Identification and characterization of low pH-triggered conformational changes in the herpes simplex virus glycoprotein B

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Identification and characterization of low pH-triggered conformational changes in the herpes simplex virus glycoprotein B

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

March 31st, 2011

By

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF LOW PH-TRIGGERED CONFORMATIONAL CHANGES IN THE HERPES SIMPLEX VIRUS GLYCOPROTEIN B

By Stephen J. Dollery, B.Sc. (Hons)

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Associate Professor, Department of Microbiology and Immunology

Herpesviruses can enter host cells by pH-dependent endocytic pathways in a cell-specific manner. The role of pH in herpesvirus endocytosis is unclear. Herpes simplex virus (HSV) is a paradigm for virus membrane fusion via a complex of glycoproteins. HSV glycoproteins B, D and the heterodimer H-L are necessary and sufficient for membrane fusion. This work analyzes the structure and function of HSV glycoproteins B, D, and H-L at neutral pH, and at the physiological low-pH encountered during endocytic entry. It is demonstrated that mildly acidic low pH triggers specific
conformational changes in HSV gB at a pH of 5.7 to 6.0. The antigenic structure of gB functional region I that is critical for fusion is specifically altered by mildly acidic pH both \textit{in vitro} and during entry into host cells. Point mutations within gB functional region 1 that block membrane fusion still allow conformational changes in region 1. This suggests that specific hydrophobic residues are essential for fusion domain insertion into the host cell membrane but not conformational change. The detected conformational changes were reversible, similar to other class III fusion glycoproteins. Exposure to mildly acidic pH directly triggered the fusion function of HSV glycoproteins and caused gB, but not other glycoproteins, to become more hydrophobic. The oligomeric conformation of gB is altered at a similar pH range.

In addition, several approaches were used to monitor gB throughout glycoprotein synthesis and maturation. It is shown that gB may cotranslationally fold and oligomerize as it is synthesized on the ribosome. As gB matures it then alters conformation and/or binding partner to form antigenically distinct populations of gB within the cell and virion. I conclude that intracellular low pH induces changes in gB conformation that, together with additional triggers such as receptor-binding, are essential for virion-cell fusion during herpesviral entry by endocytosis.
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List of abbreviations

CD = Cluster of differentiation
CMV = Cytomegalovirus
CPK = Corey, Pauling, Kultin color scheme
DEPC = Diethyl pyrocarbonate
E = Early
EBV = Epstein-Barr Virus
ER = Endoplasmic reticulum
g = (Prefix) Glycoprotein
x g = Times gravity
Grp = Glucose regulated protein
HIV = Human immunodeficiency virus
HSPG = Heparan sulphate proteoglycans
HSV-1 = Herpes simplex-1
HSV-2 = Herpes simplex-2
ICP = Infected Cell protein
IE = Immediate early
Ig = Immunoglobulin
IRF = Interferon regulatory transcription factor-1
L = Late
LPS = Lipopolysaccharide
LSCM = Laser scanning confocal microscopy
MAb = Monoclonal antibody
MOI = Multiplicity of infection
ND = Nuclear domain
$p$ = (Suffix) Protein
PAb = Polyclonal antibody
PAGE = Polyacrylamide gel
PCR = Polymerase chain reaction
PI = Post infection
PILR$\alpha$ = Paired immunoglobulin-like type 2 receptor $\alpha$
Pro A = Staphylococcal Protein A
T = Triangulation number
T7 = Bacteriophage T7
TLR = Toll like receptor
UL = Unique long
US = Unique short
VP = Viral protein
VSV = Vesicular Stomatitis Virus
VHS = Virion host shutoff protein
WT = Wild type
I. Introduction to herpesviruses

Classification and structure

The *Herpesviridae* are a large family of viruses that are traditionally identified based upon virion architecture (167). Once identified, a herpes virus is then subcategorized into one of three subfamilies based upon its tissue tropism. Nucleotide homology is now also a key method for virus identification and classification (145).

