CHARACTERIZATION OF SOLUBLE HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN D MEDIATED INFECTION

by

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The predominant mechanism of herpes simplex virus type 1 (HSV-1) entry into permissive cells involves initial virus attachment to the cells by the interaction of envelope glycoproteins gC and gB with cell surface glycosaminoglycans (GAGs), binding of envelope glycoprotein D to one of several dissimilar co-receptors, and fusion of the virus envelope with the cell membrane requiring the combined essential functions of glycoproteins gD, gB and gH/gL. The binding of gD to its cognate receptor appears to result in emission of an activating signal to the fusion apparatus which minimally consists of the other essential glycoproteins. To gain a better understanding of gD's involvement in the fusion-activating process, we took the approach of separating gD from the virus envelope to determine whether a soluble form of gD (sgD) could mediate entry of gD-deficient virus. The results showed that sgD enabled entry of gD-deficient HSV-1 into CHO-K1 cells bearing the gD receptors HVEM or nectin-1. Using mutant forms of sgD that selectively bind to one or the other receptor, we demonstrated that entry by this mechanism is receptor specific.

Investigation of the mechanism of sgD-mediated entry demonstrated that the presence of virus at the cell surface was required at the time of sgD-receptor binding, which could be explained in part by our observation that sgD rapidly dissociated from the receptor under our experimental conditions. In addition, entry was not eliminated instantaneously when receptor-bound sgD was exposed to 37ºC, suggesting that the active conformation of receptor-bound sgD

is not highly unstable. sgD was not stabilized at the cell surface or internalized in the presence of gD-deficient virus. Using lysosomotropic agents as well as protease protection assays, we obtained no reproducible evidence that sgD-mediated entry takes place by endocytosis.

Surprisingly, virus attachment to cell-surface GAGs was not required for sgD-mediated entry. Furthermore, gD-deficient virus attached to GAG-deficient cells in the absence of sgD, revealing a previously unknown binding interaction between the HSV virion and the cell. This interaction was shown to be of a less stable nature than the virus-GAG interaction, and may play a role in normal virus entry. Our results provide new tools and directions to unravel the still incompletely understood events set in motion by gD binding to its receptor.

TABLE OF CONTENTS

1. INTRODUCTION 1
1.1. HERPES SIMPLEX VIRUS 1
1.1.1. Overview 1
1.1.2. HSV disease 2
1.1.3. Virion composition 3
1.1.4. HSV life cycle 5
1.1.5. The fate of the infected cell 9
1.2. HSV-1 ENTRY 10
1.2.1. Overview 10
1.2.2. Fusion mechanisms employed by other enveloped viruses 12
1.2.3. HSV entry pathway 14
1.2.4. HSV attachment 16
1.2.5. HSV fusion 17
1.2.6. HSV binding and entry receptors 21
1.2.6.1. Heparan sulphate 21
1.2.6.2. HSV entry receptors 22
1.2.7. Essential virus components for entry 24
1.2.7.1. Glycoprotein D 24
1.2.7.2. Glycoprotein B 28
1.2.7.3. Glycoprotein H 29
2. SPECIFIC AIMS 31
3. MATERIALS AND METHODS 36
3.1. Cells and Viruses 36
3.2. Plasmids 37
3.3. Purification of sgD Proteins 39
3.4. Transient gD-Complementation Assay 39
3.5. Detection of sgD Bound to CHO-HVEM Cells by Immuno-fluorescence 40
3.6. Flow Cytometry 40
3.7. Localization of sgD After Binding at 4ºC and Shifting to 37ºC 41
3.8. Detecting Bound Virus by Western Blot Analysis 42
3.9. Detection of KΔUs3-8Z Binding to pgsA745-nectin-1 Cells by Indirect Entry Assay 43
3.10. Real-Time Quantitative PCR 43
3.11. Calculation of the Relative Efficiency of sgD-Mediated Entry 44
3.12. Treatment with Lysosomotropic Agents 45
3.13. Trypsin Protection Assay 45
3.14. Proteinase K Protection Assay 46
3.15. Rate of Entry of gD^- KΔUs3-8Z in the Absence of sgD 47
3.16. Rate of Entry of gD^- KΔUs3-8Z in the Presence of sgD 47

4. CHARACTERIZATION OF SOLUBLE GLYCOPROTEIN D MEDIATED ENTRY OF gD-DEFICIENT HSV 48
4.1. Soluble gD Mediates Entry of a gD-Deficient Virus into HSV Receptor-Expressing Cells 48
4.2. Determining the Efficiency of sgD-Mediated Entry 53
4.3. sgD-Mediated Entry of gD-Deficient HSV Requires Specific Interaction with a gD Receptor 54
5. Mechanistic Aspects of sgD-Mediated Entry 56
6. The Role of GAGs in sgD-Mediated and Wild-Type Virus Entry 65
6.1. sgD- Mediated Entry into GAG-Deficient Cells 65
6.2. Binding of gD-Deficient Virus to GAG-Deficient Cells 67
6.3. Characterization of the Novel Binding Interaction 69
7. ENDOCYTOSIS 74
7.1. Introduction 74
7.2. Results 74
7.2.1. Inhibition with lysosomotropic agents 74
7.2.2. Detection of endocytosed HSV by proteinase protection assay 79
7.2.3. Rate of entry 80
8. DISCUSSION 84
8.1. Mechanistic Aspects of sgD-Mediated Entry 86
8.2. The Role of GAGs in sgD-Mediated and Wild-Type Virus Entry 88
8.3. Endocytosis 92
8.4. SUMMARY 96
BIBLIOGRAPHY 97

LIST OF FIGURES

Figure 1. Schematic representation of the HSV-1 genome. 3
Figure 2. EM micrograph depicting HSV-1 virion structure. 5
Figure 3. Virus replication cycle. 6
Figure 4. Fusion of envelope viruses with a Type I fusion protein. 14
Figure 5. Model for HSV entry. 18
Figure 6. Crystal structure of gD bound to HVEM. 25
Figure 7. Crystal structure of gD in complex with HVEM depicting the functional regions of gD. 26
Figure 8. Construction of KΔUs3-8Z virus. 37
Figure 9. Construction of soluble gD_{287}. 49
Figure 10. Western blot analysis of purified soluble gD proteins. 50
Figure 11. sgD (wt) mediates entry of gD-deficient virions into receptor-bearing cells. 51
Figure 12. sgD and virus dose dependence of sgD-mediated infection. 52
Figure 13. Infection of CHO-HVEM and CHO-nectin-1 cells by gD-complemented KΔUs3-8Z. 53
Figure 14. sgD-mediated entry of gD-deficient HSV requires specific interaction of sgD with a gD receptor. 55
Figure 15. Mechanistic requirements of sgD-mediated entry. 57
Figure 16. Pre-adsorption of gD^- KΔUs3-8Z to CHO-HVEM cells pre-incubated with sgD improves infection efficiency. 59
Figure 17. Time required for sgD inactivation. 60
Figure 18. Detection of cell-surface-associated sgD after incubation at 37ºC. 61
Figure 19. sgD localization. 63
Figure 20. Detection by gC antibodies of gD^- virus bound to CHO-HVEM cells. 64
Figure 21. Characterization of pgsA745-nectin-1 cells. 66
Figure 22. sgD-mediated infection of GAG-deficient pgsA745-nectin-1 cells. 67
Figure 23. Binding of gD-deficient KΔUs3-8Z virus to GAG-deficient pgsA745-nectin-1 cells. 69
Figure 24. Comparison of KHZ.1 and gD^- KΔUs3-8Z binding to GAG-deficient and GAG-expressing cells. 71
Figure 25. Role of novel binding interaction in entry of gD-deficient KΔUs3-8Z. 72
Figure 26. Stability of the novel binding interaction. 73
Figure 27. Effect of lysosomotropic agents on VSV entry into CHO-HVEM and J1.1-nectin-1 cells. 78
Figure 28. Model illustrating possible higher binding effeciency of gD- virus through novel binding interaction. 90

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1. INTRODUCTION

1.1. HERPES SIMPLEX VIRUS

1.1.1. Overview

Herpes simplex virus type 1 (HSV-1) is a member of the the *Herpesviridae* family. The herpesviruses are extremely common and over 100 have been identified in a wide variety of animal species, from chickens to turtles to fish.

The *Herpesviridae* family is divided into the alpha, beta and gamma subclasses based on biological characteristics and genomic analysis. Nine human herpesviruses have been isolated thus far. Herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella zoster virus (VZV) belong to the alphaherpesvirus subfamily. Alphaherpesviruses are characterized by having a broad host range, short replication cycle, rapid spread in cell culture and the ability to establish latency in sensory neurons (154). Four human herpesviruses belonging to the Betaherpesvirus subfamily are cytomegalovirus (CMV), human herpesvirus 6A (HHV6A), human herpesvirus 6B (HHV6B) and human herpesvirus 7 (HHV7). Members of this subfamily have a host range that is confined to a specific species, a longer replication cycle, spread slowly in cell culture, and establish latency in secretory glands and lymphoreticular cells. Finally, Epstein Barr virus (EBV) and human herpesvirus 8 (HHV8 or KSHV) are the two

human Gammaherpesviruses, and are characterized by having a host range that is limited to the family level and establishing latency in the lymphoid system.

Disease caused by human herpesviruses include chickenpox and shingles caused by VZV, infectious mononucleosis caused by EBV and HCMV, Kaposi's sarcoma caused by KSHV, childhood roseola caused by HHV6A, genital herpes caused by HSV-2, and cold-sores caused by HSV-1.

1.1.2. HSV disease

HSV is an important human pathogen, and is the most extensively studied of the herpesviruses. HSV is contacted when mucosal epithelia or abraded skin is exposed to secretions containing virus from an infected individual. During the lytic cycle, the virus actively replicates in the host epithelial cells until the cells are destroyed, eliciting an immune response, which contributes to the formation of the commonly known cold sores or blisters. The virus then enters the nerve endings of the peripheral sensory nerves that innervate the primary site of infection, and travels by retrograde transport to the cell bodies of the sensory ganglia, where it enters a latent state, in which the viral genome is maintained in episomal form, and all viral gene expression is silenced [reviewed in (85)]. This allows the virus to evade immune surveillance and persist for the entire lifetime of the host. HSV can be reactivated from its quiescent state by various stimuli, such as stress, UV light, or tissue damage. Upon reactivation, the virus travels back to the site of primary infection and may cause recurrent lesions on mucosal epithelium, skin, and the cornea. Keratitis caused by recurrent corneal HSV infections is the leading cause of blindness in the United States.

Occasionally, complications such as encephalitis and disseminated infection in newborns and immunocompromised individuals can occur [reviewed in (59)].

1.1.3. Virion composition

A herpesvirus virion consists of a DNA core, an icosahedral capsid and tegument, surrounded by an envelope made up of a lipid bilayer containing various viral proteins and glycoproteins. The HSV-1 double-stranded linear DNA genome consists of 152 kbp and encodes at least 84 gene products [reviewed in (153)]. It includes unique long (U_L) and unique short (U_S) sequences that are flanked by inverted repeats on both ends (R_L and R_S). The repeats associated with the U_L region are designated ab and b'a' and the U_S region repeats as a'c' and ca (Figure 1) (66, 165).

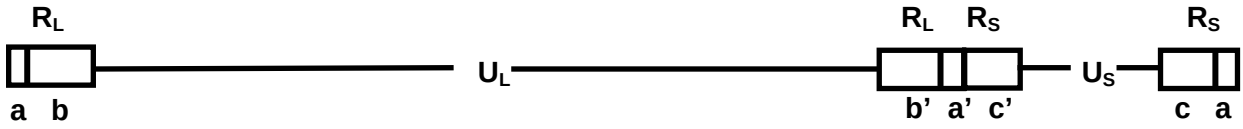


Figure 1. Schematic representation of the HSV-1 genome.
The HSV genome consists of a long (L) and short (S) component. Each of these components includes a unique sequence (U_L and U_S) flanked by inverted repeats (R_L and R_S). The repeat sequences of the long component are designated ab and b'a', and the repeat sequences of the short component are a'c' and ca (66, 165).

Several genes that lie in the repeat regions of the HSV genome exist in two copies (ICP0, ICP4 and $\gamma_1$34.5). Because of the presence of the inverted repeats, the HSV genome can readily undergo homologous recombination, causing the L and S portions to be inverted in respect to each other. Each of the four resulting isomers is found in equimolar amounts in infected cells.

The viral DNA is tighly packed in a disordered form (49, 200) and enclosed in an icosahedral capsid composed of 162 capsomers (150 hexons and 12 pentons) (77). The pentons are found at the faces and edges of the capsid, and the hexons are located at the vertices. Each hexon is comprised of 6 molecules of VP5 (virion protein), the major capsid protein, and six molecules of VP26. Eleven of the pentons are composed of a U_L19 pentamer. The twelfth penton is a fascinating structure called a portal which comprises twelve molecules of U_L6 (124). Viral DNA is packaged inside the capsid by being threaded through the portal. Three types of capsids can be isolated from cells infected with HSV, and are designated A for empty capsid, B for capsid filled with the VP22a scaffolding protein, and C for a mature, DNA-containing capsid (77).

Surrounding the capsid is an amorphous material called the tegument that includes several important viral proteins, such as VP16, which stimulates the initiation of viral gene expression [reviewed in (153)]. The virion is then enclosed by an envelope composed of a lipid bilayer, in which the HSV glycoproteins are embedded (Figure 2).

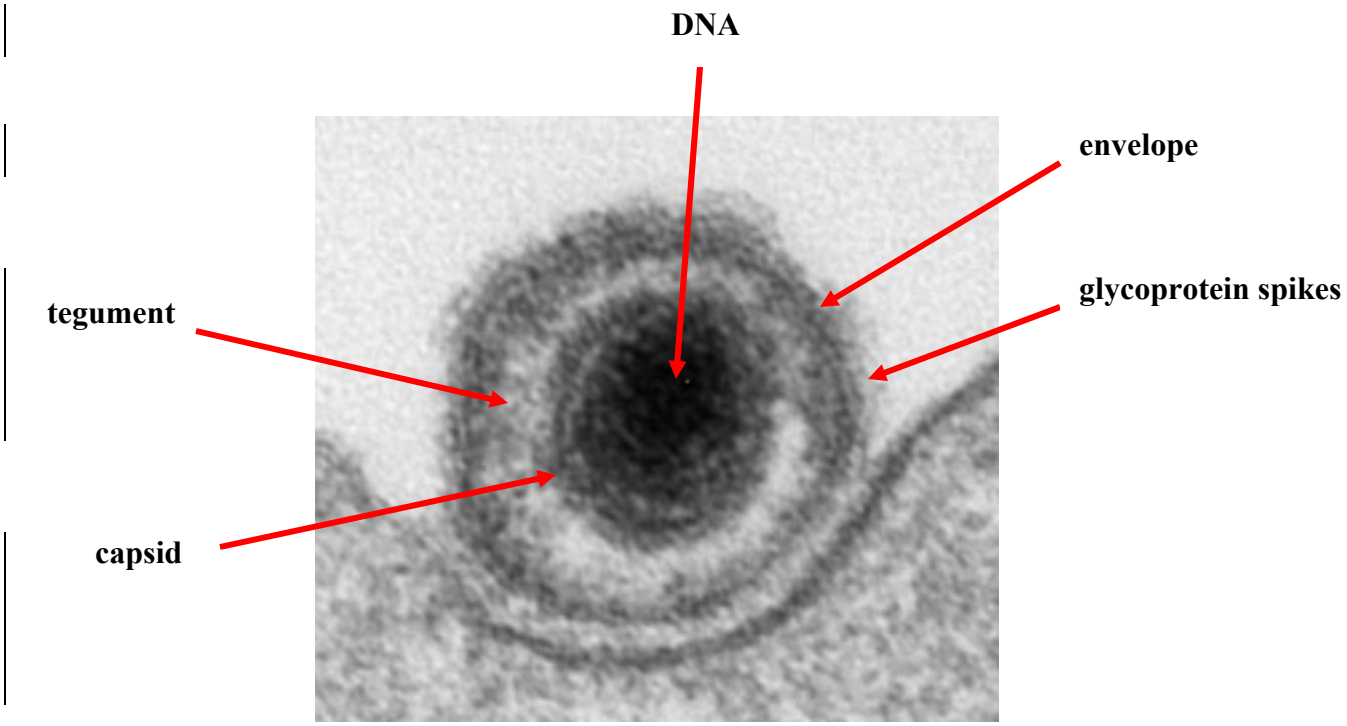


Figure 2. EM micrograph depicting HSV-1 virion structure.

The HSV virion is comprised of a double-stranded linear 152 kbp genome enclosed in an icosahedral capsid. The capsid is surrounded by an amorphous tegument, and the entire structure is enclosed in an envelope, containing the viral glycoproteins. Modified from Milne et al., J. Virology, June 2005 (119).

1.1.4. HSV life cycle

Virus enters the host cell by binding and fusion of the viral envelope with the cell surface membrane, and release of the viral capsid into the cytoplasm of the cell. A detailed description of the entry process will follow in section 1.2.

Upon entry into the cytoplasm of the cell, the capsid with the intact tegument is transported via microtubules to the nuclear membrane (103, 105, 133, 171). During transport, some of the tegument proteins dissociate from the capsid and begin to interact with the proteins in the cell and modify the cellular environment on the behalf of the virus. After reaching the

outer nuclear membrane, the capsid docks at the nuclear pore, and viral DNA is injected into the nucleus (Figure 3) [reviewed in (116)].

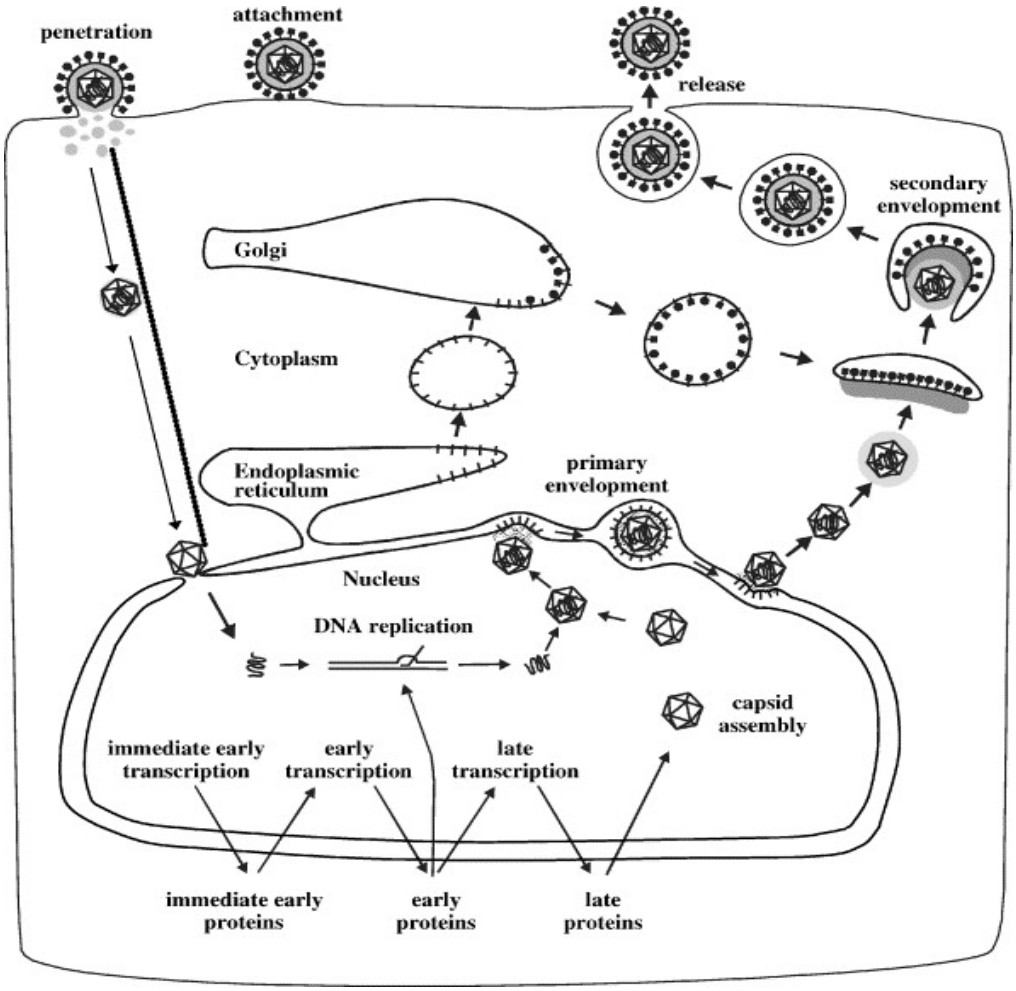


Figure 3. Virus replication cycle.

HSV entry into a host cell is initiated by binding of the virus to receptors at the cell surface, followed by fusion of the viral envelope with the cell membrane, and release of the viral capsid into the cytoplasm of the cell. The naked capsid is then transported via microtubules to the nuclear pore, where the viral DNA is injected into the nucleus. Replication of viral DNA and capsid assembly occurs in the nucleus, after which the capsid buds into the inner nuclear membrane, acquiring its first envelope, fuses with the outer nuclear membrane, and is released into the cytoplasm. The naked capsid then buds into trans-Golgi network, where it acquires its complement of glycoproteins, and the enveloped virion is transported to the cell membrane and released to the outside of the cell. Taken from Mettenleiter, T.C., Virus Research, 2004 (116).

HSV-1 replication occurs through a highly regulated cascade of gene expression [reviewed in (153)], and is broken down into three phases: α or immediate early (IE), β or early (E), and γ or late (L). All viral genes are transcribed by the cellular RNA polymerase II (1, 32). The first viral genes to be transcribed are the α or immediate early (IE) genes. α gene promoters contain many binding sites for cellular transcription factors. VP16, which is a viral transactivator that is brought into the cell in the viral tegument, plays a role in the expression of α genes (4, 14, 142). α gene expression peaks between two to four house post infection (hpi), and includes six viral proteins (ICP0, ICP4, ICP22, ICP27, ICP47 and Us1.5), most of which serve as transcription factors for the next stages of viral gene expression [reviewed in (153)]. ICP4 is a transcriptional regulator that is required for the expression of all viral proteins after the α phase (22, 37, 89, 143, 187). ICP0 is a nonspecific transactivator, and is not required for infection, but increases the production of virus from infected cells by 10- to 100-fold (160, 177).

β gene expression is induced by the production of α gene products, and peaks 4-8 hpi [reviewed in (153)]. β gene products include proteins that are involved in viral DNA replication and the production of nucleotides so that viral DNA synthesis can proceed in non-dividing cells. Several β proteins are viral DNA polymerase (U_L30), ssDNA binding protein (U_L29), ribonucleotide reductase (U_L39 and U_L40), which reduces ribonucleotides to deoxyribonucleotides, thymidine kinase (U_L23), which phosphorylates nucleosides to provide nucleoside triphospate precursors for DNA synthesis.

The final stage of viral gene expression is broken down into two phases: γ_1 and γ_2. γ_1 genes are know as the early-late genes [reviewed in (153)]. Their expression peaks relatively early in infection (8-12 hpi), and is stimulated several-fold by the synthesis of viral DNA. γ_2

gene expression requires viral DNA synthesis and peaks later on in infection (12-16 hpi). Late genes include HSV glycoproteins and capsid proteins.

Upon arrival in the nucleus, the HSV genome localizes in punctate structures called prereplicative sites near cellular ND10 structures (112). As synthesis of viral DNA progresses, the newly made HSV genomes are concentrated in globular complexes called replication compartments (80, 183).

Seven viral proteins are required for the replication of the HSV DNA (18, 194): viral DNA polymerase (U_L30) (144), ssDNA-binding protein (U_L30 or ICP8) (28), processivity factor (U_L42), origin-binding protein (U_L9) (38), and the helicase-primase complex comprising U_L8, U_L8 and U_L52 (34, 153). Three origins of replication are present in the HSV-1 genome, although only one is required for successful replication of the virus in cell culture (44, 100, 120, 184, 189). oriS lies in the repeat region of the S portion of the viral genome, and therefore exists in two copies. oriL lies in the long region of the viral DNA.

Previously it was believed that the incoming genome circularizes upon entry into the nucleus, and that this form serves as the template for HSV DNA replication (54, 141, 151). Recently, Jackson and DeLuca presented evidence that the template for HSV DNA replication during the lytic cycle is actually linear and that ICP0 controls the genome configuration upon infection (81). It was also previously believed that viral DNA is synthesized by a rolling-circle mechanism, resulting in “head-to-tail” concatemers (82). In their report, Jackson and DeLuca, suggest that instead viral DNA replication may proceed from the three origins of replication resulting in complex DNA structures which are then modified by viral proteins and homologous recombination.

Viral DNA is packaged in pre-assembled capsids in the nucleus [reviewed in (77)]. Capsid proteins are translocated to the nucleus after being synthesized in the cytoplasm, where they are assembled with the assistance of the scaffolding proteins VP22a. Concatemeric viral DNA is cleaved into single unit genomes and threaded through the portal of the capsid.

The mature capsid then embarks on its journey to reach the outside of the cell, where it will be released, and become able to infect another cell. It begins by budding into the inner nuclear membrane, where it acquires its primary envelope [reviewed in (116)]. It then fuses with the outer nuclear membrane, and is released into the cytoplasm of the cell. Next, the naked capsid buds into the trans-Golgi network, where it obtains its secondary envelope, along with most of the tegument proteins and glycoproteins. Interactions have been identified between the tegument and the glycoproteins (45, 61, 201) and between the tegument and the capsid (114, 200). According to one model, the tegument proteins serve as a bridge between the capsid and the membrane-bound glycoproteins, directing capsids to the sites of envelopment, and then allowing virions to bud into cellular organelles (87). From the trans-Golgi network, the enveloped virion is transported in a vesicle to the cell membrane. Upon arrival, the vesicle carrying the mature virion fuses with the cell membrane, and releases the virus particle to the outside of the cell.

