brains were removed and prepared for cryosectioning. Serial sections through the coronal plane were taken from the regions of the brain corresponding to the SCN, SC, and LGN (Fig. 5). Sections were immunostained with anti-HSV rabbit polyclonal antibodies and then with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies, and infected neurons were visualized by immunofluorescence.

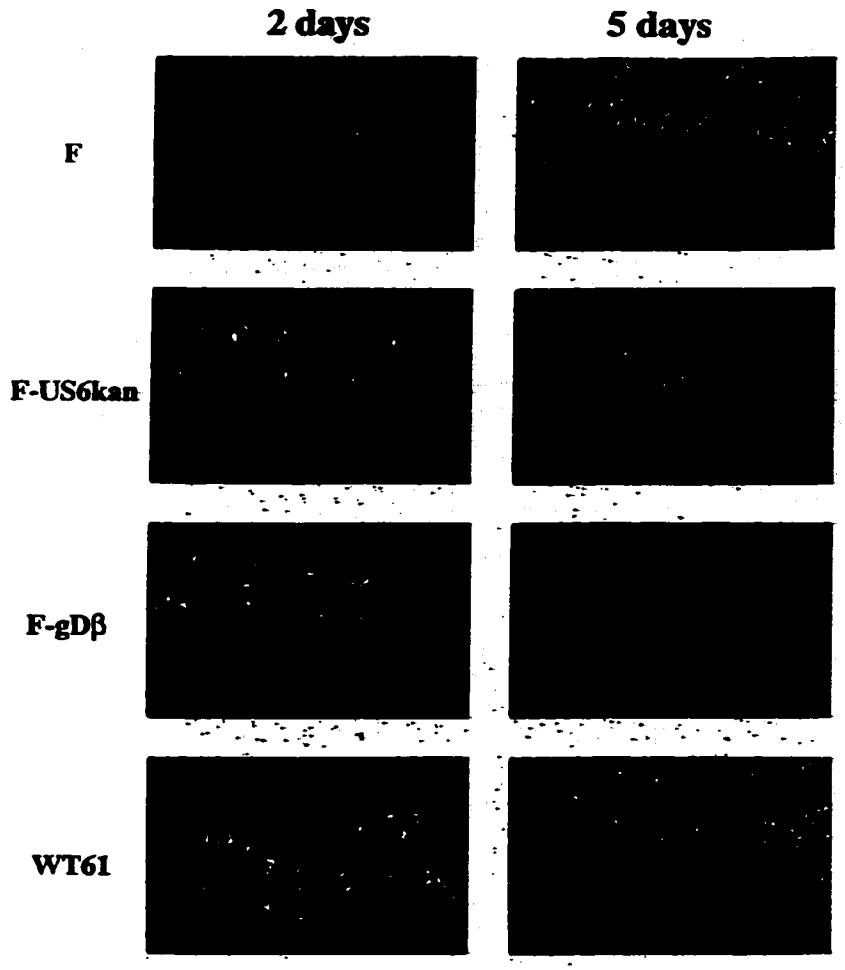


FIG. 3. Infections of the rat retina by gD⁻ mutant viruses. The wild-type HSV-1 strain F, the gD⁻ mutant (F-US6kan), the gD⁻ gI⁻ mutant (F-gDβ), or wt61 was inoculated into the vitreous body of one eye of each anesthetized rat, using 5×10^6 PFU. Stocks of both gD⁻ mutants were derived from complementing VD60 cells, which supply gD *in trans*. Two or five days after infection, the eyes were removed and the retinas were fixed and stained as described in the legend to Fig. 2.

In rats infected with wild-type HSV (F), some immunostaining was evident in the SCN after 3 days (data not shown) and was more pronounced by 5 days (Fig. 6). In the LGN, HSV-specific immunostaining first became evident 4 days after retinal infection (data not shown) and was more extensive by day 5 (Fig. 6). The majority of labeled cells in the LGN were in the dLGN, although there were a few HSV-infected cells scattered throughout the vLGN. Immunostaining in the SC became evident after 3 days and progressively became more intense over the next 2 days (data not shown and Fig. 6). The SC sections shown include the superficial layers of the colliculus which are retinorecipient neurons. It was conceivable that virus leaking from the eye could infect oculomotor neurons which project to the deeper layers of the colliculus. However, sections through these layers failed to indicate the presence of viral antigens (data not shown). In animals infected with either the gI⁻ mutant (F-US7kan) or the gE⁻ mutant (F-gEβ), immunostaining in the SCN and LGN was markedly less (fewer cells were labeled) than that observed in rats infected with wild-type HSV-1 (F) (Fig. 6). While the degrees of immunolabeling throughout the LGN for gE⁻ and gI⁻ mutants were reduced from that of the wild type, the majority of HSV-specific labeling was distributed in the dLGN in a manner similar to that of the wild-type virus. Similar amounts of immunolabeling were observed in the SCs of animals infected with either wild-type HSV-1 or the gE⁻ and gI⁻ viruses after 5 days. Therefore, mutant viruses unable to express gE or gI are restricted in their ability to spread to some retinorecipient areas of the brain.

The gJ⁻ mutant, F-US5β, infected all three retinorecipient nuclei as well as wild-type HSV-1 did (Fig. 6). As with wild-type HSV-1, immunostaining in the LGN in F-US5β-infected animals was largely restricted to the dLGN, rather than the vLGN. Together, these data suggest that gJ is not required for efficient transneuronal spread from retina to brain. In contrast, no immunostaining of any retinorecipient region of the brain was observed 5 days after infection with the gD⁻ mutant (F-US6kan) or the gD⁻ gI⁻ mutant (F-gDβ). The recombinant wt61, in which gD and gI expression were rescued, produced staining patterns very similar to that observed with wild-type F (Fig. 7). These results demonstrate that gD is essential for

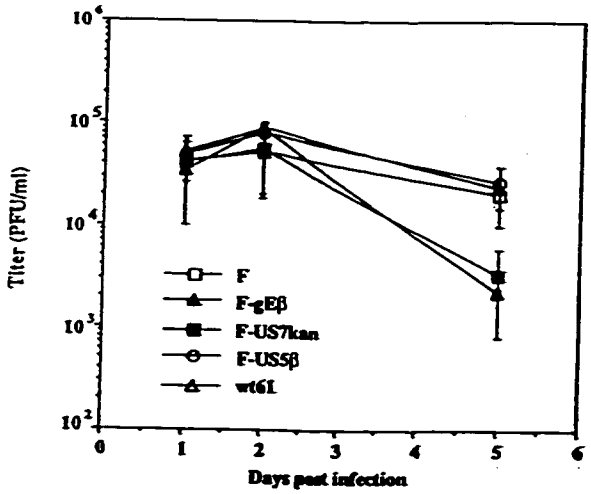


FIG. 4. Production of infectious viruses by HSV-1 gE⁻, gI⁻, and gJ⁻ mutants in the rat retina. Retinas were infected with either wild-type HSV-1 (F), the gE⁻ mutant (F-gEβ), the gI⁻ mutant (F-US7kan), the gJ⁻ mutant (F-US5β), or wt61. After 1, 2, or 5 days, the retinas were removed, washed in PBS, frozen, thawed, and then homogenized. Homogenates were titered on Vero cell monolayers.

transneuronal spread of HSV to retinorecipient neurons in the brain.

Cell-to-cell spread of gE⁻, gI⁻, and gD⁻ mutants in cultured neurons. To further investigate neuron-to-neuron spread of HSV-1, we tested the ability of the mutants to spread in cultures of primary rat sensory neurons derived from trigeminal ganglia. By culturing the neurons on a poly-D-lysine and laminin substrate, which is known to stimulate neuritic growth (22), it was possible to produce a complex array of cell-cell and cell-neurite contacts or synapses. Neurons were grown for several weeks in the presence of a mitotic inhibitor, cytosine arabinoside, so that fibroblasts and glia were killed, producing cultures that were greater than 95% neurons. This system provided an ideal model to study the spread of HSV-1 between neurons where accessory cells were absent and input multiplicities of virus could be controlled.

HSV was plated on the cultured neurons at a low multiplicity of infection so that over the course of several days, viruses spread from the initial infected cells to neighboring neurons and through the dense network of neurites. It should be noted that the virus stocks used in these experiments were titered on Vero cells and when the cells were infected with 1 PFU per neuron, less than 10% of the neurons displayed viral antigens after 12 h. All the neurons in the culture could be infected when higher multiplicities of virus were used (10 PFU per neuron) or when virus was allowed to spread through the cultures. Following 48 h of infection with either wild-type or mutant viruses, cells were fixed and then immunostained for HSV antigens or for the 160-kDa neurofilament protein. The spread of HSV through the culture was assayed by counting the number of individual cells labeled with both anti-HSV and antineurofilament antibodies (Fig. 8A). Wild-type HSV-1 (F) and wt61, infected in excess of 600 neurons after 48 h (Fig. 8B). In contrast, the gE⁻ mutant (F-gEβ) or the gI⁻ mutant (F-US7kan) infected less than half this number of neurons. The gD⁻ mutant, F-US6kan, infected still fewer neurons, although there was some spread of virus infection in the absence of gD. However, in the absence of both gD and gI, F-gDβ did not spread beyond a single infected neuron. These results are similar to those we previously described for fibroblast monolayers in which there was some virus spread in the absence of gD and no virus spread in the absence of both gD and gI (6).

Replication of both mutant and wild-type HSV-1 in neuronal cultures was also assessed by infecting the cultures and then measuring infectious virus after various times. When cells were infected with higher multiplicities of HSV-1 (10 PFU per cell), all the neurons in the culture expressed HSV antigens after a single round of replication. Under these conditions, wild-type HSV-1 and gE⁻, gI⁻, and gJ⁻ mutant viruses all produced equal quantities of infectious virus (Fig. 9A). Therefore, gE⁻, gI⁻, and gJ⁻ mutant viruses infect these neurons and produce infectious virus normally. However, when lower multiplicities of infection were used, so that only approximately 1 in 10 to 1 in 20 of the neurons were initially infected, production of infectious viruses in cultures infected with the gE⁻ and gI⁻ viruses was reduced by approximately 10-fold (Fig. 9B). Under these conditions, production of infectious virus was dependent on neuron-to-neuron spread of virus, especially after 24 h, since anti-HSV antibodies were present to neutralize any infectious virus released into the medium. Together, these data, along with our observations that gE⁻ and gI⁻ viruses spread poorly in the retina (Fig. 2), support the hypothesis that the gE-gI heterooligomer facilitates neuron-to-neuron spread. Again, we did not measure replication of the gD⁻ viruses in these latter assays, because the viruses cannot enter most cultured cells and because immunofluorescence experiments (Fig. 8) indicated that these viruses could not spread.

DISCUSSION

There is good evidence that the HSV gE-gI heterooligomer functions to bind the Fc domain of IgG (for a recent review, see reference 58). However, more recently, there has been substantial evidence presented that this glycoprotein complex plays an important role in allowing HSV to spread from cell to cell in certain cultured cells (4, 13), in epithelial and other tissues in vivo (4, 13), and in the nervous system (40, 47). No role has been demonstrated for the Fc receptor in protecting HSV from the immune system in vivo, the gE-gI binds IgG relatively weakly, and other alphaherpesviruses have gE-gI ho-

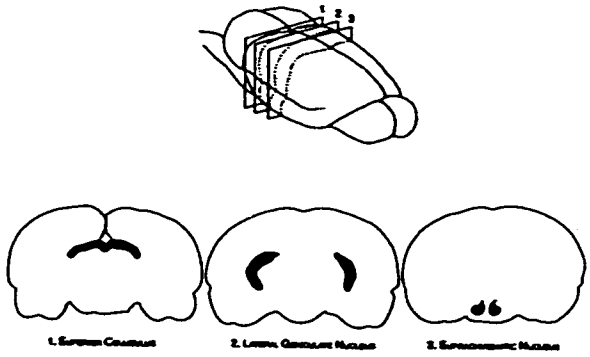


FIG. 5. Retinorecipient regions of the rat brain. Three coronal sections through the brains of rats, which had been infected in the eye with HSV-1 mutants, were taken. These sections correspond to the stereotaxic locations of the SC (section 1), or optic tectum, which is located in the midbrain, LGN (section 2), which is present in the thalamus, and SCN (section 3), which is in the hypothalamus. Each of these nuclei is highlighted and, for clarity, has been enlarged, so that they are not to scale with other regions of the brain.

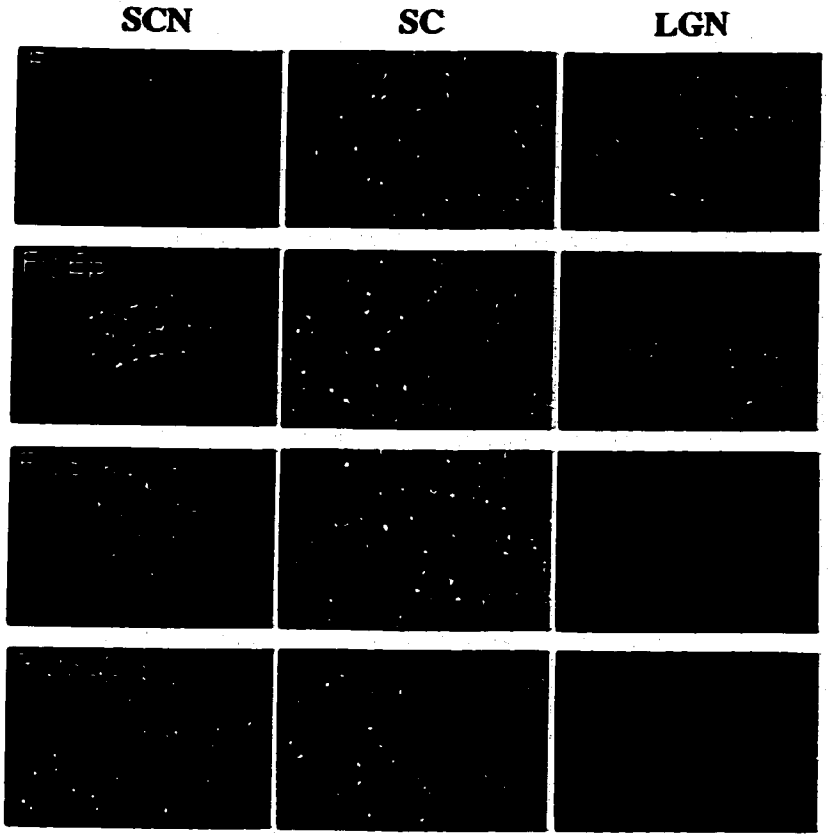


FIG. 6. Infection of retinorecipient regions of the rat brain by HSV-1 gE⁻, gI⁻, and gI⁻ mutants. Rats were infected in the vitreous body of one eye with wild-type HSV-1 (F), the gE⁻ mutant (F-gEβ), the gI⁻ mutant (F-US7lacz), or the gI⁻ mutant (F-US5β). Five days later, the animals were anesthetized and perfused with 4% paraformaldehyde. The brains were removed, postfixed overnight in 4% paraformaldehyde, equilibrated in 15% sucrose, and sectioned. The sections were stained with rabbit anti-HSV antibodies and then with FITC-conjugated anti-rabbit antibodies, and immunostaining was observed only in the contralateral regions of the brain. The sections shown are the SCN, SC, and LGN, which includes both the dLGN (shown on the right side of the micrograph) and vLGN (left side).

mologs that do not bind IgG, so it has been proposed that gE-gI primarily functions in vivo to promote cell-to-cell spread of alphaherpesviruses (58). Previous studies had indicated that HSV gE⁻ or gI⁻ mutants are defective in cell-to-cell spread in epithelial tissues of experimental animals and spread poorly into the nervous system. However, since these mutants frequently replicated poorly at the periphery and produced substantially less infectious virus, it was not clear whether gE⁻ and gI⁻ viruses had difficulty gaining entry into neurons or were defective in transneuronal spread. Our studies were designed to address this question and, specifically, to evaluate neuron-to-neuron spread of HSV-1 mutants unable to express gE or gI, as well as two glycoproteins, gD and gJ, encoded by neighboring genes.

We chose to examine the spread of HSV-1 mutants in the retina and into the retinorecipient areas of the rat brain for a number of reasons. First, the retina is a dense matrix of neuronal connections which resembles the gray matter of the brain, and this neuronal circuitry is well characterized in the rat (for a review, see reference 43). Second, the retina can be readily infected by HSV, without significant injury to the tissue, and can be readily dissected and stained. Similar injections into the mouse eye are technically more challenging and less reproducible. Third, by using the rat visual system, we could more readily compare our observations to previous results involving HSV (41, 42) and PrV (9, 10, 18, 57). The central visual pathway in the retina consists of three types of neurons aligned in series. The visual signal originates with the photoreceptors, rods and cones, found at the back of the retina in the bacillary and outer nuclear layers (Fig. 1). This signal is conveyed by bipolar cells, located in the outer plexiform and inner nuclear layers of the retina, to retinal ganglion cells in the innermost layer of the retina. Axons from ganglion cells project to the visual centers of the brain. This synaptically linked pathway is modulated by lateral connections with horizontal cells in the outer plexiform layer and with amacrine cells in the inner plexiform layer. This complex yet well-ordered architecture of cellular and synaptic connections within the retina provides an ideal model to study neuron-to-neuron transmission of HSV without many of the complications associated with peripheral or intracerebral infections.

Two days after infection with wild-type HSV-1, we observed distinct foci of infection primarily in the inner and outer nuclear and ganglion cell layers, apparently involving columns of infected neurons, suggesting that infection spreads through direct synaptic connections of the central retinal pathway.

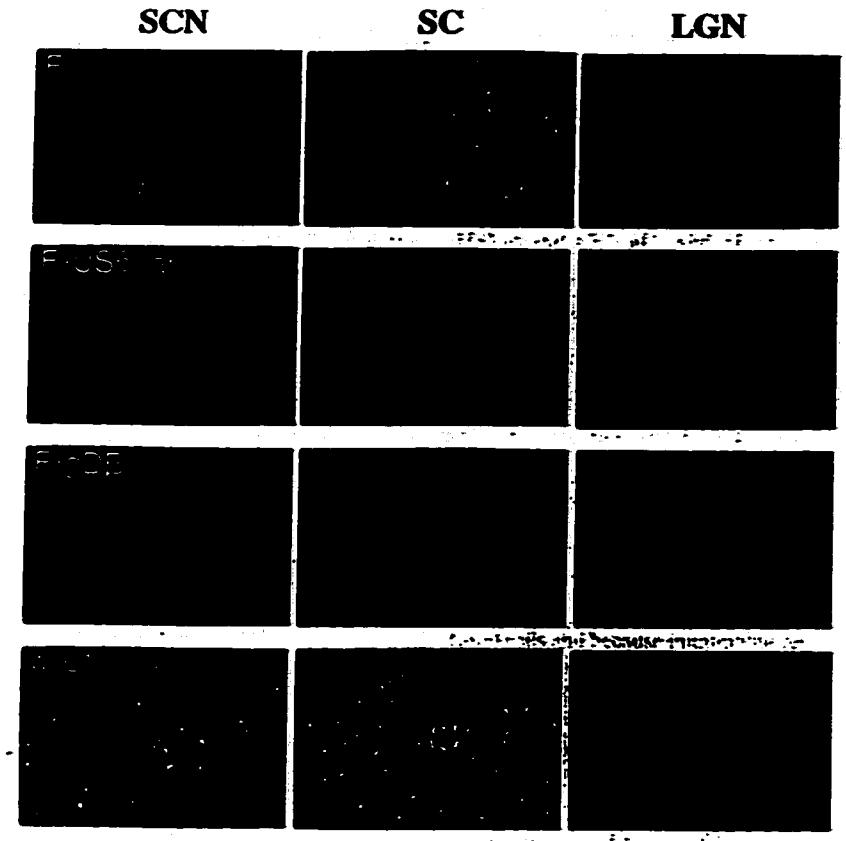


FIG. 7. Infection of retinorecipient regions in the brain by HSV-1 gD⁻ mutants. Rats were infected in one eye with wild-type HSV-1 (F), the gD⁻ mutant (F-US6kan), the gD⁻ gI⁻ mutant (F-gDβ), or wt61. Five days later, the animals were anesthetized and perfused with 4% paraformaldehyde. The brains were removed, postfixed, sectioned, and stained as described in the legend to Fig. 6.

These data are consistent with previous observations involving HSV and PrV (10, 41). The ensuing lateral spread of HSV through the retina, observed after 5 days, apparently involved infection of horizontal and amacrine cells. These cells form synaptic connections with neurons of the central pathway and also form gap junctions with other amacrine or horizontal cells (for a review, see reference 12), facilitating rapid lateral cell-to-cell spread of HSV. Müller fibers, a type of glial cell which forms tight connections with retinal neurons, may also be involved in lateral spread of HSV in the retina (28).

HSV-1 mutants unable to express gE or gI initiated infection of neurons in the rat retina, in a manner comparable to that observed with wild-type HSV-1; equal numbers of foci of infection were observed in the retina after 2 days. Therefore, in agreement with our previous observations involving cultured fibroblasts (13), it appears that the gE-gI heterooligomer is not required for entry of extracellular virus into neurons, in this case primarily neurons of the inner layers of the retina including ganglion cells. However, by 5 days after infection, it was clear that gE⁻ and gI⁻ mutants were defective in virus spread through the retinal microcircuitry; this was especially true of the lateral spread of virus. Consistent with the immunocytochemical analysis of the retina, we found that 10-fold-fewer infectious HSV-1 gE⁻ or gI⁻ mutant virus was produced after 5 days. Therefore, the gE-gI heterooligomer is required for efficient cell-to-cell spread in the retina. Mutants lacking gD also established primary infections in the retina, although the virus particles in this case were derived from complementing cells, so that gD was present. However, the gD⁻ viruses did not spread significantly, and by 5 days there was little expression of viral antigens.

We also investigated the roles of glycoproteins D, E, I, and J in transneuronal spread of HSV-1 from retinal ganglion cells to retinorecipient areas of the brain. The SC, or optic tectum, located in the midbrain, was infected equally well by gE⁻ and gI⁻ mutants and wild-type HSV-1. By contrast, the gE⁻ and gI⁻ mutants infected the LGN and SCN poorly, so that many fewer cells were infected by the mutants than by the wild-type parent strain F.

There are at least two possible explanations for the defects in the spread of the gE⁻ and gI⁻ mutants to the SCN and LGN. First, since we observed reduced spread of the mutant viruses within the retina, fewer retinal ganglion cells were infected. We expect that this more restricted pattern of HSV infection in the retina might cause fewer retinorecipient neurons in the CNS to be infected. In the rat, more than 90% of all retinal ganglion cells project to the contralateral SC (34), whereas a much smaller fraction of the ganglion cell population project to the SCN and LGN. Therefore, we suspect that the more restricted spread of gE⁻ and gI⁻ mutants in the

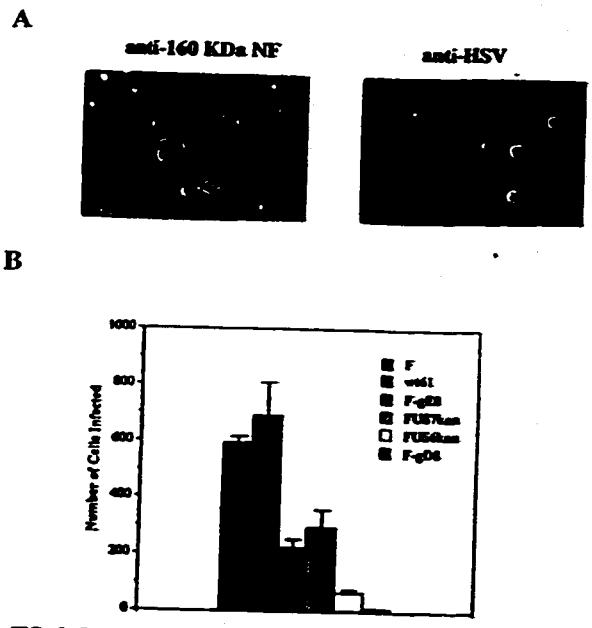


FIG. 8. Spread of HSV-1 gE⁻ gI⁻, and gD⁻ mutant viruses in cultures of dissociated rat sensory neurons. Approximately 2,000 neurons derived from dissociated rat trigeminal ganglia were plated on coverslips coated with laminin and poly-D-lysine. Cultures were treated with a mitotic inhibitor (cytosine arabinoside) which killed fibroblasts and glial cells so that cultures were produced which were greater than 95% neurons. Neuronal cultures were infected with wild-type HSV-1 (F), the gE⁻ mutant (F-gEβ), the gI⁻ mutant (F-US7kan), the gD⁻ mutant (F-US6kan), the gD⁻ gI⁻ mutant (F-gDβ), or wt61 at a low multiplicity of infection so that approximately 1 in 10 to 1 in 20 of the neurons were infected. After 2 h, the cultures were washed with PBS and overlaid with MEMN–10% FBS supplemented with 0.1% human gamma globulin (a source of anti-HSV neutralizing antibodies). After 48 h, cells were fixed briefly with 4% paraformaldehyde, incubated with both rabbit polyclonal anti-HSV-1 antibodies and a mouse monoclonal antibody directed against the 160-kDa neurofilament protein and then with FITC-conjugated anti-rabbit antibodies and rhodamine-conjugated anti-mouse antibodies. (A) Representative micrographs of neuronal cultures infected with wild-type HSV-1. (Left) Cells were stained with anti-160-kDa neurofilament (NF) antibodies. (Right) The same cells were stained simultaneously with anti-HSV antibodies. The arrows indicate cells which were stained with both anti-HSV-1 antibodies and anti-160-kDa neurofilament. (B) Cells stained with both antineurofilament and anti-HSV antibodies were counted after 48 h. The resulting values are the means of 10 separate cultures that were independently infected, stained, and counted. Error bars represent standard deviations.

retina, coupled with fewer projections to the SCN and LGN, combined to produce less infection in these regions of the brain. A second explanation, which is not mutually exclusive of the first, is that gE⁻ and gI⁻ mutants infect the SCN and LGN less efficiently because these viruses spread poorly by anterograde axonal transport to the brain or infect secondary neurons in the brain less efficiently. There is no evidence that gE⁻ or gI⁻ mutants possess defects in anterograde transport, and since there is evidence that the viral nucleocapsids and glycoproteins move independently in axons (46), it seems unlikely that the gE-gI heterooligomer would affect axonal transport (discussed further below). Reduced expression of HSV antigens in the SCN and LGN could be attributed to fewer infectious virus particles reaching these regions from retinal ganglion cells, as well as less efficient spread of virus within the SCN and LGN. It was previously reported that an HSV gE⁻ mutant spread poorly after inoculation into the brain (40). However, we favor the notion that fewer gE⁻ and gI⁻ viruses reach the SCN and LGN because we could show that there was less virus present in the retina to initiate such infections and also because the differences were observed relatively early after infection in the brain. We can draw similar conclusions about the work of Balan et al. (4), who studied HSV infection of the peripheral nervous system. HSV gE⁻ and gI⁻ mutants produced a more limited infection in the mouse ear than did wild-type HSV-1, and there was a dramatic reduction in virus spread to sensory ganglia, apparently, at least in part, because less virus was produced in the skin.

To further address the issue of whether the gE-gI hetero-

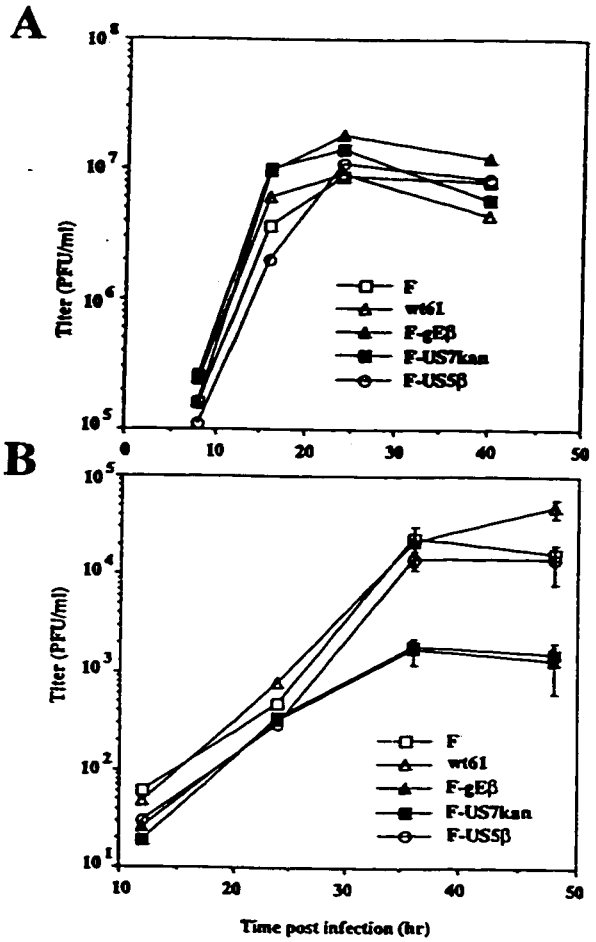


FIG. 9. Production of infectious viruses by HSV-1 gE⁻, gI⁻, and gJ⁻ mutants after infection of rat sensory neurons in culture. Neuronal cultures were established and then the cells were infected with wild-type HSV-1 (F), the gE⁻ mutant (F-gEβ), the gI⁻ mutant (F-US7kan), the gJ⁻ mutant (F-US5β), or wt61, using 10 PFU/cell in panel A or a low multiplicity of infection in panel B, so that only 1 in 10 or 1 in 20 neurons were infected initially. In panel A, 24-well dishes, containing approximately 4×10^4 neurons per well were used; in panel B, 24-well dishes, containing 2×10^3 neurons per well were used. After 2 h, the monolayers were washed with PBS and incubated in MEMN–10% FBS (A) or MEMN–10% FBS supplemented with 0.1% human gamma globulin (B) for the indicated times. At the indicated times, medium and cells (A) or cells alone (B) were collected and frozen at −70°C. In panel B, cells were washed twice with medium to remove neutralizing antibodies prior to being collected; note that only a fraction of the cells were infected after 48 h. Samples were thawed, sonicated, and titered on Vero cells.

oligomer is necessary for neuron-to-neuron spread of HSV, it was advantageous to examine the infection of neurons cultured in vitro, conditions that made it possible to control virus multiplicities and study neurons in isolation from accessory cells. Cultures of rat trigeminal ganglion neurons were established, and the cultures were treated with drugs to kill nonneuronal cells. The neurons formed an extensive network of neurites, and once infected with HSV-1, virus could spread from cell to cell without exposure to neutralizing antibodies present in the medium. Thus, we could study virus spread through a two-dimensional neuronal network of cell-cell, cell-neurite, and neurite-neurite contacts. Mutants unable to express gE or gI infected approximately 50% fewer neurons than did wild-type HSV-1 in a 48-h period, under conditions in which only a small fraction of the neurons were initially infected and viruses spread from neuron to neuron. After immunocytochemical staining, foci of virus infection could be observed in the neuron cultures, and after 24 h, the gE$^-$ and gI$^-$ viruses produced the same number of foci of infection as that produced by wild-type HSV-1. Moreover, the production of infectious viruses did not differ significantly when neuronal cultures were infected at a higher multiplicity of infection, so that every cell was infected. In contrast, when neurons were infected at a lower multiplicity of infection and spreading of virus through the medium was prevented with neutralizing antibodies, the gE$^-$ and gI$^-$ viruses produced 10-fold-less virus than wild-type HSV-1 did. These data strongly support the hypothesis that the gE-gI heterooligomer is required for efficient neuron-to-neuron spread of HSV and yet is not necessary for entry of extracellular virus particles or production of infectious virus. Note that many of the junctions formed between the cultured neurons were not synaptic junctions; however, such junctions are likely to play important roles in virus spread within the retina and brain. Nevertheless, these results, coupled with the in vivo results, are consistent with the hypothesis that gE-gI also facilitates transsynaptic spread of HSV.

The gD$^-$ mutants (F-US6kan, which lacks gD alone, and F-gDβ, which lacks gD and gI) were severely compromised in their ability to spread from neuron to neuron. Although a few neurons were infected by F-US6kan, F-gDβ did not spread beyond a single infected neuron. Therefore, we can conclude that gD is required for neuron-to-neuron spread, although there is some limited spread in its absence, as was observed with cultured human fibroblasts (6).

Our results must also be considered in light of previous detailed observations on the spread of PrV infection from the retina to the brain. Card et al. (9) observed that a gE$^-$ PrV spread poorly from the retina to certain retinorecipient regions of the rat brain, the SC, also denoted the optic tectum, and LGN. The SCN, in the hypothalamus, was infected equally well by wild-type and gE$^-$ viruses. A subsequent study indicated that a gI$^-$ mutant had a similar phenotype (57). It appeared that the gE$^-$ PrV infected fewer neurons in the retina (9), as we found here with HSV-1 gE$^-$ and gI$^-$ mutants. Both wild-type PrV, as well as gE$^-$ and gI$^-$ mutants, infected only a fraction of the retinal ganglion cells and the researchers concluded that reduced spread of PrV to the SC and LGN was related to the inability of gE$^-$ or gI$^-$ viruses to infect a subset of the ganglion cells that project to the SC and LGN. However, it was not clear from these studies whether PrV gE$^-$ and gI$^-$ mutants produced less-infectious progeny in the retina than wild-type PrV did. Even if this were the case, these observations cannot be explained by relating the results to the observation that approximately 90% of retinal ganglion cells project to the contralateral SC, because the SC was inefficiently infected by the gE$^-$ and gI$^-$ PrV. Enquist et al. (18) concluded that PrV gE and gI are not necessary for the primary infection in the retina, as we concluded for HSV gE and gI. Our studies involving cultured trigeminal ganglion neurons suggested that HSV gE$^-$ or gI$^-$ mutants could infect all of the neurons in culture when a high multiplicity of infection was used or when the virus was given sufficient time to spread in the culture. Thus, we found no evidence for selective infection of a subset of trigeminal ganglion neurons, although it is possible that there was selective growth of certain neurons in these cultures. Furthermore, we found no evidence that in the retina gE$^-$ and gI$^-$ viruses lack the ability to infect specific neurons, rather our data supports the notion that HSV gE$^-$ and gI$^-$ mutants are defective in the efficiency in which they are transmitted from any infected neuron to another neuron. However, PrV appears to differ from HSV in that the spread of virus into selected neurons in the retina or regions of the brain apparently requires gE-gI. It should also be noted that PrV gE$^-$ mutants also have defects in the spread to the CNS following intranasal infection of pigs (25, 29, 32a).

There are several important differences between HSV and PrV that must also be considered. HSV infects the rat nervous system less efficiently than does PrV, so that our studies necessitated 5- to 50-fold-more virus than was previously used in the PrV studies. Moreover, unlike PrV, which produces significant pathogenesis frequently leading to death, in our experiments, no animals infected with wild-type HSV-1 showed any overt symptoms of infection and the animals survived with apparently normal functions. There are also notable differences in the mechanisms by which PrV and HSV spread from cell to cell. Our laboratory has previously demonstrated that gD is essential for cell-to-cell spread in certain cultured cells (33), corneal epithelium (14), and the retina (this study). However, we note that there is a small amount of cell-to-cell spread in the absence of gD (6) (Fig. 8). By contrast, the PrV gD$^-$ mutants display little or no defects in virus spread in cultured cells and in the nervous systems of mice following either injection into a nerve or subcutaneous or intraperitoneal injection (1a, 44, 45).

It is by no means clear how the gE-gI heterooligomer functions to increase the efficiency of HSV cell-to-cell transmission between fibroblasts, epithelial cells, or neurons. The existing data suggest at least two mechanisms by which the gE-gI heterooligomer could affect the spread of HSV between neurons or other cell types. One possibility is that gE-gI may bind cellular proteins which act as viral receptors and which are concentrated at cell junctions or, in this case, at neuronal synapses. Thus, by binding to such proteins, gE-gI might promote cell-to-cell transmission of HSV but would be unnecessary for entry of extracellular virus particles into cells. There is evidence for transfer of HSV and other alphaherpesviruses across synaptic junctions. Electron micrographs of neurons infected in vitro or in vivo show alphaherpesviruses at synaptic junctions (8, 46), and although the viruses are not always released there, it appears that a reasonably large fraction of virus particles are released into the synaptic cleft.

There is good evidence for anterograde transport of nucleocapsids combined with independent transport of viral glycoproteins and envelopment of capsids at axon terminals (46). Preferential sorting of viral glycoproteins to the axon terminal (15) could promote envelopment and exocytosis at these sites. Penfold et al. (46) observed nodules along HSV-infected axons corresponding to patches of viral glycoproteins, suggesting exocytosis of viruses at or near axon terminals. There is also evidence that HSV can attach preferentially to synaptosomes derived from rodent brain and to glial cells but less efficiently to neuronal perikarya (55). The velocity of HSV spread

through secondary and tertiary neuronal networks in vivo (50 to 100 mm/day) strongly supports virus transmission across synapses (39, 41) but does not exclude other mechanisms of transmission. Therefore, gE-gI may promote virus entry into afferent neurons by promoting interactions between virus particles and cellular receptors concentrated at synapses. McGeoch (38) noted that gI appears to belong to a family of U_S-encoded proteins, including gD and gG, and possibly gE, with similar positioning of cysteine residues and suggested that these glycoproteins might be derived from a common ancestor. Therefore, since there is good evidence that gD facilitates virus entry into cells by interacting with a limited set of cellular receptors (for a recent review, see reference 6), gE-gI may similarly facilitate cell-to-cell spread by promoting interactions with cellular receptors concentrated at cell junctions or synapses. However, it is also important to note that in our neuronal cultures many of the cell contacts were nonsynaptic cell junctions. In the retina, spread across nonsynaptic junctions would be less likely.

