THE ROLE OF MANNOSE 6-PHOSPHATE RECEPTORS DURING HERPES
SIMPLEX VIRUS REPLICATION AND GLYCOPEPTIDE D BINDING

by

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for the Degree
Doctor of Philosophy

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THE ROLE OF MPRS DURING HSV REPLICATION
DOCTOR OF PHILOSOPHY (1997) McMASTER UNIVERSITY
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TITLE: The role of mannose 6-phosphate receptors during HSV replication and glycoprotein D binding

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ABSTRACT

The focus of my research has been to identify cellular proteins which facilitate herpes simplex virus (HSV) entry and cell-to-cell spread. Previous research had demonstrated that HSV glycoprotein gD bound to a cell surface protein which was required by the virus for entry into cells. I began efforts to identify and characterize cellular proteins which bound to a soluble form of gD which lacked the transmembrane domain and cytoplasmic tail. I demonstrated that both the 275 kDa and 46 kDa mannose 6-phosphate receptors (MPRs) bound to soluble and full-length gD. The interaction between gD and the 275 kDa MPR was mediated primarily through mannose 6-phosphate (M6P) residues present on N-linked oligosaccharides.

There was evidence that interactions between HSV and MPRs were important for virus entry. When the interaction between HSV and MPRs was blocked, HSV entry into cells was inhibited by 60% to 80%. However, HSV was able to enter into a mouse cell line that did not express MPRs. In addition to functioning during entry, there was evidence that MPRs also played a role in HSV egress or cell-to-cell spread. Blocking MPRs or preventing the addition of M6P residues to HSV glycoproteins resulted in inefficient viral egress and cell-to-cell spread. Therefore blocking the ability of HSV to interact with MPRs renders the virus less efficient at entry, cell-to-cell spread, and egress.

To determine whether MPRs functioned during HSV egress from cells, I
examined the intracellular localization of HSV gD by indirect immunofluorescence. I demonstrated that the HSV proteins gD, gI, and HSV capsids localized to the same compartment as the 275 kDa MPR. However, the intracellular retention of gD was not dependent on M6P residues. These data provide evidence that HSV proteins accumulate in intracellular compartments. These intracellular compartments represent a site involved in HSV exit from cells.
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I would like to thank my parents. Their continual support and encouragement has made much of my success possible. I would also like to thank my beautiful wife Janice. Her support, patience, and understanding have made possible the completion of this thesis. Finally, I would like to thank all the members of the cancer research group who I had the pleasure of working with. They made a difficult experience much more bearable. In particular I would like to thank Bruce Rowley, Craig Smibert, and Ian York for not only being good friends but also good role models. In addition, I feel a special word needs to be said about my friend and partner in crime Chris Counter. However, since that word is obscene I will move on to thank others. I would also like to acknowledge some special friends and colleagues Cindy Beauchamp, Peter Cheung, Kevin Dingwell, Kim Goldsmith, Lloyd Hutchinson, Peter Jugovic, Steve Primorac, Roman Tomazin, and Mark Whalley.
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<td>rpm</td>
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1 INTRODUCTION

1.0 Herpesviridae:

Members of the Herpesviridae family of animal viruses have a common virion structure, mode of replication, and the capacity to establish a latent infection (reviewed in Roizman, 1996). The Herpesvirus particle varies in size from 120 nm to 300 nm (Roizman, 1996) and is composed of a virion core, capsid, tegument, and lipid envelope. The viral core of Herpesviruses contains a double stranded DNA genome which is maintained in a torus structure (Furlong et al., 1972; Nazerian, 1974). The genome size of characterized Herpesviruses varies from between 86,000 base pairs for channel catfish virus (Cebrian et al., 1982; Wolf and Darlington, 1971) to 229,000 base pairs for human cytomegalovirus (Smith, 1956). The viral DNA genome is contained within the capsid which is a 110 nm icosahedral structure composed of 12 pentomeric capsomers and 150 hexomeric capsomers. Surrounding the capsid is the tegument (Roizman, 1996) which is an amorphous structure composed of viral proteins. The lipid envelope is derived from the host cell (Armstrong et al., 1961; Morgan et al., 1959) and contains glycosylated and nonglycosylated viral membrane proteins.

The Herpesviridae family is divided into three sub-families: alphaherpesviruses, betaherpesviruses, and gammaherpesviruses. Herpesviruses are grouped into these sub-families based on similarities in:
genome structure, tissue tropism, cytopathology, and site of latent infection (Roizman, 1996). A brief description of each of the Herpesviridae sub-families is presented below.

The beta herpesviruses are characterized by a long replication cycle, infected cells becoming enlarged, and a latent phase which is thought to be established in secretory glands, lymphoreticular cells, kidneys, or other tissues (Roizman, 1996). Human cytomegalovirus (HCMV) is the prototype for the beta herpesviruses. HCMV has the largest genome of the characterized Herpesviridae viruses consisting of 229,000 base pairs (Smith, 1956) which encodes at least 208 open reading frames (Mocarski, 1996). The replication cycle for HCMV is slow, requiring 48 to 72 hours to produce infectious viral particles (Mocarski, 1996). Clinically, HCMV suppresses the host immune system and produces asymptomatic infections but can produce tissue damage in various organs and cause cytomegalic cells (infected cells becoming very large) (Britt and Alford, 1996). HCMV is an opportunistic infectious agent that reactivates in immunocompromised individuals causing further immunosuppression as well as infecting cells and organs throughout the body and producing tissue damage (Britt and Alford, 1996).

The gamma herpesviruses are characterized by growth in lymphoblastoid cells and latency in lymphoid tissue (Roizman, 1996). Epstein-Barr virus (EBV) is the best characterized of the gamma herpesviruses. EBV has a viral genome of 172,000 base pairs which encodes approximately 100 genes (Middleton et
EBV exclusively infects primate B-lymphocytes where it establishes a persistent latent infection (Kieff, 1996). Within the latent state, the EBV genome persists as a circular DNA episome which is replicated once per cell cycle, thus providing a useful model for studying eukaryotic DNA replication (Kieff, 1996). EBV causes a number of human diseases including infectious mononucleosis, Burkitt’s lymphoma (a B-cell lymphoma), nasopharyngeal carcinoma, and hairy oral leukoplakia (Rickinson and Kieff, 1996).

The alphaherpesviruses share a number of characteristics including a short replication cycle, lytic infection, and the ability to form a latent infection in sensory ganglia (Roizman, 1996). Members of the alphaherpesviruses include herpes simplex virus (HSV) types 1 and 2, varicella zoster virus (VZV), and pseudorabies virus (PRV).

1.0.1 Herpes simplex viruses:

Among the best characterized of the human alphaherpesvirus sub-family are the closely related HSV types 1 (HSV-1) and 2 (HSV-2). The HSV particle is approximately 150 nm in diameter (Roizman and Sears, 1996). The HSV genome is composed of approximately 152,000 base pairs (Kieff et al., 1971; Wadsworth et al., 1975) arranged into two regions: the unique long (UL) and unique short (US) (reviewed in Roizman and Sears, 1996; Sheldrick and Berthelot, 1975). The UL and US regions are flanked by reiterated repeats
(Wadsworth et al., 1975). At least 78 proteins are encoded by the HSV genome including structural components of the virion, membrane proteins, transcription factors, inhibitors of host protein synthesis, enzymes involved in nucleic acid metabolism and DNA replication, and inhibitors of the immune response (Roizman and Sears, 1996). The vast majority of HSV infections are minor or asymptomatic. However, an HSV infection under rare circumstances can cause: herpetic encephalitis, herpetic keratoconjunctivitis, herpetic vulvovaginitis, atypical pneumonia, and eczema herpeticum (Whitley, 1996).

1.1 Infectious cycle of HSV:

Primary infection of humans by HSV types 1 and 2 occurs when an HSV infected individual who is producing HSV comes into contact with a seronegative individual. Primary infection of a seronegative individual usually occurs at mucosal surfaces or abraded skin or in the eye. Replication of HSV in epithelial cells, keratinocytes, fibroblasts, or endothelial cells results in infectious viral particles being released by the cell (discussed in section 1.1.7) as well as cell lysis. Clinically, a primary HSV infection results in a skin lesion which is caused by a combination of inflammatory response and virus-induced cell death (Whitley, 1996).

Following replication at the site of primary infection, the virus infects sensory neurons and capsids are transported retro-axonally (Cook and Stevens, 1973) to the sensory ganglia where the virus establishes a latent
infection (discussed in section 1.1.9) (Roizman and Sears, 1996). During HSV latency, the viral genome is maintained in neurons in the absence of clinical symptoms. At intervals, viral reactivation in sensory ganglia results in infectious HSV being conveyed by axonal transport back to a site close to the region of primary infection (Cook and Stevens, 1973). HSV may then re-initiate replication which can cause skin lesions to reoccur. The infectious cycle of an HSV infection has primarily been elucidated in cultured cells in vitro and the available data is summarized below.

1.1.1 HSV entry into cells:

HSV entry into cells is mediated by proteins in the envelope of the viral particle. The virion glycoproteins gB (UL27), gD (US6) and the hetero-oligomer gH (UL22)/gL (UL1) are all required for the production of infectious virions (Cai et al., 1987; Forrester et al., 1992; Hutchinson et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993). The remaining virion glycoproteins gC (UL44), gE (US8), gG (US4), gI (US7), gJ (US5), and gM (UL10) are not essential for replication of the virus in certain cultured cells (Baines and Roizman, 1993; Frame et al., 1986; Heine et al., 1974; Longnecker et al., 1987; Longnecker and Roizman, 1987; Longnecker and Roizman, 1986; MacLean et al., 1993) but may play important roles during virus replication and spread in vivo. In addition, there are a number of nonglycosylated virion membrane proteins including UL20 (Baines et al., 1991; MacLean et al., 1991; Ward et al., 1994), UL34
(Purves et al., 1992), and UL43 (MacLean et al., 1991) whose function during HSV replication is unclear.

The mechanism of HSV entry into host cells is thought to occur in 3 distinct phases: 1) adsorption, 2) stable attachment, and 3) penetration of the plasma membrane (Fuller and Lee, 1992; Johnson et al., 1990; Johnson and Ligas, 1988; McClain and Fuller, 1994). Although these distinct stages of entry have been defined, the role that the HSV virion surface proteins perform, the specific cellular proteins that they interact with remain largely uncharacterized.

1.1.2 Adsorption of HSV:

The first interactions between HSV and the cell surface involves adsorption of the virus to glycosaminoglycans (GAGs). GAGs are polysaccharides composed of repeating disaccharide units which are referred to as proteoglycans when attached to a protein core (reviewed in Hook et al., 1984). The composition of sugars, degree of sulphation, and site of sulphate group attachment are determinants which divide the GAGs into at least 4 categories: hyaluronate, chondroitin sulphate/dermatan sulphate, heparan sulphate/heparin, and keratan sulphate (Hook et al., 1984). Heparin and heparan sulfate are sulfated polysaccharides containing glucuronic acid, iduronic acid, and galactosamine sugars (Hook et al., 1984). The addition of heparin to preparations of HSV stocks was found to inhibit infection of cells
(Nahmias and Kibrick, 1964). However the stage at which heparin acted during the replication cycle of the virus was not defined until more recently.

The inhibition of HSV entry by heparin was investigated to determine the mechanism of viral inhibition. Both soluble heparin and heparan sulphate inhibited adsorption of HSV to cells (WuDunn and Spear, 1989). HSV infection was inhibited by up to 90% when cell lines were defective in the synthesis of heparan sulphate (Gruenheid et al., 1993; Shieh et al., 1992); cells were pretreated with soluble heparin or heparan sulphate (Lycke et al., 1991; Shieh et al., 1992; WuDunn and Spear, 1989); or heparan sulphate proteoglycans were cleaved from the surfaces of cells by enzymatic digestion (Shieh et al., 1992; WuDunn and Spear, 1989). The addition of heparin or heparan sulphate after adsorption of HSV to cells had no effect on viral entry (WuDunn and Spear, 1989) suggesting that post-adsorption stages of entry occur by a heparin-insensitive mechanism. From these data it was concluded that HSV adsorbed to heparan sulphate during entry into cells.

Initial reports on the ability of HSV to interact with heparan sulphate proteoglycans reported no effect of dermatan sulphate or chondroitin sulphate on HSV entry into Chinese hamster ovary (CHO), Hep-2, and green monkey kidney (GMK) cells (Lycke et al., 1991; Shieh et al., 1992; WuDunn and Spear, 1989). However chondroitin sulphate and dermatan sulphate partially inhibited HSV entry into a mouse L cell line that was defective for heparan sulphate proteoglycan synthesis (Banfield et al., 1995b). Furthermore, HSV entry into
normal mouse L cells was inhibited by dermalan sulphate but not chondroitin sulphate (Banfield et al., 1995b). Several explanations for the differential response of cells to chondroitin sulphate and dermalan sulphate have been proposed (Banfield et al., 1995b). It is possible that the presence of heparan sulphate on the surfaces of cells “masks” interactions of HSV with chondroitin sulphate. Alternatively, differences in the types and composition of proteoglycans present on the surfaces of different cell types may result in altered mechanisms of HSV adsorption between different cell type. Whatever the cause of cell-type differences in susceptibility to chondroitin sulphate and dermalan sulphate, they highlight the fact that HSV may utilize different cell surface components to enter into different cell types.

It is possible that the interaction of HSV with GAGs is the result of the virus binding to highly sulphated molecules carrying a negative charge. Heparin/heparan sulphate with increased levels of sulphation were more effective at inhibiting HSV adsorption to cells, supporting a model in which the level of sulphation is the primary determinant in the effectiveness of heparin/heparan sulphate at inhibiting HSV adsorption (Herold et al., 1995; Lycke et al., 1991). However dextran sulphate, a polyanion which is more highly sulphated than heparin, was unable to inhibit HSV adsorption to cells. These data suggest that the sugar groups of the GAGs or the conformation with which a particular GAG chain displays the sulphate group is important for virus binding.
The HSV glycoproteins gC and gB are responsible for binding to heparan sulphate proteoglycans (Herold et al., 1991). Only the glycoproteins gB and gC from an HSV infected cell lysate could bind to a heparin-Sepharose column (Herold et al., 1991). Virions lacking gC were 75% reduced in adsorption to cells; whereas virions that lacked gB had almost normal adsorption kinetics compared to wild-type virus (Herold et al., 1994). HSV virions which lacked both gC and gB showed almost no adsorption to the cell surface (Herold et al., 1994). It is possible that gC functions as the dominant protein for virus adsorption. In this scenario, gB functions to compensate for the loss of gC by mediating viral adsorption in the absence of gC. Alternatively gB and gC may bind to different determinants on GAG molecules (Herold et al., 1995) which may result in gB and gC differentially contributing to adsorption depending on the cell type.

Binding to GAGs is required for efficient HSV entry into cells. However, in cell lines which lack any of the major GAGs, HSV produced less than 1% the number of plaques compared to wild-type cells (Banfield et al., 1995a). Furthermore heparan sulphate had only moderate inhibitory effect on HSV entry into GAG-deficient cells (Banfield et al., 1995a). These data suggest that at low efficiency, HSV may infect cells by bypassing the adsorption stage or there may exist a GAG-independent adsorption event.

The function of HSV adsorption to GAGs during entry is unclear. It is possible that GAGs represent a receptor for HSV involved in concentrating the
virus at sites close to the plasma membrane where subsequent entry events may then occur. Alternatively the binding of HSV to GAGs may induce conformational changes in HSV glycoproteins, “activating” them for subsequent events in entry. However infection of cell lines which lacked heparan sulphate proteoglycans was not enhanced by the addition of soluble heparin suggesting that a mechanism whereby heparin is required only for the activation of viral proteins is not correct (Shieh et al., 1992). It is equally possible that soluble heparin may inappropriately “activate” HSV for later events in entry prior to binding of the virus to the cell surface. Adsorption is required for efficient entry into cells although in the absence of GAGs a small percentage of HSV virions can enter into cells.

1.1.3 Stable attachment of HSV to cellular receptors:

Following adsorption, HSV forms a heparin-insensitive attachment to a cell surface receptor (Fuller and Lee, 1992). The glycoprotein(s) responsible for binding to such a receptor have not been unequivocally identified. Since entry into cells requires virus binding to receptors (discussed in section 1.2 and 1.2.1), the glycoprotein(s) responsible for attachment of HSV must be essential for virus infectivity. The glycoproteins gB, gD, and the heteroligomer gH/gL are essential for entry of HSV into cultured cells (Cai et al., 1987; Forrester et al., 1992; Hutchinson et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993) and represent good candidates for receptor binding proteins.
Glycoprotein gD has properties of a protein which binds to a stable attachment receptor. Pretreatment of cells with UV-inactivated wild-type, gH-minus, or gB-minus HSV virions inhibited subsequent infection of cells by infectious HSV particles (Forrester et al., 1992; Johnson and Ligas, 1988; Lee and Fuller, 1993). However, pretreatment of cells with UV-inactivated HSV particles lacking gD (which adsorbed normally to cells) did not block subsequent infection of the cells by infectious HSV. UV-inactivated HSV particles were not inhibiting post-entry stages of HSV replication since UV-inactivated virions had no effect on HSV plaque formation or gene expression when applied after the cells were treated with HSV-1 (Johnson and Ligas, 1988). This suggested that gD on the surface of the virion binds to a cell surface component required for HSV entry (Johnson and Ligas, 1988). These observations were extended using soluble forms of gD1 (termed gD-1t) and gD2 (termed gD-2t) which were truncated to remove the transmembrane domain and cytoplasmic tail. Pretreatment of cells with either gD1t or gD2t prevented subsequent infection of cells by infectious HSV-1 or HSV-2 (Johnson et al., 1990). Soluble gD-1t and gD-2t bound in a saturable manner to approximately 500,000 sites/cell with a $k_d$ of 2.4X10^{-7} M (Johnson et al., 1990). These data further supported the hypothesis that gD binds to a cell surface component which is required for entry. In addition gD from HSV-1 and HSV-2
binds to an identical or similar cell surface component since type 2 gD can block HSV-1 entry and gD1 can block HSV-2 entry (Johnson et al., 1990).

It remains possible that other HSV glycoproteins mediate attachment to cells but there exist no conclusive evidence to support this. Only the glycoproteins gB, gC, and gD bound to biotinylated cell surface membranes (Kuhn et al., 1990). However it is unclear whether gC or gB binding to these membranes was mediated through their ability to bind to GAGs (Herold et al., 1991), or to other cellular factors in addition to GAGs. Although there are no reports of other HSV glycoproteins supporting viral attachment to cells this does not mean that alternate mechanisms for stable attachment of HSV do not exist. The observation that gB-negative HSV particles had similar adsorption kinetics compared to wild-type virions but a gB/gC negative virus demonstrated a more dramatic reduction in HSV adsorption than gC-negative virions alone (Herold et al., 1991) illustrates how a phenotype may be “masked” by a more dominant protein mediating the same effect. It is possible that the presence of gD in the virion envelope “masks” attachment events occurring in a gD-independent manner. However these gD-independent events can not cause entry of a substantial fraction of HSV (Ligas and Johnson, 1988).

The cellular receptor involved in HSV stable attachment is not a GAG. It was estimated that approximately 5000 UV-inactivated HSV-1 particles/cell were required to block HSV entry into cells (Johnson and Ligas, 1988). However greater than 40,000 UV-inactivated HSV particles/cell adsorbed under
these conditions (Johnson and Ligas, 1988). These data suggest that gD on the surface of the virion binds to a cell surface component which is relatively limited in number and required by the virus for entry into cells. Furthermore, soluble gD bound to the cell surface in a saturable manner and was not inhibited by removal of GAGs from the cell surface (Johnson et al., 1990). Additional evidence to suggest that GAGs are not the attachment receptor comes from research on HSV entry into swine testis cells. These cells possess functional GAGs but lack a cell surface component which mediates viral attachment (Subramanian et al., 1994). Therefore, taken together these data imply that HSV binds to a saturable cell surface component after adsorption which does not appear to be GAGs.

1.1.4 Penetration across the plasma membrane:

Following attachment to a cell surface receptor HSV penetrates the plasma membrane. Evidence favours a model in which HSV virions fuse directly with the plasma membrane rather than being taken up by endosomal vesicles followed by virion fusion with the vesicle membrane. Low pH endosomes are not required for HSV entry since weak bases, such as chloroquine, do not affect this process (Wittels and Spear, 1990). Blocking receptor mediated endocytosis by potassium depletion and hypotonic shock also had no affect on the ability of HSV to form plaques (Wittels and Spear, 1990). In addition, resistance to photo-treatment concomitant with resistance to
HSV neutralizing antibodies (DeLuca et al., 1981) and electron microscopy (EM) data (Lee an Fuller, 1993; Rosenthal et al., 1989), provided evidence that virions fuse with the plasma membrane. However these data do not rule out HSV entry into an endosomal compartment or by other mechanisms such as caveolae (Anderson et al., 1992) followed by rapid fusion at relatively neutral pH or by a pH independent mechanism.

1.1.4.1 Syncytial formation:

The mechanism of virus-induced cell-to-cell fusion, a phenomenon known as syncytial formation, may be similar, but not identical to the fusion between the virion envelope and the plasma membrane. The identification of genes bearing syncytial mutations may identify proteins which regulate or participate in fusion events. Syncytial mutations reside within: the N-terminal and C-terminal domains of gK (UL53) (Dolter et al., 1994); the cytoplasmic domain of gB (Baghian et al., 1993; Engel et al., 1993; Gage et al., 1993); the UL20 gene product (Baines et al., 1991); and the UL24 gene product (Jacobson et al., 1989). In addition to mutations which induce cell fusion, a number of mutations have been identified that block syncytial plaque formation caused by a syncytial mutation at another locus. Mutations within UL45 or in the cytoplasmic tail of gH abolished syncytial plaque formation by an HSV strain with a syncytial mutation in the gB locus (Haanes et al., 1994; Wilson et al., 1994). Furthermore, a syncytial plaque phenotype could be reverted to a wild-
type plaque morphology when HSV strains containing syncytial mutations in
either gB or gK were supplied with the corresponding wild-type protein in trans
(Hutchinson et al., 1993). Therefore, mutants of the HSV proteins gB, gH, gK,
UL20, UL24, and UL45 affect syncytial plaque formation.

Syncytial mutations cause unregulated cell-to-cell fusion but may not be
a good indicator of proteins involved in virion-cell fusion. Some syncytial
mutations are present in genes not associated with the virion envelope and may
regulate fusion during egress of HSV particles (discussed in section 1.1.7). The
gK protein is localized to the perinuclear and nuclear membranes, and is
unlikely to be present in the virion (Hutchinson et al., 1995), yet it is frequently
involved in the syncytial phenotype. Therefore gK is not likely to be involved in
virion-cell fusion but may affect the cell surface distribution of other proteins
which promote fusion. Similarly, the syncytial phenotype of viruses with
mutations in the UL20 and UL24 genes result from lack of expression of the
gene products (Baines et al., 1991; Jacobson et al., 1989; Sanders et al., 1982).
These data suggest that gK, UL20 and UL24 may be regulators of cell fusion
but do not directly participate in the process of virion-cell fusion, while gB, gH,
and UL45 may be directly involved in virion-cell fusion.

1.1.4.2 Glycoproteins involved in fusion at the cell surface

As has previously been described, gD is required for HSV penetration of
the plasma membrane. gD appears to be involved in receptor binding which is
required for subsequent fusion events to occur (discussed in section 1.1.3). At least 3 glycoproteins, gB, gH, and gL may play a direct role in virion-cell fusion. Evidence supporting a role for gB in virion-cell fusion is based on: the presence of syncytial mutations within gB; anti-gB antibodies inhibition of formation of syncytial plaques; and the requirement for gB during entry (Baghain et al., 1993; Engel et al., 1993; Gage et al., 1993). gH and gL may also function during penetration of the plasma membrane. It is unclear what essential role gH and gL perform during HSV entry, though current data points to a role for gH in a post-stable attachment step of entry. In the presence of anti-gH neutralizing antibodies HSV virions are observed by EM to be partially fused to the plasma membrane but the capsids failed to enter into cells (Fuller and Lee, 1992) suggesting that fusion had been initiated but events leading to release of the viral capsid were blocked. These studies were carried out using EM which fails to differentiate between infectious and non-infectious viral particles making the significance of these observations unclear. These data suggest that gH/gL are not required for the initiation of fusion but may function in the expansion of the fusion pore to allow release of the viral capsid into the cytosol. The exact mechanism of fusion and the glycoproteins involved remain to be positively determined although the glycoproteins gB, gD, gH, and gL are required for virion-cell fusion.
1.1.5 HSV proteins function in concert during HSV entry into cells:

During entry, the HSV glycoproteins may function in concert to promote appropriate fusion of the virus with the plasma membrane. Many viruses require a “trigger” for virion-cell fusion to occur. For example, low-pH triggers fusion of influenza virus with endosomes (reviewed in White, 1992). The presence of a sequential series of interactions between the virion and the cell surface may prevent inappropriate activation of the viral fusion machinery until the virus is attached to the cell surface. If the virus is not in close apposition to a cellular membrane when the fusion polypeptide(s) is activated, the virus may be rendered non-infectious.

Cooperativity may occur between adsorption and penetration during HSV entry into cells. GAGs are required for virion-cell fusion (Shieh and Spear, 1994). A CHO cell line deficient for GAG synthesis produced approximately 5% of the number of syncytial plaques compared to wild-type CHO cell lines when transfected with HSV DNA containing a syncytial mutation (Shieh and Spear, 1994). The addition of soluble heparin to the GAG-deficient CHO cell lines restored the ability to produce syncytial plaques to a level approximately 60% of that obtained with wild-type CHO cells. These data suggest that heparin/heparan sulfate GAGs may “activate” glycoprotein(s) responsible for cell fusion suggesting a link between adsorption and cell fusion.

The glycoproteins on the virion surface are not randomly distributed but appear to be clustered perhaps in functional units. Electron microscopy of
negatively stained HSV virions revealed that gB is present in patches on the
virion surface which appeared to exclusively contain gB, whereas gC and gD
were randomly distributed on the virion surface (Stannard et al., 1987). gB and
gC appeared as large spikes (gC approximately 24 nm and gB approximately
14 nm) whereas gD spikes were shorter (8-10 nm) (Stannard et al., 1987).
However virus particles described in these studies appeared morphologically
aberrant which cast some doubt on these results. In addition, the glycoproteins
gB (Sarmiento et al., 1979) and gD (Eisenberg et al., 1982; Gibson and Spear,
1983) formed homo-oligomers while gE/gI (Johnson et al., 1988) and gH/gL
(Hutchinson et al., 1992) formed hetero-oligomer complexes. Taken together
these data suggested that there was some order to the arrangement of
glycoproteins on the virion surface. Cross-linking studies of HSV virions have
demonstrated that gB/gC, gC/gD, gD/gB, gH/gL, gC/gL, and gD/gL are in close
proximity to each other in the virion envelope (Handler et al., 1996a; Handler et
al., 1996b). However cross-linking data are prone to artifacts and may not
provide an accurate representation of the contacts made between
glycoproteins. None of these studies examined glycoprotein organization
during the course of the entry process. It is possible that during entry, changes
in the glycoprotein organization occur resulting in novel interactions between
proteins. These data indicate that HSV glycoproteins are not in discrete units in
isolation from other glycoproteins, but are in close proximity to a number of
different glycoproteins which may allow for conformational changes in one glycoprotein to trigger functions in others.

1.1.6 HSV Replication and Capsid Assembly:

Following penetration, the viral capsid is released into the cytoplasm where it is transported to a nuclear pore (Batterson et al., 1983; Tognon et al., 1981). The viral DNA is released, probably through the nuclear pore, into the nucleus of the infected cell where DNA transcription and viral replication occurs (Roizman and Sears, 1996). Transcription of viral genes occurs in three phases: immediate early, early, and late. The immediate early or α genes are expressed in the absence of viral replication and include: the transactivators ICP0, ICP4, and ICP22 (reviewed in Roizman and Sears, 1996); a post-transcriptional regulator ICP27 (Hardy and Sandri-Goldin, 1994; McMahan and Schaffer, 1990); and the immune evasion protein ICP47 (York et al., 1994). The early or β genes are synthesized following the immediate early genes and require the presence of the ICP4 protein (Imbalzano et al., 1990; Imbalzano et al., 1991). The early genes include proteins involved in DNA replication and certain structural proteins and glycoproteins (Roizman and Sears, 1996). The late or γ genes are synthesized after viral DNA replication has occurred. The late genes include structural proteins of the virus (Roizman and Sears, 1996).
At least late in infection, HSV DNA replicates by a rolling circle mechanism which entails continuous replication of a circular genome without genome cleavage. During an HSV infection concatemers of HSV DNA have been isolated which are characteristic of rolling-circle replication (Ben-Porat and Tokazewski, 1977; Jacob et al., 1979). The presence of HSV genomes that show a variety of isomers of the $U_L$ and $U_S$ segments (Delius and Clements, 1976; Hayward et al., 1975) are most easily explained through rolling circle replication generating concatemers of DNA followed by cleavage of the concatemers into genome length pieces (Deiss et al., 1986). Inversion of the $U_L$ segment relative to the $U_S$ segment occurs through circularization of the HSV genome and recombination between reiterated repeats.

The viral DNA is packaged into capsids which are assembled in the nucleus. The outer capsid shell is composed of the viral proteins VP5 (UL19), VP19C (UL38), VP23 (UL18), and VP26 (UL35) (McNabb and Courtney, 1992; Newcomb et al., 1993; Pertuiset et al., 1989; Prevelige and King, 1993; Rixon et al., 1990; Trus et al., 1992). The inner core of the type B capsid (capsid which lacks viral DNA) contains the proteins VP21 (UL26), VP24 (UL26), and VP22a (UL26.5) (Davison et al., 1992; Liu and Roizman, 1991; Person et al., 1993). Capsid assembly begins when VP5 binds to the UL26.5 protein in the cytoplasm of infected cells and the complex is transported to the nucleus (Thomsen et al., 1995). In the nucleus it is proposed that UL26.5/VP5 dimers
spontaneously assemble to form the scaffold structure of the inner core and a framework for assembly of the outer capsid. VP5 may catalyze or provide a scaffold for the polymerization of the outer capsid. The capsid proteins appear to be sufficient for icosadeltahedral capsid assembly since purified baculovirus produced capsid proteins VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26, spontaneously assembled to form viral capsids (Thomsen et al., 1995).