1.1.5. The fate of the infected cell

Upon infection, the virus has multiple mechanisms to modify the cell environment in favor of viral replication and immune evasion [reviewed in (153)]. After entering the cell, HSV rapidly shuts off the host RNA, DNA and protein synthesis (155). The virus has two goals in doing this:

the first is to hijack the cell's protein and DNA production machinery for itself, and the second is to down-regulate cellular proteins which may interfere with its replication or allow its detection by the host's immune system. One viral protein that is involved in this effort is the virion host shutoff protein (*vhs* or U_L41) [reviewed in (169)], which is a tegument protein that starts to work immediately after the release of the viral capsid into the cytoplasm of the cell. *vhs* destabilizes cellular mRNA, and is thought to either be an RNase itself or cooperate with a cellular RNase in some manner. ICP27 is another viral protein that interferes with transcription by repressing splicing (139, 161). Other ways by which HSV affects cell function are: 1) degrades or redirects cellular proteins to perform functions to support virus replication; 2) blocks cell cycle in G1 or G2; 3) blocks apoptosis; 4) blocks antigen presentation by MHC class I; and 5) blocks the cell's dsRNA sensing mechanism [reviewed in (153)].

1.2. HSV-1 ENTRY

1.2.1. Overview

Enveloped virus entry into host cells consists of two stages: binding of the virus to the cell surface and fusion of the virus envelope with a cell membrane. Thus, two types of receptors exist that interact with virus glycoproteins. A binding receptor is one that simply allows for the binding of the virus particle to the cell surface. An entry receptor triggers a conformational change in a viral glycoprotein that leads to fusion. In some instances, both functions may be served by the same receptor.

A problem that each virus family has evolved to solve is how to trigger its fusion mechanism only after coming in contact with the host cell, since indiscriminate fusion would cause virus particles to fuse together before reaching their target cell. All enveloped viruses bear glycoproteins in their envelopes, one of which contains a fusogenic peptide that is inserted into a membrane of the host cell to initiate the fusion process between the viral envelope and a cell membrane. Most viruses express only one or two glycoproteins in their envelopes, making the identification of the fusogen-bearing glycoprotein fairly simple. HSV is a large and extremely complicated virus, containing 11 glycoproteins in its envelope, four of which are essential for entry [reviewed in (13)]. This multitude of glycoproteins greatly complicates the elucidation of the fusion and entry process. The fusogen-bearing glycoprotein normally exists in a meta-stable state, and is easily triggered to undergo a conformational change, exposing the fusion peptide, after encountering the appropriate trigger.

Enveloped viruses employ two possible routes of entry into a cell: endocytosis or fusion at the cell membrane. Viruses that take the endocytic pathway fuse with the endosomal membrane to enter the cell cytoplasm upon exposure to an acidic environment, while viruses that fuse at the cell membrane depend on a glycoprotein-receptor binding event to initiate fusion. In addition, a hybrid system of entry is utilized by several viruses which enter through a pH independent, caveolae-mediated endocytosis pathway (90, 109, 117, 131, 149, 150, 157, 176). A possible explanation for why a virus would need to take this pathway would be to gain access to a fusion-triggering co-receptor that is present only in a caveosome. Recent evidence has also been presented that several viruses are able to utilize multiple entry pathways, when the main pathway of entry is blocked (Sieczkarski 2002 – influenza, Damm & Helenius SV40?).

HSV entry is initiated by binding of viral glycoproteins B (gB) and C (gC) to heparan sulfate (HS) glycosaminoglycan (GAG) side chains of cell-surface proteoglycans (104, 166, 175, 195). This step is thought to concentrate virus at the cell surface and allow glycoprotein D (gD) to engage the entry receptor, such as HVEM (HveA), nectin-1 (HveC), or 3-O-sulfated heparan sulfate [reviewed in (13, 172, 173)]. Receptor binding by gD is believed to set in motion the fusion process, which requires gB and the gH:gL heterodimer for completion. Although it has been shown that four glycoproteins (gB, gD, gH and gL) are required for HSV entry, the individual roles of these glycoproteins are still largely unknown.

1.2.2. Fusion mechanisms employed by other enveloped viruses

Fusion of two biological membranes is a process that requires an investment of energy. In the case of fusion of an enveloped virus with a cell-membrane, the energy is derived from a large conformational change that is carried out by a viral glycoprotein [reviewed in (27)]. The conformational change is triggered either by a low pH environment in an endocytic compartment or by the binding of the glycoprotein to a cell-surface receptor, and leads to the insertion of a hydrophobic fusion peptide into the opposing cellular membrane. This event is thought to de-stabilize the membrane, and allow for the fusion process to proceed. The details of the ensuing fusion process are still largely unknown.

Two types of viral fusion proteins have been identified thus far. Type I fusion proteins form spiky projections that are positioned perpendicular to the viral envelope [reviewed in (27)]. Each protein is synthesized as a single-chain molecule, which then assemble into trimers. Each protein is cleaved by host proteases, which allows it to enter a metastable state and then carry out

the fusion process. The newly created amino terminus contains the fusion peptide, and a region near the C-terminus contains heptad repeats that form coiled-coil structures that aid in the fusion process. After encountering the appropriate trigger, the fusion peptide is inserted into the opposing membrane. The protein is then refolded into a trimeric coiled-coil, thereby drawing the two membranes into close proximity to initiate fusion (Figure 4). The influenza HA glycoprotein is the best characterized fusion protein in this class.

Type II fusion proteins lie parallel to the surface of the viral envelope, and are found in members of the Flaviviridae family [reviewed in (67)]. They are synthesized in a metastable form as heterodimers with another viral glycoprotein. Upon entry, the partner glycoprotein dissociates after being exposed to a low pH of an endosome. The fusion protein in turn undergoes a large conformational change, and enters a homotrimer conformation. The internal fusion peptide is propelled towards the endosomal membrane, initiating fusion of the two membranes.

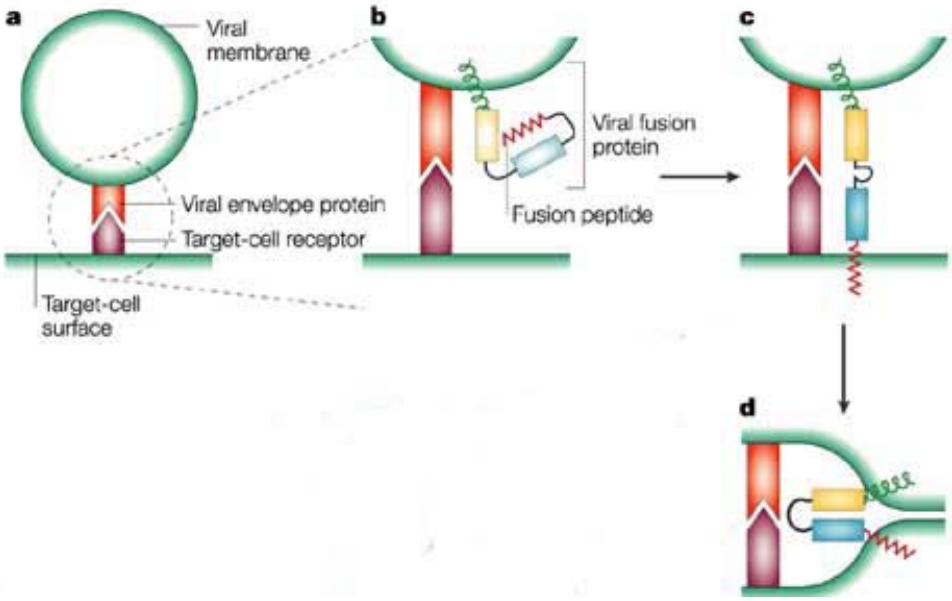


Figure 4. Fusion of envelope viruses with a Type I fusion protein.

a) A type I viral fusion protein binds to its receptor at the cell-surface, triggering a conformational change in the viral fusion protein (b-d). c) Fusion peptide is inserted into the target cell membrane. d) Formation of the trimeric coiled-coil draws the viral and cell membranes into close proximity. Modified from Colman, P.M. and Lawrence, M.C., Nature Reviews, April 2003 (27).

1.2.3. HSV entry pathway

Until recently, HSV was believed to enter all cells by fusion at the cell surface. However, several studies that have been published in the last few years showed that HSV entry into certain cell types can occur by endocytosis. Nicola et al. demonstrated that HSV entry into CHO and HeLa cells, but not Vero cells, can be inhibited by energy depletion or hypertonic medium, which inhibits endocytosis, and by lysosomotropic agents that block the acidification of endocytic vesicles (128).

Lysosomotropic drugs have traditionally been used to show whether a virus enters cells through endocytosis. They prevent endosome acidification, thereby blocking the necessary trigger that the virus requires in order to fuse with the endocytic compartment. Most of these drugs also affect the cells in various non-specific ways. Bafilomycin A1 is the most specific lysosomotropic agent available today. This drug is thought to specifically inhibit vacuolar H^+ -ATPases that are responsible for lowering the pH of endocytic vesicles (137). Nevertheless this drug is fairly new, and it is possible that future studies will show that it as well has other non-specific effects on the cell.

Specific methods to elucidate the entry pathway of a virus take advantage of dominant-negative mutants of components of the endocytosis machinery, such as Eps15, which is an essential component of clathrin coated pits (170), or dynamin, which is involved in the formation of both clathrin and caveolar vesicles (35, 129). Nevertheless, by blocking endocytosis, these methods interfere with many essential cellular processes, and can therefore indirectly affect infection. The most reliable method used today to demonstrate endocytosis or lack thereof is real-time video microscopy, which allows the tracking of a fluorescently-labelled virus particle through endocytic compartments (170).

In another study, Nicola et al. showed that endocytosis in CHO cells is receptor-independent, but that expression of a gD-receptor is required for productive infection (125). While other viruses require either receptor binding or exposure to low pH to trigger their fusion mechanism, these studies suggest that HSV requires both in certain cell types. The reason for this unusual requirement is still unknown. A more recent study showed that HSV infection of C10 murine melanoma cells takes place by yet another pathway: gD-receptor-dependent, but pH-independent, endocytosis (119). Whether or not HSV entry by these novel pathways leads to

productive infection is yet to be conclusively proven, as the current studies all relied on the use of chemical agents to block endocytosis. As previously mentioned, chemical agents can have secondary effects on the cells that interfere with infection.

1.2.4. HSV attachment

Heparan sulfate (HS) is an HSV binding receptor. Elimination of the HSV-HS interaction decreases virus infectivity, but does not abolish it altogether, indicating that fusion can still occur in its absence (62). Several lines of evidence demonstrate the binding role of HS in HSV entry. CHO cell mutants that are defective in GAG synthesis are highly resistant to HSV infection and binding of virus to these cells is greatly impaired (3, 166). Mutant viruses that are gC-deficient infect cells with a 10- to 20-fold reduced efficiency (71). 65% less gC-deficient virus particles bound to Vero cells as compared to wild type virus while the deletion of the HS binding domain of gB reduced the amount of bound virions by only 20% (96). The effect of deleting both gC and the HS binding domain of gB reduced the number of bound viruses by 80% as compared to wild type virus (96). The gD-receptor interaction is thought to bind the other 20% of virus in the absence of the gB and gC binding functions (3). The affinity of the gC-HS interaction is 30-fold higher than that of the gD-receptor interaction (158), which together with the greater abundance of HS on the surface of cells, may explain the greater role of HS in HSV binding.

Although the interaction of gB with HS appears to be unnecessary and redundant, it may be required for virus binding to certain cell types or have an additional role in entry that does not involve binding. For example, it was shown that a gC-negative virus was only able to infect

MDCK cells from the basolateral surface (163). In addition, gC and gB appear to bind to different HS structures and with different affinities (68, 181).

1.2.5. HSV fusion

Initial binding of the HSV virion to HS allows for the efficient interaction of gD with the entry receptor on the cell surface. Three structurally unrelated gD entry receptors have been identified thus far, HVEM (HveA) (121), nectin-1 (HveC) (55), and 3-O-sulfated heparan sulfate (167), and it has been demonstrated that the gD-receptor interaction is absolutely required for HSV entry (83, 84). Penetration of virus also requires the action of gB, gH, and gL. Deletion of any one of these genes results in the production of virus that is able to bind to cells but can not penetrate (10, 43, 98, 156). Neutralizing antibodies that target each of the four essential glycoproteins have been isolated, supporting the requirement for each in the entry process (60, 123, 130, 134, 136). In addition, several groups have shown that these four glycoproteins are necessary and sufficient to induce cell-cell fusion when they are expressed in the same membrane (138, 182), confirming that they constitute the minimal HSV fusion machinery. Nevertheless, it has not yet been determined how gD initiates the fusion cascade upon receptor binding, or what the individual roles of the other three essential glycoproteins are in the entry process.

According to the current model for HSV entry, binding of gD to its receptor triggers a conformational change in gD, which allows it to send a fusion signal to the other three essential glycoproteins (Figure 5) (174). An alternative hypothesis is that the gD-receptor interaction simply serves to tightly bind the viral envelope and the cell membrane, allowing for another

event to trigger fusion, such as the binding of another glycoprotein to a co-receptor. In either case, the actual fusion process is thought to be mediated by one or all of the other essential glycoproteins.

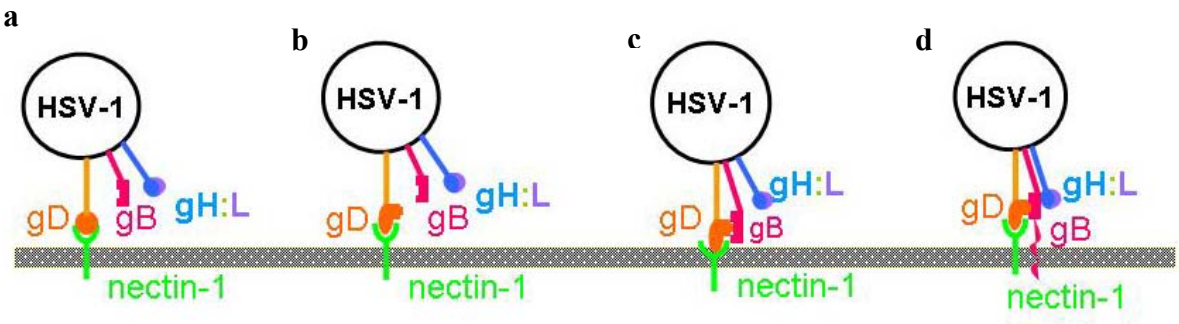


Figure 5. Model for HSV entry.

gD binds a specifice cell-surface receptor (a), undergoes a conformational change (b), and is then able to transmit the fusion signal to the other essential glycoproteins (c). Fusion process is carried out by one or more of the other essential glycoproteins (d).

gD itself is not likely to be the fusogen for several reasons. First, a viral fusogen must be anchored in the viral envelope in order to enable fusion of the two opposing membranes, and it has been demonstrated that membrane-anchorage of gD is not essential for its function. gD can function in cell fusion when its ectodomain is linked to a GPI anchor (86) and a soluble form of the gD ectodomain can mediate entry of a gD-deficient virus, as reported earlier by Cocchi et al (23), and us in the current study. Second, glycoprotein D is missing from the envelopes of β and γ herpesviruses, as well as VZV, and is therefore thought to be the initiator of fusion in HSV and not an integral part of the conserved fusion machinery.

An important study that was instrumental in establishing the cascade model of HSV entry was conducted by Fuller and Lee (46). These authors showed that virions that were attached to the cell-surface, in which gD or gH were neutralized by antibodies, had a distinctly different morphology than wild type virus. gD-inactivated virions were loosely associated with the cell surface in contrast to wild type virions, which were associated more tightly. Virions with inactivated gH appeared to be arrested at a later stage of fusion, and formed fusion bridges with the cell, suggesting that gH was involved in a fusion step that was further downstream than gD.

After the gD receptors were identified and it was conclusively shown that the gD-receptor interaction is absolutely required for HSV entry, the main questions facing the HSV entry field were:

1. Does the gD-receptor interaction serve to tightly bind the virus to the cell or does it enable gD to send a fusion signal to the other viral glycoproteins?
2. Does the fusion signal go through the viral envelope or cell membrane?
3. Which viral glycoprotein(s) does gD interact with to communicate the fusion signal?
4. How do gB and gH participate in the fusion process?
5. Which glycoprotein(s) is the fusogen?

In the last few years several studies have begun to address all of these issues. The strongest evidence against the hypothesis that states that the purpose of the gD-receptor interaction is only to tightly bind virus to the cell was provided by studies showing that both the gD receptors and gD itself can function to mediate entry in soluble form. Qing Bai and Heechung Kwon in our laboratory demonstrated that soluble nectin-1 and soluble HVEM can mediate entry of HSV containing a full complement of viral glycoproteins into gD-receptor deficient CHO-K1 cells (94). This study also provides strong evidence to show that the fusion

signal is transmitted through the viral envelope and not through the cell membrane. The current study and published work by others demonstrate that soluble gD can mediate entry of gD-deficient HSV particles into receptor-bearing cells. Since membrane anchorage of both gD or the gD receptor is thus not required for HSV entry, it can be concluded that the purpose of the gD-receptor interaction is to trigger fusion and not to tightly bind virus to the cell.

How gD is able to initiate fusion is currently the most important question facing the HSV entry field, and is under intense investigation by several laboratories. The HVEM binding domain on gD has been clearly defined (17, 30, 196, 197), and evidence for the localization of the nectin-1 binding domain has recently been presented (detailed below) (29, 106). In addition, a gD domain that is involved in entry, but not binding to either receptor was recently identified by two laboratories (detailed below) (23, 50, 198), providing further support that gD does have a non-binding role in entry. Nevertheless, a binding partner in the HSV envelope for gD has thus far not been identified.

Whitbeck and co-workers provided the first evidence of a link between gD-receptor binding and a downstream effect on one of the other essential glycoproteins (C. Whitbeck, Y Zou, R. Milne, G.H. Cohen and R.J. Eisenberg. 29th International Herpesvirus Workshop, abstr. 2.13, 2004). These authors showed that the proteinase digestion pattern of gB in the virion changed upon gD association with soluble nectin-1.

Some evidence exists for complex formation between gD and the other essential glycoproteins. First, Handler et al. demonstrated an association between gD and glycoproteins B and H by cross-linking of purified HSV, immuno-precipitation of one glycoprotein, and probing Western blots of the precipitate with antibodies against other glycoproteins (64). The results from this study demonstrated the existence of oligomers containing glycoproteins D, B and C.

Another cross-linking study conducted by Rodger et al. supported the findings of this report for wild type virus (152). However, when this group examined virus particles that lacked either glycoprotein B, C or D, they found that the cross-linking interactions between the remaining two glycoproteins remained unaltered.

In the last year, studies addressing the existence of an HSV fusogen have been published. Gianni et al. reported the presence of a potential fusion peptide and heptad repeat sequences in gH, suggesting that this molecule may be the fusogen (52, 57, 58). In addition, Galdiero et al. identified several sequences in gH that can induce fusion of large unilamellar vesicles (52, 57, 58).

1.2.6. HSV binding and entry receptors

1.2.6.1. Heparan sulphate

Heparan sulphate (HS) is thought to be expressed in all non-circulating tissues. It consists of long chains of repeating modified disaccharide units, with alternating N-sulfate or N-acetyl glucosamine (GlcN) residues and glucuronic acid (GlcA) or iduronic acid (IdoA) residues [reviewed in (88)]. Although the majority of the HSV-1 HS binding can be accounted for by electrostatic forces mediated by basic residues of gB and gC and negatively charged sulfate and carboxyl groups of the HS chains, there is a significant amount of specific interactions that exist between these molecules. In addition, not all cells that express HS necessarily contain binding sites for gB and gC. Binding of gB and gC to HS on the cell surface is dependent on a preferred arrangement of the disaccharide sugar residues on HS (42) and specific amino acid sequences on gB and gC (96, 108, 180). The HS binding domain of gB has been mapped to a lysine-rich (pK)

sequence consisting of residues 68 to 76, while three different domains of gC mediate its binding to HS (96, 168). Glycoproteins B and C appear to bind to different domains of HS, since gC but not gB binding to HS can be inhibited with several different compounds (68, 69). In addition, gB's interaction with HS seems to be more specific, since higher concentrations of sodium chloride were needed to disengage the gB-HS complex than gC-HS (181).

1.2.6.2. HSV entry receptors

Three classes of structurally unrelated HSV receptors have been identified through expression cloning (13, 25, 167). The first HSV receptor to be isolated was HVEM (Herpes Virus Entry Mediator, also referred to as HveA), belonging to the TNFα/NGF receptor family (121). Next, two HSV receptors belonging to the immunoglobulin superfamily were identified, and named nectin-1 (or HveC) and nectin-2 (or HveB) (55, 186). The last HSV-1 receptor to be discovered is a 3-O-sulfated derivative of heparin sulfate, but not much information is available about its biological role (167).

HVEM mediates entry of most strains of HSV-1 and 2 and is found on activated lymphocytes, epithelial cells, and fibroblasts, but not on neurons (172). HVEM is the principal HSV receptor on activated lymphocytes, but not in other cell types, in which the principal receptor is nectin-1. In activated lymphocytes, the cellular function of HVEM is to bind the ligands LIGHT and lymphotoxin-α (113). Studies of the physiological role of HVEM have shown that the binding of LIGHT can provide a secondary signal for T cell activation [reviewed in (33)]. In vivo studies have demonstrated that the LIGHT-HVEM interaction contributes to a CTL-mediated immune response (172). The implications of the physiological role of HVEM to HSV biology are yet to be established.

Nectin-1 serves as an entry receptor for HSV-1 and 2, pseudorabies virus, and bovine herpesvirus type 1. Nectin-1 is broadly expressed on human cell lines, organs, and tissues, such as epithelial cells, fibroblasts, and neurons. Three other nectins have been identified thus far [reviewed in (179)]. Nectins function as Ca^{2+}-independent homo- and heterophilic adhesion molecules, and interact with afadin, which connects them to the actin cytoskeleton. They can co-localize with cadherins to adherens junctions, as well as function in cell adhesion independently. Nectins consist of an amino-terminal V-domain, two C2-like domains, a transmembrane domain and a cytoplasmic tail.

Nectins form homo-cis-dimers, and this formation is essential for the formation of trans-dimers. Each nectin family member forms homo-trans-dimers, and nectins 3 and 4 also form hetero-trans-dimers with nectin-1 that are of a significantly higher affinity than the homo-trans-dimers (40, 148). The nectin-3 and 4 binding domain of nectin-1 overlaps with its gD binding domain (40).

The nectin-1 V-domain is necessary and sufficient to mediate HSV entry. A truncated form of the V-domain fused to the transmembrane region was shown to function in mediating entry, indicating that the C2-like domains are not essential (24). In addition, a soluble form of the V-domain was shown to mediate HSV entry into receptor-deficient cells (94).

Nectin-2, another member of the immunoglobulin family of receptors, mediates the entry of several HSV-1 mutants – rid1, rid2 and ANG, but does not allow entry of wild type virus (102, 186). In addition, nectin-2 mediates the entry of HSV-2 and pseudorabies virus (186).

1.2.7. Essential virus components for entry

1.2.7.1. Glycoprotein D

Glycoprotein D (gD) is a major player in the HSV entry process, and is thought to trigger the fusion cascade by binding to a cell-surface receptor. gD is expressed by all alphaherpesviruses, with the exception of VZV, and is thought to be the envelope component that confers host and tissue specificity [reviewed by (172)].

gD is a type I integral membrane glycoprotein and consists of 369 residues with an ectodomain of 316 amino acids, three N-linked oligosaccharide attachment sites (188), and 6 cysteines that form three disulfide bonds (101). The crystal structure of the gD ectodomain (the first 259 residues), unbound and bound to the HVEM receptor has been reported (Figure 6) (16). Based on these data and numerous genetic analyses, important conclusions can be drawn about the structure of this key molecule. gD_{259} consists of a central V-like immunoglobulin fold, with large extensions at the N- and C-termini. The N-terminal residues, which constitute the HVEM binding site form a hairpin upon HVEM binding. In the crystal structure of free gD, the N-terminus does not form the hairpin structure. Adjacent to the N-terminal hairpin is an α-helix, comprised of residues 224-240. Residues in the α-helix stabilize the binding of HVEM to the hairpin domain, and are also involved in nectin-1 binding and entry.