Virions of the herpesviridae consist of a core containing a linear double-stranded DNA genome that ranges from 124-230 kb in length. The genome is surrounded by an icosahedral capsid of 162 capsomers (154). A proteinaceous layer of tegument engulfs the capsid and spans out to associate with envelope proteins (106). The outermost structure of the virion is termed the viral envelope. The envelope is a host derived lipid bilayer into which viral glycoproteins are imbedded. Glycoprotein spikes protrude from the virion envelope and are the first feature of the virus to interact with the host (71).

All herpes viruses produce virions during the lytic replication cycle, destroying the host cell. Herpes viruses also undergo a latent phase in hosts that do not succumb to initial infection. During latency, genomes retain the capacity to reactivate and cause disease at a later date. Latency ensures life-long infection of the host.

The *Herpesviridae* are divided into three subfamilies based upon tissue tropism (110).

**Alphaherpesvirinae** have a rapid life cycle, efficiently destroy infected cells and are able to become latent in sensory ganglia.
**Betaherpesvirinae** have a long life cycle, infected cells frequently become enlarged and latency is commonly maintained in secretory glands, lymphoreticular cells and kidneys.

**Gammaherpesvirinae** are generally host specific and usually replicate in either B or T cells. Latency is usually maintained in lymphoid tissue.

**Distribution in nature**

There are over 200 identified herpes viruses that are highly disseminated throughout nature. Divergent sources range from marine mollusks to humans. There are eight known human herpes viruses of medical significance (Table 1) and numerous economically burdensome veterinary herpes viruses.
Table 1: The human herpes viruses

<table>
<thead>
<tr>
<th>Name</th>
<th>Sub-Family</th>
<th>Primary Target cell type</th>
<th>Latency</th>
<th>Means of Spread</th>
<th>Associated manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes simplex-1 (HSV-1)</td>
<td>Alpha</td>
<td>Mucoepithelia</td>
<td>Neuron</td>
<td>Close contact</td>
<td>Oral and genital lesions, ocular and neuronal infection.</td>
</tr>
<tr>
<td>Herpes simplex-2 (HSV-2)</td>
<td>Alpha</td>
<td>Mucoepithelia</td>
<td>Neuron</td>
<td>Close contact</td>
<td>Oral and genital lesions, ocular and neuronal infection.</td>
</tr>
<tr>
<td>Varicella Zoster virus (VZV)</td>
<td>Alpha</td>
<td>Mucoepithelia</td>
<td>Neuron</td>
<td>Respiratory and close contact</td>
<td>Chicken pox, shingles, encephalitis</td>
</tr>
<tr>
<td>Epstein-Barr Virus (EBV)</td>
<td>Gamma</td>
<td>B-lymphocyte, epithelia</td>
<td>B-lymphocytes</td>
<td>Close contact, transfusions, tissue transplant, and congenital</td>
<td>Infectious mono-nucleosis, various lymphoma</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>Beta</td>
<td>Epithelia, monocytes, lymphocytes</td>
<td>Monocytes, lymphocytes and possibly others</td>
<td>Saliva</td>
<td>Flu like symptoms. Systemic infection in neonates and immuno-compromised.</td>
</tr>
<tr>
<td>Herpes lymphotropic virus (HHV-6)</td>
<td>Beta</td>
<td>T-lymphocytes and others</td>
<td>T-lymphocytes and others</td>
<td>Respiratory and close contact</td>
<td>Roseola, encephalitis</td>
</tr>
<tr>
<td>Human herpes virus-7 (HHV-7)</td>
<td>Beta</td>
<td>T-lymphocytes and others</td>
<td>T-lymphocytes and others</td>
<td>?</td>
<td>Roseola, encephalitis</td>
</tr>
<tr>
<td>Kaposi's sarcoma-associated herpes virus (KSHV)</td>
<td>Gamma</td>
<td>Endothelial cells</td>
<td>Unknown</td>
<td>Close contact (sexual), saliva</td>
<td>Kaposi's sarcoma, Multicentric Castleman's disease, primary effusion lymphoma</td>
</tr>
</tbody>
</table>

Adapted from references (148, 186).
Medical significance of HSV

Herpes simplex viruses, HSV-1 and HSV-2, are common, contagious, incurable alphaherpesviruses. Herpes simplex derives its name from the Greek “\textit{herpein}” which means to creep. By middle age, life long HSV-1 infection is commonly estimated to be present in 80\% of individuals while HSV-2 prevalence is estimated at 30\% (31). The most common sites of primary infection are mucous membranes irrespective of virus type. Primary infection is usually the most serious. Recurrent reactivation from latency is normally only painful (187). Herpes simplex is the leading cause of sporadic fatal encephalitis, and also causes meningitis, myelitis, and radiculitis in other areas of the nervous system.