The second mechanism by which the gE-gI heterooligomer could affect cell-to-cell spread of HSV involves sorting or targeting of HSV particles to cell junctions or, in the case of neurons, to axon terminals or synapses. Although it is not known whether gE and gI are sorted to specific regions of polarized cells or neurons, these glycoproteins could promote the appearance of enveloped virus particles near cell or synaptic junctions. The recent results of Penfold et al. (46) support the view that HSV nucleocapsids and glycoproteins are independently transported to axon terminals. Therefore, it is unlikely that gE-gI specifically target capsids to axon terminals. However, these glycoproteins may promote assembly of enveloped particles or sorting of the particles to synaptic junctions by unknown processes. We are currently investigating these two potential mechanisms by which gE-gI could promote cell-to-cell spread of HSV.

ACKNOWLEDGMENTS

We thank Sandy Ball and Cory Sakai for valuable help with the retinal studies and the entire Doering laboratory for providing keys to their in the laboratory. We are also most grateful to Colin Nurse for advice in the early stages of this work and Ian York for providing F-USSβ.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (N.C.I.C.). D.C.J. was a senior research scholar of the N.C.I.C. while this study was conducted.

ADDENDUM IN PROOF

We have recently restored gE expression in F-gEβ by recombination of a wild-type gE gene. This virus, F-gEβR, produced large plaques on human fibroblasts and behaved similarly to wild-type virus in the rat visual system.

REFERENCES

1. Adler, R., J. C. Glorioso, J. Cossman, and M. Levine. 1978. Possible role of Fc receptors on cells infected and transformed by herpes virus: escape from immune cytolysis. Infect. Immun. 21:442–447.
1a. Babic, N., T. C. Mettenleiter, A. Flamand, and G. Ugolini. 1993. Role of essential glycoproteins gII and gp50 in transneuronal transfer of pseudorabies virus from the hypoglossal nerves of mice. J. Virol. 67:4421–4426.
2. Baines, J. D., and B. Roizman. 1991. The open reading frames U_L3, U_L4, U_L10, and U_L16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. 65:938–944.
3. 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.
4. 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.
5. Bell, S., M. Cranage, L. Borysiewicz, and T. Minson. 1990. Induction of immunoglobulin G by Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J. Virol. 64:2181–2186.
6. 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.
7. Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62:2596–2604.
8. Card, J. P., L. Rinaman, R. B. Lynn, B.-H. Lee, R. P. Meade, R. R. Miselis, and L. W. Enquist. 1993. Pseudorabies virus infection of the rat central nervous system: ultrastructural characterization of viral replication, transport, and pathogenesis. J. Neurosci. 13:2515–2539.
9. 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.
10. Card, J. P., M. E. Whealy, A. K. Robbins, R. Y. Moore, and L. W. Enquist. 1991. Two α-herpesvirus strains are transported differentially in the rodent visual system. Neuron 6:957–969.
11. Cook, M. L., and J. G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect. Immun. 7:272–288.
12. DeVries, S. H., and D. A. Baylor. 1993. Synaptic circuitry of the retina and olfactory bulb. Cell, 72/Neuron, 10(Suppl.):139–149.
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. Dingwell, K. S., and D. C. Johnson. Unpublished data.
15. Dotti, C. G., and K. Simons. 1990. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. Cell 62:63–72.
16. Dowter, K. W., and R. W. Veltri. 1984. In vitro neutralization of HSV-2: inhibition by binding of normal IgG and purified Fc to virion Fc receptor (FcR). J. Med. Virol. 13:251–259.
17. Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effect on social behaviour of infected cells. J. Gen. Virol. 2:357–364.
18. Enquist, L. W., J. Dubin, M. E. Whealy, and J. P. Card. 1994. Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion cell neurotropism. J. Virol. 68:5275–5279.
19. Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. 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.
20. Frank, I., and H. M. Friedman. 1989. A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J. Virol. 63:4479–4488.
21. Hanke, T., F. L. Graham, V. Lulitanond, and D. C. Johnson. 1990. Herpes simplex virus IgG receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology 177:437–444.
22. Higgins, D., P. J. Lein, D. J. Osterhout, and M. L. Johnson. 1991. Tissue culture of mammalian autonomic neurons, p. 177–205. *In* G. Banker and K. Goslin (ed.), Culturing nerve cells. MIT Press, Cambridge, Mass.
23. Hill, T. J., H. J. Field, and A. P. C. Roome. 1972. Intra-axonal location of herpes simplex virus particles. J. Gen. Virol. 15:253–255.
24. Hutchinson, L., and D. C. Johnson. 1995. Herpes simplex virus glycoprotein K promotes egress of virus particles. J. Virol. 69:5401–5413.
25. Jacobs, L., W. A. M. Mulder, J. T. Van Oirschot, A. L. J. Gielkens, and T. G. Kimman. 1993. Deleting two amino acids in glycoprotein gI of pseudorabies virus decreases virulence and neurotropism for pigs, but does not affect immunogenicity. J. Gen. Virol. 74:2201–2206.
26. Johnson, D. C., M. C. Frame, M. V. 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.
27. 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. 62:4605–4612.
28. Kaneko, A. 1979. Physiology of the retina. Annu. Rev. Neurosci. 2:169–191.
29. Kimman, T. G., N. de Wind, N. Oei-Lie, J. M. A. Pol, A. J. M. Berns, and A. L. J. Gielkens. 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen. Virol. 73:243–251.
30. 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-hydroxynonyl)adenine]. J. Gen. Virol. 67:2023–2028.
31. Kristensson, K., E. Lycke, and J. Sjostrand. 1971. Spread of herpes simplex virus in peripheral nerves. Acta Neuropathol. 17:44–53.
32. Kristensson, K., I. Nennesmo, L. Persson, and E. Lycke. 1982. Neuron to

neuron transmission of herpes simplex virus. J. Neurol. Sci. 54:149–156.
32a. Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter. 1994. Role of envelope glycoproteins gI, gp63, and gIII in the invasion and spread of Aujeszky's disease virus in the olfactory nervous pathway of the pig. J. Gen. Virol. 75:2319–2327.
33. Liges, 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.
34. Linden, R., and V. H. Perry. 1983. Massive retinotectal projection in rats. Brain Res. 272:145–149.
35. 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.
36. 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 α47 gene. J. Virol. 58:583–591.
37. Longnecker, R., and B. Roizman. 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science 236: 573–576.
38. McGeoch, D. J. 1990. Evolutionary relationships of virion glycoprotein genes in the S regions of alphaherpesvirus genomes. J. Gen. Virol. 71:2361–2367.
39. McLean, J. H., M. T. Shipley, and D. L. Bernstein. 1989. Golgi-like transneuronal retrograde labelling with CNS injections of herpes simplex virus type 1. Brain. Res. Bull. 22:867–881.
40. Neidhardt, H., C. H. Schroder, and H. C. Kaerner. 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infection. J. Virol. 61:600–603.
41. Norgren, R. B., and M. N. Lehman. 1989. Retrograde transneuronal transport of herpes simplex virus in the retina after injection in the superior colliculus, hypothalamus and optic chiasm. Brain Res. 479:374–378.
42. Norgren, R. B., J. H. McLean, H. C. Babel, A. Wander, D. L. Bernstein, and M. N. Lehman. 1992. Anterograde transport of HSV-1 and HSV-2 in the visual system. Brain. Res. Bull. 28:393–399.
43. Parnavelas, J. G., A. Dinopoulos, and S. W. Davies. 1989. The central visual pathways, p. 1–164. *In* A. Bjorklund, T. Hokfelt, and L. W. Swanson (ed.), Handbook of chemical neuroanatomy, vol. 7. Integrated systems of the CNS, part III. Elsevier Science Publishers, New York.
44. Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann. 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. 66:894–905.
45. 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.
46. Penfold, M. E. E., 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. USA 91:6529–6533.
47. 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. Acta Virol. 34:305–320.
48. Rodieck, R. W. 1979. Visual pathways. Annu. Rev. Neurosci. 2:193–225.
49. 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.
50. Ruyechan, 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. 29:677–697.
51. Simmons, A., D. Tscharke, and P. Speck. 1992. The role of immune mechanisms in control of herpes simplex virus infection of the peripheral nervous system. Curr. Top. Microbiol. Immunol. 179:31–56.
52. Smiley, J. R., D. C. Johnson, L. I. Pizer, and R. D. Everett. 1992. The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein D (gD) promoter are not essential for efficient gD transcription during virus infection. J. Virol. 66:623–631.
53. Ugolini, G., H. G. J. M. Kuypers, and A. Simmons. 1987. Retrograde transneuronal transfer of herpes simplex virus type 1 (HSV 1) from motoneurones. Brain Res. 422:242–256.
54. Ugolini, G., H. G. J. M. Kuypers, and P. L. Strick. 1989. Transneuronal transfer of herpes virus from peripheral nerves to cortex and brainstem. Science 243:89–91.
55. Vahlne, A., B. Nystrom, M. Sandberg, A. Hamberger, and E. Lycke. 1987. Attachment of herpes simplex virus to neurons and glial cells. J. Gen. Virol. 48:359–371.
56. 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.
57. Wheeby, M. E., J. P. Card, A. K. Robbins, J. R. Dubin, H.-J. Raiba, and L. W. Enquist. 1993. Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. 67:3786–3797.
58. York, I. A., and D. C. Johnson. 1995. Inhibition of humoral and cellular immune recognition by herpes simplex viruses, p. 89–110. *In* G. McFadden (ed.), Viroceptors, virokines and related immune modulators encoded by DNA viruses. R. G. Landes Company, Austin, Tex.
59. York, I. A., 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.
60. 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.

Chapter 4 Herpes simplex virus glycoproteins gE and gI accumulate at junctions between cells

4.0 Preface

In the following chapter, I carried out all the experiments described.

Chapter 4

Herpes simplex virus glycoproteins gE and gI accumulate at junctions between cells

Kevin S. Dingwell[1] and David C. Johnson[2*]

[1] Department of Biology, McMaster University, Hamilton, ON, Canada L8N 3Z5; [2]Department of Molecular Immunology and Microbiology, Oregon Health Sciences University, Portland, Oregon, USA.

Running Title: HSV gE/gI at cell junctions

Corresponding author: Room 6365
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd., L220
Portland, OR 97201
USA
Tel: (503) 494-0834
FAX: (503) 494-6862

ABSTRACT

Previous studies have demonstrated that a complex of herpes simplex virus (HSV) glycoproteins gE and gI mediate cell-to-cell spread in certain cell types (epithelial cells, fibroblasts and neurons), and yet gE/gI is not important for entry of extracellular virus. It appears that gE/gI facilitates movement of HSV from an infected cell to an uninfected cell, probably across junctional complexes formed between cells. In order to examine how gE/gI mediates cell-to-cell spread, we expressed the glycoproteins without other HSV polypeptides using recombinant adenovirus (Ad), vectors. When human HEC-1A epithelial cells were infected with these Ad vectors, gE accumulated at lateral surfaces of cells, colocalizing with the adherens junction protein β-catenin, but was not found on either the apical or basal plasma membranes. Additionally, gE/gI was excluded from tight junctions. In subconfluent monolayers, gE/gI was found at cell junctions but was absent from those lateral surfaces not in contact with another cell, a pattern identical to that of β-catenin. Similar results were obtained with HSV-infected HEC-1A cells. By contrast, HSV glycoprotein gD was found only along the apical plasma membrane domain. Expression of gE/gI did not cause any visible cytopathic effects, nor was there any redistribution of either ZO-1, or β-catenin. We observed no effect of gE/gI expression on transepithelial resistance, and there was no increase in paracellular transport of ^{14}C-inulin. Accumulation of gE at

lateral borders was not related to extensive interactions with cytoskeletal elements because gE remained largely in a Triton X-100 soluble fraction. Together these results support a model in which the gE/gI complex accumulates at cell junctions, perhaps by interacting with junctional components and, in so doing, mediates transfer of HSV across the junctions.

INTRODUCTION

Herpes simplex virus (HSV), replicates in tissues of epithelial origin such as the oral mucosa and corneal epithelium, and must spread efficiently through these tissues in order to gain access to the nervous system by way of innervating sensory axons. In epithelial tissues, cells are joined together by extensive cell-to-cell contacts or cell junctions. There are at least two routes by which HSV spreads from infected cells to neighbouring uninfected cells. First, newly replicated virions may be released from an infected cell (e.g., at the apical surface),and the virions can then adsorb to and enter nearby cells. This mode of spread would likely cause the virus to be susceptible to anti-HSV neutralizing antibodies. However, it is well known that HSV can cause secondary lesions in the mucosa of individuals producing large quantities of neutralizing antibodies, and so HSV must be able to spread by mechanisms that allow the virus to escape these antibodies. HSV virions can apparently spread directly from one cell to another across cellular junctions, which are inaccessible to anti- HSV antibodies. We refer to this mode as direct cell-to-cell spread, and it is likely that this mode of spread is an important parameter of HSV pathogenesis. Both routes of HSV

spread involve the viral membrane glycoproteins gB, gD, and gH/gL. Deletion mutants lacking either gB, gD, or gH/gL can enter cells (if the viruses have been propagated in a cell line that provides the missing glycoprotein in *trans*), but the mutants are unable to subsequently spread from the initially infected cell. Therefore cell-to-cell spread and entry of extracellular virus share many important properties. However, cell-to-cell spread also differs from entry of extracellular virus in that the former process involves HSV glycoproteins gE and gI.

We and others have shown that a complex of HSV glycoproteins, gE/gI, and its homologues in other α-herpesviruses, facilitates the spread of virions via the direct cell-to-cell route (Balan et al., 1994; Dingwell et al., 1994; Zsak et al., 1992). However, gE/gI is not required for entry of exogenous virus, and mutants exhibit growth kinetics similar to their wild type parent (Balan et al., 1994; Dingwell et al., 1994). However, if the gE^- and gI^- mutants are forced to spread directly from cell-to-cell (e.g., infection at low multiplicities of infection and in the presence of neutralizing antibodies), the plaque size of the mutants is significantly reduced compared with wild type HSV-1, and there is a concomitant decrease in virus titre (Balan et al., 1994; Dingwell et al., 1994). The gE/gI mutants are also severely attenuated *in vivo*. Both gE^- and gI^- mutants grow poorly in the cornea and ear pinna (Balan et al., 1994; Dingwell et al., 1994) and fail to spread efficiently within the nervous system and cause neurological disease (Balan et al., 1994; Dingwell et al., 1994; Dingwell et al., 1995; Kudelova et al., 1991; Neidhardt et al., 1987; Rajcani et al., 1990; Rajcani et al., 1990). Therefore, we have

concluded that the gE/gI complex plays an important or essential role in cell-to-cell spread especially *in vivo*. Since gE/gI participates exclusively in this process, and not in entry of extracellular virus, these glycoproteins provide an important molecular tool to study HSV cell-to-cell spread.

Currently, little is known about the molecular mechanisms by which gE/gI mediates cell-to-cell spread. It is important to study gE/gI function in cell types where cell-to-cell spread is efficient and that are also relevant to *in vivo* disease. Epithelial cells are one such cell type; the cells form extensive cell-to-cell contacts including tight junctions, adherens junctions, gap junctions, and desmosomes. HSV gE^- and gI^- viruses produce small plaques composed of as few as 3-10 infected cells on monolayers of cultured human epithelial cells, whereas wild type HSV can spread to 150-300 cells in the same time (Balan et al., 1994; Dingwell, unpublished). Epithelial cells are distinguished by their polarized nature: the apical and basolateral plasma membranes are physically separated by tight junctions (reviewed in Balda and Matter, 1998). There is asymmetric sorting of membrane proteins between these two plasma membrane compartments. Therefore, it would appear likely that there are distinct interactions between HSV and components of either the apical or basolateral domains. Since gE/gI is essential for efficient cell-to-cell spread in epithelial cells *in vitro*, and *in vivo*, we attempted to ascertain how gE/gI interacts with these distinct plasma membrane domains of epithelial cells.

Replication defective adenovirus (Ad), vectors carrying either the gE or gI

genes, were constructed in order to express gE/gI in the absence of other HSV polypeptides. When gE was expressed without gI in human epithelial cells, gE accumulated intracellularly, so that gI expression was required for gE to reach the cell surface. When both gE and gI were coexpressed, the gE/gI complex trafficked to and accumulated at the lateral surfaces of epithelial cells at cell junctions. However, we did not detect gE/gI accumulation at those lateral surfaces that did not form cell junctions. We propose that gE/gI plays an important role in cell-to-cell spread by binding specifically to cell junction components of epithelial cells.

Materials and Methods

Cells and viruses. HEC-1A cells (kind gift of Jay A. Nelson, Oregon Health Sciences University) were grown in RPMI medium (Biowhittaker Inc., Walkersville, MD) supplemented with 10 % (vol/vol) heat-inactivated foetal bovine serum (FBS; Biowhittaker). 293 cells (Graham et al., 1977), and Vero cells were grown in Dulbecco's modified minimal essential medium (D-MEM, Biowhittaker) supplemented with 10 % and 5% FBS respectively. HSV-1 strain F (obtained from P.G. Spear, Northwestern University Medical School, Chicago), F-US7kan (Johnson et al., 1988), F-gEß (Dingwell et al., 1994), were propagated and titred on Vero cells. AdgE, AdgI (Ad $E1^{+}/E3^{-}$ vectors, (Hanke et al., 1990), Ad($E1^{-}$)gE, Ad($E1^{-}$)g I (Ad $E1^{-}/E3^{+}$ vectors), and AdgD1($E1^{-}$) (Brunetti et al., 1998) were propagated and titred on 293 cells.

Antibodies. Monoclonal antibody (MAb) 3104, specific for gI and 3114 specific

for gE (Johnson et al., 1988), were gifts of Anne Cross and Nigel Stow (Institute of Virology, Glasgow, UK). MAb II-481 was obtained from P. G. Spear (Northwestern University Medical School, Chicago). MAb DL-6 specific for gD (Isola et al., 1989), was a gift of G.H. Cohen and R. J. Eisenberg (University of Pennsylvania, Philadelphia, PA). Purified MAb directed to ß-catenin was obtained from Transduction Laboratories (Lexington, KY), and rabbit polyclonal antibodies directed to ZO-1 were obtained from Zymed Laboratories (South San Francisco, CA). Texas Red coupled goat anti-rabbit IgG and FITC-coupled and Cy3-coupled goat anti-mouse IgG were obtained from Jackson ImmunoResearch Labs Inc. (West Grove, PA). BODIPY-coupled goat anti-FITC IgG was obtained from Molecular Probes (Eugene, OR). The FITC analogue Oregon Green was coupled to MAb 3114 using a FluoReporter® Oregon Green™ 488 protein labelling kit (Molecular Probes) kit as described by the manufacturer. Goat anti-mouse HRP was obtained from Amersham Life Sciences, Inc. (Arlington Heights, IL).

Construction of replication defective recombinant adenovirus vectors expressing either gE or gI. All restriction and DNA modification enzymes were obtained from New England Biolabs, (Beverly, MA). Plasmid DNA was prepared by using Qaigen 500 columns (Qaigen Inc., Chatsworth, Ca). The full length gE and gI genes were excised from plasmids pSV2X3gE and pSV2X3gI (Hanke et al., 1990), respectively and subcloned into plasmid pCA3 (Hitt et al., 1995), using the restriction enzymes EcoR1 and XbaI. The resulting plasmids, pCA3gE and

pCA3gI contained either the gE or gI genes under the human cytomegalovirus (HCMV) immediate early promoter followed by a SV40 poly A sequence. Recombinant adenoviruses expressing either gE [Ad(E1$^-$)gE] or gI [Ad(E1$^-$)gI], were obtained following co-transfection of either pCA3gE or pCA3gI with pBHG10 (Bett et al., 1994), into 293 cells using the calcium phosphate technique as previously described (Hitt et al., 1995). Recombinant viruses were plaque purified three times.

Labelling of cells with [^{35}S] methionine and cysteine, immunoprecipitation of proteins, and gel electrophoresis. Monolayers of HEC-1A cells grown on 35-mm dishes were infected with either HSV-1 (F) at an MOI (multiplicity of infection) of 20, or using an MOI of 400 with either Ad(E1$^-$)gE, or using 400 PFU/cell of both Ad(E1$^-$)gE and Ad(E1$^-$)gI, or were left uninfected. At 6 hr p.i., HSV infected monolayers were washed twice with DMEM lacking methionine and cysteine (DMEM$^=$)and then metabolically labelled with [^{35}S]methionine and cysteine (150 µCi/ml; NEN) in DMEM$^=$ for an additional 3 hrs. Cells infected with Ad vectors were metabolically labelled with [^{35}S]methionine and cysteine (150 µCi/ml) in DMEM$^=$ between 48-51 hrs. p.i. Cells were lysed in Nonidet P-40 (NP40)-deoxycholate (DOC) extraction buffer (1% NP40, 0.5% DOC, 50 mM Tris-Cl [pH 7.5], 100 mM NaCl) containing 2 mg/ml bovine serum albumin (BSA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at -70°C. Lysates

were thawed quickly at 37°C, spun down at high speed to pellet any insoluble materials, and gE or gI were immunoprecipitated with MAbs 3114 or 3104 respectively, for 2 hr at 4°C. Protein A-Sepharose (Pharmacia, Dorval QU) was added to the lysates and incubated for an additional 2 hrs, end-over-end at 4°C, then the protein A-Sepharose was washed three times with NP40/DOC extraction buffer, and the immunoprecipitated proteins were then after addition of one volume of loading buffer (100 mM Tris-Cl [pH 6.8], 4% SDS; 4% 2-mercaptoethanol, 20% glycerol, bromophenol blue), followed by boiling for 10 min. Samples were electrophoreced in a 12% polyacrylamide gel. The gels were fixed, enhanced with Enlightning (Dupont/NEN), dried and then exposed to Kodak XAR film.

Immunofluorescence of HSV or Ad infected HEC-1A cells. HEC-1A cells grown in Permanox coated transwell slides (Lab Tek, Naperville, IL) were infected with either HSV-1 (F) at an MOI (multiplicity of infection) of 20, or at a MOI of 400 with either Ad(E1$^-$)gE or Ad(E1$^-$)gI, coinfected with both Ad(E1$^-$)gE and Ad(E1$^-$)gI, or left uninfected. At either 8 or 11 hr p.i., HSV infected cells were fixed in 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature (RT), and then in some cases permeabilized with 0.2% Triton X-100 (TX100) for 5 min at RT. Cells infected with the Ad vectors were fixed and permeabilzed as above at 48 hr p.i. Non specific binding was blocked with blocking buffer (B buffer; PBS with 2% normal goat serum, 2% BSA, 0.02% Tween 20) for 30 min at RT. MAb directed against ß-catenin (Transduction Labs)

was added to the cells for 1 hr at RT, and then washed three times for 5 min with B buffer. Cy3 coupled secondary antibodies were then added for 1 hr at RT, and samples were washed three times for 5 min with BB. Oregon-Green coupled MAb 3114 (specific for gE) was added to cells for 1 hr at RT and then washed three times for 5 min at RT. BODIPY-coupled anti-FITC antibodies were added for 1 hr at RT, and then washed three times for 5 min. Samples were mounted using Vectashield (Vector Laboratories, Burlingame, CA), and then sealed with nail hardener (Sally Hansen), and then viewed with a Leica confocal microscope. Cells were stained with antibodies directed against gE and ß-catenin as described above. Cells were stained for ZO-1, and also for gE using rabbit anti ZO-1 antibodies and anti-gE MAb 3114, cells washed three times for 5 min with BB, followed by the addition of goat anti-mouse FITC coupled- and goat anti-rabbit Texas red-coupled antibodies for 1 hr at RT. Samples were washed three times for 5 min with BB and then mounted with Vectashield and sealed with nail hardener.

Transepithelial resistance and paracellular permeability. In order to establish a epithelial cell barrier, HEC-1A cells were grown on Millicell-CM membrane inserts (12mm diameter, 0.4 μm; Millipore, Bedford, MA), coated with rat tail collagen (Boehringer Mannheim). 5×10^5 cells were added to each insert and maintained with occasional media changes over the course of 5 days. Transepithelial resistance (TER) was measured on each of the following 5 days using a Millipore Voltohmmeter. Cell monolayers with a net resistance of greater than 400 Ωcm^2 (net resistance = total resistance - resistance of membrane alone)

were used in subsequent experiments. Monolayers were infected withAd(E1$^-$)gE and Ad(E1$^-$)gI (400 PFU/cell of each), or using 800 PFU/cell of AddlE1 or were left uninfected. Following 48 hr p.i., TER was measured. Paracellular permeability was measured at 48 hrs p.i., by adding 1μCi/ml of [^{14}C]inulin in 200 μl RPMI to the apical compartment of infected or mock infected cells. At 20 min intervals, the inserts were transferred to new wells over a total period of 2 hrs. The amount of radioactivity accumulated in the basal compartment was counted.

Western blot analysis. Proteins were subjected to electrophoresis on polyacrylamide gels, then transferred to Immobilon membranes by transblotting at 25 Volts overnight in 25 mM Tris-Cl, 190 mM glycine, and 20% methanol. Membranes were dried and incubated with 5% non fat skin milk in wash buffer (W buffer; PBS containing 0.1 % Tween 20) for 2 hr at room temperature. Primary antibodies specific for gE (II-481) were diluted in W buffer and then added to the membranes for 1 hr at room temperature. Membranes were washed once for 15 min, then three times for 5 min prior to the addition of goat anti-mouse horseradish peroxidase labelled IgG in W buffer. Membranes were incubated for 1 hr at RT, then washed as above.. Proteins were detected using an ECL kit (Amersham) using Hyperfilm ECL film (Amersham).

Determination of TX100 insolubility of gE. Monolayers of HEC-1A cells grown in 12 well dishes were co-infected with AdE1gE and AdE1gI using 400 PFU/cell or were left uninfected. At 48 hrs p.i., cells were washed twice with tris-saline (15 mM tris-Cl [pH 7.5], 150 mM NaCl), and then extracted with 300 μl

of ice cold extraction buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM Tris-Cl [pH 7.5] and 150 mM NaCl) containing TX100 concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.5, 0.75, and 1% (w/v) for 10 min. Cells were then scraped into the extraction buffer, transferred to 1.5 ml centrifuge tubes and spun at 20, 000 x g for 30 min at 4°C. Supernatants were transferred to fresh tubes and 100 µl of 4 times concentrated loading buffer (8% SDS, 8% 2-mercaptoethanol, 200 mM Tris-Cl [pH 6.8], 40% glycerol, bromophenol blue) was added. Pellets were resuspended in 400 µl of SDS loading buffer. All samples were boiled for 10 min and then run on a 7.5 % polyacrylamide gel, and western blotted.

RESULTS

Construction of replication defective Ad vectors expressing either gE or gI. In previous studies, we and others have shown an important role for gE/gI in facilitating cell-to-cell spread of HSV between polarized cells and also between non-polarized cells. However, it can be problematic to study the details of this process in cells due to the numerous cytopathic effects generated during HSV replication. HSV infection induces a rapid inhibition of host protein synthesis, alterations in the cytoskeleton leading to cell rounding, and changes in host membranes including disruption of the Golgi apparatus. Therefore, in order to avoid the pleiotropic effects of HSV replication on the host cell, and to be able to study trafficking of gE/gI in epithelial cells without the effects of other HSV polypeptides, we constructed replication defective adenovirus (Ad) vectors that expressed either gE or gI. The complete coding sequences of gE and gI were

excised from plasmids pSVXXXgE and pSVXXXgI respectively, using the restriction enzymes EcoR1 and XbaI and inserted into the shuttle vector pCA3, so that the gE and gI genes were inserted between the human cytomegalovirus (HCMV) immediate early promoter and the SV40 polyadenylation sequence, in a right to left orientation with respect to the flanking Ad E1 sequences (Figure 1). Recombinant Ad vectors Ad(E1$^-$)gE and Ad(E1$^-$)gI were isolated following co-transfection of 293 cells with either pCA3gE or pCA3gI and pBHG10 which supplies the right end of the Ad genome.

Expression of gE and gI from the recombinant viruses were assessed by infecting HEC-1A cells with either Ad(E1$^-$)gE or Ad(E1$^-$)gI using 400 PFU/cell or by coinfecting with both Ad(E1$^-$)gE and Ad(E1$^-$)gI using 800 PFU/cell in total, then labelling the cells with [^{35}S]methionine/cysteine. gE or gI were immunoprecipitated using either gE or gI specific MAbs 3114 and 3104, respectively. Two protein species from Ad(E1$^-$)gE infected cells could be immunoprecipitated using the MAb 3114, and corresponded to the immature and mature forms of gE (Fig 2A). Similarly, bands corresponding to the mature and immature forms of gI could be precipitated using MAb 3104 from Ad(E1$^-$)gI infected cell lysates. A complex of gE and gI could be precipitated from lysates of cells coinfected with both Ad(E1$^-$)gE and Ad(E1$^-$)gI especially using MAb 3104 (Figure 2A). Similar amounts of gE and gI were immunoprecipitated from cells infected with Ad(E1$^-$)gE and Ad(E1$^-$)gI as were observed in extracts from

HSV infected cells labelled with [^{35}S]methionine/cysteine between 6 to 9 hrs. p.i.

For future experiments in which we wanted to evaluate the effects of gE/gI expression on cell junctions, it was important to determine if the steady state levels of gE and gI expressed by the Ad vectors were comparable to that produced during infection with HSV. To assess this, we used Western blot analysis comparing gE expression in HSV-infected cells with Ad(E1$^-$)gE /gI-infected HEC-1A cells. Higher levels of gE accumulated in Ad(E1$^-$)gE/gI infected cells, 48 hr after infection, than were observed in HSV-infected cells, 8 hr after infection (Figure 2B). Together, these data suggested that we could efficiently express gE and gI using the recombinant Ad vectors, to levels similar or higher than that during infection with HSV.

Trafficking of gE to the cell surface requires co-expression of gI.

Nonpolarized cell lines (i.e., Vero cells) have been used extensively as a model system to examine the functions of HSV glycoproteins during viral replication. However, the role for gE/gI in facilitating viral cell-to-cell is less evident in nonpolarized cells (Dingwell et al., 1994), which lack many of the junctional complexes found in epithelial cells. In epithelial cells, the requirement for gE/gI in cell-to-cell spread is much more apparent. gE$^-$ and gI$^-$ HSV mutants spread very poorly in epithelial tissues, and *in vitro* the mutant viruses spread to a limited number of cells (i.e., 10-20 cells) compared to several hundred cells when infected with wild type HSV (Balan et al., 1994; Dingwell, unpublished; Dingwell et al.,

1994). Therefore to further characterize the role of gE/gI in cell-to-cell spread in cells relevant to *in vivo* infections, we examined the subcellular localization of gE and gI in human HEC-1A epithelial cells. HEC-1A cells were derived from the human endometrial mucosa and display all the functional characteristics of polarized epithelial cells (Ball et al., 1995): i) they efficiently establish high transepithelial resistance when grown on filter supports; ii) influenza virus and vesicular stomatitis virus (VSV), asymmetrically bud from the apical and basolateral domains respectively; iii) and there is polarized distribution of endogenous cellular proteins (e.g., polyimmunoglobulin receptor).

When cells infected with Ad(E1$^-$)gE alone and permeabilized following fixation, gE was predominantly found in a perinuclear region and often localized to one side of the nucleus, and did not appear to be present on the plasma membrane (Figure 3A) . Little or no gE could be detected on the cell surface when cells were not detergent permeabilized (Figure 3D), suggesting that gE did not reach the apical cell surface. When cells were co-infected with Ad(E1$^-$)gE and Ad(E1$^-$)gI, the staining pattern of gE changed dramatically; gE staining was primarily found at the periphery, in a ring-like pattern separating adjoining cells and there was little perinuclear staining (Figure 3B). Again, gE could not be detected on the apical membrane of cells expressing gE and gI when the cells were not detergent permeabilized (Figure 3E). Another HSV glycoprotein, gD expressed using a recombinant Ad vector, was found on the apical membrane (Figure 3F). Therefore, gE transport to the cell surface in these polarized

epithelial cells requires co-expression of gI, and together the gE/gI complex does not reach the apical surface but instead traffics to the basolateral membrane.

Ad expressed gE localizes to the lateral plasma membrane. Unlike conventional epifluorescence microscopy, confocal microscopy optically sections a cell from top to bottom, providing a three dimensional picture of a protein's distribution throughout the cell. Provided there are markers for different cellular structures (i.e., cell junctions), confocal imaging is an invaluable tool to study the dynamics of protein trafficking and accumulation to specific areas within the cell. In order to examine the distribution of gE in more detail, we infected polarized epithelial cells with Ad(E1$^-$)gE and Ad(E1$^-$)gI and stained simultaneously for gE and either ZO-1, or ß-catenin. ZO-1 is localized specifically to tight junctions and is required for maintaining the integrity of the tight junctional barrier (reviewed in Balda and Matter, 1998). Adherens junctions are located along the lateral plasma membrane just below the tight junction and are formed following the homophilic binding of cadherins molecules between neighbouring cells (reviewed in Yap et al., 1997). ß-catenin is an integral component of adherens junctions binding to the cytoplasmic tail of the cadherin molecule and with α-catenin linking the cadherin complex to the actin cytoskeleton.

Confocal sections were taken through cells expressing gE and gI (Ad vector infected), beginning at the level of the tight junctions (near the apical border), and extending toward the basal surface of cells where they were attached to the substrate. Extensive colocalization of gE and ß-catenin was

observed along the entire lateral plasma membrane of infected cells starting at a level below the tight junctions (Fig. 4). There was little or no gE in focal planes including Z)-1 staining and thus no colocalization of gE with ZO-1 (Fig 4). There was also little or no gE in the cytoplasm. When confocal sections were taken through the z-axis of gE/gI expressing cells (i.e., a plane perpendicular to the images in Figure 4 such that sections of the cell are taken from side to side rather than top to bottom), we again observed the accumulation of gE along lateral borders between cells, although in this case the images were vertical stripes of gE expressed along the lateral surfaces (Figure 5J-K). There was little or no gE found on the apical and basal plasma membrane surfaces. Moreover, gE accumulation was confined to regions along the lateral plasma membrane that did not include the tight junction. gE/gI accumulation along lateral plasma membrane was also observed using replication-competent Ad vectors (that expressed Ad E1 proteins with gE or gI inserted into the E3 region). At early times after infection (18 hr) there were no cytopathic effects of Ad virus replication on the polarized HEC-1A cells. There was no detectable redistribution of ZO-1 or ß-catenin after expression of gE and gI in cells; even when the levels of gE/I exceeded those in HSV-infected cells. When we expressed another HSV glycoprotein gD in polarized HEC-1A cells, we found that gD accumulated primarily at the apical plasma membrane (Fig. 5L). Thus gE accumulates along the lateral membrane below the level of the tight junction of polarized HEC-1A cells, in a pattern similar to the adherens junction protein ß-catenin, whereas gD is distributed primarily on

the apical plasma membrane.

As with cells infected with Ad(E1$^-$)gE and Ad(E1$^-$)gI, gE was also observed accumulating along the lateral plasma membrane in HSV-1-infected HEC-1A cells. At 8 hr after infection with wild type HSV, gE first appeared at the cell surface in small patches colocalizing with ß-catenin along all lateral junctions between cells (Figure 5D-F). By 11 hr, gE extended throughout the lateral plasma membrane (Figure 5G-I). gE staining was also evident in the cytoplasm but this staining was relatively faint and there was no gE on the apical surface of HSV infected cells. Therefore when expressed by either HSV or Ad vectors, gE is transported to the cell surface and accumulates along the lateral surface of the plasma membrane.