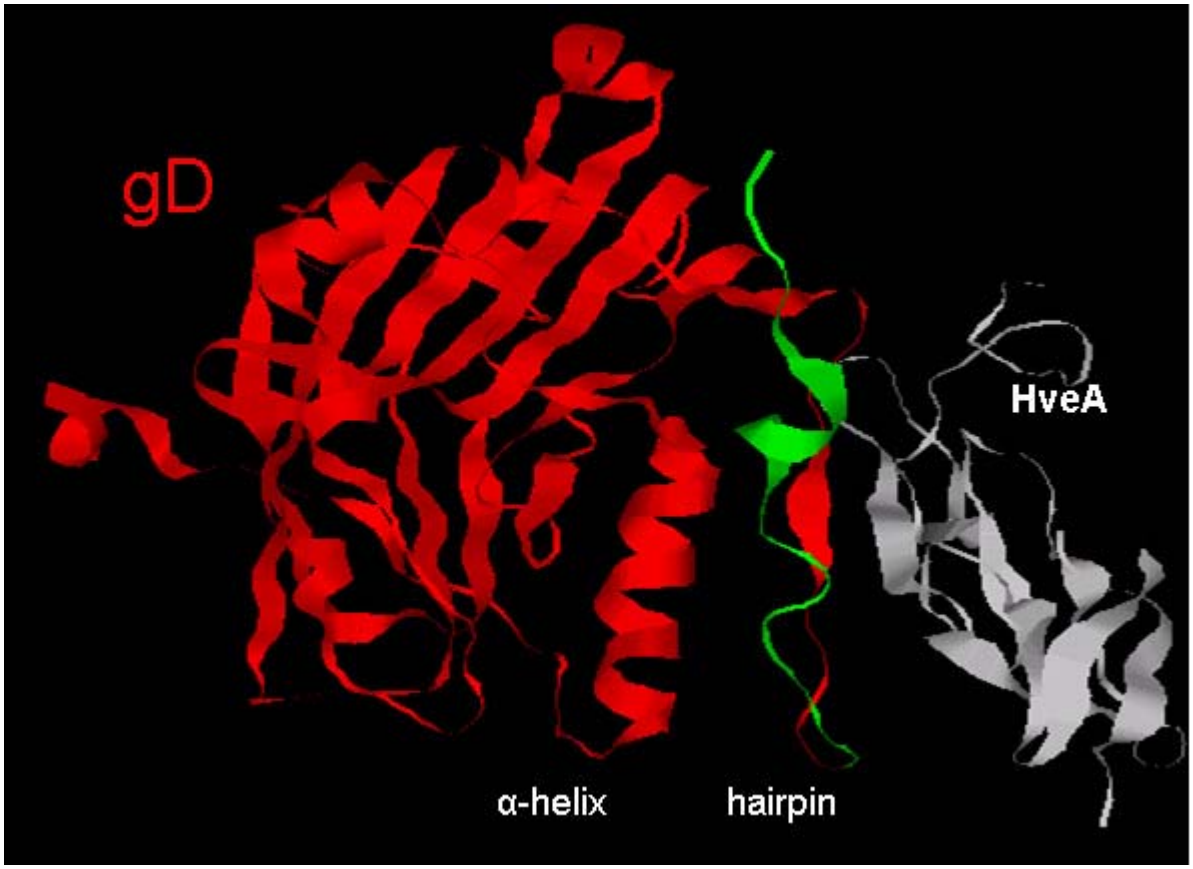


Figure 6. Crystal structure of gD bound to HVEM.

Ribbon diagram of the crystal structure of glycoprotein D bound to the HveA receptor. gD is colored in red and green, and HveA in grey. The α-helix and hairpin structures are indicated. (Image created with RasMol).

Using a genetic approach, Chiang et al. defined four functional domains of gD (20). gD mutants were constructed by linker-insertion mutagenesis and tested for their ability to complement the entry function of a gD-minus virus. The mutations that failed to complement entry were grouped into four functional regions. Region I corresponds to the hairpin domain, region II is part of the Ig-like core, region III includes the α-helix, and region IV comprises the un-crystallized portion of gD between the α-helix and the transmembrane domain (Figure 7).

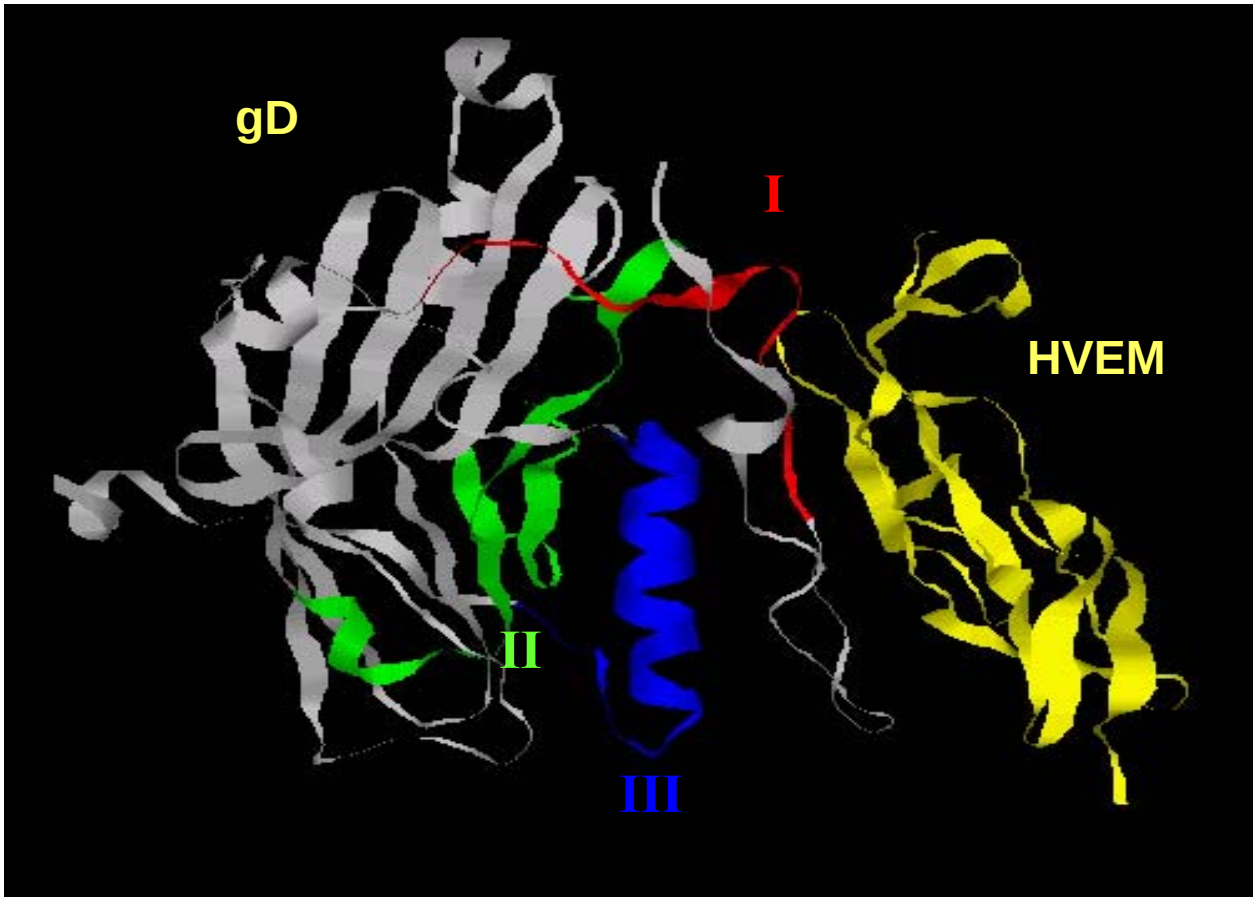


Figure 7. Crystal structure of gD in complex with HVEM depicting the functional regions of gD.

Crystal structure of gD depicting three of the four functional regions of gD defined by linker-insertion mutagenesis study conducted by Chiang et al. (20). (Image created with RasMol).

Mutational analyses have revealed distinct but overlapping regions of gD that contribute to binding of the different cognate receptors. Numerous mutants have been described that are competent for binding and entry via nectin-1, but not HVEM (17, 30, 31, 93, 102, 118, 121, 186, 191, 196, 197). Moreover, rare mutants with the reciprocal profile have now been identified as well (29, 106) (Q. Bai, W. Ali Shah, J.B. Cohen, R.J. Eisenberg, G.H. Cohen and J.C. Glorioso. Abstr. 26th International Herpesvirus Workshop, abstr. 2.10, 2001). These studies have helped to define the HVEM binding site, localized mainly to the N-terminal hairpin domain of gD, in

agreement with the crystal structure. They are beginning to bring the nectin-1 binding domain, which includes several discontinuous epitopes near the top of the α-helix, into sharper focus.

Identification of a gD domain that is involved in entry, but not receptor binding, has been a core objective in the HSV entry field for the past decade. It has been demonstrated that several monoclonal antibodies do not interfere with virus binding to HVEM, but neutralize virus entry into cells expressing this receptor (93, 127). In addition, several mutations in gD abrogated the ability of virus to enter cells, while preserving the ability of gD to bind both receptors. These studies identified regions important for entry consisting of residues 216-221, 243, 272-279, and 277-299. Recently, Cocchi et al. further defined an entry domain between residues 260 and 310 by showing that a soluble form of gD created by truncation of the ectodomain after position 285 (gD_{285}), but not gD_{260}, was able to mediate entry of a gD-deficient virus, although both molecules were capable of binding to nectin-1 and HVEM (23). These authors named this region the pro-fusion domain (PFD), and suggested that this domain is directly involved in interactions between gD and another viral glycoprotein. Zago et al. supported this finding by showing that a chimeric HSV/PrV gD can substitute for HSV gD in entry and cell-cell fusion only if the first 285 amino acids of the chimeric molecule are derived from the HSV gD (198). Since the first 261 amino acids of HSV gD were sufficient for binding to both the HVEM and the nectin-1 receptors, these results showed that a domain between residues 262 and 285 was required for entry but not receptor binding. Based on the observation that 5- and 10- amino acid deletions across this region did not identify a sub-domain that is critical for cell-cell fusion, these authors proposed that this domain is not directly involved in the still hypothetical interaction of gD with another HSV glycoprotein, but instead may constitute a flexible stalk that is involved in promoting a conformational change in gD or the proper positioning it of gD to trigger fusion.

When the crystal structure of gD was solved, it was puzzling that no major conformational change could be detected between the receptor-bound and free forms of gD. It was hypothesized that the remainder of the gD ecto-domain outside of the crystallized portion (residue 260-310) might be involved in a larger conformational change than what the crystal structure revealed. Recently, Fusco et al. demonstrated that this is indeed the case by showing that a soluble form of this region (named the PFD from a previous study) bound soluble gD truncated at amino acid 260 (gD_{260}) (50). This interaction was disrupted by binding of gD_{260} to either the HVEM or nectin-1. These authors proposed a model in which gD exists in a closed conformation before receptor binding, in which the C-terminal PFD interacts with the N-terminus, and that after receptor binding, gD enters an open conformation in which it is able to transmit the fusion signal to other glycoproteins.

The cytoplasmic and transmembrane domains of gD are dispensable for gD's entry function (41, 192). The cytoplasmic tail of gD was deleted from residue 343 and replaced with the cytoplasmic domain of CD8 with no effect on the infectivity of gD-deficient viruses complemented with this chimeric molecule. The transmembrane domain of a Golgi-resident enzyme was also substituted for that of gD. The resulting recombinant virus replicated to wild type levels.

1.2.7.2. Glycoprotein B

Glycoprotein B is another essential player in fusion and entry of HSV. It is the most highly conserved glycoprotein in the *Herpesviridae* family (135) and is the prime candidate to be the fusogen. gB consists of 904 amino acids, comprising an ectodomain of 696 amino acids, a cytoplasmic tail of 109 residues, and a 69 amino acid C-terminal hydrophobic stretch that is the

predicted membrane-anchoring domain (9). The C-terminal hydrophobic stretch is predicted to contain three 20-21 amino acid segments, which span the membrane three times (132). Various sequences in the ecto-, transmembrane, and cytoplasmic domains of gB are required for the fusion and entry functions of the virus (10, 11, 51, 72, 73, 110, 146, 185). Regions in the external and cytoplasmic domains of gB have been identified which alter the rate of entry of HSV-1 (9, 36, 51, 73). Wild-type HSV does not cause significant cell-cell fusion upon infection, whereas mutants that cause extensive polykaryocyte formation (*syn* mutants) have been readily isolated (147). *Syn* mutations have been mapped to the cytoplasmic domain of gB (9-11, 51). Taken together, these data strongly suggest that HSV fusion involves communication between the ecto- and cytoplasmic domains of gB through the predicted transmembrane region.

Glycoprotein B can be extracted from virions or cells in the form of homodimers (21, 63, 74, 162). Dimerization domains of different affinities have been mapped (74, 145), and the position of the strongest oligomerization site was narrowed down to residues 626-653 (97).

1.2.7.3. Glycoprotein H

Glycoprotein H is the third and final participant that is essential for the entry function of HSV. It forms a heterodimer with gL, which is a small, non-membrane-anchored glycoprotein (79). Complex formation with gL is required for the proper processing of gH, as well as for infectivity of the virus (79). gH consists of 838 amino acids, with an ectodomain of 786 residues, a cytoplasmic tail that is 14 residues long, and a transmembrane domain of 21 amino acids. As with gB, all three domains of gH play a crucial role in the entry process of the virus as mutations in each of the three domains have been shown to abolish infectivity (8, 52, 65, 193). *Syn* mutations map to the gL glycoprotein of the gH:L complex (99).

Recently, several fusogenic domains have been identified in gH, suggesting that this glycoprotein may participate in mediating the actual fusion process. Gianni et al. used the bioinformatics tool ENSEMBLE to identify two membrane α–helices in gH, located at positions 377-397 and 513-531 (57). Deletion of each of these domains from gH eliminated its ability to function in cell-cell fusion and to complement entry of a gH^- virus. In addition, substitution of these domains with the fusion peptides of HIV gp41 and VSV-G partially rescued the cell-cell fusion activity and infectivity of gH. In a second report from this group, a potential heptad repeat (HR) with a high probability to form a coiled coil was identified at position 443-471 by using an optimized Lupas algorithm (58). A double mutation in the HR region predicted to eliminate the ability of this sequence to form a coiled coil abolished the ability of gH to complement entry of a gH^- virus. A peptide corresponding to this sequence was also shown to inhibit HSV entry. In another report by Galdiero et al., the hydrophobicity-at-interface scale proposed by Wimley and White was used to identify six hydrophobic sequences in gH that have the potential to interact with target membranes (53). Four peptides corresponding to these sequences were able to induce fusion of large unilamellar vesicles, and experiments with mixtures of different peptides showed that these regions may act in a synergistic manner. One of the peptides identified by this group (residues 381-420) represents a region that somewhat corresponds with one of the domains identified by Gianni et al. (377-397). The effect of mutations in the regions identified by the Galdiero group on HSV infectivity or cell-cell fusion still need to be determined in order to establish the role of these domains in virus fusion.

2. SPECIFIC AIMS

According to the currently favored model for HSV entry, gD binding to a cognate receptor induces a conformational change in gD which signals activation of the fusion machinery by enabling communication with the likely effector components of the fusion apparatus, gB and/or gH:gL [reviewed in (174)]. Identifying the exact sequence of events in the complex fusion cascade has been hampered by the inability to isolate the role of individual glycoproteins in the process.

In order to overcome this problem, we took the approach of separating gD from the viral envelope and tested its ability to mediate entry in soluble form. We reasoned that if gD would have the ability to mediate entry when separated from the viral envelope, this system could be used to further dissect the early events in the HSV entry process.

Aim 1: Determine whether gD can function to mediate HSV-1 entry in soluble form.

The goal of this aim was to determine whether a soluble version of the gD ectodomain, referred to as soluble (s) gD, could complement a gD-deficient virus for entry and to compare aspects of this system with entry directed by envelope-anchored gD. The results showed that soluble gD can mediate entry of gD-deficient HSV-1 into CHO cells in a manner dependent on the presence of a cell-surface receptor for gD. In order to estimate the efficiency of sgD-mediated entry, TaqMan PCR was utilized to compare the virus particle concentrations in our stocks of gD-deficient and gD-complemented virus used in entry assays. Receptor specificity of sgD-mediated entry was demonstrated by producing sgD proteins that contained mutations previously shown to

redirect virus entry through either HVEM or nectin-1 when present in the envelope-anchored form of gD. The mutant sgDs demonstrated the same receptor-specificity profile as their envelope-anchored counterparts. It was concluded that anchorage of gD in the virus envelope is not essential for its role in entry.

Aim 2: Characterize the mechanism of sgD-mediated entry

The goal of this aim was to utilize our system, in which gD separated from the virus particle was able to mediate entry, to determine the order of interactions between gD, the gD-receptor and the gD-deficient virion containing the other essential glycoproteins. This part of our study was initiated by conducting a series of pre-incubations of sgD and the gD-deficient virus with receptor-bearing CHO cells. The results showed that while the pre-incubation of virus with the cells supported sgD-mediated entry, pre-incubation of sgD with the cells completely eliminated entry. Since gD is thought to undergo a conformational change upon receptor binding in order to transmit the fusion signal to the other essential glycoproteins, we hypothesized that sgD may fall back to an inactive conformation upon receptor binding in the absence of the other virion glycoproteins. Upon further investigation, the results showed that although ample amounts of sgD were present at the cell surface immediately after pre-incubation, sgD rapidly dissociated from its receptor after excess sgD was removed from the media. In addition, the results demonstrated that receptor-bound sgD was not inactivated instantaneously upon exposure to the permissive temperature of 37ºC, indicating that if sgD does undergo a conformational change upon receptor binding, the conformational change is of a stable nature.

As an alternative approach to identify interactions between receptor-bound sgD and the virus, the possibility that sgD was stabilized at the cell-surface in the presence of the virus was investigated. The results showed no evidence of sgD stabilization.

Finally, in light of the recent evidence that virus entry into CHO cells occurs by endocytosis, sgD internalizion in the presence of virus was tested. The results provided no evidence of sgD internalization in the presence or absence of virus.

Aim 3: Determine GAG-dependence of sgD-mediated entry.

To determine whether sgD-mediated infection was dependent on virus binding to cell-surface GAGs, a cell line (pgsA745-nectin-1) was generated that is GAG-deficient but expresses nectin-1. The results showed that these cells were susceptible to sgD-mediated entry, demonstrating that sgD-mediated entry is not dependent on the presence of GAGs at the cell surface.

Aim 4: Determine the binding mechanism utilized by gD-deficient virus on GAG$^-$ cells.

The occurrence of virus entry into the pgsA745-nectin-1 cells in the absence of GAGs and virion gD indicated that viral-cellular attachment took place independent of the known binding events between the virus and cellular nectin-1 or GAGs. We reasoned that this could be due to either the formation of a sgD-mediated attachment bridge between virion glycoproteins and cell surface nectin-1, or to the existence of a previously unknown, GAG- and gD-independent binding event between the virus and the cell.

Binding of gD-deficient virions to GAG-deficient cells was tested in the absence and in the presence of sgD by Western blot. The results showed that the gD-deficient virus was capable of

binding to GAG-deficient cells in the absence of sgD, demonstrating the existence of a novel binding mechanism.

Characterization of the novel binding interaction indicated that i) it is of a less stable nature than the virus-GAG interaction, ii) it is also employed by gD^+ virus, and iii) virus appears to bind better through this interaction in the absence of gD.

Aim 5: Define the pathway of sgD-mediated entry.

In light of the recent demise of the long-held paradigm that HSV enters all cells by fusion at the cell-surface, we wished to investigate the entry pathway of gD-deficient HSV in the presence of sgD. To this end, we first attempted to reproduce the results of Nicola et al. demonstrating inhibition of virus entry into CHO cells by lysosomotropic agents. Our results yielded inconsistent data for HSV, while the same drugs consistently inhibited entry of the control virus, VSV, whose entry is well-established to occur by endocytosis. We thus were unable to apply this method to investigate the sgD-mediated entry pathway.

We then attempted to show endocytosis of wild type virus by using a protease protection assay, as described by Milne et al. (119). Our results did not show any glycoprotein internalization, which would have indicated that endocytosis did occur in our system.

Finally, we tried to utilize the sgD-mediated entry system to demonstrate the internalization of gD-deficient virus in the absence of sgD, based on the observation by Nicola et al. that wild-type virus is rapidly internalized into CHO cells in the absence of gD receptors (126). This assay showed that if internalization is occurring, it is at a rate that is significantly reduced from the reported $t_{1/2}$=9 min.

In conclusion, we found no evidence of endocytosis of wild type or gD-deficient virus into CHO cells. Further studies are required to clarify the discrepancy between these results and those of Nicola and co-workers.

3. MATERIALS AND METHODS

3.1. Cells and Viruses

Vero and 293T cells were obtained from the ATCC. VD60, a gD-complementing cell line, was a gift from Dr. David C. Johnson (Oregon Health Sciences University) (98). Vero, VD60 and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Gibco). Chinese hamster ovary cells (CHO-K1), and CHO-K1 cells stably expressing HVEM or nectin-1 were kindly provided by Dr. Patricia Spear (Northwestern University), and grown in F-12K medium (Gibco) supplemented with 10% FBS. CHO-nectin-1 and CHO-HVEM cells were maintained under selection with 400 μg/ml G418 (Gibco). pgsA745 cells that are derived from CHO-K1 and are defective in GAG synthesis (39) were obtained from the ATCC (CRL-2242). pgsA745-nectin-1 cells were derived from the pgsA745 cell line by transfection with a full-length nectin-1 cDNA expression plasmid (178) and selection of hygromycin B resistant clones. pgsA745-nectin-1 cells were maintained under hygromycin B selection. J1.1-nectin-1 cells were derived by stably transfecting J1.1-2 cells (kindly provided by Dr. Gabriella Campadelli-Fiume) with the nectin-1 plasmid described above (178), and selecting hygromycin B resistant clones.

KΔUs3-8Z is derived from HSV-1 KOS. This virus contains a *lacZ* reporter gene under transcriptional control of a human cytomegalovirus (HCMV) promoter in place of the unique short (U_S) region 3 through 8 genes of the HSV-1 genome, including the glycoprotein D (U_S6) gene (Figure 8)(2). KΔUs3-8Z was propagated and titered on VD60 cells and passaged through Vero cells to obtain gD-deficient virions. KHZ.1 is a KOS-derived virus containing an HCMV-

lacZ expression cassette in the thymidine kinase locus (115). QOZHG is a replication-defective derivative of KOS (19). This virus does not produce four of the immediate early proteins (ICP4, 22, 27 and 47) due to deletions and substitutions in its genome. It contains the enhanced green fluorescent protein (EGFP) and β–galactosidase reporter genes, and is grown on ICP4 and 27 complementing Vero-derived 7B cells (107). VSV expressing GFP was kindly provided by Dr. Patricia W. Dowling and Dr. Ira Bergman (University of Pittsburgh School of Medicine) and has been previously described (7).

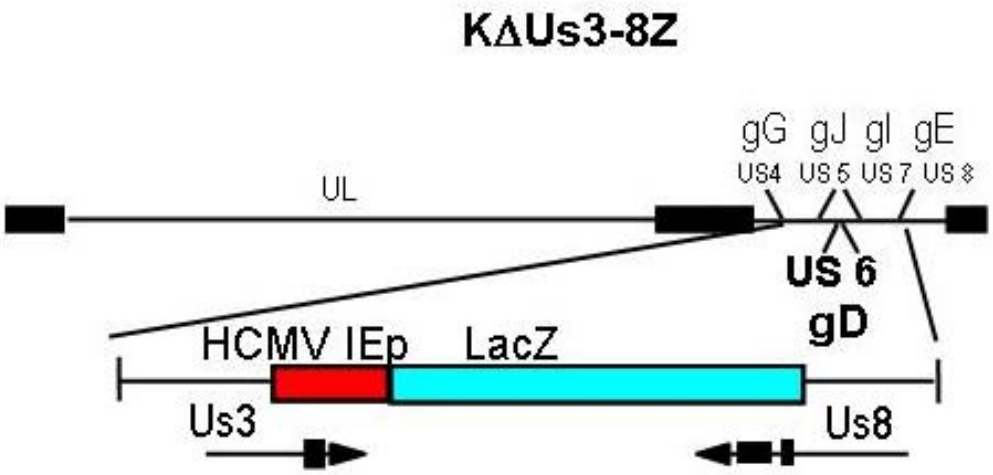


Figure 8. Construction of KΔUs3-8Z virus.
LacZ gene under the HCMV promoter was inserted into the Us3-8 locus of KOS, replacing gD, as well as several other non-essential glycoproteins.

3.2. Plasmids

The HSV-1 *Sac*I fragment containing the gD promoter and coding sequence was cloned into pSP72 (Promega, Madison, WI) and the resulting plasmid designated pgDSac.

In order to create an expression plasmid for soluble gD, the transmembrane region was removed from pgDSac after amino acid 287 by digesting pgDSac with NarI and EcoRI and replacement of this segment with annealed complementary oligonucleotides specifying 6 histidine residues followed by a stop codon (primer 1: 5' CG CAC CAT CAC CAT CAC CAT TAG TTT AAA CGG GGG 3'; primer 2: 5' A ATT CCC CCG TTT AAA CTA ATG GTG ATG GTG ATG GTG 3'). The truncated gD-His_6 sequence was excised from a selected recombinant by digestion with HindIII and EcoRI and placed under transcriptional control of the HCMV promoter of expression vector pcDNA3.1+ (Gibco-Invitrogen, Carlsbad, CA). The resulting plasmid, $psgD_{287}$, was confirmed by DNA sequencing.

Mutant L25P and R222N,F223I full-length gD constructs were generated in pgDSac using the Gene Editor in vitro site-directed mutagenesis kit (Promega). The mutants were authenticated by DNA sequencing and tested in a transient complementation assay for their ability to complement gD-deficient KΔUs3-8Z virus (Q. Bai, W. Ali Shah, J.B. Cohen, R.J. Eisenberg, G.H. Cohen and J.C. Glorioso. Abstr. 26th International Herpesvirus Workshop, abstr. 2.10, 2001). In order to transfer the mutations from pgDSac to $psgD_{287}$, an acceptor plasmid ($psgD_{287}\Delta$) was first derived from $psgD_{287}$ by deletion of an internal 216-bp BstZ17I-NaeI fragment. The desired mutations were then transferred from the pgDSac-based plasmids as HindIII-PpuMI fragments, restoring the deleted region in the acceptor plasmid. Recombinants were identified by digestion with FspI, which cuts in the deleted region and distinguishes between pgDSac and $psgD_{287}$.