Neonatal infection occurs in around 1 to 2000-5000 births and is frequently lethal. HSV is the predominant cause of genital ulcerative disease (190). HSV is accepted as a major contributing factor in the spread of HIV. Increased contact with blood at areas of ulceration, and increased concentration of HIV containing CD4+ cells at the site of HSV infection, are believed to play contributing roles. A review of 19 studies showed that people with HSV-2 have a 3 fold increased risk of contracting HIV (61). The same study estimates, that in certain populations, up to 60 percent of HIV infections can be attributed to HSV. A large fraction of the 33 million people living with HIV will have contracted HIV due to HSV infection.
Antiviral Drugs

There are several effective antiviral drugs against HSV. Acyclovir was the first to be identified, numerous analogues are now available that are increasingly potent. Acyclovir is a guanosine analogue prodrug that once metabolized inhibits viral DNA polymerase (31, 91). Derivatives of acyclovir work via a comparable mechanism. acyclovir and derivatives function only to manage infection. Acyclovir-resistant HSV is a frequent cause of morbidity in the immunocompromised. Alternative drugs with different modes of action are desired. The availability of current antivirals has not diminished the prevalence of herpes simplex viruses. This may be because HSV can be transmitted from an asymptomatic host.

Vaccines

There are many candidate vaccines that fall under four main groups, some of which have proved relatively successful in animal models. A vaccine for genital herpes is still desirable. However, pharmaceutical companies interest in this area has waned, apparently due to the success of antiviral drugs. The main groups of possible vaccine are, wild-type, inactivated, subunit and genetically engineered (live). Wild-type vaccines have proven unsuccessful and in inactivated vaccines long-term protection has not been established. Subunit vaccines and genetically engineered vaccine areas remain the most likely strategies at proving successful (145). A subunit vaccine had some success in phase III clinical trials.
Herpes simplex structure

DNA Core

HSV-1 and 2 have genomes 152kb and 155kb in length respectively (109). The genome is densely wrapped into a liquid crystalline state. Spermidine and spermine may neutralize the phosphates. Both HSV genomes are structurally similar and contain unique long and unique short (UL and US) regions that invert to form four isoforms during replication (109). Immediate early genes map to near the termini of UL/US regions. Early and late genes including glycoprotein-encoding genes are dispersed within the UL and US components (Figure 1).
Figure 1. HSV-1 map indicating the positions of important glycoproteins

HSV glycoproteins are arrayed across the entire genome. Positions of glycoproteins used in this study are indicated. The scale is in map units. UL is the unique long region of the genome, US the unique short.
Capsid

Herpes simplex virus is 180-225 nm in size with a capsid approximately 100 nm in diameter. The capsid has T=16 symmetry with a T=4 lattice structure (154). The capsid is predominantly made of viral proteins (VP) 5, 26, 23, and 19 (174, 195). VP5 is the major capsid protein, with 5 protomers present in pentons and 6 in hexons. VP26 forms rings above hexomers while VP23 and VP19 link adjacent capsomers (Figure 2).

Tegument

HSV tegument proteins are multifunctional. Functions include capsid transport, and adaptation of the cell to virus production prior to capsid reaching the nucleus. Proteins such as the Virion Host Shutoff protein down regulate host protein synthesis while proteins such as VP16 are transcriptional activators of HSV (15). Tegument proteins also have suggested roles in capsid transport, docking at the nuclear pore and DNA translocation. It is known that upon virus binding there are signaling events that cause destabilization of VP16 (111). Tegument protein ICP0 has also been shown to have a role in regulating the proteasome dependent entry of HSV (45).