Although the distribution of gI was not examined, we would predict that gI would also accumulate along the lateral plasma membrane in a manner similar to gE. This suggestion is based on a number of observations: first, gE and gI are known to form a complex, that together can act as a receptor for the Fc domain of IgG (Johnson et al., 1988). Only the gE/gI complexes immunoprecipitated with nonimmune rabbit serum from HSV infected cell lysates, rather than gE or gI singly. When we expressed gE and gI together in HEC-1A cells using the Ad vectors, nonimmune rabbit serum coimmunoprecipitated both gE and gI (data not shown), suggesting that a functional complex was formed in these cells. In Vero cells, most of the gE is associated with gI and vice versa (Johnson et al., 1988). Secondly, gE accumulation at lateral membranes of polarized HEC-1A cells

required coexpression of gI. Together, these data strongly suggest that gE and gI are transported together to the plasma membrane where the gE/gI complex accumulates along lateral membranes. Further characterization of gI's subcellular distribution will be performed in the near future.

gE binds to cell junctions. In subconfluent monolayers of HEC-1A cells, ß-catenin staining was concentrated at cell junctions, and was not found on those lateral membranes not in contact with another cell (Figure 5B and H). In both AdgE/gI and HSV infected cells, gE staining was similarly found at these sites of cell-cell contact, colocalizing with ß-catenin (Figure 5A-C; G-I). Little or no gE was observed on those surfaces not in contact with another cell. The fact that gE was not found on those lateral surfaces not in contact with other cells was surprising since in subconfluent cells, tight junctions do not form and there is no physical barrier precluding the diffusion of lipids and proteins between the apical and basolateral plasma membrane domains. Thus in the absence of a tight junction barrier, cell surface glycoproteins (i.e, gE) would be able to diffuse to all surfaces, including membranes that do not form cell junctions. Lateral diffusion of proteins within the plasma membrane can also be restricted or inhibited by protein-protein interactions with higher order structures on the membrane (Mays et al., 1994). This latter type of inhibition to protein diffusion likely accounts for our observations with ß-catenin. In subconfluent cells, adherens junctions are forming, producing stable protein-protein complexes at sites between neighbouring cells. Thus ß-catenin, via its interactions with the cadherins, would

be restricted to regions of the plasma membrane engaged in the formation of cell-cell junctions. Similarly, the accumulation of gE/gI only at cell junctions, and not at junction-free lateral membranes, suggests that gE/gI is interacting with cellular components at sites of cell-cell contact.

gE is not bound to cytoskeletal elements. In polarized epithelia, the accumulation of cellular proteins at cell junctions often involves binding of the proteins with components of the cortical cytoskeleton (reviewed in Mays et al., 1994). The Na^+, K^+-ATPase for example, localizes to sites of cell-cell contact through a stable association with ankyrin and fodrin, two components of the cortical membrane cytoskeleton (Hammerton et al., 1991; Morrow et al., 1989; Nelson and Veshnock, 1987). This interaction correlates with an increase in the resistance of the Na^+, K^+-ATPase to extraction with nonionic detergents (Hammerton et al., 1991). Since gE/gI apparently binds to cell junctions, it was of interest to determine if the localization of these glycoproteins to cell junctions was dependent upon an association with the cortical cytoskeleton (i.e, whether gE/gI became detergent insoluble). Monolayers of HEC-1A cells were coinfected with Ad($E1^-$)gE and Ad($E1^-$)gI, and cell extracts were made using increasing concentrations of TX100, from 0% to 1% (w/v). Cell extracts were centrifuged at high speed to pellet the insoluble cytoskeletal fraction. The soluble (supernatant) and insoluble (pellet) fractions were denatured in buffer containing 2% SDS and separated by SDS polyacrylamide electrophoresis. Proteins were transferred to nitrocellulose and gE detected using anti-gE MAb II-481. As expected, all of the

gE was found within the insoluble fraction (P), when the extraction buffer did not contain TX100 (Figure 6). As the TX100 concentration was increased, we observed a concomitant change in the solubility of gE. At 0.05% TX100, greater than half of gE was found within the soluble fraction (S), and with 0.1% TX100 and higher detergent concentrations, most or all the gE was in the soluble fraction. It is known that ß-catenin is insoluble in 0.5% TX100 (Hinck et al., 1994; Nathke et al., 1994). Therefore, the accumulation of the gE/gI complex at sites of cell-cell contact is not related to associations of gE/gI with the detergent insoluble cortical cytoskeleton.

Expression of gE does not disrupt epithelial junctions. Previously, it has been reported that HSV infection alters junctional complexes of polarized epithelial cells leading to an increase in paracellular permeability and a redistribution of the cell junction markers ZO-1 (tight junction), B-cadherin and ß-catenin (adherens junction), and the actin cytoskeleton (Maidji et al., 1996). The HSV-dependent disruption of cell junctions was attributed to the expression of gE, based on the observation that an HSV gE^- mutant did not disrupt junctions as effectively as wild type HSV (Maidji et al., 1996). In order to determine whether gE/gI could disrupt cell junctions, HEC-1A cells were coinfected with Ad($E1^-$)gE and Ad($E1^-$)gI and the distribution of two cell adhesion molecules ZO-1 and ß-catenin were evaluated by confocal immunofluorescence. Confocal sections at the level of the tight junction (e.g., near the top of the cell) showed the characteristic circumferential pattern of ZO-1 staining at the periphery of cells (Figure 4E).

Similarly, with confocal sections below tight junctions, we observed the typical staining pattern of ß-catenin at cell junctions (Figure 4B). We could not detect any redistribution of either ZO-1 or ß-catenin following expression of gE/gI using the Ad vectors, suggesting that gE/gI does not disrupt of cell junctions even after 24 or more hr of expression. Similarly, there was no redistribution of ZO-1 or ß-catenin until 12 hr after infection with HSV-1 at a time when the cytoskeleton of HEC-1A cells were disrupted and cells rounded up.

It was conceivable that gE/gI expression could alter polarized cells without obviously changing the distribution of tight junction proteins. The integrity of tight junctions can be measured experimentally by an increase in transepithelial resistance when a voltage is applied to one side of an epithelial monolayer. To ascertain if gE/gI could alter tight junctions and inhibit their function, transepithelial resistance of HEC-1A cells grown on filter supports was measured following coinfection with either Ad(E1$^-$)gE and Ad(E1$^-$)gI, or with AddlE1, a control virus that does not express gE or gI. There were no significant differences in transepithelial resistance after expression of gE/gI using the Ad vectors (Figure 7A), and in all cases, monolayers maintained a resistance greater than 400 Ωcm^2, similar to that observed with uninfected (mock) cells.. These measurements were made 48 hr after infection of the monolayers with Ad(E1$^-$)gE and Ad(E1$^-$)gI, so that the quantity of gE/gI produced in the cells was higher than that observed in HSV-infected cells 8 hr after infection (Figure 2).

We also examined the ability of polarized HEC-1A monolayers expressing gE and gI to exclude the paracellular transport of [^{14}C] labelled inulin (a small molecule of approximately 5000 Da), from the apical to basolateral compartments. [14C]inulin was added to the apical compartment of the monolayers coinfected with either the Ad(E1$^-$)gE and Ad(E1$^-$)gI, or infected with AddlE1, or left uninfected and the accumulation of radiolabel in the basal compartment measured. With membrane inserts alone, the labelled inulin was able to accumulate rapidly in the basal compartment with maximal levels achieved within 20 min (Figure 7B, membrane). Movement of [^{14}C] inulin across monolayers of uninfected HEC-1A cells was markedly reduced. Similarly, there was little paracellular movement of the label in cells infected with either Ad(E1$^-$)gE and Ad(E1$^-$)gE or with AddlE1 (Figure 5B). Therefore, the expression of gE/gI alone does not affect the ability of polarized HEC-1A cells to maintain functional tight junctions, nor does it lead to a redistribution of either tight junction or adherens junction protein components.

Figure 1: Construction of Ad(E1$^-$)gE and Ad(E1$^-$)gI. The full length gE and gI genes were and subcloned into the plasmid pCA3 forming pCA3gE and pCA3gI respectively. The gE or gI genes were flanked by the human cytomegalovirus (HCMV) immediate early promoter and the SV40 poly A sequence, such that gE and gI transcription occurred in the opposite direction with respect to the flanking Ad E1 sequences. Recombinant adenoviruses expressing either gE [Ad(E1$^-$)gE] or gI [Ad(E1$^-$)gI], were obtained following co-transfection of either pCA3gE or pCA3gI with pBHG10 into 293 cells using the calcium phosphate technique. Recombinant viruses were plaque purified three times.

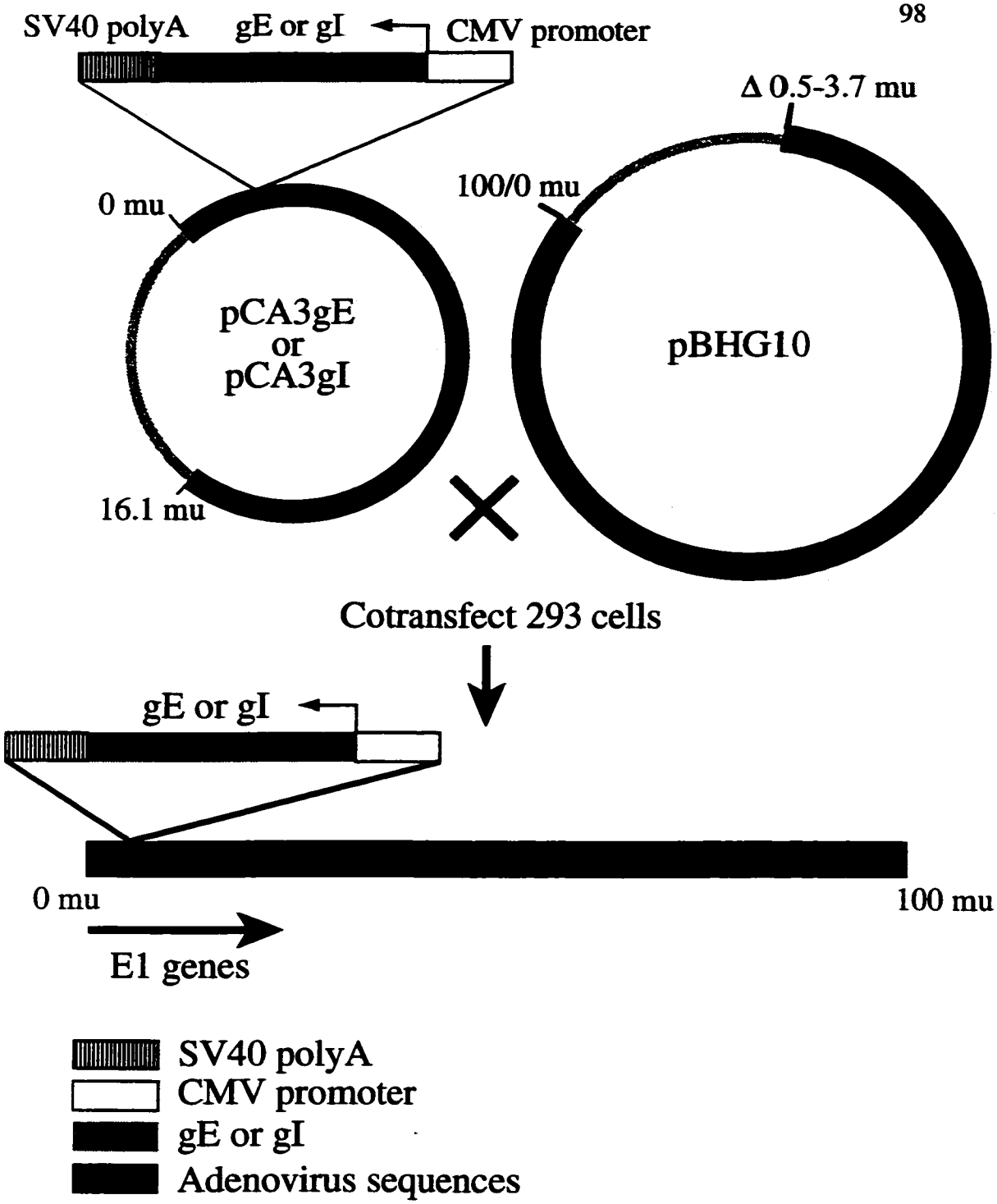

SV40 polyA
gE or gI
CMV promoter
Δ 0.5-3.7 mu
100/0 mu
0 mu
pCA3gE
or
pCA3gI
pBHG10
16.1 mu
Cotransfect 293 cells
gE or gI
0 mu
100 mu
E1 genes
SV40 polyA
CMV promoter
gE or gI
Adenovirus sequences

Figure 2: Expression of gE and gI by recombinant adenovirus vectors. (A): Human HEC-1A cells were infected at an MOI of 400 with either Ad(E1$^-$)gE, Ad(E1$^-$)gI, coinfected with Ad(E1$^-$)gE/gI or infected at an MOI of 20 with HSV-1. Ad infected cells were labelled after 48 hr while HSV infected cells were labelled at 8 hrs, both for 3 hrs, with [35S]methionine and cysteine. Cells were lysed in NP40/DOC extraction buffer, and extracts were immunoprecipitated with MAbs specific for gE (3114) or gI (3104). (B) HEC-1 cells were either co-infected with AdE1gE/gI at an MOI of 400 or with HSV at an MOI of 20. At either 48 hrs (Ad) or 8 hrs (HSV-1), cells were lysed in boiling SDS loading buffer. Samples were separated by polyacrylamide electrophoresis, transferred to membranes and then incubated with the gE specific MAb II-481. Blots were washed and then incubated with horseradish peroxidase coupled anti-mouse antibodies and developed using ECL chemiluminescence.

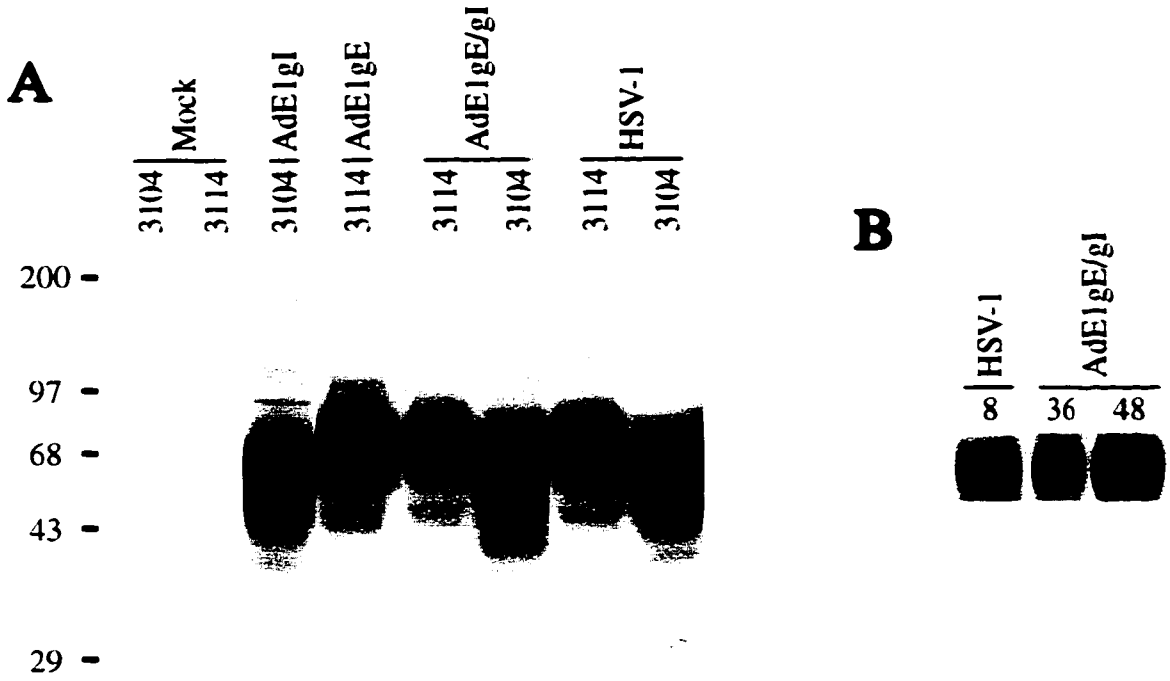

A
3104
Mock
3114
3104
AdE1gI
3114
AdE1gE
3114
AdE1gE/gI
3104
3114
HSV-1
3104
200
97
68
43
29
B
HSV-1
8
AdE1gE/gI
36
48

Figure 3: gE requires gI to reach the cell surface. HEC-1 cells were infected with either Ad(E1$^-$)gE (A, D), Ad(E1$^-$)gE/gI (B,E) or AdgD(E1$^-$), and then fixed after 48 hrs p.i. Cells were permeabilized (A,B,C) or untreated (C,D,E) prior to incubation with MAbs 3114 against gE (A,B,D,E) or DL-6 against gD (C,F) for 1 hr at RT. Samples were washed and then incubated with FITC-coupled goat anti-mouse antibodies, washed, and then viewed using an Nikon epifluorescence microscope.

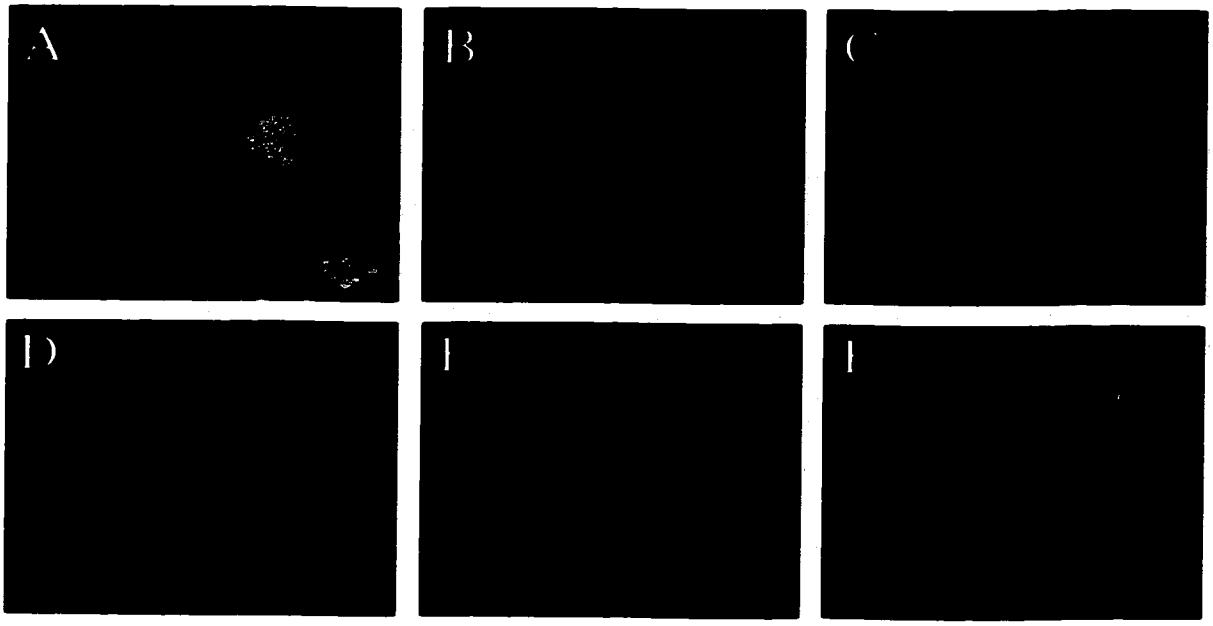

A
B
C
D
E
F

Figure 4: gE co-localizes with the cell adhesion marker ß-catenin. HEC-1A cells were co-infected with Ad(E1$^-$)gE/gI and at 48 hrs p.i., were fixed and permeabilized. Cells were incubated sequentially with antibodies directed against ß-catenin followed by Cy3 coupled goat anti mouse antibodies (B,C). Cells were washed again and then incubated with the gE MAb 3114 coupled to the FITC analogue Oregon Green for 1 hr, washed and then incubated with BODIPY-coupled goat anti-FITC antibodies (A,C). In addition, cells were incubated simultaneously with MAb 3114 specific for gE (D, F) and rabbit antibodies against ZO-1 (E, F). Samples were washed and then incubated with FITC-coupled goat anti-mouse, and Texas red-coupled goat anti-rabbit antibodies for 1 hr, then washed and mounted. All samples were viewed using a Leica confocal microscope.

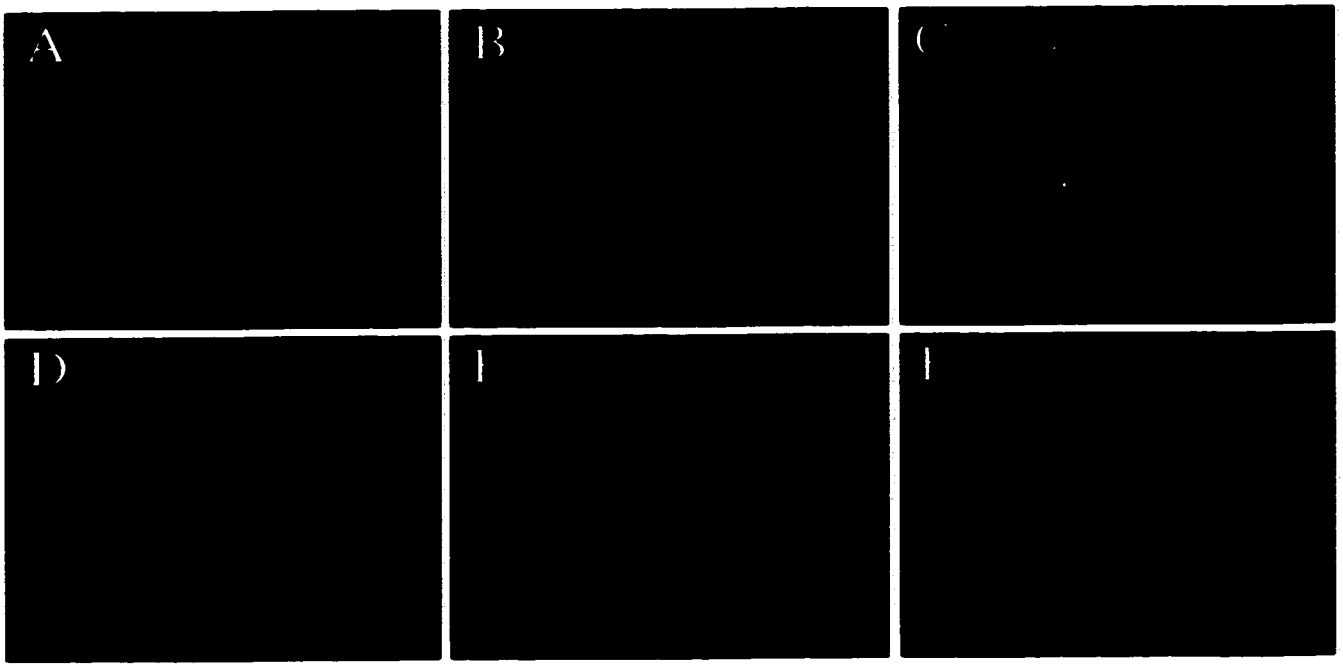

A
B
C
D
E
F

Figure 5: gE specifically accumulates at lateral borders between cells. Confluent (J-L) or subconfluent (A-I) monolayers were infected with either AdgE/gI (A-C, J-K) or AdgD(E1$^-$) (L) at an MOI of 400 or with HSV-1 (D-I) at an MOI of 20. At either 22 hr (A-C, J-L), 8 hr (D-F), or 11 hrs (G-I), after infection, cells were fixed and permeabilized. Samples were sequentially incubated with ß-catenin antibodies, washed, and then incubated with Cy3 coupled goat anti-mouse antibodies (A-I). This was followed by an incubation with 3114 (gE specific MAb) coupled to Oregon Green, and then the cells were washed, and incubated with BODIPY coupled goat anti-FITC. Alternatively, cells were incubated with either 3114 (J), or 3114 and ZO-1 antibodies (K), or DL-6 (gD specific) and ZO-1 antibodies (L). Samples were washed and then incubated with either Texas red goat anti-mouse (J), or both FITC-coupled goat anti-mouse and Texas red-coupled goat anti-rabbit antibodies (K-L). All samples were viewed by confocal microscopy. Samples J-L were viewed through the Z-axis. Sample J channel was switched from red to green to keep gE staining colour consistent.

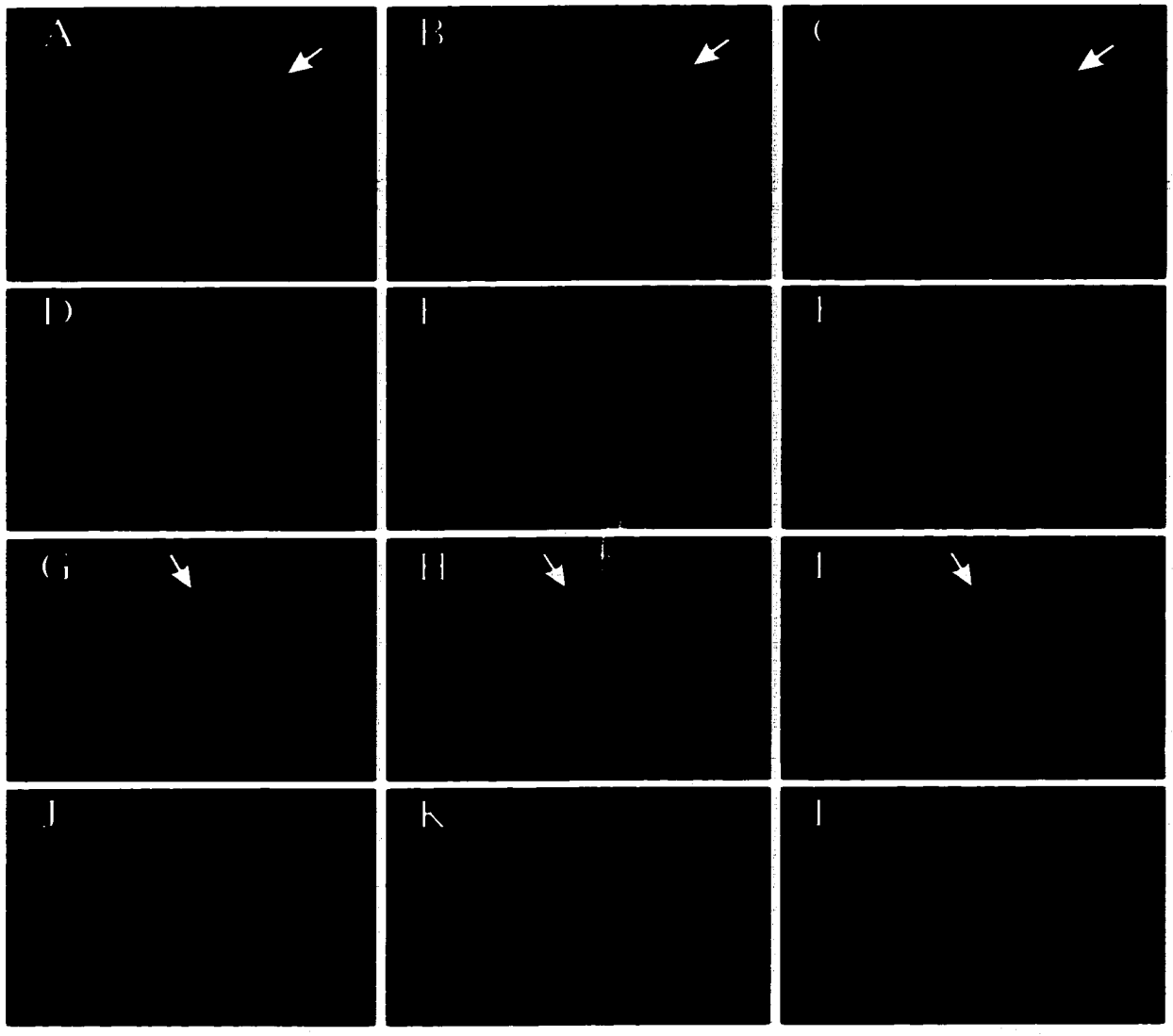

A
B
C
D
E
F
G
H
I
J
K
L

Figure 6: gE does not associate with the detergent insoluble cytoskeleton. Monolayers of HEC-1A cells were infected with both AdE1gE and gI for 48 hrs, then washed with tris saline, and then lysed for 10 min at 4 C in extraction buffer contained increasing amounts of TX100. Cells were scraped into the lysis buffer, and all samples were centrifuged at 20, 000 x g for 30 min. Loading buffer was added to both the supernatant and pellet and then boiled for 10 min. Samples were subjected to electrophoresis using a 7.5 % polyacrylamide gel, transferred to nitrocellulose and then incubated with MAb II-481 specific for gE. The mouse antibodies were detected by horseradish peroxidase coupled rabbit anti-mouse antibodies using an Amersham ECL chemiluminescence kit.

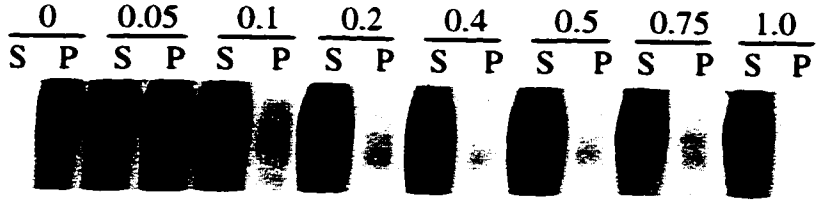
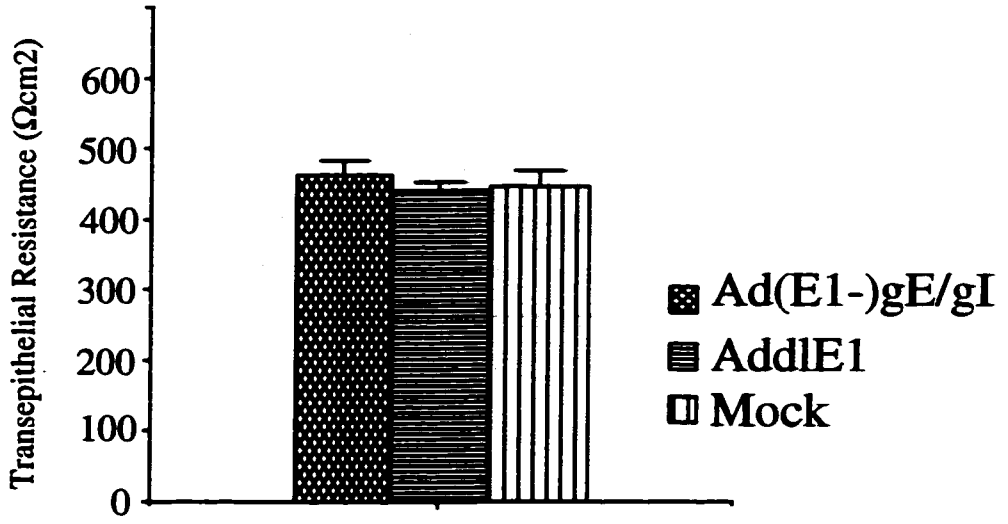

0
0.05
0.1
0.2
0.4
0.5
0.75
1.0
S P S P S P S P S P S P S P S P

Figure 7: gE/gI expression does not disrupt the functional integrity of tight junctions. HEC-1A cells were grown on filter supports for 5 days until the transepithelial resistance was greater than 400 Ωcm^2. Cells were coinfected with Ad($E1^-$)gE and Ad($E1^-$)gI using 400 PFU/cell of each, or infected with AddlE1 using 800 PFU/cell or left uninfected. At 48 hr after infection, the integrity of tight junctions was measured by transepithelial resistance (A) and paracellular permeability (B). For paracellular permeability, [^{14}C]inulin was added to the apical compartment and at various times following addition the labelled inulin, the apical chamber was moved to a new well. Samples were taken from each basolateral chamber and radioactivity measured. In both A and B, three different wells were used for each measurement; error bars represent the standard deviation.

A


Transepithelial Resistance (Ωcm2)
600
500
400
300
200
100
0
Ad(E1-)gE/gI
AddlE1
Mock

B

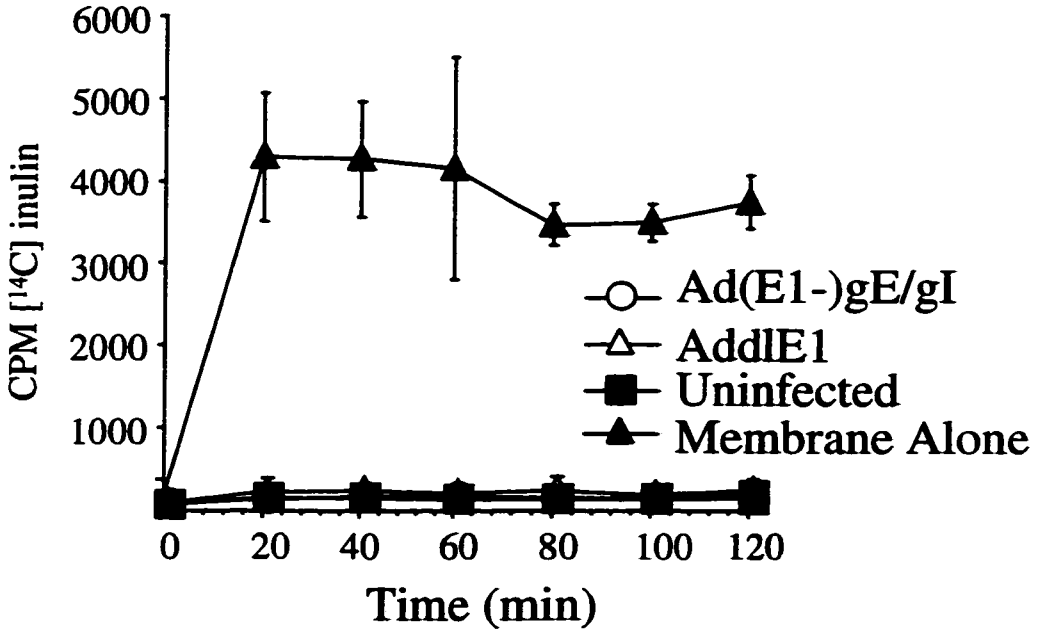
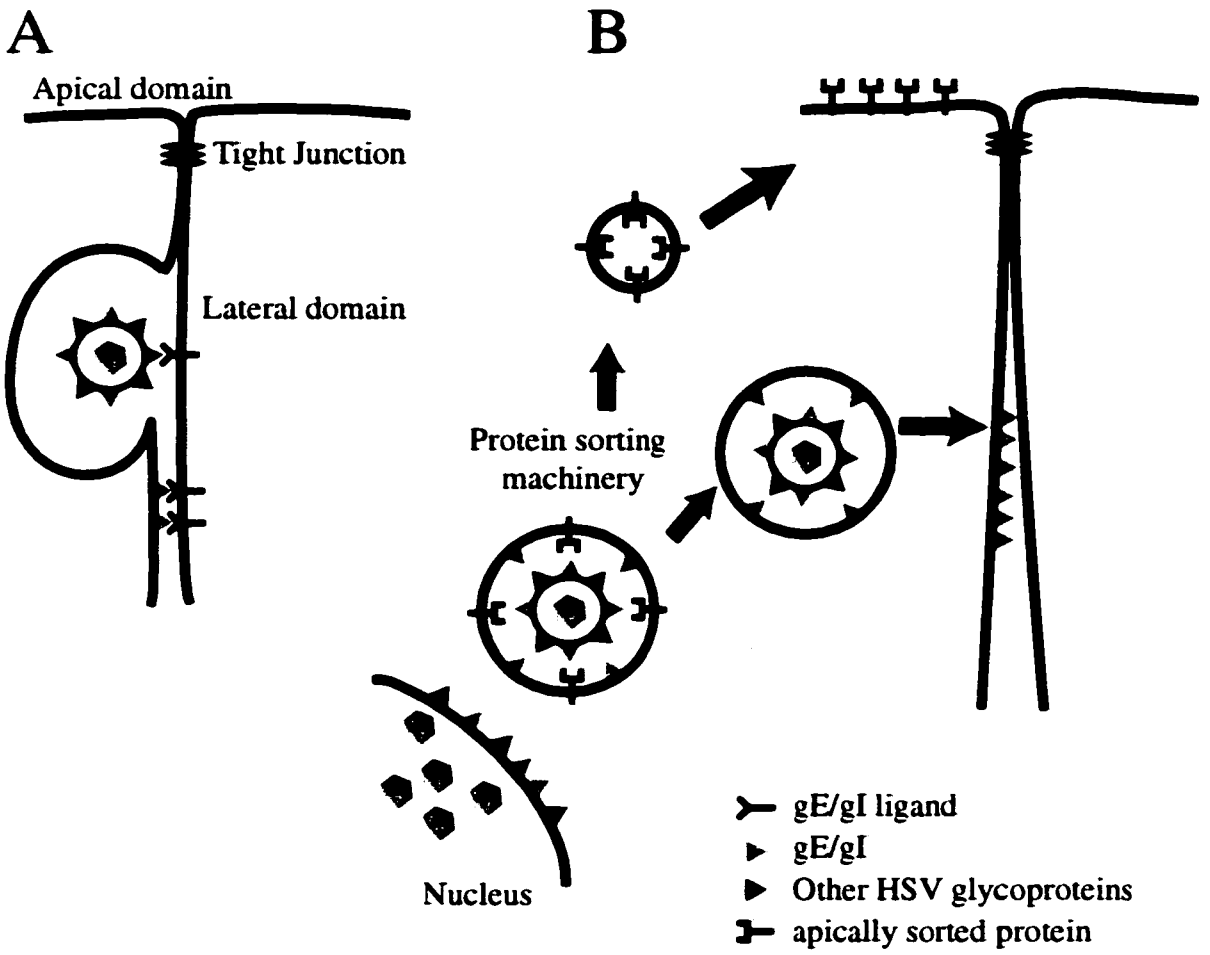

CPM [14C] inulin
6000
5000
4000
3000
2000
1000
0
20
40
60
80
100
120
Time (min)
Ad(E1-)gE/gI
AddlE1
Uninfected
Membrane Alone

Figure 8: Models for gE/gI function in cell-to-cell spread (A) The accumulation of gE/gI at lateral junctions between cells suggest that gE/gI may facilitate HSV cell-to-cell spread by binding to cellular ligands found at cell junctions. Transfer of virus across the junction is enhanced by binding of gE/gI within the virion envelope (top) or in the plasma membrane (bottom) to cellular ligand(s). The ligand could be a component of either the infected cell or on the plasma membrane of the adjoining uninfected cell. (B) The alternative model predicts that gE/gI acts as trafficking signal to direct enveloped particles to the lateral plasma membrane. This places the virion in a position largely resistant to neutralizing anti-HSV antibodies. However, the two models are not mutually exclusive, since both trafficking and ligand binding by gE/gI may be important for HSV cell-to-cell spread.