3.3. Purification of sgD Proteins

293T cells were transfected with $psgD_{287}$, $psgD_{287}$(L25P) and $psgD_{287}$(R222N,F223I) plasmids using LipofectAMINE-PLUS (Gibco-Invitrogen). After 3 days at 37°C, supernatants were collected and loaded onto ProBond (Gibco-Invitrogen) nickel-chelating columns for purification of the His-tagged proteins according to the manufacturer's protocol. Proteins were concentrated by using a Centricon YM-10 centrifugal filter device (Amicon, Bedford, MA) and dialyzed against PBS at 4°C overnight.

The molecular weights of each protein were estimated by Western blot analysis. 15 µl of each protein were electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to an Immobilon^{TM} PVDF membrane (Millipore, Billerica, MA). The membrane was blocked for 1 h in 5% milk/PBST at room temp, then probed with polyclonal anti-gD antibody R7 (kindly provided by Drs. G.H. Cohen and R.J. Eisenberg, University of Pennsylvania) (92) and an anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-His HRP-conjugated antibody (Santa Cruz Biotechnology). The membrane was developed using Amersham ECL kit (Amersham, Piscataway, NJ). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA).

3.4. Transient gD-Complementation Assay

Vero cells were seeded in a 6-well plate and were either mock-transfected or transfected with gDSacI using LipofectAMINE-Plus reagent (Gibco-Invitrogen). Cells were infected for 2 h at 37ºC with gD-complemented KΔUs3-8Z at MOI=3 24 h after transfection, and then washed with

0.1M glycine (pH 3.0) for 1 min at room temperature to deactivate extra-cellular virus. Virus was harvested 1 day post infection. The supernatant was discarded and the cells were scraped in a volume of 200 µl serum-free DMEM. Cells were sonicated and the cell debris was pelleted by low-speed centrifugation. CHO-nectin-1 cells were infected with 25 µl supernatant and CHO-HVEM with 50 µl for 3 h. Infected cells were visualized after 24 h by X-gal staining.

3.5. Detection of sgD Bound to CHO-HVEM Cells by Immuno-fluorescence

Sub-confluent monolayers of CHO-HVEM cells in a 48-well plate were incubated with 1000 ng of sgD for 1 h at 37ºC. Cell were then washed and fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 5 minutes at room temperature. Cells were then washed and incubated with a pool of anti-gD monoclonal antibodies at a 1:500 dilution (75) for 1 h at room temperature, washed, and incubated for another hour with an anti-mouse Cy3 conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO), diluted 1:500. Cells were visualized with a Nikon diaphot TMS fluorescent microscope under a 20X magnification (Nikon, Melville, NY) and photographed with a Leica Microsystems AG DFC 300F digital camera.

3.6. Flow Cytometry

Mouse monoclonal antibody CK41 (92) at a 1:250 dilution was used to identify surface nectin-1 in adherent cell cultures. Antibody binding and washes were performed at 4ºC in PBS containing 1% horse serum (Gibco). Cell bound CK41 was detected using FITC-conjugated anti-mouse antibody (Sigma-Aldrich) and analyzed on a FACSCalibur (Becton-Dickinson, San Diego, CA).

3.7. Localization of sgD After Binding at 4ºC and Shifting to 37ºC

sgD was incubated with confluent monolayers of CHO-HVEM cells for 2 h at 4ºC in a 24-well dish. The samples that included gD$^-$ KΔUs3-8Z were then washed, and incubated with the virus for an additional 1.5 h at 4ºC. Following the 4ºC incubation, cells were washed with cold buffer and either lysed right away or shifted to 37ºC for various amounts of time in the presence of 37ºC F12 media containing aprotinin and leupeptin, and then lysed with 50 µl PARP buffer (6 M urea, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, and 5% β-mercaptoethanol) (140) per well. At each time point duplicate wells were treated with 100 ug/ml TPCK-treated trypsin (Sigma-Aldrich) for 2.5 min at 37ºC before lysing, and the trypsin was quenched with 200 ug/ml soybean trypsin inhibitor (Sigma-Aldrich). Supernatant was collected from the cells that were shifted to 37ºC before lysis buffer was added. Each sample was sonicated for 20 sec with a Fisher Scientific 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH) to break up the cellular DNA and prepared for Western blot analysis. 25 µl of each lysate and 25 µl of the supernatants were electrophoresed on a 10% SDS-polyacrylamide gel and the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 1 h in 5% milk/PBS at room temperature, then probed with R7 polyclonal anti-gD antiserum at a 1:5,000 dilution in 2% milk/PBS at 4ºC overnight, and the membrane was washed three times in PBS for 10 min each. Goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was then incubated with the membrane at a 1:2,000 dilution in 2% milk/PBS for 1 h at room temperature, membranes were washed three times in PBS for 10 min each, and developed using Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL).

3.8. Detecting Bound Virus by Western Blot Analysis

Virus (gD$^-$ KΔUs3-8Z or KHZ.1) was adsorbed to confluent monolayers of pgsA745-nectin-1, CHOK1 or CHO-HVEM cells in 24-well plate for 2 h at 4ºC, either in the absence or presence of 1 μg sgD. The cells were washed with cold buffer and lysed immediately with 50 μl PARP buffer. Bound virus was detected by Western blot analysis. Each sample was sonicated for 20 sec with a Fisher Scientific 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH) to break up the cellular DNA. 25 μl of each lysate was electrophoresed on a 10% SDS-polyacrylamide gel and the proteins were transferred to an Immobilon^{TM} PVDF membrane (Millipore, Billerica, MA). The membrane was blocked for 1 h in 5% milk/PBST at room temp, and bound virus was detected with NC1 polyclonal rabbit anti-VP5 antiserum (26) (kindly provided by Drs. G.H. Cohen and R.J. Eisenberg, University of Pennsylvania) at a 1:5,000 dilution in 5% milk/PBST at room temp for 1.5 h, followed by goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at a 1:2,000 dilution. Membranes were developed using Amersham ECL kit (Amersham).

As a control for the amount of protein loaded in each lane, membranes were re-probed with a mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA) at a 1:5,000 dilution.

3.9. Detection of KΔUs3-8Z Binding to pgsA745-nectin-1 Cells by Indirect Entry Assay

gD$^-$ KΔUs3-8Z was adsorbed to confluent monolayers of pgsA745-nectin-1 cells in 48-well plate for 2 h at 4ºC, either in the absence of presence of 1000 ng sgD. The cells were washed with cold buffer, and 1000 ng sgD was added in 37ºC media to allow adsorbed virus to penetrate. Cells were infected at 37ºC for 3 h, sgD was replaced by media, and cells were stained with X-gal after 16 h.

3.10. Real-Time Quantitative PCR

Viral preparations were quantified for the immediate-early gene ICP47. All assays were conducted in 50 μL PCR volumes containing viral samples (2 μL), 200 nM of each primer, 200 nM Probe, and 25 μL TaqMan® 2X Universal Master Mix (PE Applied Biosystems, Foster City, CA). Primer sequences for ICP47 (forward- CAC GAC ATG CTT TTC CCG A, and reverse- TTC CCG CAG GAG GAA CG), were designed using the Primer Express program (PE Applied Biosystems). The TaqMan probe for detection of ICP47 (CGC CGG TCG CCT CGA CGA) was labeled with fluorescent reporter dye 6-carboxyfluorescein (6-FAM) at the 5' end and the quencher dye carboxytetramethylrhodamine (TAMRA) at the 3' end (PE Applied Biosystems). All PCR reactions were set up in a MicroAmp Optical 96-well Reaction Plate (PE Applied Biosystems). Amplification conditions were 2 min at 50°C and 10 min at 95°C for the first cycle, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The TaqMan probes were cleaved during the amplification of target sequence generating fluorescent emission specific for FAM-labeled probes. All samples and standards were run at least in duplicate and each run contained several negative (reaction mix with no sample and a sample known to contain no HSV

DNA) and positive controls (samples known to contain HSV sequences, a plasmid and a HSV vector). Standards curves for viral gene ICP47 were generated using 10-fold serial dilutions of plasmids known to contain the respective target sequences. Efficiency of ICP47 primer-probe set was confirmed previously by side-by-side TaqMan PCR runs with other HSV genes of other KOS-derived viruses including ICP27 and gD that have been qualified by negative staining electron microscopy analysis. The emission data was collected in real-time from an ABI PRISM® 7000 Sequence Detector System (SDS) and analyzed using Sequence Detector Software (PE Applied Biosystems).

Each purified virus sample was diluted at in TE (10mM Tris-HCL, pH 7.5, 1mM EDTA) and filtered twice in sterile Pall® Acrodisc® 32 mm Syringe Filter with 1.2 μm Supor membrane® (Pall Life Sciences, East Hills, NY). Genomic DNA of each sample was isolated by using QIAamp® DNA Micro Kit (Qiagen Inc., Santa Clara, CA) using the conditions specified by the manufacturer.

3.11. Calculation of the Relative Efficiency of sgD-Mediated Entry

Using the TaqMan standard curves described above, the particle concentrations of our virus stocks were determined to be $8.39 \pm 1.26 \times 10^9$/ml for gD$^-$ KΔUs3-8Z and $9.03 \pm 1.91 \times 10^{10}$/ml for gD-complemented KΔUs3-8Z. For each preparation, the transduction efficiency on CHO-HVEM cells (particles/blue cells) was then established as approximately 1.1×10^3 for the gD-stock in the presence of sgD versus 1.1×10^2 for the gD-complemented stock. Thus, the difference in transduction efficiencies between the two stocks was estimated as approximately 10-fold.

3.12. Treatment with Lysosomotropic Agents

Stock solutions of ammonium chloride (1.5 M; Sigma-Aldrich) and chloroquine (50 mM; Sigma-Aldrich) were prepared immediately prior to use. Monensin stock solution (75 mM; Sigma-Aldrich) was prepared in ethanol and stored at -20ºC. Bafilomycin A1 (160 µM; Sigma-Aldrich) and concanamycin A (50 µM; Sigma-Aldrich) stock solutions were prepared in DMSO, aliquoted and stored at -20ºC. Cells in 96-well plates were pre-incubated with final concentrations of drugs for 30 min in a total volume of 30 µl F12 media. 3 µl virus was added per well, and cells were infected for 6-10 h at 37ºC in the presence of drug, depending on the virus used. Infected cells were detected by X-gal staining, ONPG assay or observation of EGFP expression.

3.13. Trypsin Protection Assay

QOZHG at MOI=50 was adsorbed to CHO-HVEM cells in a 48-well plate for 2 h at 4ºC. Cells were washed and either lysed immediately with PARP buffer (6 M Urea, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol) (140) (6 M urea, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, and 5% b-mercaptoethanol), or shifted to 37ºC for 10 or 30 min, and then lysed. Duplicate samples were treated with TPCK-trypsin (100 µg/ml) for 2.5 min after the 10 or 30 min incubations. Trypsin was quenched with soybean trypsin inhibitor (200 µg/ml), cells were scraped, pelleted, and re-suspended in 50 µl PARP buffer per well.

The presence of glycoproteins in the lysates was detected by Western blot analysis. 30 µl of each sample were electrophoresed on a 10% SDS-polyacrylamide gel and the proteins were

transferred to an Immobilon™ PVDF membrane (Millipore, Billerica, MA). The membrane was blocked for 1 h in 5% milk/PBST at room temperature, then probed with R7 rabbit polyclonal antiserum at a 1:5,000 dilution in 5% milk/PBST at room temp for 1.5 h, and the membrane was washed three times in PBST for 10 min each. Goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was then incubated with the membrane at a 1:2,000 dilution for 1 h at room temperature in 5% milk/PBST, membrane was washed three times in PBST for 10 minutes each, and developed using Amersham ECL kit (Amersham). Afterwards, the membrane was stripped and re-probed with a pool of anti-gB monoclonal antibodies (1:5,000 dilution (110) and again with anti-VP5 NC1 antibody (1:1,000 dilution).

3.14. Proteinase K Protection Assay

A stock solution of proteinase K (10 mg/ml) was prepared in Ham's F12 medium, aliquoted, and stored at -20ºC. A stock solution of PMSF (100 mM) was prepared in ethanol, aliquoted and stored at -20ºC. KHZ.1 at MOI=20 was adsorbed to CHO-HVEM cells in a 24-well plate for 2 h at 4ºC. Cells were washed and either kept at 4ºC with serum-free media, or shifted to 37ºC for 10 or 30 min, and then transferred to 4ºC with 4ºC serum-free media. Duplicate samples were treated with proteinase K (2 and 4 mg/ml) for 1 h at 4ºC, and proteinase K was quenched with PMSF (4 mM). Cells were scraped, pelleted, and re-suspended in 50 µl PARP buffer per well.

The removal of virus particles from the cell surface by proteinase K and internalization of glycoproteins was detected by Western blot analysis. 30 µl of each sample were electrophoresed on a 10% SDS-polyacrylamide gel and the proteins were transferred to an Immobilon™ PVDF membrane (Millipore, Billerica, MA). The membrane was blocked for 1 h in 5% milk/PBST at

room temperature, then probed with anti-VP5 NC1 antibody (1:5,000 dilution) in 5% milk/PBST at room temperature for 1.5 h, and the membrane was washed three times in PBST for 10 min each. Goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was then incubated with the membrane at a 1:2,000 dilution for 1 h at room temperature in 5% milk/PBST, membrane was washed three times in PBST for 10 min each, and developed using Amersham ECL kit (Amersham).

3.15. Rate of Entry of gD$^-$ KΔUs3-8Z in the Absence of sgD

gD$^-$ KΔUs3-8Z was adsorbed to CHO-HVEM cells for 2 h at 4ºC. Cells were washed and 500 ng sgD was either added immediately or after various amounts of time that the cells were incubated at 37ºC in the absence of sgD. The degree of infection was quantified by ONPG 16 h after infection. The amount of internalized virus in the absence of sgD was extracted from the observed decrease in entry over time, since gD$^-$ virus was shown to be non-infectious in the absence of sgD.

3.16. Rate of Entry of gD$^-$ KΔUs3-8Z in the Presence of sgD

gD$^-$ KΔUs3-8Z was adsorbed to CHO-HVEM cells for 2 h at 4ºC. Cells were washed and 500 ng sgD in 37ºC media was added to each well. Cells were shifted to 37ºC, and wells were treated with 0.1M glycine after various amounts of time to neutralize virus that had not yet been internalized. The degree of infection was quantified by ONPG 16 h after infection.

4. CHARACTERIZATION OF SOLUBLE GLYCOPROTEIN D MEDIATED ENTRY OF gD-DEFICIENT HSV

4.1. Soluble gD Mediates Entry of a gD-Deficient Virus into HSV Receptor-Expressing Cells

To determine whether glycoprotein D has the ability to mediate HSV entry when taken out of the context of the viral envelope, a soluble form of gD (sgD) was generated by truncation of the ectodomain after amino acid 287 (Figure 9), and the ability of this protein to induce entry of the gD-deficient virus KΔUs3-8Z (2) was examined. Virions lacking gD were produced by infection of Vero cells with gD-complemented KΔUs3-8Z, removal of extracellular virus, and collection of cell lysates 3-5 days later.

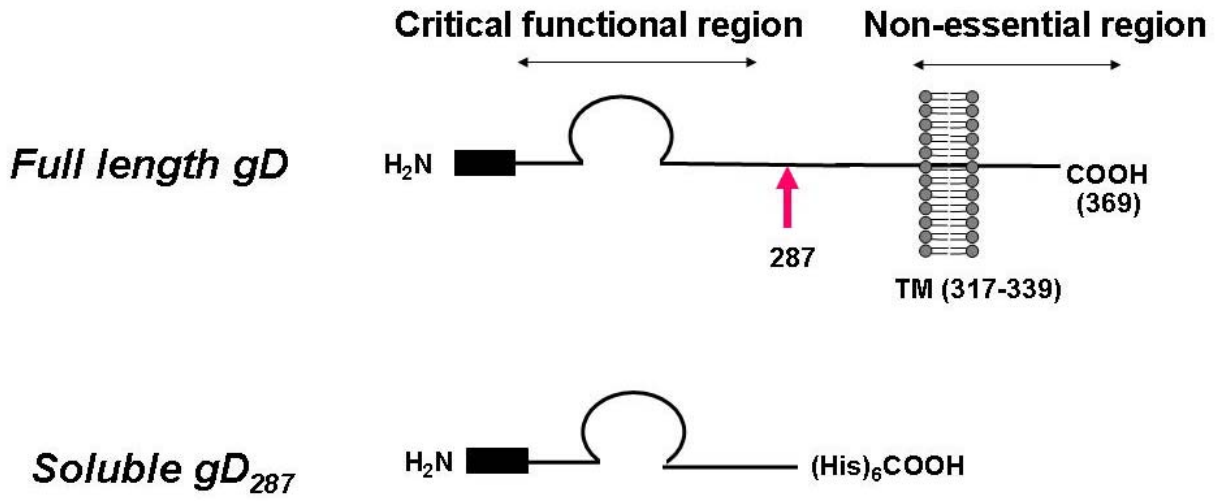


Figure 9. Construction of soluble gD_{287}.

sgD_{287} was constructed by truncating the full-length gD after amino acid 287. This construct comprises the critical functional region of the gD ectodomain, which is necessary and sufficient for HSV entry.

Soluble gD proteins used in this study were prepared by transfection of 293T cells with suitable mammalian expression constructs and recovery of the histidine-tagged products by passage of the growth media over Ni^{2+} affinity columns. Each purified protein was detected at a molecular size of approximately 38kD on Western blots using a His tag-specific antibody (Figure 10).

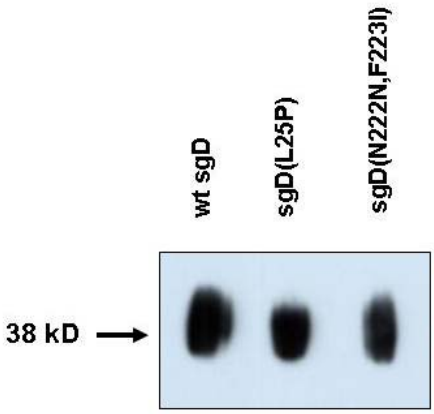


Figure 10. Western blot analysis of purified soluble gD proteins.

His-tagged wild-type (wt) and mutant sgD proteins were purified as described in Materials and Methods, and electrophoresed on a 10% SDS polyacrylamide gel. Proteins were detected with an anti-His HRP-conjugated antibody. Wild type and mutant sgD proteins were detected at a molecular size of approximately 38kD.

To assay sgD for its ability to mediate entry of gD-deficient KΔUs3-8Z, CHO-K1 cells and derivative lines expressing HVEM or nectin-1 were incubated for 3 h with the virus in the presence of the soluble protein. Entry was visualized 16 h later by staining of the cells for β-galactosidase activity expressed by the virus. Figure 11 demonstrates that sgD enabled entry of the gD-deficient virus into CHO-HVEM and CHO-nectin-1 cells, but not CHO-K1 cells. The degree of infection increased linearly with both the virus dose and the amount of sgD used (Figure 12). sgD-mediated infection of CHO-nectin-1 cells was less efficient than infection of CHO-HVEM cells, a finding that did not correlate with the relative susceptibility of these two cell lines to infection by virus harboring gD in its envelope (Figure 13).

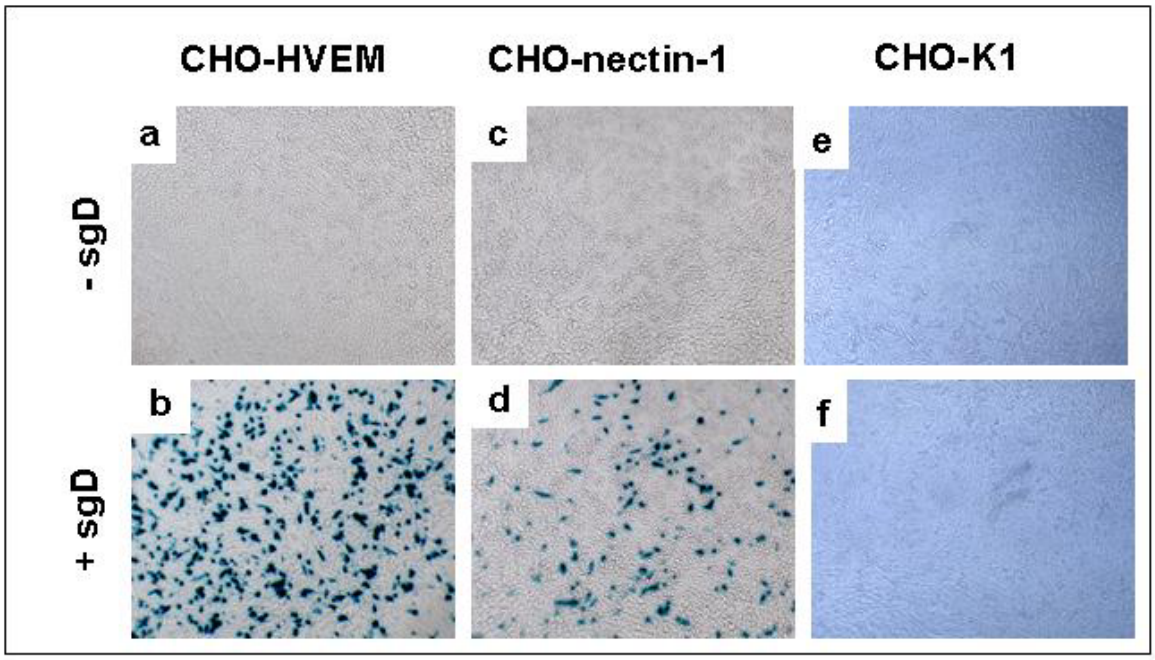


Figure 11. sgD (wt) mediates entry of gD-deficient virions into receptor-bearing cells.

Confluent monolayers of CHO-HVEM (a and b), CHO-nectin-1 (c and d) and CHO-K1 (e and f) cells in 96-well plates were incubated with gD-deficient KΔUs3-8Z virus, which contains the *lac*Z gene driven by the HCMV promoter (2), in the presence or absence of 500 ng sgD for 3 hours at 37ºC. Infected cells were identified by X-gal staining 16 hours after infection.

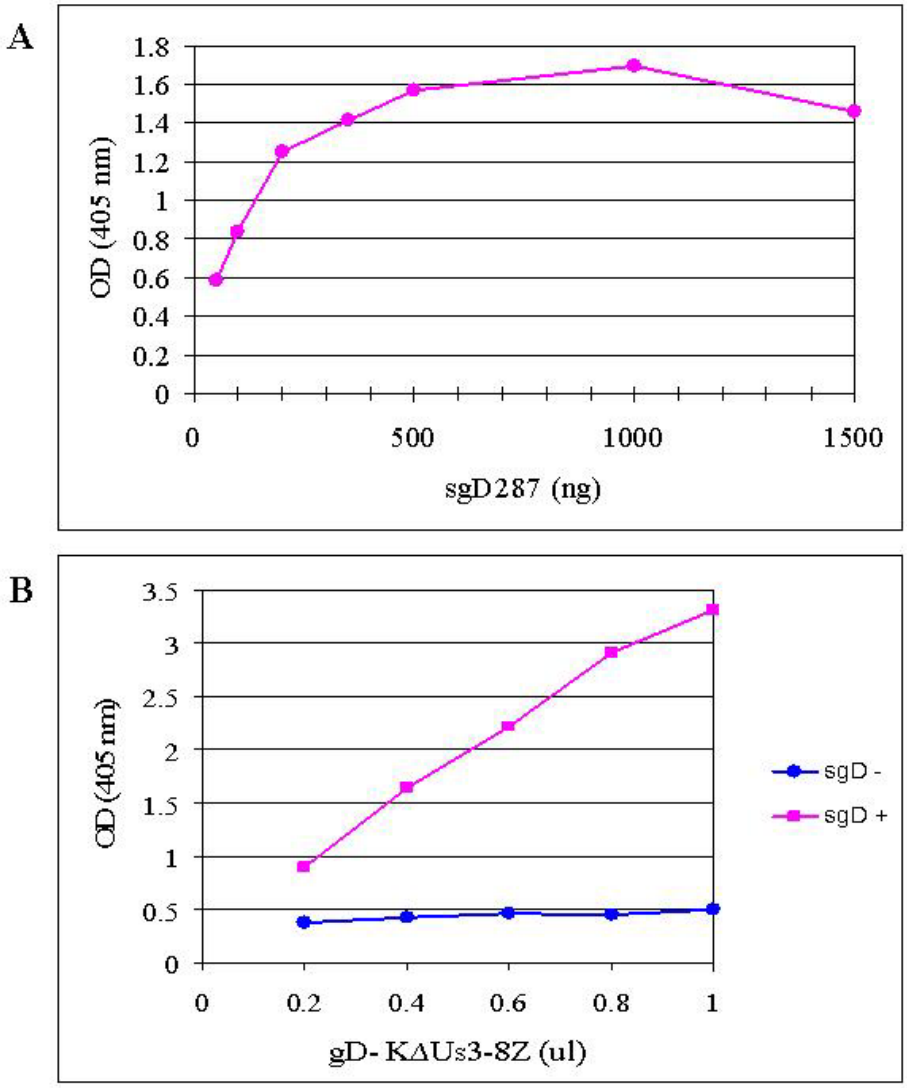


Figure 12. sgD and virus dose dependence of sgD-mediated infection.

CHO-HVEM cells were infected using 1 µl gD-deficient KΔUs3-8Z and varying amounts of sgD (A) or gD-deficient KΔUs3-8Z in the presence of 500 ng sgD (B). Entry was quantified by measuring β-galactosidase activity using the ONPG assay.