Envelope

The outermost layer of the virion is a host-derived lipid bilayer termed the viral envelope. There are around 11 virally encoded glycoproteins and numerous other non-glycosylated proteins that are embedded in the envelope (145). It is believed that several host proteins may also be incorporated into the virion although the exact
proteins and their functions are controversial. Cryoelectron tomography revealed that there are on average 659 glycoprotein spikes per HSV virion. The glycoproteins are arranged in a non-random fashion, forming clusters that predominate on one side of the envelope (71).
Figure 2: Cryoelectron tomograph of a HSV virion

A) A surface rendering of a HSV tomogram. Shown in dark blue is the virus envelope, in yellow glycoprotein spikes protrude from the surface and appear arranged in a non-random fashion.

B) A segmented rendering showing the capsid in light blue, tegument proteins in orange, and again in dark blue the virus envelope with yellow glycoproteins. Pp is the proximal pole; dp the distal pole. The scale bar is 100 nm.

(Image reproduced with permission from the publisher (71))
Viral lifecycle

Viral entry

Virus entry is the first and possibly the most critical step in the viral replication process (1). Viral glycoproteins are responsible for the first interactions with the host cell. gB, gD and gH-gL are essential for infection (135, 176). Other proteins, such as gC and UL45, are known to augment entry or regulate membrane fusion (49, 80, 177). The role of around 15 other membrane proteins in the endocytic entry of HSV is as yet unstudied (49, 106). The first step in HSV entry is non-essential but augments entry. gC and gB reversibly bind to heparan sulphate proteoglycans (HSPGs) (79, 172). This functions to concentrate virions to the cell surface. The second step is an irreversible ligand-binding event. gD binds with a cognate receptor. gD receptors identified include nectins 1 and 2, HVEM, and 3-0 sulfated heparin sulphate (28, 97, 117, 126, 158, 173). It is currently believed that nectin-1 and HVEM are the most important receptors in viral pathogenesis. Upon receptor binding, gD undergoes a conformational change from a prefusion closed conformation to an open pro-fusion conformation, allowing further interactions to occur. However, the subsequent sequence of events is unclear.

It is known that upon receptor binding, gD interacts with gB and gH-gL either sequentially or in a complex (7, 8, 68). gB is also known to bind to cellular receptors PILRα and non-muscle myosin IIA. (5, 6, 153). The sequence of gB receptor binding in relation to gD receptor binding is as yet unevaluated. The role of non-muscle myosin IIA in directing fusion is also unevaluated. Additional cues for fusion are likely, and possibly
direct the site of membrane fusion. Following fusion triggering events, either gH-gL or gB or a heterologous complex execute fusion of host and viral membranes. This may be initiated through a state of hemi-fusion in which the outer leaflets of the two membranes become one, but the inner leaflets remain separate until further conformational changes in the fusion machinery occur (169). Pore formation then follows. The pore then expands prior to complete de-envelopment and capsid and tegument release into the cytoplasm. There are at least two possible sites of capsid release into the cell (discussed in the HSV entry pathway section).

After de-envelopment the capsid is transported through the cytoplasm to the nucleus by anterograde microtubule transport (Figure 3) (155). Capsid transport is proteasome dependent (45). DNA enters the nucleus through pores, and importin-β helps translocate DNA into the nucleus (129).

The nucleus is the site of viral transcription, DNA replication, encapsidation and the starting location of capsid egress (138). The nucleus is rearranged and compartmentalized to facilitate these events. There is breakdown of the nucleolar membrane. ICP0 causes the disruption of ND10 structures leading to conversion to pre-replication compartments (107, 159). DNA first appears near ND10 sites, there then forms replication compartments that coalesce and eventually fill the nucleus, condensation of host cell chromatin is also observed as this is occurring, it is believed that this also contributes to the host gene shutoff (118). Later on in infection the chromatin disperses around the nucleus and the nuclear lamina is disrupted (23, 130). Host proteins such as p53, DNA recombination and repair proteins, and IRF-3 are recruited to replication sites, however their roles remain to be elucidated (43, 188).
**Viral gene expression**

There are around 80 genes expressed in a highly regulated cascade (82, 145). Host RNA polymerase II transcribes all the viral genes in the nucleus (4).

At 2-4 hr PI, in the absence of any other protein production “immediate early” (IE) gene transcripts are produced. IE genes usually encode transcription factors such as VP16 that stimulate “Early” (E) gene expression. Between 4-8 hrs early gene products are produced. This requires IE transcription factors but no DNA replication (83). Early gene products are involved in the viral DNA metabolism (145).