Packaging of the viral DNA into the type B capsids requires sequences within the reiterated repeats of the HSV genome and occurs by a “head-full” mechanism (reviewed in Roizman and Sears, 1996). Within each of the reiterated repeats (which flank the UL and US regions of the HSV genome) is a sequence composed of direct repeats and unique sequences termed the a sequence (Wadsworth et al., 1975). HSV viral DNA packaging begins when a single a sequence of a concatemer of HSV DNA binds to the capsid. Uptake of viral DNA into the capsid continues until the capsid is full (a phenomenon known as the “head-full” mechanism) and another a sequence in the same orientation as the first a sequence is encountered (one complete genome) (Deiss et al., 1986). The “head-full” mechanism ensures that partial HSV genomes or multiple genomes are not incorrectly packaged into the capsid.
1.1.7 HSV egress:

The HSV egress pathway begins with envelopment of the viral nucleocapsids and ends with release of viral particles from infected cells. It is well accepted that HSV capsids in the nucleus become initially enveloped by budding through the inner nuclear lamella (Darlington and Moss, 1968). The mechanisms by which these enveloped HSV particles reach the cell surface is controversial. The nuclear membrane predominantly contains immature HSV glycoproteins whose oligosaccharides have not been modified by Golgi-derived enzymes (Ali et al., 1987; Avitable et al., 1994; Compton and Courtney, 1984; Gilbert and Ghosh, 1993; Torrisi et al., 1992) whereas extracellular virions contain fully processed oligosaccharides (Compton and Courtney, 1984). These data suggest that the HSV virion must either lose its nuclear derived envelope and reacquire an envelope with mature glycoproteins or, viral particles travel through the Golgi and have the oligosaccharides on their glycoproteins modified while embedded in the virion surface. In addition, models for HSV egress must explain the presence of non-enveloped capsids in the cytoplasm of infected cells (Penfold et al., 1994). These capsids may be part of the HSV egress pathway or represent a "dead-end" pathway of non-infectious capsids which are aberrantly de-enveloped and never re-enveloped.
1.1.7.1 HSV egress by the exocytic pathway:

Treatment of HSV infected cells with monensin, which blocks budding of vesicles from the Golgi, caused enveloped HSV virions to accumulate in Golgi-derived vesicles (Johnson and Spear, 1982). This led Johnson and Spear (1982) to propose a model in which enveloped HSV virions in the perinuclear space follow the secretory pathway to the cell surface. The glycoproteins associated with the virion envelope were predicted to be modified as the viral particle transits through the endoplasmic reticulum (ER) and Golgi. In this model unenveloped capsids in the cytoplasm of infected cells are the result of inappropriate fusion between organelle membranes and HSV particles (Campadelli-Fiume et al., 1991) or are the result of reinfection of the cell by newly released infectious viral particles. In general, it is a rare event to observe nucleocapsids in the process of envelopment at the inner nuclear envelope suggesting that the envelopment process occurs rapidly. However partly enveloped cytoplasmic capsids are often observed by EM. This suggests that either cytoplasmic capsid envelopment is a slow process or, that these partially enveloped capsids observed by static EM represent viral particles in a “dead-end” egress pathway not involved in the production of infectious HSV particles.

1.1.7.2 HSV egress by deenvelopment/reenvelopment:

An alternate mechanism for herpesvirus egress has been proposed based on a model for egress of VZV, a related virus to HSV. EM studies of VZV
infected cells documented the presence of VZV particles only within cytoplasmic vesicles containing \[^3H\]fucose label (Jones and Grose, 1988). This sugar is incorporated onto glycoproteins in the Golgi apparatus, therefore VZV particles are not present in cytoplasmic vesicles derived from the nuclear envelope or ER. These data lead Jones and Grose (1988) to propose a model in which enveloped VZV particles in the perinuclear space lose their lipid envelope as they bud through the outer nuclear lamella. The unenveloped VZV capsids in the cytoplasm reacquired an envelope by budding into cytoplasmic vesicles. Therefore it was suggested that VZV egress may occur by a deenvelopment/reenvelopment model. Another alphaherpesvirus which may undergo deenvelopment/reenvelopment is PRV. Studies involving the drug brefeldin A (which blocks ER to Golgi transport) suggested that PRV egress occurs through deenvelopment at the ER followed by reenvelopment of the PRV capsid in the Golgi region (Whealy et al., 1991). Interestingly, EM studies of PRV reenvelopment is thought to occur by wrapping of a membranous vesicle around the viral capsid. When the vesicle membrane has surrounded the capsid, fusion between vesicle membranes occurs resulting in an enveloped viral particle inside a cytoplasmic vesicle.

Both the models of Johnson and Spear (1982) and Jones and Grose (1988) largely rely on EM (static) pictures of the virus egress pathway and it is impossible to determine the chain of events from such images, ie what appears to be a capsid undergoing deenvelopment could easily be mistaken as
reenvelopment of the capsid. In addition, EM can not differentiate between infectious and non-infectious particles making interpretation of what is important during egress and what is a “dead-end” egress pathway difficult. However there is some biochemical evidence to suggest that deenvelopment/reenvelopment occurs during HSV egress. The addition of an ER retention signal to gH, which caused the retention of gH in the ER/nuclear membrane as assessed by an inability of N-linked oligosaccharides to be processed to complex type oligosaccharides, resulted in the production of non-infectious viral particles that lacked gH in the cell culture supernatant (Browne et al., 1996). Since it was predicted that the gH became incorporated into the viral envelope upon budding into the perinuclear space, the virus must lose this envelope and reacquire a viral envelope in the Golgi, trans Golgi network (TGN), or cytoplasmic vesicles to explain these observations. However, the interpretation of these data were made difficult because it was not proven that the ER-retained gH reached the inner nuclear envelope or became associated with viral particles. Support for the deenvelopment/reenvelopment model has also come from studies of HSV-infected neurons. In neurons unenveloped capsids travel along the axons and become enveloped at the axonal termini (Penfold et al., 1994). This may represent a specialized mechanism of HSV egress in neurons, or may demonstrate that unenveloped capsids are part of the general egress pathway for HSV. Further evidence to suggest that HSV virions lose their lipid envelope derived from the inner nuclear membrane
comes from analysis of the lipid content of HSV virions. The lipid composition of extracellular HSV virions was different from the composition of the nuclear membranes (Van Genderen et al., 1994; Steinhart et al., 1981). These data suggest that HSV virions lose their nuclear envelope during the egress pathway. However it is possible that lipids are inserted or modified in the virion envelope as the virus transits through the ER/Golgi. These data support a model in which HSV egress occurs by deenvelopment/reenvelopment.

1.1.7.3 Transport of HSV virions in cytoplasmic vesicles to the cell surface:

Both models of HSV egress (described in section 1.1.7.1 and 1.1.7.2) do not identity the cytoplasmic vesicles containing enveloped HSV particles. VZV, as does HSV, accumulates in cytoplasmic vesicles which were assumed to be secretory vesicles (Cook and Stevens, 1970; Gershon et al., 1973; Jones and Grose, 1988). There is evidence to suggest that VZV may accumulate in endosomal vesicles. Studies involving quantitative EM suggested that unenveloped VZV capsids acquire their envelope by budding into the TGN (Gershon et al., 1994). Vesicles containing VZV particles subsequently moved from the TGN but did not follow the secretory pathway to the cell surface. Instead, the cytoplasmic vesicles containing VZV virions were acidic (Gershon et al., 1994) suggesting that virions were accumulating in endosomes. In human fibroblast cells in culture, the VZV particles within cytoplasmic vesicles
appear to be largely degraded (Gabel et al., 1989; Gershon et al., 1994). Like VZV, a proportion of the HSV particles remain cell-associated and are found to accumulate in cytoplasmic vesicles with a variety of morphologies (Dick and Rosenthal, 1995).

1.1.7.4 Viral proteins involved in HSV egress:

The viral proteins involved in HSV egress have not been well defined. HSV virions lacking gK showed a dramatic decrease in the number of enveloped HSV particles in the perinuclear space and at the cell surface, with a concomitant increase of unenveloped capsids in the cytoplasm of infected cells (Hutchinson and Johnson, 1995). Conversely, overexpression of gK resulted in accumulation of viral particles in the perinuclear space (Hutchinson and Johnson, 1995). These data coupled with the known syncytial point mutations in gK (Bond and Person, 1984; Dolter et al., 1994; McGeoch et al., 1988; Pogue-Geile et al., 1984; Pogue-Geile and Spear, 1987; Read et al., 1980; Ruyechan et al., 1979) support a model in which gK prevents fusion of enveloped viral particles with the outer nuclear envelope.

The UL20 gene may also function during HSV egress. Deletion of the UL20 gene had no effect on HSV entry or replication but few extracellular viruses were produced (Baines et al., 1991). Cells infected with a UL20-negative mutant accumulated viral particles in the perinuclear space (Baines et al., 1991). Therefore, UL20 is thought to be important for egress of the virus.
from the perinuclear space. The exact role which gK and UL20 perform during
HSV egress will be difficult to resolve until the mechanism of HSV egress is
better understood. But mutations in these genes and a better understanding of
the gene products may also provide a better picture of the egress pathway.

1.1.8 HSV cell-to-cell spread:

Cell-to-cell spread of HSV involves the spread from an infected cell to an
uninfected cell and may occur by two distinct mechanisms (Dingwell et al.,
1994). Cell-to-cell spread may occur by release of viral particles at the cell
surface followed by infection of neighbouring cells by the same mechanism as
HSV entry (described in sections 1.1.1). The presence of infectious HSV
particles in cell-culture supernatants of infected cells provides support for this
model.

Evidence for the presence of an alternate mechanism has been reported
based on the existence of HSV proteins which are dispensable for HSV entry
into cells but are required for production of a wild-type plaque size. This
alternate pathway of HSV cell-to-cell spread requires the essential HSV
glycoproteins gB, gD, and gH/gL since no plaques are produced in their
absence (Cai et al., 1987; Forrester et al., 1992; Ligas and Johnson, 1988;
Roop et al., 1993). gE-negative, gl-negative, or gM-negative HSV virions or an
HSV strain carrying a recombinant gD lacking the N-linked oligosaccharide
sites, produced small plaques, ie spread poorly from cell-to-cell yet, penetration
or entry into cells was unaffected (Balan et al., 1994; Davis-Poynter et al., 1994; Dingwell et al., 1994; Dingwell et al., 1995; MacLean et al., 1993; Sodora et al., 1991). Since gE and gl are not required for normal entry of HSV into cells, it was proposed that these glycoproteins play a role in cell-to-cell spread (Dingwell et al., 1994).

gE and gl may promote cell-to-cell spread in regions where two cells are closely apposed to one another (Dingwell et al., 1994). The basis for this hypothesis comes from experiments with neutralizing antibodies against HSV. These antibodies had a much greater inhibitory effect on HSV plaque numbers when the cells were infected with either a gE or gl negative virus compared to wild-type virions (Dingwell et al., 1994). It was proposed that wild-type virions can spread from cell-to-cell across cell junctions (which protect the virus from neutralizing antibodies) (Dingwell et al., 1994) and that in the absence of gE or gl, this process is less efficient (Dingwell et al., 1994). Therefore cell-to-cell spread of gE-negative or gl-negative virions must occur largely through the release of infectious virions into cell-culture supernatants. This renders the virus susceptible to neutralizing antibodies, whereas in normal HSV infections gE and gl promote egress of viral particles to cell junctions or cell contact points, a mechanism insensitive to neutralizing antibodies.

The study of virion-cell fusion, viral egress, and cell-to-cell spread are complicated by the fact that it is often difficult to determine which phenomenon is being studied. For instance, if HSV infection of cells produces a small plaque
phenotype, is this the result of reduced efficiency of viral egress or of cell-to-cell spread of the virus? Static studies which utilize EM data lack the ability to differentiate between non-infectious and infectious viral particles making interpretation of the results difficult. Since the fraction of infectious particles may be 5% of the total particles produced (Herold et al., 1991), the vast majority of viral particles observed are, in fact, non-infectious. Until assays are developed which differentiate between the effects of virion-cell fusion, viral egress, and cell-to-cell spread it will remain difficult to determine the mechanism behind these phenomena.

1.1.9 HSV infection of neurons and establishment of latency:

HSV produces a latent infection. Latency has been defined as the persistence of the virus in the host in the absence of clinical symptoms (Fraser et al., 1992). Upon replication at the site of initial HSV infection at the mucosa, viral particles are released from infected epithelial cells. These particles infect innervating sensory neurons and viral capsids are transported by retrograde axonal transport to the innervating ganglia (Kristensson et al., 1986; Lycke et al., 1984; Stevens and Cook, 1971) where HSV may undergo viral replication. After a short period of time, no replicating virus can be detected in the infected ganglia and the HSV genome is largely transcriptionally silent (Roizman and Sears, 1996).
Neurons represent the primary site of latency for HSV but the mechanism of establishment and reactivation of this state in infected individuals is not well understood. During latency, the HSV genome is maintained in an extrachromosomal state as a circular molecule (Deshame and Fraser, 1989; Mellerick and Fraser, 1987) which expresses latency-associated transcripts (LATs) (Stevens et al., 1988). There are at least three overlapping LATs which are encoded antisense to the ICP0 gene (Croen et al., 1987; Rock et al., 1987; Stevens et al., 1987). LATs are not required for HSV replication, establishment, or maintenance of latency (Block et al., 1990; Dobson et al., 1989; Ho and Mocarski, 1989; Javier et al., 1988; Lieb et al., 1989; Steiner et al., 1989), however their deletion causes inefficient HSV reactivation from latency (Lieb et al., 1989; Steiner et al., 1989).

Reactivation of HSV from the latent state occurs under a variety of conditions including stress, or tissue damage (Roizman and Sears, 1996). An in vitro system was developed to examine the mechanism of HSV infection upon reactivation from latency in neurons. This system involved 2 chambers separated by an agarose barrier. Neurons were grown in the central chamber and the axons grew through the agarose barrier and made contact with epidermal cells growing in the outer chamber. HSV infection of the neuron cell bodies in the inner chamber resulted in enveloped and unenveloped HSV particles in the neuronal soma while unenveloped capsids were exclusively found in axonal termini making contact with epidermal cells (Penfold et al.,
1994). These data are consistent with the hypothesis that upon reactivation from latency, unenveloped HSV capsids are transported anterograde to the axonal termini. Viral glycoproteins are also transported to the axonal termini where the unenveloped capsid buds through the plasma membrane to form an enveloped HSV particle at peripheral tissues close to the site of primary viral infection. This model predicts that the HSV infectious cycle is re-initiated at the mucosal surface.

1.2 Viral Receptors:

Viruses are obligate intracellular parasites which require host cellular machinery for propagation. To initiate a productive infection extracellular viruses have evolved a number of strategies to traverse host cell membranes and gain entry into cells. Virus entry requires specific interactions between virus and host cell rather than simple contact between virus particle and the plasma membrane. Viral particles must bind to a cell-surface receptor followed by interactions with cellular membranes which result in delivery of the capsid to the cytoplasm. What follows is a description of the salient features of viral-cell surface interactions.

1.2.1 General Summary of Receptors for Viruses:

The first interaction between viruses and the host cell is the binding of the virion to cell surface receptors. Viruses utilize a broad spectrum of cell-surface
components including proteins, carbohydrates, and glycolipids to attach to the surfaces of cells (reviewed in: Carrasco, 1994; Hayward, 1994; Lentz, 1990; Marsh and Helenius, 1989). The absence of the receptor or the inability of the virus to bind to cell-surface components results in inability of the virus to infect the cell (Knipe, 1996). The presence and distribution of viral receptors is a key determinant of viral tropism. Viruses with a restricted host range may bind to species-specific molecules. For example, poliovirus binds to a member of the immunoglobulin superfamily called PVR (Mendelsohn et al., 1989). A mouse homolog of the PVR protein does not mediate poliovirus entry (Morrison and Racaniello, 1992). Similarly viruses which are restricted in their ability to replicate in particular cell types often utilize viral receptors which are restricted to these cell types. For example, EBV is restricted to infecting cells which express the EBV receptor, C3d complement receptor (Fingeroth et al., 1984; Frade et al., 1985; Reisert et al., 1985; Tanner et al., 1987). Viruses that possess a broad host-range must be able to bind to cell-surface components distributed on a variety of species and cell-types or use multiple receptors. For example, influenza virus binds sialic acid residues present on virtually all mammalian cells (Herrler et al., 1985; Paulson et al., 1979; Suzuki et al., 1985).

Based on the requirement for virus binding to cell surface receptors it is not surprising that viruses have adopted multiple cell surface receptors to: enter into a variety of different cell types; provide functional redundancy; or allow for a broader host range. Table 1.1 provides a partial list of viruses from a number of
<table>
<thead>
<tr>
<th>Host Cell Receptor</th>
<th>Virus</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF receptor (Cao et al., 1995)</td>
<td>Measles Virus</td>
<td>Paramyxoviridae</td>
</tr>
<tr>
<td>GP135 and GP25 integrins (Wickham et al., 1993)</td>
<td>Virus</td>
<td>Family</td>
</tr>
<tr>
<td>Membrane cofactor protein (CD46) (Dong et al., 1993)</td>
<td>Measles Virus</td>
<td>Paramyxoviridae</td>
</tr>
<tr>
<td>Bgp2 (Nedelec et al., 1994)</td>
<td>Adenovirus Type 2</td>
<td>Adenoviridae</td>
</tr>
<tr>
<td>MCP3-MCP4 (MHCII or Bgp1a) &amp; MHCII (MHCII or Bgp1b)</td>
<td>Mouse hepatitis virus</td>
<td>Coronaviridae</td>
</tr>
<tr>
<td>CD2K (Gumper et al., 1995)</td>
<td>Group</td>
<td>Picornaviridae</td>
</tr>
<tr>
<td>VLCR (Gumper et al., 1995)</td>
<td>Human rhinovirus minor</td>
<td>Family</td>
</tr>
<tr>
<td>Low density lipoprotein receptor (LDLR) (Hoyer et al., 1994)</td>
<td>Virus (HIV-1)</td>
<td>Human Immunodeficiency Virus</td>
</tr>
</tbody>
</table>

Table 1.1: Viruses which utilize multiple cellular receptors
different families that have been shown to bind to more than a single receptor. Table 1.1 provides evidence that binding to multiple cellular proteins is not a unique feature of very complex viruses but is a general property of most viral families.

With the growing list of viruses that bind to multiple cellular proteins, it will become important to understand how a virion interacts with these proteins as it enters the cell. Viruses may utilize multiple independent receptors which provide functional redundancy during entry. For example, the human rhinovirus minor group binds to the low density lipoprotein receptor (LDLR) (Hofer et al., 1994) as well as related proteins VLDLR and α2MR/LRP expanding its host range (Gruenberger et al., 1995) (described in section 1.2.2). A number of questions will need to be addressed to understand the mechanism of entry of viruses into cells bearing multiple but independent viral receptors such as: does the virus preferentially utilize some receptors over others; what are the effects on viral entry when removing or blocking the ability of the virus to interact with a subset of its receptors; is their cooperativity in binding to multiple cellular receptors?

Viruses can also enter cells using several receptors in a sequential fashion. Binding of the virus to one cellular protein may induce conformational changes in viral proteins allowing for interactions to occur with other cellular proteins during entry. Such viruses require the presence of each of the
individual receptors to enter a single cell. Examples of the use of receptors in a sequential fashion are human immunodeficiency virus (HIV) and adenoviruses (described in more detail in sections 1.2.3.1 and 1.2.3.2).

1.2.2 Multiple independent cellular receptors:

Human rhinoviruses are members of the Picornaviridae family and have been divided into 2 groups based on receptor binding specificity (Abraham and Colonno, 1984; Uncapher et al., 1991). The major group binds to ICAM-1 (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989; Tomassini and Colonno, 1986) and the minor group of rhinoviruses (MGR) to the human low density lipoprotein receptor (LDLR) (Hofer et al., 1994). Normal human fibroblasts grown under conditions that suppress LDLR synthesis were approximately 100-fold less infectable by MGR than cells grown under conditions which stimulate LDLR synthesis (Hofer et al., 1994). Surprisingly, virus progeny obtained from fibroblasts which lacked LDLR, derived from patients with familial hypercholesterolemia (FH), were similar to levels obtained from normal fibroblasts. Furthermore downregulation of LDLR in FH fibroblasts had no effect on MGR entry into these cells (Hofer et al., 1994). Since FH fibroblasts synthesize no LDLR and yet produced similar levels of infectious progeny compared to normal fibroblasts, there must be an alternate receptor for MGR entry.
It was postulated that proteins related to LDLR might be utilized by MGR (Gruenberger et al., 1995). Synthesis of these proteins might be upregulated in FH fibroblasts to compensate for the absence of LDLR and explain the infectability of FH fibroblasts by MGR. It was shown that MGR bound to α2-macroglobulin receptor/LDLR-related protein (α2MR/LRP) (Gruenberger et al., 1995). Entry of MGR could be inhibited into FH fibroblasts by 90% when α2MR/LRP receptors were blocked however, no inhibition of MGR entry into normal fibroblasts was observed under these conditions (Gruenberger et al., 1995). These data suggest that the LDLR receptor is the preferred receptor for MGR entry into cells. However in the absence of LDLR, rhinoviruses utilize α2MR/LRP and the recently identified very low density lipoprotein receptor (VLDLR) (Gruenberger et al., 1995). It is possible that the affinity of the virus for LDLR is higher than for α2MR/LRP and VLDLR.

1.2.3 Multiple sequential cellular receptors:

1.2.3.1 Human immunodeficiency virus:

An intriguing picture of the mechanism by which HIV interacts with the cell surface has recently emerged and has provided important insights into how viruses utilize multiple sequential cell surface receptors.
The first characterized receptor for HIV was the CD4 antigen which is bound by the gp120 molecule of HIV (Claphan et al., 1989; Dalgleish et al., 1984; Maddon et al., 1986). Introduction of the human CD4 gene into human T lymphocytes, B lymphocytes, and epithelial cells resulted in the cells becoming susceptible to HIV infection (Maddon et al., 1986). However, the presence of human CD4 protein on the surface of murine cells was not sufficient to mediate entry of HIV, suggesting that other receptors or cofactors present on the surface of human cells were required (Maddon et al., 1986).

Recently several cofactors or secondary receptors which allow HIV to enter cells have been identified. All strains of HIV can infect primary human CD4-positive T lymphocytes (Collman et al., 1990; Maddon et al., 1986; Sweet et al., 1991). Isolates of HIV derived from asymptomatic HIV patients can also infect macrophages but are less efficient in infecting transformed T cells (M-tropic strains of HIV) (Cheng-Mayer et al., 1988). As the HIV disease progresses towards the development of symptoms of acquired immune deficiency syndrome (AIDS) there is often an alteration of viral tropism. HIV is less able to infect primary macrophages in culture and gains the ability to infect transformed T cell lines (T-tropic) (Connor and Ho, 1994; Schuitemaker et al., 1992). M-tropic and T-tropic viruses utilize different host cofactors for entry into cells. M-tropic viruses interact with the seven-transmembrane G protein-coupled chemokine receptor, CC-CKR-5 (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996) whereas T-tropic viruses utilize a
related protein called fusin (Feng et al., 1996). Transfection of human CC-CKR-5 or fusin into mouse cells that expressed CD4 resulted in infection of the cells by HIV (Feng et al., 1996). These data demonstrate that in addition to binding to CD4 molecules, HIV also requires binding to chemokine receptors such as CC-CKR-5 or fusin and that these secondary receptors are required for entry.

One of the most exciting observations to emerge from studying HIV interactions with the secondary cofactors is that the virus alters its tropism during the course of disease. This switch in tropism is correlated with amino acid changes in the gp120 protein (Cheng-Mayer et al., 1988; Cheng-Mayer et al., 1990; Chesebro et al., 1992; Hwang et al., 1991; O'Brain et al., 1990; Westervelt et al., 1991; Westervelt et al., 1992; Willey et al., 1994). It appears that infection of the host is initiated by M-tropic viruses since individuals which lack a functional CC-CKR-5 cofactor (which is responsible for HIV entry into M-tropic but not T-tropic viruses), are resistant to HIV infection (Liu et al., 1996). However much of the HIV research has centered around T-tropic strains of HIV which have been adapted to grow in the laboratory but may not be representative of the virus which is involved in the primary infection of humans. This should provide a cautionary message to researchers in the field since viruses adapted to grow in cultured cells may have altered their receptor specificity through changes in their receptor binding protein in order to replicate in certain cultured cells.
While CD4 and CC-CKR-5 or fusin clearly mediate entry into some cells, a number of cell types derived from the nervous system, liver, endothelial cells, stromal tissues, and blood vessels that lacked detectable levels of CD4 can be infected by HIV (Chiodi et al., 1987; Clapham et al., 1989; Dewhurst et al., 1987; Harouse et al., 1989; Li et al., 1990; Moses et al., 1993; Tateno et al., 1989; Werner et al., 1990). Thus HIV must utilize cellular receptors other than CD4 and chemokine receptors to enter into cells. A number of alternate HIV receptors have recently been described. The HIV glycoprotein gp120 bound to the glycolipid galactosyl ceramide and antibodies directed against galactosyl ceramide inhibited HIV entry into neural and epithelial cells (Fantini et al., 1993; Harouse et al., 1991; Yahi et al., 1992). In addition, HIV entry into human brain capillary endothelial cells occurred by a CD4-independent and galactosyl-ceramide independent manner (Moses et al., 1993). Alternate HIV receptors have been proposed such as a: 45 kDa mannose binding lectin (Curtis et al., 1992); Fc receptors (Takeda et al., 1988); 260 kDa protein (Ma et al., 1994); 180 kDa protein on glioma cells (Kozlowski et al., 1991); and sulfatide in neuroblastoma cell lines (Bhat et al., 1991; Harouse et al., 1989; Harouse et al., 1991). The ability of these putative viral receptors to mediate HIV entry have not been adequately tested, therefore the biological significance of these proteins is not clear.

HIV represents one of the clearest examples of the complexity of virus binding to cellular receptors. gp120 on the surface of HIV virions binds to CD4,
galactosyl-ceramide, and potentially 4 other cellular proteins. Upon binding to its cellular receptor, gp120 undergoes a conformational change (Doranz et al., 1996) that allows it to bind to a cofactor or secondary receptor leading to fusion with the plasma membrane. The ability to interact with a particular cofactor changes during the course of the disease as the variable region (V3) of gp120 changes (Choe et al., 1996). Early in the course of disease HIV gp120 variants bind better to CC-CKR-5 but later on they bind better to fusin.

1.2.3.2 Adenoviruses

Adenoviruses are nonenveloped DNA viruses which also represent examples of viruses that utilizes at least 2 receptors sequentially during entry into cells (Nemerow et al., 1994). Internalization of adenovirus type 2 (Ad2) occurs by receptor-mediated endocytosis (Chardonnet and Dales, 1970; FitzGerald et al., 1983). Ad2 attaches to the cell surface via the virion fiber protein to unknown receptors (Devaux et al., 1987; Devaux et al., 1990). Following attachment, additional interactions between Ad2 and the cell surface are required, since Ad2 could attach to but not enter some cell types (Silver and Anderson, 1988). The penton base of Ad2 is composed of 5 identical subunits each containing an Arg-Gly-Asp (RGD) sequence (reviewed in Nemerow et al, 1994) which can be bound by a number of integrins such as $\alpha_5\beta_1$, $\alpha_{v\beta_3}$, and most integrins containing an $\alpha_v$ subunit (Cheresh and Spiro, 1987; Hynes,
1992). Soluble forms of penton bases and antibodies directed to \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins inhibited Ad2 entry into cells but did not block viral attachment (Bai et al., 1994; Wickham et al., 1993) whereas soluble fiber protein blocked attachment (Philipson et al., 1968). These data suggest that Ad2 utilizes sequential interactions to enter into cells: initially the virus attaches to the cell surface by an as yet unidentified cellular factor via the Ad2 fiber protein. Following attachment, Ad2 penton bases interact with \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins, an interaction required for entry of the virus.

In addition to integrins the penton bases may bind to other cellular receptors. Ad2 bearing mutations within the penton base gene which alters the RGD coding region entered non-adherent cells with normal kinetics as compared to wild-type Ad2 (Bai et al., 1993). Interestingly, entry of Ad2 containing an altered RGD sequence showed delayed entry kinetics into adherent epithelial cells (Bai et al., 1993). In addition, cell lines defective in \( \alpha_v \)-integrin synthesis were partially infectable by Ad2 and antibodies against \( \alpha_v \)-integrins only partially inhibited Ad2 infection of cells (Wickham et al., 1993). These data support a model in which adenoviruses use receptors other than RGD-binding integrins for internalization.
1.3 Cellular receptors for HSV:

HSV types 1 and 2 can infect a wide range of cultured cell types including fibroblasts, epithelial cells, endothelial cells, keratinocytes, and neurons from a variety of species, including rat and mice (reviewed in Spear, 1993). Therefore the receptor for HSV must be present on a wide variety of cell types from many mammalian species, or the virus must utilize multiple cellular proteins for entry into cells.

A number of candidates for cell surface receptors which mediate HSV stable attachment have been proposed. The fibroblast growth factor (FGF) receptor was reported as a candidate cellular receptor for HSV-1 (Baird et al., 1990; Kaner et al., 1990). It was proposed that the HSV virion bound FGF which allowed binding of HSV/FGF to the FGF receptor (Baird et al., 1990). However subsequent experiments have shown that FGF, which binds to heparan sulfate GAGs, blocked adsorption of HSV to heparan sulphate moieties (Mirda et al., 1992; Muggeridge et al., 1992; Shieh and Spear, 1991). Furthermore cell lines which lacked the FGF receptor could be infected by HSV (Mirda et al., 1992; Muggeridge et al., 1992; Shieh and Spear, 1991). Another HSV receptor has recently been proposed. A 62 kDa protein was identified which was bound by anti-idiotype antibodies against an epitope on gD (Huang and Campadelli-Fiume, 1996). Levels of inhibition of HSV entry into cells by anti-idiotype antibodies varied from 50 to 100% (Huang and Campadelli-Fiume, 1996). However, anti-idiotype antibody approaches frequently produce
antibodies which block viral entry by nonspecific means for example by crossreacting with cellular proteins.

Recently a CHO cell line which was resistant to HSV entry was made susceptible to HSV penetration when a human cDNA was expressed in the cells (Montgomery et al., 1996). The protein product of the human cDNA was identified as a novel member of the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family designated HVE M. HVE M may represent a receptor for HSV entry into CHO cells, swine cells, and human peripheral blood mononuclear cells since anti-HVE M antibodies inhibited HSV entry into these cells as shown by a decrease in immediate early promoter activity. However anti-HVE M antibodies and soluble HVE M did not significantly inhibit HSV entry into human HeLa cells (Montgomery et al., 1996) suggesting that other cellular receptors must exist to allow entry of HSV into human adherent cells, cells that are most important for HSV infection in vivo.

1.3.1 Properties of the HSV receptor binding protein gD:

I was interested in identifying cellular receptors which were bound by the HSV glycoprotein D (gD). As has already been described (section 1.1.3) gD has properties of a receptor binding protein. The characteristics of gD are summarized below.

gD expressed by HSV type 1 (gD1) is a 394 amino acid protein encoded by the US6 open reading frame (McGeoch et al., 1985). Cleavage of the 25
amino acid signal sequence from gD results in a 369 amino acid mature protein (Watson et al., 1982). The amino-terminus of gD1 is composed of a 314 amino acid extracellular domain which is modified with 3 N-linked oligosaccharides (Cohen et al., 1983; Sodora et al., 1989; Watson et al., 1982) as well as 2 or 3 O-linked oligosaccharides (Johnson and Spear, 1983; Serafini-Cessi et al., 1983). gD1 contains a single transmembrane domain comprising amino acids 315 to 339 and a short cytoplasmic tail extending from amino acids 340 to 369 (McGeoch et al., 1985). gD contains 6 cysteine residues whose position within the extracellular domain of the protein is conserved in all gD homologs sequenced (Flowers et al., 1991; Lasky and Dowbenko, 1984; Petrovskis et al., 1986; Ross and Binns, 1991; Ross et al., 1991; Tikoo et al., 1990; Watson, 1983; Watson et al., 1982). Through testing pairs of cysteine deletions, it was suggested that cysteines 1 and 5, cysteines 2 and 6, and cysteines 3 and 4 formed disulfide bonds (Long et al., 1992). gD1 shares approximately 85% amino acid identity with the 368 amino acid HSV type 2 gD (gD2) (McGeoch et al., 1985; Minson et al., 1986; Watson, 1983; Watson et al., 1982).