A
Apical domain
Tight Junction
Lateral domain
B
Protein sorting machinery
Nucleus
gE/gI ligand
gE/gI
Other HSV glycoproteins
apically sorted protein

DISCUSSION

HSV normally replicates and spreads extensively in the mucosal epithelium prior to viral invasion of the peripheral nervous system, where the virus can establish a latent infection. Although, the mechanisms regulating the spread of HSV through epithelial tissues are poorly understood, experiments with HSV mutants *in vitro* and *in vivo,* suggested that cell-to-cell spread of virus through these tissues was dependent upon the HSV glycoprotein complex gE/gI (Balan et al., 1994; Dingwell et al., 1994). However, it was clear that gE/gI was not involved in entry of extracellular virus particles. One mechanism by which gE/gI could promote cell-to-cell spread is through interactions with cellular ligands found at junctions between cells. In an effort to further examine these interactions between gE/gI and the junctions formed between cultured cells, we expressed gE and gI to high levels in polarized human epithelial cells in the absence of other HSV polypeptides using replication defective Ad vectors.

Here, we report for the first time that HSV gE requires gI expression in order for the gE/gI complex to reach the cell surface of epithelial cells. When gE was expressed alone in polarized HEC-1A cells, we found that gE accumulated within the cell in a perinuclear distribution, and very little gE, if any, reached the cell surface. In contrast, gE, when expressed together with gI in epithelial cells, was transported to the basolateral plasma membrane. This result might not be too surprising given that most of the gE in HSV infected cells is complexed with gI.

However, previous studies demonstrated that HSV gE was transported efficiently to the cell surface when expressed without gI in either mouse L cells, human R970 and HeLa cells (Bell et al., 1990; Hanke et al., 1990; Rosenthal et al., 1987). gE homologues of other α-herpesviruses such as feline (FHV), and bovine (BHV) herpes viruses required coexpression of the respective gI genes, for efficient gE/gI transport to the cell surface (Mijnes et al., 1996; Whitbeck et al., 1996). In the absence of gI expression, the BHV and FHV gE homologues were retained in the ER as immature glycoproteins, and remained endoglycosidase H sensitive. ER retention is common for many viral membrane glycoproteins in which complex formation is required for transport, and acts as a quality control mechanism to ensure proper protein folding (reviewed in Doms et al., 1993). HSV gL, for example, must be coexpressed with gH for correct folding, processing and transport of the gH/gL complex to the cell surface (Hutchinson et al., 1992). In an analogous manner, HSV gI may assure proper folding and processing of gE, permitting the gE/gI complex to leave the ER and be transported to the cell surface, but this effect of gI appears to be cell specific. Studies by Hanke et al., (1990) showed that the level of gE expressed on the surface of human R970 cells using a gE expressing Ad vector was not significantly different from that of HSV-infected cells. In contrast, immunofluorescent flow cytometry analysis of stable HEp-2 cell lines expressing gE, showed that greater than 90% of gE expressed by these cells remained in the cytoplasm, while less than 10% was found on the cell surface (van Vliet, 1993). Infection of the gE expressing clones

with a HSV gE$^-$ mutant (which expressed HSV gI), resulted in the transport of almost all of the gE to the cell surface (van Vliet, 1993). Similarly VZV gE conformation as measured by conformation dependent MAbs, varies when expressed on the cell surface of VZV infected cells in the absence of VZV gI. In HeLa cells, VZV gE was transported efficiently to the cell surface and was antigenically similar to gE when coexpressed with gI (Yao et al., 1993), while in human melanoma cells, gE in the absence of gI, was not recognized by some gE specific MAbs (Mallory et al., 1997). Therefore, the cellular process that regulates gE folding, processing and export from the ER may be subtly distinct in different cell types.

Analysis by confocal microscopy showed that gE/gI accumulated along the lateral plasma membranes of HEC-1A epithelial cells, extensively colocalizing with the adherens junction protein ß-catenin. gE was concentrated along the lateral plasma membrane below the level of the tight junction, and was not detected on either the apical or basal plasma membranes. Similar results were observed following infection of HEC-1A cells with wild type HSV-1. Previously, gE and other HSV glycoproteins have been reported to accumulate at either the basolateral or apical domains of certain polarized cells (Nielsen et al., 1991; Srinivas et al., 1986). However, in each case, all the HSV glycoproteins expressed after virus infection were either apically or basolaterally sorted; there was no evidence of differential targeting between the various glycoproteins (Nielsen et al., 1991; Srinivas et al., 1986). In our experiments, HSV gD, expressed in HEC-1A

cells using a replication-defective Ad vectors was predominantly sorted to the apical domain, while gE/gI was specifically found on the lateral plasma membrane. We currently don't understand this differential sorting of gE/gI and gD. We are currently determining whether basolateral sorting of gD requires expression of other HSV polypeptides.

The accumulation of gE/gI along the lateral membranes of polarized epithelial cells suggests that there may be specific sorting mechanisms that cause the gE/gI complex to be delivered to the basolateral membrane domain. Basolateral sorting in polarized cells is mediated by a variety of signals in the cytoplasmic tails of membrane glycoproteins (reviewed in Matter and Mellman, 1994; Mellman, 1996) . Many of these sequence motifs are degenerate, overlapping with signals that cause proteins to be incorporated into clathrin coated pits or endosomes, including tyrosine based motifs (e.g., YXXϕ; where ϕ is any bulky-hydrophobic amino acid) and alternatively, di-leucine motifs (Matter and Mellman, 1994; Mellman, 1996). The macrophage IgG Fc receptor, for example, is sorted to the basolateral domain by the same di-leucine motif that mediates endocytosis (Hunziker and Fumey, 1994). At this time we do not know what signal is responsible for basolateral sorting of gE/gI, however the cytoplasmic domain of gE contains the YADW sequence which conforms to the YXXϕ-based motifs involved in basolateral sorting. gI contains a di-leucine motif at the C-terminus of the cytoplasmic domain. Whether these sequence motifs within gE and gI act as sorting signals causing gE/gI to be delivered to the lateral membrane

is the topic of continuing research in our lab.

Recently, it was suggested that HSV gE and gI may promote cell-to-cell transmission of HSV in polarized epithelial cells by specifically disrupting junctional complexes between cells (Maidji et al., 1996). HSV gE$^-$ and gI- mutants produced smaller plaques on epithelial cells and there were marked differences in both the redistribution of cell junction makers and changes in paracellular permeability when compared to cells infected with wild type HSV (Maidji et al., 1996). However, the redistribution of cell junction components and the disruption of cell junctions was observed after several rounds of HSV infection, and the proportion of cells infected with the gE$^-$ and gI$^-$ mutants was approximately 10 fold lower than wild type virus. Therefore, these observations could be due to a reduced level of viral induced cell rounding or disruption of cytoskeletal elements that occurs because the gE- and gI- mutants do not spread in the cultured cells. In polarized HEC-1A cells expressing gE/gI, ZO-1 staining remained as a distinct band near the apical border, and there was no evidence of ZO-1 redistribution, demonstrating that tight junction integrity was not altered by gE/gI expression. Since it is formally possible that the integrity of tight junctions could be altered without affecting the distribution of ZO-1, we measured transepithelial resistance and paracellular permeability across monolayers of polarized HEC-1A cells made to express gE and gI. er We could not detect affects of gE/gI expression on tight junction function when compared to monolayers either infected with a control Ad vector or left uninfected. It could be argued though, that the level of

expression of the Ad vectors was not sufficient to disrupt tight junctions. However, Western blot analysis of the steady state levels of gE showed that gE expression by the Ad vector in HEC-1A cells was higher than the gE expression achieved following infection with wild type HSV. Therefore, at least in HEC-1A cells gE/gI expression does not disrupt cell junctions in any manner measured by these techniques. Maidji et al., used different cells, human retinal pigment epithelial cells, and these results could be related to differences in the effects of gE/gI in these different cells.

A striking observation in our analysis of gE/gI distribution in epithelial cells was specific accumulation of gE/gI at cell junctions but not at lateral surfaces no involved in junctions. This was observed in cells made to express gE/gI and during HSV-infection of HEC-1A cells. Since these cells are not polarized, domain specific sorting signals cannot account for our observations. Membrane proteins should be fully capable of diffusing between free lateral domains and neighbouring junctional domains, and thus the accumulation of gE/gI at junctions must be accounted for by some mechanism to restrict the gE/gI diffusion to plasma membrane domains that do not contain cell junctions. Several membrane proteins such as the Na^+, K^+-ATPase (Hammerton et al., 1991), and the voltage-sensitive K^+ channel Kv2.1(Scannevin et al., 1996), accumulate at lateral junctions based on their association with the cortical cytoskeleton (reviewed in Mays et al., 1994). Kv2.1 for example, accumulates at lateral junctions in non-confluent monolayers as soon as cell contacts are made and adherens

junctions begin to form (Scannevin et al., 1996). Despite this similarity with Kv2.1, we could not detect any evidence that gE associated with the cytoskeleton based on gE's solubility in Triton X-100 . Therefore, the gE/gI complex is likely binding to certain components of epithelial cell junctions other than those associated with the cytoskeleton.

This is the first direct evidence that gE/gI interacts directly with epithelial cell junctions. The nature of the interaction of gE/gI with cell junctions is unclear, however, there are at least two mechanisms which could account for the lateral clustering and accumulation of the gE/gI complex to cell junctions. First, gE could be associated with protein components of cell junctions such as cell adhesion molecules. ß-catenin is a soluble protein but localizes to cell junctions based on its ability to bind to cadherins. Second, gE/gI in the lateral membranes of one cell could associate with cellular ligands found on the opposing cell's plasma membrane. The close apposition between these two plasma membranes would place gE/gI into a position so as to interact with a cellular ligand on an adjoining cell. A similar mechanism is thought to occur during the assembly of gap junctions (reviewed in Laird, 1996). In mouse hepatocytes, developing sites of cell-cell contact acted as foci for connexin interactions between neighbouring cells (Fujimoto et al., 1997). When adherens junctions were disrupted in these cells, there was a concomitant loss of connexin interactions and connexin were no longer found at the cell surface (Fujimoto et al., 1997).

The accumulation of gE/gI at cell junctions provides an important clue as to how gE/gI might facilitate the transfer of HSV from cell-to-cell. Here we propose two models to accommodate our recent findings (Figure 8). Model A suggests that gE/gI binds to cellular ligands probably on the surface of a neighbouring cell, and through these interactions, facilitates transfer of HSV across cell junctions. Whether gE/gI facilitates cell-to-cell spread when found within the plasma membrane at cell junctions (bottom part of 8A), or as part of the virion envelope (top part of 8A) is not clear as yet. It is clear that gE/gI is incorporated into the plasma membrane and cell junctions as well as being present in the virion envelope. PrV mutants in which gE was expressed but was not incorporated into the virion, formed small plaques on monolayers of bovine epithelial cells (Tirabassi et al., 1997), suggesting that gE must be in the virion envelope in order to facilitate PrV cell-to-cell spread. However, these PrV mutants expressed gE genes which lacked the cytoplasmic domains, and so the small plaque phenotype could be due to other defects in gE function (e.g., interactions with cellular proteins). Regardless of whether gE/gI is part of the virion envelope or plasma membrane, gE/gI might facilitate transfer by forming a favourable environment for gB, gD, gH/gL to function in promoting fusion between the virion envelope and the plasma membrane of the apposing cell. Model B would suggest that gE/gI acts as a trafficking signal to ensure that enveloped particles are transported to cell junctions rather than to the apical plasma membrane. Targeting of virions to cell junctions, rather than the apical surface may protect

HSV to some degree from neutralization by anti-HSV antibodies. These two models are not necessarily mutually exclusive. Therefore it will be important in the future to separate gE/gI's trafficking signals from the ability of the complex to bind to cell junctions.

ACKNOWLEDGEMENTS:

We thank Kenneth Fish, Jodi Engstrom, and Sally Hansen for their patience and help with the confocal microscopy. We are grateful to the members of the Johnson and Jay Nelson labs for many helpful discussions. Support for this research was provided by grants from the Medical Research Council of Canada (MRC), and the National Cancer Institute of Canada (NCIC). K.S. D. held a research studentship from the MRC and D. C. J. was a senior research scholar of the NCIC during the course of this work.

REFERENCES:

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (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*, 1245-58.

Balda, M. S., and Matter, K. (1998). Tight Junctions. J Cell Sci *111*, 541-547.

Ball, J. M., Moldoveaunu, Z., Melsen, L. R., Kozlowski, P. A., Jackson, S., Mulligan, M. J., Mestecky, J. F., and Compans, R. W. (1995). A polarized human endometrial cell line that binds and transports polymeric IgA. In Vitro Cell Dev Biol *31*, 196-206.

Bell, S., Cranage, M., Borysiewicz, L., and Minson, T. (1990). Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J Virol *64*, 2181-6.

Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (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.

Brunetti, C. R., Dingwell, K. S., Wale, C., Graham, F. L., and Johnson, D. C. (1998). Herpes simplex virus gD and virions accumulate in endosomes by mannose-6-phosphate dependent and independent mechanisms. J. Virol *(in press)*.

Dingwell, K. (unpublished). .

Dingwell, K. S., Brunetti, C. R., Hendricks, R. L., Tang, Q., Tang, M., Rainbow, A. J., and Johnson, D. C. (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J Virol *68*, 834-45.

Dingwell, K. S., Doering, L. C., and Johnson, D. C. (1995). Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J Virol *69*, 7087-98.

Doms, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. (1993). Folding and assembly of viral membrane proteins. Virology *193*, 545-562.

Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A., Ohokuma, A., and Shibata, Y. (1997). Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. J Cell Sci *110*, 311-22.

Graham, F. L., Smiley, J., Russel, W. C., and Nairn, R. (1977). Characteristics of a

human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-72.

Hammerton, R. W., Krzeminski, K. A., Mays, R. W., Ryan, T. A., Wollner, D. A., and Nelson, W. J. (1991). Mechanism for regulating cell surface distribution of Na+,K(+)-ATPase in polarized epithelial cells [see comments]. Science *254*, 847-50.

Hanke, T., Graham, F. L., Lulitanond, V., and Johnson, D. C. (1990). Herpes simplex virus IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology *177*, 437-44.

Hinck, L., Nathke, I. S., Papkoff, J., and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. J Cell Biol *125*, 1327-40.

Hitt, M., Bett, A. J., Addison, C. L., Prevec, L., and Graham, F. L. (1995). Techniques for human adenovirus vector construction and characterization. In Methods in Molecular Genetics, K. W. Adolph, ed. (Orlando, Fl: Academic Press), pp. 13-30.

Hunziker, W., and Fumey, C. (1994). A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. EMBO *13*, 2963-2969.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C., and Johnson, D. C. (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*, 2240-50.

Isola, V. J., Eisenberg, R. J., Siebert, G. R., Heilman, C. J., Wilcox, W. C., and Cohen, G. H. (1989). Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. J Virol *63*, 2325-34.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M., and Stow, N. D. (1988). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J Virol *62*, 1347-54.

Kudelova, M., Kostal, M., Cervenakova, L., Rajcani, J., and Kaerner, H. C. (1991). Pathogenicity and latency competence for rabbits of the herpes simplex virus type 1 ANGpath gC and gE defective mutants. Acta Virol *35*, 438-49.

Laird, D. W. (1996). The life cycle of a connexin: gap junction formation, removal, and degradation. J Bioenerg Biomembr *28*, 311-8.

Maidji, E., Tugizov, S., Jones, T., Zheng, Z., and Pereira, L. (1996). Accessory human cytomegalovirus glycoprotein US9 in the unique short component of the viral genome promotes cell-to-cell transmission of virus in polarized epithelial cells. J Virol *70*, 8402-10.

Mallory, S., Sommer, M., and Arvin, A. M. (1997). Mutational analysis of the role of glycoprotein I in varicella-zoster virus replication and its effects on glycoprotein E conformation and trafficking. J Virol *71*, 8279-8288.

Matter, K., and Mellman, I. (1994). Mechanisms of cell polarity: sorting and transport in epithelial cells. Curr Opin Cell Biol *6*, 545-554.

Mays, R. W., Beck, K. A., and Nelson, W. J. (1994). Organization and function of the cytoskeleton in polarized epithelial cells: a component of the protein sorting machinery. Curr Opin Cell Biol *6*, 16-24.

Mellman, I. (1996). Endocytosis and molecular sorting. Ann Rev Cell Dev Biol *12*, 575-625.

Mijnes, J. D., van der Horst, L. M., van Anken, E., Horzinek, M. C., Rottier, P. J., and de Groot, R. J. (1996). Biosynthesis of glycoproteins E and I of feline herpesvirus: gE-gI interaction is required for intracellular transport. J Virol *70*, 5466-75.

Morrow, J. S., Cianci, C. D., Ardito, T., Mann, A. S., and Kashgarian, M. (1989). Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin-Darby canine kidney cells in intact renal tubule cells. J Cell Biol *108*, 455-465.

Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J., and Nelson, W. J. (1994). Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. J Cell Biol *125*, 1341-52.

Neidhardt, H., Schroder, C. H., and Kaerner, H. C. (1987). Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. J Virol *61*, 600-3.

Nelson, W. J., and Veshnock, P. J. (1987). Ankyrin binding to (Na+ + K+)ATPase and implications for the organization of membrane domains in polarized cells. Nature *328*, 533-6.

Nielsen, L. N., Whitley, R. J., and Chatterjee, S. (1991). Apical expression of herpes simplex virus type 2 glycoproteins in human neuroblastoma cells. Virology *185*, 908-10.

Rajcani, J., Herget, U., and Kaerner, H. C. (1990). Spread of herpes simplex virus (HSV) strains SC16, ANG, ANGpath and its glyC minus and GlyE minus mutants in DBA-2 mice. Acta Virol *34*, 305-20.

Rajcani, J., Herget, U., Kostal, M., and Kaerner, H. C. (1990). Latency competence of herpes simplex virus strains ANG, ANGpath and its gC and gE minus mutans. Acta Virol *34*, 477-86.

Rosenthal, K. L., Smiley, J. R., South, S., and Johnson, D. C. (1987). Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes. J Virol *61*, 2438-47.

Scannevin, R. H., Murakoshi, H., Rhodes, K. J., and Trimmer, J. S. (1996). Identification of a cytoplasmic domain important in the polarized expression and clustering of the Kv2.1 K+ channel. J Cell Biol *135*, 1619-1632.

Srinivas, R. V., Balachandran, N., Alonso-Caplen, F. V., and Compans, R. W. (1986). Expression of herpes simplex virus glycoproteins in polarized epithelial cells. J Virol *58*, 689-93.

Tirabassi, R. S., Townley, R. A., Eldridge, M. G., and Enquist, L. W. (1997). Characterization of pseudorabies virus mutants expressing carboxy- terminal truncations of gE: evidence for envelope incorporation, virulence, and neurotropism domains. J Virol *71*, 6455-64.

van Vliet, K. (1993). Function of herpes simplex virus Fc- and C3-receptors a case of molecular mimicry. In Eijkman-Winkler Laboratory of Medical Microbiology (The Netherlands: Utrecht University), pp. 137.

Whitbeck, J. C., Knapp, A. C., Enquist, L. W., Lawrence, W. C., and Bello, L. J. (1996). Synthesis, processing, and oligomerization of bovine herpesvirus 1 gE and gI membrane proteins. J Virol *70*, 7878-84.

Yao, Z., Jackson, W., Forghani, B., and Grose, C. (1993). Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus-T7 RNA polymerase transfection system. J Virol *67*, 305-14.

Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. Ann Rev Cell Dev Biol *13*, 119-46.

Zsak, L., Zuckermann, F., Sugg, N., and Ben-Porat, T. (1992). Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell

transmission. J Virol *66*, 2316-25.

5 DISCUSSION

5.1 State of Knowledge Before My Studies Began

When I began my Ph. D. studies, little was known about the function of gE/gI in the life cycle of HSV. gE^- and gI^- HSV mutants could be grown in culture with no appreciable deleterious effects on viral replication, and therefore both gE and gI were termed "non-essential". Since it seemed unlikely that HSV would conserve "non-essential" genes, attention centred around a role for gE/gI as a modulator of the host immune response. Several groups including our own had shown that gE/gI could act as a receptor for the Fc domain of immunoglobulin G (IgG), and could protect HSV infected cells and virions *in vitro* from complement-dependent immune lysis (reviewed in section 1.4.2.2). However, there was no evidence that gE/gI acted as an IgG Fc receptor *in vivo*, and this evidence is still lacking five or six years later. There was some evidence that gE/gI affected HSV pathogenesis (reviewed in 1.4.2.3), but it remained unclear whether this was related to the loss of Fc receptor activity or to some other activity of the proteins.

In an effort to determine whether gE/gI dependent Fc receptor activity protected HSV *in vivo*, a collaboration was set up between our lab and that of Dr. Robert Hendricks (University of Illinois at Chicago), who was studying HSV pathogenesis in the eye. Using mouse and rabbit corneal models, where virus infection was initiated in the cornea and spread to the nervous system, we showed that the gE^- and gI^- mutants were markedly less pathogenic than wild

type virus, an observation that could not be explained by the loss of Fc receptoractivity. The differences in pathogenicity between wild type HSV and the mutants were evident prior to the appearance of anti-HSV IgG, and gE/gI binds mouse IgG poorly or not at all (Carter and Easty, 1981; Johansson et al., 1985). I also showed that gE^- and gI^- mutants were much less able to spread directly from cell-to-cell in monolayers of cultured human fibroblasts (Chapter 2). I extended these findings, showing that HSV gE/gI was required for spread of HSV into and in the nervous system (Chapter 3). The molecular mechanism(s) by which gE/gI facilitates cell-to-cell spread are still not well characterized, however, we suspected that gE/gI acted at the cell surface and specifically at cell junctions promoting movement of virus between cells. Therefore, to further characterize the details of how gE/gI functions in cell-to-cell spread, I examined the effects of gE/gI expression in polarized human epithelial cells (Chapter 4). Epithelial cells were chosen since the phenotype of the gE/gI mutants was most severe in these cells *in vitro* and because HSV infects and spreads through epithelial cells *in vivo*. I demonstrated that when expressed alone, gE accumulated in a perinuclear region within the cell and required the concomitant expression of gI to reach the cell surface. I also showed that gE/gI was specifically transported to the basolateral domain of epithelial cells, where it accumulated along the lateral surface of the plasma membrane. In addition, I found that gE/gI accumulation was restricted to only those lateral junctions in contact with another cell. This provided substantial evidence that gE/gI binds to cellular ligand(s) found at

cellular junctions in order to facilitate HSV cell-to-cell transmission. Together, these studies provide a framework for future examination of the process of HSV cell-to-cell spread. In subsequent pages, I will discuss the mechanism(s) involved in HSV cell-to-cell spread, and the roles that gE/gI may have in this process. I will also discuss the various mechanisms of cell-to-cell spread used by other viruses including other members of the α-herpesvirus family.

During the initial course of my investigation into the role of HSV gE/gI in cell-to-cell spread, several reports were published on the pseudorabies virus (PrV) gE and gI homologues of HSV. PrV causes a disease in pigs similar to that of HSV in humans, infecting the nasal and pharyngeal mucosa which is followed by viral uptake into the central nervous system (reviewed in Ben-Porat and Kaplan, 1985). It was known that attenuated vaccine strains of PrV (i.e. Bartha strain) did not express several PrV glycoproteins, among them the PrV homologues of HSV gE and gI (then referred to as gI and gp63, respectively) (Mettenleiter et al., 1985; Petrovskis et al., 1986). Expression of both PrV gE and gI appeared to be required for the full expression of virulence following intracerebral infections of chickens (Mettenleiter et al., 1988). Moreover, the PrV Bartha strain exhibited altered neurotropism and decreased neurovirulence in the rat visual system (Card et al., 1991). These data led two groups to investigate how PrV gE/gI may be involved in viral spread. Zsak et al., (1992), found that PrV gE$^-$ mutants produced small plaques on chicken embryo fibroblasts in the presence of PrV neutralizing antibodies. Based on this and other experiments, they proposed that PrV gE

promoted the transmission of virus by the direct cell-to-cell route (Zsak et al., 1992). Similarly, studies by Card and colleagues demonstrated that a gE deletion within PrV could account for the observed changes to the neurotropism and virulence of the Bartha strain compared to a fully virulent wild type PrV strain (Card et al., 1992). There was some evidence that HSV gE$^-$ mutants had reduced pathogenicity (section 1.4.2.3), but an association between the loss of gE expression and defects in viral spread was not recognized, nor was it known if observations with PrV gE$^-$ and gI$^-$ mutants could be extended to HSV. My studies showing that HSV gE- and gI- mutants spread poorly in fibroblasts and epithelial cells confirmed what was found with PrV gE- mutants. In addition I have added to our understanding of the role of gE/gI in HSV pathogenesis and mechanisms by which gE/gI promotes α-herpesvirus cell-to-cell spread by showing that gE/gI localize to cell junctions and function in cell-to-cell spread between neurons.

5.2 Cell-to-Cell Spread of Virus

5.2.1 Biological Importance

The principle definition of HSV pathogenicity is the ability of HSV to replicate in epithelial tissues, gain access to the nervous system and establishes a latent infection (Whitley, 1996). The human host, however, is armed with a series of defences to protect itself from either a primary HSV infection or if infection does take place, to limit and prevent secondary infections (reviewed in Roitt, 1988). Primary HSV infections are inhibited by IgA present within the

external body fluids of the host. This IgA acts by inhibiting viral adsorption and infection of epithelial cells. In the event of viral infection of epithelial cells, IgE can bind to viral antigens which can lead to mast cell activation (degranulation). Mast cell degranulation releases immune mediators that recruits agents of the host immune response to the site of infection such as IgG, complement and immune cells (e.g., T-cells). IgG neutralizes HSV by either stereochemically inhibiting binding of virus to cell surface receptors or by inducing both complement-dependent and -independent immune lysis of HSV infected cells, while T-cells are directly cytotoxic to virus infected cells. In addition, during HSV recrudescent disease, HSV is faced with high levels of anti-HSV antibodies and a primed population of immune cells that can be rapidly expanded following exposure to the viral antigens. In spite of these host defences, HSV replicates and spreads quickly and efficiently through the skin and mucosal epithelium of humans causing ulcerative and vesicular lesions.

HSV has evolved an efficient means of propagating and disseminating viral progeny despite the host immune defences. One simple, but exceedingly important aspect of HSV biology is its ability to replicate and spread quickly and efficiently from infected cells to uninfected neighbours. First, rapid replication and spread of progeny virus to uninfected cells makes HSV less susceptible to immune recognition and cytolysis. Second, efficient transmission of virus directly from an infected to uninfected cell without appearance of virus in the extracellular compartment would decrease the likelihood of neutralization by IgG and

complement.

5.2.2 Cell-to-Cell Spread of α-herpesviruses

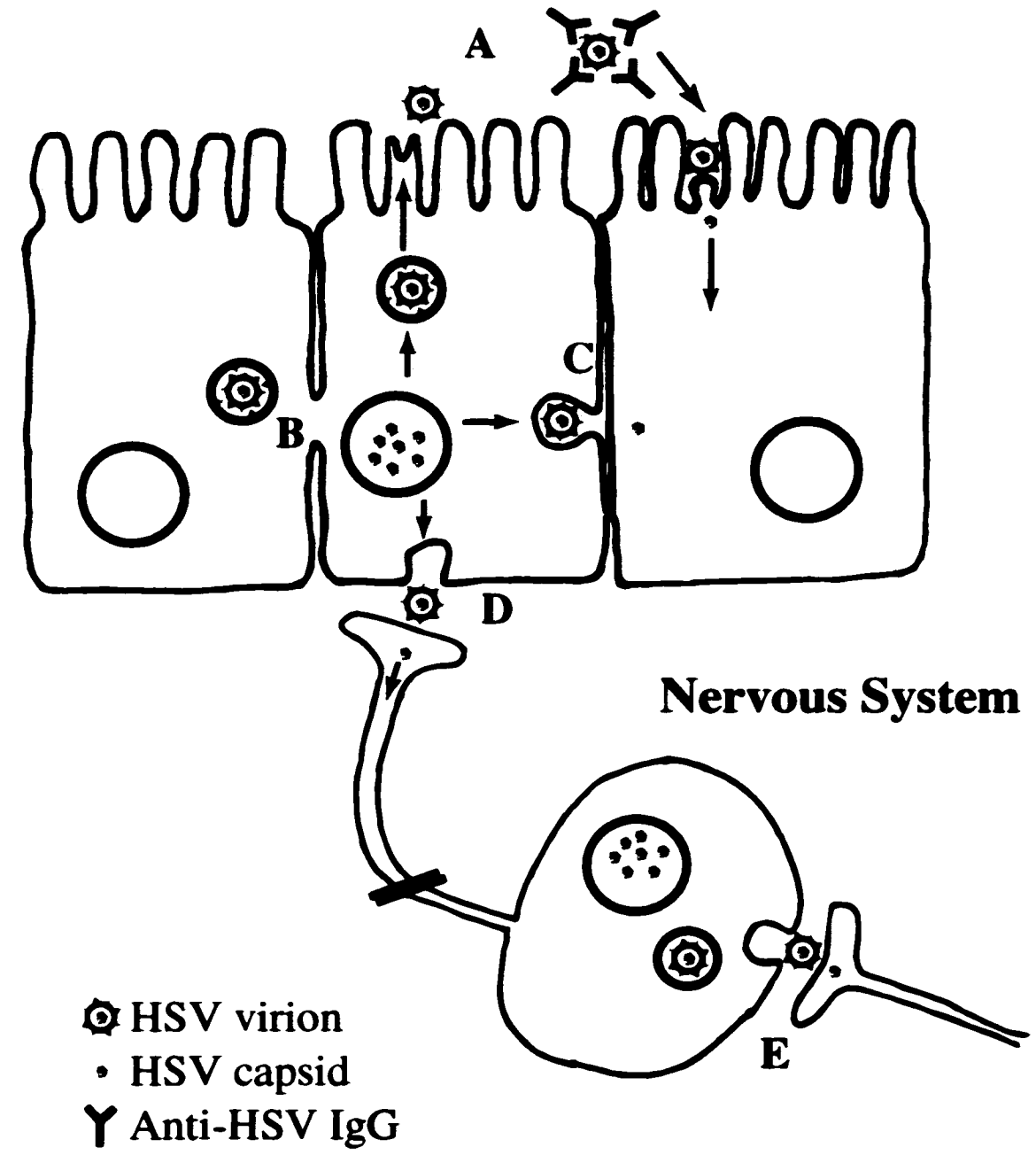
For the most part, studies of HSV spread, and the spread of other α-herpesviruses, have been focused on the release of virus from cells into the extracellular compartment, followed by binding to, and infection of neighbouring uninfected cells. Direct cell-to-cell transmission of HSV has many similar properties since virions are released from cells but probably into spaces between cells that are protected by cell junctions. Although many of the proteins involved in egress and entry of extracelluar virus are also involved in cell-to-cell spread of HSV, the effects of these of these proteins in cell-to-cell spread may be subtly different for the two different processes. In addition, α-herpesviruses have evolved proteins e.g., gE/gI, that are apparently involved solely in facilitating cell-to-cell transmission and do not affect entry of extracellular virus. Other viral proteins, essential for entry of extracellular virus, play a less important role in cell-to-cell spread.

For α-herpesviruses, and likely other viruses which replicate in a variety of different tissues, the mechanisms that allow efficient cell-to-cell spread must work efficiently within well in different cell types. HSV, for example, replicates and spreads between epithelial cells and fibroblasts in the epithelial mucosa, in the skin, and in the eye, and is taken up by axons of innervating sensory neurons and then spreads through the nervous system. This cycle of HSV replication illustrates the requirement for the virus to develop mechanisms which will

efficiently transmit progeny between and within cellular environments.

There are at least five different ways by which α-herpesviruses are spread from cell-to-cell (Figure 5.1): i) spread by extracellular virus (Fig 5.1A), ii) cell-cell fusion (Fig 5.1 B), iii) direct cell-to-cell spread without fusion (i.e., between epithelial cells; Fig 5.1 C), iv) neuron-to-neuron spread (i.e., trans-synaptic transfer; Fig 5.1 E)), and v) epithelial-to-neuron transmission (Fig 5.1 D). Some of these mechanisms of cell-to-cell spread share common properties with one another.

Figure 5.1 Possible mechanisms of HSV transmission. A) Extracellular virus may be released from an infected cell which can then bind to and enter. This mode of spread is particularly susceptible to neurtralizing anti-HSV antibodies; B) HSV infection can induce syncytia formation in which infected cells recruit neighbouring uninfected cells into large polykaryocytes following fusion of the cells' plasma membranes; C) direct cell-to-cell transmission of HSV involves transfer of infectious virus across junctions formed between infected and uninfected cells; D) at the site of primary infection HSV spreads from epithelial tissues to neurons, whether it occurs in a manner similar to A or C is not known; E) HSV spreads directly across the synaptic cleft either in the retrograde or anterograde directions, retrograde spread of virus is depicted here;


Peripheral Epithelia
A
B
C
D
Nervous System
E
HSV virion
HSV capsid
Anti-HSV IgG

5.2.2.1 Extracellular Virus

The cell-to-cell spread of HSV by the extracellular route is the simplest of mechanisms, yet remains very important for the dissemination of HSV from one individual to another. This process involves the release of virus particles from an infected cell into the extracellular space and body fluids (i.e., saliva) and subsequent adsorption and entry into uninfected cells (refer to Fig 5.1 A). However, the release of extracellular virus can be inefficient with some cells. Electron micrographs of HSV infected cells grown in culture frequently show cell surfaces encrusted with virions and less than 10% of infectious virus is released into the medium of HSV infected monkey Vero cells. VZV particles remain exclusively cell associated, and attempts at producing infectious cell free VZV in culture have remained unsuccessful (reviewed in Cohen and Straus, 1996). Spread of α-herpesviruses by extracellular routes likely plays a less important role in tissues where cells are closely associated, e.g. in solid tissues. Release of virus into the extracellular space also places the virus at risk of neutralization by anti-viral antibodies. In plaque assays with wild type HSV and PrV, the presence of neutralizing antibodies at the time of infection can inhibit plaque formation, yet if the antibodies are present in the medium after virus has enters cells there is little effect on the size of plaques produced (Dingwell et al., 1994; Zsak et al., 1992). In spite of this vulnerability to antibodies, cell free or extracellular virus is likely to be the principal means for viral transmission between individuals. Primary HSV-1 infections of the epithelial mucosa usually results following direct contact with

ulcerative lesions or infected secretions (Corey and Spear, 1986). The efficiency of spread between individuals through contact with infected secretions is not known.

5.2.2.2 Cell-Cell Fusion (syncytia formation)

HSV infection of certain cultured cells can induce syncytia formation in which infected cells can recruit neighbouring uninfected cells into large polykaryocytes following fusion of the cells' plasma membranes (see section 1.2.1.6; Fig 5.1 B). The importance of HSV induced cell-cell fusion in viral transmission *in vivo* is unclear. Although fused cells are seen in HSV lesions, very few of the HSV clinical isolates derived from these lesions produce syncytia in cultured cells. The majority of syncytial strains of HSV arise spontaneously during laboratory passage of the virus and are due to mutations in a number of HSV membrane proteins and glycoproteins (see section 1.2.1.6). The problem of reconciling the low frequency of syncytial strains in clinical samples of HSV with the high levels of syncytial viruses in lab stocks may be related to the fact that syncytial strains are less able to replicate and propagate *in vivo*. In addition, syncytial strains of HSV may cause cell-cell fusion in one cell type, yet be unable to do so in others. Thus, the polykaryocytes observed *in vivo*, may involve cells that are readily fused by HSV. Hence, it becomes important to study HSV induced cell-cell fusion, and the role that this mechanism may play in HSV cell-to-cell transmission, in a biologically relevant cell type.