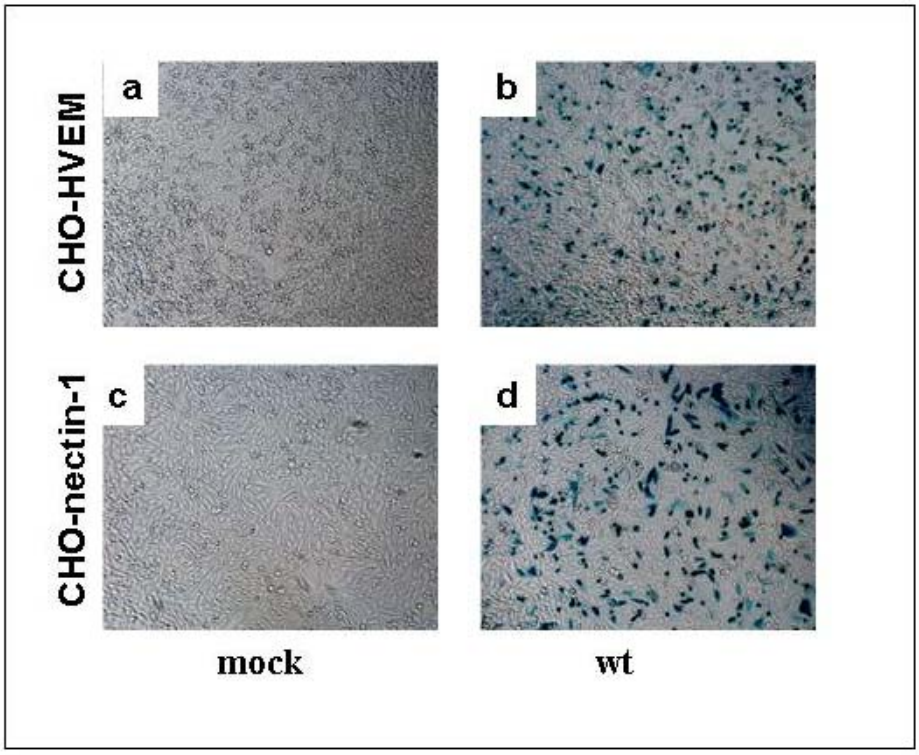


Figure 13. Infection of CHO-HVEM and CHO-nectin-1 cells by gD-complemented KΔUs3-8Z.

Virus was produced by infecting mock- or gD(wt)-transfected Vero cells with gD-complemented KΔUs3-8Z at MOI=3 and harvesting lysates 24 h post infection. a, b) CHO-HVEM cells were infected with 50 µl out of a total of 200 µl lysate. c, d) CHO-nectin-1 cells were infected with 25 µl lysate. Infected cells were visualized by X-gal staining 16 h post infection. The number of infected cells was comparable on CHO-HVEM and CHO-nectin-1 cells.

4.2. Determining the Efficiency of sgD-Mediated Entry

Given the requirement for three components in sgD-mediated infection compared to two in normal infection, it was anticipated that sgD-mediated infection would be substantially less efficient than normal infection. To determine the relative efficiency of sgD-mediated infection versus infection by gD-containing gD virus, quantitative TaqMan PCR was used to estimate the

number of virus particles needed in each case to produce a given number of transduced CHO-HVEM cells (see Materials and Methods). The results showed a 10.6 fold reduction in transducing events per viral particle for gD-deficient KΔUs3-8Z in the presence of sgD compared to a KΔUs3-8Z preparation harvested from gD-complementing VD60 cells. Thus it was estimated that the efficiency of sgD-mediated infection is approximately one order of magnitude lower than that mediated by virion gD.

4.3. sgD-Mediated Entry of gD-Deficient HSV Requires Specific Interaction with a gD Receptor

HSV-1 entry into cells depends on the interaction of virion gD with a cognate cell-surface receptor. To confirm that sgD-mediated entry of gD-deficient virus likewise required a specific interaction between sgD and a cognate receptor, receptor-specific mutant versions of the soluble protein were tested. The L25P mutation in gD impairs virus entry through HVEM without diminishing entry via nectin-1 (197). In addition, a rare gD mutant that is defective for binding and entry via nectin-1 but competent for binding and entry via HVEM was previously isolated in our laboratory [gD(R222N,F223I)] (Q. Bai, W. Ali Shah, J.B. Cohen, R.J. Eisenberg, G.H. Cohen and J.C. Glorioso. Abstr. 26th International Herpesvirus Workshop, abstr. 2.10, 2001). To determine whether these mutations affected sgD-mediated infection, sgD(L25P) and sgD(R222N,F223I) were prepared and tested for their ability to mediate entry of gD-deficient KΔUs3-8Z into gD-receptor bearing cells. As shown in Figure 14, sgD(L25P) displayed a substantially reduced ability to mediate entry into CHO-HVEM cells (compare panels c and e) while its ability to mediate entry into CHO-nectin-1 cells was unaltered, if not enhanced, compared to wild-type sgD (panels d and f). Conversely, sgD(R222N,F223I) mediated entry into

CHO-HVEM, but not CHO-nectin-1 cells (panels g and h). These results supported the conclusion that sgD-mediated entry of gD-deficient virus involves specific interaction of the soluble mediator with a cognate cellular receptor, similar to virion gD interaction with a cognate receptor during normal infection.

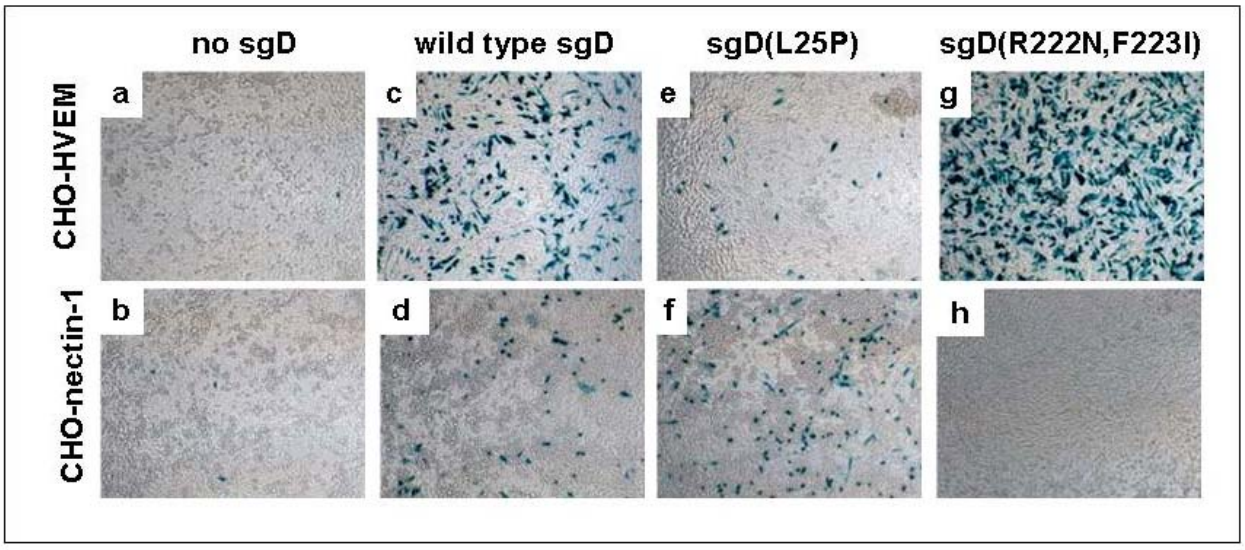


Figure 14. sgD-mediated entry of gD-deficient HSV requires specific interaction of sgD with a gD receptor.

gD-deficient KΔUs3-8Z was incubated with CHO-HVEM and CHO-nectin-1 cells in the absence of sgD (a and b) or in the presence of wild type sgD (c and d), sgD (L25P) (e and f), or sgD (R222N,F223I) (g and h) for 3 h at 37°C. Infected cells were identified by X-gal staining 16 hours after infection.

5. Mechanistic Aspects of sgD-Mediated Entry

The availability of a functional 3-component system presented the opportunity to investigate the order of interactions between gD, the receptor and the other virion glycoproteins. We asked if sgD first needs to bind to its cell-surface receptor in order to function as a stable receptor for the gD^- HSV virion. According to the current model for HSV entry, gD undergoes a conformational change upon receptor binding, which allows it to interact with another virion glycoprotein and thereby initiate the fusion cascade Our experiments were designed to determine whether this conformational change is of a stable or transient nature.

CHO-HVEM cells were incubated with sgD for 1 h at 37°C, washed several times to remove free sgD, and exposed to gD-deficient KΔUs3-8Z at 37°C. The results showed that infection was reduced to background levels under these conditions (Figure 15C), compared to readily detectable infection when sgD and the virus were added to the cells either simultaneously (Figure 15B) or in the reverse order (Figure 15D). Washing the cells prior to the addition of gD-deficient virus did not remove adsorbed sgD since cell-associated sgD was clearly observed at this stage by immuno-fluorescence using a pool of monoclonal anti-gD antibodies (75) (Figure 15F). These results suggested that sgD may undergo a transient conformational change and fall back to an inactive state before virus accumulation at the cell surface is adequate for sgD-mediated entry.

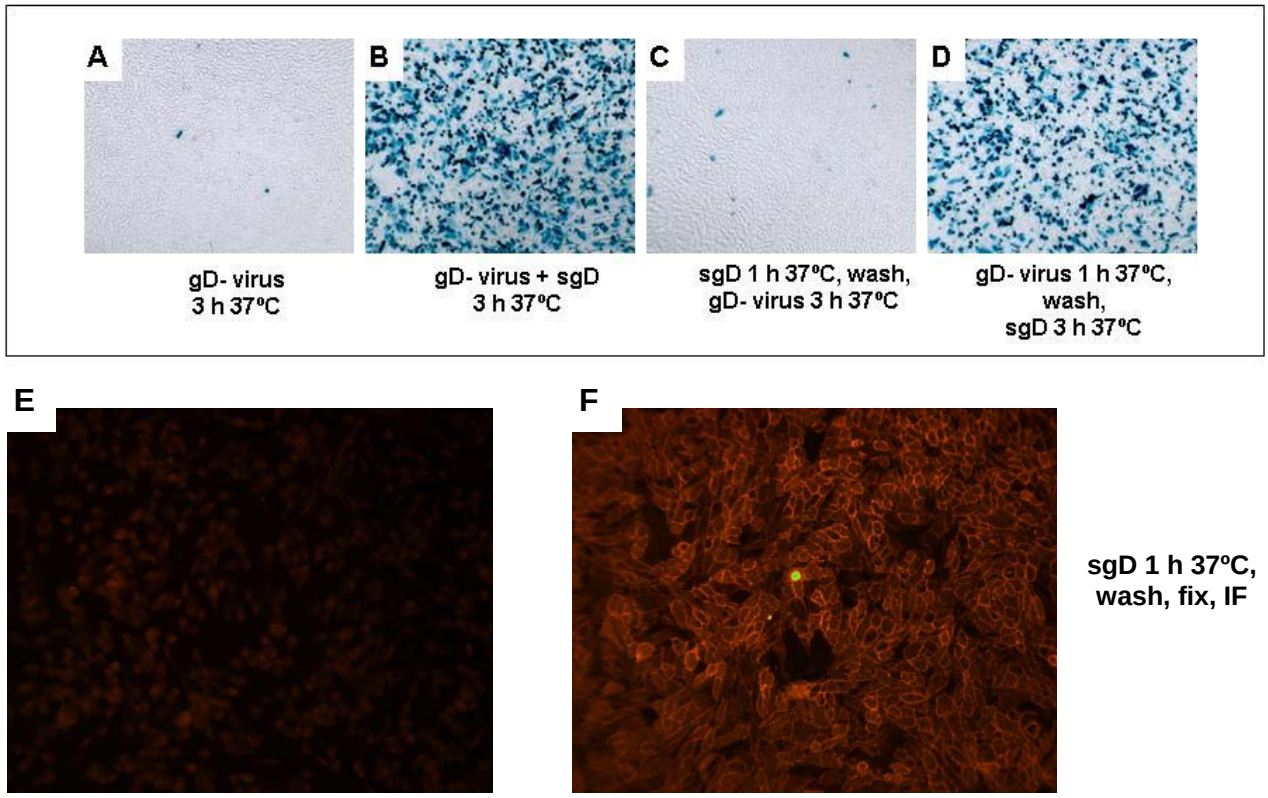


Figure 15. Mechanistic requirements of sgD-mediated entry.

Entry of gD$^-$ KΔUs3-8Z virus was assayed on CHO-HVEM cells under various conditions, and sgD binding to the cells was examined by immunofluoresence. (A) Cells were incubated with gD$^-$ KΔUs3-8Z alone at 37ºC for 3 h. (B) sgD and gD$^-$ KΔUs3-8Z were added to cells simultaneously and incubated with the cells for 3 h at 37ºC. (C) Cells were pre-incubated with sgD at 37ºC for 1 h, washed, and incubated with gD$^-$ KΔUs3-8Z for 3 h. (D) Cells were pre-incubated with KΔUs3-8Z for 1 h at 37ºC, washed, and incubated with sgD at 37ºC for 3 h. (E, F CHO-HVEM cells were incubated for 1 h at 37ºC without (E) or with sgD (F), the cells were washed with buffer and then fixed for 5 min with 2% paraformaldehyde, and sgD was detected at the cell surface by sequential incubation with a pool of monoclonal anti-gD antibodies (75) and Cy3-conjugated anti-mouse secondary antibody.

To test this interpretation, sgD binding to the cells was performed at 4ºC in an attempt to separate receptor binding from secondary events, such as conformational changes, tha may occur only at 37ºC. After removal of free sgD with cold buffer, gD-deficient virus was added to the

monolayer and the cells were shifted to 37ºC for the usual 3 h infection. By this protocol, infection remained much lower than following simultaneous addition of virus and sgD (Figure 16, compare A and B). This result was consistent with instability of the receptor-induced active conformation of sgD, diminishing the availability of the active form prior to abundant virus adsorption to the cells. To determine whether saturated virus attachment preceding the shift to 37ºC would restore entry, we incubated the cells sequentially with sgD and gD-deficient KΔUs3-8Z at 4ºC, washing after each incubation, and then raised the temperature to 37ºC. Under these conditions, the infection level approached that observed after simultaneous addition of virus and sgD (Figure 16, compare A and C). Together, these results indicated that sgD binding to HVEM produces a receptor for gD-deficient virus that is unstable at 37^{o}C. Our results do not show whether this receptor requires the increased temperature to assume its active state, but they do suggest that this active state is short-lived at the higher temperature.

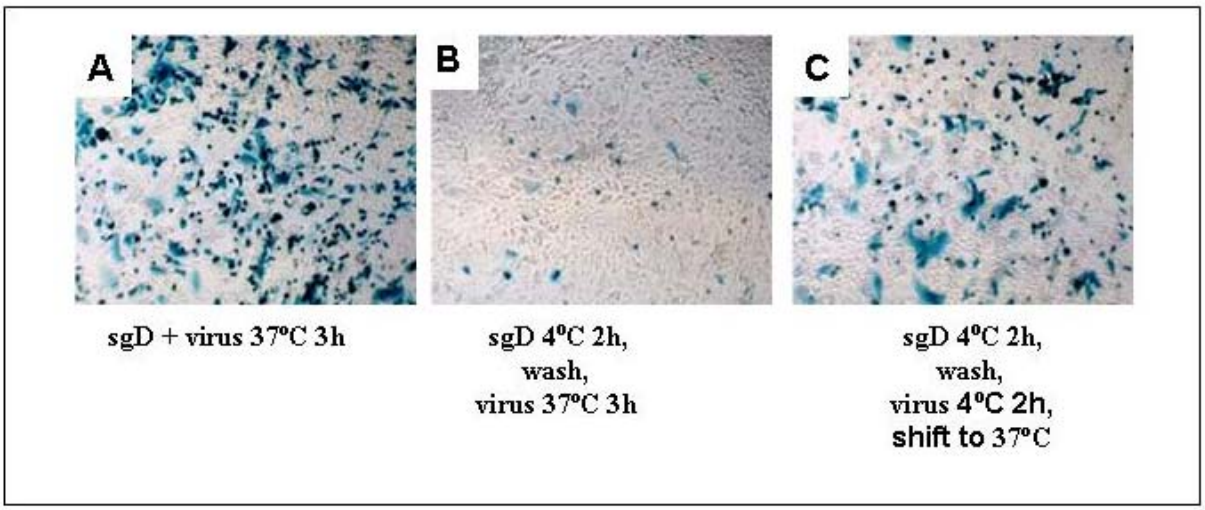


Figure 16. Pre-adsorption of gD$^-$ KΔUs3-8Z to CHO-HVEM cells pre-incubated with sgD improves infection efficiency.

(A) sgD and gD$^-$ KΔUs3-8Z were added to CHO-HVEM cells simultaneously and incubated with the cells for 3 h at 37ºC. (B) Cells were pre-incubated with sgD for 2 h at 4ºC and then washed to remove unbound sgD. gD$^-$ KΔUs3-8Z was added and the cells were incubated at 37ºC for 3 h. (C) Cells were pre-incubated with sgD for 2 h at 4ºC, washed, and incubated with gD$^-$ KΔUs3-8Z for an additional 2 h at 4ºC. The cells were then washed again and shifted to 37ºC for 3 h. After the 3 h infection period, fresh media was added to all wells, and the cells were X-gal staining 16 h later.

To determine the inactivation kinetics of receptor-bound sgD, we shifted the cells to 37ºC for various times between the sgD and virus adsorption steps performed at 4^{o}C. Our results showed that entry was diminished after 5 min at 37ºC, and completely eliminated after 30 min (Figure 17). Thus, the inactivation was not instantaneous, as would be expected for an inactivating conformational change, suggesting that the receptor-bound, active conformation of sgD was stable at the infection temperature. Thus, we hypothesized that other processes were responsible for the decreasing activity of pre-bound sgD at 37^{o}C, such as internalization of the sgD-receptor complex, sgD dissociation, or degradation at the cell surface.

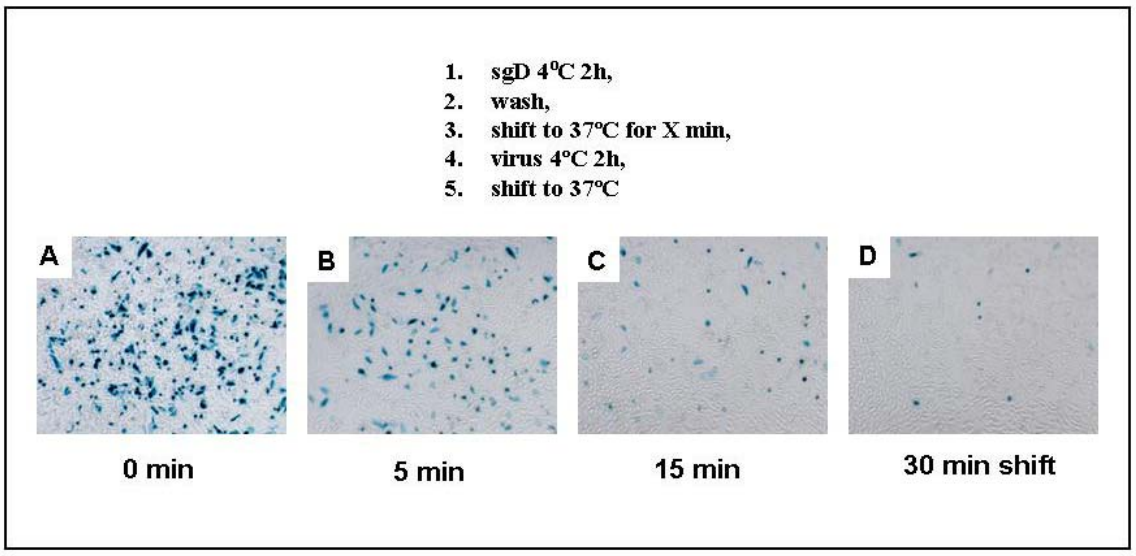


Figure 17. Time required for sgD inactivation.

CHO-HVEM cells were pre-incubated with sgD for 2 h at 4ºC, the cells were washed, and either gD^- virus was added right away and incubated with the cells for 2 h at 4ºC (A), or the cells were shifted to 37ºC for 5 (B), 15 (C) or 30 min (D), and then incubated with the virus for 2 h at 4ºC. All cells were shifted to 37ºC to allow for infection.

To explore these options, we determined whether sgD binding to the cells was stable at 37ºC in the absence of excess sgD in the media. CHO-HVEM cells were incubated with sgD for 2 h at 4ºC and washed to remove free sgD. The cells were then either fixed immediately (Figure 18A), or shifted to 37ºC for 30 min and then fixed (Figure 18B). Cell-associated sgD was detected by immuno-fluorescence using a pool of monoclonal anti-gD antibodies (75). The results showed a dramatic reduction in signal following incubation at 37ºC (Figure 18B). The same phenomenon was observed using CHO-nectin-1 cells, demonstrating that the effect was not limited to HVEM-bound sgD (Figure 18C and D).

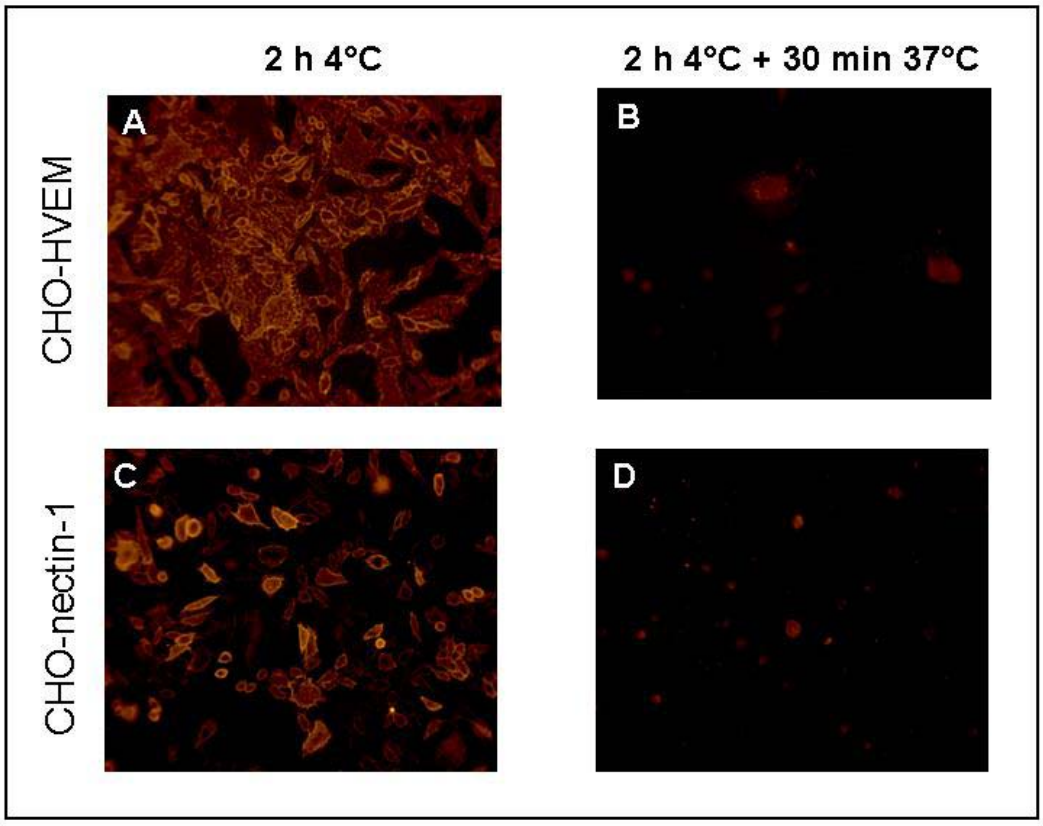


Figure 18. Detection of cell-surface-associated sgD after incubation at 37ºC.

sgD was bound to CHO-HVEM (A and B) or CHO-nectin-1 (C and D) cells at 4ºC for 2 h. Cells were washed with cold buffer, and either fixed immediately (A and C) or shifted to 37ºC for 30 min and then fixed (B and D). Receptor-bound sgD was detected at the cell surface with a pool of monoclonal anti-gD antibodies (75), as described earlier.

In light of recent evidence showing endocytic HSV uptake into CHO cells (126, 128), we were interested in distinguishing whether the observed loss of cell-associated sgD at 37^{o}C could be ascribed to receptor-mediated endocytosis or to other processes, such as proteolytic degradation at the cell surface or dissociation from the cognate receptor in the absence of abundant free sgD. To this end, CHO-HVEM cells were incubated with sgD at 4ºC for 2 h, free sgD was removed, and the cells were lysed either immediately or after incubation at 37ºC for 10

or 30 minutes. Duplicate samples were treated in succession with trypsin and soybean trypsin inhibitor prior to cell lysis to enable identification of internalized sgD. Western blot analysis using gD-specific antibodies showed a loss of cell-associated sgD over time (Figure 19A, lanes 2, 6, 10). Trypsin effectively removed all sgD from the cell surface (Figure 19A, lane 4), and no trypsin-resistant material was detected at either the 10- or 30-min time points (Figure 19A, lanes 8 and 12); in separate experiments, the effectiveness of soybean trypsin inhibitor in eliminating all trypsin activity prior to cell lysis was confirmed (Figure 19C, compare lanes 2 and 4). To determine whether the loss of cell-surface sgD over time could be attributed to dissociation from the receptor, media samples were included in the Western blot analysis of Figure 19A (lanes 14, 15). sgD was detected in both samples, each representing one-eighth of the total supernatant at the given time point. Judging from a separate experiment using TCA precipitation to collect sgD from the entire supernatant, the amount of sgD released into the media after 30 min at 37^{o}C was approximately equal to the amount of cell-associated sgD at the start (Figure 19B, compare lanes 1 and 3). These results demonstrated that sgD dissociated from the HVEM receptor after excess sgD was removed from the media, thereby providing a satisfactory explanation for the observation that pre-bound sgD could mediate entry of pre-attached virus, but not of virus that was added at the infection temperature.