The earliest functional studies with gD were carried out using monoclonal antibodies. Antibodies directed to gD neutralized an HSV infection and apparently inhibited a post-adsorption step (Fuller and Spear, 1987; Highlander et al., 1987; Minson et al., 1986; Para et al., 1985). In addition to inhibiting entry, incubation of infected cells with certain anti-gD monoclonal antibodies inhibited the formation of HSV plaques (Highlander et al., 1987;
Minson et al., 1986). Furthermore, fusion of infected cells with uninfected cells by syncytial viruses could be blocked when antibodies specific for gD were added to cells (Highlander et al., 1987; Minson et al., 1986; Noble et al., 1983). These data suggested that gD may function in post-adsorption stages of HSV entry, as well as cell-to-cell spread and syncytial formation. However, some caution must be used in interpreting these studies because blocking antibodies can cause pleiotropic effects on viruses that do not always indicate the stage that is being inhibited. This is based on the fact that proper entry requires that events preceding entry occur normally, i.e., fusion events require viral attachment stages. For example, antibodies specific for both the haemagglutinin-neuraminidase (HN) and F proteins of paramyxovirus prevented fusion (Merz et al., 1980; Miura et al., 1982). It was subsequently shown that the F protein is responsible for fusion whereas the HN protein binds to the cell surface and brings the viral and cellular membranes into close apposition which is required for subsequent fusion events (White et al., 1983).

Glycoprotein D is essential for HSV entry into cells. A gD-null HSV-1 virus, FgDB, was constructed in which gD sequences were replaced by the β-galactosidase gene (Ligas and Johnson, 1988). FgDB required propagation on complementing cell lines (i.e., VD60 cells), that produced gD in trans for infectivity. FgDB, lacking gD, was produced on Vero cells and this virus adsorbed normally but was unable to penetrate cells and initiate an infection (Ligas and Johnson, 1988). Brief treatment of cells that had been incubated
with FgDb lacking gD with the fusogen polyethylene glycol resulted in entry of
the virus and establishment of a productive infection (Ligas and Johnson, 1988)
suggesting that gD negative virions were able to bind to the cell surface but
were unable to fuse with the plasma membrane. These data suggested that gD
functions after adsorption at a stage which is required for HSV entry into cells.
As has previously been described, experiments involving UV-inactivated HSV
virions lacking gD (Johnson and Ligas, 1988), and soluble gD (Johnson et al.,
1990) provided evidence that HSV gD bound to a limited number of cell surface
receptors (discussed in section 1.1.3) which were required by HSV for entry into
cells.

The 3 N-linked oligosaccharide sites of HSV-1 gD are dispensable for
infection of cultured cells. A mutant form of HSV-1, termed QAA was
constructed which had the wild-type gD sequence replaced with gD lacking the
three N-linked oligosaccharide sites. QAA entered monkey Vero cells with
similar efficiency and kinetics as wild-type virus and produced similar levels of
intracellular and extracellular viral particles (Sodora et al., 1991). QAA showed
no defects in establishing, maintaining, or reactivating from latency in an in vivo
mouse eye model of infection (Tal-Singer et al., 1994). However QAA infection
of Vero cells produced a smaller plaque than wild-type HSV suggesting that N-
linked oligosaccharides on gD are important for HSV egress or cell-to-cell
spread (Sodora et al., 1991). Taken together these data suggest that N-linked
oligosaccharide sites on gD may be involved in cell-to-cell spread or viral
egress \textit{in vitro} but are dispensable for virus spread in mice as well as production of infectious virus in cultured cells and in mice.

A series of mutations within the gD gene have yielded information regarding functional domains of gD. Linker insertion or deletion mutations were tested for their ability to complement a gD-negative HSV strain. Deletion of region 231-235 (Feenstra \textit{et al.}, 1990) or 28-63 (Muggeridge \textit{et al.}, 1990) of gD resulted in properly folded proteins which were unable to complement the gD-negative HSV strain, FgDβ. Conversely, deletion of gD residues 7-21, 300-305, 338-369, or the cytoplasmic tail of gD (residues 340-369) were dispensable for gD function in cultured cells (Feenstra \textit{et al.}, 1990; Muggeridge \textit{et al.}, 1990).

Four functional regions within gD were identified by linker insertion mutations which were required for complementation of FgDβ (Chiang \textit{et al.}, 1994). These regions are: region I composed of amino acids 27-43; region II, amino acids 126-161; region III, amino acids 225-246; and region IV, amino acids 277-310 (Chiang \textit{et al.}, 1994).

There is evidence consistent with the idea that HSV gD performs multiple functions during HSV replication in cells. Soluble gD containing a mutation within region I was unable to inhibit HSV entry into cells when the glycoprotein was produced in insect cells (Nicola \textit{et al.}, 1996). Therefore, mutations within region I abolish the ability of soluble gD to bind to a cell surface receptor. However, soluble forms with mutations in regions II, III, or IV were able to block
HSV entry but did not allow virus replication when the full-length mutant gD's were expressed in the virus (Nicola et al., 1996). These data are intriguing since the majority of the HSV neutralizing antibodies bind to regions II and III (Fuller and Spear, 1985; Fuller and Spear, 1987; Highlander et al., 1987; Minson et al., 1986; Para et al., 1985). Therefore region I may represent a critical domain for cellular receptor binding while regions II, III, or IV may be required for other aspects of HSV replication. However, only a single insertion mutation within each of the four identified functional regions was cloned into soluble gD and tested for its ability to inhibit HSV entry (Nicola et al., 1996). It remains possible that some of the other identified functional regions within the gD gene are involved in receptor binding but that this phenotype may require additional region II or III mutations to be expressed. These data are consistent with the hypothesis that gD participates in stages of HSV entry other than receptor binding, for example membrane fusion. Alternatively, sequences within gD which are not essential for receptor binding may be required for incorporation of gD into the virion and thus would be required for production of infectious virions.

The phenomenon known as gD-mediated interference occurs in cell lines which constitutively express gD. These cells were resistant to infection by HSV-1 (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989). gD-mediated interference may represent a mechanism to prevent super-infection of HSV infected cells by newly released HSV particles. The primary protein in the
virion particle which mediates interference is gD. Certain viruses, e.g., strain ANG, or virus mutants which can overcome interference, e.g., rid1, express forms of gD (often with mutations around residues 25 or 27) which when transferred to other viruses can produce progeny which can enter gD-expressing cells.

There are two hypotheses which have been proposed to explain gD-mediated interference: 1) gD on the surface on the cell interacts with an HSV receptor; or 2) gD produced by the cell interacts with gD in the virion (Campadelli-Fiume et al., 1988; Dean et al., 1994). If gD on the cell surface is binding to a cellular receptor then it is predicted that single amino acid changes within virion gD which abolish gD-mediated interference are the result of altered receptor binding by virion gD. Therefore, virion gD containing point mutations and wild-type cell surface gD each bind to different cellular determinants or with different efficiencies and thus cell surface expressed gD no longer blocks virion gD from binding to a cell surface receptor. This hypothesis can be tested. If gD-mediated interference is overcome by altered receptor binding specificity then point mutations in both virion and cell surface gD should result in each protein having the same altered receptor binding specificity and therefore gD-mediated interference should occur. However, gD-mediated interference did not occur when a point mutation within gD which abolished gD-mediated interference was present both in virion gD and cell-surface gD (Campadelli-Fiume et al., 1990). Therefore these data argue against point mutations in gD circumventing gD-mediated interference by altering receptor specificity.
It is possible that gD-mediated interference results when sequences within gD present on the cell surface bind to sequences within gD on the virion (Campadelli-Fiume et al., 1990; Dean et al., 1994; Johnson and Spear, 1989). In this scenario, the point mutations in gD which abolish gD mediated interference are altering a homotypic gD binding domain (Campadelli-Fiume et al., 1990; Dean et al., 1994). However no biochemical evidence exists to prove that homotypic interactions occur between cell surface and viral gD.

Recent data has provided evidence to suggest that gD-mediated interference may not be important for HSV replication and may be artifactual. HSV strain ANG is resistant to gD-mediated interference (Dean et al., 1994). The gD protein from strain ANG contains naturally occurring amino acids substitutions at position 25 and 27, as well as other differences in the gD sequence (Dean et al., 1994) suggesting that, whatever the function of gD-mediated interference, it is not essential for HSV replication. In addition, a cell line expressing the HSV gene US11 which encodes a RNA binding protein (Roller and Roizman, 1994) was resistant to HSV infection (Roller and Roizman, 1994). This resistance was overcome by mutations within gD (Roller and Roizman, 1994). Since US11 is an intracellular protein, and therefore not predicted to reach the cell surface, it is possible that during the construction of both US11 and gD expressing cell lines, other alterations occurred within the cells which prevented entry of most HSV strains but could be overcome by
mutations within gD. It is unclear what role if any gD-mediated interference is
performing during an HSV infection.

1.3.2 HSV glycoproteins are multi-functional:

Many of the HSV glycoproteins are multi-functional. As their host adapt
and change, a virus must adapt to retain the precarious balance between host
and parasite. Viruses have a relatively small genome compared to their host
species, so changes and adaptations must predominantly occur within the
framework of existing viral genes. The virus could obtain gene functions which
allow the virus to become more efficient at infecting its host by: "incorporating"
into the viral genome host genes that modulate a particular activity; gene
duplication and alteration within the viral genome; or modification of existing
viral genes to carry out additional viral functions. This latter strategy would be
the most economical for the virus in terms of managing its resources. Having
multi-functional proteins minimizes the size of the viral genome thereby: 1)
preventing packaging problems of the genome into the viral capsid; 2) making
viral replication more efficient; 3) allowing viral proteins to be synthesized more
efficiently. It is not surprising that HSV has numerous examples of
glycoproteins which perform multiple functions.

The list of multi-functional HSV glycoproteins is extensive. As has
already been described, gD functions as a receptor binding protein, it may
prevent HSV re-infection, and may be involved in additional events required for
entry. gB is involved in binding to heparan sulphate proteoglycans during adsorption of HSV to the surfaces of cells (Herold et al., 1991) and probably promotes fusion between viral and cellular membranes. gC mediates adsorption of HSV to the surfaces of cells (Herold et al., 1991) and binds to the C3b complement receptor perhaps protecting HSV infected cells from complement-dependent mediated cell lysis (Friedman et al., 1984; McNearney et al., 1987). gE and gl form a complex that binds to the Fc region of IgG molecules (Johnson and Feenstra, 1987; Johnson et al., 1988) perhaps protecting the virion or HSV infected cells from antibody dependent complement lysis (Dowler and Veltri, 1984; Frank and Friedman, 1989, Hanke et al., 1990). Recently a number of reports have suggested that gE and gl play a role in HSV cell-to-cell spread of the virus, functions independent of IgG binding. Mutants of HSV or PRV lacking gE or gl were impaired in their ability to spread in cultured cells and in different tissues including the nervous system (Balan et al., 1994; Card et al., 1992; Dingwell et al., 1994; Dingwell et al., 1995). Defects in virus spread in vivo of these viruses occurred prior to the appearance of anti-HSV IgG (Dingwell et al., 1994). gE-negative and gl-negative viruses demonstrate normal entry kinetics into cells indicating that gE/gl are not required for entry of extracellular virus particles (Balan et al., 1994; Dingwell et al., 1994). It is possible that Fc binding of gE/gl is related to their ability to promote cell-to-cell spread because these proteins may bind to other members of the immunoglobulin supergene family. Alternatively, Fc binding
and cell-to-cell spread or egress activity may be mediated through different
domains within the gE/gI molecule.

1.4 Experimental Rationale:

At the time when the experiments described in this thesis were initiated,
there were no good candidates for stable attachment receptors for HSV.
Experimental evidence, presented in section 1.1.3 supported the notion that gD
bound to a cellular receptor. Previous experiments have shown that soluble gD
can bind to cell surface components in a saturable manner and block the ability
of HSV to enter into cells (Johnson et al., 1990). In addition, soluble gD bound
to approximately 500,000 cell surface sites with a $k_d$ of 2.3X10^{-7} M (Johnson et
al., 1990). These data suggested that soluble gD was binding to a cell surface
component required by HSV types 1 and 2 for entry into cells. Using soluble gD
as a ligand, I began research to identify cellular proteins which bound to soluble
gD. The results of these experiments are described in Chapter 2. Having
identified a cellular protein, the 275 kDa mannose 6-phosphate receptor (MPR),
to which gD binds, I conducted experiments to determine the role that this
protein plays in an HSV infection. The outcome of these experiments are
outlined in Chapter 3. I demonstrated that anti-MPR antibodies, a soluble form
of MPR, and a MPR ligand inhibited HSV entry. In addition, I showed that MPR
ligands and the absence of mannose 6-phosphate (M6P) residues inhibited
HSV egress or cell-to-cell spread. Subsequent experiments were directed at
the effects of M6P residues on gD and the role of MPRs in determining
intracellular trafficking of gD and affecting virus egress. These experiments are
detailed in Chapter 4.
2 Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors.

2.0 Preface:

In the following publication, D.C. Johnson, K. Dingwell and I carried out the experiments in Figures 2.1, 2.2, and 2.3. Figures 2.4, 2.6, 2.7, and 2.8 were performed by myself including the purification of the 275 kDa MPR. The purified protein was sent to Chiron Corporation for N-terminal micro-sequencing by F.R. Masiarz (Figure 2.5). W. Gregory and R.L. Burke provided us with the soluble gD-2t used in the purification experiments. S. Kornfeld carried out the sugar analysis described in Table 2.1 on material I purified from HSV infected cells.
Herpes simplex viruses (HSV) use multiple and sequential receptors to enter host cells. HSV glycoprotein D (gD) has been implicated in binding to cellular receptors that facilitate virus penetration into cells. We used soluble forms of gD that were expressed in Chinese hamster ovary cells to characterize and identify a putative cellular receptor for HSV as the 275-kDa mannose 6-phosphate/insulin-like growth factor II receptor. Soluble gD also bound to the 46-kDa cation-dependent mannos-6-phosphate (Man-6-P) receptor and was extensively modified with Man-6-P residues on its Asn-linked oligosaccharides. Additionally, soluble gD was a high affinity substrate for N-acetylglucosamine-1-phosphate transferase, the first enzyme in the biosynthetic pathway for the addition of Man-6-P residues to lysosomal enzymes. The membrane form of gD immunoprecipitated from HSV-infected cells also bound to the 275-kDa mannos-6-phosphate/insulin-like growth factor II receptor, albeit poorly, and only a small fraction of the membrane gD was modified with Man-6-P. Notwithstanding this low level of mannose phosphorylation, the interaction between gD and Man-6-P receptors may play a role in some aspect of virus entry or egress.

Mammalian viruses have been shown to utilize a large number of cell surface components as virus receptors (reviewed in Refs. 1 and 2). Virus receptors facilitate entry of viruses into cells by promoting (i) the initial concentration of virus particles on the cell surface, (ii) targeting of viruses to specific domains of the cellular membranes, (iii) close proximity between viral and cellular membranes, or (iv) activation of viral components required for entry. There are a number of examples of viruses that bind to simple cell surface carbohydrate structures, e.g., influenza virus binds to sialic acid before being incorporated into endosomes (reviewed in Refs. 3 and 4). Similarly, Sendai virus binds to sialic acid but fuses directly with the plasma membrane at neutral pH (5). Other viruses may utilize cell surface proteins that are relatively restricted and, thus, the host range of these viruses is largely limited by the distribution of the receptors. For example, the human immunodeficiency virus uses the CD4 molecule to enter CD4+ T lymphocytes and, although other surface molecules may be necessary for human immunodeficiency virus entry and alternate receptors have been described, the host range of human immunodeficiency virus is largely limited to cells that express CD4 (6–8). There is also growing evidence that a large group of mammalian viruses use multiple receptors to enter cells. For example, a virus may use distinct molecules to enter different cell types, or it may use several receptors in a sequential fashion to promote attachment and entry into cells. Adenovirus type 2 uses fiber proteins, extending from penton base proteins on the surfaces of adenovirus capsids, to attach to unknown cellular receptors, yet penton base proteins interact with vitronectin receptors promoting entry into cells (9–11).

Herpes simplex viruses (HSV) have the capacity to infect many different cell types from a broad range of mammalian species, and there is evidence that HSV utilize multiple and sequential receptors to enter cells. HSV encode upward to 70 polypeptides (12), and there are at least 11 membrane glycoproteins that all have the potential to promote receptor interactions on a variety of cell types (reviewed in Refs. 13 and 14). Initially, HSV adsorbs onto heparan sulfate glycosaminoglycans (15–17) and chondroitin sulfate glycosaminoglycans, which are abundant components of the cell surface. Cell surface glycosaminoglycans appear to be required for efficient entry of HSV, and their role may be to concentrate virus particles on the cell surface or to cause cell surface activation in some manner.

In addition to binding to cell surface glycosaminoglycans, HSV entry into cells requires interactions with other cell surface components that are more restricted in number. Virus mutants lacking gB, gD, gH, or gL, for example, adsorb normally onto the cell surface but cannot enter cells (19–22). Cells treated with a relatively small number (~5,000/cell) of UV-inactivated HSV particles were resistant to infection with infectious HSV, and these virus particles blocked virus entry into cells rather than virus adsorption or postentry stages of virus replication (23, 24). In contrast, UV-inactivated HSV particles lacking gD were unable to block infection of the cells. These results suggest that gD is required in the envelope of HSV in order for the virus particles to interact with a limited number of cell surface sites.
HSV gD Binds to Mannose 6-Phosphate Receptors

required for entry. Supporting these conclusions, cell lines constitutively expressing gD on the cell surface were found to be resistant to infection by HSV (25, 26). Presumably, the gD sequesters a protein that is required for viral entry. Soluble forms of HSV type 1 (HSV-1) gD and HSV type 2 (HSV-2) gD were able to block virus entry into cells by binding to satureable receptors on the cell surface (27). Together, these studies provide compelling evidence that gD interacts with cell surface molecules that are required for entry of HSV, although it is clear that other HSV glycoproteins, gB and gHgL, are also required for HSV entry (19–22).

Here we present evidence that soluble forms of HSV-1 gD, HSV-2 gD, and, to a lesser extent, gD from virus-infected cells interact with the 27-kDa mannos 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor and are modified with Man-6-P residues. These findings are consistent with Man-6-P receptors acting as cellular receptors for HSV.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Vero, 293, CHO, and human R970 cells were grown in a minimal essential medium (Life Technologies, Inc.) supplemented with 7% fetal bovine serum (FBS). Normal human fibroblasts were grown in an minimal essential medium with 10% FBS; PC12 cells (obtained from ATCC) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% horse serum; and human keratinocytes were grown in Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. SF-9 cells were propagated in Grace's medium (Life Technologies, Inc.) supplemented with 0.33% lactalbumin hydrolysate, and 19% FBS. HSV-1 strain F was obtained from P. G. Spear (now at Northwestern University, Chicago, IL).

Materials—Bovine serum albumin (BSA), 0.25 M Tris, pH 7.4, 2 M EDTA, 0.25 M phenanthroline, 1 M phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin was used as a membrane fraction. Human placental membranes and membranes derived from rat brain were prepared as previously described (35), and the membrane fraction was resuspended in 40 mM imidazole HCl, pH 7.4, 200 μM NaCl and frozen at -70 °C. The proteins were transferred into nitrocellulose membranes (Schleicher and Schuell) by electrophoresis in 20 mM Tris-HCl, 150 mM glycine, pH 8.3, 20% methanol (transfer buffer) for 6 h at 45 V using a Bio-Rad transfer apparatus. The nitrocellulose membranes were incubated for 12–18 h with PBS containing 1% skim milk (Carnation, powdered), 0.5% BSA (Sigma) and then with PBS containing 0.6% fish gelatin (Sigma), 0.5% BSA (PBS/gelatin/BSA) for 1 h. The nitrocellulose was incubated with biotinylated gD-11, gD-21, or gB-2t (1 × 10⁻⁹ cpm/ml, 0.1 μg/ml) in PBS/gelatin/BSA for 3–4 h at room temperature. The membranes were washed 3 times with PBS/gelatin/BSA, dried, and exposed to X-Omat film (Eastman Kodak Co.).

470 kDa proteinase F and other diazytardustase—Mammanse derived from approximately 2 × 10⁶ Vero cells were extracted with 2 mg/ml octyl glucoside and 2 mg/ml CHAPS and treated with endoglycosidase F (2.7 μg/ml for 2h at 37 °C). Similarly, 0.3 μg of gD-2t were incubated with endoglycosidase F (0.25, 0.5, 0.75, or 1 units/50 μl reaction) or neuraminidase (0.05, 0.1, or 0.15 units/50 μl reaction) in 50 mM NaH₂PO₄/NaOH, pH 7.4, for 2 h at 37 °C. The proteins were mixed with loading buffer, and samples were separated on 4–15% polyacrylamide gels.

Purification and Sequence Analysis of gD Binding Protein from Fetal Kidney—Supplied by Dr. M. E. Stoddard (U.S. Department of Agriculture, Peoria, IL), a solution of pentamannos phosphatase Affinity Columns—Pentamannos phosphatase was prepared as described by Slodki et al. (38) from yeast Pichia (Hansenula) holstii phosphomannan, which was supplied by Dr. M. E. Stoddard (U.S. Department of Agriculture, Peoria, IL). A solution of pentamannos phosphatase in water (300–400 mg/ml) was coupled to CNBr-Sepharose by the procedure described by Jeffay et al. (39).

Purification of 275-kDa M6P/IGF-II Receptor and 46-kDa CD-MPR from Bovine Testes and M6P/IGF-II Receptor from FBS—The M6P/ IGF-II receptor and the CD-MPR were purified from bovine testis. Briefly, a membrane extract was prepared from 400 g of bovine testis as previously described (40) and applied to a pentamannos phosphate-Sepharose column. The column was washed overnight with 50 mM imidazole containing 0.5% Triton X-100, pH 7.5, 200 mM NaCl, 0.05% Triton X-100, then with wash buffer containing 1 mg/ml octyl glucoside, and then eluted with wash buffer containing 1 mg/ml octyl glucoside, 5 mM NaCl, pH 6.5, 0.5 M NaCl, 5 mM EDTA, diluted with an equal volume of this buffer, and applied to a pentamannos phosphate-Sepharose column (41). The column was washed with 50 mM imidazole, pH 6.5, 150 mM NaCl, and the soluble M6P/IGF-II receptor eluted in 50 mM imidazole, pH 6.5, 150 mM NaCl, 6 M Mann-6-P.

Detection of Viral gD Immobilized to Nitrocellulose by Soluble [125I] M6P/IGF-II Receptor—The membrane form of gD was prepared by incubating approximately 1 × 10⁶ Vero cells with HSV using 6 plaque-forming units/cell. The cells were lysed in 50 ml of 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl (Non- idet P-40/glycine) for 1 h at 4 °C. The extracts were clarified by centrifugation at 80,000 × g for 1 h and then mixed with 40 μl of mAb D6 ascites fluid for 1 h at 4 °C. Protein A-Sepharose beads were added and incubated for an additional 2 h at 4 °C. The beads were washed 5 times with E. Coli P-40/glycine deoxycholate buffer and resuspended in 2% SDS, 2% β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, bromophenol blue. The samples were boiled for 5 min, and the proteins were resolved on 12% N,N'-diallyltartardiamide cross-
HSV gD Bind to Mannose 6-Phosphate Receptors

RESULTS

Soluble gD-1t and gD-2t Bind to Large Cellular Membrane Glycoprotein—In order to identify cellular proteins with which gD interacts, membranes were fractionated from Vero cells, Vero cells, and human fibroblasts, and proteins were extracted from these membranes using low concentrations (0.1-0.2%) of SDS in the absence of reducing agents. The proteins were subjected to electrophoresis in polyacrylamide gels that did not contain SDS and transferred to nitrocellulose, which was subsequently incubated with 125I-labeled gD-1t or gD-2t. A single cellular protein, with an apparent molecular mass of approximately 180 kDa, was detected on these ligand blots with either gD-1t or gD-2t. Fig. 1A. Binding of labeled gD to the cellular protein was inhibited by a 50-fold molar excess of unlabeled gD-2t (Fig. 1B, lane 2), and [125I]gD-2t did not bind to the 180-kDa protein (Fig. 2). The 180-kDa protein was not detected in the cytoplasmic fraction from Vero cells (Fig. 2). It was extracted from membrane fractions with nonionic detergent but not with 2 M NaCl or 0.1% sodium deoxycholate, and the protein partitioned into a Triton X-114 phase (results not shown), suggesting that the protein was an integral membrane glycoprotein. In addition, the protein was sensitive to endo-

180-kDa Protein Was Present in Cellular Membranes Derived from a Variety of Different Mammalian Cell Lines and Tissues and in Fetomaternal Serum—HSV can infect a broad range of cell types from a variety of human and animal tissues, and therefore, cellular receptors for HSV should be broadly distributed. Fig. 3 shows that the 180-kDa cellular protein could be detected with [125I]gD-2t in membrane extracts from all of the mammalian cell lines tested as well as from a number of tissue samples, including human placenta and rat brain. The protein was not detected in SP-2 insect cells. A protein with a slightly faster electrophoretic mobility (~165 kDa) was detected in FBS and not in adult human or bovine sera, and this protein did not require detergents for its solubility.

KCl Binding Protein—Based on the observation that gD-2t could bind to a membrane protein immobilized on nitrocellulose, we attempted to purify the 180-kDa protein from human placental membranes and from FBS using gD-tethers. A monoclonal antibody to protein A-Sepharose. Solubilized placental membranes were incubated with gD-2t and then with monoclonal antibodies specific for gD (D6) or gB (15S2B). The antibody-gD complexes were precipitated using protein A-Sepharose. The precipitates were solubilized by boiling in 3% SDS and 3% β-mercaptoethanol and separated by SDS-polyacrylamide gel electrophoresis. A protein with an apparent
molecular mass of approximately 240 kDa was precipitated with gD-2t (Fig. 4). This protein was not detected when the gD-2t was omitted or when a gD-specific antibody, 15B8, or no antibody was used. When a similar purification scheme was applied to FBS, a protein with an apparent molecular mass of approximately 220 kDa was detected (Fig. 4). Contaminating proteins derived from the mouse ascites fluid and placental membranes were well separated from the large cellular protein by electrophoresis. The 240-kDa protein detected in this experiment by silver staining had a significantly slower electrophoretic mobility than the 180-kDa cellular protein detected on ligand blots, where cell extracts were subjected to electrophoresis in low concentrations of SDS and without reduction (Fig. 1). However, the two bands appeared to be identical because cell extracts or FBS depleted of the 240- or 220-kDa proteins by incubation with gD-2t, DLI, and protein A-Sepharose also displayed reduced levels of the 180- or 165-kDa proteins detected using [125I]gD-2t on ligand blots (results not shown). Therefore, it appeared that reduction and denaturation of the protein was responsible for the differences in gel mobility.

Amino Acid Sequence Analysis of gD Binding Protein from FBS—In order to identify the 180-kDa gD binding protein and obtain amino acid sequence information, we attempted to purify the protein from human placental membranes. However, the 180-kDa membrane protein was difficult to solubilize in a form where it would bind to gD affinity matrices or ion exchange resins (not shown). Therefore, we elected to purify the smaller, soluble protein from FBS using gD-2t tethered through monoclonal antibody DLI. The gD binding protein was purified from 40 ml of FBS as described above. Following polyacrylamide gel electrophoresis and transfer to a PVDF membrane, the protein was subjected to amino acid sequence analysis. An unambiguous determination of the first 9 amino acids of the protein was obtained, and the sequence was compared with a protein data bank (Fig. 5A). This search indicated that the aminoterminal sequence of the gD binding protein was identical to residues 48–56 of the 275-kDa bovine M6P/IGF-II receptor previously reported by Lobel et al. (48). The M6P/IGF-II receptor is known to possess a large aminoterminal signal sequence of 47 residues, and thus, it was not surprising that the sequence of the gD binding protein began at residue 48. In addition, it has previously been demonstrated that the M6P/IGF-II receptor is removed from the surfaces of cells by proteolytic cleavage near the transmembrane domain (47) and is found as a soluble protein in fetal serum (48). Amino acid composition analysis of the gD binding protein produced a pattern of amino acids similar to that predicted from the nucleic acid sequence of the M6P/IGF-II receptor gene (Fig. 5B), although some small differences would be expected because the signal sequence, transmembrane domain, and cytoplasmic domain would be absent from the soluble receptor. These results support the conclusion that the gD binding proteins in FBS and placental membranes were the soluble and membrane forms of the M6P/IGF-II receptor.

gD-2t Binding to Both 46-kDa Cation-dependent Man-6-P Receptor and 275-kDa M6P/IGF-II Receptor—Two Man-6-P receptors have been described, a 46-kDa CD-MPR and the 275-kDa M6P/IGF-II receptor (reviewed in Ref. 49). To investigate the possibility that gD binds to both MPRs, the two receptors were purified from bovine testes (46). Preparations containing both receptors were denatured in SDS, reducing agents were subjected to electrophoresis, and gels were stained with Coomassie Brilliant Blue (Fig. 6B). Alternatively, receptor preparations were subjected to electrophoresis under nondenaturing conditions, transferred to nitrocellulose, and incubated with [125I]gD-2t. Soluble gD-2t bound to both the 46-kDa CD-MPR and the 275-kDa M6P/IGF-II receptor (Fig. 6A). The M6P/IGF-II receptor from bovine testes displayed an apparent molecular mass of ~200 kDa in Fig. 6A rather than 180 kDa, presumably related to the variable electrophoretic mobility of receptors from various tissues (Fig. 4). It is not clear why the gD-2t did not bind to the 46-kDa CD-MPR in experiments involving extracts from cultured cells (e.g. Fig. 2), although the CD-MPR may not have been efficiently extracted under these conditions.

Soluble M6P/IGF-II Receptor Binds to Both Soluble gD-2t and Viral gD—To assess interactions of the M6P/IGF-II receptor with soluble gD produced in CHO cells and with gD produced in HSV-infected cells, we measured the binding of the soluble M6P/IGF-II receptor to these proteins on ligand blots.
A.

**gD Binding protein**

**M6P/IGF-II receptor**

![Image of amino acid sequence analysis](image)

**Fig. 5.** Amino acid sequence analysis of the gD binding protein purified from fetal bovine serum. The gD binding protein was purified from 80 ml of fetal bovine serum using 40 μg of gD-2t in mAb 1D6, and protein A-Sepharose and proteins eluted from protein A-Sepharose beads as described under "Experimental Procedures." Proteins were subjected to electrophoresis, transferred to a PVDF membrane, which was stained with Coomassie Brilliant Blue, and the band containing the large protein was excised. A 75% of the PVDF membrane was subjected to amino acid sequence analysis, and a search of the Protein Identification Resource data base indicated that there was sequence identity across a 9-amino acid stretch beginning at residue 48 of the bovine M6P/IGF-II receptor (46). Amino acids shown in outline were not unambiguously determined, and a question mark indicates a position where no determination could be made. B, 25% of the PVDF membrane containing the gD binding protein was hydrolyzed with 1 N HCl for 24 h at 105 °C, and the amino acid composition was determined as described under "Experimental Procedures." A comparison between the amino acid composition of the gD binding protein and that predicted for the bovine M6P/IGF-II receptor (46) is shown. Note that errors are expected for glycine and serine, which commonly contaminate samples derived from SDS-polyacrylamide gels, and the gD binding protein lacks the transmembrane and cytoplasmic domains of the M6P/IGF-II receptor. ND, not determined.

B.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Predicted composition of</th>
<th>Composition of gD binding protein</th>
</tr>
</thead>
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<tr>
<td></td>
<td>gD binding protein</td>
<td></td>
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![Image of soluble gD-2t binding](image)

**Fig. 6.** Soluble gD-2t binds to both the M6P/IGF-II receptor and the CD-MPR. The M6P/IGF-II receptor and the CD-MPR were purified from bovine testes using immobilized phosphotyrosine chromatography as described under "Experimental Procedures." A, proteins were mixed with 0.1% SDS and 10% glycerol, subject to electrophoresis on an 15% polyacrylamide gel, transferred to nitrocellulose, and probed with [125I]gD-2t. B, purified proteins were mixed with 2% SDS, 1% β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, and 10% glycerol and then separated on 4–15% polyacrylamide gels and stained with Coomassie Brilliant Blue.