Late gene expression peaks after viral DNA replication (39). Viral DNA is known to circularize rapidly after infection and DNA replication is thought to occur via rolling circle replication. Late genes are typically structural components of the virion including viral glycoproteins.

**Capsid assembly**

Capsid assembly occurs in the nucleus. VP5, the major capsid protein, forms hexon and penton capsomers. VP26 forms at the tips of hexons (122). The minor capsid proteins, VP19C and VP23, hold capsomers together. These form hetero-oligomers in a 1:2 ratio. The capsid forms around a scaffold protein U26.5. Proteolytic degradation of the scaffold protein is an essential step in capsid maturation (47). A portal is formed at one vertex of the capsid. This is comprised of 12 units of UL6 and is believed to associate through interactions with VP26. Translocation of DNA into the capsid occurs through the portal vertex. The encapsidation of DNA relies upon numerous proteins but the core elements are UL28, UL15 and UL33, which form a
terminase complex (16). The terminase cleaves concatemeric DNA into unit length genomes and drives DNA through the portal.

Egress

Capsids are too large to egress through the nuclear pore and so egress though the nuclear membrane via envelopment and de-envelopment (56). Following assembly, capsids align along the inner-nuclear membrane and undergo a process known as primary envelopment. UL31 and UL34 are thought to disrupt nuclear laminins helping capsids bud into the perinuclear space (140). These capsids gain a primary envelope in the process. For de-envelopment, gB and gH-gL act redundantly to fuse the primary envelope with the outer nuclear membrane, releasing the capsid into the cytoplasm. US3 kinase is believed to regulate fusion mediated by gB via phosphorylation of gB’s cytoplasmic tail.

Transport of the capsid through the cytoplasm to the site of secondary envelopment is mediated via microtubular transport. Attachment to microtubules may be via UL37p, ICP0 and VP1/2 (46). In neuronal cells egress of membrane associated capsids may be dependent upon the tegument protein UL36p (155). UL20p/gK and UL11p are needed for the cytoplasmic envelopment of virions (64, 105). Secondary envelopment occurs as the capsid buds into a vesicle that contains markers of the trans-Golgi network and endosomal membranes (76). Release of virus into the extra-cellular space occurs as the vesicle fuses with the plasma membrane (175).
Figure 3. HSV life cycle

1. Envelope glycoproteins interact with cell surface molecules; virions accumulate and are endocytosed into a lysosome terminal endocytic pathway.

2. At the appropriate low pH the viral envelope fuses with the vesicular membrane releasing capsid and tegument into the cytoplasm. Fusion is dependent on gB, gD, gH-gL, gD receptor. Low pH is essential but the role is unclear.

3. There is proteasome dependent capsid transport to the nuclear membrane. Capsid docks at a nuclear pore; genomic DNA is released and imported.

4. The HSV gene expression cascade ensues. This culminates in DNA replication and packaging of unit length genomes into nascent capsids.

5. Egress begins. Capsids undergo gH- and gB-dependent envelopment/de-envelopment to escape the nucleus. Capsids are then transported along microtubules to the site of secondary envelopment.

6. Parallel to capsid egress (red arrows), viral membrane glycoproteins are synthesized on the ribosomes of the endoplasmic reticulum. They are transported to the Golgi apparatus where they undergo complex glycosylation.

7. Glycoproteins progress through the secretory pathway to a site of assembly.
8. Additional tegument proteins are added to capsids in the cytoplasm. Capsids are then re-enveloped as they bud into vesicles containing markers of the trans Golgi and endosomes. Virions at this point are now infectious but trapped in the cell.

9. Virions are then either released via exocytosis or released during cell lysis.
Immune modulation

Herpes simplex viruses go unnoticed in their hosts for extended periods of time. Herpes simplex carefully modulates the host immune response to evade clearance. It is still unknown why HSV is periodically able to overcome the immune system and cause outbreaks while at other times it is suppressed. The ability of the virus to become latent is an excellent way for the virus to evade the immune system. There are no known interactions with the immune system during latency.