To test whether the presence of gD-deficient KΔUs3-8Z would either stabilize sgD at the cell-surface or cause its internalization, gD^{-} virus was included at each of the time points described above. The amount of gD^{-} virus applied to the wells was the same as that normally used for infection of CHO-HVEM cells and was more than sufficient to detect bound virus by immunofluorescence using a pool of monoclonal anti-gC antibodies (76) (Figure 20). Our results showed that sgD was not detected in the interior of the cell in the presence of the virus (Figure

19A lanes 9 and 13). In addition, sgD was not detectably stabilized at the cell surface in the presence of virus (Figure 19A, lanes 7 and 11).

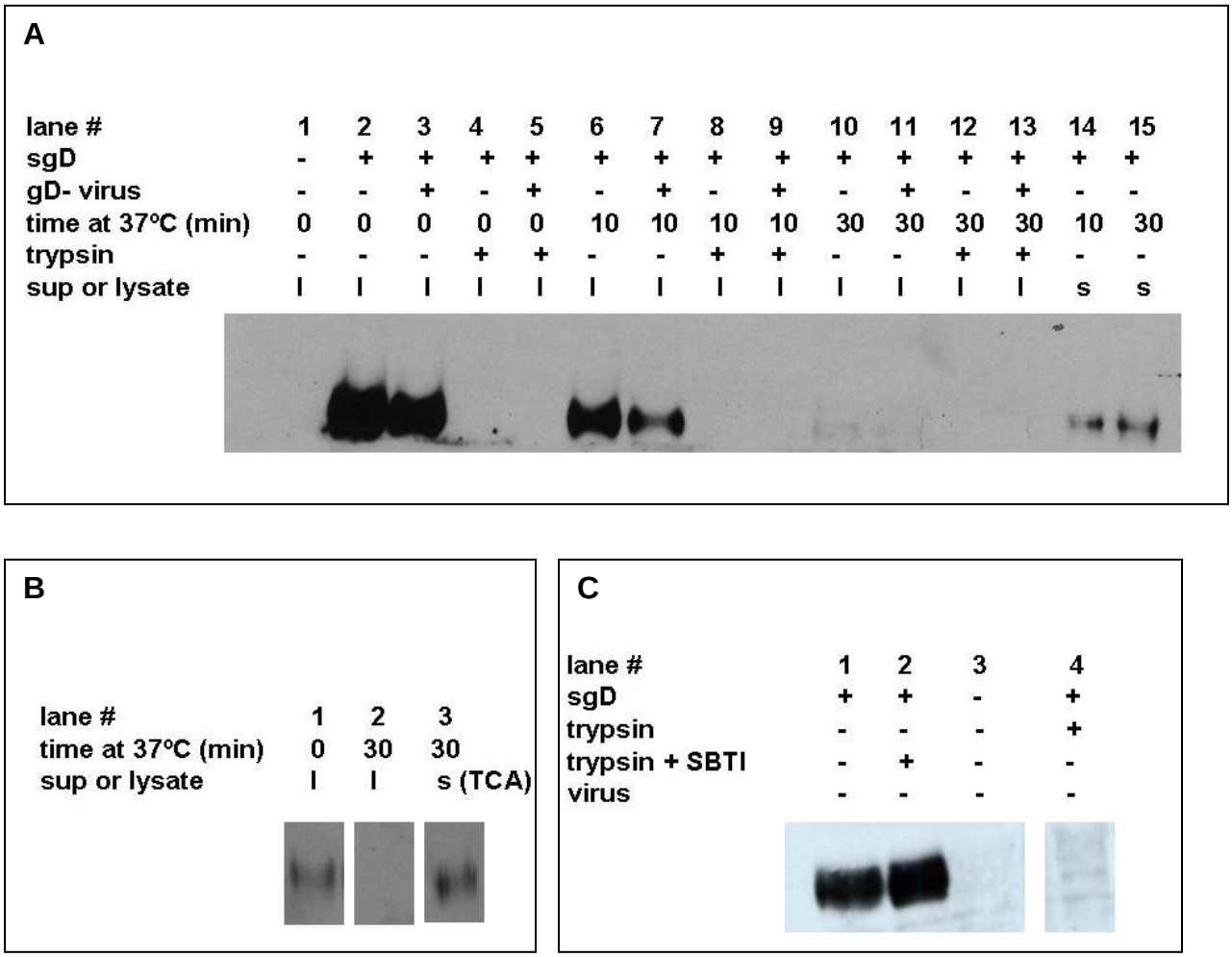


Figure 19. sgD localization.

(A) sgD was bound to CHO-HVEM cells for 2 h at 4ºC either alone (lanes 2, 4, 6, 8, 10 and 12) or in the presence of gD-deficient virus (lanes 3, 5, 7, 9, 11 and 13). Cells were washed with cold buffer and either lysed immediately (lanes 1-5) or incubated with fresh, pre-warmed media at 37ºC for 10 (lanes 6-9) or 30 min (lanes 10-13) before lysis. To identify internalized sgD (lanes 4, 5, 8, 9, 12 and 13), cell-bound sgD was removed with 100 ug/ml TPCK trypsin, followed by quenching with soybean trypsin inhibitor, prior to cell lysis. Supernatants were collected from reactions 6 and 10 at the end of the 37ºC incubation period (10 and 30 min, respectively), and a fraction ($1/8^{th}$) of each was loaded on the gel (lanes 14 and 15). Lysate from CHO-HVEM cells without sgD or virus was applied as a control (lane 1). (B) sgD was bound to CHO-HVEM cells for 2 h at 4ºC. The cells were then washed and lysed either immediately (lane 1) or after incubation at 37ºC for 30 min with fresh, pre-warmed medium (lane 2). The medium was collected at the end of the 37ºC incubation and its protein content precipitated with TCA (lane 3).

(C) sgD was adsorbed to CHO-HVEM cells for 2 h at 4ºC, and the cells were washed with cold buffer to remove unbound sgD. Lane 1, cells were collected by scraping and centrifugation, and resuspended in lysis buffer. Lane 2, trypsin (100 μg/ml) and soybean trypsin inhibitor (SBTI, 200 μg/ml) were added to the cells simultaneously before collecting the cells. Lane 3, lysate of CHO-HVEM cells without sgD. Lane 4, CHO-HVEM cells pre-incubated with sgD, as above, were incubated with trypsin in pre-warmed medium at 37ºC for 2.5 min. SBTI was then added, and the cells were collected and resuspended as above.

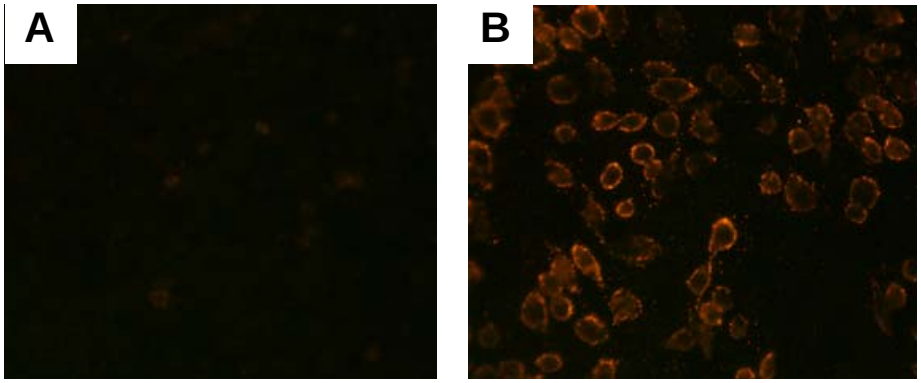


Figure 20. Detection by gC antibodies of gD$^-$ virus bound to CHO-HVEM cells.

gD-deficient KΔUs3-8Z was adsorbed to CHO-HVEM cells for 2 h at 4ºC. Cells were then washed with cold media to remove unbound virus, and fixed with 2% paraformaldehyde. Bound virus was detected by incubation with a pool of anti-gC monoclonal antibodies (B) (76) followed by anti-mouse Cy3 conjugated secondary antibody. Cells alone, incubated with the same antibodies, were used as a control (A).

6. The Role of GAGs in sgD-Mediated and Wild-Type Virus Entry

6.1. sgD- Mediated Entry into GAG-Deficient Cells

Since gD-deficient HSV is unable to bind to cells through the gD-receptor interaction, we expected that the presence of GAGs at the cell surface would be an absolute requirement for the purpose of virus attachment in sgD-mediated entry. To test this suggestion, a GAG-deficient cell line expressing nectin-1 (pgsA745-nectin-1) was generated. This line was derived by stable transfection of pgsA745 cells, a CHO mutant line that is defective in GAG synthesis (39), with a nectin-1 expression plasmid. Flow-cytometry analysis using nectin-1-specific monoclonal antibody CK41 (92) confirmed the presence of nectin-1 on the surface of pgsA745-nectin-1 cells. Quantitative analysis further demonstrated that the proportion of cells expressing surface nectin-1 was substantially greater for the pgsA745-nectin-1 line than for the GAG-expressing CHO-nectin-1 line used in earlier parts of our study (Figure 21A and B). In addition, pgsA745-nectin-1 cells expressed more surface nectin-1 per cell than their CHO-nectin-1 counterparts.

To examine the HSV susceptibility of this new cell line, a virus that had the full complement of envelope glycoproteins, including gD, was used. KHZ.1 is a KOS-derived virus containing a lacZ reporter gene in the thymidine kinase (tk) locus (115). The results showed that GAG^+ CHO-nectin-1 cells were substantially more susceptible to KHZ.1 infection than pgsA745-nectin-1 cells (Figure 21C and D), consistent with the well-documented finding that efficient HSV infection depends on virus attachment to cell-surface GAGs (3, 62, 70, 71, 96,

166). These results suggested that elevated nectin-1 levels do not fully compensate for the absence of GAGs in HSV infection.

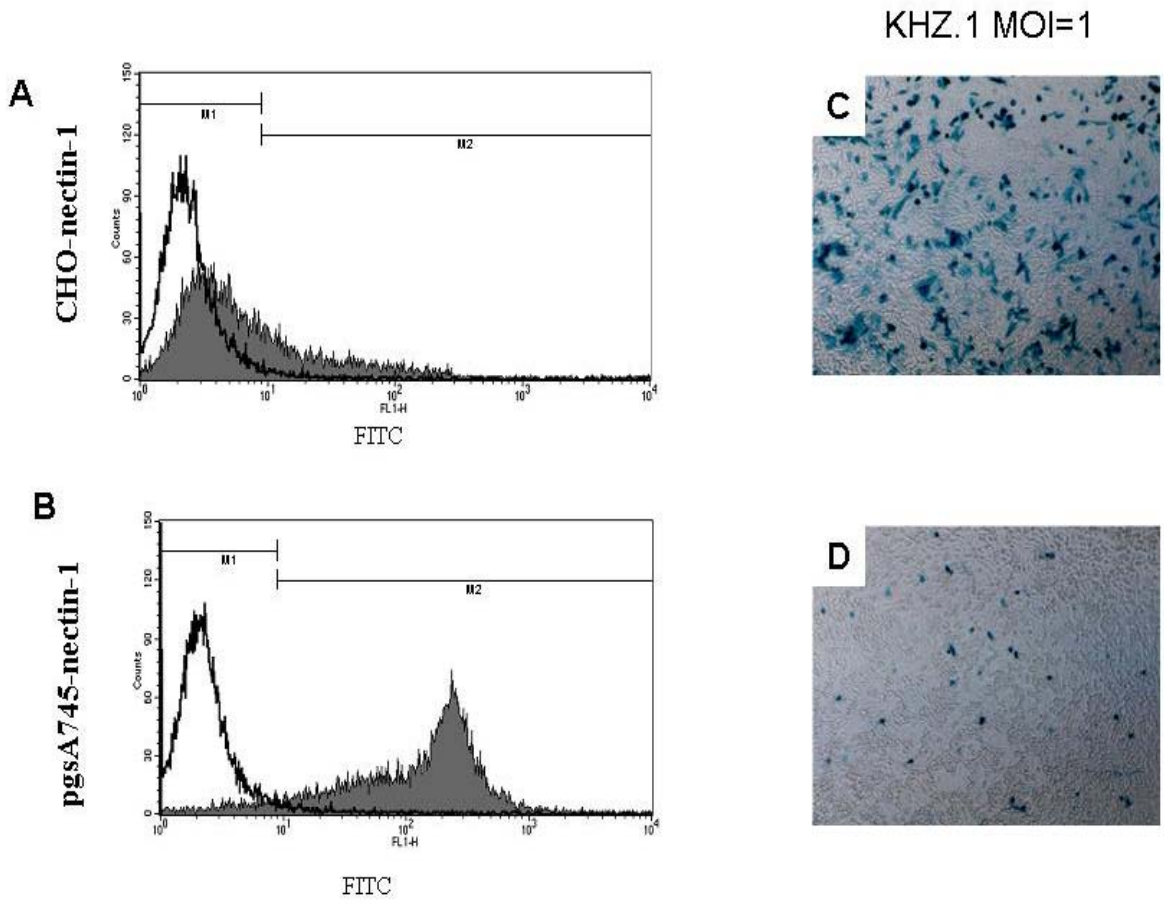


Figure 21. Characterization of pgsA745-nectin-1 cells.

CHO-nectin-1 (A) and pgsA745-nectin-1 (B) cells were incubated with CK41 monoclonal antibody at 4ºC, followed by an anti-mouse FITC conjugated antibody. Receptor levels on each cell line were quantified by flow cytometry. Entry of a KOS-derived virus containing a *lacZ* expression cassette in the tk locus, KHZ.1 (115), was tested on CHO-nectin-1 (C) and pgsA745-nectin-1 (D) cells by infecting cells for 2 hours at 37ºC at an MOI of 1. Infected cells were visualized by X-gal staining 16 hours post infection.

We next tested the ability of gD-deficient KΔUs3-8Z to enter the GAG-deficient pgsA745-nectin-1 cells in the presence of sgD was tested. Remarkably, pgsA745-nectin-1 cells were permissive for sgD-mediated entry despite the absence in this system of any known

mechanism for virus attachment (Figure 22C and D). Moreover, infection was typically more abundant with these GAG-deficient cells than with the GAG-expressing CHO-nectin-1 line (Figure 22, compare D and F). One possible explanation for the increased susceptibility of our pgsA745-nectin-1 cells to sgD-mediated infection is the elevated levels of nectin-1 on these cells, perhaps suggesting a greater role for the gD receptor in sgD-mediated infection than in infection utilizing virion gD.

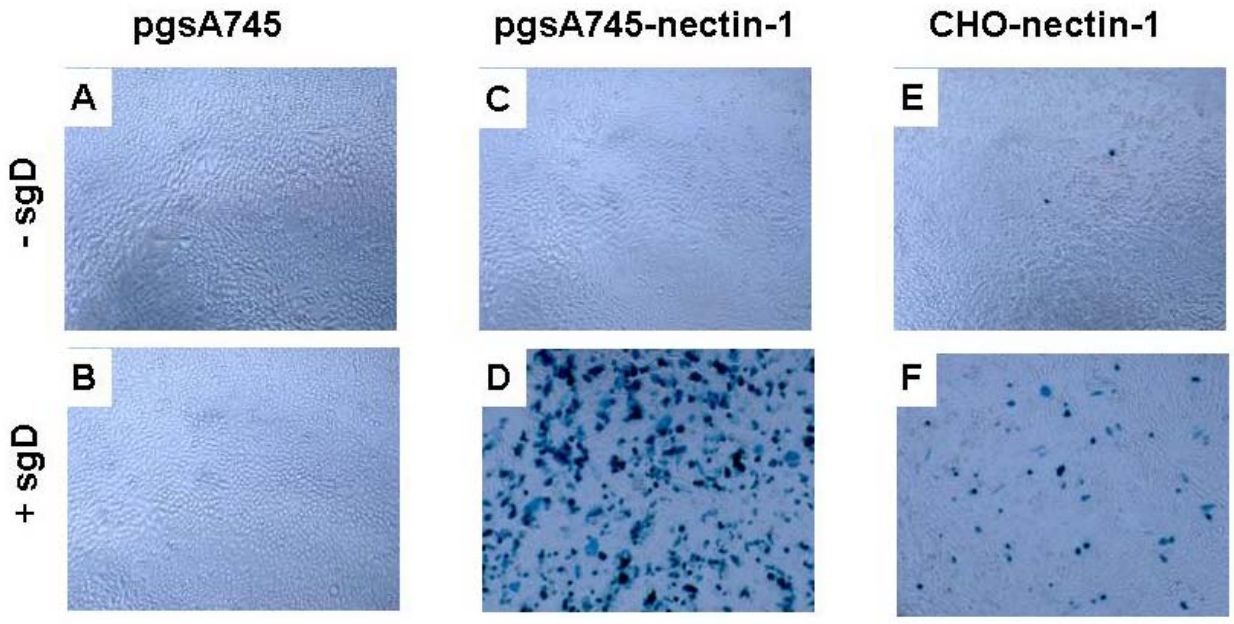


Figure 22. sgD-mediated infection of GAG-deficient pgsA745-nectin-1 cells.

gD-receptor-deficient pgsA745 (A and B), pgsA745-nectin-1 (C and D), and CHO-nectin-1 (E and F) cells were infected with gD-deficient KΔUs3-8Z virus in the presence or absence of sgD for 3 h at 37°C. Entry was detected by X-gal staining the cells after 16 h.

6.2. Binding of gD-Deficient Virus to GAG-Deficient Cells

The unexpected occurrence of virus entry in the absence of GAGs and virion gD suggested that virus attachment to the cell could take place independent of the known virus binding

mechanisms. We sought to determine whether this phenomenon reflected (i) the formation by sgD of an attachment bridge between virion glycoproteins and cell-surface receptors, specifically nectin-1, or (ii) a novel binding interaction between the virus and cell that was independent of gD or GAGs. To discriminate between these two possibilities, the amount of gD-deficient virus bound to pgsA745-nectin-1 cells was measured by Western blot analysis. The cells were incubated with gD-deficient KΔUs3-8Z for 2 h at 4ºC in the presence or absence of sgD, washed to remove unbound virus and sgD, and lysed. Western-blot probing with NC1 antibody reactive with the major capsid protein VP5 (26) revealed cell-associated virus (Figure 23A, lane 3), implying a previously unknown binding interaction. The presence of sgD did not detectably alter the amount of bound virus (lane 2), indicating that sgD interaction with its receptor did not significantly contribute to virus binding at 4^{o}C. These results suggested that sgD-mediated infection of pgsA745-nectin-1 cells involved an sgD-independent binding step utilizing a novel interaction between the virus and cells.

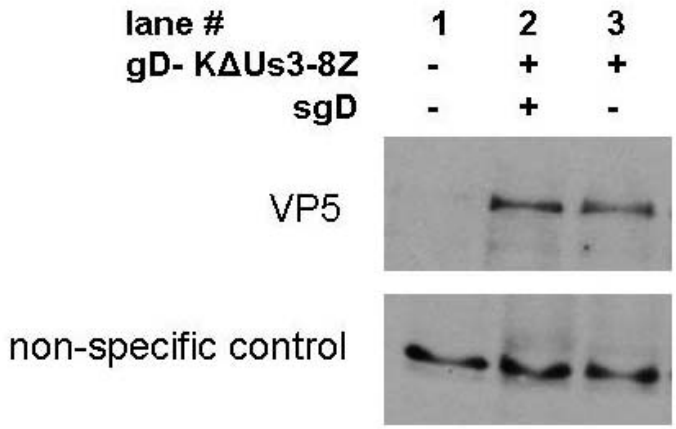


Figure 23. Binding of gD-deficient KΔUs3-8Z virus to GAG-deficient pgsA745-nectin-1 cells.

pgsA745-nectin-1 cells were incubated with gD-deficient KΔUs3-8Z alone (lane 3) or in the presence of sgD (lane 2) at 4°C for 2 h. Cells were then washed, lysed and analyzed by Western blot using anti-VP5 antibody NC1 (26). A prominent, non-specific cellular band is shown as a loading control.

6.3. Characterization of the Novel Binding Interaction

To determine whether the ability to bind GAG-deficient cells was unique to gD-deficient HSV, the amount of gD-deficient KΔUs3-8Z and KHZ.1 bound to GAG^+ and GAG^- cells was determined. KHZ.1 was adsorbed to pgsA745, pgsA745-nectin-1, CHO-K1 and CHO-nectin-1 cells at 4ºC for 2 h, unbound virus was removed, and cell lysates were analyzed by Western blot using anti-VP5 antibody. The results showed that virus containing envelope-anchored gD, like gD-deficient virus, bound to GAG-deficient pgsA745 cells (Figure 24A, lane 3), although much less abundantly than to GAG-containing CHO-K cells (Figure 24A, lanes 1 and 2). KHZ.1 binding to nectin-1 expressing pgsA745-nectin-1 cells was improved over binding to nectin-1

deficient pgsA745 cells (Figure 24A, compare lanes 3 and 4), demonstrating that the nectin-1-gD interaction does play a role in virus attachment in the absence of GAGs, although not clearly in the presence of GAGs (Figure 24A, compare lanes 1 and 6 or lanes 2 and 5). In agreement with the KHZ.1 entry data (Figure 21), the amount of KHZ.1 bound to nectin-1 bearing pgsA745 cells was lower than the amount bound to GAG-expressing CHO-K1 cells (Figure 24A, compare lanes 1 and 4), showing that elevated levels of nectin-1 do not compensate for the absence of GAGs in HSV binding.

Since sgD-mediated entry was not affected by the absence of cell-surface GAGs, the amounts of gD-deficient virus bound to GAG^+ and GAG^- cells were compared. gD-deficient KΔUs3-8Z was adsorbed to GAG-deficient pgsA745 and pgsA745-nectin-1 cells and to GAG-expressing CHO-K1 and CHO-nectin-1 cells. Bound KΔUs3-8Z was detected by Western blot as described above. The results showed that the presence of cell-surface GAGs did contribute to the binding of gD-deficient virus (Figure 24B, compare lanes 1 and 2, lanes 3 and 4). Thus, the comparable levels of sgD-mediated entry into GAG^+ and GAG- nectin-1 expressing cells could not be attributed to equal virus binding. Interestingly, the ratio of virus bound to CHO-K1 compared to pgsA745 appeared to be much higher for KHZ.1 than for gD-deficient KΔUs3-8Z (Figure 24A, compare lanes 1 and 3, with Figure 24B, lanes 1 and 2). It is uncertain whether this difference reflects increased binding of gD-deficient KΔUs3-8Z to pgsA745 cells compared to KHZ.1, decreased binding to CHO-K1 cells, or a combination of these two factors.

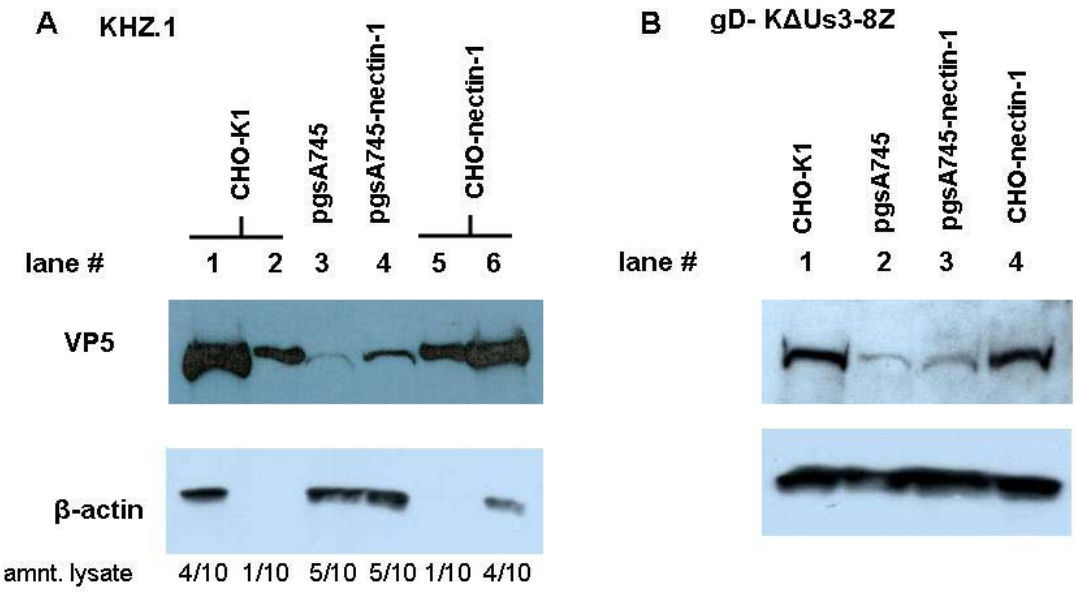


Figure 24. Comparison of KHZ.1 and gD$^-$ KΔUs3-8Z binding to GAG-deficient and GAG-expressing cells.