The membrane form of HSV-1 gD (viral gD) was prepared by immunoprecipitation from cell extracts of HSV-1-infected cells using the anti-gD monoclonal antibody 1D6. Viral gD and soluble gD-2t were resolved on polyacrylamide gels in low concentrations of SDS, and the proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose and incubated with [125I]labeled soluble M6P/IGF-II receptor. As shown in Fig. 7, both soluble gD-2t and viral gD bound [125I]-labeled M6P/IGF-II receptor. In this experiment, 100-fold more viral gD was loaded onto the gel than gD-2t, and the binding of [125I]M6P/IGF-II receptor was approximately half of that observed with gD-2t when the blots were analyzed using a PhosphorImager. Other experiments involving both HSV-1 and HSV-2 gD yielded similar results so that, on average, soluble gD-2t bound approximately 200-fold more M6P/IGF-II receptor than viral gD immunoprecipitated from HSV-infected cells.

The M6P/IGF-II receptor is known to bind Man-6-P residues present on high mannose-type Asn-linked oligosaccharides of lysosomal enzymes. To determine if Asn-linked oligosaccharides of gD were necessary for the binding of gD to the M6P/IGF-II receptor, gD-2t was treated with endoglycosidase F to remove Asn-linked oligosaccharides or with neuraminidase to remove only sialic acid residues. Treated and untreated gD-2t were resolved on polyacrylamide gels, transferred to nitrocellulose, and probed with [125I]-labeled soluble M6P/IGF-II receptor. The receptor bound poorly to gD-2t, which had been treated with endoglycosidase F, whereas treatment of gD-2t with neuraminidase had little or no effect on binding (Fig. 8A). To confirm that endoglycosidase F treatment of gD-2t did not cause degradation of gD-2t, parallel nitrocellulose blots were probed with gD-specific rabbit polyclonal antibody and [125I]-labeled protein A, and equivalent quantities of gD were observed in all the lanes (Fig. 8B). These results are consistent with the hypothesis that binding of gD-2t to the M6P/IGF-II receptor requires the presence of Asn-linked oligosaccharides on gD.

**Determination of Man-6-P Content of Soluble and Viral gD**—In order to determine directly the Man-6-P content of soluble and membrane forms of gD, CHO cells expressing soluble gD-2t and R970 cells infected with HSV-1 were labeled with [2-14C]mannose, and the gD was isolated by immunoprecipitation. The [14C]mannose-labeled Asn-linked oligosaccharides of gD were then analyzed for their content of complex and high mannose-type species and for the extent of phosphorylation of the latter type of oligosaccharide. Phosphorylation was determined by ion exchange chromatography on QAE-Sephadex as previously described (44). The results of two of these analyses are summarized in Table I. The secreted gD-2t con-
HSV gD Binds to Mannose 6-Phosphate Receptors

Fig. 8. Binding of the M6P/IGF-II receptor is dependent upon the presence of N-linked oligosaccharides on gD-2t. Soluble gD-2t was treated with 0.1 M lactose, 0.25, 0.5, or 1 unit of endoglycosidase F with 0.25, 0.5, or 0.75 units of neuraminidase for 3 h at 37°C. The gD-2t was mixed with 1% SDS, 1% glycerol and then resolved on 4–10% polyacrylamide gels and transferred to nitrocellulose membranes. N-trioctylurea containing treated or untreated gD-2t was incubated with soluble (19)M6P/IGF-II receptor (A1) or a gD-specific rabbit polyclonal antisera and 125I-labeled protein A (B) and then washed and exposed to x-ray film.

tained 58% high mannose oligosaccharides, and almost all of these molecules had either 1 or 2 Man-6-P residues. Since gD contains three Asn-linked oligosaccharides (S5), one would expect that at least two, and possibly a portion of all three, of the oligosaccharides on each gD molecule would be phosphorylated. By contrast, gD immunoprecipitated from HSV-infected cells contained primarily complex-type oligosaccharides (91.6%), and the high mannose species were predominantly neutral. In this experiment, 0.32% of the total Asn-linked oligosaccharides from viral gD were phosphorylated. Similar analyses of viral gD were performed in five additional experiments where cells were labeled with [3H]mannose at a variety of times post-infection and using various chase protocols, and in all instances only a small fraction of the total oligosaccharides derived from viral gD were phosphorylated. An average of about 0.1% of the Asn-linked oligosaccharides were modified, so that 0.3% or as many as 1% of the viral gD molecules would be expected to contain phosphorylated high mannose-type oligosaccharides. There appear to be thousands of gD molecules/virus particle. In addition, other HSV glycoproteins may also be modified with Man-6-P, and, therefore, the physiological importance of these observations is not yet clear.

It has been reported that glycoproteins expressed by various zoster virus (another herpesvirus) contain Man-6-P residues in complex-type oligosaccharides rather than on their high mannose-type oligosaccharides (S5). We were unable to detect any Man-6-P in the complex-type oligosaccharides of HSV gD.

Soluble gD-2t is a high affinity substrate for N-Acetylglucosaminyl-1-phosphotransferase. When soluble gD-2t was first dephosphorylated with E. coli alkaline phosphatase and then tested as a substrate for partially purified A. castellanti N-acetylglucosaminyl-1-phosphotransferase, the gD-2t was found to be an excellent substrate. The K_m of the reaction was 15.4 µM, with a V_max of 25 pmol of GlcNAc-PO_4 transferred per hour, giving a relative catalytic efficiency (V_max/K_m) of 1623. This compares with a relative catalytic efficiency of 1225 for cation D, an authentic lysosomal enzyme (S2). Using similar assay conditions, no phosphorylation of gD-2t was detected.

Table 1

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Complex</th>
<th>HM</th>
<th>NHP</th>
<th>HM + 1PM</th>
<th>HM + 2PM</th>
<th>PO_4</th>
</tr>
</thead>
<tbody>
<tr>
<td>viral gD</td>
<td>91.6</td>
<td>8.4</td>
<td>96.2</td>
<td>3.1</td>
<td>0.7</td>
<td>0.32</td>
</tr>
<tr>
<td>gD-2t</td>
<td>42.0</td>
<td>58.0</td>
<td>0.9</td>
<td>79.2</td>
<td>12.9</td>
<td>57.5</td>
</tr>
</tbody>
</table>

* The calculated molar percent of complex oligosaccharides versus HM oligosaccharides were high. All oligosaccharides contain an average of 5.1 mannose residues, and the complex-type oligosaccharides contain 3 mannose residues.

* The percent of HM oligosaccharides that were found to contain NHP, HM + 1PM, and HM + 2PM is indicated.

* The percent of total oligosaccharides (both HM and complex) that were phosphorylated is indicated.

DISCUSSION

Soluble forms of HSV gD, gD-4t, and gD-2t bind to the surfaces of mammalian cells and block HSV entry into cells (27). Here, we demonstrated that soluble gD molecules bind to a large cellular glycoprotein that was present in membranes derived from a panel of human, monkey, and rodent cells and to a somewhat smaller protein in fetal bovine serum. The large membrane protein was not detected with a soluble form of another HSV glycoprotein, gB-2t. We used gD-2t as an affinity ligand to partially purify the gD binding protein from fetal bovine serum, and amino acid sequence analysis established that it was the M6P/IGF-II receptor. A truncated form of the M6P/IGF-II receptor has been previously shown to accumulate in fetal serum (48, 53, 54), presumably as a result of cleavage from the surfaces of cells (47). In addition, we found that soluble gD-2t bound to the 46-kDa cation-dependent MFR.

An analysis of the Asn-linked oligosaccharides of gD-2t showed that 58% of its oligosaccharides were of the high mannose type, and virtually all of these contained 1 or 2 Man-6-P residues. Since gD contains three Asn-linked oligosaccharides (S5), one might expect that most or all soluble molecules would contain Man-6-P residues. Further, gD-2t was found to be a high affinity substrate for the phosphotransferase of A. castellanti, which recognizes a common protein recognition marker in lysosomal enzymes (S2). This result indicates that gD-2t is a good substrate for the phosphotransferase in intact cells and supports the view that phosphorylation of gD is not fortuitous and plays some role in HSV replication.

In contrast to the observations with soluble gD-2t, only a small fraction (0.3–1%) of gD derived from HSV-infected cells was modified with Man-6-P. This may explain the observation that approximately 200-fold more viral gD was required than gD-2t in order to obtain equivalent binding of the soluble M6P/IGF-II receptor. Assuming that 100% of soluble gD-2t molecules contain at least one phosphorylated oligosaccharide, one would predict that viral gD would be 1/200 as potent as gD-2t if 0.5% of viral gD molecules contain at least one phosphorylated oligosaccharide. It is unlikely that a substantial fraction of viral gD oligosaccharides are initially phosphorylated.
and then dephosphorylated in infected cells since one would expect that neutral high mannose oligosaccharides would be produced. Viral gD contained 92% complex-type oligosaccharides and, thus, it appears that these molecules reached the Golgi apparatus, where oligosaccharide processing as well as phosphorylating enzymes are located.

It is not clear why gD expressed in HSV-infected cells is phosphorylated less efficiently than soluble gD produced in transfected CHO cells. The most likely explanation is that truncation of the transmembrane domain of gD increases recognition and modification by phosphorylase. It is possible that the phosphotransferase can more efficiently recognize gD or the gD domain required for interaction with phosphorylase when the protein is truncated. Supporting this view, a membrane-anchored form of cathepsin D was modified much less efficiently with Man-6-P than was soluble cathepsin D in transfected cells. Alternatively, these differences may be related to differences between the cell types. The transfected CHO cells were engineered to secrete relatively large amounts of soluble gD, and these cells may be substantially different from HSV-infected human cells. Unfortunately, CHO cells are very difficult to infect with HSV, making direct comparisons between transfected and infected CHO cells impractical. A less likely alternative is that there are differences in the folding of soluble and viral gD molecules that affect recognition by phosphotransferase. Arguing against this possibility, numerous anti-gD monoclonal antibodies that recognize conformationally dependent epitopes can bind to soluble gD (29, 35).

A previous report suggested that varicella zoster virus glycoproteins modified with Man-6-P may cause virus particles to be diverted from the secretory pathway to prelysosomal compartments by virtue of interactions with MPRs (51). Egress of herpesvirus particles from the nuclear envelope to the cell surface may involve a second envelopment process at the Golgi apparatus (55, 56), or, alternatively, enveloped virus particles may transit through the Golgi, or even prelysosomal membranes, en route to the plasma membrane (57). In either case, herperviruses face a substantial hurdle in exiting host cells once the initial envelopment occurs at the nuclear membrane, and, therefore, intracellular sorting machinery such as the Man-6-P/MPR system may play a critical role in virus egress.

Although the Man-6-P content of gD immunoprecipitated from virus-infected cells is relatively low, this may not accurately reflect the level of phosphorylation of viral gD molecules. For example, the majority (>90%) of gD appears to be associated with various cellular membranes and not with the viral envelope (58). Despite extensive efforts, we have been unable to precipitate sufficient quantities of [3H]mannose-labeled gD from extracellular virus particles in order to analyze phosphorylation of virion gD. However, HSV particles or glycoproteins may well interact with MPRs, e.g., on the cell surface during virus entry into cells or during virus egress. HSV glycoproteins other than gD may be phosphorylated, and there are hundreds or thousands of copies of each glycoprotein/glycoprotein virus particle. MPRs are relatively conserved and widely distributed on mammalian cells (reviewed in Refs. 59 and 49). These properties are consistent with the extended host range of HSV, which can infect most mammalian cells. Recently, we observed that a MPR ligand and anti-MPR antibodies could reduce the size and number of HSV plaques, and cell-to-cell spread of HSV was markedly reduced on Pseudo-burler fibroblasts, where phos-

REFERENCES

38. S. Kornfeld, unpublished observations.
40. R. L. Burke, unpublished observations.
HSV gD Binds to Mannose 6-Phosphate Receptors

2.2 Segue

In this chapter I described the identification of a cellular protein which binds to soluble gD-2t. The cellular gD binding protein was identified as the 275 kDa MPR. I further demonstrated that the interaction between gD and the 275 kDa MPR was mediated through M6P residues present on the N-linked oligosaccharides on soluble gD and to a lesser extent on full-length gD. The M6P-modification of gD lead me to test whether gD-2t could interact with other M6P-binding proteins. I showed that in addition to the 275 kDa MPR, gD also bound to the 46 kDa MPR. In the following chapter, I tested the role that the MPRs play in HSV entry and cell-to-cell spread of the virus.
3 Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission

3.0 Preface:

In the following publication, T. Ludwig and B. Hoflack provided us with the MPR-deficient mouse fibroblasts and R.L. Burke provided us with soluble gD-2t. D.C. Johnson and myself carried out the experiment described in Figure 3.1. I carried out the experiments described in Figures 3.2, 3.3, 3.4, 3.5, and 3.7. The experiment depicted in Figure 3.6 was carried out by K. Dingwell and myself.
Role of Mannose-6-Phosphate Receptors in Herpes Simplex Virus Entry into Cells and Cell-to-Cell Transmission

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Herpes simplex virus (HSV) glycoprotein D (gD) is essential for virus entry into cells, is modified with mannos-6-phosphate (M-6-P), and binds to both the 275-kDa M-6-P receptor (MPR) and the 46-kDa MPR (C. R. Brunetti, R. L. Burke, S. Kornfeld, W. Gregory, K. S. Dingwell, F. Masljarz, and D. C. Johnson, J. Biol. Chem. 269:17807–17814, 1994). Since MPRs are found on the surfaces of mammalian cells, we tested the hypothesis that MPRs could serve as receptors for HSV during virus entry into cells. A soluble form of the 275-kDa MPR, derived from fetal bovine serum, inhibited HSV plaques on monkey Vero cells, as did polyclonal rabbit anti-MPR antibodies. In addition, the number and size of HSV plaques were reduced in cells treated with bovine serum albumin conjugated with phenylasparagine-phosphate (PM-P04-BSA), a bulky ligand which can serve as a high-affinity ligand for MPRs. These data imply that HSV can use MPRs to enter cells; however, other molecules must also serve as receptors for HSV because a reasonable fraction of virus could enter cells treated with even the highest concentrations of these inhibitors. Consistent with the possibility that there are other receptors, HSV produced the same number of plaques on MPR-deficient mouse fibroblasts as were produced on normal mouse fibroblasts, but there was no inhibition with PM-P04-BSA with either of these embryonic mouse cells. Together, these results demonstrate that HSV does not rely solely on MPRs to enter cells, although MPRs apparently play some role in virus entry into some cell types and, perhaps, act as one of a number of cell surface molecules that can facilitate entry. We also found that HSV produced small plaques on human fibroblasts derived from patients with pseudo-Hurler’s polydystrophy, cells in which glycoproteins are not modified with M-6-P residues and yet production of infectious HSV particles was not altered in the pseudo-Hurler cells. In addition, HSV plaque size was reduced by PM-P04-BSA; therefore, it appears that M-6-P residues and MPRs are required for efficient transmission of HSV between cells, a process which differs in some respects from entry of exogenous virus particles.

Viral receptors are molecules which facilitate binding of viruses to the surface of cells and entry into the cells. Ideally, if a virus is dependent on specific receptors for penetration into host cells, antibodies or ligands which bind to the receptor should inhibit virus infection. Additionally, cell lines which lack the putative receptor should be resistant to infection, and if these cells are made to express the receptor, e.g., by transfection, the cells can then be infected by the virus. However, these optimum criteria are frequently not satisfied, and there is growing evidence that many animal viruses utilize multiple pathways to enter host cells. For example, reoviruses enter different cell types by using distinct receptors (9, 11), and human immunodeficiency virus (HIV) appears to be able to enter cells by a CD4-independent mechanism (42) as well as CD4-independent mechanisms (19, 25, 58). There are also viruses that use multiple receptor molecules sequentially to enter cells. For example, adenovirus type 2 (Ad2) utilizes fiber proteins, which extend from penton bases at the icosahedral vertices of the viral capsid, to attach to or adsorb onto cells (14, 46), whereas penton bases promote internalization by interacting with vitronectin integrins (62). Antibodies specific for the vitronectin receptor reduced Ad2 internalization but not viral attachment, and ligands for vitronectin integrins inhibited Ad2 entry, although the inhibition was often not complete, suggesting that other molecules may also promote entry of Ad2 (62).

Herpes simplex viruses (HSV) can infect a very broad range of cell types in vivo and in vitro, including fibroblasts, keratinocytes, epithelial cells, glial cells, and neurons of rodent, rabbit, monkey, or human origin, although infection of rodent cells is frequently less efficient (48). Therefore, cellular receptors for HSV must be ubiquitous or HSV utilizes multiple receptors or entry pathways with different cells. HSV encodes at least 12 membrane glycoproteins, 4 of which, gB, gD, gH, and gL, are required for entry of the virus into all cultured cells that have been tested to date (6, 20, 38, 49); however, there is mounting evidence that other glycoproteins may be involved in HSV entry into diverse cell types, especially more highly differentiated cells. For example, gE and gI play a role in cell-to-cell transmission of HSV across junctions formed between human fibroblasts (1a, 15) and between neurons (14a). In addition, there is evidence for multiple HSV receptors on polarized epithelial cells (50). Therefore, it is likely that HSV uses multiple and cell-specific receptors for different cell types.

There is also good evidence that HSV uses distinct cell surface receptors in a sequential fashion to enter cells. Initially, the virus adsorbs onto heparan sulfate glycosaminoglycans (GAGs) (51, 64) and certain chondroitin sulfate GAGs (36), which are very numerous components of the extracellular matrix and plasma membrane. This interaction appears to be mediated primarily by gC (27) and may serve to concentrate the virus on the cell surface or activate the virus in some
manner so that subsequent interactions can occur. There is evidence that following the initial adsorption step, HSV interacts with receptors which are required for virus entry and which are much more limited in number than the sites to which the virus can adsorb. Addison et al. (1) and later Johnson and Miggins (31) found that cells treated with approximately 5,000 UV-inactivated virus particles per cell were resistant to infection with HSV. The inhibition of virus entry by UV-inactivated HSV particles was mediated at the level of virus entry and not at postentry stages of replication, e.g., transport to the nucleus or early transcription, because UV-inactivated HSV added to cells after adsorption of infectious HSV at 4°C failed to inhibit synthesis of early HSV proteins, yet both types of virus particles could enter cells (31). In contrast to virus particles containing gD, UV-inactivated virus particles lacking gD failed to prevent entry of infectious HSV type 1 (HSV-1) or HSV-2, suggesting that gD is required in order that virus particles can interact with this limited set of cell surface receptors required for virus entry. Supporting this hypothesis, cell lines constitutively expressing relatively high levels of gD were resistant to infection by HSV (7, 32). Presumably, gD produced by the cell sequesters a cellular protein required for viral entry, although mutations which overcome the interference phenotype can alter viral gD, and thus other explanations have been proposed (8, 13). In addition, gD-2 would also be able to bind to the cell surface in a saturable manner, the binding was dependent on cell surface proteins, and soluble gD inhibited HSV entry into cells without affecting virus adsorption (50). Together, these results provide compelling evidence that HSV gD acts to engage a set of cell surface receptors which are relatively restricted in number and which facilitate virus entry into cells at a stage subsequent to virus adsorption onto more numerous cell surface GAGs. Whether any of the other HSV proteins which are required for virus entry act as receptors binding proteins is not clear; however, there is evidence that HSV uses distinct receptors to enter different cell types (50).

Recently we demonstrated that HSV gD bound to both the 275-kDa cation-independent mannose-6-phosphate (M-6-P) receptor (CD-MPR) and the 46-kDa cation-dependent M-6-P receptor (CD-MPR) and was modified with M-6-P (5). Both M-6-P receptors (MPRs) sort glycoproteins modified with M-6-P to lysosomes by binding the lysosomal enzymes in the trans-Golgi compartment and diverting the enzymes into the endosomal pathway (reviewed in reference 12). MPRs are also found on the surfaces of cells, almost exclusively in clathrin-coated pits, and there the receptors also bind extracellular lysosomal enzymes and direct them to endosomes and lysosomes (reviewed in references 34 and 35). Accumulation of MPRs cytoplasmically between the Golgi, endosomes, and the plasma membrane. The cell surface 275-kDa CI-CD-MPR also serves as a receptor for insulin-like growth factor II (IGF-II) (41, 44).

In this study, we investigated the role of MPRs in the entry of HSV into cultured cells and in cell-to-cell spread of the virus. A synthetic ligand or antibodies which bind to MPRs and a soluble form of the 275-kDa CI-CD-MPR inhibited HSV entry and production of HSV plaques on monkey cells. However, mouse cells lacking both the 46-kDa CD-MPR and the 275-kDa CI-CD-MPR could be infected by HSV. Therefore, MPRs are not absolutely required for HSV entry into cells but may represent one of several pathways by which HSV enters cells. We also obtained evidence that M-6-P modification of HSV glycoproteins play a role in cell-to-cell spread of the virus by using human fibroblasts incapable of phosphorylating mannose residues.

**MATERIALS AND METHODS**

**Cells.** Monkey Vero cells were grown in a minimal essential medium (o-MEM; Gibco Laboratories, Burlington, Ontario, Canada) supplemented with 7% fetal bovine serum (FBS; Biotrak, Mississauga, Ontario, Canada). Human skin fibroblasts derived from a healthy person (CIM0020) or a pseudo-Hunter patient (GM3391) were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J., and the cells were grown in o-MEM supplemented with 20% FBS. Human epidermal tumor cells were grown in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% FBS. Mouse fibroblasts were derived from embryos of wild-type (BALB/c) mice or from mice lacking the 275-kDa CI-CD-MPR or both the 275-kDa CI-CD-MPR and the 46-kDa CD-CD-MPR 13.5 days after gestation, and the cells were propagated in DMEM supplemented with 20% FBS as previously described (39).

**Viruses.** Wild-type HSV-1 strain F, obtained from P. G. Spear (then at the University of Chicago), and wild-type HSV-2 strain 333 (obtained from B. Roizman, University of Chicago) and the FHSV-1 mutant QAA, which lacks all three N-linked glycosylation sites in gD (53), obtained from G. H. Cohen and R. J. Eisenberg (University of Pennsylvania, Philadelphia), were all propagated and titrated on Vero cell monolayers. The HSV gFQAA mutant indicates F-US572 and F-QD, which is also gFQAA, were propagated and titrated on Vero cells which express gD (58). Visceral stromatitis virus (VSV) strain Indiana was a generous gift from L. Prevec (McMaster University, Hamilton, Ontario, Canada).

**Reagents and antibodies.** Phosphoglucomutase, bovine serum albumin (BSA), M-6-P, and bovine serum albumin (BSA)-40-kDa, were obtained from Sigma Chemical Co. (St. Louis, Mo.). [3H]Phosphatidylcholine and [3H]Phosphatidylethanolamine were obtained from New England Nuclear (Mississauga, Ontario, Canada). Soluble gD-2, which lacks the transmembrane domain and cytoplasm (8, 13), was included in addition to gD-1 and gD-2. Soluble gD-2 was able to bind to the cell surface in a saturable manner, the binding was dependent on cell surface proteins, and soluble gD inhibited HSV entry into cells without affecting virus adsorption (50). Together, these results provide compelling evidence that HSV gD acts to engage a set of cell surface receptors which are relatively restricted in number and which facilitate virus entry into cells at a stage subsequent to virus adsorption onto more numerous cell surface GAGs. Whether any of the other HSV proteins which are required for virus entry act as receptors binding proteins is not clear; however, there is evidence that HSV uses distinct receptors to enter different cell types (50).

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In this study, we investigated the role of MMPs in the entry of HSV into cultured cells and in cell-to-cell spread of the virus. A synthetic ligand or antibodies which bind to MMPs and a soluble form of the 275-kDa CD-MPR inhibited HSV entry and production of HSV plaques on monkey cells. However, mouse cells lacking both the 46-kDa CD-MPR and the 275-kDa CI-CD-MPR could be infected by HSV. Therefore, MMPs are not absolutely required for HSV entry into cells but may represent one of several pathways by which HSV enters cells. We also obtained evidence that M-6-P modification of HSV glycoproteins play a role in cell-to-cell spread of the virus by using human fibroblasts incapable of phosphorylating mannose residues.
ROLE OF MPRs IN HSV INFECTION

RESULTS

A soluble form of the 275-kDa MPR inhibits HSV-2 plaque production. To investigate the role of MPRs in entry of HSV into cells, we attempted to block infectivity of HSV by incubating the virus with a soluble form of the 256-kDa MPR. FBS contains a soluble form of the 275-kDa MPR (and not the 46-kDa MPR) which is produced by proteolytic release of the extracytoplasmic domain of the receptor and has an apparent molecular mass of 225 kDa (10); this MPR is designated the 225-kDa MPR. The precise cleavage site has not been defined, but the soluble MPR binds both M-4-P-modified proteins and IgG-I. The 225-kDa MPR was purified from FBS by PM-PO₄ affinity chromatography (16, 37). PM-PO₄ affinity chromatography yields a high degree of purification of either the 225-kDa MPR or the 275- and 46-kDa glycosylated MPRs (25, 37, 49a). This high degree of purity (>94%) (49a) produced in a single step is related to the fact that columns can be washed extensively and then eluted in the same buffer with the addition of 5 or 6 mM M-6-P. Contaminants (e.g., bound by ionic interaction) are not specifically eluted under these conditions. After SDS-polyacrylamide gel electrophoresis of the MPR preparations from FBS, a major protein species of approximately 225 kDa was detected by Coomassie blue staining (Fig. 1, inset). We also observed a minor protein band migrating slightly faster than the 225-kDa soluble MPR, which is probably a different form of the 225-kDa MPR, because soluble GD-2t interacted with this protein on ligand blot (not shown).

Preparations of HSV-2 derived from cell culture supernatants were mixed with soluble MPR (0 to 6 M) corresponding to 0 to 1.5 mg/ml and then the HSV-2 protein mixture was plated on Vero cell monolayers for 90 min. After this incubation, the cells were washed and incubated with the appropriate concentrations of the soluble 225-kDa MPR for another 24 h; then the cell monolayers were stained, and viral plaques were counted. There were reductions in the numbers of HSV-2 plaques produced on Vero cell monolayers with increasing concentrations of soluble 225-kDa MPR, but there was no inhibition with similar concentrations of BSA (Fig. 1). Because of the availability of limited amounts of the soluble MPR, and because we found it necessary to incubate cells both before and after virus adsorption, these experiments were not performed with replicate wells. Instead, numerous experiments using different preparations of the protein were performed in the same
In four experiments, HSV-2 plaques were inhibited in number by 45 to 75% when virus was treated with 6 μM MPR. Preparations of the 225-kDa MPR which had been heat denatured did not affect the number of HSV-2 plaques, and there was no evidence that the purified 225-kDa MPR produced any toxicity to cells which could be cultured in the presence of 6 μM MPR for 5 days without changes in morphology or growth (results not shown). In other experiments, similar inhibition of HSV-1 plaques was observed when virus was mixed with soluble 225-kDa MPR (not shown).

Rabbit anti-MPR antibodies inhibit production of HSV-2 plaques. To characterize the role of MPRs in HSV entry further, polyclonal anti-MPR antibodies were produced in rabbits. The 275- and 46-kDa MPRs were purified from bovine testes by PM-PO affinity chromatography as previously described (16, 37). Again, a high degree of purity was obtained in a single step. SDS-polyacrylamide gel electrophoresis of these preparations, followed by Coomassie blue staining, demonstrated that the 275- and 46-kDa MPRs were by far the predominant proteins in these preparations (Fig. 2A). There were, however, faint traces of contaminating proteins of approximately 70 kDa in these preparations. Rabbits were injected with purified MPRs or, in parallel, with soluble gD21 (30).

When the purified MPRs were subjected to electrophoresis, the anti-MPR antibodies reacted with the 275- and 46-kDa MPRs on Western blots (Fig. 2B). In addition, when Vero cells were radiolabeled with 35Smethionine and 35S sulfate, the anti-MPR serum precipitated both the 275- and 46-kDa MPRs as well as a protein of approximately 85 kDa (Fig. 2C). This protein was also detected with an anti-MPR serum from another laboratory (49a). This 85-kDa protein may represent a proteolytic product of the 275-kDa MPR, a cross-reactive cellular protein, or a protein contaminant of the original MPR preparation. The anti-MPR sera were of relatively low titer compared with anti-gD sera. For example, in dot blot assays in which gD21 or purified MPRs were bound to nitrocellulose, anti-MPR antibodies could be detected at a dilution of 1:500, whereas anti-gD21 antibodies could be detected at a dilution of 1:10,000. We expect that these differences in antibody titers are related to difficulties in producing high-titer sera to the relatively conserved MPRs. In contrast, gD is highly antigenic. IgG fractions were purified from the anti-MPR, preimmune, and anti-gD sera.

The effects of anti-MPR antibodies were tested by incubating the IgG with Vero cell monolayers for 30 min at 32°C and then adding HSV-2 for 90 min at 37°C. Unabsorbed virus and unbound sera were removed, and then the cells were washed with citrate buffer (pH 3.0) to inactivate HSV-2 which had adsorbed but not entered cells. The cell monolayers were in-
cubated for an additional 36 h, the cells were stained, and plaques were counted. Anti-MPR IgG inhibited production of HSV-2 plaques by greater than 55% at the highest concentrations used (400 μg/ml) (Fig. 2D) and was not otherwise toxic to cells. Similar results were obtained in other experiments with purified anti-MPR serum described previously (18), and there was no effect of the anti-MPR antibodies if the antibodies were added after virus entry into cells (not shown). However, we cannot discount the possibility that the anti-MPR antibodies inhibited virus entry by binding to a cross-reactive protein, such as the 85-kDa protein that we observed in Fig. 2C. Anti-GD IgG was more effective in blocking plaque formation, with 30% inhibition observed at the lowest concentration tested (25 μg/ml), and no plaques detected at 100 μg/ml. There was little or no effect on the number of HSV-2 plaques when Vero cells were incubated with IgG derived from preimmune sera at 400 μg/ml (Fig. 2D). Similar inhibition of HSV-2 plaques was observed on monolayers of bovine MDBK cells treated with anti-MPR sera (not shown).

Inhibition of HSV plaques by PM-PO₄-BSA. Binding of lysosomal enzymes to cell surface MPRs can be at least partially inhibited by M-6-P, albeit the affinity of both MPRs for monomeric M-6-P is relatively low (Kₘ of 8 × 10⁻⁹ M). Diphenylbutylated oligosaccharides bind to the 275- and the 46-kDa MPRs with higher affinities (2 × 10⁻⁷ and 2 × 10⁻⁹ M, respectively) suggesting that MPRs bind M-6-P residues at more than a single site (17, 29). We observed no inhibition of HSV plaque formation when cells and virus were incubated with 10 μM M-6-P or with 1 μM IGF-II (not shown). To evaluate a ligand which is bulky and binds to both MPRs with higher affinity than does M-6-P, we purified PM-PO₄ from yeast phosphorylated, and conjugated this material to BSA, producing PM-PO₄-BSA, which binds specifically and with high affinity to cell surface MPRs (4, 57). The extent of BSA modification with PM-PO₄ was estimated by 30 to 35 PM-PO₄ molecules per a protein molecule, as judged from the substantial decrease in electrophoretic mobility observed on SDS-polyacrylamide gels (85 to 90 kDa versus 88 kDa for unmodified BSA) (Fig. 3A, inset).