During lytic infection HSV modulates the host’s response at many levels. HSV ICP0 has been shown to be important in blocking the production of numerous interferons (54, 104, 114). HSV has also been shown to reduce Jak1 and Stat2 activation in the infected cell. The reduction was again linked to ICP0 and also to the VHS gene (32). Interaction with Toll receptors at both the cell surface, and in endosomes is one of the first interactions of virus with its host cell. Activation of Toll receptors can result in multiple cascades of antiviral activity. HSV is thought to associate directly (bind) or activate indirectly (viral DNA) with TLR1, 2, 3, 7, and 9. TLR2 maybe particularly important in HSV encephalitis (100). HSV has the ability to inactivate both classical and alternative components of the complement pathway. This has the effect of protecting both cell free virus and virus in infected cells. HSV-1 gC binds to the complement protein C3b and it is thought that this activity can block the otherwise inevitable cascade (62, 84). HSV ICP47 is believed to down regulate surface MHC-1 thereby blocking CD8+ T-cell activation (193). HSV ICP22 has been shown to diminish CD4+ cell activation (14). HSV-infected dendritic cells do not undergo maturation even in the presence of additional stimuli such as LPS and may enter an
apoptotic state (89, 151). These effects have been attributed partially to the actions of the VHS and ICP27 proteins (145). HSV has been shown to exhibit Fc receptor like activity and can bind Ig. This activity has been attributed to glycoproteins G and I. This has been shown to protect infected cells from antibody-mediated cytotoxicity (51). It was also observed that an antibody complex forms when the Fab region of antibody binds glycoprotein and Fc region of antibody binds Fc receptor. This complex was also shown to make HSV resistant to neutralization by the classical pathway of complement neutralization (60).

**Multiple HSV entry pathways**

The majority of animal virus families take advantage of endocytosis to accomplish cell entry (131). For many years, it was thought that HSV entered cells exclusively by fusing with the cell membrane with no requirement for endocytosis. More recently it was demonstrated that HSV entry also proceeds via endocytosis. The entry pathway utilized is now known to be cell type dependent. Active endocytosis is necessary for HSV entry into several experimental cell types, but most importantly HSV was shown to enter its primary site of infection, epithelial cells, via endocytosis (124, 125, 127). In contrast, entry into other neuronal cells and fibroblast cells, occurs by direct penetration of the plasma membrane and has no apparent requirement for endocytosis (36, 116, 124, 125, 127).

Both the endocytic and non-endocytic entry pathways share a number of features. Study of the kinetics of initial uptake, trafficking, penetration, and virion capsid delivery to the nucleus indicate, that entry by an endocytic mechanism is rapid, efficient and leads to productive infection (127), as is the case for direct penetration at the cell
surface. The completion of the entry process via either pathway requires participation of gB and gD and the gH-gL (36, 127, 161). Binding of virion gD to any one of its cognate receptors is a required component of the HSV entry process (28, 40, 162). In the non-endocytic pathway, HSV engages gD receptors at the cell surface and the capsid penetrates directly into the cytosol. In the endocytic entry pathway, capsid penetration is spatially distinct from cell surface binding. The enveloped virion is first taken up from the cell surface in a process termed internalization. This step is essential for successful endocytic entry but does not occur in the case of direct penetration at the plasma membrane. Internalization of HSV is rapid but is not mediated by any of the known gD receptors (127). Endocytosed HSV traverses a lysosome-terminal endosomal pathway that becomes lower in pH as the virus approaches the lysosome. Endosomal low-pH is needed for endocytic entry but its role is not clear. Trafficking of the virus to the site of intracellular penetration is also independent of gD receptors. However, interaction with a gD receptor, either at the plasma membrane or at an internal membrane, is required for release of the capsid into the cytosol. In the absence of receptor interaction, virions are trapped within endocytic compartments and ultimately undergo lysosomal degradation (127).

Common properties of viruses that utilize pH-dependent entry pathways include (i) entry by an endocytic mechanism, (ii) requirement of endosomal low pH for entry; (iii) inactivation of entry function by low-pH pretreatment of isolated particles, and (iv) activation of membrane fusion function by acid pH (52). HSV fulfills the first three of these four criteria, at least in certain cell types (124, 125, 127). HSV entry into cells that support an endocytic entry pathway is susceptible to inhibition by lysosomotropic
agents, which elevate the normally acidic pH of endosomes (125). However, HSV penetration at the surface (of Vero cells, for example) is not inhibited by such treatment (125, 191) and is considered pH independent. Consistent with a role for pH in membrane fusion, treatment of purified HSV particles with a mildly acidic pH of 4.5 to 5.5 irreversibly inactivates entry activity (125).