Another question that arises is whether HSV induced cell-cell fusion is

actually a mechanism of viral transmission between cells. HSV induced cell-cell fusion does not require the production or release of infectious virus, and hence, the recruitment of a neighbouring uninfected cell into a polykaryocyte does not necessarily result in spread of virus to an uninfected host, and initiation of a new round of replication. However, limited cell fusion may be a rapid means to spread virus laterally between cells and through tissues. Since virus induced cell-cell fusion recruits a neighbouring uninfected cell, progeny virus would be able to infect cells which were not previously in contact with the originally infected cell. Consequently, only one round of replication would be required for virus to spread to and infect the third outlying cell, instead of two rounds of viral replication. Thus, limited cell-cell fusion in addition to direct cell-to-cell spread (see below), would be an efficient means to amplify and spread virus quickly through epithelial tissues. Moreover, this increased speed by which virus is spread may improve the likelihood of virus infecting axons of sensory neurons prior to viral clearance by the host immune response.

5.2.2.3 Direct Cell-to-Cell Spread Between Similar Cells (without fusion).

Direct cell-to-cell transmission of HSV appears to involve the transfer of infectious virus across junctions formed between infected and uninfected cells (Fig 5.1 C). This mechanism of virus spread is likely an important route of cell-to-cell spread *in vivo,* as HSV is largely cell associated and because virus remains resistant to neutralization by anti-HSV antibodies. In monolayers of cultured

cells, HSV readily replicates and spreads between cells even in the presence of neutralizing serum. This observation may account for the ability of HSV to produce secondary lesions in patients that have high anti-HSV antibody titres. Thus by spreading in a manner resistant to antibody neutralization, HSV can replicate and spread through epithelial tissues of individuals and cause secondary lesions even under conditions of a primed humoral immune response. The mechanisms by which HSV spreads across cell junctions remain unclear, and will be discussed in more detail in section 5.4.

5.2.2.4 Direct Neuron-to-Neuron Spread (trans-synaptic transfer).

Numerous studies have established the ability of HSV to spread efficiently along direct synaptic pathways (Bak et al., 1977; Kristensson et al., 1982; Norgren and Lehman, 1989; Norgren et al., 1992; Ugolini et al., 1987; Ugolini et al., 1989). Although it is unclear how HSV is spread between neurons, the ability of HSV to follow established synaptic connections, suggest that the virus spreads directly across the synaptic cleft, in a manner largely resistant to antibody neutralization (Price et al., 1982; discussed in more detail in section 5.4.3; Fig 5.1 E). This characteristic has led to the use of HSV and other α-herpesviruses as transneuronal tracers, in mapping synaptic pathways *in vivo* (reviewed in Kuypers and Ugolini, 1990). Conventional transneuronal tracers such as nontoxic tetanus toxin fragments, horseradish peroxidase, and the lipophilic dye DiI, are limited for extensive mapping through second and third order neurons as only a small fraction of the tracer is transferred trans-synaptically.

α-herpesviruses however, are well suited for tracing experiments because they are self-amplifying cell markers. Virus replication within each neuron ensures a high signal for detection using conventional immunohistochemical techniques.

Trans-synaptic transfer of HSV in humans is uncommon, as HSV spread is usually restricted to ganglionic neurons where the virus establishes a latent infection. Any spread from these latent sites predominantly leads to anterograde transport of virus back to peripheral tissues, yet in rare instances, virus spreads in the retrograde direction further into the CNS. The result can be catastrophic for affected individuals; herpes simplex encephalitis (HSE) has a mortality rate of 70% or greater if left untreated (Roizman and Kaplan, 1992). HSE is the leading form of sporadic encephalitis (Picard et al., 1993). What is puzzling is the low incidence of HSE. The chance of contracting HSE is approximately 2.3 in a million, yet between 70-80% of the population suffers from mucocutaneous HSV lesions and presumably harbour latent virus in their trigeminal ganglia (Whitley, 1996). Thus there must be distinct mechanisms in place to restrict transport and replication of HSV within the brain. As infections in the brain does not allow virus to spread between individuals, it makes greater evolutionary sense for HSV to limit spread to only first order neurons so that infected hosts are not killed, and virus can spread to other individuals by moving back to the mucosal tissues.

5.2.2.5 Epithelia-Neuron Spread.

In order to establish a latent infection in ganglia, HSV must first infect sensory axons within peripheral mucocutaneous tissues. At the site of the

primary infection there is an epithelial-neuronal spread of infectious HSV (Fig 5.1 D). Likewise following reactivation, virus spreads from the axon to epithelial cells. The mechanism by which HSV spreads from an epithelial cell to a neuron has not been well characterized. It is not known whether virus spreads in the form of extracellular virus or by direct transfer across an axonal connection with the epithelial cell. The study of this mode of viral spread is difficult in an animal model since it is both hard to visualize the process and difficult isolating it from the other forms HSV cell-to-cell transmission. However, the development of an *in vitro* system by Penfold and colleagues (1994), may provide the means to address this mechanism of cell-to-cell spread (Penfold et al., 1994). This *in vitro* system is a modified dual Campenot chamber (Campenot, 1977), in which human foetal dorsal root ganglia are cultured inside a central inner chamber (a cloning cylinder sealed with silicon grease to a coverslip), with autologous skin explants grown in an exterior chamber. Axons, extending from the ganglionic neurons, pass underneath the cloning cylinder into the exterior chamber where the axons develop connections with epidermal keratinocytes. Once these connections are made, neurons can be infected with virus, and the spread of HSV from axon to epidermal cells can be studied in detail.

When neurons within the cloning cylinder were infected with HSV, enveloped particles were later observed in the cytoplasm (Penfold et al., 1994), similar to previous results of HSV infected human, mouse, and rat neurons (Card et al., 1993; Hill et al., 1972; Kristensson et al., 1974; Lycke et al., 1988; Lycke et al.,

1984). However, only unenveloped capsids were observed within axons at the epidermal-axon interface (Penfold et al., 1994). This observation was surprising, in light of the current paradigm of α-herpesvirus egress (see 1.2.2.11) in which viral nucleocapsids are transported to cell surface within internal cell membrane cisternae. Naked nucleocapsids were often seen within axons in preparations of neurons infected *in vitro* and *in vivo*, but it was thought that these capsids represented somatopetally transported virus (i.e, virus that has infected an axon and was in the process of being transported to neuronal cell body); while enveloped capsids appeared within the axon shortly after viral replication, and thus, these particles were believed to be progeny virus being transported from the neuron to the periphery (Card et al., 1993; Hill et al., 1972; Kristensson et al., 1974; Lycke et al., 1988; Lycke et al., 1984). Since the epidermal cells were efficiently infected in the Penfold system, the spread of HSV from neuronal soma to axon tips in contact with epidermal cells may represent a novel mechanism of viral assembly, egress, and spread. Scanning electron micrographs displayed the presence of nodules concentrated at the surface of axon terminals which labelled with gold-conjugated anti-gD antibodies. From these data, it was suggested that egress of HSV at axon terminals may have occurred by an exocytosis mechanism in which virus budded into patches of viral glycoproteins at the axon surface (Penfold et al., 1994). However, as the emergence of viral particles from the axon were not observed, it remains to be determined whether viral egress could occur by exocytosis. Nevertheless, this system promises to be invaluable in elucidating

the steps of viral transfer from neuron to epithelial cells. Questions concerning the susceptibility of emerging HSV virions to neutralizing antibodies can be addressed by adding antibodies to the external chamber, and, in addition, neurons can be infected with HSV mutants in order to test the roles of viral glycoproteins during HSV transmission.

5.3 Cell-to-Cell Spread By Other Enveloped Viruses.

All enveloped viruses that grow and spread in solid tissues are confronted by similar obstacles that must be overcome in order to successfully spread from cell-to-cell. The strategies may vary from one virus to another, depending on the objective: for example a virus may spread from cell-to-cell using one mechanism if the infected and uninfected cells are in close apposition, while a different mechanism may be used to infect cells found at a distance. Many enveloped viruses have developed similar mechanisms of spread due to a strong selective pressure elicited by the host's immune response. Here I describe some of the mechanisms for direct cell-to-cell spread by two enveloped viruses from different virus families, human immunodeficiency virus (HIV), and vaccinia virus. I will discuss how these two viruses have evolved mechanisms to solve the problem of how to move from one cell to a closely apposing cell, and how these approaches are similar to the strategies of cell-to-cell transmission of HSV.

5.3.1 Human Immunodeficiency Virus

HIV is a member of the lentivirus family of retroviruses (reviewed in Luciw, 1996). The viral RNA is packaged within a nucleocapsid that is surrounded by a

lipid envelope derived from host cell's plasma membrane. The site of assembly of HIV is not well characterized. Either RNA is packaged into nucleocapsids in the cytoplasm where the complex then moves to the plasma membrane, or capsid assembly occurs at the plasma membrane. In either case, nucleocapsids acquire their viral envelopes following budding through the host cell plasma membrane. Together, virions are approximately 100 nm in diameter. HIV predominantly infects $CD4^{+ve}$ cells such as T-cells, and cells of the monocyte-macrophage lineages. In some cases, HIV can infect epithelial and endothelial cells that do not express detectable levels of CD4 (reviewed in Hirsch and Curran, 1996). The spread of HIV among cells and between individuals may take on different forms (reviewed in Phillips, 1994). First, HIV may spread as cell-free virus secreted by infected circulating mononuclear cells. Alternatively, cell-cell fusion may occur between infected cells and neighbouring uninfected cells. Finally, HIV may be transmitted by direct cell-to-cell spread, without fusion, between circulating mononuclear cells, or between mononuclear cells and the epithelium.

In infected individuals there are two pools of virus: cell associated and cell-free virus. In tissue culture, most susceptible cells can be efficiently infected with both HIV viral pools, however, there is considerable debate over which pool plays the more significant role in viral dissemination *in vivo*. Unfortunately, quantitative analysis of the levels of each pool in either blood, semen, or secretions has revealed little as to the importance of one versus the other. Nonetheless, recent studies have suggested that cell mediated cell-to-cell spread may have an

important role in the HIV growth cycle (Phillips, 1994).

Similar to HSV, direct cell-to-cell spread of HIV may take place following cell-cell fusion, as syncytia formation of HIV infected cells has been readily observed in culture. When T-cells were infected with H9 HIV-infected cells, viral proteins and unintegrated DNA were detected within hours, and further examination of these cultures demonstrated the development of polykaryocytes (Sato et al., 1992). In contrast, when the cells were infected with extracellular virus, viral DNA and protein synthesis were not evident in T-cells until several days after infection nor was there any evidence of syncytia formation. This suggests that HIV induced cell-cell fusion may play a critical role in efficiently spreading the virus among cells. In addition, syncytial spread of HIV between cells may protect virus from anti-HSV antibody neutralization. In studies in which peripheral blood lymphocytes (PBL), from HIV patients were mixed with mitogen activated PBL from uninfected individuals, HIV specific antibodies had no effect on syncytial HIV induced spread (Gupta et al., 1989). Thus, HIV can use cell-cell fusion to efficiently spread to other cells, in a manner which is invisible to antibody neutralization. In spite of these observations in culture, *in vivo* observations of HIV induced syncytia are rare (Hirsch and Curran, 1996), and therefore, the significance of cell-cell fusion as a mechanism of HIV transmission remains unclear.

α-herpesviruses spread within epithelial tissues, whose cells are held together by highly ordered structural complexes, producing extensive stretches

of plasma membrane contact between cells. During direct cell-to-cell spread of HSV, virus appeared to cross these junctions in a manner largely resistant to antibody neutralization (Dingwell et al., 1994). For HIV, which infects circulating immune cells that do not normally initiate extensive sites of cell-cell contact, the release and spread of virus between cells would seem to be highly susceptible to antibody neutralization. It is apparent, however, that HIV has developed a second, antibody neutralization-resistant form of cell-to-cell spread that does not require fusion between cells. Infection of epithelial cells is difficult with cell-free or extracellular virus but can be achieved efficiently following the addition of chronically HIV-infected T-cells (Tan et al., 1993). This may be related to the observation that there is polarized budding of HIV from the infected T-cells and monocytes, and this budding can be directed toward uninfected cells (Fais et al., 1985; Pearce-Pratt et al., 1994; Perotti et al., 1996; Phillips and Bourinbaiar, 1992; Tan et al., 1993). Transmission electron microscopy studies demonstrated a polarized budding and release of HIV to regions of the T-cells or monocyte plasma membranes involved in cell-cell contact with the epithelial cell (Fais et al., 1985; Pearce-Pratt et al., 1994; Perotti et al., 1996; Phillips and Bourinbaiar, 1992; Tan et al., 1993). In addition, virus was concentrated on the plasma membrane of microvilli-like structures that had developed at these sites of polarized budding (Tan et al., 1993). Viral spread could not be blocked by the addition of anti-gp120 serum (Phillips and Bourinbaiar, 1992). The intercellular adhesion molecule (ICAM-1), was also concentrated at these polarized sites, and

co-localized with HIV matrix protein p18 (Fais et al., 1985), and similar observations (polarized distribution of ICAM-1 and virion assembly) were made in single HIV infected $CD4^+$ cells not in contact with other cells (Fais et al., 1985).

Lymphocyte and mononuclear cell immune functions are regulated, in part, by the specific interactions of cell adhesion molecules ICAM-1 and its ligand, leucocyte function-associated antigen (LFA-1) (Springer, 1990). Inhibition of ICAM-1 /LFA-1 binding specifically decreases the capacity of HIV to spread from cell-to-cell (Hildreth and Orentas, 1989). Together, these observations suggest that HIV has created an artificial epithelial-like cell junction between the infected and uninfected cells, where virus can spread efficiently in a microenvironment devoid of anti-HIV antibodies. First, the polarized budding of HIV at the site of cell-cell contact increases the chance of virions attaching to and entering the neighbouring uninfected cells. Second, coupled with the microvilli development at sites of cell-cell contact, the effective surface area available for virus-cell and cell-cell interactions are increased, which, in addition to improving the chances of virion binding to cell surface receptors, also creates a confined site for cell-to-cell spread allowing the virus to evade immune neutralization by anti-HIV antibodies. Finally, the induced polarized accumulation of ICAM-1 at sites of virion maturation and budding may facilitate HIV cell-to-cell spread by increasing the chance of an infected cell binding to $LFA\text{-}1^{+ve}$ cells.

5.3.2 Vaccinia Virus

Vaccinia virus is a member of the *poxviridae* family of large DNA viruses. Virions appear as smooth rounded rectangles (approximately 350nm x 270nm), that contain a double stranded genome of 130 kbs encoding up to 200 open reading frames (ORFs) (reviewed in Moss, 1996). Viral replication occurs in the cytoplasm producing a variety of enveloped forms of progeny virus. The first infectious viruses formed are referred to as intracellular mature virions (IMV), and acquire their lipid envelope following envelopment by membrane cisternae of the intermediate compartment between the endoplasmic reticulum (ER), and the Golgi complex (Sodeik et al., 1993). Budding of IMVs into the *trans*-Golgi network produce the second enveloped form of intracellular virions called intracellular enveloped viruses (IEV), which have a total of 4 membrane envelopes (Schmuelz et al., 1994). There are two forms of extracellular virions which are produced after fusion of the outer IEV envelope with the plasma membrane: the first is referred to as cell-associated enveloped viruses (CEVs), and remain attached to the plasma membrane (Blasco and Moss, 1991). The second form of extracellular virus are virions released entirely from cells and are referred to as extracellular enveloped virions (EEVs) (Payne, 1980). The relative roles of the various forms of the virus: IMVs, IEVs, CEVs, EEVs, in propagating infections have been the subject of intense effort over the last several years, and several important insights have been made into the unique mechanisms of vaccinia cell-to-cell spread.

EEVs are the principle form of enveloped vaccinia viruses involved in long

range cell-to-cell transmission of vaccinia virus to other cells both *in vivo* and *in vitro* (Boulter and Appleyard, 1973; Payne, 1980). Long range transmission of vaccinia virus is measured *in vitro* as the ability of virus strains to produce secondary plaques. In liquid overlays in which neutralizing antibodies are absent, smaller secondary plaques develop, which together appear comet-like, emanating from the central primary plaque. Comet shaped plaque distributions (i.e., long range spread) correlated directly with the quantity of EEV produced; those strains that produced high levels of EEVs exhibited higher numbers of secondary plaques (i.e., larger cometlike-shaped), while those strains that made low levels of EEVs, produced little if any secondary plaques and hence smaller comets (Payne, 1979; Payne, 1980). However, low EEV producing strains can produce large comets if the infected cells are treated during plaque development with low levels of trypsin. Trypsin released the cell-bound CEVs, essentially producing EEVs capable of long range viral dissemination (Blasco and Moss, 1992). In contrast to comet formation (i.e., formation of secondary plaques), primary plaque formation is independent of the quantity of EEVs produced, as primary plaques were of identical size between high and low EEV producing vaccinia strains. Therefore, another infectious form of the virus, either INV or CEVs must be involved in direct cell-to-cell spread. Vaccinia virus mutants with lesions that affect several membrane glycoproteins did not produce CEVs and displayed a small plaque phenotype, yet, these mutants produced normal levels of INVs suggesting that CEVs were required for vaccinia cell-to-cell transmission and

plaque production (Blasco and Moss, 1991; Duncan and Smith, 1992; Rodriguez and Smith, 1990; Schmutz et al., 1991; Wolffe et al., 1993). In addition, while EEV specific antibodies block comet formation, they had no effect on primary plaque production (Boulter and Appleyard, 1973). Since CEVs are cell associated, they are in the appropriate position to mediate cell-to-cell spread, and CEVs are often present in confined spaces between the plasma membranes of infected and adjacent uninfected cells. Recent studies have shown that CEVs associated with vaccinia virus-induced microvilli are essential for efficient vaccinia virus cell-to-cell transmission (Wolffe et al., 1997).

Vaccinia virus infection of susceptible cells induced the formation of large actin based microvilli (actin bundled by α-actinin, fibrin and filamin) with a single IEV virion at the tip (Cudmore et al., 1995; Hiller et al., 1979; Krempien et al., 1981; Stokes, 1976). As the actin tails formed, IEVs were propelled with a speed of 2.8 $\mu m\ min^{-1}$ toward the cell surface where the outer envelope of the IEV fused with the plasma membrane. The CEVs remained associated with the microvilli, and as the actin tail grew, the microvilli projected the CEVs into neighbouring cells (Cudmore et al., 1995). The A34R glycoprotein has been shown to be involved in the induction of viral microvilli, suggesting that A34R may be involved in actin tail nucleation at the surface of the viral particle (Wolffe et al., 1997). The failure of the A34R mutants to induce viral microvilli resulted in the decreased ability of vaccinia to spread from cell-to-cell and form primary plaques. Thus, by inducing the formation of actin bundles and microvilli, and becoming

associated with this cellular machinery, vaccinia ensures itself an efficient means of cell-to-cell spread.

While the mechanisms of cell-to-cell spread for HIV, vaccinia, and HSV are quite different, these viruses have all exploited the benefit of close membrane apposition to increase the efficiency of viral transmission. HIV specifically buds from the plasma membrane at sites of cell-cell contact rather than budding nonspecifically at other regions of the plasma membrane. Vaccinia has used the cellular cytoskeleton to extend viral progeny into neighbouring uninfected cells. Finally, HSV glycoprotein complex gE/gI binds to components of cell junctions to facilitate viral cell-to-cell spread. No matter what the specific mechanism, the desired outcome is still the same: viral progeny is spread efficiently across cellular membranes from an infected to an uninfected neighbour in a manner that is largely resistant to anti-viral neutralizing antibodies.

5.4 Mechanistic Properties of HSV Direct Cell-to-Cell Spread

Cell-to-cell spread likely occurs through a number of different mechanisms which invariably requires viral components used in both entry of extracellular virus and viral egress. Separating the roles of these viral components in either egress or entry from their functions in cell-to-cell spread has proven difficult. The plaque assay as a measure of cell-to-cell spread does not specifically measure cell-to-cell spread, making it difficult to ascribe a function in cell-to-cell transmission to a viral protein. For example, small plaque phenotypes can be due to any number of defects in viral replication, defects in entry of extracellular virus, viral

gene expression, DNA replication, capsid envelopment, or egress of virions to the cell surface. Studies of cell-to-cell spread *in vivo* must also be evaluated with some care, due to the many influences of the host immune response on viral replication. In addition, the study of neuron-to-neuron spread is complicated by the fact that infection of the nervous system often requires previous infection of peripheral tissues. Despite these obstacles, several of the key components in HSV cell-to-cell spread have been determined, including proteins which specifically facilitate this process.

5.4.1 Viral Components Required For Cell-to-Cell Spread.

All of the glycoproteins (gB, gD, gH/gL), required for entry of cell-free (extracellular) virus are also required for cell-to-cell spread. Mutants in any of these glycoproteins do not form plaques on noncomplementing cell lines (Cai et al., 1988; Forrester et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993), and monoclonal antibodies against gB, gD, and gH can inhibit development of plaques (Buckmaster et al., 1984; Highlander et al., 1988; Highlander et al., 1987; Minson et al., 1986). There is some evidence that these glycoproteins may act differently in cell-to-cell spread than in viral penetration. Several complement-independent neutralizing anti-gB monoclonal antibodies that prevented viral penetration, did not inhibit plaque development, even when used at concentrations well in excess of what was required to restrict viral penetration (Navarro et al., 1992). Similarly, neutralizing anti-gD monoclonal antibodies, had no effect on the ability of HSV to form plaques on Vero cell monolayers (i.e, failed

in plaque reduction assays; (Buckmaster et al., 1984). However, these antibodies may not gain access to viral glycoproteins which are found at cell junctions. Therefore, these observations could be due to an inability of the antibodies to bind to the neutralizing epitopes of gB or gD when they are expressed either at the lateral membrane of infected cells, or on the virion envelope when virions are present at cell junctions. However, experiments with antibody-resistant HSV gH mutants argue against this explanation (Gompels et al., 1991). In an effort to determine the epitopes of gH that are involved in viral entry and cell-to-cell spread, HSV mutants were derived which were resistant to neutralization and plaque reduction by the anti-gH MAb LP11 (Buckmaster et al., 1984; Gompels et al., 1991). The gH sensitivity to LP11-mediated neutralization was determined by infecting Vero-F6 cells (express wild type gH), with the HSV gH mutants and then plaquing the infectious virus produced on Vero cells in the presence of LP11. Since the virus produced in this assay will have wild type gH within the virion envelope, the sensitivity of HSV to LP11 neutralization can be measured. Likewise, the gH sensitivity to LP11-mediated plaque reduction was determined by infecting Vero cells with HSV gH mutants and then plaquing progeny virus on Vero-F6 cells expressing wild type gH on the cell surface. Surprisingly, some gH mutants were sensitive to neutralization but insensitive to plaque inhibition while others were the opposite, sensitive to plaque reduction but insensitive to neutralization. This suggests that the conformation of gH on the surface of cells and the virion envelope are different, and hence gH may function differently in

cell fusion during entry of extracelluar virus than during cell-to-cell spread. These results also suggest that transfer of virus across cell junctions does not occur by transfer of extracellular virus since the ability of MAbs to neutralize infectivity does not correlate with plaque reduction (Buckmaster et al., 1984; Highlander et al., 1988; Navarro et al., 1992).

Several gB MAbs which have limited neutralization activities can strongly inhibit viral cell-to-cell spread (Highlander et al., 1988). Therefore, gB, gD, and gH/gL are likely to be involved at specialized intercellular contacts and promote virion envelope fusion with the neighbouring cell. It is interesting to note that MAb LP11 which neutralized HSV as well as inhibited cell-to-cell spread, had no effect on the development of infectious centres (Buckmaster et al., 1984), supporting the hypothesis that HSV preferentially spreads at specialized sites of cell-cell contact between infected and uninfected cells.

The ability of gD to mediate cell-to-cell spread depends in part on the presence of N-linked oligosaccharides. The gD QAA mutant which lacks N-linked sugars, produced smaller plaques on both Vero cells and human fibroblasts (Brunetti et al., 1995; Sodora et al., 1989; Sodora et al., 1991). The small plaque phenotype could not be ascribed to structural differences between QAA gD and wild type gD, and viral replication (i.e., production of infectious virus) did not appear to be affected by the mutations in gD (Sodora et al., 1991). There was no significant difference in the kinetics of viral penetration into Vero cells between the QAA mutant and the wild type virus, nor were there any differences in the

yields of either extracellular or cell-associated virus (Sodora et al., 1991). Together, these data suggest that gD functions in cell-to-cell spread in a different way than in viral penetration which does not require gD oligosaccharides.

The role for N-linked sugars on gD became clearer following the report by Brunetti et al., (1994), who showed that HSV gD could bind to both the cation-independent and -dependent MPRs (Brunetti et al., 1994) and that these receptors could mediate viral entry into primate cells (Brunetti et al., 1995). HSV entry could be partially blocked by either antibodies to MPR, soluble MPR, or by pentamannose-phosphate, a synthetic ligand for MPR (Brunetti et al., 1995). MPRs sort lysosomal enzymes at the *trans*-Golgi network to the endosomal pathway, by binding to N-linked oligosaccharides of enzymes which have been modified with mannose-6-phosphate (M-6-P) residues (reviewed in Dahms et al., 1989). Efficient cell-to-cell transmission of HSV required MPRs as well as the addition of M-6-P to gD; similar numbers of plaques were formed by HSV on human fibroblasts deficient in mannose phosphorylation (pseudo-Hurler cells), yet the plaque size was significantly smaller compared to normal fibroblasts (Brunetti et al., 1995). The QAA mutant also formed small plaques smaller plaques on both pseudo-Hurler and normal fibroblasts. These data suggest that phosphorylation of mannose residues on the N-linked sugars of gD is required for efficient HSV cell-to-cell spread and a lack of phosphorylation of gD sugars is responsible for the reduced replication of HSV in pseudo-Hurler cells. Although MPRs were not absolutely required for HSV entry, these data clearly demonstrated that both

MPRs and M-6-P residues on N-linked sugars of gD are important for some aspect of gD function in HSV cell-to-cell transmission, which may be different from the penetration of extracellular virus.

The PrV gD homologue is required for entry of extracellular virus, but is less important in viral cell-to-cell transmission (Peeters et al., 1993; Rauh and Mettenleiter, 1991). PrV gD^- mutants, previously grown on gD complementing cells, can infect gD^- cells and spread from cell-to-cell *in vitro* and *in vivo* (Peeters et al., 1992; Peeters et al., 1993). PrV gD is not required for trans-synaptic spread; gD mutants spread transneuronally into the CNS following infection of the nasal epithelium of pigs (Mulder et al., 1996), and into second order neurons following infection of the motor hypoglossal (XII) nerves of mice (Babic et al., 1993). Although HSV gD appears to be very important in cell-to-cell transmission, HSV gD^- mutants can also form minute plaques on normal human fibroblasts (three to five cells over a period of 56 hrs, while wild type virus produces plaques in the order of several hundred infected cells;(Brunetti et al., 1995). Together, these data on the roles of gD illustrate that the mechanism of cell-to-cell spread is, at least at some level, distinct from entry of extracellular virus.

Glycoprotein gM (UL10) is dispensable in cell culture and it has been suggested that gM plays a role in direct cell-to-cell spread *in vitro* and *in vivo* (Baines and Roizman, 1991; Baines and Roizman, 1993; Davis-Poynter et al., 1994; MacLean et al., 1991; MacLean et al., 1993). HSV gM^- mutants form

smaller plaques on Vero cell monolayers, and yields of infectious virus following infection at low MOIs (e.g., 0.001 pfu/cell) were 10 fold lower than with wild type virus (MacLean et al., 1991). However, Baines and Roizman (1991) reported 10 fold lower yields of virus with a gM$^-$ mutant when Vero and BHK cells were infected at high MOIs (5 pfu/cell) (Baines and Roizman, 1991). This casts some doubt as to whether gM plays a role in cell-to-cell transmission, or whether gM is required for some other aspect of virus replication. Recent studies with a PrV gM$^-$ mutant suggests that gM may play a role earlier in entry of extracellular virus as gM$^-$ mutants displayed a marked delay in viral penetration (Dijkstra et al., 1996).

The HSV glycoproteins gE and gI are the only viral glycoproteins that play a role in viral cell-to-cell spread and not apparently in entry of extracellular virus or egress of virus to the cell surface. We and others have showed that the HSV gE/gI complex was required for efficient cell-to-cell spread in fibroblasts and epithelial cells in culture, and in epithelial tissues *in vivo* (Balan et al., 1994; Dingwell et al., 1994). gE/gI function in facilitating cell-to-cell spread was first described in PrV and subsequent studies have established that gE/gI's role in facilitating cell-to-cell is conserved among all α-herpesviruses examined to date (Mallory et al., 1997; Mijnes et al., 1997; Rebordosa et al., 1996; Zsak et al., 1992).

Restricted spread of HSV or PrV gE$^-$ or gI$^-$ mutants could theoretically be due to any number of defects in viral replication, and therefore a detailed analysis of the replicative abilities of the gE$^-$ and gI$^-$ mutants was necessary. Kinetic

analysis of viral penetration into fibroblasts (Fig 2.8) and expression of early genes (Fig 2.9) showed that the gE$^-$ and gI$^-$ mutants did not have defects in adsorption onto the cell surface or in entry of extracellular virus. There were also no differences in the particle-to-pfu ratio between mutant viruses and the wild type parent (Balan et al., 1994). gE$^-$ or gI$^-$ HSV infected fibroblasts produced similar levels of both cell free and cell associated virus in single step growth curves (Fig 2.6). However, when either fibroblasts (Fig 2.7) or epithelial cells (Balan et al., 1994), were infected with the mutants using a low MOI, either in the presence or absence of neutralizing HSV-antibodies, viral yields were 10 (no antibodies) to 200 fold (+ antibodies) lower than with wild type HSV-1. For wild type viruses, the presence of neutralizing antibodies had little effect on plaque sizes, yet antibodies produced a substantial reduction in plaque size with the gE$^-$ and gI$^-$ mutants. As antibodies in the medium would theoretically neutralize extracellular virus particles released from infected cells, these results suggested that the primary mode of cell-to-cell spread of wild type HSV occurs across cell junctions in a manner resistant to antibody neutralization. It was also likely that the gE$^-$ and gI$^-$ mutant viruses spread poorly across these junctions. The studies by Balan et al (1994) lead to similar conclusions and involve independently generated gE$^-$ and gI$^-$ mutant viruses derived from a different wild type HSV-1 strain SC16 (Balan et al., 1994).

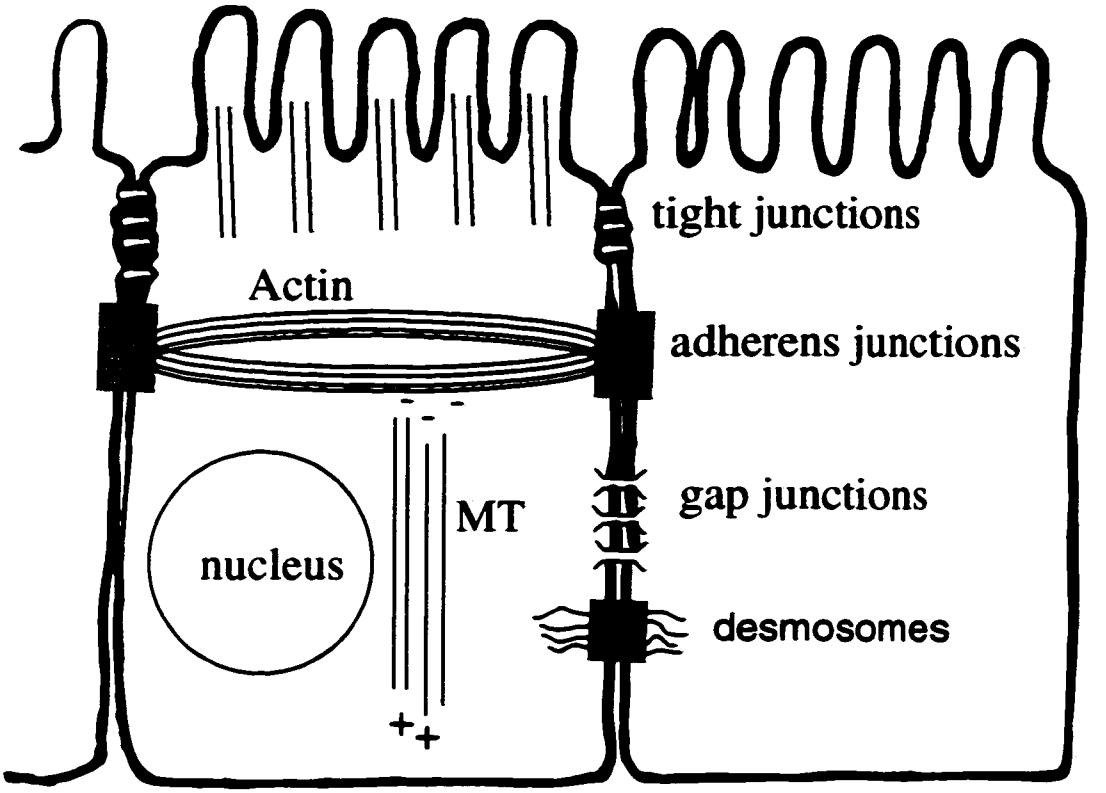
5.4.2 HSV Spread Across Epithelial Cell Junctions

Epithelial cell junctions are structures formed as neighbouring cells form complex protein-protein linkages between themselves. Among these structures are the adherens junctions, tight junctions, desmosomes, and gap junctions (Fig 5.2). One of the major structures that establish and maintain the integrity of cell junctions are adherens junctions, made up of the Ca^{+2}-dependent cadherins (reviewed in Ranscht, 1994; Yap et al., 1997). Cadherin mediated adhesion is regulated by the binding of cadherin ectodomains between neighbouring cells, forming a molecular zipper, thus binding the two cells together (Shapiro et al., 1995). Cadherins are linked to the actin cytoskeleton through interactions between their C-terminal domains and cytoplasmic proteins known as catenins (α-, ß-, and γ-catenin). ß-catenin binds to the distal region of the cadherin cytoplasmic domain and also binds to α-catenin, which in turn couples the cadherin-catenin complex to the cytoskeleton.

The formation of adherens junctions leads to rapid cellular remodelling of the cytoskeleton which provides essential cues required for epithelial polarization (reviewed in Drubin and Nelson, 1996; Gumbiner, 1990; Mays et al., 1994). Two microtubule networks develop: the first is a dense mat of short, randomly oriented microtubules located between the apical plasma membrane and Golgi apparatus, with a second set of bundled microtubules oriented longitudinally in the cell, with their plus ends directed toward the basal surface of the cell (Bacalloa et al., 1989; Bre et al., 1990). Microfilament bundles and other actin filaments reorganize to

subcellular regions within the epithelial cell. Each apical microvillus is composed of an actin filament core that extends into a meshwork of actin and other cytoskeletal components collectively called the terminal web, located underneath the apical plasma membrane (reviewed in Mooseker, 1985). Perhaps the most visible change to the actin cytoskeleton is the formation of prominent actin bundles encircling the apex of the lateral plasma membrane and these actin bundles are associated with tight junctions and adherens junctions (Kartenbeck et al., 1991). The assembly of all additional junctional complexes at the lateral surfaces between cells, including desmosomes, gap junctions, and tight junctions, depends upon the prior establishment of cadherin-mediated adherens junctions (Gumbiner and Simons, 1988; Gumbiner et al., 1988; Jongen et al., 1991; Marrs et al., 1995; Wheelock and Jensen, 1992). Tight junctions encircle the apical border at the apex of the lateral plasma membrane domain, forming both a morphological and physical border between the apical and basolateral plasma membrane domains (Balda and Matter, 1998). Once formed, tight junctions establish and maintain epithelial polarity by acting as a physical barrier to the paracellular transport of ions and small molecules, as well as the diffusion of proteins and lipid between the apical and basolateral plasma membranes.

Figure 5.2 Structure of epithelial cell junctions. At sites of cell-cell contact in epithelial cells, discrete junctions are formed. Tight junctions encircle the apical border at the apex of the lateral plasma membrane domain, forming both a morphological and physical border between the apical and basolateral plasma membrane domains (Balda and Matter, 1998). Once formed, tight junctions establish and maintain epithelial polarity by acting as a physical barrier to the paracellular transport of ions and small molecules, as well as the diffusion of proteins and lipid between the apical and basolateral plasma membranes. Below the tight junction are the Ca^{+2} dependent cadherin-mediated adherens junctions. Adherens junction formation is required for the formation of all other junctional complexes. Gap junctions form intercellular channels that allow for the free diffusion of ions between the cytoplasms of neighbouring cells. Desmosomes are cadherin-like junctions that provide structural and tensile strength to epithelial tissues. The distribution of microtubules (MT) and actin are also depicted.


tight junctions
Actin
adherens junctions
gap junctions
MT
nucleus
desmosomes

From our analysis of the gE$^-$ and gI$^-$ mutant viruses in Chapters 2 and 3, it appeared that gE$^-$ and gI$^-$ mutants spread poorly across cell junctions. We postulated that in order to facilitate HSV cell-to-cell transmission, gE/gI might be acting at cell junctions, possibly by binding to some ligand(s) that are concentrated at cell junctions. However, HSV causes a number of deleterious effects on cells, especially late after infection, most significantly the rounding up and separation of cells from one other. Therefore, the study of how gE/gI functions at cell junctions was made difficult by these HSV-induced cytopathic effects (CPE). In an effort to characterize gE/gI function in the absence of other HSV polypeptides, and without viral CPE, we chose to express gE and gI using replication-defective adenovirus vectors.