CHO-K1 (lanes 1 and 2 in A, lane 1 in B), pgsA745 (lane 3 in A, 2 in B), pgsA745-nectin-1 (lane 4 in A, 3 in B) and CHO-nectin-1 (lanes 5 and 6 in A, 4 in B) cells were incubated with gD$^+$ KHZ.1 at MOI=100 (A) or gD$^-$ KΔUs3-8Z (B) for 2 h at 4ºC. The cells were then washed to remove un-bound virus, lysed, and analyzed by Western blot. Bound virus was detected by probing membranes with the anti-VP5 antibody NC1 (26). 2/5th of the total lysate was loaded in lanes 1 and 6 and 1/10th in lanes 2 and 5 of gel A. ½ of the total lysate was loaded in all the other lanes. B-actin was used as a loading control.

To test the role of the GAG- and gD-independent cell association of gD-deficient virus in sgD-mediated infection, we examined whether virus entry could be observed when the binding and entry steps were separated. As shown in Figure 25, entry was severely reduced when gD-deficient virus was incubated with pgsA745-nectin-1 cells at either 37ºC for 1 h (panel C) or at 4ºC for 2 h (panel D) prior to the removal of free virus and infection at 37^{o}C in the presence of sgD. Entry was marginally increased when sgD was included in the virus-binding step at 4ºC (panel E), and somewhat further enhanced when fresh sgD was additionally included at the 37ºC infection stage (panel F). These results suggested that gD-deficient virus that is bound to GAG$^-$,

nectin-1 expressing cells through the novel binding interaction becomes rapidly unavailable to interact with sgD that is added subsequently.

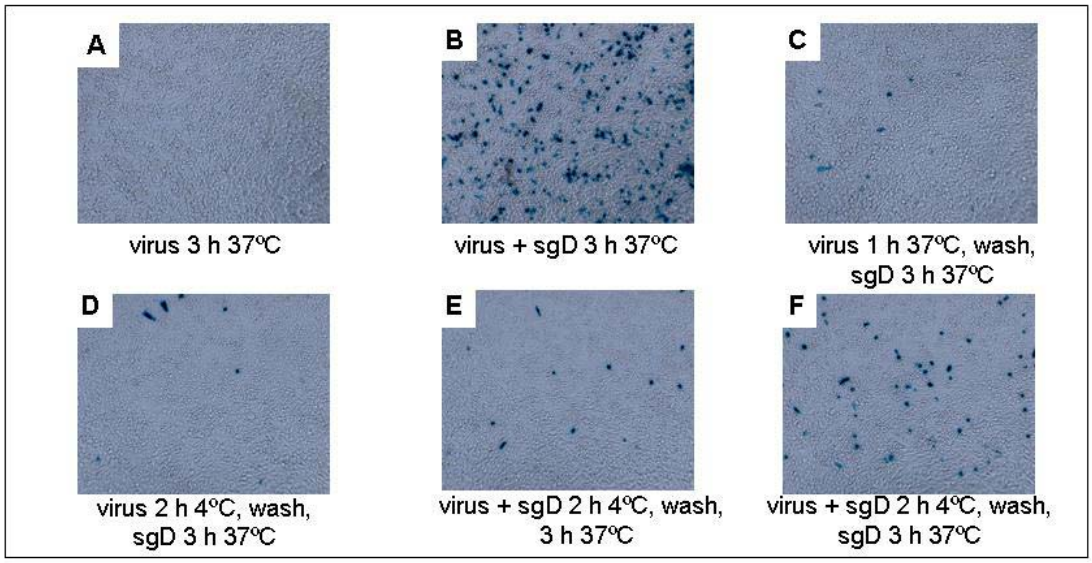


Figure 25. Role of novel binding interaction in entry of gD-deficient KΔUs3-8Z.

sgD-mediated entry into pgsA745-nectin-1 cells was analyzed under various conditions. (A) Virus alone was added to cells for 3 h at 37ºC. (B) Virus and sgD were added together for a 3 h infection period at 37ºC. (C) Virus was pre-incubated with cells for 1 h at 37ºC, cells were washed, and sgD was added for 3 h at 37ºC. (D) Virus was adsorbed to cells at 4ºC for 2 h, unbound virus was removed by washing the cells with cold buffer, and sgD was added for 3 h at 37ºC. (E) Virus was adsorbed to cells in the presence of sgD for 2 h at 4ºC, cells were washed, and shifted to 37ºC for 3 h. (F) Virus was adsorbed to cells in the presence of sgD for 2 h at 4ºC, cells were washed, and fresh sgD was added to the cells for 3 h at 37ºC. Infected cells were identified by X-gal staining 16 h after infection.

We hypothesized that these observations may be due to a less stable nature of the novel binding interaction, when compared to the virus interaction with cell-surface GAGs. To investigate this hypothesis, the dissociation of gD-deficient KΔUs3-8Z from CHO-HVEM cells and pgsA745-nectin-1 cells was compared. gD-deficient KΔUs3-8Z was adsorbed to the cells at 4ºC for 2 h, unbound virus was removed and the cells were either lysed immediately or shifted to 37ºC for 10 or 30 min and then lysed. The amount of virus associated with the cells was

determined by Western blot analysis as described above. Virus associated with the GAG-deficient cells decreased gradually over time at 37ºC (Figure 26B) while the amount of virus associated with GAG-expressing cells remained constant (Figure 26A). To determine whether this difference could also be observed with virus containing envelope-anchored gD, the experiment was repeated with KHZ.1 using CHO-K1 and pgsA745 cells. The results were similar to those obtained with gD-deficient virus (Figure 26C and D), demonstrating that binding through the novel binding interaction is less stable than GAG-mediated binding of HSV.

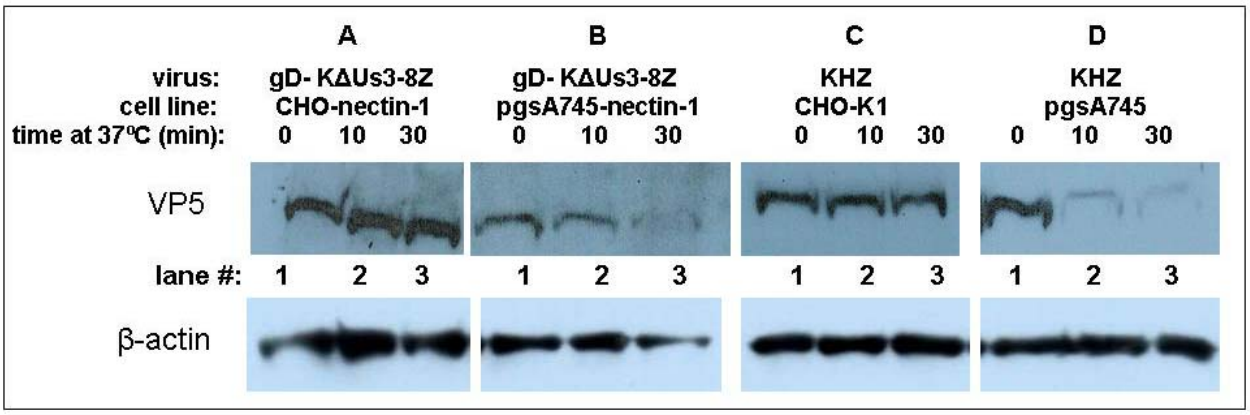


Figure 26. Stability of the novel binding interaction.

CHO-HVEM (A), pgsA745-nectin-1 (B), CHO-K1 (C), and pgsA745 (D) cells were incubated with gD-deficient KΔUs3-8Z (A and B) or KHZ.1 at MOI=100 (C and D) for 2 h at 4ºC, washed to remove un-bound virus, and either lysed immediately (lanes 1) or shifted to 37ºC for 10 (lanes 2) or 30 min (lanes 3), and then lysed. ½ of all the lysates were analyzed by Western blot. Membranes were probed with anti-VP5 NC1 antibody to identify the amount of bound virus in each sample, and with anti-β-actin as a loading control.

7. ENDOCYTOSIS

7.1. Introduction

As stated earlier, several studies have recently presented evidence suggesting that HSV entry into certain cell types, including CHO cells, occurs through endocytosis (56, 119, 125, 126, 128). These studies suggested that HSV infection of CHO cells occurs through a pH-dependent endocytic pathway by showing that lysosomotropic drugs inhibit productive infection, while entry into Vero cells, in which the original HSV entry pathway studies were conducted, was not affected (126, 128). In one study, Nicola et al. showed that internalization into CHO cells does not require the presence of a gD-receptor (128). Since a gD-receptor is required for productive infection of these cells, these authors hypothesized that the receptor is required for release of virus from the endocytic compartment. In light of these observations, we wished to determine whether the pathway of sgD-mediated entry into CHO cells was different from that used by gD-containing virus with the aim of gaining a better understanding of the role of gD in endocytosis and fusion at the cell surface or endosomal membrane.

7.2. Results

7.2.1. Inhibition with lysosomotropic agents

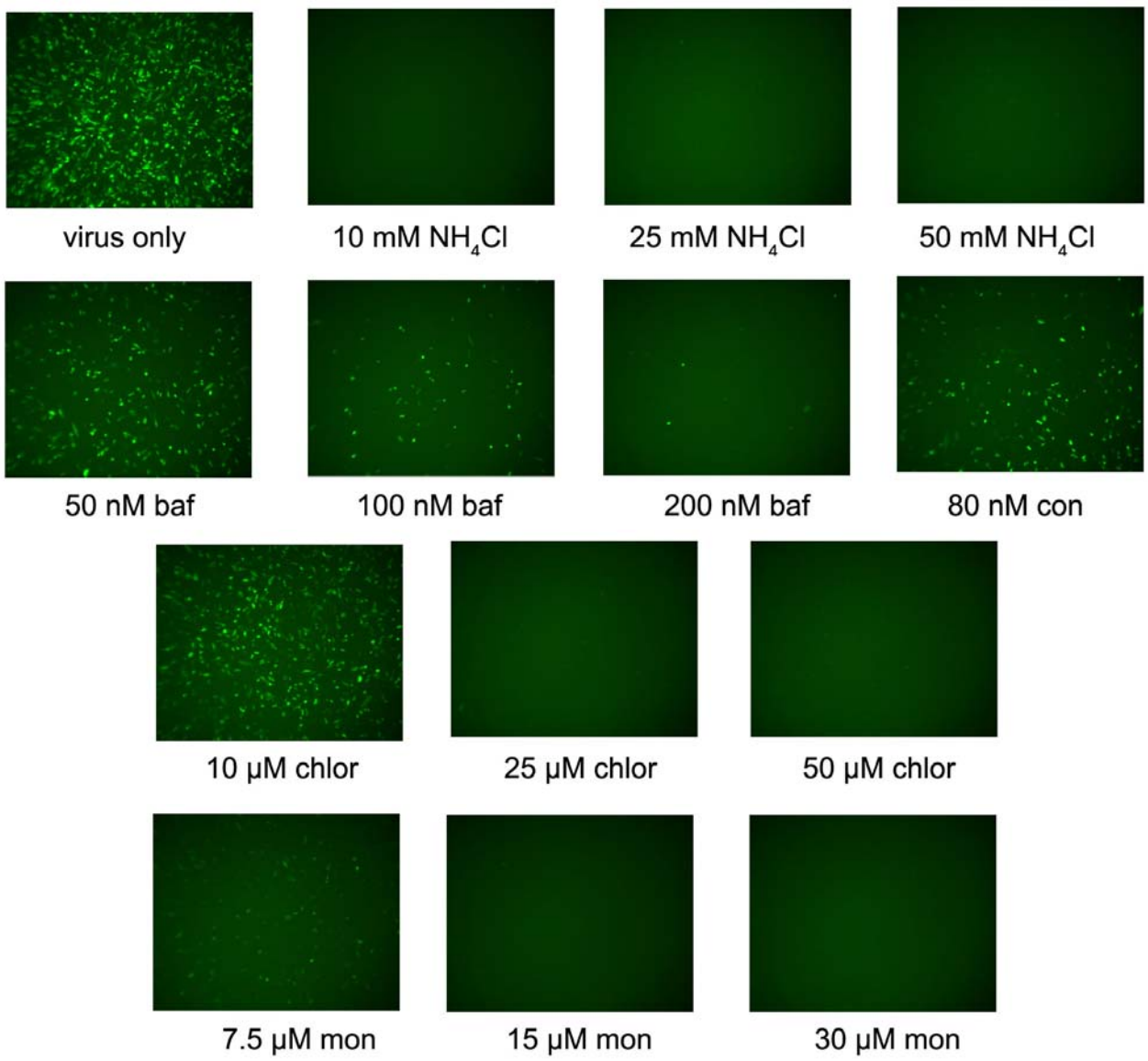
As the first step in our investigation of the sgD-mediated entry pathway, we attempted to reproduce the observations of Nicola et al. (126, 128) that productive HSV infection of CHO

cells is blocked by lysosomotropic agents. To this end, several recombinant viruses that contain envelope anchored gD were tested [KHZ.1, the replication-defective recombinant QOZHG, and gD-complemented KΔUs3-8Z] for the effects of NH_4Cl and monensin, using the same protocol and conditions as was described by Nicola et al. (126). Cells were pre-incubated with the drugs, virus was added, and β–gal expression was assayed by X-gal staining or ONPG 6-9 h after initiating infection, depending on the virus. The results with each virus varied greatly from experiment to experiment, from no inhibition at all on CHO-HVEM and Vero cells, to partial inhibition on both cell lines (data not shown). In most instances in which partial inhibition was observed on CHO-HVEM cells, some inhibition, although to a lesser degree, was also evident on Vero cells.

Given the variability observed with NH_4Cl and monensin, the repertoire of lysosomotropic agents was expanded to include the specific vacuolar ATPase inhibitors bafilomycin A1 and concanamycin A, and the weak base chloroquine, which prevents endosomal acidification by buffering the endosomal pH. Bafilomycin A1 yielded results that were similar to those obtained with NH_4Cl and monensin, and a high concentration of 400 nM was required to achieve even partial inhibition. Concanamycin A and chloroquine did not inhibit entry of any of the viruses tested into either cell line. Numerous conditions were tested, including various drug concentrations, pre-incubation times, glycine treatment after infection, pH adjustments, and inclusion of serum, none of which improved the reproducibility of these experiments. J1.1-nectin-1 cells created in our laboratory were also extensively tested. A similar line that was created in the laboratory of G. Campadelli-Fiume is reportedly resistant to inhibition by lysosomotropic agents and is infected by HSV via fusion at the cell surface (56). As with CHO-HVEM and Vero cells, inconsistent results were obtained with our J1.1-nectin-1 cells (data not

shown). In contrast, endocytic entry of VSV (111) was easily confirmed using the same drugs, as illustrated by representative data in Figure 27. In these experiments, CHO-HVEM and J1.1-nectin-1 cells were pre-incubated with drugs for 30 min and infected with green fluorescent protein (GFP)-expressing VSV at an MOI of 1 in the continued presence of drugs for 1 h. Entry was detected at 7 hpi. Our results showed that VSV infection was consistently inhibited at low concentrations of the different drugs. The lone exception was concanamycin A, which required a relatively high concentration (80 nM) to achieve partial inhibition. Thus, in our experience, treatment of cells with lysosomotropic agents is a reliable approach to establishing the entry pathway of VSV, but not HSV.

A


virus only
10 mM NH_4Cl
25 mM NH_4Cl
50 mM NH_4Cl
50 nM baf
100 nM baf
200 nM baf
80 nM con
10 µM chlor
25 µM chlor
50 µM chlor
7.5 µM mon
15 µM mon
30 µM mon

B

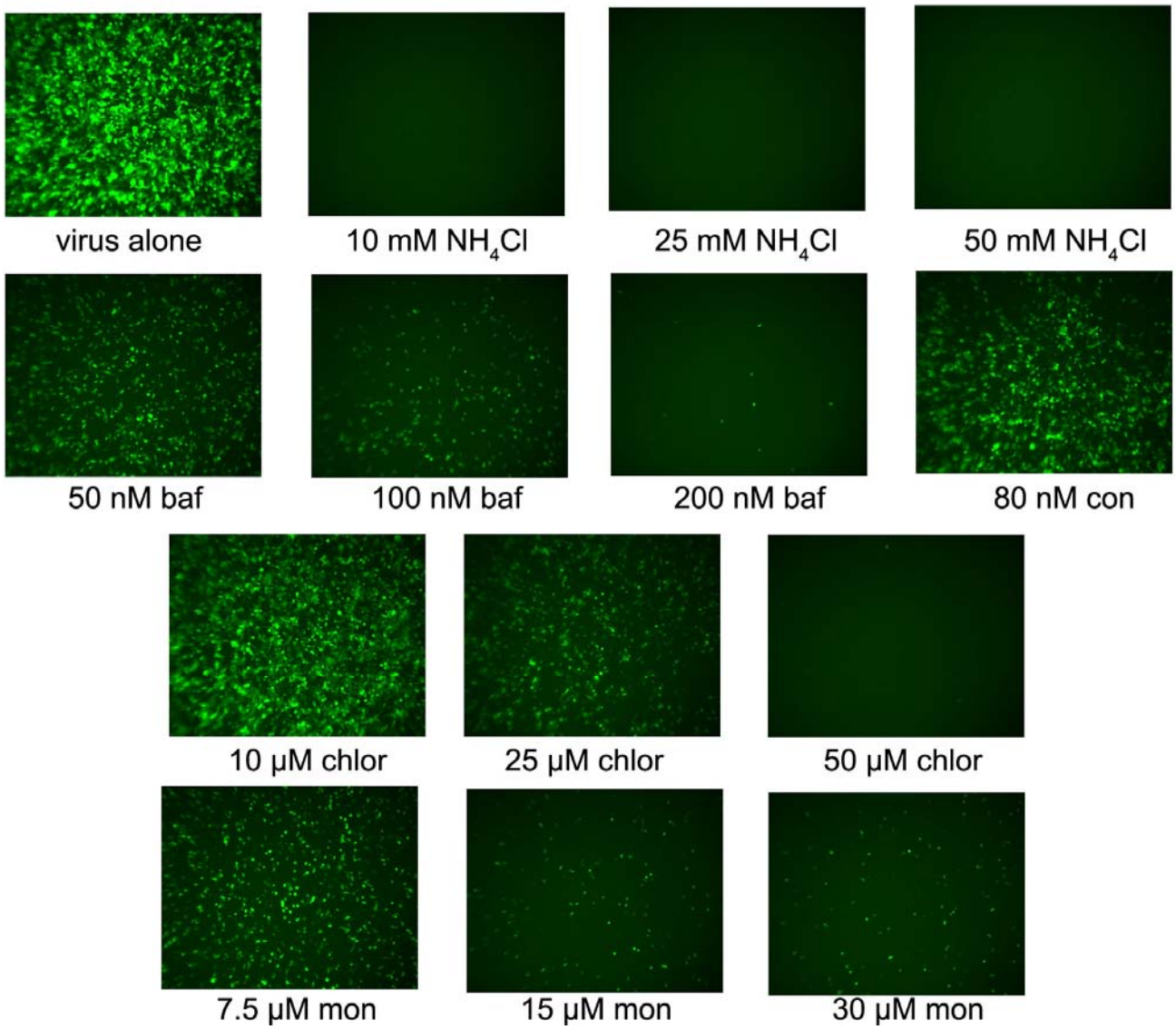


Figure 27. Effect of lysosomotropic agents on VSV entry into CHO-HVEM and J1.1-nectin-1 cells.

CHO-HVEM (A) and J1.1-nectin-1 (B) cells were pre-incubated with different lysosomotropic agents at the indicated concentration for 30 min. The pre-incubation media were then removed and GFP-expressing VSV (MOI=1) was added to the cells along with the previous concentration of drug. Cells were incubated at 37ºC for 7 h, and GFP expression was visualized under a fluorescent microscope.

7.2.2. Detection of endocytosed HSV by proteinase protection assay

We next attempted to detect HSV endocytosis by utilizing an approach described by Milne et al. (119). This approach is based on the principle that if the entire virus particle is taken up into an endocytic vesicle, then viral envelope glycoproteins should be detectable inside the cell shortly after the initiation of infection. These internalized glycoproteins should be resistant to protease treatment of intact cells, whereas virus fusion at the cell surface should leave the viral glycoproteins susceptible to protease digestion. Accordingly, HSV recombinant QOZHG at an MOI of 50 was adsorbed to CHO-HVEM cells at 4ºC for 2h, cells were washed to remove unbound virus, and either lysed immediately or shifted to 37ºC for 10 or 30 min, and then lysed. At the 10 and 30 min time points prior to cell lysis, duplicate samples were treated with trypsin, as described earlier (Figure 19), to remove viral glycoproteins exposed at the cell surface as either envelope components of intact virus or as a result of envelope fusion with the cytoplasmic membrane. Cell lysates were then analyzed by Western blot using antibodies for gB and gD. Internalized glycoproteins were not detected (Figure 24, lanes 3 and 5) despite the reportedly rapid kinetics ($t_{1/2}$=9 min) of endocytic HSV uptake by CHO cells (128).

After binding virus to the cells, we were unable to completely remove all virus particles from the cell surface using trypsin or proteinase K (data not shown). As a consequence, it could not be rigorously demonstrated that virus particles, if not their envelope glycoproteins, had indeed penetrated the cells after the different incubations at 37^{o}C. Nevertheless, since it is well established that HSV enters cells with a $t_{1/2}$ of approximately 10 min (78, 91, 128, 171), it may be assumed that virus penetration did occur under our conditions. Thus, neither protease protection nor treatment with lysosomotropic agents provided solid evidence for endocytic HSV infection of CHO cells. Given the discrepancy between our results and published data, these

procedures were not used to examine the route of sgD-mediated entry or explore the role of gD in HSV endocytosis.

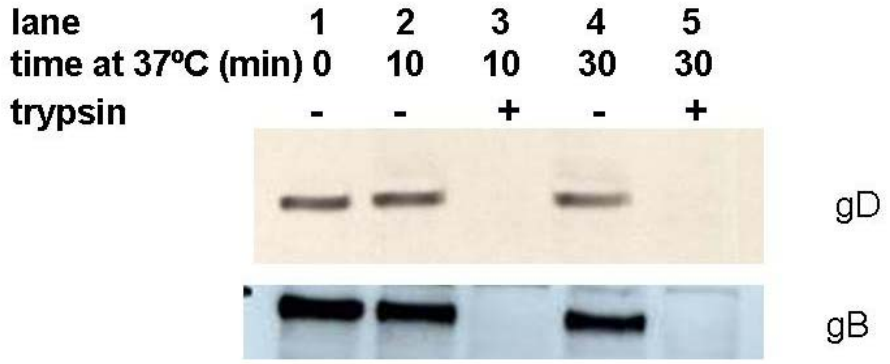


Figure 24. Detection of internalized glycoproteins.

QOZHG at MOI=50 was adsorbed to CHO-HVEM cells for 2 h at 37ºC. Cells were then washed and either lysed immediately (lane 1), or shifted to 37ºC for 10 (lanes 2 and 3) or 30 min (lanes 4 and 5) and then lysed. Duplicate samples of temperature-shifted cells for 10 and 30 minutes were treated with trypsin (lanes 3 and 5) to allow detection of internalized glycoproteins. Lysates were analyzed by Western blot. gD was detected with R7 antibody (92) and gB with a pool of anti-gB monoclonal antibodies (110).

7.2.3. Rate of entry

Our sgD-mediated entry system was used to approach the endocytosis issue from a different angle. We reasoned that if gD^+ HSV is internalized into CHO cells independently of the gD receptor, as proposed by Nicola and colleagues (128), then a gD-deficient virus is likely internalized by the same mechanism and at the same rate, whether in the presence or absence of sgD. An assay was devised in which the inverse of the rate of virus entry was measured. In this assay, it wasdetermined how much virus remains available at the cell surface for interaction with sgD at various times during incubation of attached virus at 37°C. This assay is based on the principle that endocytosed gD-deficient virus alone is unable to escape from endosomes and thus

will not express its reporter gene on entry. In contrast, virus that enters upon subsequent addition of sgD will be able to reach the nucleus and produce a reporter signal. Thus, the signal that was detect would be a measure for the amount of virus that remained at the cell surface at the time of sgD addition following incubation of virus-coated cells at 37°C. Since virus binding to GAG^+ cells is stable at 37°C (see Figure 25), this signal is also an inverse measure for the amount of virus internalized prior to the addition of sgD. Using this approach, we sought to confirm the conclusion by Nicola et al. (128) that HSV is internalized into CHO cells in a receptor-independent manner at a $t_{1/2}$ rate of 9 min.

gD-deficient virus was attached to CHO-HVEM cells at 4ºC for 2 h, the cells were washed, shifted to 37ºC for various amounts of time, and then sgD was added to allow productive infection by virus that remained available at the cell-surface. The results showed that there was a very gradual decline over time at 37ºC in the amount of virus that was present at the cell surface (Figure 25). Our data suggest a $t_{1/2}$ of approximately 90 min for sgD- and receptor-independent internalization of gD-deficient virus, with the understanding that this number is an underestimate if virus dissociation from the cells over this longer period of time is no longer negligible. It is possible that the discrepancy between our results and those of Nicola et al. betray that gD may have a role in HSV endocytosis that is independent of its receptor-binding role. Moreover, it is possible that any of the other glycoproteins that are deleted in our gD-deficient recombinant virus (gG, gJ, gI, gE) is important for efficient endocytic uptake.