We then tested the ability of PM-PO₄-BSA to block HSV entry into cells. Vero cells were incubated with various concentrations of PM-PO₄-BSA for 40 min, and then HSV-I or a control virus, VSV, was added (~10 PFU/2-cm² well) for a further 60 min in the presence of PM-PO₄-BSA. Unbound virus was removed, and the cells were washed and incubated for 36 h (HSV-I) or 18 h (VSV) in the presence of the appropriate concentration of PM-PO₄-BSA. In the experiment shown, there was a reduction in the number of HSV-I plaques produced by over 80% when cells were treated with 4.5 μM (corresponding to ~400 μg/ml) PM-PO₄-BSA throughout the experiment and of 75% when the cells were treated with 2.3 μM PM-PO₄-BSA (Fig. 3A). Cells treated with similar concentrations of BSA alone showed no inhibition of HSV plaque production (not shown). When PM-PO₄-BSA was present only during the first 100 min (Fig. 3A), there was little or no inhibition of HSV plaques, suggesting that virus adsorption onto the cells was not inhibited by this treatment and virus particles could enter cells once PM-PO₄-BSA was removed. Similarly, the number of HSV plaques was not altered when PM-PO₄-BSA was added after virus entry into cells (Fig. 3A), suggesting that PM-PO₄-BSA does not block postentry stages of HSV replication in cells. In these experiments, replicate wells were not analyzed because materials were limited; however, three experiments using different preparations produced inhibition of HSV-I ranging from 61 to 85%. Treatment of Vero cells with PM-PO₄-BSA not only inhibited the number of HSV plaques produced on the cells but also substantially reduced the sizes of HSV-I(F) plaques produced in the presence of 1.1 or 2.3 μM PM-PO₄-BSA (Fig. 4). In contrast to the inhibition of HSV, PM-PO₄-BSA did not inhibit production of plaques by VSV (Fig. 3A). PM-PO₄-BSA was not toxic to cells and produced no detectable alteration in the growth or morphology of uninfected Vero cells over several days (results not shown).

There was also evidence that PM-PO₄-BSA inhibited the initial entry of HSV-I into cells, rather than some other aspect of virus replication required to form plaques. Cells were treated with various concentrations of PM-PO₄-BSA, then infected with HSV at 10 PFU per cell, and subsequently radiolabeled with [³⁵S]methionine. The HSV-I early protein thymidine kinase was immunoprecipitated from cell extracts as a measure of virus entry into cells (Fig. 3B and C). In this experiment, when PM-PO₄-BSA was kept continuously present, there was inhibition of early protein synthesis (Fig. 3C), but when the PM-PO₄-BSA was removed after virus adsorption (Fig. 3B), some fraction of the HSV-I entered the cells once the inhibitor was removed and there was little or no inhibition of thymidine kinase expression.
The experiments in Fig. 3 and 4 involved HSV-1, whereas the results in Fig. 1 and 2 involved HSV-2. We have not detected differences in the entry of HSV-1 and HSV-2 into cells: UV-inactivated HSV-2 blocks entry of HSV-1 and vice versa (31). HSV-1 gD blocks entry of both HSV-2 and HSV-1, and HSV-2 gD inhibits both HSV-1 and HSV-2 (30), and both proteins bind to MPRs (5). Therefore, we assume that HSV-1 and HSV-2 are functionally interchangeable in these particular experiments, and for technical reasons, e.g., the anti-MPR serum was compared with serum directed to HSV-2 gD, we primarily used HSV-2 in early experiments and HSV-1 in the later experiments. Given limited quantities of material, it was frequently difficult to compare HSV-1 and HSV-2 extensively, and in actuality, it may be more important to compare the effects of these inhibitors on different cell types.

Infection of MPR-deficient mouse fibroblasts by HSV. To further assess the role of MPRs in HSV entry into cells, we studied mouse fibroblasts unable to express one or both of the
MPRs (39). These fibroblasts were derived from embryos of mice lacking the 275- and 46-kDa MPRs. There is evidence that lysosomal enzymes are missorted in these cells and secreted rather than being targeted to lysosomes. We infected fibroblasts derived from wild-type (BALB/c) mice, mice lacking the 275-kDa MPR, or mice lacking both the 46- and 275-kDa MPRs with HSV-1 (F) or with VSV; 2 h later, the cells were labeled with [35S]methionine. To quantitate virus entry into the cells, the HSV early protein thymidine kinase or the VSV N protein were immunoprecipitated from cell extracts. Similar amounts of thymidine kinase were produced in HSV-infected wild-type, 275-kDa MPR-deficient, and 46/275-kDa MPR-deficient fibroblasts (Fig. 5A). The levels of VSV N protein produced in all three cell lines were also similar. In other experiments, there was no significant difference in protein expression in the number of HSV plaques produced on wild-type and MPR-deficient fibroblasts. Because the plaques formed on these embryonic mouse fibroblasts were not large, it was difficult to ascertain whether the plaques formed on MPR-deficient fibroblasts were smaller than those formed on the normal mouse fibroblasts.

The ability of PMPO4,BSA to inhibit HSV entry was tested on both normal and MPR-deficient mouse fibroblasts. These experiments provided a suitable control for toxicity of PMPO4,BSA on virus and cells and also tested whether MPRs play a role in HSV entry into these mouse fibroblasts. PMPO4,BSA did not inhibit HSV plaque formation on either normal mouse fibroblasts or 46/275-kDa MPR-deficient fibroblasts, but there was inhibition on Vero cells in the same experiment (Fig. 5B). In other experiments, PMPO4,BSA had no effect on HSV entry into normal mouse fibroblasts or 46/275-kDa MPR-deficient fibroblasts as assessed by expression of thymidine kinase in the cells (data not shown). These results further confirm that HSV does not rely on MPRs to enter these embryonic mouse fibroblasts. These observations also suggest that there are differences in entry of HSV into monkey Vero cells and these mouse fibroblasts. In the experiment shown, the number of HSV plaques produced on both types of mouse fibroblasts was lower than produced on Vero cells, suggesting that infection of the mouse cells may be less efficient. In addition, since there was no measurable effect of PMPO4,BSA on either the mouse cells or virus, the results further support our belief that this inhibitor is not generally toxic to cultured cells or to HSV replication.

In the absence of gD phosphorylation, HSV produces small plaques on fibroblasts monolayers. Fibroblasts derived from patients with pseudo-Hurler polydystrophy possess very low levels of N-acetylglucosamine-1-phosphotransferase activity, which is required for phosphorylation of mannose residues on lysosomal enzymes (35, 47). Modification of lysosomal enzymes with M-6-P in pseudo-Hurler fibroblasts is reduced to 3% of the level found in normal fibroblasts (60A). There is little or no information on the status of MPRs in pseudo-Hurler fibroblasts, although one would not expect alterations in these proteins. Presumably, HSV gD, which has three N-linked glycosylation sites, would be inefficiently modified with M-6-P in pseudo-Hurler fibroblasts. To determine the effects of incomplete phosphorylation of gD, fibroblasts derived from a pseudo-Hurler patient (GM3391) or fibroblasts from a healthy parent of this patient (GM0080) were infected with HSV-1, and virus plaques were allowed to form over a period of 56 h. Wild-type HSV-1(F) produced similar numbers of plaques on both pseudo-Hurler fibroblasts and normal human fibroblasts. However, smaller plaques were produced by HSV-1(F) on pseudo-Hurler fibroblasts (10 to 20 cells infected) compared with plaques produced on normal human fibroblasts (>100 infected cells) (Fig. 6A and C); similar results were obtained with HSV-2 (not shown). As a control in these experiments, we used HSV-1 QAA, a mutant derived from HSV-1(F) in which gD coding sequences were altered so that none of the three N-linked oligosaccharides are added (34). Since there are no N-linked oligosaccharides and O-linked oligosaccharides cannot be modified with M-6-P (34), it is very likely that the QAA
FIG. 6. HSV plaques on normal and pseudo-Hurler fibroblasts. Monolayers of normal (norm) or pseudo-Hurler (P.hur.) fibroblasts were infected with wild-type HSV-1 strain F or HSV-1 mutant QAA, which expresses a form of gD lacking N-linked oligosaccharides. F-US6kan, which does not express gD, or F-gDB, which does not express gD and gI. F-US6kan and F-gDB virus preparations were derived from VDH6 cells. Cells were stained with crystal violet after 56 h, and representative plaques were photographed.
gD molecule is not modified with M-6-P in both pseudo-Hurler and normal fibroblasts. The QAA mutant produced small plaques (10 to 20 infected cells) on both normal and pseudo-Hurler fibroblasts (Fig. 6B and D). These observations supported the notion that the small-plaque phenotype of wild-type HSV-1 on pseudo-Hurler cells was related to lack of phosphorylation of gD rather than to other effects.

An HSV-1 mutant unable to express gD, F-U56kan, also produced very small plaques on normal human fibroblasts; three to five cells were infected after 56 h (Fig. 6E). A second HSV-1 gD mutant, F-gdB, which is also unable to express gD, was unable to spread beyond a single cell (Fig. 6F). Note that the F-U56kan and F-gdB virus stocks used to infect these fibroblasts were derived from complementing VD60 cells which supply gD in trans, and thus viruses initiating the infection possessed gD. These results demonstrated that HSV-1 can spread, albeit inefficiently, from cell to cell without gD and produce microscopic plaques. However, efficient spread of HSV requires gD molecules, and defects in glycosylation or the addition of M-6-P to gD (and perhaps other HSV glycoproteins) markedly reduced cell-to-cell spread. The observation that F-gdB (which lacks gD as well as gD) was unable to form any type of plaque, i.e., to spread at all, is consistent with previous observations that the gE-gH hetero-oligomer contributes substantially to the ability of HSV to spread from cell to cell in monolayers of human fibroblasts (15).

It was possible that the small plaques produced by HSV-1 on pseudo-Hurler fibroblasts were related to defects in virus replication in the cells. To address this point, normal and pseudo-Hurler fibroblasts were infected with wild-type HSV-1(F) at 10 PFU per cell; at various times after infection, the cells and growth media were harvested and infectious HSV-1 was quantitated by using plaque assays on Vero cells. The kinetics of production of infectious HSV-1 were similar on both normal and pseudo-Hurler fibroblasts, and the final amounts of virus produced were also similar (Fig. 7). These data suggested that the small-plaque phenotype of HSV-1 on pseudo-Hurler fibroblasts was due to a defect in cell-to-cell spread of virus and not due to defects in virus replication in the cells. In addition, the results show that HSV-1 derived from pseudo-Hurler fibroblasts, in which gD is inefficiently modified with M-6-P, produces normal numbers of plaques on Vero cell monolayers.

**DISCUSSION**

There is considerable evidence that HSV gD is required for virus entry into cells, promoting virus interactions with a relatively restricted set of cell surface receptors at a stage subsequent to adsorption onto cell surface GAGs. To identify the cellular receptors with which gD interacts, we developed a ligand blot assay, and using this assay coupled with gD affinity chromatography, we purified a large cellular membrane protein which was identified as the 275-kDa MPR (5). Purified MPRs could bind to both soluble and viral gD molecules, though the binding to viral gD was low and only a small fraction of viral gD was modified with M-6-P, whereas virtually every molecule of soluble gD was phosphorylated. It is possible that gD molecules in the virion envelope are more extensively modified with M-6-P than gD extracted from virus-infected cells; however, efforts to analyze gD from virions have, to date, been unsuccessful. Nevertheless, there are thousands of gD molecules per virion, and thus a relatively low level of phosphorylation may have important biological implications for HSV. It is also possible that gD is inefficiently modified with M-6-P in order to promote escape of newly produced virions so that virus particles are not retained in endosomal or lysosomal membranes or on cell surface MPRs. We note that a small fraction of varicella-zoster virus (VZV) glycoproteins are also modified with M-6-P, and it has been hypothesized that this causes VZV to remain primarily cell associated (22). Like VZV, HSV remains primarily cell associated. In analyzing the phosphorylation of gD, we observed that soluble gD-2t could be modified in vitro by N-acetylgalactosamine-1-phosphotransferase, the enzyme responsible for the addition of M-6-P residues, and the relative catalytic efficiency of this reaction was greater than that for cathepsin D, an authentic lysosomal enzyme (5). These results suggest that modification of gD, and perhaps other herpesvirus glycoproteins, with M-6-P is not fortuitous and has important implications for the biology of HSV and other herpesviruses.

To investigate the role of MPRs in HSV entry into monkey Vero cells, we investigated the effects of a synthetic ligand of MPR, anti-MPR antibodies, and a soluble form of the 275-kDa MPR. Incubation of HSV with soluble MPR inhibited plaque production by as much as 75% in some experiments, though the inhibition varied from 25% in other experiments. On the assumption that only a small fraction of virion gD molecules are modified by M-6-P residues (5), it is likely that those gD molecules that are modified are monophosphorylated rather than diglycosylated. Since proteins substituted with only a single M-6-P residue bind to surface MPRs with lower affinities than proteins with multiple M-6-P substitutions, we would expect that soluble MPR binds to viral gD molecules with an affinity of only ~8 μM (17, 59, 60). Given this consideration, it may not be surprising that there was only partial inhibition of HSV entry and that this required 6 μM soluble MPR.

Polyclonal antisera raised against both the 275- and 46-kDa MPRs inhibited HSV plaque production by up to 55%. In interpreting the results of these sera, a number of points must be considered. It is well known that only a small fraction of MPRs (5 to 10%) are present on the cell surface at any one time and that the receptors recycle rapidly (23, 45, 56). Thus, MPR ligands or anti-MPR antibodies may be rapidly removed from the cell surface by recycling. Furthermore, MPRs are
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highly conserved among mammalian species, and the titers of our anti-MPR sera were about 20-fold lower than titers of anti-gD sera prepared in parallel and which more effectively inhibited HSV plaque production. In comparing these two sets of antisera, it should be made clear that the anti-gD antibodies can neutralize HSV or block entry by binding directly to virus particles, which is unlikely to be the case for anti-MPR antibodies, which must bind to surface MPRs constantly recycling from within cells. Furthermore, it has been difficult to inhibit binding and uptake of M-6-P-modified proteins by more than 50% with similar anti-MPR antibodies (57). Recent observations with adenosviruses highlight the difficulty of inhibiting virus entry by using antireceptor antibodies. Anti-integrin antibodies used at concentrations similar to those used in this study (100 to 500 μg/ml) caused only partial inhibition of adenosvirus entry into cells (43, 46), perhaps because adenosviruses can enter by using other receptors, although other explanations are also possible because cells lacking receptors are much more resistant to adenosvirus.

Inhibition of HSV plaque production and virus entry into cells was also observed when cells were treated with PM-PO4-

BSA, which acts as a high-affinity, relatively bulky ligand for surface MPRs. As with soluble MPR, concentrations of 1.1 to 4.5 μM PM-PO4-

BSA were required to inhibit HSV entry and plaque formation by 61 to 82%. Again, there was still a substantial fraction of HSV which could enter cells treated with even the highest concentrations of PM-PO4-

BSA tested. These inhibitory concentrations are similar to those of soluble gD-2i (2 to 4 μM) required to inhibit HSV entry into cells and plaque production (30). Given that the affinity of soluble gD-2i for cell surface receptors is only moderate (Kd ≈ 0.25 μM [30]), it is perhaps not surprising that 10- to 20-fold-higher concentrations of gD-2i, or PM-PO4-

BSA, might be required to inhibit interactions between virus and MPRs, interactions that are likely to be highly multimeric. Similar concentrations of soluble gB-2i, which is not modified with M-6-P (5), did not alter plaques (30).

To inhibit HSV entry into cells, it was necessary to keep PM-PO4-

BSA present continuously, as was also the case with soluble MPR. We found that there was little or no effect on plaque formation if PM-PO4-

BSA was present during the period in which cells and virus were incubated together but was removed when the virus inoculum was removed. We interpret these results to indicate that virus particles linger on surface GAGs and enter cells when MPRs are no longer blocked. Similarly, soluble gD must be kept continuously present in order to inhibit virus entry and soluble gD had no effect on HSV adsorption onto the cell surface (30), suggesting, again, that HSV bound on surface GAGs can enter cells once soluble gD is removed. In addition, the continuous presence of PM-PO4-

BSA was not toxic to cells and did not inhibit virus replication at a postentry stage because there was no inhibition by PM-PO4-

BSA, which was continuously present 60 min after addition of HSV. It might be suggested that PM-PO4-

BSA, which possesses a negative charge, can inhibit HSV entry analogously to heparin, by inhibiting the ability of virus to adsorb onto cell surface heparan sulfate GAGs (55, 64). However, there are important differences in how PM-PO4-

BSA and heparin act. Heparin will effectively inhibit HSV adsorption onto the cell surface, if present only during the period when virus and cells are incubated together (64), and yet PM-PO4-

BSA must be kept continuously present in order to inhibit HSV. In addition, there was no effect of PM-PO4-

BSA on mouse fibroblasts, cells which presumably contain heparan GAGs since heparin blocks HSV infection of mouse cells (24). Therefore, our results with three inhibitors, PM-PO4-

BSA, soluble MPR, and anti-MPR antibodies, support the hypothesis that HSV utilizes MPRs to enter cells and that this occurs at a postadsorption step. However, since in none of these experiments could we completely inhibit HSV entry or plaques, it is probable that other receptors or pathways must also function to mediate entry into human and monkey cells.

We also found that mouse fibroblasts lacking both MPRs were as efficiently infected by HSV as fibroblasts which express MPRs, and virus produced normal numbers of plaques on the MPR-negative cells. At face value, these results appear to contradict the results with soluble MPR, MPR ligands, and antibodies, arguing that HSV does not require MPRs to enter cells. However, it appears that there are MPR-independent entry pathways in monkey and human cells, and therefore these entry mechanisms may be predominant in these embryonic mouse fibroblasts. Indeed, M-6-P-independent uptake of phosphorylated lysosomal enzymes is quite important compared with M-6-P dependent uptake in these mouse embryonic fibroblasts (27a). Consistent with the idea that HSV enters these mouse cells primarily by MPR-independent pathways, little or no inhibition of HSV plaque production by PM-PO4-

BSA was observed on these fibroblasts. In addition, HSV plaque production on these mouse cells was frequently less efficient than with Vero cells. However, it is also clear that gD is important in entry into these mouse cells because gD-negative viruses could not enter the cells and recombinant gD-2i reduced the number of HSV plaques produced on these cells (4a). These results are consistent with the notion that gD has other functions, e.g., in membrane fusion (21), or binds to other receptors.

The phenotype of the QAA mutant, which lacks N-linked oligosaccharides and thus M-6-P residues on gD, is very different from that of HSV-1 gD-negative mutants. QAA produces normal numbers of plaques and penetrates into cultured monkey cells with similar kinetics to that of wild-type HSV-1 (54). Furthermore, HSV derived from pseudo-Hurler fibroblasts, which presumably lacks most or all M-6-P, can infect Vero cells and produce normal numbers of plaques. Thus, loss of phosphorylation of gD (and perhaps other HSV glycoproteins) causes effects different from those caused by ligand or antibody blocking of MPRs. Although we do not understand these results, they may suggest that MPR ligands and anti-MPR antibodies act not simply by binding to surface MPRs, blocking their availability to HSV. It is possible that the effects of inhibitors are indirect; for example, the inhibitors may sterically hinder other molecules that HSV uses to enter cells.

One way of explaining these results is to consider the observation that MPRs are primarily localized to cell surface coated pits so that the they are highly restricted in terms of distribution and mobility (45). Anti-MPR antibodies and ligands added to the cell surface presumably bind to coated pits. If one assumes that HSV can use coated pits as part of its entry pathway, inhibition of HSV entry by MPR antibodies or MPR ligands may be explained by an indirect effect on coated pits. In this scenario, other HSV receptors may reside in coated pits and MPR ligands or anti-MPR antibodies may limit accessibility of these receptors. There is no published evidence suggesting that coated pits play a role in HSV entry into cells, and in contrast to this notion, there is evidence that HSV can enter cells by fusion of the viral envelope with the plasma membrane. Normally, viruses which use coated pits become endocytosed and enter the cytoplasm after fusion in low pH endosomes (26, 61). However, HSV does not appear to require low pH to fuse with cellular membranes because chloroquine does not block the virus (63). In addition, there is electron microscopic evidence for fusion of HSV particles with the plasma membrane (21),
ROLE OF MPRs IN HSV INFECTION

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3.2 Addendum

The following sections relate to data which appeared as “data not shown” in Brunetti et al., 1995 (Chapter 3).

3.2.1 PM-PO4-BSA has similar inhibitory affects on HSV-1 and HSV-2:

To determine whether HSV-1 and HSV-2 behave similarly with respect to their interaction with MPRs, I tested the ability of PM-PO4-BSA to inhibit entry of HSV-1 and HSV-2 into cells. Vero cells were treated with either 0, 2, 4, 8, or 16 µM of PM-PO4-BSA for 30 min at 37°C. The cells were subsequently infected with either HSV-1 strain F (■) or HSV-2 strain 333 (●) for 60 min at 37°C. The medium was removed, the cells were washed, and overlaid with fresh medium containing the appropriate concentration of PM-PO4-BSA and 0.1% human gamma globulin. The cells were incubated at 37°C for 36 hours to allow plaques to develop, then they were stained with crystal violet and the plaques were counted.

The results demonstrated that HSV-1 strain F and HSV-2 strain 333 showed similar levels of inhibition of plaque formation by PM-PO4-BSA (Figure 3.8). These data provide evidence that both HSV-1 and HSV-2 display a similar response to reagents which block the ability of HSV to interact with MPRs.
Figure 3.8: Inhibition of HSV-1(F) and HSV-2(333) plaque production by the MPR ligand PM-PO₄-BSA. Vero cells growing in 6-well dishes were treated with either 0, 2, 4, 8, or 16 μM of PM-PO₄-BSA and incubated for 30 min at 37°C followed by infection for 60 min with either HSV-1(F) (-■-) or HSV-2(333) (-○-) at 37°C. The cells were washed and overlaid with medium containing the appropriate concentration of PM-PO₄-BSA and 0.1% human gamma globulin. 36 hours later the cells were stained with crystal violet and plaques were counted.
3.2.2 Deglycosylated soluble gD retains the ability to inhibit HSV entry into cells:

To determine whether oligosaccharides on gD are important for inhibiting HSV entry into cells, soluble gD-2t was treated with an endoglycosidase to remove N-linked oligosaccharides and tested for its ability to inhibit HSV entry. PNGase F (endoglycosidase F) treatment removes complex and high mannose N-linked oligosaccharides from glycoproteins. Soluble gD-2t (obtained from Chiron Corporation, Emeryville, CA) was either mock-treated or incubated with PNGase F (New England Biolabs, Mississauga, Ont.) under conditions described by the manufacturer for 5 hours at 37°C. Approximately 5 µg of the mock-treated (gD-2t) or PNGase F-treated gD-2t (gD-2t+PNGase F) were separated on a 12% polyacrylamide gel and the proteins were visualized by Coomassie brilliant blue staining. Figure 3.9 inset shows that treatment of soluble gD-2t with PNGase F resulted in cleavage of the N-linked oligosaccharides from gD so that the majority of the material had a faster electrophoretic mobility.

To determine whether deglycosylated gD-2t inhibited HSV entry into cells, either 0, 50, 100, or 200 µg/ml of deglycosylated or mock treated gD was applied to a 12-well dish of confluent Vero cells and the cells were incubated for 30 min at 4°C. Approximately 200 plaque forming units (PFU)/well of HSV-1(F) was added to the cells which were incubated for an additional 60 min at 4°C.
The medium and virus were removed and fresh medium supplemented with the appropriate concentration of mock treated (-■-) or deglycosylated (-▲-) gD-2t was added followed by incubation of the cells at 37°C for 60 min. The medium was removed and fresh medium containing 0.2% human gamma globulin was added and the cells were incubated at 37°C for 48 hours to allow plaques to develop. The cells were stained with crystal violet and the plaques were counted. Figure 3.9 shows that both mock treated and deglycosylated gD-2t effectively inhibited HSV entry into cells. These data provide evidence that N-linked oligosaccharides are not necessary for the inhibition of HSV entry by soluble gD.
Figure 3.9: Deglycosylated soluble gD-2t blocks HSV-1(F) entry into cells. Soluble gD-2t was either mock treated or treated with PNGase F for 5 hours at 37°C. The soluble gD-2t was separated on a 12% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue (inset). Either 0, 50, 100, or 200 µg/ml of mock-treated (-■-) or deglycosylated (-▲-) gD-2t was applied to a 12-well dish of Vero cells for 30 min at 4°C. The cells were incubated with HSV-1(F) for 60 min at 4°C. The medium was removed and fresh medium containing the appropriate concentration of mock-treated or deglycosylated gD-2t was added and the cells were incubated at 37°C for 60 min. The medium was replaced with medium containing 0.1% human gamma globulin. 48 hours later, the cells were stained with crystal violet and plaques were counted and their number in treated samples was expressed as a percentage of the total number of plaques in the untreated sample.
3.2.3 PM-PO$_4$-BSA does not inhibit HSV entry into mouse fibroblasts:

Based on the observation that PM-PO$_4$-BSA had no effect on the ability of HSV to enter into normal mouse fibroblasts or mouse fibroblasts which lacked both the 275 kDa and 46 kDa MPRs (Chapter 3), we tested other mouse cell lines to determine if this phenomenon was a unique property of normal mouse fibroblasts or a more general property of mouse cells. Normal mouse fibroblasts, mouse fibroblasts that lacked both the 275 kDa and 46 kDa MPR (Ludwig et al., 1994), mouse L cells which lacked the 275 kDa MPR (D9) (Gabel and Foster, 1986), D9 cells which had been stably transfected with the bovine 275 kDa MPR (CC2), or D9 cells transfected with the bovine 275 kDa MPR which lacked the cytoplasmic tail (Dd4) (Lobel et al., 1989) were incubated for 60 min at 37°C with either 0 or 4 μM of PM-PO$_4$-BSA. The cells were infected with HSV-1(F) by adding virus in the presence of PM-PO$_4$-BSA and incubated for an additional 90 min at 37°C. The virus and medium were removed and the cells were treated with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 minute to neutralize extracellular virions. The cells were then resuspended in fresh medium for an additional 30 min at 37°C. The medium was removed and the cells labelled with 50 μCi/ml of [³⁵S]cysteine and [³⁵S]methionine in Dulbecco’s medium lacking cysteine and methionine for 3 hours at 37°C. The cells were lysed in NP40/DOC (1% Nonidet P-40, 0.5%
sodium deoxycholate, 50 mM Tris pH 7.5, 100 mM NaCl) and the HSV thymidine kinase (tk) protein was immunoprecipitated using a polyclonal rabbit antiserum (a gift of Dr. William Summers, Yale University, New Haven, Conn.) and protein-A Sepharose beads (Pharmacia, Baie d'Urfe, Quebec). The proteins were separated on a 10% polyacrylamide gel.

Similar levels of thymidine kinase were produced in all the various cells whether or not 4 μM PM-PO$_4$-BSA was present (Figure 3.10). These data support previous observations (Chapter 3) that PM-PO$_4$-BSA had no effect on HSV entry into mouse cells.
**Figure 3.10:** PM-PO$_4$-BSA had no effect on HSV entry into mouse cells.

Monolayers of normal mouse fibroblasts, mouse fibroblasts lacking the 275 kDa and 46 kDa MPR, mouse L cells deficient for the 275 kDa MPR (D9), D9 cells expressing the bovine 275 kDa MPR (CC2), or D9 cells expressing the bovine 275 kDa MPR which lacked a cytoplasmic tail (Dd4) were incubated in the presence (+) or absence (-) of 4 μM PM-PO$_4$-BSA for 60 min at 37°C. The cells were infected with HSV-1(F) at 1 PFU/cell and incubated at 37°C for 90 min. The cells were treated with citrate buffer and labelled with [$^{35}$S]cysteine and [$^{35}$S]methionine (50 μCi of each/ml) for 3 hours. HSV thymidine kinase (tk) was immunoprecipitated from HSV-infected cell extracts. Proteins were separated on a 10% polyacrylamide gel.
3.3 Segue

In this chapter I described the potential role which MPRs play in an HSV infection. Using either a soluble form of the 275 kDa MPR, antibodies against both the 275 kDa and 46 kDa MPR, or PM-PO₄-BSA (a bulky ligand which binds to MPRs with high affinity) I inhibited HSV entry into cells by 60 to 80%. Surprisingly this inhibition was observed on primate cell lines but not on cell lines of mouse origin. In addition, I showed that blocking MPRs or preventing the addition of M6P residues to HSV glycoproteins resulted in inefficient cell-to-cell spread of the virus. Taken together these observations suggest that MPRs are not essential for HSV infection of cells. However blocking the ability of HSV to interact with MPRs renders the virus less efficient at both entry and egress/cell-to-cell spread. In the following chapter I examined the potential role of MPRs in HSV egress.
4 Carbohydrate analysis and intracellular localization of herpes simplex virus gD1

4.0 Preface

In the following publication, I constructed the plasmid pCA3gD1t and pCA4gD1 which were rescued into adenovirus vectors by J. Rudy from Dr. F. Graham’s lab. I carried out the experiments depicted in Table 4.1 and Figures 4.2, 4.3, and 4.5. Figure 4.4 was a collaborative effort between myself and C. Wale.
Soluble and full-length HSV gD are modified with M6P residues and accumulate in endosomes or TGN

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Running Title: Intracellular targeting of HSV gD

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ABSTRACT

Herpes simplex virus (HSV) gD was shown to bind the 275 kDa and 46 kDa mannose 6-phosphate receptors (MPRs) primarily through mannose 6-phosphate residues (M6P) present on N-linked oligosaccharides of gD (C.R. Brunetti, R.L. Burke, S. Kornfeld, W. Gregory, K.S. Dingwell, F. Masiarz, and D.C. Johnson, J.Biol. Chem. 269:17067-17074, 1994). Based on the impaired ability of HSV to form plaques on cells in which the virus was prevented from interacting with MPRs, we proposed that MPRs may function during HSV egress from cells (C.R. Brunetti, R.L. Burke, B. Hoflack, T. Ludwig, K.S. Dingwell, and D.C. Johnson, J.Virol. 69:3517-3528, 1995). To further analyze the amount of M6P modified oligosaccharides on gD and to examine the intracellular distribution of gD, we expressed gD and truncated gD using adenovirus vectors. Approximately 2.8% of the oligosaccharides on full-length gD and 10.1% of those on soluble gD1t were modified with M6P residues. This fraction of gD when present in the virion envelope may affect intracellular targeting or egress of virus particles. Therefore we examined the intracellular distribution of gD expressed in cells infected with HSV or using recombinant adenovirus vectors. Immunofluorescent staining demonstrated that gD localized to a compartment which also contained the 275 kDa MPR and the transferrin receptor suggesting that this compartment was either an endosomal or trans Golgi network compartment. In addition, cell fractionation experiments provided evidence that this compartment was not lysosomal. However, the role of MPRs in this
localization was unclear since localization of gD was not dependent on M6P residues, and other HSV proteins, such as gI which lack M6P residues, were also found in this intracellular compartment. In addition, HSV virions accumulated in this compartment. Therefore endosomes or the trans Golgi network may be part of the HSV egress pathway.
Introduction:

Herpes simplex virus (HSV) glycoprotein D (gD) is essential for virus entry, cell fusion, and cell-to-cell spread. It binds to proteins or other molecules which are relatively restricted in number on the cell surface and which are required for HSV entry. UV-inactivated wild-type but not HSV virions lacking gD could block subsequent entry of infectious HSV particles into cells (Johnson and Ligas, 1988). Other experiments confirmed that this inhibition was mediated at the cell surface and not internally. Furthermore, treatment of cells with soluble forms of gD blocked the ability of HSV-1 and HSV-2 to infect cells (Johnson et al., 1990). These data suggest that gD binds to a cell surface receptor required for HSV entry.