When both gE and gI were expressed without other HSV polypeptides in human polarized epithelial cells (HEC-1A cells), gE accumulated along lateral junctions between cells and colocalized with the adherens junction protein ß-catenin (Fig 4.4 C). Very little or no gE/gI was found on either the apical or basal domains of the plasma membrane (Fig 4.5 J-K) while another HSV glycoprotein, gD, was found predominantly on the apical surface (Fig 3.5 L). Similar observations were made with HSV -infected cells, in which gE/gI was observed exclusively on lateral surfaces of cells. This suggests that gE/gI was specifically sorted to the basolateral domain of polarized epithelial cells.

5.4.2.1 Basolateral Protein Sorting of gE/gI

The polarized distribution of membrane proteins in epithelial cells is established by three different mechanisms: i) the intracellular sorting and trafficking of proteins to basolateral versus apical plasma membrane domains, ii) retention of proteins in specific plasma membrane domains, and iii) physical restriction of proteins by tight junctions.

Targeting of membrane proteins to the basolateral plasma membrane is regulated by sorting signals within the cytoplasmic domain. Many of these protein sorting motifs are degenerate in that they either overlap or constitute signals involved in sorting to clathrin coated pits or endosomes (reviewed in Keller and Simons, 1997; Matter and Mellman, 1994; Mellman, 1996). Signals involved in sorting to basolateral domains can be: i) tyrosine based, in which the critical tyrosine is paired with a bulky-hydrophobic amino acid (YXXϕ) or ii) dileucine based (LL or LI), or iii) other signals not based on tyrosine nor leucine residues.

The finding that gE/gI is present on the lateral surfaces of polarized HEC-1A cells would support the hypothesis that gE and/or gI have basolateral targeting signals. gE contains a consensus tyrosine-based motif $_{472}$YADW$_{475}$, while gI has a consensus dileucine-based motif $_{388}$LL$_{389}$ within their respective cytoplasmic domains (McGeoch et al., 1985). It is not known at this time whether these signals regulate the intracellular targeting of gE/gI to the lateral plasma membrane of HEC-1A cells, targeting to endosomes, or whether these signals play any role in gE/gI-dependent HSV cell-to-cell transmission. However, these

tyrosine-based motifs are conserved among gE homologues of at least two other α-herpesviruses, PrV and VZV, suggesting that these sequences may play an important role in gE/gI function.

Within the cytoplasmic domain of VZV gE there are two tyrosine-based motifs and an acidic cluster of amino acids which coupled with two serine residues constitutes a casein kinase II (CKII) phosphorylation site (Alconada et al., 1996; Yao et al., 1993). Mutagenesis of these sequences suggested that both elements were required for TGN localization of VZV gE and recycling of gE from the cell surface (Alconada et al., 1996; Zhu et al., 1995; Zhu et al., 1996). These trafficking signals are also found in other proteins localized to the TGN such as the endoprotease furin (Jones et al., 1995). The cycling of furin from the TGN to the cell surface is dependent upon the phosphorylation of the CKII site within furin's C-terminal tail. In a non-phosphorylated state, furin remained predominantly TGN associated, but upon CKII-dependent phosphorylation of the serine residues (within a motif similar to that in VZV gE), furin was transported to the cell surface (Jones et al., 1995). Mutagenesis of the CKII site in VZV gE, abrogated gE localization to the TGN, suggesting that phosphorylation of gE, similar to furin, is important for gE trafficking. Indeed, localization of VZV gE to the TGN may have important implications in the egress of VZV from infected cells. It has been proposed by a number of groups that VZV egress from infected cells follows an envelopment-deenvelopment pathway (see section 1.2.2.11), in which naked VZV nucleocapsids acquired their final envelope by budding into the TGN

(Gershon et al., 1994; Jones and Grose, 1988; Zhu et al., 1995). TGN accumulation of VZV gE, which is the most abundant VZV glycoprotein produced in infected cells (Grose and Litwin, 1988), would be consistent with a role of the TGN during VZV egress, and a central role for gE in this process.

Analysis of the HSV and PrV gE genes revealed that both gE glycoproteins have CKII consensus sequences in their cytoplasmic tails downstream of the putative tyrosine-based sorting signals (McGeoch et al., 1985; Petrovskis et al., 1986), and further, gE has been shown to be phosphorylated on serine residues (Edson, 1993; Edson et al., 1987). These trafficking signals within the α-herpesvirus gE sequences are the most conserved elements within the C-terminal domains, strongly suggesting that these signals play an important role in gE function. It is important to note however, that the role of the tyrosine-based motif and the CKII site within the VZV gE gene were assessed following transient transfections of gE expressing constructs in Cos-7 and HeLa cells (Alconada et al., 1996; Yao et al., 1993). In contrast, our analysis of HSV gE trafficking was conducted by expressing gE using both Ad vectors and HSV in polarized epithelial cells. Note that loss of gE expression has little effect on HSV cell-to-cell spread in cells other than epithelial cells. Therefore it will be of great interest to determine: i) how VZV gE is transported in epithelial cells; ii) whether the tyrosine-based motifs and the CKII site regulate gE trafficking of all α-herpesviruses; and iii) whether these or other C-terminal motifs play any role in gE-dependent cell-to-cell spread.

5.4.2.2 gE/gI Binds to Components of Epithelial Cell Junctions

The most significant finding during our analysis of HSV gE trafficking in polarized epithelial cells, was our observation that gE accumulated only at sites of cell-cell contact (Fig 4.5 A-C, G-H). In subconfluent HEC-1A cells, many cells have lateral plasma membrane domains not in contact with another cell, and we found gE localized only along those lateral plasma membrane domains in contact with other cells, in a pattern similar to the adherens junction protein ß-catenin. As the subconfluent epithelial cells in my experiments were not fully polarized (i.e, tight junctions were not able to form around the entire cell), lateral diffusion of gE could occur between the apical and basolateral domains. Our observation that gE was specifically restricted to cell junctions are consistent with the hypothesis that gE is being physically retained at sites of cell-cell contact. Many proteins which are retained at the lateral plasma membrane of epithelial cells, do so based on a physical interactions with the cytoskeleton (Nelson and Veshnock, 1987). This feature is most often assessed by extracting proteins with non-ionic detergents (e.g., Triton X-100) in which cytoskeletal elements or proteins bound to them remain insoluble. We did not find evidence that gE/gI was associated with the cytoskeleton. Together, these data strongly suggest that gE/gI is specifically associating with a component of epithelial cell junctions that can be extracted with nonionic detergent.

Our original observations that gE/gI participates in cell-to-cell spread involved fibroblasts (Chapter 2), while those of Balan et al.,(1994) involved

epithelial cells. In both cases, gE$^-$ and gI$^-$ viruses spread poorly between cells. Fibroblast cell-cell junctions are not often considered to be highly ordered structural complexes such as seen with epithelial cell-cell contacts. However, studies by Yonemura et al (1995) have suggested that mini-adherens junctions are formed at the tips of thin cellular processes joining NRK fibroblasts . P-cadherin, α-catenin, ZO-1 and vinculin, proteins found in epithelial cell junctions were specifically concentrated at these fibroblast adherens junctions (Yonemura et al., 1995). Although fibroblasts do not develop tight junctions and become polarized, the junctions formed between these cells share the basic characteristics of epithelial cell junctions. Therefore, it is likely that gE/gI functions analogously at epithelial and at fibroblast cell junctions.

5.4.2.3 Models for How gE/gI Facilitates Direct HSV Cell-to-Cell Spread

There are several possible mechanisms by which gE/gI could be facilitate HSV cell-to-cell spread (refer to Figure 4.8). First, in panel A, gE/gI may be localized at cell junctions and may interact with cellular ligand(s) and, through these interactions, facilitate transfer of virus from an infected cell to an uninfected cell. In this model gE/gI could work either within the plasma membrane directly (top part of 4.8A), or as part of the virion envelope (lower part of 4.8A). In both cases, gE/gI might facilitate transfer of virus from one cell to a neighbouring cell by priming the cell junctions for gB, gD, gH/gL function. For example, gE/gI could act by sequestering a cellular ligand that might either interfere with virion entry into an

adjacent cell or release of the virion into the extracellular compartment. Alternatively, gE/gI, when bound to its cellular ligand, may promote cell-to-cell spread by playing an active part in regulating the fusion of the virion envelope with the plasma membrane of the neighbouring cell. There is some evidence that gE and gI contributes directly to the fusion of cell membranes as some gE^- and gI^- deletion mutants, that are associated with syncytial (fusion-inducing) HSV strains, are unable to cause cell fusion, apparently because they lack gE or gI (Balan et al., 1994; Chatterjee et al., 1989; Davis-Poynter et al., 1994). Thus, gE/gI might act as a cofactor to gB, gD, and gH/gL during virion binding or membrane fusion. However, the requirement for gE and gI in syn^- strains is not universal, as some gE^- and gI^- HSV mutants retain their syncytial phenotype (i.e, can still cause fusion of cells) (Johnson et al., 1988; Neidhardt et al., 1987).

The subcellular location of a putative gE/gI ligand is not clear, gE could bind to a cellular ligand within the plasma membrane of either the infected or uninfected cell. We suspect that the ligand is concentrated at cell junctions based on the accumulation of gE at cell junctions. In this case, the gE/gI binding protein could very well be a cell adhesion molecule (CAM), or other protein that is concentrated at sites of cell-cell contact.

An alternative model which might explain the accumulation of gE/gI at sites of cell-cell contact and a role for gE/gI in cell-to-cell spread would suggest that the gE/gI complex acts intracellularly to target virion egress specifically to the lateral plasma membrane (refer to Figure 4.8, panel B). By targeting the virus

to sites of cell-cell contact, there would be a greater likelihood of cell-to cell transmission of HSV, in an environment inaccessible to antibody neutralization. The presence of tyrosine-based and dileucine-based trafficking motifs within the cytoplasmic domains of gE and gI, coupled with the accumulation of gE/gI at the lateral plasma membrane of polarized epithelial cells are consistent with this model. In addition, we have some evidence obtained using electron microscopy that there are marked differences in the subcellular distributions of enveloped particles when gE is absent from HSV infected cells (Brunetti et al.,). The distribution of enveloped HSV particles in MDBK (bovine) cells infected with a gE^- mutant HSV-1 was significantly skewed toward accumulation of enveloped particles in the cytoplasm rather than at the cell surface (Brunetti et al.,). Enveloped particles produced in cells infected with wild type HSV-1 were more commonly found on the cell surface. A more detailed study of the intracellular distribution of enveloped virions in polarized human epithelial cells, and of HSV carrying gE mutants with defects in trafficking signals, might help to discriminate between these two models. However, the two models need not be mutually exclusive. Even if gE/gI affects trafficking of enveloped particles to sites of cell-cell contact, gE/gI could, in addition, facilitate the transfer of virions across cell junctions. Therefore, gE/gI could be considered as a funnel, directing virions to the most appropriate membrane (i.e, the plasma membrane of the adjoining cell) and then fostering fusion between the virion envelope and the plasma membrane.

5.4.3 HSV Spread from Neuron-to-Neuron

Previous work showed that there was attenuated virulence of HSV gE$^-$ and gI$^-$ mutants in peripheral epithelial tissues. However it was also demonstrated that the gE$^-$ and gI$^-$ mutants spread poorly either into or within the nervous system (Balan et al., 1994; Dingwell et al., 1994; Kudelova et al., 1991; Neidhardt et al., 1987; Rajcani et al., 1990; Rajcani et al., 1990). Moreover, there was previous evidence that gE and gI were involved in neuronal spread and neurovirulence in studies with PrV gE$^-$ and gI$^-$ mutants (Card et al., 1992; Card et al., 1991; Mettenleiter et al., 1985; Mettenleiter et al., 1988; Whealy et al., 1993). Considerable effort has been made since these original observations in evaluating the importance of gE/gI to α-herpesvirus transneuronal spread (Dingwell et al., 1995; Enquist et al., 1994; Kaashoek et al., 1996; Kritas et al., 1995; Kritas et al., 1994; Kritas et al., 1994; van Engelenburg et al., 1994; van Engelenburg et al., 1995).

5.4.3.1 Structure of Neuronal Synapses

Neuronal synapses are dynamic sites of cell-cell contact that regulate the transfer of information between these excitable cells. Synaptic connections between cells can take on a wide variety of shapes, sizes and functions, depending upon the neuronal components involved (i.e., axon, dendrite, cell body) and the makeup of these synapses govern both the direction and mode of communication between neurons (reviewed in Jessell and Kandel, 1993). The chemical

synapse is probably the best characterized of the known synaptic connections and involves the polarized (i.e., unidirectional), transmission of a signal from one cell to another. Neurotransmitters or neuropeptides which are released from presynaptic membrane act as signals across synapses, and elicit a receptor-mediated response in the postsynaptic membrane. These signals are normally unidirectional in that neurotransmitter release is restricted to the presynaptic cell, and receptors are found only on the postsynaptic cell. However, some chemical synapses are bidirectional (i.e., reciprocal transmission), as both membranes share pre- and post synaptic elements (e.g., dendrodentritic connection between dendrites of two different neurons). The alternative form of signal transmission is electrical, which by nature, is also bidirectional. Here, depolarization of neighbouring neurons is mediated by an ionic current passing though gap junctions that connect the neurons at sites of cell contact.

The mechanisms that regulate the release of neurotransmitters and peptides at the presynaptic membrane, share many characteristics with the trafficking of intracellular membrane proteins, and the processes of endocytosis and exocytosis in non-neuronal cells (reviewed in Kelly, 1993; Mellman, 1996; Rothman, 1994; Sudhof, 1995). Many of the molecules required for the fusion of intracellular or extracellular membranes are also active at the synapses, e.g., N-ethylamleimide-sensitive factor (NSF) and α-SNAP (soluble NSF attachment protein) are associated together in a high molecular weight fusion complex and act together in a manner similar to that in non-neuronal cells (O'Connor et al.,

1994; Rothman, 1994).

The primary structure of CNS synaptic connections has remained essentially uncharacterised compared to the relatively detailed analysis of the neuromuscular junction (Burden, 1998). However it is clear that the synaptic complex is built around an neuron-neuron cell junction which shares many characteristics with epithelial cell junctions e.g., desmosomes, gap junctions, and adherens junctions. Electron micrographical studies of synaptic and epithelial junctions noted similar plasma membrane thickenings with underlying membrane densities that were directly or indirectly associated with the cytoskeleton (Borysenko and Beringer, 1989; Peters et al., 1991; Yap et al., 1997). Both junctions have tightly apposed cell membranes (~ 20 nm) that serve as sites for cell-cell communication (e.g., through gap junctions). Recent evidence suggests that epithelial and synaptic junctions also share Ca^{+2} dependent cadherin-based adherens junctions (Fannon and Colman, 1996; Uchida et al., 1996).

5.4.3.2 gE/gI Facilitates Neuron-to-Neuron Spread

We found that unlike wild type HSV, gE^- and gI^- mutant viruses did not cause lethal encephalitis in mice following replication in the corneal epithelium (Table 1.2). Although we did not carefully quantitate the amount of gE^- and gI^- mutants that reached the CNS, our data showed that the spread of the mutant viruses into the brain was markedly reduced. Using the ear pinea of mice as the site of peripheral infection, Balan and colleagues found that virus titres of gE^- and

gI$^-$ mutants were four logs lower in the trigeminal ganglion than the wild type parent virus, and furthermore, the mutants could not be detected in the brains of infected mice (Balan et al., 1994). In both of sets of experiments, the decreased spread within nervous system by the mutant virus may be related to a local decrease of viral replication in the peripheral epithelial tissues innervated by the sensory nervous system. Hence the decreased neuronal spread of the mutants may have been partially due to decreased virus produced in epithelial tissues and available to infect sensory axons. However, it is also likely that the gE$^-$ and gI$^-$ mutants have a defects in spread of virus from epithelial tissues to neurons. To further characterize whether gE/gI was involved in facilitating neuron-to-neuron spread and to reduce the effects of viral replication in peripheral epithelial tissues, we chose an intra-ocular route of HSV infection, in which neurons would be directly infected. We measured virus spread between neurons of the retina to neurons in the visual centres of the brain. The visual system model had been used previously by others to examine transneuronal spread of α-herpesviruses into the CNS (Card et al., 1990; Card et al., 1991; Kristenson et al., 1974; McLean et al., 1989; Norgren and Lehman, 1989; Norgren et al., 1992).

Wild type and gE$^-$ and gI$^-$ mutant viruses were injected into the vitreous body of the rat eye, and we followed the spread of virus in the retina and into the brain by taking sections of the infected retina and brain at various times after infection. We found that the gE$^-$ and gI$^-$ mutants showed a marked decrease in

their ability to spread within all the layers of the retina, compared to wild type HSV-1 that soon infected the majority of the retinal neurons (Fig 3.2). Both gE^- and gI^- mutants replicated within the retina to titres 10 fold lower than wild type virus (Fig 3.4), and also failed to spread efficiently to retinorecipient regions in the brain (Fig 3.6) i.e., regions of the brain that receive direct synaptic connections via the optic nerve. Together, these data suggested that the gE/gI complex was important in facilitating neuron-to-neuron spread.

HSV can be transmitted in either the retrograde direction (i.e., from axon toward cell body) or in the anterograde direction (from cell body to axon) and in many cases the principal direction of viral spread is strain dependent (LaVail et al., 1997). Anterograde transport for the most part appears to be slower than retrograde spread, for both HSV and PrV, and the viruses also show a preference for retrograde transport (Enquist, 1995; Kuypers and Ugolini, 1990; McLean et al., 1989; Ugolini et al., 1989). With most strains of HSV, intracerebral injections of virus results in labelling of second order neurons that is consistent with infection via retrograde transport of virus. It is unclear why α-herpesviruses should have a preference for retrograde transport, since the virus is transported in both directions during the life cycle of the virus *in vivo*. A number of studies suggest that PrV gE/gI may have some role in anterograde transport. Infection and spread of gE^- and gI^- PrV mutants in the olfactory pathway of pigs was significantly attenuated compared to spread with wild type PrV (Kritas et al., 1994), suggesting that the glycoprotein mutants have a defect in anterograde

spread. Likewise, when rats were infected in the retina, PrV gE$^-$ and gI$^-$ mutants displayed a tropism toward specific retinal recipient regions in the brain (a form of anterograde transneuronal transport) (Card et al., 1992; Whealy et al., 1993). This tropism appears to be related to the nature of the synaptic connections of the retinal ganglion cell axon with neurons in the optic centres of the brain and not viral entry into subsets of retinal ganglion cells. Co-infection of retinal ganglion cells with both gE$^-$ and gI$^-$ mutants led to infection of all visual centres, a pattern identical to wild type virus (Enquist et al., 1994). It is important to note however, that HSV gE$^-$ and gI$^-$ mutants did not display this tropism in the rat visual system. HSV gE$^-$ and gI$^-$ mutants displayed a reduced ability to spread between neurons within the retina and from the retina to certain retinal recipient regions in the brain (the suprachiasmatic nucleus (SCN) and the lateral geniculate nucleus (LGN)). The optic tectum receives the vast majority of retinal axons connections, while a much smaller proportion of axons project to the SCN and the LGN (Lindon and Perry, 1983; Parnavelas et al., 1989). We suspect that the reduced spread of the mutants to the brain is likely a result of fewer retinal ganglion cells becoming infected, coupled with a reduced number of ganglion cell neurons projecting to the LGN and SCN. Therefore, it is unlikely that HSV gE/gI are involved solely in facilitating anterograde transneuronal transport of HSV, but plays a more universal role in viral spread from neuron-to-neuron.

Our *in vitro* analysis of gE/gI in promoting neuron-to-neuron spread

clearly showed that gE/gI had an important role in this mode of viral transmission. Both gE^- and gI^- mutants spread poorly through cultures of trigeminal ganglion neurons (Fig 3.8 and 3.9), and produced 10 fold lower viral yields compared to wild type HSV when neurons were infected at low MOIs. Although there were likely synaptic connections established between these neurons in culture, neuron-neuron spread of HSV would have also occurred at sites of neurite-neurite, neurite-neuron, and neuron-neuron contact. Similar observations were observed following infection of the rat retina. We observed distinct foci of HSV spread within the retina, so that it appeared that virus passed trans-synaptically through both the lateral and central signalling pathways (see Fig 3.1). However, as the wild type virus was able to spread trans-synaptically through the retina and infect virtually every neuron, the gE^- and gI^- mutants spread to only a limited number of synaptically linked neurons. How gE/gI promotes spread between neurons remains unclear. However, with the recent discovery of cadherin molecules at synaptic junctions (Fannon and Colman, 1996; Uchida et al., 1996), it is tempting to speculate that gE/gI might function in neurons in a similar manner as in epithelial cells to promote cell-to-cell spread across cell-cell junctions that do not differ dramatically from epithelial cell junctions (see section 5.4.2.3).

5.4.3.3 Models for How gE/gI Facilitates HSV Neuron-to-Neuron Spread

The molecular mechanism by which gE/gI promotes neuron-to-neuron spread of HSV has not been explored. However, the structural similarities between synapses and epithelial cell junctions and the polarized trafficking of glycoproteins between axons and dendrites (Dotti and Simons, 1990), and the requirement of gE/gI for trans-synaptic spread of HSV-1 suggests that gE/gI might function in neurons in a manner similar to that in epithelial cells.

The first model by which gE/gI promotes cell-to-cell spread of HSV in epithelial cells suggests that gE binds to a cellular ligand found at cell junctions, and thereby facilitates transport of HSV across the cell junction (refer to fig 4.8A). A similar mechanism could be evoked for gE/gI in trans-synaptic virus spread. Here, gE/gI within the virion envelope or within the synaptic membrane would bind to a ligand on the apposing synaptic membrane in order to facilitate HSV spread. However, in contrast to models in which gE/gI functions in the virion envelope, recent evidence suggests that PrV gE/gI facilitates transneuronal spread as part of the synaptic membrane (Tirabassi et al., 1997). PrV mutants did not incorporate mutant gE molecules with C-terminal deletions into the virion envelope (or at least gE could not be detected in virions), yet the mutant gE was able to mediate trans-synaptic spread of PrV to all retinal regions of the brain (Tirabassi et al., 1997). This study also found, that the PrV C-terminal gE mutants formed small plaques on MDBK cells. Therefore, PrV gE/gI may promote

transneuronal spread in a manner different from cell-to-cell spread between epithelial cells.

PrV gE promoted anterograde transfer of virus from retinal ganglion cell axons to retinorecipient neurons in the brain. Recall that retrograde transport of HSV involves transfer of virus from neuron cell bodies to axons, and therefore virus passes from the postsynaptic membrane to the presynaptic membrane. In anterograde transport, virus passes from the axon or presynaptic membrane to dendrite or neuron, the postsynaptic membrane. We have no evidence as to whether HSV gE specifically facilitates retrograde versus anterograde transport, (i.e., gE^- and gI^- mutants spread poorly *in vitro* and in the retina where HSV could have spread in both directions). However, the mechanism of gE/gI activity in promoting trans-synaptic transport and the proteins that gE/gI may bind may also be different, depending upon which side of the synapse gE/gI is found. Unlike epithelial cells in which apposing membranes could be assumed to share the same complement of membrane proteins, typical chemical synapses likely have different protein ensembles at the pre- and post-synaptic membranes. At the presynaptic membrane, action potentials depolarize the membrane, inducing the fusion of synaptic vesicles with the presynaptic membrane which then releases neurotransmitters into the synaptic cleft (reviewed in Jessell and Kandel, 1993; Kelly, 1993). Following fusion, synaptic vesicles rapidly recycle and replenish their neurotransmitter content from cytoplasmic stores. The post-synaptic membrane contains ion channels, neurotransmitter receptors and

transporters not found on presynaptic membranes and which are required to receive the incoming signal. The extent of exocytosis and endocytosis are also significantly decreased (Jessell and Kandel, 1993). However, in anterograde and retrograde transneuronal transport of HSV, gE/gI could be interacting with structural components of the synapse that are shared by both pre- and post-synaptic membranes (i.e., cadherin or gap junction proteins). Therefore, it will be important to determine whether gE/gI is differentially transported in neurons (i.e, dendrites versus axons) and whether gE/gI accumulates at synaptic connections similar to epithelial cell junctions.

It is also possible that gE/gI may selectively target virus particles to synaptic junctions. However, this model predicts that gE/gI may play some role in anterograde versus retrograde transport. The data from experiments by Penfold et al. (1994), suggested that in anterograde viral transport, viral nucleocapsids and glycoproteins are transported separately to axon terminals where capsid envelopment and exocytosis takes place (see section 5.2.2.5). In retrograde transport, egress of viral particles may take place as in non-neuronal cells in which enveloped particle are transported to the cell surface within internal membrane cisternae of the exocytic pathway. In this case, gE/gI could direct enveloped particles to sites of cell-cell contact (e.g., synapses), similar to its model B described for epithelial cells (Fig 4.8B). However, it is much more difficult to envision a function for gE/gI in directing virions to axon terminals if the model of Penfold et al., of separate transport of capsids and glycoproteins to axons is

correct. Since the C-terminal domain of PrV gE is dispensable for anterograde transport (Tirabassi et al., 1997), gE could not interact with tegument or capsid proteins to promote capsid envelopment or budding at the axon plasma membrane. However, it is formally possible that interactions between gE/gI and the nucleocapsid could occur by virtue of the C-terminal domain of gI. Therefore, mutagenesis of gE and gI, particularly the removal of both C-terminal domains, may help to determine whether gE/gI facilitates trans-synaptic spread of virus as part of the synaptic membrane.

5.4.3.4 Contribution of gE/gI to Neurovirulence

All of the HSV and PrV gE^- and gI^- mutants display a marked attenuation in neurovirulence following infection of the CNS in several animal models (Card et al., 1992; Dingwell et al., 1994; Jacobs et al., 1994; Kimman et al., 1994; Kritas et al., 1995; Kritas et al., 1994; Kritas et al., 1994; Kudelova et al., 1991; Mettenleiter et al., 1985; Mettenleiter et al., 1988; Neidhardt et al., 1987; Rajcani et al., 1990; Rajcani et al., 1990). Virulence is defined as the degree of pathogenicity (i.e, extent of disease), induced by a microorganism and is commonly measured as the mean time to death after infection or mean time to appearance of symptoms (reviewed in Card and Enquist, 1995). Although this may be the definition of virulence, the source or cause of virulence is another matter all together. In the vast majority of cases, the degree of neurovirulence of HSV strains and mutants has correlated with the ability of virus to spread through the nervous system of the host. Those HSV mutants that were defective in viral replication or spread

displayed an attenuated phenotype. The first evidence that gE$^-$ mutants were less neurovirulent came from LD_{50} (50% lethal dose) experiments in which mice were intracerebrally injected with gE$^-$ mutants or wild type virus (Neidhardt et al., 1987). The wild type virus LD_{50} was 1 PFU/mouse compared to and LD_{50} of 1 x 10^5 PFU/mouse for the gE$^-$ mutant. In our experiments, HSV gE$^-$ and gI$^-$ mutants were markedly less neurovirulent in the mouse than wild type virus. Corneal inoculation of mice with wild type HSV resulted in encephalitis and death. In contrast, mice infected with the gE$^-$ and gI$^-$ mutants showed no outward signs of encephalitis and did not die. We attributed the decreased neurovirulence to defects in the ability of the gE$^-$ and gI$^-$ mutants viruses to spread from cell-to-cell, and into and through the nervous system. In fact, very little of the gE$^-$ and gI$^-$ mutants could be detected in the trigeminal ganglia, and the mutants could not be detected in the brains of infected mice (Balan et al., 1994). PrV gE$^-$ and gI$^-$ mutants show a similar decrease in neurovirulence compared to wild type virus following infection of the rat visual system (Card et al., 1992; Whealy et al., 1993). These mutants spread poorly to the brain, and the onset of symptoms of mutant infected rats took 40 hrs longer than wild type virus infected animals. In addition there was a reduced pathogenesis in rats infected with the PrV gE$^-$ and gI$^-$ mutants compared to wild type infected animals (ie., less neuronal and glial lysis). This is as one would predict if the extent of spread and cell lysis equated to

neurovirulence.

In an attempt to separate neurovirulence from neurotropism, Tirabassi and colleagues (1997), found that PrV mutants with the cytoplasmic domain truncated spread as well as wild type PrV in the rat visual system. Surprisingly the mutants exhibited an attenuated neurovirulent phenotype. This suggests that the N-terminus of gE is sufficient to allow PrV to infect and spread in the nervous system yet the virus is less neurovirulent without the cytoplasmic domain of gE. How PrV gE causes the virus to be virulent remains to be determined. Moreover, it is not known if HSV gE acts in the same way. HSV is much less pathogenic in the rat than PrV, and in our experiments, in which we infected the rat retina with wild type HSV strain F, the animals showed no outward symptoms of disease even after five days after infection. If gE/gI is sufficient to engender disease in animals, it may be possible to investigate how this occurs by expressing gE and gI alone in the CNS using adenovirus (Ad) vectors. Adenoviruses have proven to be efficient non-toxic vectors for expressing foreign genes in the nervous system of several different animal models (reviewed in Slack and Miller, 1996).

5.5 The gE/gI Fc Receptor Activity and Its Relationship to Cell-to-Cell Spread

The gE/gI complex was first identified based on its ability to act as a receptor for the Fc domain of IgG (Baucke and Spear, 1979; Johnson and Feenstra, 1987; Johnson et al., 1988; Watkins, 1964). Subsequent studies evaluated the relative importance of either gE or gI in this activity (Bell et al.,

1990; Dubin et al., 1990; Hanke et al., 1990). While gE/gI expression may be able to protect HSV infected cells and virions from complement mediated-immune lysis in experiments in tissue culture (Dubin et al., 1991; Frank and Friedman, 1989; Van Vliet et al., 1992), there has been no evidence that gE/gI can protect HSV *in vivo*. Our studies and those of Balan et al., (1994) are consistent with the hypothesis that Fc receptor activity is not obviously important during primary infections in peripheral tissues (i.e, corneal epithelium or ear pinea). The attenuated growth and spread observed with the gE^- and gI^- mutants in the epithelium occurred prior to the appearance of anti-HSV antibodies (Balan et al., 1994; Dingwell et al., 1994), and gE/gI binds to mouse IgG poorly or not at all (Johansson et al., 1985). In plaque assays on monolayers of primary human fibroblasts, we found that the gE^- and gI^- mutants consistently produced smaller plaques compared to wild type HSV, whether in the presence of neutralizing or non-neutralizing rabbit or mouse antibodies (Figure 2.10). Therefore, there are marked defects in cell-to-cell spread which are independent of antibodies. Moreover, HSV gE/gI binds relatively weakly to the Fc domain of IgG. In binding experiments with [^{125}I] labelled rabbit IgG, the quantity of label bound to gE/gI expressing cells was 100 to a 10,00 fold lower than the amount of label originally added to the cells , and over 30% of the label which bound to cells, constituted nonspecific binding (Hanke et al., 1990). The affinity of gE/gI for the Fc domain of IgG increased when IgG was heat-aggregated or was bound to antigen (Baucke and Spear, 1979; Dorval et al., 1979), suggesting that higher

affinity binding of gE/gI to aggregated IgG was due to the multivalency of IgG complexes. This is also illustrated by assays in which IgG-coated red blood cells were added to gE/gI expressing cells. Under these conditions, the background binding to uninfected cells was less than 2% compared to gE/gI infected cells, and a much higher percent of the input IgG bound to the cells (Hanke et al., 1990). Fc receptor activity by gE/gI is not well conserved among the α-herpesviruses family, and it has been difficult or impossible to show Fc receptor activity by the gE/gI homologues of PrV, BHV, and VZV (Litwin et al., 1990; Whitbeck et al., 1996; Zuckermann et al., 1988). Together, these data suggest that HSV gE/gI can act as an Fc receptor although it is not clear whether this apparently low affinity binding alters HSV replication or pathogenesis *in vivo*. It is likely that gE/gI's primary role is to promote cell-to-cell spread of HSV. In fact Baucke and Spear (1979) commented: "The fact that detection of the binding activity has so far relied on the use of IgG, however, does not necessarily imply that the purpose of the virus-induced receptor is to interact with IgG." (Baucke and Spear, 1979). Thus gE/gI could bind to a member of the IgG superfamily, even one that is localized to cell junctions.

If gE/gI does act as an Fc receptor, it would likely be important during recrudescence disease. High serum titres of anti-HSV antibodies are present throughout the development of secondary lesions, and thus gE/gI dependent Fc receptor activity may be an additional benefit to ensure efficient viral dissemination, over and above the ability of the complex to facilitate cell-to-cell spread.

Weeks et al., (1997) have been able to separate the Fc binding activity of gE from that of cell-to-cell spread. The domain responsible for cell-to-cell spread had not been determined, but previous analysis mapped gE Fc receptor activity to amino acids 235-380 (Basu et al., 1995; Dubin et al., 1994). Insertion of four amino acids at position 399 produced a mutant form of gE that retained its ability to facilitate HSV spread from cell-to-cell in keratinocyte monolayers, but one which lacked Fc binding activity (Weeks et al., 1997). Therefore, this HSV-1 mutant provides a means to test the importance of gE/gI Fc receptor activity to HSV replication *in vivo*.

5.6 Future Directions

There are a number of potential mechanisms that could be involved in gE/gI mediated cell-to-cell spread. My work on HSV gE/gI, and that of others on α-herpesvirus gE/gI homologues have provided a basis to propose and test a number of hypotheses. Results from studies in which gE/gI was expressed in polarized HEC-1A cells have raised a number of questions concerning the activity of gE/gI in HSV cell-to-cell spread with respect to the distribution and trafficking of gE/gI within epithelial cells. Is gE/gI required at the lateral junctions in order to facilitate cell-to-cell spread; does gE/gI target virions to cell junctions; what is the gE/gI ligand found at cell junctions; does gE/gI facilitate cell-to-cell spread as part of the virion envelope or as part of the plasma membrane? To address several of these questions I have proposed the following experiments.

5.6.1 Experiments Testing Models of gE/gI Function in Epithelial Cells

One question that does arise from the two models proposed (refer to Figure 4.8), is whether gE/gI's ability to mediate cell-to-cell spread depends upon accumulation of gE/gI at cell junctions. Therefore, if gE/gI were to be targeted to the apical domain, would the complex be able to facilitate cell-to-cell transmission? To address this question, the putative gE cytoplasmic targeting signals could be mutated by replacing the tyrosine and tryptophan residues within the consensus sequence $_{472}$YADW$_{475}$ with alanine residues, and rescuing these mutations into the HSV gE- mutant F-gEß. The subcellular distributions of the mutant gE proteins would be assessed by confocal microscopy of mutant HSV infected HEC-1A cells. If the mutants were distributed on the apical plasma membrane, cell-to-cell spread would then be assayed by plaque size on human epithelial cells. An alternative to mutating the trafficking signals would be to exchange the cytoplasmic domain of gE with the cytoplasmic domain of a membrane protein known to be sorted to the apical plasma membrane of polarized epithelial cells. However, the cytoplasmic domain of gE may have additional functions including the ability of gE to be incorporated into the virion envelope (Tirabassi et al., 1997), and therefore this mutation may not only mislocalize the protein but also cause it to be excluded from the virion envelope. The incorporation of mutant forms of gE into the virion envelope would be determined by Western blotting membrane extracts from purified virions with the gE specific

MAb II-481. In addition, the contribution of gI to gE/gI trafficking must also be considered during these experiments. gI is required for gE/gI to reach the cell surface in polarized epithelial cells, and gI also has putative basolateral trafficking motifs ($_{388}$LL$_{389}$) at the carboxy-terminus of its cytoplasmic domain. Altering the trafficking of gE/gI to the apical surface in polarized epithelial cells might also address the question of whether gE/gI targets HSV to cell junctions, and whether this is necessary for HSV cell-to-cell spread. Preliminary data suggested that the distribution of enveloped virions in epithelial cells infected with an HSV-1 gE-mutant was skewed toward an accumulation of enveloped particles in the cytoplasm, in contrast to wild type infected cells, where enveloped virions were distributed at the cell surface (Brunetti et al.,). Thus, a detailed analysis of electron micrographic sections of epithelial cells infected with HSV gE mutants with defects in intracellular trafficking or accumulation at cell junctions may provide some insight into whether gE/gI acts to transport virions to cell junctions.

A careful interpretation of all the results is critical for discriminating between models A and B (Fig 4.8). If trafficking of gE to the apical surface does not alter the ability of HSV to spread from cell-to-cell, this would suggest that gE activity does not function while part of the lateral plasma membrane domain, nor does gE promote trafficking of virions to cell junctions. Moreover, if Western blotting experiments showed that mutant forms of gE were associated with virions, this would suggest that gE might promote transport of HSV across cell junctions as a component of the virion envelope. The alternative result (e.g., gE

trafficking to the apical surface affected cell-to-cell spread) would not necessarily discriminate between the two models. The electron micrographs of particle distributions may not be sufficient to determine if gE-mediated trafficking of enveloped particles does occur. In addition, it would be impossible to discriminate whether gE acted as part of virion envelope or lateral plasma membrane.