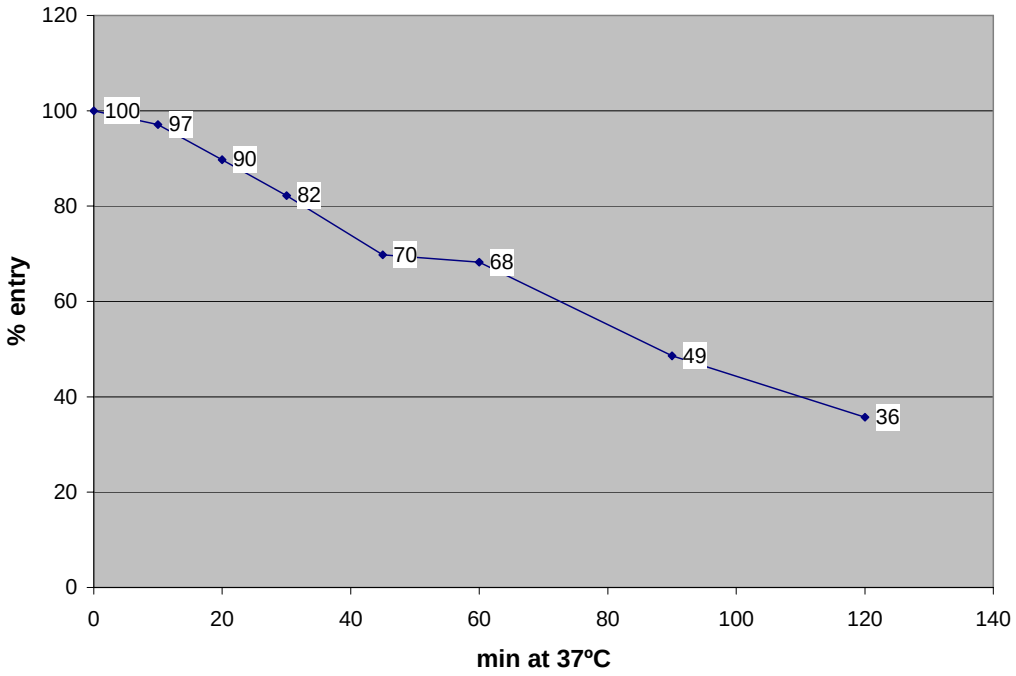


Figure 25. Rate of entry of gD$^-$ KΔUs3-8Z in the absence of sgD.

gD$^-$ KΔUs3-8Z was adsorbed to CHO-HVEM cells at 4ºC for 2 h. Cells were then washed and either sgD was added immediately in 37ºC media, or 37ºC media alone was added for various periods of time before adding sgD. Cells were infected for 3 h following the addition of sgD, and infection was quantified by ONPG assay 16 h post infection.

To determine the rate of entry of gD-deficient virus in the presence of sgD, gD-deficient virus was bound to CHO-HVEM cells for 2 h at 4ºC, cells were washed, sgD was added and cells were shifted to 37ºC to allow infection. At various time points, cells were glycine treated to inactivate virus that remained at the cell surface. Infection was measured after 16 h by ONPG assay. The results showed that the rate of sgD-mediated entry is comparable to that reported for wild-type virus (Figure 26).

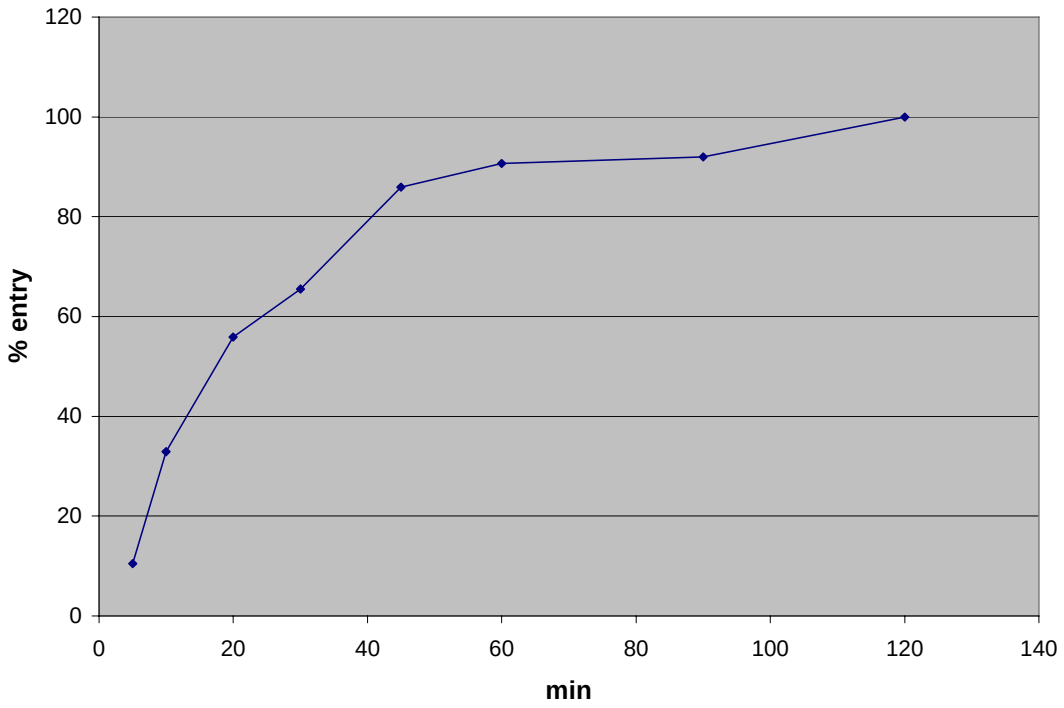


Figure 26. Rate of sgD-mediated entry.

gD$^-$ KΔUs3-8Z was adsorbed to CHO-HVEM cells for 2 h at 4ºC, and the cells were washed. sgD was added to all wells in 37ºC media, and the cells were glycine treated after various amounts of time. Infection was monitored by ONPG assay 16 h post infection.

8. DISCUSSION

HSV entry into cells is initiated by the binding of viral envelope components gB and gC to glycosaminoglycan moieties on the cell surface. Subsequent binding of viral envelope gD to a cell-surface co-receptor, such as HVEM, nectin-1, or 3-OST-3 modified HS, is thought to launch a cascade of events promoting fusion of the viral envelope with the cell membrane. The central goal of the current study was to establish and characterize a system that could aid in gaining a better understanding of the early events in HSV entry. By separating gD from the viral envelope, interactions of gD with other viral components involved in HSV entry could potentially be identified and studied. The results showed that gD is functional in mediating virus entry as a soluble molecule, demonstrating that envelope-anchorage of gD is not essential for HSV entry. sgD-mediated entry was less efficient than entry by wild type virus and required sgD recognition of a gD cognate receptor. sgD rapidly dissociated from its receptor into sgD-free media at 37ºC in the presence or absence of gD-deficient virus, and no evidence was obtained for cellular uptake of sgD in the presence of virus. Finally, sgD-mediated entry did not require cell surface GAGs and evidence was obtained that attachment of gD-deficient virus to GAG-deficient cells as well as attachment of gD^+ virus to GAG- and gD receptor-deficient cells occurred via a novel interaction that was less stable than the interaction of either gD^+ or gD^- virus with cell-surface GAGs.

While this study was in progress, Cocchi and co-workers reported a related set of findings (23). In their study, a soluble gD molecule truncated at position 285 was found to be active in mediating entry of gD-deficient virus particles into HSV-susceptible cells, whereas soluble gD truncated at position 260 was not. Since both of these molecules were previously

shown to bind to the gD receptors HVEM and nectin-1 (93, 159, 190), these results indicated that a region between residues 260 and 285 is essential for communication of gD with the fusion machinery. Whether this region is directly involved in interaction with other essential envelope glycoproteins is unknown.

The experiments described in our study support and extend the findings of Cocchi et al. by characterizing sgD-mediated entry into a different target cell line using a different virus strain and by defining additional characteristics of the system. Our results extend the published findings by demonstrating that gD receptor-deficient CHO cells were resistant to sgD-mediated infection. In addition, the receptor specificity of sgD-mediated infection was predictably altered by mutations in sgD that selectively abolish binding to nectin-1 (L25P) or HVEM (R222N,F223I). Together, these results constituted solid evidence that sgD-mediated infection requires a specific binding interaction between the soluble molecule and a cognate receptor.

This study further adds to the previous publication by including an estimate of the efficiency of sgD-mediated infection. Since gD-deficient HSV is not infectious in the absence of sgD and thus can not be titered, an estimate was obtained of the number of virus particles in our preparations using quantitative PCR compared to a stock of gD-complemented KΔUs3-8Z of known titer. A ratio of HSV genomes:infectious particles was then calculated for each virus. Although this procedure does not distinguish between fully and incompletely assembled particles, the results suggested that sgD-mediated infection was approximately 10-fold less efficient than normal infection. Since sgD-mediated infection requires three components, compared to two in normal infection, a decrease in efficiency was anticipated.

sgD_{287} was used in our work because this type of truncated gD is known to have a greater affinity for HVEM and nectin-1 than the complete ectodomain (93, 159). Cocchi et al. report that

sgD truncated at position 285 (gD_{285t}) is as active in mediating gD-deficient virus entry via human nectin-1 as sgD truncated at position 306 (gD_{306t}) (23), suggesting that residues 286-306 compensate for the reduced receptor-binding activity of the longer molecule by increasing the efficiency of a subsequent step in the entry process.

8.1. Mechanistic Aspects of sgD-Mediated Entry

In initial experiments, we sought to utilize our system of sgD-mediated infection to clarify the order in which gD, the HSV virion, and the gD-receptor must interact to effect virus entry. We observed that entry was highly impaired when the virus was added subsequent to sgD binding to CHO-HVEM cells at 37ºC. Entry efficiency was recovered only when sgD and the virus were sequentially bound to CHO-HVEM cells at 4ºC, thereby allowing both components to be present at the cell-surface at the time of the temperature shift to 37ºC. Further investigation suggested that receptor-bound sgD was not inactivated instantaneously at 37ºC. These results indicated that entry required the immediate presence of cell-attached virus at the time that receptor-bound sgD was exposed to the infection temperature. Possible explanations included: 1) sgD must interact with the receptor and virion simultaneously in order to perform its fusion-triggering function, as suggested by Cocchi et al (23), and 2) sgD becomes rapidly unavailable for interaction with subsequently added virus due to dissociation from its receptor, degradation, or internalization. Without disproving the first possibility, our results indicated that the second was applicable to our experimental conditions. We showed that sgD rapidly dissociated from the cell surface at 37ºC after excess sgD was removed from the media. After 30 min, sgD was identified exclusively in the supernatant of the cells and could not be detected at the cell surface or interior.

This characteristic was unaltered by the presence of gD-deficient virus during the sgD binding step at 4ºC, indicating that cell-associated virus did not stabilize the sgD-cell interaction or promote sgD internalization during the subsequent incubation at 37^{o}C.

Due to the rapid dissociation of sgD from the cell surface in sgD-free media at 37^{o}C, our study could not distinguish whether receptor binding may mediate a stable or transient change in sgD that enables communication with gD-deficient virus. Recent evidence supports a model in which (i) a C-terminal portion of the gD ectodomain (residues 260-285) in the free form of gD folds over and binds to sequences in the N-terminal region (residues 1-260), and (ii) receptor binding dissolves this interaction (50). One suggested scenario is that upon receptor binding, gD enters an "open" conformation, enabling a yet to be defined domain of the molecule to interact with one of the other essential viral glycoproteins. In the case of soluble gD, it is possible that the open conformation is unstable in the absence of the remaining viral glycoproteins and enters an inactive state in which it is no longer able to interact with gD-deficient virus particles added at a later time. This possibility is consistent with our observations that sgD-mediated entry is abolished when the soluble molecule is bound to the cells at 37^{o}C prior to virus addition or when cell-bound sgD is exposed to the infection temperature at the time of virus addition, before abundant virus accumulation at the cell surface. Moreover, it is consistent with the observation that infection returned to normal levels when sgD and the virus were sequentially attached to the cells prior to raising the temperature. However, proper interpretation of these results is hampered by the observed dissociation of sgD from its receptor under our experimental conditions. Thus, additional information or alternate approaches are required to characterize the active state of sgD.

It has previously been reported that approximately 50% of wild type virus adsorbed to CHO cells enters the cells within 30 min at 37^{o}C, while the other 50% presumably remains attached to the cell surface (128). Following binding of sgD and gD^{-} virus to CHO-HVEM cells at low temperature, it was therefore expected that a substantial amount of sgD would remain stably associated with the cell surface if receptor-bound sgD forms a stable interaction with adsorbed virus at 37^{o}C. We did not see evidence for sgD stabilization at the cell surface in the presence of virus (Figure 19A). In addition, no evidence was obtained for sgD internalization in the presence or absence of virus (Figure 19A). These results argued that either the putative interaction between receptor-bound sgD and cell-bound virus was insufficient to retain sgD at the cell surface, or that the fraction of virus that fails to enter at 30 min is defective for interaction with receptor-bound sgD. Although our observations suggested that sgD was not internalized along with the virus, it should be noted that these assays are usually performed at much higher levels of infection (119) than attainable using gD-deficient virus combined with sgD. Hence, any endocytic uptake of sgD coincident with virus entry may have escaped detection. For future studies, radiolabelled virus can be used to increase the sensitivity of these assays.

8.2. The Role of GAGs in sgD-Mediated and Wild-Type Virus Entry

By analogy to wild-type HSV infection, it was anticipated that sgD-mediated infection would be enhanced by virus binding to cell-surface GAGs. Surprisingly, we observed that GAG-deficient CHO-K1 cells expressing nectin-1 were at least as susceptible to sgD-mediated infection as GAG-bearing CHO-nectin-1 cells. By comparing the amounts of gD-deficient virus bound to

pgsA745-nectin-1 cells in the absence and presence of sgD, evidence was obtained for GAG- and sgD-independent virus attachment, suggesting the existence of a previously unknown attachment mechanism.

Interestingly, the ratio of virus bound to GAG-expressing CHO-K1 cells compared to GAG-deficient pgsA745 cells appeared significantly higher for KHZ.1 (Figure 24A, lanes 1 and 3) than for gD-deficient KΔUs3-8Z (Figure 24B, lanes 1 and 2). This observation points to either an increased ability of gD^- virus to bind to GAG^- cells or a reduced ability of gD^- virus to bind to GAG^+ cells, as compared to virus containing the full complement of envelope glycoproteins (illustration in Figure 28). One possible model to explain these results is that the novel binding function may represent a downstream interaction in the fusion cascade whose formation is normally controlled by the gD-receptor interaction (Figure 29). In the absence of gD, binding through the novel binding interaction may be more efficient. In any event, the absence of GAGs had a smaller effect on gD^- virus attachment than on KHZ.1 attachment. Together with the substantially higher levels of nectin-1 on our pgsA745-nectin-1 line compared to CHO-nectin-1 cells (Figure 21), we believe that this may explain the counterintuitive observation of greater sgD-mediated entry into the GAG-deficient pgsA745-nectin-1 cells than into their GAG-bearing counterparts (Figure 22).

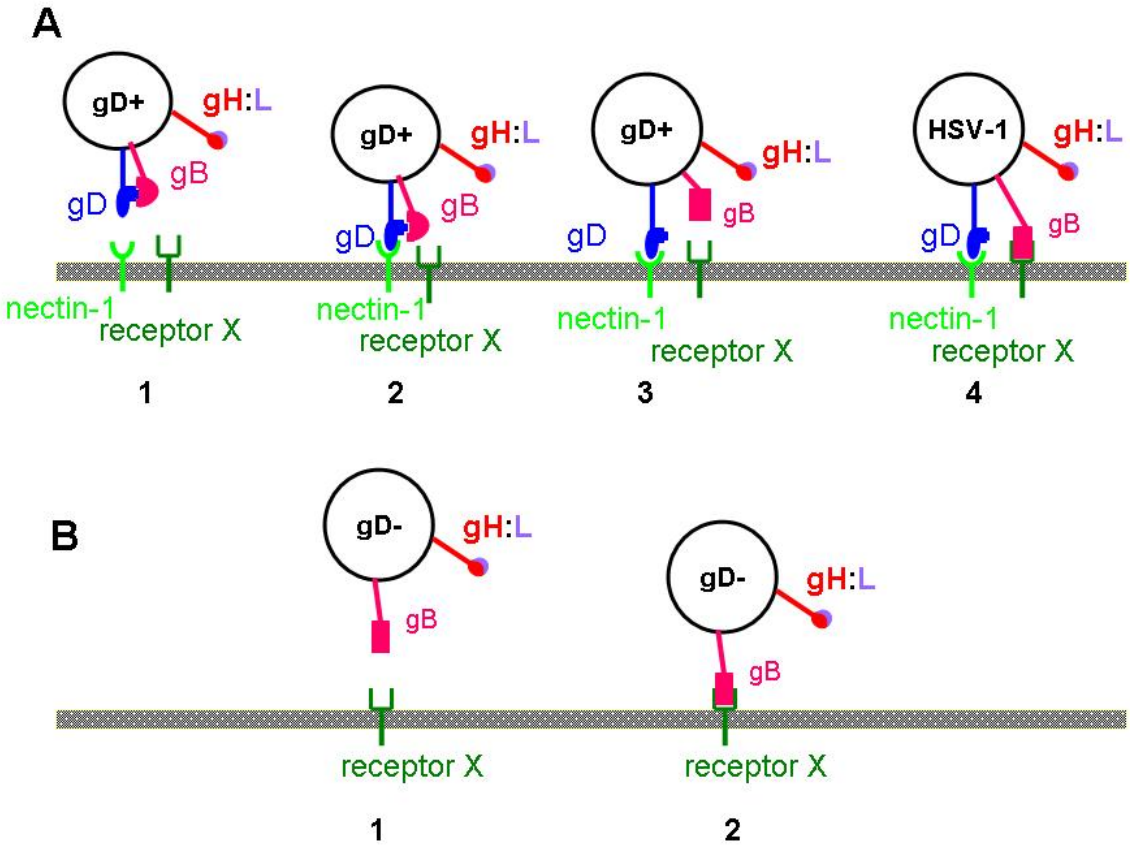


Figure 28. Model illustrating possible higher binding effeciency of gD- virus through novel binding interaction.

(A) In an HSV virion containing a full complement of envelope glycoproteins, gD may interact with gB, holding it in a conformation in which gB is unable to interact with its coreceptor (1). Upon binding to a cell-surface receptor (2), gD may release gB (3), allowing gB to enter a conformation in which gB is now able to bind to its coreceptor (4). (B) In an HSV virion which lacks gD in its evelope, gB is not bound by gD (1), and is free to interact with its receptor (2).

In spite of the ability of our gD-deficient virus to bind to GAG-deficient cells, no entry was detected when the virus was pre-adsorbed at 37ºC or 4ºC, the cells were washed, sgD was added, and the cells were shifted to 37ºC for a 3 hour infection period (Figure 25C and D). For comparison, normal levels of entry were observed when GAG-positive CHO-HVEM cells were tested by the same protocol (Figure 15D). Thus the stability of HSV binding through the novel

binding interaction with HSV bound through GAGs was compared. Our results indicated that both "wild-type" (KHZ.1) and gD-deficient virus dissociated from GAG-deficient cells during incubation of 10-30 min at 37ºC, while no dissociation was detected for either virus from GAG-expressing cells (Figure 26). These observations demonstrated that the novel binding interaction does not attach HSV to cells as stably as GAGs. Thus it is likely that gD-deficient HSV pre-incubated with GAG-deficient pgsA745-nectin-1 cells dissociates from the cells before the subsequently added sgD is able to attach to its receptor and trigger fusion.

Although the significance of the novel attachment function under normal conditions is not known, it is conceivable that it represents an essential co-receptor or plays a prominent role in virus attachment to GAG-deficient cells. In support of this notion, Bender et al. have obtained evidence for a novel binding function of gB and have linked this function to the fusion process by demonstrating that soluble gB blocks virus infection of GAG-deficient cells (5). Although it remains to be seen whether the interaction we observe involves gB, this is a likely possibility. Future studies will be designed to investigate this possibility by using a gB^- virus in our assays.

8.3. Endocytosis

In light of recent evidence suggesting that HSV entry into certain cell types occurs through endocytosis and not fusion at the cell-surface, we sought to define the pathway of sgD-mediated entry in order to determine whether this system could be used to study the role of gD in endocytosis. Our use of lysosomotropic agents to confirm endocytic entry of gD^+ HSV into receptor-bearing CHO but not Vero cells yielded inconsistent results that did not support published observations (119, 126, 128). In contrast, all of the drugs that were tested consistently inhibited entry of a control virus, VSV, whose entry pathway is well established to be mediated by endocytosis. Complete inhibition of VSV infection was observed at low concentrations of these drugs, with the exception of concanamycin A which required a higher concentration than commonly used.

Nicola and co-workers used several reliable methods to demonstrate that entire HSV particles are internalized into CHO cells (126, 128). Indeed, endocytic HSV internalization that does not lead to productive infection has previously been documented (12, 199). The main evidence presented by Nicola et al. for productive infection by endocytosed HSV was their observation that infection was inhibited by various agents that inhibit the acidification of the endocytic vesicles or prevent endocytic uptake from the cell-membrane. In the hands of Nicola et al., these drugs completely inhibited HSV entry into CHO cells without negative effects on entry into Vero cells, whose HSV entry pathway has been firmly established to be fusion at the cell surface (47, 48, 122). In our hands, when a certain drug was observed to inhibit infection of CHO cells to a significant degree, inhibition was also observed on Vero cells, although to a

somewhat lesser degree. Based on this experience, we feel that it can not be concluded that productive entry into one cell type is through endocytosis and into the other through fusion at the cell surface. In support of this notion, no evidence was presented by Nicola et al. showing virions fusing with endocytic vesicles in their EM study, while virions fusing at the cytoplasmic membrane were readily observed with Vero cells (182). As mentioned in the introduction, the use of endocytosis inhibiting drugs is not considered to be an ideal method to determine whether endocytosis leads to productive infection of a virus, since most of these drugs have non-specific effects on the cell. While we accept the finding that HSV can be endocytosed by CHO cells, we suggest that the verdict is still out whether this pathway leads to productive infection.

An alternative possibility is that productive infection of CHO cells can occur via multiple pathways. HSV is a large and complex virus that utilizes redundant mechanisms to accomplish many tasks. In their study, Nicola et al. used CHO cells that are stably transformed with the *lacZ* gene under control of the HSV ICP4 promoter, and a virus that is slightly different from the ones used in our study. The balance between endocytosis and fusion at the cell membrane may be quite sensitive in the case of HSV, and use of a slightly different cell line and virus strain may tip the balance one way or the other.

We attempted to obtain evidence for HSV endocytosis by examining protection of envelope glycoproteins from trypsin digestion at early stages of infection (Figure 24). This assay was based on the principle that if a complete virus particle is internalized into an endocytic vesicle, the glycoproteins will be protected from external enzyme digestion. Our results clearly showed that gB and gD were not internalized in our cells (Figure 24), suggesting that enveloped particles were not endocytosed in our system. Although rapid HSV internalization has been demonstrated in many studies (78, 91, 128, 171), we were unable to directly demonstrate that

virus particles were indeed internalized in some form at 10 or 30 min after the initiation of infection. This was due to incomplete protease sensitivity of virus attached to the cell surface to removal by trypsin or proteinase K. Thus, although protease-resistant VP5 was observed after incubation of virus-coated cells for 10-30 min at 37^{o}C, it could not be concluded with certainty whether any fraction of this signal represented internalized virus. Additional experiments are required to optimize the conditions in order to conclude that entire glycoprotein-bearing virus particles are not found inside the cells in our system.

Finally, we took advantage of our sgD-mediated entry system to explore the reported receptor-independent endocytic internalization of HSV into CHO cells (128). We reasoned that if 50% of infectious virus is internalized after 9 min in a receptor-independent manner, but can not escape from the endosomal compartment in the absence of a gD receptor (128), then productive sgD-mediated infection should be drastically reduced by prior incubation of cell-attached virus at 37ºC. In other words, 50% of pre-attached virus should be endocytosed after 9 min at 37^{o}C and thus be unavailable to sgD added at that time to mediate productive infection. This assay showed that the rate of virus removal from the cell surface was at least 10-fold slower than the reported rate of receptor-independent endocytosis.

Several explanations exist for this result. One that is consistent with the results that were obtained with the other methods that we attempted to use to address the endocytosis question, is that infectious virus does not enter our cells by endocytosis, and simply dissociates slowly over time. Another possibility is that by measuring the amount of virus left at the cell surface at each time point instead of the amount of virus that has already entered the cell, our assay does not truly represent the rate of internalization. Addition of sgD at the various time points could stimulate entry of additional virus that would not have entered the cell initially. A third

possibility that requires further investigation is that the rate of endocytosis in the absence of sgD is reduced. This would be interesting, since Nicola et al. reported that the rate of endocytosis does not depend on the presence of a gD-receptor.

The least invasive method to conclusively show which pathway is taken by productively infecting virus is real-time microscopy, which can be used to follow virus particles whose capsids and/or envelopes have been fluorescently labeled (95, 164). In addition, dominant-negative mutants which interfere with clathrin-mediated endocytosis can be used, such as Eps15Δ95/295 or Dyn^{K44A} (6, 15). These methods should prove useful in the future to settle the ongoing dispute over the pathway of HSV-1 entry.

8.4. SUMMARY

Characterization of a system in which HSV-1 glycoprotein D provided *in trans* was shown to mediate entry of gD-deficient virions offers new opportunities to dissect the molecular interactions occurring during the earliest stages of HSV infection. In this study, we demonstrated that sgD-mediated entry requires direct interaction of sgD with a gD cognate receptor. Moreover, we showed that sgD-mediated entry is neither dependent on nor enhanced by the presence of GAGs on the cell surface, although we confirmed that cell-attachment of our gD-deficient virus was enhanced by these cellular structures. Thus, our findings raise the possibility that GAGs, while promoting attachment, may negatively affect the entry process. We extended these observations by showing that HSV can utilize a previously unknown interaction for gD- and GAG-independent attachment to cells. Identification of the viral and cellular components of this novel interaction, as well as definition of the role of this interaction in normal HSV infection, are among the future goals derived from this study.

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