HSV types 1 and 2 can infect a wide range of cultured cell types including fibroblasts, epithelial cells, endothelial cells, keratinocytes, and neurons (reviewed in Spear, 1993). Therefore, HSV must utilize ubiquitously expressed cell surface receptors or multiple receptors. A number of potential gD binding proteins have been characterized and may represent such multiple receptors. We reported that gD was able to interact with the 275 kDa and the 46 kDa mannose 6-phosphate receptors (MPRs) (Brunetti et al., 1994). Both a soluble form of gD and to a lesser extent full-length gD were modified with mannose 6-phosphate (M6P) residues (Brunetti et al., 1994). Blocking the ability of HSV virions to interact with MPRs using antibodies, ligands, or a soluble form of the 275 kDa MPR decreased HSV entry into monkey Vero cells.
by 60% to 80% (Brunetti et al., 1995). These data suggested that MPRs represent a cellular receptor for HSV which is utilized by the virus during entry into cells. However HSV could enter into mouse fibroblast cells which were entirely lacking in MPRs, and, ligands which bound to MPRs had no effect on virus entry or replication in these cells (Brunetti et al., 1995). Therefore MPRs may represent a cell surface receptor for HSV entry into primate but not rodent cells. There is also evidence to suggest that the ability of gD to interact with MPRs is involved in HSV egress or cell-to-cell spread. An HSV mutant which expresses a form of gD lacking N-linked oligosaccharides and thus M6P residues, was inefficient in spreading from cell-to-cell (Sodora et al., 1991). In addition, a small plaque size was produced when wild type HSV infected a cell line defective for addition of M6P residues or when MPRs were blocked with bulky ligands (Brunetti et al., 1995). Normal numbers of HSV particles were produced in both instances but it was not clear whether the small HSV plaque size was related to defects in viral egress or spread from cell-to-cell. Therefore, MPRs apparently function during HSV entry into, egress from, or cell-to-cell spread in primate cells.

Other cellular proteins that are bound by HSV gD have been described. Anti-idiotype antibodies produced using a gD specific monoclonal antibody, reacted with a 62 kDa cellular protein (Huang and Campadelli-Fiume, 1996). HSV entry into cells was inhibited by 50% to 100% when the cells were pre-incubated with the antibodies. However it is unclear whether anti-idiotype
antibodies mimic the receptor binding domain of gD and thus what role this protein plays during HSV entry into cells. Another potential gD binding protein has recently been reported. CHO cells are normally resistant to HSV entry and transfection of a human cDNA into CHO cells yielded cells that could be infected by HSV (Montgomery et al., 1996). The human cDNA was identified as a novel member of the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family designated HVEM (Montgomery et al., 1996). Recently, it was found that gD bound to soluble HVEM in vitro (G. Cohen and R. Eisenberg, personal communication), suggesting that HVEM acts as a receptor for gD. Transfection of HVEM cDNA into CHO and swine cells permitted HSV entry (Montgomery et al., 1996). This entry could be inhibited by anti-HVEM antibodies and soluble HVEM. In contrast, anti-HVEM antibodies and soluble HVEM did not significantly inhibit HSV entry into human HeLa cells (Montgomery et al., 1996) suggesting that other cellular receptors must exist for virus entry into human adherent cells, i.e. cells that are the normal hosts for HSV in vivo.

HSV egress is the process by which unenveloped capsids in the nucleus acquire a lipid envelope and reach the cell surface. HSV egress begins when unenveloped capsids bud through the inner nuclear envelope to acquire a lipid envelope. The mechanism by which enveloped HSV particles in the perinuclear space are released from cells is controversial. Experiments involving monensin, which blocked vesicles from budding from the Golgi
suggested that enveloped HSV capsids in the perinuclear space retained their envelope during transport through the Golgi and on to the cell surface (Johnson and Spear, 1982). By this model, glycoproteins are processed by Golgi enzymes while imbedded in the virion envelope during transit to the cell surface.

There is some evidence to suggest that HSV egress does not occur by a single envelopment step. Electron micrographs of cells infected with varicella zoster virus (VZV) (Jones and Grose, 1988) and pseudorabies virus (PRV) (Whealy et al., 1991) have provided evidence that HSV egress occurs by a deenvelopment/reenvelopment mechanism. This model proposes that enveloped herpesvirus particles within the perinuclear space lose their lipid envelope by fusing with the outer nuclear envelope or ER producing unenveloped HSV capsids in the cytoplasm. The capsid reacquires a lipid envelope by budding into cytoplasmic vesicles, probably Golgi membranes, containing fully processed HSV glycoproteins. The cytoplasmic vesicles containing enveloped HSV particles subsequently fuse with the plasma membrane to release infectious viral particles. There is evidence supporting this model. The lipid composition of extracellular HSV virions was different than that of nuclear membranes and more similar to Golgi membranes (Van Genderen et al., 1994; Steinhart et al., 1981) suggesting that virions found in the extracellular space have lost their nuclear-derived lipids. Further support came from experiments involving a recombinant form of HSV-1 gH engineered
to contain an ER retention sequence (Browne et al., 1996). Retention of gH in
the ER/nuclear membrane was associated with the production of non-infectious
extracellular viral particles lacking gH (Browne et al., 1996). If the mutant gH
was incorporated into the viral envelope at the nuclear membrane, the virus
must lose this envelope and reacquire a new envelope lacking gH in the Golgi,
or other cytoplasmic vesicles. The significance of these observations is unclear
since the presence of ER-retained gH in the nuclear envelope was not directly
demonstrated. At present therefore we cannot be sure whether HSV egress
occurs by a deenvelopment/reenvelopment mechanism or by a single
envelopment event occurring at the inner nuclear envelope.

With many herpesviruses, including HSV and VZV, a substantial fraction
of virus particles are found inside cells in cytoplasmic vesicles (Cook and
Stevens, 1970; Darlington and Moss, 1968; Gershon et al., 1973; Jones and
Grose, 1988; Schwartz and Roizman, 1969). Quantitative electron microscopy
was used to support the hypothesis that unenveloped VZV capsids acquired
their envelope by budding into the TGN and were subsequently transported to
endosomes/lysosomes (Gershon et al., 1994). Many of the VZV particles found
in cytoplasmic vesicles appeared to be partially degraded (Gabel et al., 1989;
Gershon et al., 1994) as might be expected in lysosomes. Electron microscopy
analysis does not distinguish between infectious and non-infectious viral
particles and so it is not clear whether infectious virions accumulate in
lysosomes. Like VZV, HSV-infected cells release only a small fraction of viral
particles that are produced by cells (Dick and Rosenthal, 1995). Most HSV particles accumulate in cytoplasmic vesicles with a variety of morphologies and of unknown derivation (Darlington and Moss, 1968; Schwartz and Roizman, 1969).

Since we have shown that at least one HSV glycoprotein, gD, is modified with M6P it was of interest to determine whether gD could be targeted to endosomes and perhaps lysosomes. Similarly, it was of interest to determine whether HSV particles were transported into the endosomal/lysosomal compartment. Using recombinant adenovirus (Ad) vectors we expressed gD, as either a membrane bound or a soluble form, in cells without other HSV polypeptides. In cells infected with these Ad vectors, both forms of gD colocalized with the 275 kDa MPR in an endosomal/TGN compartment but were not present in lysosomes. Similarly, gD in HSV infected cells also colocalized with the 275 kDa MPR. Moreover, virus nucleocapsids accumulated in the endosome/TGN compartment. However this accumulation was not dependent on M6P alone.

Materials and Methods

Cells:

Human R970 cells (ATCC) were propagated in α-minimal essential medium (Life Technologies, Inc) supplemented with 7% fetal bovine serum
(FBS). MRC-5 human fibroblasts (ATCC) were grown in Dulbecco's medium (Life Technologies, Inc) supplemented with 10% FBS. 293 cells were propagated in minimal essential medium F11 supplemented with 10% horse serum (Graham et al., 1977).

**Viruses and antibodies:**

Wild-type HSV-1 strain F was obtained from P.G. Spear (Northwestern University, Chicago). The QAA virus is an HSV-1 mutant that lacks the 3 N-linked oligosaccharide sites on gD (Sodora et al., 1989) and was obtained from G.H. Cohen and R.J. Eisenberg (University of Pennsylvania, Philadelphia). Rabbit anti-MPR serum specific for both the 275 kDa and 46 kDa mannose 6-phosphate receptor was prepared as previously described (Brunetti et al., 1995). Monoclonal antibody (mAb) DL6 (Isola et al., 1989) and rabbit antiserum NC-1 (Cohen et al., 1980) were a gift from G.H. Cohen and R.J. Eisenberg (University of Pennsylvania, Philadelphia, PA). Monoclonal antibody LP2 was obtained from A. Minson (Cambridge University). Rabbit antiserum which recognizes human TAP (Hill et al., 1995) was obtained from H. Ploegh (MIT). Monoclonal antibody 3104 which recognizes gI was a generous gift from A. Cross and N.Stow (Institute of Virology, Glasgow, United Kingdom) (Johnson et al., 1988). Mouse anti-transferrin receptor was obtained from Sigma (Mississauga, Canada).
Reagents:

[2-3H]mannose, [35S]cysteine, and [35S]methionine were purchased from DuPont NEN (Mississauga, Ontario). Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Endoglycosidase H1 (Endo H) was obtained from New England Biolabs (Burlington, Ontario). Bovine serum albumin (BSA), FITC isomer 1, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (Mississauga, Canada). γ-interferon was obtained from Gibco (Burlington, Ont).

Construction of replication defective recombinant adenovirus vectors expressing gD1 or gD1t:

Plasmids pCA3 and pCA4 contain the left end (16%) of the Ad5 genome with a deletion in the E1 region and differ in the orientation of the cloning poly linker (Hitt et al., 1995). The full-length gD-1 gene encoding amino acids 1 to 394 or a truncated version of the gD-1 gene, lacking the transmembrane domain and the cytoplasmic tail and encompassing amino acids 1 to 312 (gD1t) (from HSV-1 strain KOS) were cloned into pCA4 and pCA3, respectively. In these plasmids the gD gene was connected to the HCMV immediate early promoter and was inserted into the E1 region of Ad5. The plasmids were denoted pCA3gD1t (truncated gD gene) and pCA4gD1 (full-length gD gene).
Co-transfection of the pCA3gD1t or pCA4gD1 vectors with pBHG10 in 293 cells produced recombinant AdgD1t(E1-) and AdgD1(E1-).

**Affinity purification of anti 275 kDa MPR antibodies:**

Soluble mannose 6-phosphate receptor (MPR) was purified from FBS as previously described (Brunetti et al., 1995). Approximately 1 mg of purified soluble MPR was coupled to CNBr-activated Sepharose 4B beads (Pharmacia, Baie d'Urfé, Quebec) according to the protocol supplied by the manufacturer to produce MPR-Sepharose. Anti-MPR serum was diluted 1 to 15 with 10 mM Tris pH 7.5 and applied to the MPR-Sepharose beads. The column was washed extensively with 500 mM NaCl, 10 mM Tris pH 7.5. Antibodies bound to the MPR-Sepharose column were eluted using 100 mM glycine pH 2.5 and precipitated by adding an equal volume of ammonium sulphate and incubating at 4°C for 6 h. The antibodies were pelleted by spinning at 10,000Xg for 30 min and the pellet was resuspended in PBS and dialyzed against PBS for 24 h at 4°C.

**Immunoprecipitation:**

Extracts of radiolabelled cells were made using Nonidet P-40 (NP40)-sodium deoxycholate (DOC) buffer (1% NP40, 0.5% DOC, 50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing 2 mg/ml BSA and 1 mM PMSF. The extracts were clarified by centrifuging at 100,000 X g for 60 min at 4°C.
Antibodies were mixed with the extracts and the samples were stored on ice for 60 min. Protein A-Sepharose beads (Pharmacia, Baie d'Urfé, Quebec) were added and the samples were incubated for a further 2 h at 4°C. The protein A-Sepharose beads were washed three times with NP40/DOC buffer and resuspended in 50 mM Tris pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, bromophenol blue, and 2% β-mercaptoethanol. The samples were boiled for 5 min to elute the proteins which were then loaded onto 10% N,N'-diallyltartardiamide cross-linked polyacrylamide gels. The gels were dried and exposed to Kodak XAR film.

**Labelling of gD with [3H]mannose and analysis for M6P:**

Approximately 1.5 X 10⁷ R970 cells were infected with AdgD1t(E1-) or AdgD1(E1-) using 10 PFU/cell in Dulbecco's supplemented with 2% FBS and incubated at 37°C for 48 h. Cells were labelled for 4 h at 37°C in Dulbecco's medium containing 10% of the normal level of glucose, 1% FBS, and 30 μCi/ml of D-[2-3H]mannose. The medium was removed and the cells were chased for 3 h at 37°C in medium containing normal levels of glucose. The medium was removed and the cells were lysed in NP40/DOC buffer containing 2 mg/ml BSA and 1 mM PMSF. gD was immunoprecipitated using a mixture of mAb DL6 and LP2 and eluted from the beads by resuspending in 2% SDS, 100 mM Sodium Citrate pH 5.5 and boiling for 10 min. The eluted proteins were incubated with 5000 units of Endo H for 6 h at 37°C. The sample was diluted to 2 ml with 2 mM
Tris base and applied to a Centricon-30 membrane (Amicon, Beverly, MA) so that the liberated oligosaccharides passed through the membrane and the core glycoprotein was retained. The oligosaccharides were subjected to mild acid hydrolysis by boiling for 30 min in the presence of 0.02 N HCl. The oligosaccharides were diluted to 10 ml and applied to a 1 ml QAE-Sephadex ion exchange column (Pharmacia, Baie d’Urfé, Quebec). Oligosaccharides without M6P residues do not bind to the ion-exchange column, whereas those bearing 1 and 2 M6P residues were eluted with Tris base containing 20 mM and 70 mM NaCl, respectively (Baranski et al., 1990; Cantor and Kornfeld, 1992).

**Cell fractionation:**

Approximately 1.5 X 10^7 MRC-5 human fibroblasts grown on a 150-mm tissue culture dish were washed with PBS and scraped into 10 ml of PBS. The cells were pelleted at 1000 X g for 5 min and the cells were resuspended in 1.5 ml of homogenization buffer (HB) (0.25 M sucrose, 1 mM EDTA, pH 7.5) and stored on ice for 5 min. The cells were homogenized using a Dounce homogenizer and the nuclei and unbroken cells were pelleted at 4000Xg for 20 min. The supernatant was diluted in HB buffer and Percoll to produce a 12 ml solution with a final Percoll concentration of 18%. The gradient was centrifuged for 30 min at 20,000 rpm in a 50Ti rotor (Beckman Instruments Inc., Palo Alto., CA) at 4°C, fractionated into twelve 1 ml fractions collected from the bottom of
the tube, and the fractions analyzed for radioactivity or enzyme markers.
Lysosomes are enriched in the bottom fractions of the gradient while ER, Golgi, endosomes, and plasma membranes are enriched in the top fractions of the gradient (Green et al., 1987).

**Enzymatic assays on cell fractions:**

Lysosomal β-hexosaminidase activity was determined (as described in Green et al., 1987) for each fraction of the 18% Percoll gradient. 100 μl of a 1 ml fraction was diluted to 1 ml with 1.67 mM p-nitrophenyl N-acetyl β-glucosaminide (Sigma Chemical Co, Mississauga, Ontario), 50 mM sodium citrate pH 4.5, 0.1% Triton X-100 and incubated at 37°C for 60 min. The reaction was terminated by the addition of 200 μl of 1 M sodium carbonate pH 10 and the absorbance was read at 400 nm.

Golgi galactosyltransferase activity was determined (as described in Green et al., 1987) by diluting fractions with 50 mM Tris-HCl pH 7.6, 20 mM MnCl₂, 0.4% Triton X-100, 0.0031 nmoles [¹⁴C]UDP-galactose (DuPont NEN, Mississauga, Ontario), 17.5 μg ovalbumin (Sigma Chemical Co, Mississauga, Ontario) and incubating at 37°C for 60 min. 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA) was added to each sample followed by incubation on ice for 30 min. The samples were passed through a Whatman GF/C glass fiber membrane filters and the filters were washed 3 times with 5% (w/v) TCA and
placed under a heat light to dry. 5 ml of toluene (Fisher Scientific) was added to each sample and radioactivity was quantified by Cerenkov counting.

**Cell surface labelling:**

Approximately 1.5 X 10^7 MRC-5 fibroblasts grown on a 150 mm^2 tissue culture dish were overlaid with an 8 ml solution of PBS containing 1.2 mg lactoperoxidase (Sigma Chemical Co, Mississauga, Ontario), 1 mM CaCl_2, 1 mM MgCl_2 and 1 mCi Na^{125I}. Every 2 min for 16 min, approximately 120 µl of 0.1% H_2O_2 was added to the cells. The cells were then incubated for 8 min at room temperature, washed 3 times with PBS containing 1 mM CaCl_2, 1 mM MgCl_2, 5 mg/ml BSA, 1% FBS followed by fractionation on an 18% Percoll gradient as described above. One ml fractions were collected and analyzed by a liquid scintillation counter.

**Coupling of FITC to rabbit antibodies:**

Approximately 1 ml of rabbit anti-serum NC-1 was adjusted to pH 8.0 by the addition of 100 µl of 1 M Tris pH 8.0. The antibody solution was mixed with 1 ml of protein A-Sepharose beads and incubated overnight at 4℃. The protein A-Sepharose beads were poured into a column which was washed with 10 ml of 100 mM Tris pH 8.0 followed by 10 ml of 10 mM Tris pH 8.0, and the IgG eluted with 100 mM glycine pH 3.0. The purified anti-NC-1 IgG
(approximately 2 mg/ml) was dialyzed overnight at 4°C with 100 mM carbonate/bicarbonate buffer pH 9.0. Approximately 0.025 mg of a 1 mg/ml solution of FITC in DMSO was added to 300 µl of purified NC-1 IgG and the mixture was incubated at room temperature for 2 hours. The conjugated IgG was separated from the uncoupled FITC by using a Sephadex G50 superfine (Pharmacia, Baie d’Urfé, Quebec) gel filtration column.

**Confocal Immunofluorescence:**

Approximately 1 X 10⁶ cells grown on glass coverslips were infected with AdgD1(E1-) or AdgD1t(E1-) at an MOI of 100 for 48 h. Alternatively, cells were infected with either HSV-1(F) or QAA at an MOI of 10 for 8 or 16 h. The cells on coverslips were fixed in 4% paraformaldehyde for 10 min followed by 2 washes with PBS, and permeabilization with 0.2% Triton X-100 in PBS for 5 min followed by 2 washes with PBS. The cells were incubated for 1 h at room temperature in 1% BSA, 2% goat serum, 0.02% Triton X-100 in PBS (blocking buffer) then incubated with the primary antibodies diluted in blocking buffer for 1 h at room temperature. The samples were washed 3 times with blocking buffer and then incubated with secondary goat anti-rabbit Texas Red and goat anti-mouse FITC antibodies diluted in blocking buffer. Alternatively, the cells were prepared as described above except that they were incubated with anti-275 kDa MPR antibodies and with Texas Red conjugated goat anti-rabbit IgG. The cells were washed, incubated with blocking buffer containing 10% rabbit serum,
then stained with the FITC conjugated anti-NC1 IgG. All cells were washed with H₂O and the coverslips were air-dried mounted on glass microscope slides and viewed on a Zeiss confocal microscope.

Results:

Construction of recombinant adenovirus vectors expressing gD1 or gD1t:

HSV infection causes shutoff of host protein synthesis (Roizman et al., 1965; Summers et al., 1975; Sydiskis and Roizman, 1966), alteration of cytoskeleton, rounding of cells, and disruption of the Golgi apparatus in some cell types (Campadelli-Fiume et al., 1993). To examine the processing and targeting of gD in the absence of HSV proteins and HSV-induced cytopathology, we constructed recombinant adenovirus vectors expressing gD. Either soluble gD encoding amino acids 1 to 312 (encompassing the extracellular domain of gD but lacking the transmembrane domain and cytoplasmic tail) or membrane bound gD, encoding the entire protein, amino acids 1 to 394, were coupled to the HCMV promoter and inserted into the E1A region of adenovirus (Ad) producing a non-replicating virus (see Figure 1). E1A deleted adenoviruses can produce substantial levels of recombinant protein without expression of adenovirus proteins or marked effects on infected human cells (Hitt et al., 1995). Recombinant adenoviruses were denoted AdgD1(E1-),
expressing full-length gD, and AdgD1t(E1-), expressing a truncated soluble form of gD.

To test for expression of gD, human R970 cells were infected with either AdgD1(E1-) or AdgD1t(E1-) for 44 h. The cells were metabolically labelled with $^{35}$S]cysteine/methionine for 2 h and gD was immunoprecipitated. Figure 2A shows that AdgD1(E1-) and AdgD1t(E1-) produced membrane-bound and soluble forms of gD of the appropriate sizes. gD1t does not contain a membrane anchor and, therefore, it was likely that a fraction of the mature gD1t protein was secreted. To determine the level of intracellular versus extracellular gD1t, R970 cells were infected for 44 hours with AdgD1t(E1-), and labelled with $^{35}$S]cysteine/methionine for 30 min, followed by a 2 hour chase period. The gD1t was immunoprecipitated from either the cell culture supernatant or detergent extracts of cells. Figure 2B showed that most of the total gD1t was secreted from the cell. Quantifying the amount of gD1t present in Figure 2B using a PhosphorImager revealed that greater than 80% of the soluble gD1t was secreted from the cell though steady-state levels of soluble gD in the cells was higher (not shown). The majority of the intracellular gD1t was found in the immature form (pgD1t) and was probably retained intracellularly since it remained cell associated after a 5 h chase period (data not shown). Conversely, the extracellular gD1t migrated slower in the SDS polyacrylamide gel and was probably of the mature, fully processed form. These data
demonstrate that both AdgD1(E1-) and AdgD1t(E1-) express gD proteins of the predicted size, and soluble gD1t is largely secreted.

**M6P content of gD proteins expressed by AdgD1(E1-) and AdgD1t(E1-):**

Previously, we found that approximately 58% of the oligosaccharides associated with a soluble form of gD produced in recombinant CHO cell lines and secreted into the cell culture supernatant, were modified with M6P residues (Brunetti *et al.*, 1994). By contrast, only about 0.3% of the oligosaccharides on full-length membrane bound gD produced in HSV infected R970 cells, were modified with M6P residues (Brunetti *et al.*, 1994). It was not clear whether this dissimilarity was due to differences in the cells, the proteins or virus infection of the cells. To determine the amount of M6P associated with soluble versus full-length gD in the same cells, we analyzed the M6P content of gD produced by AdgD1(E1-) and AdgD1t(E1-) in human R970 cells. The cells were labelled with [3H]mannose for 4 h beginning 40 h after infection then the label was chased for 3 h. gD was immunoprecipitated from detergent extracts of the cells and from the medium, then treated with Endo H to release high mannose oligosaccharides. The liberated oligosaccharides were separated from the core glycoprotein using a Centricon-30 membrane and were subjected to mild acid hydrolysis to remove GlcNAc residues covalently attached to M6P residues. To separate uncharged oligosaccharides (without M6P) from charged
oligosaccharides (containing M6P) a QAE-Sephadex ion exchange column was used. In this separation, oligosaccharides with no M6P residues were eluted with low salt and oligosaccharides bearing one or two M6P residues were eluted using increasing NaCl concentrations (Baranski et al., 1990; Cantor and Kornfeld, 1992).

Approximately 8.4% of the membrane bound gD1 was modified with M6P residues, if one assumes that there are three N-linked oligosaccharides per gD protein (Table 1). The large fraction of gD1t which was secreted from cells was modified less extensively (2.4% - Table 1), whereas the smaller fraction of gD1t associated with cells was extensively modified with M6P residues (30.3% - Table 1). These results confirm our previous observations that soluble and membrane bound forms of gD can both be modified with M6P.

gD and virus particles colocalize with MPRs:

Since both full length and soluble gD were at least partially modified with M6P residues it was reasonable to believe that some of the gD in infected cells or cells made to express gD would bind to MPRs and be directed to an endosomal compartment in cells. Targeted transport to endosomes can also be affected by other M6P-independent mechanisms and gD may be localized to endosomes by these pathways. Immunofluorescence experiments were performed to determine whether intracellular gD colocalizes with the 275 kDa MPR. R970 cells were infected with either AdgD1(E1-) or AdgD1t(E1-), fixed
and permeabilized, then incubated with rabbit antibodies specific for the 275 kDa MPR and mouse monoclonal antibodies directed to gD. The primary antibodies were detected using fluorescent secondary antibodies, goat anti-mouse FITC and goat anti-rabbit Texas Red (275 kDa MPR). In AdgD1(E1-) infected cells, gD1 was found on the cell surface as well as an intracellular compartment which was also stained by anti-275 kDa MPR antibody (Figure 3a). The fraction of soluble gD1t that was associated with cells was largely localized in a perinuclear region, rather than on the cell surface as with membrane-bound gD, but again there was substantial co-localization with the 275 kDa MPR (Figure 3b).

We extended these studies to HSV-infected cells. A substantial fraction of HSV gD was found in cytoplasmic vacuoles that colocalized with the 275 kDa MPR (Figure 3c). The colocalization of gD to the MPR-positive compartment was a relatively stable event. Cells treated for 5 h with concentrations of cycloheximide sufficient to block protein synthesis retained this pattern of gD colocalization with the 275 kDa MPR (data not shown). The 275 kDa MPR is localized to both the TGN and late endosomal compartments suggesting that gD is also localizing to both of these intracellular compartments. To further define the intracellular localization of gD, HSV-infected cells were stained with anti-gD antibodies and anti-transferrin receptor antibodies. The transferrin receptor is found on the cell surface and inside cells, almost exclusively in the early endosomal compartment rather than in the TGN (Hopkins and Trowbridge,
1983; Peranen et al., 1995; Schmid et al., 1988; Yamashiro et al., 1984). There was partial colocalization of HSV gD with the transferrin receptor (Figure 3d) supporting the hypothesis that a fraction of gD in infected cells is in an endosomal compartment.

To determine whether this localization was a general property of HSV glycoproteins or the result of specific intracellular targeting or retention of gD, the subcellular distribution of gl was determined. gl was found at the cell surface as well as inside cells in membranes that contained the 275 kDa MPR (Figure 3e). These data demonstrate that gD and gl are both localized to compartments that contain the 275 kDa MPR.

Since gl (which does not contain M6P residues) and gD both colocalize to 275 kDa MPR containing compartments, it was of interest to determine whether colocalization of gD was dependent on M6P residues. Cells were infected with HSV(QAA), an HSV mutant that expresses a form of gD which lacks all 3 N-linked oligosaccharides and therefore M6P residues. QAA gD was found to colocalize with the 275 kDa MPR (Figure 3f). Therefore M6P residues and N-linked oligosaccharides on gD are not completely responsible for the intracellular localization of gD to compartments which contain the 275 kDa MPR.

The intracellular localization of gD and other HSV glycoproteins prompted us to examine whether the 275 kDa MPR containing intracellular compartment functioned during HSV egress. To determine whether HSV
capsids also localized to the 275 kDa MPR containing compartments, the intracellular distribution of VP5, the major capsid protein (Newcomb et al., 1993; Trus et al., 1992) was examined. VP5 protein is synthesized in the cytoplasm of HSV infected cells and transported into the nucleus for incorporation into HSV capsids (Thomsen et al., 1995). Therefore the presence of VP5 in cytoplasmic vesicles should reflect protein incorporated into HSV virions in the process of virus egress. Immunofluorescence was used to determine whether VP5 colocalized with the 275 kDa MPR. VP5 colocalized in the cytoplasm of HSV infected cells with the 275 kDa MPR containing compartment (Figure 4a). The anti-VP5 antibodies stained virus infected but not uninfected cells (Figure 4b). These data suggest that HSV particles, are found in a 275 kDa MPR positive compartment either during HSV egress from the cell or as a deadend pathway.

**Soluble gD1t and full-length gD1 are not transported to lysosomes:**

The immunofluorescence experiments suggested that gD and the 275 kDa MPR colocalize. Since MPRs target proteins to lysosomes, we investigated whether gD was delivered to lysosomes. Lysosomes are relatively dense subcellular organelles which can be readily separated from other organelles, e.g. ER, Golgi, plasma membrane, endosomes, using Percoll density gradient centrifugation (Green et al., 1987; Rohrer et al., 1995). AdgD1(E1-) and AdgD1t(E1-) infected cells were labelled with [35S]cysteine/methionine for 30 min then the label was chased for 2 h. The cells were disrupted using a
Dounce homogenizer and cellular membranes were applied to an 18% Percoll gradient, which was fractionated, and each fraction was analyzed for markers of specific organelles. In addition, gD was immunoprecipitated from each fraction. Figure 5 shows that lysosomes were found predominantly at the bottom of the gradient (β-hexosaminidase activity) while the Golgi (galactosyltransferase activity), plasma membrane ([125I]-cell surface labelling), and ER (TAP protein) were present at the top of the gradient in less dense fractions (Figure 5). Both gD1t and gD1 expressed by recombinant Ad vectors were not present in lysosomal fractions. In addition, gD produced in HSV-1(F) infected cells showed a similar distribution on Percoll gradients (Figure 5). These data suggest that gD does not accumulate in lysosomes.
Figure 1: Features of recombinant adenovirus vectors expressing gD1 or gD1t. Full-length (gD1) or truncated versions of gD (gD1t) were inserted into the E1A region of the Ad5 genome under the control of the human cytomegalovirus (HCMV) promoter in the right to left orientation relative to the direction of transcription of E1. AdgD1(E1-) encodes the full length gD1 encompassing amino acids 1 to 394. AdgD1t(E1-) expresses a soluble form of gD1 which lacks the transmembrane domain and cytoplasmic tail corresponding to amino acids 1 to 312.
Figure 2: Recombinant adenovirus vectors express soluble gD1t or full-length gD1. (A) Human R970 cells were infected with 10 PFU/cell of AdgD1(E1-) or AdgD1t(E1-). At 44 h post-infection, the cells were labelled for 2 h with $[^{35}\text{S}]$cysteine/methionine. Detergent extracts of the cells were made and gD was immunoprecipitated from cellular extracts using monoclonal antibody DL6 as described in Materials and Methods. (B) Human R970 cells were infected with AdgD1t(E1-) using 10 PFU/cell for 44 h, then the cells were labelled for 30 min with $[^{35}\text{S}]$cysteine/methionine. The media was removed and fresh unlabelled media was added and the cells were incubated for an additional 2 h. The gD present in the cell culture supernatant or in detergent extracts of the cells was immunoprecipitated with a mixture of DL6 and LP2 monoclonal antibodies as described under Materials and Methods. The proteins were separated using a 12% SDS-polyacrylamide gel.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein Analyzed</th>
<th>Cell Line</th>
<th>% Complex*</th>
<th>% HM*</th>
<th>% 1M6P</th>
<th>%2M6P</th>
<th>% gD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdgD1t</td>
<td>gD1t (media)</td>
<td>R970</td>
<td>98.5</td>
<td>1.5</td>
<td>0.8</td>
<td>-</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* The calculated percentages represent molar percentages based on the assumption that high mannose (HM) oligosaccharides contain an average of 5.1 mannose residues, and complex type oligosaccharides contain 3 mannose residues.