To test if gE/gI functions at cell junctions and not as part of the virion envelope, gE/gI must be inhibited from entering the virion envelope but still retain the ability to traffic to, and accumulate at cell junctions. This could be achieved using a tetracycline-(TC) controlled expression system, in which gE and gI would be transiently expressed in human epithelial cells and then infected with a HSV gE-/gI- virus. Stable epithelial cell lines expressing the tetracycline-controlled transactivator (tTA) would be generated. For inducible expression of gE and gI, replication defective adenoviruses (Ad) would be constructed with either gE or gI under control of the TC-response element (AdTCgE or AdTCgI). Tetracycline could be used to induce a pulse of gE and gI, so that gE/gI could be subsequently chased to cell junctions after tetracycline is removed. Following the appearance of gE/gI at cell junctions without further synthesis of gE and gI, the cells would be infected with either wild type HSV or gE^-/gI^- mutant, and then plaque sizes would be assessed a few days later. This would allow us to determine if gE/gI which is part of the plasma membrane and accumulated at cell junctions can mediate HSV cell-to-cell spread, without gE/gI in the virion envelope.

There are a number of problems with this system. First the half life of gE

might be too short so that after infection with HSV, gE/gI would disappear from cell junctions before plaques developed. This might be circumvented by expressing high levels of gE and gI, using high MOI infections with the Ad vectors. Experiments with the Ad(E1$^-$)gE/gI infections (Chapter 4) were carried out using an MOI of 400 (effective MOI of 800), without any appreciable toxic effect to the cells. The second problem, might involve endocytosis of gE/gI from the cell surface and subsequent incorporation into the virion envelope. VZV and PrV gE homologues are rapidly endocytosed from the plasma membrane via clathrin coated vesicles (Olson and Grose, 1997; Tirabassi et al., 1997), and in the case of PrV gE, endocytosis appeared to be required for gE incorporation into the virion envelope (Tirabassi et al., 1997).

Another important question that should be addressed is whether gE/gi binds to a cellular protein at cell junctions and the identity of this protein. Identification of this protein or proteins would be of tremendous importance to our understanding of how HSV spreads from cell-to-cell. Preliminary experiments involving Ad(E1$^-$)gE/gI or HSV infected HEC-1A cells have indicated that there are cellular proteins that coimmunoprecipitate with gE. In order to purify these cellular proteins that apparently bind to gE/gI, lysates from AdgE/gI infected HEC-1A cells, would be passed over a gE-antibody affinity column. Putative gE/gI binding proteins would be eluted from the column and separated on a polyacrylamide gel, then transferred to PVDF membrane. The protein band corresponding to the gE/gI binding proteins would be excised and then subject-

ed to amino acid sequence analysis. An alternative approach would be to use soluble forms of gE/gI produced by baculovirus vectors (bacgE/gI). Baculovirus vectors have been recently been constructed in our lab that express soluble forms of gE and gI. Once large amounts of bacgE/gI have been purified, the complex would be coupled to sepharose, and used as an affinity column. Lysates from HEC-1A cells would be passed through the gE/gI affinity column, and gE/gI binding proteins purified and sequenced as described above. Once a protein has been identified, it would be important to ensure that the protein is required for gE-mediated cell-to-cell spread. The first experiments would be plaque reduction experiments in which cell-to-cell spread of HSV (i.e, plaque size) would be assessed in the presence or absence of antibodies directed to the protein. In addition, if the protein were to have a known ligand, we could determine if the protein would also inhibit plaque production of HSV-1.

5.6.2 Is gE/gI Fc Receptor Activity Important for HSV In Vivo

The ability to separate the activities of gE/gI in IgG Fc receptor binding activity from its role in cell-to-cell spread has proven difficult. The recent development of an HSV gE mutant that retains cell-to-cell spread activity but does not bind IgG, should allow us to separate these two functions. I would propose to determine the relative contributions of gE/gI's Fc receptor activity and cell-to-cell spread to HSV replication *in vivo* using a rabbit eye model of HSV infection. This animal model has many of the characteristics necessary to test the requirement of gE/gI IgG Fc receptor activity *in vivo*: i) HSV can replicate and

spread efficiently through the rabbit corneal epithelium, ii) the virus can establish a latent infection in the TG, iii) latent infections can be reactivated following iontophoresis of epinephrine in the cornea (Hill et al., 1990), and importantly, iv) the HSV gE/gI complex can bind IgG by its Fc domain (Johansson et al., 1985). Rabbit corneas could be infected with wild type HSV-1, a gE- HSV mutant, or an HSV gE mutant lacking IgG Fc receptor activity, but retaining the ability to mediate cell-to-cell spread. The abilities of these viruses to cause lesions within the eye, and spread to the trigeminal ganglion could be assessed. In addition, the efficiency by which wild type and mutant viruses develop secondary lesions after reactivation and the progression of the lesion could also be determined. Since the gE mutant lacking Fc binding activities would spread from cell-to-cell spread normally, any differences in either the size or number of lesions could be ascribed to the loss of Fc receptor activity. This should correlate with the presence of nonimmune or even with HSV-specific antibodies. This strategy, however, would evaluate the role of Fc receptor activity alone but would not address the combined contribution of the cell-to-cell spread function and Fc binding activity. To examine this issue, the domain of gE required for cell-to-cell spread must also be mapped, and then mutant viruses constructed which cannot mediate cell-to-cell spread, yet which retain Fc binding activity.

One problem with studying the role of gE/gI during secondary infections using mutants that have defects in cell-to-cell spread is that such mutants will likely be less able to infect neurons and so there will be less latent HSV in the

nervous system. To generate mutants that would not express gE, a cre/loxP recombination system would be used to remove gE but only after infection of the nervous system (Gu et al., 1994). The wild type gE gene would be flanked by lox P sites, such that the gE gene would be removed by cre-mediated recombination. To temporally control the expression of cre recombination activity, cre coding sequences would be driven by the PrV LAT promoter inserted into terminal and long repeat region (R_L) of HSV, replacing one of the endogenous HSV LAT promoters. The PrV LAT promoter was active only in neuronal cells when inserted into the R_L region of HSV, while little or no promoter activity can be detected in non neuronal cells (i.e., rabbit fibroblasts; (Huang et al., 1994). The tissue specific expression of cre in neurons by the PrV LAT promoter, would permit recombination and excision of the gE gene following HSV infection of neurons within the trigeminal ganglion. Mutant forms of gE that lacked Fc receptor activity or which alter cell-to-cell spread could be expressed in addition to the lox-flanked wild type gE by inserting the mutant gE genes into the gJ (US5) locus, which has been shown to be dispensable for HSV replication *in vivo* (Balan et al., 1994; Dingwell et al., 1995). Together, using this system, approximately the same number of neurons would be infected by wild type and mutant viruses in the primary infection but following reactivation, the relative roles of gE could be determined. This would allow us to separate the relative roles of IgG Fc receptor activities versus the requirement for cell-to-cell spread activity during secondary infections when anti-HSV IgG is present.

An alternative approach is to passively transfer anti-HSV IgG into HSV naive rabbits prior to infection with wild type HSV or with HSV gE mutants lacking Fc receptor activity. However, these anti-HSV antibodies may neutralize the virus before it has a chance to start an infection. Delaying the transfer of IgG may be a partial solution, but it may be hard to discriminate differences in pathogenicity between the wild type and the gE mutant once the viruses have begun to spread within the epithelium and into the nervous system.

5.7 Conclusions

HSV has developed specific mechanisms to efficiently disseminate viral progeny among different hosts and among cells within its host. During the course of a viral growth cycle, HSV encounters a variety of cell junctions and the virus must pass across these junctions in order to spread from cell-to-cell in solid tissues, important sites of virus replication. Even though many of the proteins involved in viral entry and egress are required for spread from cell-to-cell, gE/gI facilitates cell-to-cell transmission in epithelial tissues and the nervous system, without apparently being involved in entry of extracellular virus. gE/gI acts exclusively in cell-to-cell spread, and so provides a molecular handle in this process for the first time. The mechanism by which HSV spreads from cell-to-cell remains poorly characterized, however, my work has provided some clues as to how gE/gI might promote this process.

6. REFERENCES

Ackermann, M., Longnecker, R., Roizman, B., and Pereira, L. (1986). Identification, properties, and gene location of a novel glycoprotein specified by herpes simplex virus 1. Virology *150*, 207-20.

Addison, C., Rixon, F. J., Palfreyman, J. W., O'Hara, M., and Preston, V. G. (1984). Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology *138*, 246-59.

Adler, R., Glorioso, J. C., Cossman, J., and Levine, M. (1978). Possible role of Fc receptors on cells infected and transformed by herpesvirus: escape from immune cytolysis. Infect Immun *21*, 442-7.

Alconada, A., Bauer, U., and Hoflack, B. (1996). A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network. EMBO J *15*, 6096-6110.

Alford, C. A., and Britt, W. J. (1990). Cytomegalovirus. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. e. a. Howley, eds. (New York, New York: Raven Press Ltd.), pp. 1981-2010.

Allen, J. M., and Seed, B. (1989). Isolation and expression of functional high affinity Fc receptor cDNAs. Science *243*, 378-380.

Arsenakis, M., Hubenthal-Voss, J., Campadelli-Fiume, G., Pereira, L., and Roizman, B. (1986). Construction and properties of a cell line constitutively expressing the herpes simplex virus glycoprotein B dependent on functional alpha 4 protein synthesis. J Virol *60*, 674-82.

Avitabile, E., Ward, P. L., Di Lazzaro, C., Torrisi, M. R., Roizman, B., and Campadelli-Fiume, G. (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*, 7397-405.

Babic, N., Mettenleiter, T. C., Flamand, A., and Ugolini, G. (1993). Role of essential glycoproteins gII and gp50 in transneuronal transfer of pseudorabies virus from the hypoglossal nerves of mice. J Virol *67*, 4421-6.

Bacalloa, R., Antony, C., Karsenti, E., Stelzer, E., and Simons, K. (1989). The subcellular organization of Madin-Darby canine kidney cells during the formation of a polarized epithelium. J Cell Biol *109*, 2817-2832.

Baines, J. D., and Roizman, B. (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*, 938-44.

Baines, J. D., and Roizman, B. (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*, 1441-52.

Baines, J. D., and Roizman, B. (1992). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J Virol *66*, 5168-74.

Baines, J. D., Ward, P. L., Campadelli-Fiume, G., and Roizman, B. (1991). The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J Virol *65*, 6414-24.

Bak, I. J., Markham, C. H., Cook, M. L., and Stevens, J. G. (1977). Intraaxonal transport of Herpes simplex virus in the rat central nervous system. Brain Res *136*, 415-29.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (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*, 1245-58.

Balda, M. S., and Matter, K. (1998). Tight Junctions. J Cell Sci *111*, 541-547.

Banfield, B. W., Leduc, Y., Esford, L., Visalli, R. J., Brandt, C. R., and Tufaro, F. (1995). Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. Virology *208*, 531-9.

Baringer, J. R., and Swoveland, P. (1973). Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med *288*, 648-650.

Bastian, R. R., Rabson, A. S., Yee, C. L., and Tralka, T. S. (1972). Herpes virus hominis: Isolation from human trigeminal ganglia. Science *178*, 306-307.

Basu, S., Dubin, G., Basu, M., Nguyen, V., and Friedman, H. M. (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 glyco-

protein I. J Immunol *154*, 260-7.

Batterson, W., and Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J Virol *46*, 371-7.

Baucke, R. B., and Spear, P. G. (1979). Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. J Virol *32*, 779-89.

Bell, S., Cranage, M., Borysiewicz, L., and Minson, T. (1990). Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J Virol *64*, 2181-6.

Ben-Porat, T., and Kaplan, A. S. (1985). Molecular biologyy of pseudorabies virus. In The herpesviruses, Roizman, ed. (New York: Plenum Publishing Corp), pp. 105-173.

Ben-Porat, T., and Tokazewski, S. (1977). Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology *79*, 292-301.

Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000- Dalton outer envelope protein. J Virol *65*, 5910-20.

Blasco, R., and Moss, B. (1992). Role of cell-asociated enveloped vaccinia virus in cell-to-cell spread. J Virol *66*, 4170-4179.

Bloom, D. C., Devi-Rao, G. B., Hill, J. M., Stevens, J. G., and Wagner, E. K. (1994). Molecular analysis of herpes simplex virus type 1 during epinephrine- induced reactivation of latently infected rabbits in vivo. J Virol *68*, 1283-92.

Bloom, D. C., Stevens, J. G., Hill, J. M., and Tran, R. K. (1997). Mutagenesis of a cAMP response element within the latency-associated transcript promoter of HSV-1 reduces adrenergic reactivation. Virology *236*, 202-7.

Borysenko, M., and Beringer, T. (1989). Functional Histology, Third Edition (Boston: Little, Brown, and Company, Inc.).

Boulter, E. A., and Appleyard, G. (1973). Differences between extracellular and intracellular froms of poxvirus and their implications. Prog Med Virol *16*, 86-108.

Bourkas, A. E., and Menezes, J. (1979). Studies on the induction of IgG-Fc receptors and synthesis of IgM in primary and chronically-infected lymphoid

(Raji) cells by herpes simplex virus. J Gen Virol *44*, 361-71.

Braun, D. K., Pereira, L., Norrild, B., and Roizman, B. (1983). Application of denatured, electrophoretically separated, and immobilized lysates of herpes simplex virus-infected cells for detection of monoclonal antibodies and for studies of the properties of viral proteins. J Virol *46*, 103-12.

Bre, M.-H., Pepperkok, R., Hill, A. M., Levilliers, N., Ansorge, W., Steltzer, E. H. K., and Karsenti, E. (1990). Regulation of microtubule dynamics and nucleation during polarization in MDCK II cells. J Cell Biol *111*, 3113-3021.

Brown, S. M., MacLean, A. R., Aitken, J. D., and Harland, J. (1994). ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells in vitro. J Gen Virol *75*, 3679-86.

Browne, H., Bell, S., Minson, T., and Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. J Virol *70*, 4311-6.

Brunetti, C. R., Burke, R. L., Hoflack, B., Ludwig, T., Dingwell, K. S., and Johnson, D. C. (1995). Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. J Virol *69*, 3517-28.

Brunetti, C. R., Burke, R. L., Kornfeld, S., Gregory, W., Masiarz, F. R., Dingwell, K. S., and Johnson, D. C. (1994). Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. J Biol Chem *269*, 17067-74.

Brunetti, C. R., Dingwell, K. S., and Johnson, D. J. unpublished.

Buchman, T. G., Simpson, T., Nosal, C., Roizman, B., and Nahmias, A. J. (1980). The structure of herpes simplex virus DNA and its application to molecular epidemiology. Ann N Y Acad Sci *354*, 279-90.

Buckmaster, E. A., Gompels, U., and Minson, A. (1984). Characterisation and physical mapping of an HSV-1 glycoprotein of approximately 115 X 10(3) molecular weight. Virology *139*, 408-13.

Burden, S. J. (1998). The formation of neuromuscular synapses. Genes Dev *12*, 133-48.

Butcher, M., Raviprakash, K., and Ghosh, H. P. (1990). Acid pH-induced fusion of cells by herpes simplex virus glycoproteins gB an gD. J Biol Chem *265*, 5862-8.

Bzik, D. J., Fox, B. A., DeLuca, N. A., and Person, S. (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*, 185-90.

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

Cai, W. H., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion [published erratum appears in J Virol 1988 Nov;62(11):4438]. J Virol *62*, 2596-604.

Cai, W. Z., Person, S., DebRoy, C., and Gu, B. H. (1988). Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. J Mol Biol *201*, 575-88.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F., and Roizman, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J Virol *62*, 159-67.

Campadelli-Fiume, G., Avitabile, E., Fini, S., Stirpe, D., Arsenakis, M., and Roizman, B. (1988). 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-Fiume, G., Farabegoli, F., Di Gaeta, S., and Roizman, B. (1991). Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J Virol *65*, 1589-95.

Campbell, M. E., Palfreyman, J. W., and Preston, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J Mol Biol *180*, 1-19.

Campenot, R. B. (1977). Local control of neurite development by nerve growth factor. Proc Natl Acad Sci U S A *74*, 4516-9.

Card, J. P., and Enquist, L. W. (1995). Neurovirulence of pseudorabies virus [published erratum appears in Crit Rev Neurobiol 1995;9(4):preceding 311]. Crit Rev Neurobiol *9*, 137-62.

Card, J. P., Rinaman, L., Lynn, R. B., Lee, B. H., Meade, R. P., Miselis, R. R., and Enquist, L. W. (1993). Pseudorabies virus infection of the rat central nervous system: ultrastructural characterization of viral replication, transport, and pathogenesis. J Neurosci *13*, 2515-39.

Card, J. P., Rinaman, L., Schwaber, J. S., Miselis, R. R., Whealy, M. E., Robbins, A. K., and Enquist, L. W. (1990). Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. J Neurosci *10*, 1974-94.

Card, J. P., Whealy, M. E., Robbins, A. K., and Enquist, L. W. (1992). Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J Virol *66*, 3032-41.

Card, J. P., Whealy, M. E., Robbins, A. K., Moore, R. Y., and Enquist, L. W. (1991). Two alpha-herpesvirus strains are transported differentially in the rodent visual system. Neuron *6*, 957-69.

Carter, C., and Easty, D. L. (1981). Expermimental ulcerative herpetic keratitits. I. Systemic immune responses and resistance to corneal infection. Br J Ophthalmol *65*, 77-81.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS- related body-cavity-based lymphomas [see comments]. N Engl J Med *332*, 1186-91.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma [see comments]. Science *266*, 1865-9.

Chatterjee, S., Koga, J., and Whitley, R. J. (1989). A role for herpes simplex virus type 1 glycoprotein E in induction of cell fusion. J Gen Virol *70*, 2157-62.

Cheung, P., Banfield, B. W., and Tufaro, F. (1991). Brefeldin A arrests the maturation and egress of herpes simplex virus particles during infection. J Virol *65*, 1893-904.

Chou, J., and Roizman, B. (1985). Isomerization of herpes simplex virus 1 genome: identification of the cis-acting and recombination sites within the domain of the a sequence. Cell *41*, 803-11.

Cohen, J. I., and Straus, S. E. (1996). Varicella-zoster virus and its replication. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. e. a. Howley, eds. (Philadelphia: Lippincott - Raven Publishers), pp. 2525-2546.

Cook, M. L., and Stevens, J. G. (1973). Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect Immun *7*, 272-88.

Cooper, N. R. (1994). Early events in human herpesvirus infection of cells. In Cellular receptors for animal viruses, W. E., ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 365-388.

Corey, L., and Spear, P. G. (1986). Infections with herpes simplex viruses (1). N Engl J Med *314*, 686-91.

Corey, L., and Spear, P. G. (1986). Infections with herpes simplex viruses (2). N Engl J Med *314*, 749-57.

Costa, J., Yee, C., Nakamura, Y., and Rabson, A. (1978). Characteristics of the Fc receptor induced by herpes simplex virus. Intervirology *10*, 32-9.

Costa, R. H., Cohen, G., Eisenber, R., Long, D., and Wagner, E. (1984). Direct demonstration that the abundant 6-kilobase herpes simplex virus type 1 mRNA mapping between 0.23 and 0.37 map units encodes the major capsid protein VP5. J Virol *49*, 287-292.

Cudmore, S., Cossart, P., Griffiths, G., and Way, M. (1995). Actin-based motility of vaccinia virus. Nature *378*, 636-8.

Cushing, H. (1905). The surgical aspects of major neuralgia of the trigeminal nerve. JAMA *44*, 1002-1008.

Dahms, N. M., Lobel, P., and Kornfeld, S. (1989). Mannose 6-phosphate receptors and lysosomal enzyme targeting. J Biol Chem *264*, 12115-12118.

Davis-Poynter, N., Bell, S., Minson, T., and Browne, H. (1994). Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J Virol *68*, 7586-90.

Davison, M. D., Rixon, F. J., and Davison, A. J. (1992). Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. J Gen Virol *73*, 2709-2713.

Deatly, A. M., Spivack, J. G., Lavi, E., and Fraser, N. W. (1987). RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. Proc Natl Acad Sci U S A *84*, 3204-8.

Debroy, C., Pederson, N., and Person, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. Virology *145*, 36-48.

Deiss, L. P., Chou, J., and Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J

Virol *59*, 605-18.

Deiss, L. P., and Frenkel, N. (1986). Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated a sequence. J Virol *57*, 933-41.

Delius, H., and Clements, J. B. (1976). A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J. Gen. Virol. *33*, 125-133.

Desai, P. J., Schaffer, P. A., and Minson, A. C. (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*, 1147-56.

Dickler, H. B. (1974). Studies of the human lymphocyte receptor for heat-aggregated or antigen- complexed immunoglobulin. J Exp Med *140*, 508-22.

Dijkstra, J. M., Visser, N., Mettenleiter, T. C., and Klupp, B. G. (1996). Identification and characterization of pseudorabies virus glycoprotein gM as a nonessential virion component. J Virol *70*, 5684-8.

Dingwell, K. S., Brunetti, C. R., Hendricks, R. L., Tang, Q., Tang, M., Rainbow, A. J., and Johnson, D. C. (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J Virol *68*, 834-45.

Dingwell, K. S., Doering, L. C., and Johnson, D. C. (1995). Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J Virol *69*, 7087-98.

Doerig, C., Pizer, L. I., and Wilcox, C. L. (1991). An antigen encoded by the latency-associated transcript in neuronal cell cultures latently infected with herpes simplex virus type 1. J Virol *65*, 2724-7.

Dolter, K. E., Ramaswamy, R., and Holland, T. C. (1994). Syncytial mutations in the herpes simplex virus type 1 gK (UL53) gene occur in two distinct domains. J. Virol. *68*, 8277-81.

Dorval, G., Bourkas, A. E., and Menezes, J. (1979). Comparative studies on the expression of Fc-receptors, Ia antigens, and beta 2-microglobulin following in vitro herpes simplex virus infection of human lymphoid cells. Clin Exp Immunol *37*, 477-85.

Dotti, C. G., and Simons, K. (1990). Polarized sorting of viral glycoproteins to the

axon and dendrites of hoppocampal neurons in culture. Cell *62*, 63-72.

Dowler, K. W., and Veltri, R. W. (1984). In vitro neutralization of HSV-2: inhibition by binding of normal IgG and purified Fc to virion Fc receptor (FcR). J Med Virol *13*, 251-9.

Drubin, D. G., and Nelson, W. J. (1996). Origins of cell polarity. Cell *84*, 335-344.

Dubin, G., Basu, S., Mallory, D. L., Basu, M., Tal-Singer, R., and Friedman, H. M. (1994). Characterization of domains of herpes simplex virus type 1 glycoprotein E involved in Fc binding activity for immunoglobulin G aggregates. J Virol *68*, 2478-85.

Dubin, G., Frank, I., and Friedman, H. M. (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*, 2725-31.

Dubin, G., Socolof, E., Frank, I., and Friedman, H. M. (1991). Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J Virol *65*, 7046-50.

Duncan, S. A., and Smith, G. L. (1992). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. J Virol *66*, 1610-21.

Edson, C. M. (1993). Phosphorylation of neurotropic alphaherpesvirus envelope glycoproteins: herpes simplex virus type 2 gE2 and pseudorabies virus gI. Virology *195*, 268-70.

Edson, C. M., Hosler, B. A., and Waters, D. J. (1987). Varicella-zoster virus gpI and herpes simplex virus gE: phosphorylation and Fc binding. Virology *161*, 599-602.

Efstathiou, S., Minson, A. C., Field, H. J., Anderson, J. R., and Wildy, P. (1986). Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J Virol *57*, 446-55.

Eisenberg, R. J., Ponce de Leon, M., Friedman, H. M., Fries, L. F., Frank, M. M., Hastings, J. C., and Cohen, G. H. (1987). Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. Microb Pathog *3*, 423-35.

Ejercito, P. M., Kieff, E. D., and Roizman, B. (1968). Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. J Gen Virol *2*, 357-64.

Enquist, L. W. (1995). Circuit-specific infection of the mammalian nervous system. ASM news *61*, 633-638.

Enquist, L. W., Dubin, J., Whealy, M. E., and Card, J. P. (1994). Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion cell neurotropism. J Virol *68*, 5275-9.

Fais, S., Capobianchi, M. R., Abbate, I., Castilletii, C., Gentile, M., Fei, P. C., Ameglio, F., and Dianzani, F. (1985). Unidirectional budding of HIV-1 at the site of cell-to-cell contact is associated with co-polarization of intercellular adhesion molecules and HIV-1 viral matrix protein. Aids *9*, 329-335.

Falke D, Knoblich A, and Muller S (1985). Fusion from without induced by herpes simplex virus type 1. Intervirology *24*, 211-9.

Fannon, A. M., and Colman, D. R. (1996). A model for central synaptic junctional complex formation based on the differenctial adhesive specificities of the cadherens. Neuron *17*, 423-434.

Farrell, M. J., Hill, J. M., Margolis, T. P., Stevens, J. G., Wagner, E. K., and Feldman, L. T. (1993). The herpes simplex virus type 1 reactivation function lies outside the latency-associated transcript open reading frame ORF-2. J Virol *67*, 3653-5.

Fenwick, M. L., and Everett, R. D. (1990). Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. J Gen Virol *71*, 2961-7.

Feorino, P. M., Shore, S. L., and Reimer, C. B. (1977). Detection by indirect immunofluorescence of Fc receptors in cells acutely infected with Herpes simplex virus. Int Arch Allergy Appl Immunol *53*, 222-33.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol *66*, 341-8.

Frank, I., and Friedman, H. M. (1989). A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J Virol *63*, 4479-88.

Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Seidel, C. A., and Cines, D. B. (1984). Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature *309*, 633-5.

Friedman, H. M., Wang, L., Fishman, N. O., Lambris, J. D., Eisenberg, R. J., Cohen,

G. H., and Lubinski, J. (1996). Immune evasion properties of herpes simplex virus type 1 glycoprotein gC. J Virol *70*, 4253-60.

Fries, L. F., Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Hammer, C. H., and Frank, M. M. (1986). Glycoprotein C of herpes simplex virus 1 is an inhibitor of the complement cascade. J Immunol *137*, 1636-41.

Fuller, A. O., and Lee, W. C. (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*, 5002-12.

Fuller, A. O., Santos, R. E., and Spear, P. G. (1989). Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J Virol *63*, 3435-43.

Fuller, A. O., and Spear, P. G. (1987). Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc Natl Acad Sci U S A *84*, 5454-8.

Gershon, A. A., Sherman, D. L., Zhu, Z., Gabel, C. A., Ambron, R. T., and Gershon, M. D. (1994). Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the trans-Golgi network. J Virol *68*, 6372-90.

Gibson, W., and Roizman, B. (1972). Proteins specified by herpes simplex virus. 8. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J Virol *10*, 1044-52.

Gompels, U. A., Carss, A. L., Saxby, C., Hancock, D. C., Forrester, A., and Minson, A. C. (1991). Characterization and sequence analysis of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. J Virol *65*, 2393-2401.

Goodman, J. L., and Engel, J. P. (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.

Goodpasture, E. W. (1929). Herpectic infection with a special reference to involvement of the nervous system. Medicine (Baltimore) *8*, 223-243.

Grose, C., and Litwin, V. (1988). Immunology of the varicella-zoster glycoproteins. J Infect Dis *157*, 877-881.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-

specific gene targeting [see comments]. Science *265*, 103-6.

Gumbiner, B. (1990). Generation and maintenance of epithelial cell polarity. Curr Opin Cell Biol *2*, 881-887.

Gumbiner, B., and Simons, K. (1988). A functional assay for proteins involved in establishing an epithelial occluding barrier: Identification of a uvomorulin-like polypeptide. J Cell Biol *102*, 457-468.

Gumbiner, B., Stevenson, B., and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. J Cell Biol *107*, 1575-1587.

Gupta, P., Balachandran, R., Ho, M., Enrico, A., and Rinaldo, C. (1989). Cell-to-cell transmission of human immunodeficiency virus type 1 in the prescence of azidothymidine and neutralizing antibody. J. Virol *63*, 2361-2365.

Haanes, E. J., Nelson, C. M., Soule, C. L., and Goodman, J. L. (1994). The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. J Virol *68*, 5825-34.

Handler, C. G., Cohen, G. H., and Eisenberg, R. J. (1996). Cross-linking of glycoprotein oligomers during herpes simplex virus type 1 entry. J Virol *70*, 6076-82.

Handler, C. G., Eisenberg, R. J., and Cohen, G. H. (1996). Oligomeric structure of glycoproteins in herpes simplex virus type 1. J Virol *70*, 6067-70.

Hanke, T., Graham, F. L., Lulitanond, V., and Johnson, D. C. (1990). Herpes simplex virus IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology *177*, 437-44.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C., and Roizman, B. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc Natl Acad Sci U S A *72*, 4243-7.

Helenius, A., Morein, B., Fries, E., Simons, K., Robinson, P., Schirrmacher, V., Terhorst, C., and Strominger, J. L. (1978). Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. Proc Natl Acad Sci U S A *75*, 3846-50.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R., and Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell

surface heparan sulphate and glycoprotein B. J Gen Virol *75*, 1211-22.

Herold, B. C., WuDunn, D., Soltys, N., and Spear, P. G. (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*, 1090-8.

Herrler, G., Rott, R., and Klenk, H. D. (1985). Neuraminic acid is involved in the binding of influenza C virus to erythrocytes. Virology *141*, 144-7.

Highlander, S. L., Cai, W. H., Person, S., Levine, M., and Glorioso, J. C. (1988). Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J Virol *62*, 1881-8.

Highlander, S. L., Sutherland, S. L., Gage, P. J., Johnson, D. C., Levine, M., and Glorioso, J. C. (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J Virol *61*, 3356-64.

Hildreth, J. E. E., and Orentas, R. J. (1989). Involvement of a leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. Science *244*, 1075-1078.

Hill, J. M., Sedarati, F., Javier, R. T., Wagner, E. K., and Stevens, J. G. (1990). Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology *174*, 117-25.

Hill, T. J., Field, H. J., and Roome, A. P. C. (1972). Intra-axonal location of herpes simplex virus particles. J Gen Virol *15*.

Hiller, G., Weber, K., Schneider, L., Parajsz, C., and Jungwirth, C. (1979). Interaction of assembled progeny pox viruses with the cellular cytoskeleton. Virology *98*, 142-153.

Hirsch, M. S., and Curran, J. W. (1996). Human immunodeficiency viruses. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. Howley, eds. (Philadelphia, PA: Lippincott-Raven), pp. 1953-1976.

Ho, D. Y. (1992). Herpes simplex virus latency: molecular aspects. Prog Med Virol *39*, 76-115.

Ho, D. Y., and Mocarski, E. S. (1989). Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc Natl Acad Sci USA *86*, 7596-7600.

Honess, R. W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral

proteins. J Virol *14*, 8-19.

Hope, R. G., and Marsden, H. S. (1983). Processing of glycoproteins induced by herpes simplex virus type 1: sulphation and nature of the oligosaccharide linkages. J Gen Virol *64*, 1943-53.

Hope, R. G., Palfreyman, J., Suh, M., and Marsden, H. S. (1982). Sulphated glycoproteins induced by herpes simplex virus. J Gen Virol *58*, 399-415.

Huang, A. S., and Wagner, R. R. (1966). Penetration of herpes simplex virus into human epidermoid cells. Proc Soc Exp Biol Med *116*, 863-869.

Huang, C. J., Rice, M. K., Devi-Rao, G. B., and Wagner, E. K. (1994). The activity of the pseudorabies virus latency-associated transcript promoter is dependent on its genomic location in herpes simplex virus recombinants as well as on the type of cell infected. J Virol *68*, 1972-6.

Huang, T., and Campadelli-Fiume, G. (1996). Anti-idiotypic antibodies mimicking glycoprotein D of herpes simplex virus identify a cellular protein required for virus spread from cell to cell and virus-induced polykaryocytosis. Proc Natl Acad Sci U S A *93*, 1836-40.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C., and Johnson, D. C. (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*, 2240-50.

Hutchinson, L., Graham, F. L., Cai, W., Debroy, C., Person, S., and Johnson, D. C. (1993). Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology *196*, 514-31.

Hutchinson, L., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K promotes egress of virus particles. J Virol *69*, 5401-13.

Hutchinson, L., Roop-Beauchamp, C., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. J Virol *69*, 4556-63.

Jacob, R. J., Morse, L. S., and Roizman, B. (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 the four isomeric arrangements of viral DNA. J Virol *29*, 448-57.

Jacob, R. J., and Roizman, B. (1977). Anatomy of herpes simplex virus DNA VIII.

Properties of the replicating DNA. J Virol *23*, 394-411.

Jacobs, L., Mulder, W. A., Priem, J., Pol, J. M., and Kimman, T. G. (1994). Glycoprotein I of pseudorabies virus (Aujeszky's disease virus) determines virulence and facilitates penetration of the virus into the central nervous system of pigs. Acta Vet Hung *42*, 289-300.

Jacobson, J. G., Martin, S. L., and Coen, D. M. (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*, 1839-43.

Javier, R. T., Stevens, J. G., Dissette, V. B., and Wagner, E. K. (1988). A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology *166*, 254-7.

Jayachandra, S., Baghian, A., and Kousoulas, K. G. (1997). Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. J Virol *71*, 5012-24.

Jessell, T. M., and Kandel, E. R. (1993). Synaptic transmission: A bidirectional and self-modifiable form of cell-cell communication. Cell/Neuron *72/10*, 1-30.

Johansson, P. J., and Blomberg, J. (1987). Absence of host-cell influence on binding specificity of herpes simplex virus type 1 induced Fc receptor. Acta Pathol Microbiol Immunol Scand [C] *95*, 113-6.

Johansson, P. J., and Blomberg, J. (1990). Characterization of herpes simplex virus type 1-induced Fc receptor in its interaction with rabbit immunoglobulin G (IgG). Apmis *98*, 685-94.

Johansson, P. J., Hallberg, T., Oxelius, V. A., Grubb, A., and Blomberg, J. (1984). Human immunoglobulin class and subclass specificity of Fc receptors induced by herpes simplex virus type 1. J Virol *50*, 796-804.

Johansson, P. J., Myhre, E. B., and Blomberg, J. (1985). Specificity of Fc receptors induced by herpes simplex virus type 1: comparison of immunoglobulin G from different animal species. J Virol *56*, 489-94.

Johansson, P. J., Schroder, A. K., Nardella, F. A., Mannik, M., and Christensen, P. (1986). Interaction between herpes simplex type 1-induced Fc receptor and human and rabbit immunoglobulin G (IgG) domains. Immunology *58*, 251-5.

Johansson, P. J. H., Nardella, F. A., Sjoquist, J., Schroder, A. K., and Christensen, P. (1989). Herpes simplex type 1-induced Fc receptor binds to the C(gamma)2-C(gamma)3 interface region of IgG i the area that binds staphylococcal protein A. A. Immunol *66*, 8-13.

Johnson, D. C., Burke, R. L., and Gregory, T. (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 Virol *64*, 2569-76.

Johnson, D. C., and Feenstra, V. (1987). Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J Virol *61*, 2208-16.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M., and Stow, N. D. (1988). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J Virol *62*, 1347-54.

Johnson, D. C., and Ligas, M. W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J Virol *62*, 4605-12.

Johnson, D. C., and Spear, P. G. (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*, 1102-12.

Johnson, D. C., and Spear, P. G. (1983). O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell *32*, 987-97.

Johnson, R. M., and Spear, P. G. (1989). Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J Virol *63*, 819-27.

Jones, B. G., Thomas, L., Molloy, S. S., Thulin, C. D., Fry, M. D., Walsh, K. A., and Thomas, G. (1995). Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. EMBO J *14*, 5869-5883.

Jones, F., and Grose, C. (1988). Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. J Virol *62*, 2701-11.

Jongen, W. M. F., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M., and Yamasaki, H. (1991). Regulation of connexin 43-mediated gap junctional intercellular communication by Ca+2 in mouse epidermal cells is controlled by E-cadherin. J Cell Biol *114*, 545-555.

Kaashoek, M. J., van Engelenburg, F. A., Moerman, A., Gielkens, A. L., Rijsewijk, F. A., and van Oirschot, J. T. (1996). Virulence and immunogenicity in calves of thymidine kinase- and glycoprotein E-negative bovine herpesvirus 1 mutants. Vet Microbiol *48*, 143-53.

Kaerner, H. C., Schroder, C. H., Ott-Hartmann, A., Kumel, G., and Kirchner, H. (1983). Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a nonpathogenic herpes simplex virus type 1 virus strain in mouse brain. J Virol *46*, 83-93.

Kartenbeck, J., Schemlz, M., Franke, W. W., and Geiger, B. (1991). Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured in low Ca+2 ion medium. J Cell Biol *113*, 881-892.

Katz, J. P., Bodin, E. T., and Coen, D. M. (1990). Quantitative polymerase chain reaction analysis of herpes simplex virus DNA in ganglia of mice infected with replication-incompetent mutants. J Virol *64*, 4288-4295.