† Value obtained by taking the total % of M6P modified oligosaccharides and multiplying by 3, the number of N-linked oligosaccharides on gD.
Figure 3: HSV glycoproteins accumulate in intracellular vacuoles. R970 cells grown on glass coverslips were infected with 100 PFU/cell of AdgD1(E1-) (A) or AdgD1t(E1-) (B) for 44 hours or infected with 10 PFU/cell of HSV-1(F) (C, D, E) or HSV(QAA)(F) for 8 or 11 hours. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The following antibodies were applied to cells: (A, B, C, F) affinity purified rabbit anti-275 kDa MPR and DL6 (monoclonal anti-gD antibody); (D) rabbit anti-gD and mouse anti-transferrin receptor; (E) affinity purified rabbit anti-275 kDa MPR and 3104 (monoclonal anti-gI antibody). The secondary antibodies goat anti-rabbit texas red and goat anti-mouse FITC were applied to cells. The cells were washed and viewed using a Zeiss confocal microscope.
Figure 4: The HSV capsid protein VP5 colocalizes with the 275 kDa MPR. R970 cells growing on glass coverslips were infected with HSV-1(F) (a) or were mock (b) infected. 16 hours later, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated for 60 min with blocking buffer. Affinity purified rabbit anti-275 kDa MPR antibodies were incubated on the cells for 60 min followed by 3 washes of the cells with blocking buffer. The primary antibody was detected by incubating the cells with goat anti-rabbit texas red. The secondary antibody was removed and the cells were incubated with 10% rabbit serum for 60 min. FITC-conjugated NC-1 (rabbit anti-VP5) was incubated on cells for 60 min followed by 3 washes in blocking buffer. The cells were viewed using a Zeiss confocal microscope.
B: mock treated R970 cells
**Figure 5:** Subcellular fractionation of AdgD1t(E1-), AdgD1(E1-), or HSV-1(F) infected cells. Human MRC-5 fibroblasts were infected with AdgD1(E1-) or AdgD1t(E1-) using 20 PFU/cell for approximately 48 hours or were infected with HSV-1(F) for 8 h. The cells were either left untreated, labelled for 30 min with $^{35}$S)cysteine/methionine followed by incubation for 2 hours in unlabelled media, or labelled with $^{125}$I]Na and lactoperoxidase as described under Materials and Methods. The cells were disrupted using a Dounce homogenizer and cellular material was applied to an 18% Percoll gradient and centrifuged for 30 min. The gradients were fractionated from the bottom. Fractions from untreated cells were assayed for galactosyltransferase activity (-○-) (Golgi marker) and β-hexosaminidase activity (-■-) (lysosomal marker). Fractions from cysteine/methionine labelled cells were immunoprecipitated using a gD-specific monoclonal antibody or TAP (ER marker) protein using rabbit anti-TAP serum. Fractions from cells labelled with $^{125}$I (-▲-) (plasma membrane) were analyzed by a liquid scintillation counter.
AdgD1(E1)

AdgD1t(E1)

HSV-1(F)

TAP
Discussion:

Previous studies have shown that both soluble gD and full-length gD were modified with M6P and bound to both the 275 kDa and 46 kDa MPRs (Brunetti et al., 1994). Phosphorylation of mannose residues on high mannose N-linked oligosaccharides of glycoproteins occurs in the Golgi by the action of GlcNAc-phosphotransferase in the Golgi (reviewed in Kornfeld, 1990). This enzyme recognizes specific sequences within target proteins, primarily lysosomal acid hydrolases (reviewed in Kornfeld, 1990). Clearly, both soluble gD and full-length gD contain sequences which allow GlcNAc-phosphotransferase recognition. Over half of the oligosaccharides from soluble gD-2t secreted by recombinant CHO cells were modified with M6P residues, and soluble gD-2t was as good a substrate for GlcNAc-phosphotransferase as authentic lysosomal acid hydrolases (Brunetti et al., 1994). Since gD has 3 N-linked oligosaccharides, it is likely that virtually every soluble gD-2t molecule produced in CHO cells was modified with M6P. In contrast, analysis of the N-linked oligosaccharides derived from the membrane bound form of gD purified from R970 cells 14 h post-HSV infection revealed that only about 1% of the glycoproteins were modified with M6P (Brunetti et al., 1994). Although there appeared to be large differences in the levels of M6P modified oligosaccharides on soluble versus full-length gD, comparison of the amount of M6P on these two proteins was made difficult by the fact that proteins were produced in different cell types, under very different experimental conditions and with HSV infection
in one case and not in the other. However, the difference in M6P modification could also be related to the preferential phosphorylation of mannose residues of glycoproteins that are soluble, like lysosomal enzymes, rather than membrane-bound.

To examine the phosphorylation of full-length versus soluble gD in the same cells, the proteins were expressed using Ad vectors. Approximately 2.4% of the soluble gD1t purified from the extracellular media of AdgD1t(E1-) infected cells were modified with M6P residues. Although only a small fraction of gD1t was retained in cells, this protein was modified with M6P at a higher frequency (30.3%), more similar to that observed in CHO cells secreting gD1t. It is possible that intracellular gD1t is retained in cells by virtue of its M6P modification.

Since the bulk of the gD1t (greater than 80%) is secreted from cells, only a small proportion of the total oligosaccharides on soluble gD1t, made in R970 cells, were modified with M6P residues. This differs from the situation with soluble gD-2t produced by CHO cells where virtually all the molecules were modified (Brunetti et al., 1994). It is possible that the high levels of M6P-modified oligosaccharides on soluble gD-2t produced in CHO cells may be related to a much more active GlcNAc-phosphotransferase in these cells compared to R970 cells. Alternatively, the CHO/gD-2t expressing cells which were selected for overexpression of gD-2t may have developed a tendency to hyper-phosphorylate soluble gD-2t. It is not clear how this would increase
production of soluble gD-2t, but it is known that these cells produce gD better as the cells begin to lyse.

When full-length, membrane-bound gD was produced during an AdgD1(E1-) infection, gD1 was more highly modified with M6P residues (8.4%) than the gD1 produced in HSV infected cells (1%) (Brunetti et al., 1994). Full-length gD produced in HSV-infected cells may be inefficiently modified with M6P residues because of cytotoxic effects of HSV infection. HSV shuts down host protein synthesis (Roizman et al., 1965; Summers et al., 1975; Sydiskis and Roizman, 1966) possibly reducing levels of enzymes responsible for M6P addition. In addition, HSV causes dissolution of the Golgi in some cell types (Campadelli-Fiume et al., 1993) which may alter the function of certain Golgi enzymes including GlcNAc-phosphotransferase. It is important to note that the analysis of oligosaccharides on HSV gD was carried out using gD produced relatively late after HSV infection (14 h) (Brunetti et al., 1994). Infectious HSV virions begin to be produced by 12 h post-infection (Dingwell et al., 1994; Sodora et al., 1991), and therefore M6P-modified gD may leave the cell after virus egress reducing the pool of cell-associated gD modified with M6P-residues. It is not yet clear whether gD associated with virions is modified with higher or lower amounts of M6P.

Even though only 1-2% of the gD in the virion may be modified with M6P, there are thousands of copies of gD per virion and, thus, virus particles would contain many M6P residues. Since we do not know what the level of
M6P-modified glycoproteins are in the virion envelope, M6P may well play
important roles during HSV infection of cells. We have previously demonstrated
important effects of MPRs in HSV entry into cells and cell-to-cell spread
(Brunetti et al., 1995). VZV, a related alphaherpesvirus, possesses at least 4
glycoproteins modified with M6P residues at levels between 0.5% and 2%
(Gabel et al., 1989). It has been proposed that the M6P on these glycoproteins
is important during VZV egress from cells (Gabel et al., 1989), though this has
not been directly tested. During virus egress, VZV glycoproteins appeared to be
retained in the TGN where envelopment of unenveloped cytoplasmic VZV
capsids has been proposed to occur (Gershon et al., 1994; Zhu et al., 1996).
These enveloped capsids are not transported to the cell surface, instead, VZV
particles are transported to vacuoles which have been identified as late
endosomes or lysosomes (Gershon et al., 1994). It is possible that the M6P-
modified glycoproteins are, in part, responsible for the diversion of VZV particles
from the secretory pathway to the lysosomal pathway and this may also be the
case for HSV.

Our previous observations demonstrated an interaction between gD and
MPRs and we showed negative effects of inhibiting these interactions on virus
entry and cell-to-cell spread or virus egress. This led us to examine whether gD
and HSV were present in an endosomal/TGN compartment. gD produced
during an HSV or an AdgD1(E1-) infection was found to have a perinuclear
distribution which partially colocalized with the 275 kDa MPR. Approximately
50% to 70% of the 275 kDa MPR was found in endosomes, a small fraction (5 to 10%) was on the cell surface, while the majority of the remaining 275 kDa MPR was distributed within the TGN (Geuze et al., 1988; Klumperman et al., 1993; Griffiths et al., 1988; Dintzis et al., 1994). Therefore, the colocalization of gD with the 275 kDa MPR suggests that gD and HSV are present in the TGN and endosomal compartments. Confirming the notion that gD is present in endosomes, HSV gD was also partially colocalized with the transferrin receptor, a marker for early endosomes (Hopkins and Trowbridge, 1983; Peranen et al., 1995; Schmid et al., 1988; Yamashiro et al., 1984). However, gD was not present in lysosomes as determined by cell fractionation experiments.

The intracellular localization of gD with compartments that stain with anti-275 kDa MPR/anti-transferrin receptor antibodies did not depend upon modification with M6P residues. gD was found to colocalize with the 275 kDa MPR when cells were infected with HSV(QAA), which expresses a form of gD that lacks N-linked oligosaccharides and therefore cannot have M6P residues. In addition, gD colocalized with the 275 kDa MPR in HSV infected pseudo-Hurler cells, which cannot add M6P residues to glycoproteins (data not shown). Since QAA gD and gD expressed using Ad vectors both accumulate in an intracellular compartment with the 275 kDa MPR, this localization does not require M6P residues or other HSV proteins.

Another HSV glycoprotein, gI, was also found in the MPR-positive compartment. In addition, we found HSV nucleocapsid proteins accumulating
in either the TGN or endosomal compartments. The presence of HSV virions and proteins suggests that the TGN/endosomal compartment may be involved in HSV egress perhaps as a site of HSV reenvelopment or intracellular accumulation of HSV particles. If HSV egress occurs by a deenvelopment/reenvelopment mechanism then deenvelopment of HSV virions at the outer nuclear envelope produces unenveloped cytoplasmic capsids. Endosomes/TGN may serve as a site of capsid envelopment. If HSV egress involves maintaining its nuclear-derived envelope as it moves through the Golgi, then virions may be directed to endosomes. In most cultured cells, the majority of infectious HSV particles remain cell associated with only a small proportion of virions being released into the extracellular environment (Dick and Rosenthal, 1995) and so endosomes may be a site in which virions accumulate.

Our results have demonstrated that HSV proteins accumulate in either the TGN or endosomes but not lysosomes. This intracellular accumulation does not require M6P residues on gD but may involve other M6P-independent mechanisms. It is possible that the intracellular accumulation of HSV proteins has important implications for HSV biology. The intracellular retention of HSV proteins could have implications for the mechanism of HSV egress or intracellular retention of HSV virions.
References:


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4.2 Segue

In this chapter I described the construction of two adenovirus vectors, AdgD1(E1-) and AdgD1t(E1-). These vectors and HSV were used to investigate M6P addition and intracellular targeting of gD. I showed that approximately 8% of full-length gD produced during an AdgD1(E1-) infection was modified with M6P residues, a level significantly greater than full-length gD produced during an HSV infection. Immunofluorescence experiments were used to demonstrate that gD, gI, and VP5 had a perinuclear distribution which colocalized with a TGN/endosomal marker and this localization was not dependent on M6P residues and did not involve lysosomes. The following discussion will attempt to put these and previous observations into the context of an HSV infection.
5 DISCUSSION

5.0 Overview:

The focus of my thesis has been the identification of cellular proteins which are involved in entry of HSV and cell-to-cell spread of the virus. The entry of HSV into cells is clearly a complex process, involving numerous interactions between the virion and the cell surface (reviewed in section 1.1.2, 1.1.3, and 1.1.4). When the experiments described in this thesis were initiated, there were no candidate HSV receptors.

Of the HSV glycoproteins that have been identified, gD is the most probable candidate for one mediating binding to a cell surface receptor (discussed in section 1.1.3). The data presented in this thesis describe the identification of a cellular receptor, the 275 kDa MPR, which was bound by soluble gD-2t (Chapter 2). I showed that soluble gD-2t and gD from HSV infected cells were both modified with M6P residues which allowed them to bind to both the 275 kDa and 46 kDa MPRs (Table 2.1, Table 4.1 and Figure 2.6) although in the case of gD produced in infected cells, only a small fraction of the protein was modified with M6P. In light of these findings, I investigated the role of MPRs during HSV infection of cells. Antibodies, ligands, and soluble 275 kDa MPR reduced the capacity of HSV to enter into cells as assessed by plaque assays (Chapter 3). In addition, when HSV was prevented from interacting with MPRs, plaques of small size were produced (Chapter 3). These data suggest
that MPRs play important roles in HSV entry and egress or cell-to-cell spread \textit{in vitro}. Although the importance of MPRs in the HSV infectious cycle \textit{in vivo} remains unclear, the data will be critically discussed below.

5.1 Difficulties in Identifying HSV receptors:

There are a number of characteristics of HSV and its replication cycle which present unique problems in identifying cellular receptors. 1) HSV virions form numerous interactions with the cell surface including binding to GAGs, other cell surface receptors, as well as non-specific interactions (non-specific binding refers to binding of a ligand to all components possessing at least 100-fold lower affinity than specific interactions - Limbird, 1986). These numerous interactions make it difficult to use the whole virion as a probe for viral receptors. 2) A powerful method used to identify viral receptors involves the use of entry-defective cell lines that can be transfected with cDNAs isolated from an infectable cell. cDNAs that convert uninfectable cells to those that can be infected can be reisolated and characterized. However, HSV can infect almost any cultured cell. For those cell lines which HSV can not infect, for example CHO cells, the cells often have additional defects at other stages of HSV replication making assays which rely on viral replication difficult. 3) HSV replication results in cell lysis. Using infection as an assay for the conversion of an uninfectable cell into an infectable cell will result in lysis of the target cells,
preventing their further analysis. All of these factors have combined to make identification of receptors for HSV type 1 and 2 difficult.

To circumvent these difficulties, I attempted to identify cellular receptors required for HSV entry by using soluble gD-2t. This form of the protein was a useful reagent for this purpose because: 1) it inhibited HSV entry into cells and there was evidence that it bound to an essential cellular factor required for virus entry (Johnson et al., 1990); 2) gD-2t was antigenically identical to full-length HSV gD as assessed by a number of different gD monoclonal antibodies (R.L. Burke personal communication), suggesting that it was properly folded; 3) gD-2t bound to the cell surface in the absence of GAGs suggesting that gD was not involved in adsorption onto GAGs (Johnson et al., 1990). Therefore, it appeared that soluble forms of gD might allow identification of receptors involved in stable attachment.

In spite of these advantages, there are several problems in using soluble gD-2t. This form of the protein bound to the cell surface relatively weakly (Johnson et al., 1990), compared to other receptor-ligand interactions. For example, soluble gD-2t bound to the cell surface with an equilibrium dissociation constant of 2.3X10^-7 M (Johnson et al., 1990). Other viral proteins involved in binding cell surface receptors can bind with higher affinity, eg gp120 binds to CD4 with an equilibrium dissociation constant of 4X10^-9 M (Laskey et al., 1987) and EBV gp350 binds to CR2 with an equilibrium dissociation constant of 1.2X10^-8 M (Tanner et al., 1988). In spite of this weak binding of
soluble gD-2t to the cell surface, the HSV virion apparently possesses a number of mechanisms to strengthen the interaction between the viral particle and the cell surface. For example, there are 1000's of copies of gD in the virion envelope which should bind to multiple copies of a cellular receptor. This is known as multivalent binding and allows viruses to bind to cell surface receptors through low affinity interactions but with high avidity (Roitt, I.M., 1991). In addition, the affinity between HSV virions and the cell surface may be increased through interactions mediated by a number of different viral proteins with different cell surface ligands. For example, HSV binding to the cell surface could be strengthened by interactions between gB and gC and GAGs, between gD and several stable attachment receptors, and between other HSV glycoproteins and unidentified components. Therefore, it may be difficult to assess the specific contribution of binding of soluble gD to its ligand in the absence of these additional interactions. Another potential problem with using soluble gD is that the absence of a transmembrane domain and cytoplasmic tail creates a new carboxy-terminus for this protein. This new terminus may form interactions with cell-surface proteins which do not occur with the full-length gD protein. Finally, full-length gD can form dimers (Eisenberg et al., 1982; Gibson and Spear, 1983) which may be required for cellular receptor binding, but it is unclear whether soluble gD can also do so.
5.2 gD binds to the 275 kDa MPR:

A ligand blot assay was developed to identify interactions between soluble gD-2t and cellular proteins. This assay involved the separation of proteins on polyacrylamide gels under semi-denaturing conditions, followed by transfer of the cellular proteins to nitrocellulose membranes which were then probed with $^{125}$I-labelled soluble gD-2t. Using this protocol we detected a single cellular protein of approximately 180 kDa which was bound by gD-2t but not by gB-2t (Figure 2.2). The 180 kDa gD binding protein was found in a number of cell lines (i.e. neurons, keratinocytes, and fibroblasts), consistent with the viral tropism of HSV (Figure 2.1 and Figure 2.3). Other than the 180 kDa protein, no other cellular proteins were consistently detected using $^{125}$I-labelled soluble gD-2t or gD-1t on ligand blots. Therefore, this 180 kDa protein was an excellent candidate as a receptor for gD, and for HSV.

To attempt to purify the gD binding protein for N-terminal microsequencing, I developed a gD affinity column by tethering soluble gD-2t to protein A-Sepharose beads using a monoclonal antibody which bound to the carboxy-terminus of soluble gD-2t (gD/protein A-Sepharose). Proteins from human placental membranes were extracted with detergents and passed over the gD/protein A-Sepharose column and bound proteins were eluted by boiling the gD/protein A-Sepharose beads in buffer containing SDS and β-mercaptoethanol. The eluted proteins were separated on polyacrylamide gels
and visualized by silver staining. A single protein of approximately 240 kDa was detected in the presence but not the absence of soluble gD-2t (Figure 2.4).

The 240 kDa protein was compared to the 180 kDa gD binding protein detected using ligand blots. By passing a cellular extract over the gD/protein A-Sepharose column, the 240 kDa gD binding protein would be removed from the cell extract. When the depleted extract was tested in ligand blot assays, no gD binding proteins could be detected. Since the 240 kDa and 180 kDa gD binding proteins both bound to the gD/protein A-Sepharose column, it appeared that they were identical. The difference in electrophoretic mobility or apparent molecular weight of these proteins was related to the fact that the 240 kDa protein was reduced, whereas the 180 kDa protein was not.

Ligand blot and gD/protein A-Sepharose column assays also detected a gD binding protein in fetal bovine serum (FBS) (Figure 2.3 and Figure 2.4). This protein had a faster electrophoretic mobility than that protein isolated from human placental membranes (Figure 2.3 and Figure 2.4). The most likely explanation for this difference is that the FBS-derived protein was proteolytically cleaved, producing a soluble form. I decided to purify the gD binding protein from FBS since its soluble nature meant that I could purify it in the absence of detergents.

I purified the gD binding protein from FBS using the gD/protein A-Sepharose column followed by separation of proteins by molecular weight on a polyacrylamide gel and the proteins were transferred to a PVDF membrane. A
fragment of PVDF membrane containing the gD binding protein was subjected to N-terminal micro-sequencing, and comparison of the amino acid sequence with a protein sequence data bank produced a match with the bovine 275 kDa MPR (Figure 2.5).

**5.3 Mannose 6-phosphate receptors**

The 275 kDa MPR otherwise referred to as the insulin-like growth factor II/cation-independent mannose 6-phosphate receptor (IGF-II/C1-MPR) is a multi-functional protein that binds insulin-like growth factor II (IGF-II), as well as glycoproteins with N-linked oligosaccharides modified with mannose 6-phosphate (M6P) residues. Structurally, the 275 kDa MPR contains a 2269 amino acid extracellular domain, a 23 amino acid transmembrane domain and a 163 amino acid cytoplasmic tail (Figure 5.1). The extracellular domain consists of 15 repeated subdomains each composed of approximately 147 amino acids (reviewed in Dahms et al., 1989; Dahms, 1996; Kornfeld, 1992). The extracellular domains share between 16% to 38% amino acid identity with each other though extracellular domain 13 contains an additional 43 amino acids with a motif found in fibronectin, factor XII, and a bovine seminal fluid protein (Dahms, 1996). The function of these additional 43 amino acids is currently unknown.

A single 275 kDa MPR protein binds two M6P residues (Tong and Kornfeld, 1989). To determine the location of the M6P binding sites within the
Figure 5.1

Schematic of the 275 kDa and 46 kDa MPR. Adopted from Dahms, 1996.
extracellular domain of the 275 kDa MPR, a series of proteolytic fragments of the protein were generated. By this method, the first M6P binding site was localized to extracellular domains 1 through 3, and the second within domains 7 through 9 (Figure 5.1) (Westlund et al., 1991; Dahms et al., 1993). The IGF-II binding site does not overlap with the 2 M6P binding regions but is located within domain 11 (Dahms et al., 1994; Garmroudi and MacDonald, 1994; Schmidt et al., 1995). It is unclear whether binding of IGF-II to the 275 kDa MPR affects its ability to bind M6P residues.

By binding glycoproteins containing M6P, the 275 kDa MPR targets soluble lysosomal acid hydrolases to lysosomes (reviewed in Kornfeld, 1992; Kornfeld and Mellman, 1989). Mannose residues on glycoproteins are modified with a phosphate group in the cis Golgi (reviewed in section 5.5 below) and then recognized by the 275 kDa MPR when the M6P-modified glycoprotein reaches the trans Golgi network (TGN) or the cell surface. The 275 kDa MPR-M6P modified glycoprotein complexes are transported to late endosomes where acidic pH causes the dissociation of M6P-modified proteins from the 275 kDa MPR. The M6P-modified proteins are then transported to lysosomes, while the 275 kDa MPR recycles back to the TGN or plasma membrane (summarized in Figure 5.2).

The intracellular distribution of the 275 kDa MPR was examined by immunogold labelling, and approximately 50% to 70% of the MPR was found localized to endosomes or other cytoplasmic vesicles that were not the TGN.
Figure 5.2

Cycling of the 275 kDa MPR between TGN, endosome, and plasma membrane. (Kornfeld, 1990; Dahms et al., 1989).
The remaining 275 kDa MPR was distributed between the TGN (15% to 35% of the total 275 kDa MPR) and the cell surface (10% of the total 275 kDa MPR) (Geuze et al., 1988; Klumperman et al., 1993). Other studies have confirmed that the bulk of the 275 kDa MPR was present in late endosomes in normal rat kidney cells (Griffiths et al., 1988) and in CV1 cells (Conibear and Pearse, 1994). Additional studies on the intracellular distribution of the 275 kDa MPR were performed by detecting the protein with fluorescently labelled antibodies and examining its distribution using a confocal microscope. Again, the majority of the 275 kDa MPR within CHO cells was localized to perinuclear tubular structures identified as late endosomes (Dintzis et al., 1994). However, the intracellular distribution of the 275 kDa MPR may vary depending on the cell type. For example in a bovine fibroblast cell line, the 275 kDa MPR was almost exclusively localized to the TGN (Glickman et al., 1989).

In addition to the 275 kDa MPR, there is a second MPR, a 46 kDa cation-dependent MPR (46 kDa MPR or CD-MPR). Structurally, the 46 kDa MPR contains a 159 amino acid extracellular domain, a 25 amino acid transmembrane domain and a 67 amino acid cytoplasmic domain (Figure 5.1). The extracellular domain shares between 14% to 28% sequence identity with each of the repeating domains found within the 275 kDa MPR (Dahms, 1996). The two MPRs probably arose from a common ancestral gene which underwent gene duplication and divergence to generate two distinct MPRs.
The 46 kDa MPR is also responsible for targeting M6P-modified ligands to lysosomes. Although the 46 kDa MPR is present at the cell surface, it does not appear to bind M6P-modified ligands on the cell surface but does bind ligands in the TGN (Kornfeld, 1992; Stein et al., 1987b; Tong and Kornfeld, 1989). The 46 kDa MPR has a single M6P binding site (Distler et al., 1991; Tong and Kornfeld, 1989) but it can dimerize (Dahms and Kornfeld, 1989; Stein et al., 1987a) to bind diphosphorylated oligosaccharides (Tong and Kornfeld, 1989).

5.4 M6P-dependent binding of gD to the 275 kDa MPR:

The interaction between gD and the 275 kDa MPR was dependent on the presence of M6P in gD. Removal of the oligosaccharides on soluble gD-2t abolished most or all of the binding to the 275 kDa MPR in a ligand blot (Figure 2.8). Furthermore, the ability of soluble and membrane-bound forms of gD to interact with the 275 kDa MPR was directly proportional to the percentage of the oligosaccharides which were modified with M6P. Soluble gD-2t, which contained 180-fold more M6P than membrane bound gD (Table 2.1) bound 200-fold more of the soluble 275 kDa MPR than membrane-bound gD (Figure 2.7). Therefore, the only interaction between gD and the 275 kDa MPR which was detected required the presence of M6P residues on gD. If interactions that did not depend on M6P were also involved in gD binding to the 275 kDa MPR,
these interactions must occur with a significantly lower affinity than M6P-MPR interactions or may not be detectable by ligand blots.

It was possible that gD interacts with MPRs in a M6P-independent manner. To demonstrate such potential interactions in the absence of M6P residues, $^{125}$I labelled soluble gD produced by a recombinant baculovirus in insect cells (which lack the ability to modify oligosaccharides with M6P residues) was mixed with soluble 275 kDa MPR and applied to a gel filtration column. Soluble gD and 275 kDa MPR eluted in separate fractions suggesting that the two proteins were not interacting (results not shown). An alternative approach involved adsorption of soluble 275 kDa MPR to a plastic substrate. No soluble $^{125}$I labelled gD lacking M6P residues could bind to the adsorbed 275 kDa MPR. Although these results suggest that gD and MPRs cannot bind to each other in the absence of M6P residues on gD, it remains possible that the buffer conditions used during these assays were inappropriate to allow gD-MPR interactions that were M6P-independent. Alternatively, sequences absent in the truncated gD or 275 kDa MPR proteins may be essential for interactions between the two proteins. Taking all the data into account, it appears that M6P residues on gD represent the primary mechanism for association between gD and MPRs, at least in my in vitro experiments.
5.5 The mechanism of M6P addition:

The mechanism of M6P addition to glycoproteins is not a random event. The generation of a M6P residue on N-linked oligosaccharides occurs by a series of enzymatic reactions. High mannose N-linked oligosaccharides are added to glycoproteins by an *en bloc* transfer of the preformed high mannose oligosaccharide from a dolichol phosphate lipid carrier to Asn-X-Ser or Asn-X-Thr residues as a cotranslation event in the ER (reviewed in Stryer, 1988). Following transfer of high mannose oligosaccharides to a protein in the ER, the glycoprotein is transported to the Golgi complex where the oligosaccharide chain is modified by one of two pathways (Figure 5.3). The majority of oligosaccharides have their mannose residues trimmed back by the action of mannosidase I in the cis Golgi, which is the first step in the conversion of a high mannose-type oligosaccharide to a complex-type oligosaccharide (reviewed in Kornfeld and Mellman, 1989). However, oligosaccharides associated with soluble lysosomal acid hydrolases are not converted from a high mannose-type to a complex-type oligosaccharide, instead these high mannose oligosaccharides are modified by the enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) in the cis Golgi (reviewed in Kornfeld and Mellman, 1989). GlcNAc-phosphotransferase transfers GlcNAc-1-P to the C6 hydroxyl group of a mannose residue and represents the first enzyme in the pathway leading to the generation of a M6P residue (Figure 5.3). The action of the GlcNAc-phosphotransferase inhibits mannosidase I from
acting on the carbohydrate chain (Varki and Kornfeld, 1980), thereby preventing the conversion of an oligosaccharide bearing a M6P residue from a high mannose-type to a complex-type oligosaccharide. Following the addition of a GlcNAc-1-P group to a mannose residue, the enzyme N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (α-GlcNAcase) removes the GlcNAc residue leaving a M6P residue (Figure 5.3) (Kornfeld and Mellman, 1989).

The modification of glycoproteins with M6P residues is a very specific reaction which is highly restricted to soluble lysosomal acid hydrolases. GlcNAc-phosphotransferase is responsible for the specificity of this reaction by binding to specific target sequences which are brought together upon proper folding of the protein (Lang et al., 1984; Reitman and Kornfeld, 1981; Waheed et al., 1982). Studies using fibroblasts derived from patients with I-cell disease or pseudo-Hurler polydystrophy provided additional evidence that protein sequences form the GlcNAc-phosphotransferase recognition domain. These patients have a defective GlcNAc-phosphotransferase which fails to modify oligosaccharides with M6P (Reitman et al., 1981). GlcNAc-phosphotransferase from normal and pseudo-Hurler polydystrophy patients had similar activities towards carbohydrate molecules which were not attached to proteins (Varki et al., 1981). However, GlcNAc-phosphotransferase derived from pseudo-Hurler polydystrophy patients had a markedly reduced activity with lysosomal acid
Figure 5.3

Generation of a M6P residue on an N-linked oligosaccharide chain (* represents sites of potential M6P addition. Sialic acid, Sia; Galactose, Gal; N-acetylglucosamine, GlcNAc; Mannose, Man; Asparagine, Asn)
hydrolases compared to the normal enzyme (Lang et al., 1985; Varki et al., 1981). These data suggest that GlcNAc-phosphotransferase from patients with pseudo-Hurlers polydystrophy has a defective substrate recognition domain that poorly recognizes protein domains of lysosomal enzymes.

To determine the amino acids required for GlcNAc-phosphotransferase binding, a series of amino acid substitutions were carried out between the lysosomal acid hydrolase cathepsin D and pepsinogen. The latter proteins are related aspartyl proteases sharing an overall similarity in tertiary structure and approximately 45% amino acid identity (reviewed in Davies, 1990). Cathepsin D is modified with M6P residues and targeted to lysosomes, whereas pepsinogen is secreted from the cell and lacks M6P residues (Baranski et al., 1990). To determine regions within cathepsin D responsible for M6P addition, cathepsin D sequences were transferred to the homologous region of pepsinogen and the pepsinogen/cathepsin D hybrid protein was analyzed for the acquisition of M6P residues (Baranski et al., 1991; Cantor et al., 1992). A lysine at position 203 and a stretch of amino acids between 265 and 292 of cathepsin D were required for M6P addition to a pepsinogen/cathepsin D hybrid protein (Baranski et al., 1991). Further evidence to suggest that lysine residues are important for GlcNAc-phosphotransferase recognition comes from studies of cathepsin L. Modification of lysine residues on cathepsin L by sulfo-N-hydroxysuccinimide acetate, which did not obviously affect protein folding, inhibited the addition of phosphate groups to mannose residues (Cuozzo and
Sahagian, 1994). Computer modelling of the pepsinogen/cathepsin D hybrid protein showed that lysine 203 and residues 265-292 are folded close to each other to form a large continuous extended region on the surface of the hybrid protein. These data suggest that the GlcNAc-phosphotransferase binding site was composed of distantly spaced amino acids in the primary sequence, involving a critical lysine residue, which are brought together by folding.

5.6 Role of MPRs during HSV entry:

The following sections attempt to examine and explain the effects that reagents which block HSV-MPR interactions have on HSV entry.

5.6.1 Inhibition of HSV entry using ligands and antibodies which block MPRs:

A general approach in our lab to assess the ability of antibodies and ligands to block HSV entry into cells is the reduction in the number of HSV plaques caused by the inhibitor. An HSV plaque is formed when a single viral particle infects a cultured cell growing in a monolayer. Successive rounds of HSV induced cell lysis and infection of neighbouring cells result in the production of a plaque. Therefore, the generation of an HSV plaque is the culmination of entry, replication, egress, and cell-to-cell spread events. A reduction in the number of HSV plaques would occur if a reagent affected any of these stages of HSV replication.
The presence of soluble 275 kDa MPR, PM-PO$_4$-BSA, or anti-275 kDa/46 kDa MPR antibodies resulted in a reduction in the number of HSV plaques on Vero cells (Chapter 3). There is evidence that PM-PO$_4$-BSA affected the number of HSV plaques by blocking entry of HSV into cells but not subsequent stages of HSV replication. The addition of PM-PO$_4$-BSA, after HSV had been allowed to enter into cells, had no effect on HSV plaque numbers (Figure 3.3) suggesting that PM-PO$_4$-BSA did not reduce plaque numbers by inhibiting post-entry events.

Soluble 275 kDa MPR, anti-275 kDa/46 kDa MPR antibodies, and PM-PO$_4$-BSA were required throughout the period of HSV plaque production to achieve maximum inhibition of plaque formation (Chapter 3). The continuous presence of an inhibitor of HSV entry is not an unusual requirement when maximum inhibition of HSV entry is required. This requirement was also seen in experiments using soluble gD-2t as an inhibitor of entry, where it is well established that inhibition is through blockage of this process (Johnson et al., 1990). In the presence of soluble gD-2t, HSV could still adsorb normally to GAGs and remained bound during washes with physiological buffers (Johnson et al., 1990). When the block to HSV entry is removed, the adsorbed HSV can presumably resume the entry process and infect the cell. Therefore an effective inhibitor of HSV entry might be predicted to be required continuously to prevent entry of virus which remains adsorbed onto cell surface GAGs.
5.6.2 Quantity of inhibitors required to block HSV entry into human cells:

The partial inhibition of HSV plaque production required micromolar concentrations of PM-PO₄-BSA or soluble 275 kDa MPR (Chapter 3). This raises the question of whether the amount of inhibitor used to block HSV entry was in excess of the amount required to completely occupy both the 275 kDa and 46 kDa MPRs at the cell surface. The equation of fractional occupancy can be used to determine the fraction of cell surface receptors which are occupied at a given ligand concentration. Fractional occupancy is defined as:

\[
\frac{\text{concentration of ligand}}{\text{concentration of ligand} + k_d}
\]

Using this equation, when the concentration of ligand is equal to the equilibrium dissociation constant (k_d), the fraction of receptors which is occupied is 50%. To achieve close to 99% occupancy of a cell surface receptor requires a concentration of ligand equal to 100 times the k_d. Johnson et al., (1990) demonstrated a requirement of 4 μM of soluble gD-2t to inhibit HSV entry into cells. They also determined that soluble gD-2t bound to the cell surface with a k_d of 2.3X10⁻⁷ M (0.23 μM). Therefore, inhibition of HSV entry resulted when (fractional occupancy = 4 μM/[4 μM+0.23 μM]X100%) 95% of the gD receptors
were bound. These data suggest that a high percentage of HSV cellular receptors must be blocked in order to inhibit HSV entry into cells.

Using the equation of fractional occupancy, I determined whether the concentration of soluble 275 kDa MPR and PM-PO\textsubscript{4}-BSA was sufficient to specifically inhibit HSV entry into cells. Approximately 6 \( \mu \)M of soluble 275 kDa MPR was required to achieve 70\% inhibition of HSV plaque numbers (Figure 3.1). The \( k_d \) for the 275 kDa MPR binding to M6P residues varies depending on the number of M6P residues present on an oligosaccharide chain. We do not know how extensively gD in the virion is modified with M6P and thus, it is unclear whether oligosaccharides on the surface of the virion are modified with 0, 1, or 2 M6P residues per oligosaccharide chain. If gD on the surface on the virion contains monophosphorylated oligosaccharides which bind to the 275 kDa MPR with a \( k_d \) of 7X10\(^{-6}\) M (7 \( \mu \)M) (Tong and Komfeld, 1989; Tong \textit{et al.}, 1989; Distler \textit{et al.}, 1991) then 6 \( \mu \)M soluble 275 kDa MPR would result in 46\% of the monophosphorylated gD being bound by soluble 275 kDa MPR. However, if gD on the surface of the virion contains diphosphorylated oligosaccharides (which bind to the 275 kDa MPR with an equilibrium dissociation constant of 0.002 \( \mu \)M, Tong \textit{et al.}, 1989) then 6 \( \mu \)M soluble 275 kDa MPR is approximately 30 times the amount required to achieve close to 100\% occupancy of gD on the surface of the virion suggesting that it is probably
blocking entry through non-specific interactions. I found that most of the gD in cells is monophosphorylated (Table 2.1), and assuming that this is the case in virions, this might suggest that the amount of soluble 275 kDa MPR required to partially inhibit HSV entry is not excessive.

The $k_d$ of PM-PO$_4$-BSA (diphosphorylated oligosaccharides) binding to the 275 kDa MPR is approximately $3 \times 10^{-9}$ M (0.003 μM) (Braulke et al., 1987). To achieve partial inhibition of HSV plaque numbers it was necessary to use 2.3 μM PM-PO$_4$-BSA, which is far in excess of the amount of PM-PO$_4$-BSA required to saturate binding to the 275 kDa MPR. It is possible that high levels of PM-PO$_4$-BSA blocked HSV entry into cells by binding to additional cell surface components other than MPRs (non-specific binding). Alternatively, approximately 5-10% of MPRs are found on the cell surface (Geuze et al., 1988; Klumperman et al., 1993) and the cell surface half-life is only 5 min (Oka and Czech, 1986), so that MPRs are continuously internalized. This continuous turnover of MPRs may be responsible for the requirement for high levels of PM-PO$_4$-BSA to maintain close to 100% occupancy of cell-surface 275 kDa MPR. Finally, it is possible that PM-PO$_4$-BSA was also required to block the ability of HSV to bind to the 46 kDa MPR. Although the ability of the 46 kDa MPR to bind to M6P-modified ligands at the cell surface has not been demonstrated (Stein et al., 1987b; Tong and Kornfeld, 1989; Kornfeld, 1992), HSV may utilize the 46 kDa MPR to enter cells. The $k_d$ for the 46 kDa MPR binding to PM-PO$_4$-BSA is
approximately $2 \times 10^{-7}$ M (0.2 μM) (Tong and Kornfeld, 1989; Tong et al., 1989) indicating that 2.3 μM PM-PO$_4$-BSA would occupy approximately 92% of the cell surface 46 kDa MPRs. These data suggest that although relatively high levels of PM-PO$_4$-BSA were required to inhibit HSV entry, the levels may not be unreasonably high considering the unique characteristics of the MPRs.

Approximately 300 μg/ml of anti-275 kDa/46 kDa MPR antibodies were required to achieve a 60% reduction in the number of HSV plaques (Figure 3.2). Although this antibody concentration may appear high, there are numerous examples of viruses for which similar levels of polyclonal or monoclonal antibodies are required to achieve inhibition of viral entry (summarized in Table 5.1).

5.6.3 MPRs are not required for HSV entry into murine cells

A number of pieces of experimental evidence have suggested that although MPRs may function during HSV entry into monkey Vero cells, they do not function during HSV entry into murine cells. HSV can enter into murine cells that do not express MPRs (Figure 3.5 and Figure 3.10). HSV infection of normal or 275 kDa/46 kDa MPR deficient mouse fibroblasts resulted in similar levels of HSV early gene expression (Figure 3.5) suggesting that HSV entry was not reduced in the absence of MPRs. In addition, PM-PO$_4$-BSA had no
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effect on HSV entry into normal mouse fibroblasts, 275 kDa/46 kDa MPR
deficient mouse fibroblasts, or mouse L cells as assessed by HSV early gene
expression or plaque formation (Figure 3.5 and Figure 3.10). The inability of
PM-PO$_4$-BSA to affect both of these processes in murine cells provided
evidence that PM-PO$_4$-BSA is not inhibiting HSV plaque production on Vero
cells through some toxic effect toward the HSV virion, or some aspect of HSV
replication. Furthermore, these data suggested that the 275 kDa and 46 kDa
MPRs are not required for HSV entry into murine cells.

Normally HSV does not enter or replicate efficiently in murine cells.
Approximately 40% fewer plaques were formed on mouse fibroblasts compared
to Vero cells when similar numbers of infectious HSV particles were applied to
each cell type (Figure 3.5). In addition, HSV produces much fewer infectious
progeny on these cells. These data suggest that HSV plaque production was
less efficient in murine cells and the cells are not good hosts for the virus. To
determine whether the reduction in the number of HSV plaques was the result
of defects in entry, perhaps due to the absence of viral receptors, the kinetics of
HSV entry into mouse fibroblasts and Vero cells could be determined and
compared. HSV particles would be pre-adsorbed to cells at 4°C to allow virions
to attach to cells but not enter. The cells would then be shifted to 37°C for
different periods of time to allow virus to enter into cells. After the various
incubation periods, extracellular virions would be neutralized with an acid
wash. The number of HSV plaques produced after various incubation periods would be expressed as a percentage of the total number of HSV virions which entered into cells which were treated with a mock wash step. These data would provide a measure of the relative rate at which HSV virions can enter into mouse versus monkey cells.

Murine cells can provide a useful system to test whether anti-275 kDa/46 kDa MPR antibodies and soluble 275 kDa MPR were inhibiting HSV entry by blocking other HSV receptors. There is no evidence to suggest that MPRs function during HSV entry into murine cells. This lack of inhibition of HSV entry in these cells by soluble 275 kDa MPR and anti-275 kDa/46 kDa MPR antibodies would provide evidence that these inhibitors of HSV entry into Vero cells were mediating their effects through blocking the ability of HSV to interact with MPRs. These data would also provide further evidence that murine cells are insensitive to inhibitors that block interactions between HSV and MPRs on monkey or human cells.

5.6.4 HSV entry into human or monkey cells by M6P-independent mechanisms:

In addition to the observation that MPRs do not function as a receptor for HSV entry into murine cells, there is also evidence to suggest that M6P residues are not required for HSV entry into human or monkey cells. Sodora et al., (1991) showed that QAA-HSV (which contains a recombinant gD lacking N-
linked oligosaccharides and thus M6P residues) and wild-type HSV had similar entry kinetics into Vero cells. These data suggested that M6P residues on gD were not required for HSV entry into primate cells since the only interaction I could demonstrate between gD and MPRs depended on M6P residues (discussed in section 5.4). One caveat of this conclusion is that the Sodora et al., 1991 experiments and my experiments were carried out by first titering the stocks of wild-type and QAA HSV and then determining the entry kinetics of similar numbers of infectious QAA or wild-type HSV virions (Sodora et al., 1991). If M6P residues were required for HSV entry into cells, then viral particles isolated from QAA infected cells should be less infectious. By titering the viral stocks and placing similar numbers of infectious wild-type and QAA virions onto cells, Sodora et al., (1991) may have compensated for a reduced ability of QAA virus to enter into cells by applying a greater number of QAA particles. To determine whether QAA virions were less infectious then wild-type virions, the number of HSV particles should be quantified by EM and followed by titering the HSV stocks on Vero cells to determine the ratio of virus particles to plaque forming units (PFUs).

To determine whether M6P residues are involved in HSV entry into cells, HSV virions could be isolated from HSV infected normal or GlcNAc-phosphotransferase defective human fibroblasts. These cells should produce HSV virions which are structurally similar to those produced by normal fibroblasts except they would lack M6P residues. The number of viral particles
produced by defective fibroblasts would be quantified using EM. If HSV particles generated in defective cells produce fewer HSV plaques on Vero cells compared to a similar number of particles produced on normal fibroblasts, this would suggest that M6P residues function during HSV entry.

5.6.5 HSV utilizes multiple cell surface receptors to enter into cells:

To explain why MPRs may not be absolutely essential for HSV entry into cells, one might hypothesize that HSV utilizes multiple and redundant cellular receptors for this process. Blocking the ability of HSV to interact with MPRs might not completely inhibit HSV entry, if cells possess alternate receptors. Recently several HSV attachment receptors have been identified in addition to the 275 kDa and 46 kDa MPR. A 62 kDa cellular protein was identified using anti-idiotypic antibodies raised against an epitope on gD. Antibodies specific for the 62 kDa cellular protein partially inhibited HSV entry into cells (Huang and Campadelli-Fiume, 1996). However, the 62 kDa protein has not been purified or characterized and therefore it is unclear whether this protein represents an attachment receptor bound by HSV gD. Another HSV receptor, HVEM, a member of the TNF/NGF family has been identified (Montgomery et al., 1996). Antibodies specific for HVEM or a soluble form of HVEM inhibited HSV entry into CHO cells transfected with HVEM cDNA. However antibodies directed to HVEM did not inhibit HSV entry into human HeLa cells (Montgomery et al.,
1996). HeLa cells may possess multiple HSV cellular receptors and consistent with this, Montgomery et al., (1996) reported the identification of other HeLa cell cDNAs which mediated HSV entry into CHO cells. Therefore, antibodies against HVEM or soluble HVEM may not affect HSV entry into HeLa cells because the virus utilizes alternate cell surface receptors. If HSV utilizes HVEM and other cellular receptors to enter human cells and, ligands and antibodies had no effect on this entry, then, antibodies and ligands directed to MPRs should not affect HSV entry. This might suggest that anti-MPR antibodies and ligands which extensively inhibited HSV entry, might be operating through non-specific mechanisms. However it is also possible that HVEM is not used as a receptor by HSV for entry into HeLa cells.

MPRs may represent one method for HSV attachment to cells while HVEM represents an alternative mechanism. If this hypothesis were correct, a mixture of antibodies directed to both the 275 kDa/46 kDa MPRs and HVEM should show increased inhibition of HSV entry compared to either antibody alone. It would also be informative to determine whether HVEM antibodies could inhibit HSV entry into mouse fibroblasts, a cell type which apparently lacks an MPR-dependent pathway for HSV entry.

There is additional experimental evidence to support the hypothesis that human cells possess multiple non-GAG receptors for HSV. HSV strains rid1 and ANG (discussed in section 1.3.1) are able to efficiently enter into human cell lines (Dean et al., 1994) yet cannot enter CHO cells or CHO cells
transfected with HVEM (Montgomery et al., 1996). Therefore HSV strains rid1 and ANG can enter into human cells even though the evidence suggests that rid1 and ANG do not use HVEM as a cell surface receptor. These data provide strong evidence that HSV can utilize a number of different cell surface components, GAGs, MPRs, HVEM and other receptors for entry into cells.

5.6.6 Soluble gD binds to cell surface receptors other than MPRs:

Soluble gD-2t blocks HSV entry into cells by a M6P-independent mechanism. I found that deglycosylated soluble gD-2t inhibited HSV entry into cells in a similar fashion to fully glycosylated gD-2t (Figure 3.9). This suggests that M6P residues are not required for soluble gD-2t to inhibit HSV entry into cells. Either soluble gD binds to cell surface components other than MPRs, or can bind to MPRs in a M6P-independent fashion. However, all of my data excludes the latter (section 5.4).

The ligand blot assay does not exclude the possibility that soluble gD-2t is binding to cell surface receptors other than MPRs. Soluble gD-2t bound purified 275 kDa and 46 kDa MPRs (Figure 2.6 and Table 2.1) but not to 46 kDa MPRs present in cell extracts (unpurified) using ligand blot assays (Chapter 2). It is possible that the inability to detect 46 kDa MPR in these blots was related to the affinity of the 275 kDa and 46 kDa MPRs to diphosphorylated oligosaccharides (Munier-Lehmann et al., 1996). The majority of the M6P-modified oligosaccharides on soluble gD-2t were monophosphorylated (Table
2.1) and bound to MPRs with an equilibrium dissociation constant of 7\times 10^{-6} \text{ M} (Distler et al., 1991; Tong and Komfeld, 1989; Tong et al., 1989). However, approximately 20\% of the oligosaccharides bearing M6P residues on gD-2t were diphosphorylated (Table 2.1) and these can bind to the 275 kDa MPR with an equilibrium dissociation constant of 2\times 10^{-9} \text{ M} (Tong et al., 1989) versus 2\times 10^{-7} \text{ M} for binding to the 46 kDa MPR (Tong and Komfeld, 1989; Tong et al., 1989). Perhaps the ligand blot assay is incapable of detecting interactions between gD and MPRs with equilibrium dissociation constants greater than 10^{-9} \text{ M}. Alternatively, the inability to detect unpurified 46 kDa MPR on ligand blots may result from difficulties in obtaining sufficient quantities of the 46 kDa MPR relative to the 275 kDa MPR because the 46 kDa MPR may be present in lower quantities or, may be more difficult to extract from membranes. The inability to detect soluble gD binding to the 46 kDa MPR derived from semi-purified membrane extracts provides evidence that the ligand blot assay does not detect all potential gD cellular receptors. Therefore, in addition to the 275 kDa and 46 kDa MPRs, other cellular proteins may exist which also bind to soluble gD-2t.

Since soluble gD-2t can bind to MPRs and it mediates inhibition of HSV entry by binding to cell surface proteins in addition to MPRs, the Scatchard analysis data of soluble gD-2t binding to the cell surface (Johnson et al., 1990) must reflect this. The Scatchard analysis results published by Johnson et al., (1990) were consistent with soluble gD-2t binding to a single cellular protein. However the non-specific binding of radiolabelled gD to the cell surface varied
from 20% to 70% of the total counts (Johnson et al., 1990). Therefore, the high level of non-specific binding makes it difficult to draw definitive conclusions about whether soluble gD binds to one or more cell surface components.

There are a number of possible explanations why Scatchard analysis data suggested that soluble gD-2t bound to a single-cell surface receptor. I have noted variability in preparations of soluble gD in their binding ability to MPRs, and it is possible that the soluble gD used in the binding experiments of Johnson et al., (1990) contained low levels of M6P. An alternative hypothesis is that soluble gD-2t binds to MPRs and a second cellular receptor with a similar or identical equilibrium dissociation constant, or to a cellular protein with a low affinity, too low to be seen in binding experiments. Approximately 80% of M6P-modified oligosaccharides on soluble gD-2t were monophosphorylated (Table 2.1) which would bind to MPRs with an equilibrium dissociation constant of 7X10^-6 M (Distler et al., 1991; Tong and Kornfeld, 1989; Tong et al., 1989) which is similar to 2.3X10^-7 M found for gD-2t binding to cells (Johnson et al., 1990). These data suggest that MPRs are among the cellular components to which soluble gD-2t binds on the cell surface. To investigate the possibility that gD binds to other cell surface components in addition to MPRs, the equilibrium dissociation constant for soluble gD-2t binding to mouse fibroblasts, which either possess or lack the 275 kDa and 46 kDa MPRs (Ludwig et al., 1994), could be determined. If soluble gD-2t were binding to both MPRs and a second cellular receptor, soluble gD-2t should bind with the same equilibrium
dissociation constant to both normal and MPR-deficient fibroblasts. However, it should bind to fewer cell surface sites on MPR-deficient fibroblasts since one of its cell surface receptors is absent.

5.6.7 Different strains of HSV may have a different capacity to utilize MPRs as a receptor:

Another potential reason why MPRs may not be essential for HSV entry into cells is that variants of HSV may have been selected, during virus propagation in cultured cells, so that these laboratory strains have altered receptor binding specificity. It has been shown that HIV receptor binding specificity becomes altered (described in section 1.2.3.1). Primary infection of individuals by HIV requires the cofactor CC-C3H-5, whereas most laboratory strains of HIV utilize fusin as a cofactor (Liu et al., 1996). Similarly, MPRs may have a more dominant role in entry of primary isolates of HSV into cells. Most laboratory HSV strains are propagated on cells grown in fetal bovine serum which contains 2 to 8 mg/liter of soluble 275 kDa MPR (Valenzano et al., 1995). These quantities of soluble 275 kDa MPR in FBS could potentially inhibit primary isolates of HSV just as purified 275 kDa MPR, at much higher concentrations, inhibited entry of laboratory strains into Vero cells (Figure 3.1). Primary isolates of HSV, which initially use the 275 kDa MPR as their cellular receptor may be converted to sub-strains which utilize receptors other than the 275 kDa MPR during propagation in fetal calf serum. In support of this
hypothesis, Dick and Rosenthal (1995) compared a highly passaged HSV strain KOS with two primary isolates of the virus. There was very little processing of high mannose oligosaccharides on HSV glycoproteins gD and gC from primary isolates compared to strain KOS. Since M6P residues are found attached to only high mannose oligosaccharides and not to processed, complex oligosaccharides, it is possible that the primary isolates may also contain more M6P. Therefore, these primary isolates may also bind more tightly to MPRs.

The ability of HSV to utilize a particular cellular receptor may be influenced by strain and sero-type specific factors. For example HSV-2(333) can enter into normal CHO cells with a reasonable efficiency whereas HSV-1(KOS) entered CHO cells poorly (Montgomery et al., 1996). In addition, HSV strain ANG, a naturally occurring HSV variant, lacked the ability to utilize HVEM during entry into CHO cells (Montgomery et al., 1996) whereas, a number of other HSV-1 and HSV-2 strains can utilize HVEM for this process (Montgomery et al., 1996). Together these data suggest that variants of HSV may possess different receptor binding specificity and that certain strains of HSV may be more likely to use MPRs than others.

5.7 Role of MPRs during HSV egress or cell-to-cell spread

In addition to the possible role of MPRs in HSV entry, there are a number of pieces of experimental evidence that suggest that MPRs may also be
involved in HSV egress or cell-to-cell spread. 1) Infection of cells with 
HSV(QAA), an HSV mutant which lacked N-linked oligosaccharides on gD, 
resulted in a reduced plaque size compared to wild-type HSV (Sodora et al., 
1991). 2) Incubation of HSV infected cells with PM-PO₄-BSA resulted in a 
reduced plaque size compared to BSA treated cells (Figure 3.4). 3) HSV 
infection of GlcNAc-phosphotransferase deficient human fibroblasts, which 
were unable to modify oligosaccharides with M6P residues, resulted in a small 
plaque size compared to normal fibroblasts (Figure 3.6). Since similar levels of 
total infectious virions were produced in normal and GlcNAc-
phosphotransferase deficient fibroblasts (Figure 3.7), the small plaque size may 
be the result of either inefficient cell-to-cell spread, inefficient release of 
infectious HSV virions from the cell, or defects in virus egress to the cell surface. 
In addition, the plaques produced by wild-type HSV in GlcNAc-
phosphotransferase-defective fibroblasts were similar to those produced by 
HSV(QAA) on both GlcNAc-phosphotransferase-defective and normal 
fibroblasts (Figure 3.6) supporting the view that the small plaque size was 
related to the inability of M6P residues to be added to gD, rather than some 
other defect in virus replication in the cells.

It is unclear whether the small HSV plaque size was the result of defects 
in HSV egress from cells or an inability of HSV to spread from cell-to-cell. No 
assays have been developed which can differentiate between these two
possibilities. However it is very clear that plaque size is reduced, and thus, I will propose a function for MPRs during HSV cell-to-cell spread or egress from cells.

If MPRs function in HSV entry into cells (discussed in section 5.6), they may also function during the analogous process of cell-to-cell spread. One mechanism by which cell-to-cell spread occurs involves production of infectious virions followed by infection of neighbouring cells by entry of extracellular particles (described in section 1.1.7.1). Since there exists evidence that blocking the ability of HSV to utilize MPRs affects entry, it remains possible that a similar mechanism may affect HSV spread from cell-to-cell. However, the cell-to-cell spread of HSV in these plaque assays is unlikely to involve extracellular virions because these were neutralized by using anti-HSV antibodies.

MPRs may also function during HSV egress. The 275 kDa and 46 kDa MPRs are responsible for targeting cellular proteins to endosomes and may function during HSV egress, to similarly target HSV glycoproteins or virions. HSV gD, gI, and the capsid protein VP5 (which can be used as a marker for HSV viral particles) were detected in intracellular compartments that contained the 275 kDa MPR which is a marker for TGN and endosomal compartments (Bomsel et al., 1990; Griffiths et al., 1988; Gruenberg et al., 1989; van Weert et al., 1995). This localization could have important implications on the mechanism of HSV egress. The majority of infectious HSV particles remain cell associated with only a small proportion of virions being released into the extracellular environment (Dick and Rosenthal, 1995). Therefore, infectious
HSV virions apparently accumulate in cell-derived vesicles in the cytoplasm (Darlington and Moss, 1968; Schwartz and Roizman, 1969). It is possible that enveloped HSV particles reaching the TGN, can become sorted into vesicles which reach the cell surface, as well as being retained within the TGN and endosomal compartment where viral particles are stored.

It is possible that HSV egress occurs by a deenvelopment/reenvelopment model (discussed in section 1.1.7.2). This model suggests that enveloped viral particles residing in the perinuclear space can undergo deenvelopment at the outer nuclear membrane and are released as naked capsids into the cytoplasm. Reacquisition of a viral envelope is thought to occur at some unidentified cytoplasmic vesicle. One of the criticisms of deenvelopment/reenvelopment models is that it would be less efficient than models in which the virion retains its envelope acquired at the nuclear envelope. Unenveloped HSV capsids in the cytoplasm must be transported to the proper cytoplasmic vesicles containing viral glycoproteins for envelopment to occur. The localization of fully processed HSV proteins to endosomes or TGN, which may be adjacent to the nuclear membrane, might promote this process.

If HSV egress occurs by reenvelopment of HSV capsids at a TGN/endosomal compartment, viral proteins which regulate HSV egress may in some way ensure that fusion of enveloped HSV particles within the perinuclear space occurs at regions of the outer nuclear membrane which are adjacent to
endosomes/TGN. Perhaps HSV glycoprotein gK is involved in this function. The absence of gK results in accumulation of unenveloped capsids in the cytoplasm of HSV infected cells (Hutchinson et al., 1995). It is possible that gK prevents fusion of enveloped HSV particles in the perinuclear space with regions of the nuclear envelope which are not adjacent to endosomes/TGN. In the absence of gK, deenvelopment would occur anywhere on the outer nuclear envelope resulting in unenveloped capsids in the cytoplasm at regions which are distant from membranes competent for reenvelopment. Although this hypothesis is speculative, it is consistent with what we know.

The intracellular localization of HSV proteins was not dependent on M6P residues. A mutant form of gD which could not be modified with M6P residues (due to the absence of N-linked oligosaccharides) colocalized with the 275 kDa MPR (Figure 4.3). In addition, the colocalization of gD and the 275 kDa MPR occurred in fibroblasts with a defect in GlcNAc-phosphotransferase (Chapter 4). These data suggest that M6P residues were not the only reason that gD colocalized with the 275 kDa MPR. Therefore, no evidence exists to suggest that the intracellular accumulation of HSV proteins was dependant on the 275 kDa MPR.

It seems likely that egress of at least some fraction of HSV particles involves the TGN/endosomal compartment since I observed HSV capsid proteins in TGN/endosomes (Figure 4.4). If this is the case, reagents which would disrupt the function of MPRs or alter endosomes may indirectly affect
HSV egress from cells. In GlcNAc-phosphotransferase-defective cells, lysosomal acid hydrolases failed to be targeted to lysosomes (Hasilik et al., 1981; Reitman et al., 1981). Therefore intracellular material that is destined for degradation in the lysosomes remains undegraded, resulting in accumulation of proteins and polysaccharides within the endocytic system (Ludwig et al., 1993; reviewed in Neufeld, 1991). It is possible that the small plaque size produced in GlcNAc-phosphotransferase-defective cells results from the accumulation of cellular debris in the endocytic pathway and disruption of HSV egress, rather than the absence of M6P residues. Although HSV proteins accumulate in an intracellular compartment which also contains MPRs it is unclear whether this colocalization is somehow related to the small plaque phenotype observed in GlcNAc-phosphotransferase-defective cells.

5.8 Does gD possess M6P-independent endosomal targeting sequences?

gD and other HSV proteins may have sequences that function in a M6P-independent manner to deliver the protein to endosomes. gD and gI were both found to accumulate in endosomes/TGN and this did not require M6P residues (Chapter 4). This suggests that gD and gI contain sequences which cause their intracellular accumulation in endosomes/TGN. Alternatively, these glycoproteins could accumulate in the TGN/endosomes because other viral proteins accumulate there.
One mechanism for endosomal/lysosomal targeting of integral membrane proteins requires the presence of a tyrosine-X-X-hydrophobic amino acid sequence within the cytoplasmic tail. This motif is found in the 275 kDa MPR, 46 kDa MPR, transferrin receptor, and the lysosomal proteins Igp-A (LAMP-1) and Igp-B (LAMP-2) (Guarnieri et al., 1993; Honing and Hunziker, 1995; Kornfeld, 1992). In addition, the gE homolog of VZV has a 4 amino acid sequence (including a tyrosine residue) within it's cytoplasmic domain which is responsible for intracellular retention of the protein within the TGN (Zhu et al., 1996). Other lysosomal targeting mechanisms also exist. The lysosomal integral membrane protein LIMP-II has a tyrosine-lacking cytoplasmic tail which is responsible for lysosomal targeting (Vega et al., 1991). Even though there appears to be no recognizable targeting motif within the cytoplasmic tail of gD, it remains possible that gD interacts with endosomal/lysosomal targeting machinery.

There is evidence for M6P-independent mechanisms by which lysosomal acid hydrolases are transported to lysosomes. For example, a lymphoblastoid cell line with a defect in GlcNAc-phosphotransferase preventing addition of M6P can properly transport approximately 45% of cathepsin D to lysosomes (Glickman and Kornfeld, 1993). Amino acids 188 to 265 including a lysine at position 203 of cathepsin D conferred lysosomal targeting by a M6P-independent mechanism (Glickman and Kornfeld, 1993). The M6P-independent targeting signal has some similar properties to the GlcNAc-
phosphotransferase recognition domain (section 5.5). Since soluble gD-2t was as good a substrate for GlcNAc-phosphotransferase as authentic lysosomal acid hydrolases (Chapter 2), these data suggest that the GlcNAc-phosphotransferase binding site is present in both full-length and soluble gD. Therefore, gD could also be transferred to endosomes by M6P-independent mechanisms. However, such mechanisms have not been identified. It is possible that M6P-independent lysosomal enzyme binding is mediated by a cellular protein with a similar intracellular distribution as MPRs (ie plasma membrane, TGN, and endosomes) which could act as a cellular receptor for gD or be involved in intracellular accumulation of gD.

To determine whether HSV entry required M6P-independent lysosomal targeting receptors, cathepsin D could be used to block HSV entry into cells. Cathepsin D is modified with M6P residues, which might block HSV binding to MPRs, and also can bind to the M6P-independent lysosomal targeting pathway. Therefore, if HSV entry into cells involves MPRs and M6P-independent lysosomal targeting receptors, then cathepsin D might be able to inhibit HSV entry into cells.

5.9 Conclusions:

In this thesis I have identified potential receptors acting to mediate entry of HSV into cells, the 275 kDa and 46 kDa MPRs. A number of important conclusions can be drawn from this work. 1) I have identified two proteins, the
275 kDa and 46 kDa MPRs which are bound by gD, an essential component of the HSV entry pathway. 2) HSV gD is modified with M6P residues and binds to MPRs by virtue of these M6P residues. 3) HSV does not require MPRs to enter into mouse cells but is dependent on MPRs to enter primate cell lines. 4) HSV egress or the process of cell-to-cell spread is reduced or inhibited in cells unable to modify glycoproteins with M6P residues or when gD cannot be modified with M6P. 5) HSV glycoproteins and virus particles accumulate within endosomes and the TGN. Future research may focus on determining whether endosomes are important for some aspect of the production of infectious HSV particles and whether MPRs or other targeting mechanisms cause virions to be localized there for some important biological property.
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