Keller, P., and Simons, K. (1997). Post-Golgi biosynthetic trafficking. J Cell Science *110*, 3001-3009.

Kelly, R. B. (1993). Storage and release of neurotransmitters. Cell/Neuron *72/10*, 43-53.

Kerbel, R. S., and Davies, A. J. (1974). The possible biological significance of Fc receptors on mammalian lymphocytes and tumor cells. Cell *3*, 105-112.

Kieff, E. D., Bachenheimer, S. L., and Roizman, B. (1971). Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol *8*, 125-32.

Kimman, T. G., De Wind, N., De Bruin, T., de Visser, Y., and Voermans, J. (1994). Inactivation of glycoprotein gE and thymidine kinase or the US3-encoded protein kinase synergistically decreases in vivo replication of pseudorabies virus and the induction of protective immunity. Virology *205*, 511-8.

Klein, R. J., Friedman-Kien, A. E., and DeStefano, E. (1979). Latent herpes simplex virus infections in sensory ganglia of hairless mice prevented by acycloguanosine. Antimicrob Agents Chemother *15*.

Kostavasili, I., Sahu, A., Friedman, H. M., Eisenberg, R. J., Cohen, G. H., and Lambris, J. D. (1997). Mechanism of complement inactivation by glycoprotein C of herpes simplex virus. J Immunol *158*, 1763-71.

Koyama, A. H., and Uchida, T. (1984). Inhibition of multiplication of herpes simplex virus type 1 by ammonium chloride and chloroquine. Virology *138*, 332-5.

Koyama, A. H., and Uchida, T. (1987). The mode of entry of herpes simplex virus type 1 into Vero cells. Microbiol Immunol *31*, 123-30.

Krempien, U., Schneider, L., Hiller, G., Weber, K., Katz, E., and Jungwirth, C. (1981). Conditions for pox virus-specific microvilli formation studied during synchronized virus assembly. Virology *113*, 556-564.

Kristenson, K., Ghetti, B., and Wisniewski, H. M. (1974). Study on the propagation of herpes simples virus (type 2) into the brain after intraocular injection. Br. Res. *69*, 189-201.

Kristensson, K., Ghetti, B., and Wisniewski, H., M. (1974). Study on the propagation of herpes simplex virus (type 2) into the brain after intraocular injection. Br Res *69*, 189-201.

Kristensson, K., Lycke, E., Roytta, M., Svennerholm, B., and Vahlne, A. (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-hydroxynonyl)adenine]. J Gen Virol *67*, 2023-2028.

Kristensson, K., Lycke, E., and Sjostrand, J. (1971). Spread of herpes simplex virus in peripheral nerves. Acta Neuropathol (Berl) *17*, 44-53.

Kristensson, K., Nennesmo, L., Persson, L., and Lycke, E. (1982). Neuron to neuron transmission of herpes simplex virus. Transport of virus from skin to brainstem nuclei. J Neurol Sci *54*, 149-56.

Kritas, S. K., Nauwynck, H. J., and Pensaert, M. B. (1995). Dissemination of wild-type and gC-, gE-and gI-deleted mutants of Aujeszky's disease virus in the maxillary nerve and trigeminal ganglion of pigs after intranasal inoculation. J Gen Virol *76*, 2063-6.

Kritas, S. K., Pensaert, M. B., and Mettenleiter, T. C. (1994). Invasion and spread of single glycoprotein deleted mutants of Aujesky's disease virus (ADV) in the trigeminal nervous pathway of pigs after intranasal inoculation. Vet. Microbiol *40*, 323-334.

Kritas, S. K., Pensaert, M. B., and Mettenleiter, T. C. (1994). Role of envelope glycoproteins gI, gp63 and gIII in the invasion and spread of Aujeszky's disease

virus in the olfactory nervous pathway of the pig. J Gen Virol *75*, 2319-27.

Kudelova, M., Kostal, M., Cervenakova, L., Rajcani, J., and Kaerner, H. C. (1991). Pathogenicity and latency competence for rabbits of the herpes simplex virus type 1 ANGpath gC and gE defective mutants. Acta Virol *35*, 438-49.

Kumel, G., Schroder, C. H., and Kaerner, H. C. (1986). Neurovirulence and latency in inbred mice of two HSV-1 intrastrain variants of divergent pathogenicity. Med Microbiol Immunol (Berl) *174*, 313-24.

Kuypers, H. G., and Ugolini, G. (1990). Viruses as transneuronal tracers. Trends Neurosci *13*, 71-5.

Lafferty, W., Remington, M., Winter, C., Fahnlander, A., and Corey, L. (1985). Natural history of concomitant pharyngeal and genital HSV infection: influence of viral type and anatomic site on recurrence rates. Clin Res *33*, 408A (abstract).

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, E., Berman, P., Gregory, T., and Capon, D. J. (1987). Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell *50*, 975-85.

LaVail, J. H., Topp, K. S., Giblin, P. A., and Garner, J. A. (1997). Factors that contribute to the transneuronal spread of herpes simplex virus. J Neurosci Res *49*, 485-96.

Lee, G. T., Para, M. F., and Spear, P. G. (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*, 41-9.

Lee, W. C., and Fuller, A. O. (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*, 5088-97.

Lehner, T., Wilton, J. M., and Shillitoe, E. J. (1975). Immunological basis for latency, recurrences and putative oncogenicity of herpes simplex virus. Lancet *2*, 60-2.

Leib, D. A., Bogard, C. L., Kosz-Vnenchak, M., Hicks, K. A., Coen, D. M., Knipe, D. M., and Schaffer, P. A. (1989). A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J Virol *63*, 2893-900.

Leib, D. A., Coen, D. M., Bogard, C. L., Hicks, K. A., Yager, D. R., Knipe, D. M.,

Tyler, K. L., and Schaffer, P. A. (1989). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J Virol *63*, 759-68.

Liesegang, T. J. (1992). Biology and molecular aspects of herpes simplex and varicella zoster virus infections. Ophthalmology *99*, 781-799.

Ligas, M. W., and Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol *62*, 1486-94.

Lindon, R., and Perry, V. H. (1983). Massive retinaltectal projection in rats. Brain Res *272*, 145-149.

Litwin, V., Sandor, M., and Grose, C. (1990). Cell surface expression of the varicella-zoster virus glycoproteins and Fc receptor. Virology *178*, 263-72.

Longnecker, R., Chatterjee, S., Whitley, R. J., and Roizman, B. (1987). Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc Natl Acad Sci U S A *84*, 4303-7.

Longnecker, R., and Roizman, B. (1987). Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science *236*, 573-6.

Longnecker, R., and Roizman, B. (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. J Virol *58*, 583-91.

Luciw, P. (1996). Human immunodeficiency viruses and their replication. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. Howley, eds. (Philadelphia, PA: Lippincott-Raven), pp. 1881-1952.

Lycke, E., Hamark, B., Johansson, M., Krotochwil, A., Lycke, J., and Svennerholm, B. (1988). Herpes simplex virus infection of the human sensory neuron. An electron microscopy study. Arch Virol *101*, 87-104.

Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A., and Ziegler, R. (1984). Uptake and transport of herpes simplex virus in neurites of rat dorsal root ganglia cells in culture. J Gen Virol *65*, 55-64.

MacLean, C. A., Dolan, A., Jamieson, F. E., and McGeoch, D. J. (1992). The myristylated virion proteins of herpes simplex virus type 1: investigation of their role in the virus life cycle. J Gen Virol *73*, 539-47.

MacLean, C. A., Efstathiou, S., Elliott, M. L., Jamieson, F. E., and McGeoch, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. J Gen Virol *72*, 897-906.

MacLean, C. A., Robertson, L. M., and Jamieson, F. E. (1993). Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo. J Gen Virol *74*, 975-83.

Mallory, S., Sommer, M., and Arvin, A. M. (1997). Mutational analysis of the role of glycoprotein I in varicella-zoster virus replication and its effects on glycoprotein E conformation and trafficking. J Virol *71*, 8279-8288.

Marrs, J. A., Andersson-Fisone, C., Jeong, M. C., Cohen-Gould, L., Zurzolo, C., Nabi, I. R., Rodriguez-Boulan, E., and Nelson, W. J. (1995). Plasticity in epithelial cell phenotype: modulation by expression of different cadherin cell adhesion molecules. J Cell Biol *129*, 507-19.

Marsh, M., and Helenius, A. (1989). Virus entry into animal cells. In Advances in virus research, M. K, M. F. A. and S. A. J., eds. (New York: Academic Press, Inc. Harcourt Brace Jovanovich, Publishers), pp. 107-151.

Matter, K., and Mellman, I. (1994). Mechanisms of cell polarity: sorting and transport in epithelial cells. Curr Opin Cell Biol *6*, 545-554.

Mays, R. W., Beck, K. A., and Nelson, W. J. (1994). Organization and function of the cytoskeleton in polarized epithelial cells: a component of the protein sorting machinery. Curr Opin Cell Biol *6*, 16-24.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J Gen Virol *69*, 1531-74.

McGeoch, D. J., Dolan, A., Donald, S., and Rixon, F. J. (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J Mol Biol *181*, 1-13.

McLean, J. H., Shipley, M. T., and Bernstein, D. I. (1989). Golgi-like, transneuronal retrograde labelling with CNS injections of herpes simplex virus type 1. Brain Res Bull *22*, 867-81.

McNabb, D. S., and Courtney, R. J. (1992). Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading

frame. J Virol *66*, 2653-2663.

McNearney, T. A., Odell, C., Holers, V. M., Spear, P. G., and Atkinson, J. P. (1987). Herpes simplex virus glycoproteins gC-1 and gC-2 bind to the third component of complement and provide protection against complement- mediated neutralization of viral infectivity. J Exp Med *166*, 1525-35.

McTaggart, S. P., Burns, W. H., White, D. O., and Jackson, D. C. (1978). Fc receptors induced by herpes simplex virus. I. Biologic and biochemical properties. J Immunol *121*, 726-30.

Mellerick, D. M., and Fraser, N. W. (1987). Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology *158*, 265-75.

Mellman, I. (1996). Endocytosis and molecular sorting. Ann Rev Cell Dev Biol *12*, 575-625.

Mettenleiter, T. C., Schreurs, C., Zuckermann, F., Ben-Porat, T., and Kaplan, A. S. (1985). Pseudorabies virus avirulent strains fail to express a major glycoprotein. J Virol *56*, 307-311.

Mettenleiter, T. C., Schreurs, C., Zuckermann, F., Ben-Porat, T., and Kaplan, A. S. (1988). Role of glycoprotein gIII of pseudorabies virus in virulence. J Virol *62*, 2712-7.

Mijnes, J. D. F., Lutters, B. C. H., Vlot, A. C., van Anken, E., Horzinek, M. C., Rottier, P. J. M., and de Groot, R. J. (1997). Structure-function analysis of the gE-gI complex of feline herpesvirus: Mapping of gI domains required for gE-gI interaction, intracellular transport, and cell-to-cell spread. J Virol *71*, 8397-8404.

Minson, A. C., Hodgman, T. C., Digard, P., Hancock, D. C., Bell, S. E., and Buckmaster, E. A. (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*, 1001-13.

Mitchell, W. J., Lirette, R. P., and Fraser, N. W. (1990). Mapping of low abundance latency-associated RNA in the trigeminal ganglia of mice latently infected with herpes simplex virus type 1. J Gen Virol *71*, 125-32.

Mitchell, W. J., Steiner, I., Brown, S. M., MacLean, A. R., Subak-Sharpe, J. H., and Fraser, N. W. (1990). A herpes simplex virus type 1 variant, deleted in the promoter region of the latency-associated transcripts, does not produce any

detectable minor RNA species during latency in the mouse trigeminal ganglion. J Gen Virol *71*, 953-7.

Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell *87*, 427-36.

Mooseker, M. S. (1985). Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. Annu Rev Cell Biol *1*, 209-242.

Morgan, C., Rose, H. M., Holden, M., and Jones, E. P. (1959). Electron microscopic observations on the development of herpes simplex virus. J Ex Med *2*, 643-656.

Morgan, C., Rose, H. M., and Mednis, B. (1968). Electron microscopy of herpes simplex virus. I. Entry. J Virol *2*, 507-16.

Moss, B. (1996). Poxviridae: The viruses and their replication. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. Howley, eds. (Philadelphia, PA: Lippincott-Raven), pp. 2637-2672.

Mulder, W., Pol, J., Kimman, T., Kok, G., Priem, J., and Peeters, B. (1996). 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*, 2191-200.

Nakamura, Y., Costa, J., Tralka, T. S., Yee, C. L., and Rabson, A. S. (1978). Properties of the cell surface Fc-receptor induced by herpes simplex virus. J Immunol *121*, 1128-31.

Navarro, D., Paz, P., and Pereira, L. (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.

Neidhardt, H., Schroder, C. H., and Kaerner, H. C. (1987). Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. J Virol *61*, 600-3.

Nelson, W. J., and Veshnock, P. J. (1987). Ankyrin binding to (Na+ + K+)ATPase and implications for the organization of membrane domains in polarized cells. Nature *328*, 533-6.

Nesburn, A. B., Cook, M. L., and Stevens, J. G. (1972). Latent herpes simplex virus. Isolation from rabbit trigeminal ganglia between episodes of recurrent

ocular infection. Arch Ophthalmol *88*, 412-7.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Ye, Z., and Brown, J. C. (1994). Cell-free assembly of the herpes simplex virus capsid. J Virol *1994*, 6059-6063.

Norgren, R. B., and Lehman, M. N. (1989). Retrograde transneuronal transport of herpes simplex virus in the retina after injection in the superior colliculus, hypothalamus and optic chiasm. Brain Res *479*, 374-378.

Norgren, R. B., Jr., McLean, J. H., Bubel, H. C., Wander, A., Bernstein, D. I., and Lehman, M. N. (1992). Anterograde transport of HSV-1 and HSV-2 in the visual system. Brain Res Bull *28*, 393-9.

Novotny, M. J., Parish, M. L., and Spear, P. G. (1996). Variability of herpes simplex virus 1 gL and anti-gL antibodies that inhibit cell fusion but not viral infectivity. Virology *221*, 1-13.

O'Connor, V., Augustine, G. J., and Betz, H. (1994). Synaptic vesicle exocytosis: molecules and models. Cell *76*, 785-787.

Olson, J. K., and Grose, C. (1997). Endocytosis and recycling of varicella-zoster virus Fc receptor glycoprotein gE: internalization mediated by a YXXL motif in the cytoplasmic tail. J Virol *71*, 4042-54.

Para, M. F., Baucke, R. B., and Spear, P. G. (1982). Glycoprotein gE of herpes simplex virus type 1: effects of anti-gE on virion infectivity and on virus-induced fc-binding receptors. J Virol *41*, 129-36.

Para, M. F., Baucke, R. B., and Spear, P. G. (1980). Immunoglobulin 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*, 512-20.

Para, M. F., Goldstein, L., and Spear, P. G. (1982). Similarities and differences in the Fc-binding glycoprotein (gE) of herpes simplex virus types 1 and 2 and tentative mapping of the viral gene for this glycoprotein. J Virol *41*, 137-44.

Parnavelas, J. G., Dinopoulos, A., and Davies, S. W. (1989). The central visual pathways. In Handbook of chemical neuroanatomy. Integrated systems of the CNS part III, A. Bjorklund, T. Hokflet and L. Swanson, eds. (New York: Elsevier Science Publishers), pp. 1-164.

Payne, L. G. (1979). Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. J Virol *31*, 147-55.

Payne, L. G. (1980). Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virology *50*, 89-100.

Pearce-Pratt, R., Malamud, D., and Phillips, D. M. (1994). Role of the cytoskeleton in cell-to-cell transmission of human immunodeficiency virus. J Virol *68*, 2898-905.

Peeters, B., de Wind, N., hooisma, M., Wagenaar, F., Gielkens, A., and Moormann, R. (1992). Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J Virol *66*, 894-905.

Peeters, B., Pol, J., Gielkens, A., and Moormann, R. (1993). Envelope glycoprotein gp50 of pseudorabies virus is essential for virus entry but is not required for viral spread in mice. J Virol *67*, 170-7.

Pellett, P. E., Kousoulas, K. G., Pereira, L., and Roizman, B. (1985). Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J Virol *53*, 243-53.

Penfold, M. E., Armati, P., and Cunningham, A. L. (1994). Axonal transport of herpes simplex virions to epidermal cells: evidence for a specialized mode of virus transport and assembly. Proc Natl Acad Sci U S A *91*, 6529-33.

Perotti, M. E., Tan, X., and Phillips, D. M. (1996). Directional budding of human immunodeficiency virus from monocytes. J Virol *70*, 5916-21.

Peters, A., Palay, S. L., and Webster, H. d. F. (1991). The fine structure of the nervous system: Neurons and their supporting cells (New York: Oxford University Press).

Petrovskis, E. A., timmins, J. G., Gierman, T. M., and Post, L. E. (1986). Deletions in vaccine strains of pseudorabies virus and their effects on synthesis of glycoproteins gp63. J Virol *60*, 1166-1169.

Petrovskis, E. A., Timmins, J. G., and Post, L. E. (1986). Use of lambda gt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella- zoster virus glycoproteins. J Virol *60*, 185-93.

Phillips, D. M. (1994). The role of cell-to-cell transmission in HIV infection [editorial]. Aids *8*, 719-31.

Phillips, D. M., and Bourinbaiar, A. S. (1992). Mechanism of HIV spread from lymphocytes to epithelia. Virology *186*, 261-73.

Picard, F. J., Poland, S. D., and Rice, G. P. A. (1993). New developments with herpesviruses and the nervous system. Curr Opin Neurol Neurosur *6*, 169-175.

Poffenberger, K. L., and Roizman, B. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J Virol *53*, 587-95.

Pogue-Geile, K. L., and Spear, P. G. (1987). The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP. Virology *157*, 67-74.

Preston, V. G. (1990). Herpes simplex virus activates expression of a cellular gene by specific binding to the cell surface. Virology *176*, 474-82.

Price, R. W., Rubenstein, R., and Khan, A. (1982). Herpes simplex virus infection of isolated autonomic neurons in culture: viral replication and spread in a neuronal network. Arch Virol *71*, 127-40.

Rajcani, J., Herget, U., and Kaerner, H. C. (1990). Spread of herpes simplex virus (HSV) strains SC16, ANG, ANGpath and its glyC minus and GlyE minus mutants in DBA-2 mice. Acta Virol *34*, 305-20.

Rajcani, J., Herget, U., Kostal, M., and Kaerner, H. C. (1990). Latency competence of herpes simplex virus strains ANG, ANGpath and its gC and gE minus mutans. Acta Virol *34*, 477-86.

Ramaswamy, R., and Holland, T. C. (1992). In vitro characterization of the HSV-1 UL53 gene product. Virology *186*, 579-87.

Ranscht, B. (1994). Cadherins and catenins: interactions and functions in embryonic development. Curr Opin Cell Biol *6*, 740-746.

Rauh, I., and Mettenleiter, T. C. (1991). Pseudorabies virus glycopreoteins gII and gp50 are essential for virus penetration. J Virol *64*, 5348-5346.

Read, G. S., and Frenkel, N. (1983). Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate early) viral polypeptides. J Virol *46*, 498-512.

Rebordosa, X., Pinol, J., Perez-Pons, J. A., Lloberas, J., Naval, J., Serra-Hartmann, X., Espuna, E., and Querol, E. (1996). Glycoprotein E of bovine herpesvirus type 1 is

involved in virus transmission by direct cell-to-cell spread. Virus Res *45*, 59-68.

Reeves, W. C., Corey, L., Adams, H. G., Vontver, L. A., and Holmes, K. K. (1981). Risk of recurrence after first episodes of genital herpes. Relation to HSV type and antibody response. N Engl J Med *305*, 315-9.

Rixon, F. J. (1993). Structure and assembly of herpesviruses. Sem Virol *4*, 87-93.

Rixon, F. J., Davison, M. D., and Davison, A. J. (1990). Identification of the genes encoding two capsid proteins of herpes simplex virus type 1 by direct amino acid sequencing. J Gen Virol *71*, 1211-1214.

Rock, D. L., and Fraser, N. W. (1983). Detection of HSV-1 genome in central nervous system of latently infected mice. Nature *302*, 523-5.

Rock, D. L., and Fraser, N. W. (1985). Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J Virol *55*, 849-52.

Rodriguez, J. F., and Smith, G. L. (1990). IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res *18*, 5347-51.

Roitt, I. (1988). Essential Immunology, Sixth Edition (Oxford, UK: Blackwell Scientific Publications).

Roizman, B., Aurelian, L., and Roane, P. R. J. (1963). The multiplication of herpes simplex virus. I. The programming of viral DNA duplication in HEp-2 cells. Virology *21*, 482-498.

Roizman, B., and Kaplan, L. J. (1992). Herpes simplex viruses, central nervous system, and encephalitis. In Molecular Neurovirology, R. P. Roos, ed. (Totowa, NJ: Humana Press Inc.), pp. 3-23.

Roizman, B., and Roane, P. R. J. (1964). Multiplication of herpes simplex virus. II. The relation between protein syntesis and the duplication of viral DNA in infected HEp-2 cells. Virology *22*, 262-269.

Roizman, B., and Roane, P. R. J. (1961). A physical difference between two strains of herpes simplex virus apparent on sedimentation in cesium chloride. Virology *15*, 75-79.

Roizman, B., and Sears, A. E. (1996). Herpes simplex viruses and their replication. In Fields Virology, B. N. Fields, D. M. Knipe, P. M. Howley and e. al., eds. (Philadelphia: Lippincott - Raven Publishers), pp. 2231-2296.

Roop, C., Hutchinson, L., and Johnson, D. C. (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*, 2285-97.

Rosenthal, K. L., Smiley, J. R., South, S., and Johnson, D. C. (1987). Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes. J Virol *61*, 2438-47.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55-63.

Saharkhiz-Langroodi, A., and Holland, T. C. (1997). Identification of the fusion-from-without determinants of herpes simplex virus type 1 glycoprotein B. Virology *227*, 153-9.

Sanders, P. G., Wilkie, N. M., and Davison, A. J. (1982). Thymidine kinase deletion mutants of herpes simplex virus type 1. J Gen Virol *63*, 277-295.

Sato, H., Orenstein, J. M., Dimitrov, D., and Martin, D. (1992). Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology *186*, 712-724.

Schmuelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G., and Griffiths, G. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J Virol *68*, 130-147.

Schmutz, C., Payne, L. G., Gubser, J., and Wittek, R. (1991). A mutation in the gene encoding the vaccinia virus 37,000-M(r) protein confers resistance to an inhibitor of virus envelopment and release. J Virol *65*, 3435-42.

Schranz, P., Neidhardt, H., Schroder, C. H., and Kaerner, H. C. (1989). A viable HSV-1 mutant deleted in two nonessential major glycoproteins. Virology *170*, 273-6.

Schroder, A. K., Nardella, F. A., Mannik, M., Johansson, P. J., and Christensen, P. (1987). Identification of the site on IgG Fc for interaction with streptococci of groups A, C and G. Immunology *62*, 523-7.

Schwartz, J., and Roizman, B. (1969). Concerning the egress of herpes simplex virus from infected cells: electron and light microscope observations. Virology *38*, 42-9.

Sears, A. E., Hukkanen, V., Labow, M. A., Levine, A. J., and Roizman, B. (1991).

Expression of the herpes simplex virus 1 alpha transinducing factor (VP16) does not induce reactivation of latent virus or prevent the establishment of latency in mice. J Virol *65*, 2929-35.

Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J.-F., Als-Nielsen, J., Colman, D. R., and Hendrickson, W. A. (1995). Structural basis of cell-cell adhesion by cadherins. Nature *374*, 327-337.

Shieh, M. T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol *116*, 1273-81.

Shopes, B., Weetall, M., Holowka, D., and Baird, B. (1990). Recombinant human IgG1-murine IgE chimeric Ig. Construction, expression and binding to Fc-gamma receptors. J Immunol *145*, 3842-3848.

Simmons, A., Tscharke, D., and Speck, P. (1992). The role of immune mechanisms in control of herpes simplex virus infection of the peripheral nervous system. Curr Top Microbiol Immunol *179*, 31-56.

Simmons, A., and Tscharke, D. C. (1992). Anti-CD8 impairs clearance of herpes simplex virus from the nervous system: implications for the fate of virally infected neurons. J Exp Med *175*, 1337-44.

Sjogren, H. O., Hellstrom, I., Bansal, S. C., and Hellstrom, K. E. (1971). Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen--antibody complexes. Proc Natl Acad Sci U S A *68*, 1372-5.

Slack, R. S., and Miller, F. D. (1996). Viral vectors for modulating gene expression in neurons. Curr Opin Neurobiol *6*, 576-83.

Smibert, C. A., Johnson, D. C., and Smiley, J. R. (1992). Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. J Gen Virol *73*, 467-70.

Smiley, M. L., Hoxie, J. A., and Friedman, H. M. (1985). Herpes simplex virus type 1 infection of endothelial, epithelial, and fibroblast cells induces a receptor for C3b. J Immunol *134*, 2673-8.

Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machamer, C. E., van't Hof, W., van Meer, G., Moss, B., and Griffiths, G. (1993). Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. J Cell Biol *121*, 521-541.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol *136*, 1007-21.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell *91*, 119-26.

Sodora, D. L., Cohen, G. H., and Eisenberg, R. J. (1989). Influence of asparagine-linked oligosaccharides on antigenicity, processing, and cell surface expression of herpes simplex virus type 1 glycoprotein D. J Virol *63*, 5184-93.

Sodora, D. L., Eisenberg, R. J., and Cohen, G. H. (1991). Characterization of a recombinant herpes simplex virus which expresses a glycoprotein D lacking asparagine-linked oligosaccharides. J Virol *65*, 4432-41.

Spear, P. G. (1993). Entry of alphaviruses into cells. Semin. Virol. *4*, 167-180.

Spear, P. G. (1993). Membrane fusion induced by herpes simplex virus. In Viral fusion mechanisms, B. J., ed. (Boca Raton, Florida: CRCPress, Inc.), pp. 201-232.

Spivack, J. G., and Fraser, N. W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection in mice [published erratum appears in J Virol 1988 Feb;62(2):663]. J Virol *61*, 3841-7.

Springer, T. A. (1990). Adhesion receptors of the immune system. Nature *346*, 425-434.

Stackpole, C. W. (1969). Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumour transplants maintained at low temperature. J. Virol. *4*, 75-93.

Stanberry, L. R., Kern, E. R., Richards, J. T., Abbott, T. M., and Overall, J. C., Jr. (1982). Genital herpes in guinea pigs: pathogenesis of the primary infection and description of recurrent disease. J Infect Dis *146*, 397-404.

Stannard, L. M., Fuller, A. O., and Spear, P. G. (1987). Herpes simplex virus glycoproteins associated with different morphological entities projecting from the virion envelope. J Gen Virol *68*, 715-25.

Steiner, I. (1996). Human herpes viruses latent infection in the nervous system. Immunological Reviews *152*, 157-73.

Steiner, I., Spivack, J. G., Deshmane, S. L., Ace, C. I., Preston, C. M., and Fraser, N.

W. (1990). A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J Virol *64*, 1630-8.

Steiner, I., Spivack, J. G., Lirette, R. P., Brown, S. M., MacLean, A. R., Subak-Sharpe, J. H., and Fraser, N. W. (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. Embo J *8*, 505-11.

Steiner, I., Spivack, J. G., O'Boyle, D. R. d., Lavi, E., and Fraser, N. W. (1988). Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J Virol *62*, 3493-6.

Stevens, J. G. (1989). Human herpesviruses: a consideration of the latent state. Microbiol Rev *53*, 318-32.

Stevens, J. G., and Cook, M. L. (1971). Latent herpes simplex virus in spinal ganglia of mice. Science *173*, 843-5.

Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L., and Feldman, L. T. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science *235*, 1056-9.

Stokes, G. V. (1976). High-voltage electron microscope study of the release of vaccinia virus from whole cells. J Virol *18*, 636-43.

Sudhof, T. C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature *375*, 645653.

Tal-Singer, R., Seidel-Dugan, C., Fries, L., Huemer, H. P., Eisenberg, R. J., Cohen, G. H., and Friedman, H. M. (1991). Herpes simplex virus glycoprotein C is a receptor for complement component iC3b. J Infect Dis *164*, 750-3.

Tan, X., Pearce-Pratt, R., and Phillips, D. M. (1993). Productive infection of a cervical epithelial cell line with human immunodeficiency virus: implications for sexual transmission. J Virol *67*, 6447-52.

Thomsen, D. R., Roof, L. L., and Homa, F. L. (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.

Tirabassi, R. S., Townley, R. A., Eldridge, M. G., and Enquist, L. W. (1997). Characterization of pseudorabies virus mutants expressing carboxy- terminal

truncations of gE: evidence for envelope incorporation, virulence, and neurotropism domains. J Virol *71*, 6455-64.

Tognon, M., Furlong, D., Conley, A. J., and Roizman, B. (1981). Molecular genetics of herpes simplex virus. V. Characterization of a mutant defective in ability to form plaques at low temperatures and in a viral fraction which prevents accumulation of coreless capsids at nuclear pores late in infection. J Virol *40*, 870-80.

Torrisi, M. R., Di Lazzaro, C., Pavan, A., Pereira, L., and Campadelli-Fiume, G. (1992). Herpes simplex virus envelopment and maturation studied by fracture label. J Virol *66*, 554-61.

Trousdale, M. D., Steiner, I., Spivack, J. G., Deshmane, S. L., Brown, S. M., MacLean, A. R., Subak-Sharpe, J. H., and Fraser, N. W. (1991). In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J Virol *65*, 6989-93.

Turner, A., Bruun, B., Minson, T., and Browne, H. (1998). Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system [In Process Citation]. J Virol *72*, 873-5.

Uchida, N., Honjo, Y., Johnson, K. R., Wheelock, M. J., and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. J Cell Biol *135*, 767-79.

Ugolini, G., Kuypers, H. G., and Simmons, A. (1987). Retrograde transneuronal transfer of herpes simplex virus type 1 (HSV 1) from motoneurones. Brain Res *422*, 242-56.

Ugolini, G., Kuypers, H. G., and Strick, P. L. (1989). Transneuronal transfer of herpes virus from peripheral nerves to cortex and brainstem. Science *243*, 89-91.

van Engelenburg, F. A., Kaashoek, M. J., Rijsewijk, F. A., van den Burg, L., Moerman, A., Gielkens, A. L., and van Oirschot, J. T. (1994). A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. J Gen Virol *75*, 2311-8.

van Engelenburg, F. A., Kaashoek, M. J., van Oirschot, J. T., and Rijsewijk, F. A. (1995). A glycoprotein E deletion mutant of bovine herpesvirus 1 infects the same limited number of tissues in calves as wild-type virus, but for a shorter period. J Gen Virol *76*, 2387-92.

van Genderen, I. L., Brandimarti, R., Torrisi, M. R., Campadelli, G., and van Meer, G. (1994). The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. Virology *200*, 831-6.

Van Vliet, K. E., De Graaf-Miltenburg, L. A., Verhoef, J., and Van Strijp, J. A. (1992). Direct evidence for antibody bipolar bridging on herpes simplex virus-infected cells. Immunology *77*, 109-15.

Wagner, E. K., Devi-Rao, G., Feldman, L. T., Dobson, A. T., Zhang, Y. F., Flanagan, W. M., and Stevens, J. G. (1988). Physical characterization of the herpes simplex virus latency- associated transcript in neurons. J Virol *62*, 1194-202.

Walev, I., Wollert, K. C., Weise, K., and Falke, D. (1991). Characterization of fusion from without induced by herpes simplex virus. Arch Virol *117*, 29-44.

Warner, N. L., Harris, A. W., and Gutman, G. A. (1975). Membrane immunoglobulin and Fc receptors of murine T and B cell lymphomas. pp. 203-16. In: Seligmann M, et al., ed. Membrane receptors of lymphocytes. Amsterdam, North-Holland Publ,.

Watkins, J. F. (1964). Adsorption of sensitized sheep erythrocytes to HeLa cells infected with herpes simplex virus. Nature (London) *202*, 167-178.

Watkins, J. F. (1965). The relationship of the herpes simplex haemadsorption phenomenon to the virus growth cycle. Virology *26*, 746-53.

Weber, P. C., Levine, M., and Glorioso, J. C. (1987). Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science *236*, 576-9.

Wechsler, S. L., Nesburn, A. B., Zwaagstra, J., and Ghiasi, H. (1989). Sequence of the latency-related gene of herpes simplex virus type 1. Virology *168*, 168-172.

Weeks, B. S., Sundaresan, P., Nagashunmugam, T., Kang, E., and Friedman, H. M. (1997). The herpes simplex virus-1 glycoprotein E (gE) mediates IgG binding and cell-to-cell spread through distinct gE domains. Biochem Biophys Res Commun *235*, 31-5.

Weise, K., Kaerner, H. C., Glorioso, J., and Schroder, C. H. (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*, 1909-19.

Westmoreland, D., and Watkins, J. F. (1974). The IgG receptor induced by herpes

simplex virus: studies using radioiodinated IgG. J Gen Virol *24*, 167-78.

Whealy, M. E., Card, J. P., Meade, R. P., Robbins, A. K., and Enquist, L. W. (1991). Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J Virol *65*, 1066-81.

Whealy, M. E., Card, J. P., Robbins, A. K., Dubin, J. R., Rziha, H. J., and Enquist, L. W. (1993). Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J Virol *67*, 3786-97.

Wheelock, M. J., and Jensen, P. J. (1992). Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. J Cell Biol *117*, 415-25.

Whitbeck, J. C., Knapp, A. C., Enquist, L. W., Lawrence, W. C., and Bello, L. J. (1996). Synthesis, processing, and oligomerization of bovine herpesvirus 1 gE and gI membrane proteins. J Virol *70*, 7878-84.

Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce de Leon, M., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. J Virol *71*, 6083-93.

White, J. M. (1992). Membrane fusion. Science *258*, 917-24.

Whitley, R. J. (1996). Herpes simplex viruses. In Fields Virology, B. N. Fields, D. M. Knipe and P. M. e. a. Howley, eds. (Philadelphia: Lippincott - Raven Publishers), pp. 2297.

Whitley, R. J., Levin, M., Barton, N., Hershey, B. J., Davis, G., Keeney, R. E., Whelchel, J., Diethelm, A. G., Kartus, P., and Soong, S. J. (1984). Infections caused by herpes simplex virus in the immunocompromised host: natural history and topical acyclovir therapy. J Infect Dis *150*, 323-9.

Wiger, D., and Michaelsen, T. E. (1985). Binding site and subclass specificity of the herpes simplex virus type 1-induced Fc receptor. Immunology *54*, 565-72.

Wildy, P. (1973). Herpes: history and classification. In The Herpesviruses, A. S. Kaplan, ed. (New York: Academic press), pp. 1-25.

Wilson, D. W., Davis-Poynter, N., and Minson, A. C. (1994). Mutations in the cytoplasmic tail of herpes simplex virus glycoprotein H suppress cell fusion by a

syncytial strain. J Virol *68*, 6985-93.

Wittels, M., and Spear, P. G. (1991). Penetration of cells by herpes simplex virus does not require a low pH- dependent endocytic pathway. Virus Res *18*, 271-90.

Wolffe, E. J., Isaacs, S. N., and Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination [published erratum appears in J Virol 1993 Sep;67(9):5709-11]. J Virol *67*, 4732-41.

Wolffe, E. J., Katz, E., Weisberg, A., and Moss, B. (1997). The A34R glycoprotein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. J Virol *71*, 3904-15.

WuDunn, D., and Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol *63*, 52-8.

Yao, Z., Jackson, W., and Grose, C. (1993). Identification of the phosphorylation sequence in the cytoplasmic tail of the varicella-zoster virus Fc receptor glycoprotein gpI. J Virol *67*, 4464-73.

Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. Ann Rev Cell Dev Biol *13*, 119-46.

Yasuda, J., and Milgrom, F. (1968). Hemadsorption by herpes simplex-infected cell cultures. Int Arch Allergy Appl Immunol *33*, 151-70.

Yonemura, S., Itoh, M., Nagafuchi, A., and Tsukita, S. (1995). Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells. J Cell Sci *108*, 127-142.

York, I. A., and Johnson, D. C. (1994). Inhibition of humoral and cellular immune recognition by herpes simplex viruses. In Viroceptors, virokines and related immune modulators encoded by DNA viruses, G. McFadden, ed.: R. G. Landes Company), pp. 89-110.

York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L., and Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell *77*, 525-35.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A., and Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to

the trans-Golgi network. J Virol *69*, 7951-9.

Zhu, Z., Hao, Y., Gershon, M. D., Ambron, R. T., and Gershon, A. A. (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 cytosolic domain of the molecule. J Virol *70*, 6563-75.

Zsak, L., Zuckermann, F., Sugg, N., and Ben-Porat, T. (1992). Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J Virol *66*, 2316-25.

Zuckermann, F. A., Mettenleiter, T. C., Schreurs, C., Sugg, N., and Ben-Porat, T. (1988). Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J Virol *62*, 4622-6.