The essential glycoproteins of herpes simplex entry

**Envelope glycoprotein B**

Envelope glycoprotein B is a single pass type I membrane glycoprotein. It is approximately 904 amino acids long depending upon the strain of virus. gB is expressed late in the lytic cycle of replication and is believed to function as a homotrimer. gB is conserved among all herpesviruses. In all cases studied to date, it plays multiple roles in viral entry, including receptor binding and membrane fusion. The crystal structure of an ectodomain fragment of HSV type 1 (HSV-1) gB revealed an elongated, rod-like structure containing hydrophobic internal fusion loops in domain I (Figure 4) (78). This structure bears striking architectural homology to the low pH, postfusion form of G glycoprotein from vesicular stomatitis virus (VSV-G) (Figure 5) (142, 144). Both the gB and G structures have features of class I and class II fusion proteins and are thus designated class III proteins (180). Antigenic analysis of gB has mapped antibodies to several regions of gB (Table 2). Mutations that polarize hydrophobic residues in putative fusion loops of gB result in defective membrane fusion in a cell-cell fusion assay (74). Fusion loop mutants also result in reduced liposome
binding (73). gB along with gH is needed for nuclear egress of the capsid through the outer nuclear membrane. Mutations in the fusion loops of gB also diminish capsid egress (192).

In synthesis and maturation, gB is known to oligomerize in the ER (35). It is minimally glycosylated in the ER and is further glycosylated in the Golgi. gB binds to chaperones calnexin, Grp78, and calreticulin in the ER (102). Disruption of the secretory apparatus results in loss of incorporation of gB into virions (76, 88).

**Envelope glycoprotein D**

Herpes simplex gD is approximately 394 amino acids in length (Figure 6). It is essential for membrane fusion and entry into the host cell. gD is found only in alphaherpesviruses although other subfamilies of herpes viruses may have non homologous proteins that function in a similar way. Structurally gD consists of a receptor binding domain, an Ig like core, and a C-terminal pro-fusion domain (Figure 6). gD is believed to form a homooligomer, possibly a homodimer. Functionally, gD is a receptor binding protein that can utilize one of several cognate receptors (98, 117, 156, 160). Nectin-1 and HVEM are believed to be the most important in pathogenesis (161). During receptor binding, gD undergoes a conformational change that moves the C-terminal region away from the receptor binding site (99). It is believed that this conformational change converts gD into a profusion conformation. The C-terminal region, sometimes referred to as the pro-fusion domain, may interact with gB/ gH-gL or both (8, 10). Data for gD interactions with gB/gH-gL that lead up to fusion are not
comprehensive. There are many antibodies to specific regions of gD. Those used in this study are listed in Table 3.

**Envelope glycoproteins H and L**

gH-gL is a heterodimer that occurs at a one to one ratio. gH-gL is an essential component of the fusion machinery and has homologues conserved throughout the known human herpesviruses (25, 135, 176). gH is the largest component of the complex. It is 838 amino acids in length (108, 133). gH is a type one single pass glycoprotein with both N and O linked glycosylations. Sections of gH are reasonably hydrophobic and were believed to be components of a fusion glycoprotein. The crystal structures of several herpes virus gH-gLs have been resolved, revealing a structure dissimilar to any known fusion glycoprotein (Figure gH-gL) (34). It is likely that HSV gH-gL functions as a regulator of other fusion components (34).

gL is a 224 amino acid, non membrane anchored protein that associates with gH during synthesis and folding in the endoplasmic reticulum. It is essential for the correct folding and incorporation of gH into the virion and cell plasma membrane. gL is tightly associated with gH in the crystal structure. The structure of HSV-2 gH-gL revealed a “boot” shaped molecule (34). gH has three distinctly folded domains H1, H2 and H3 with gL adding a single domain to heterooligomer. The domains have been termed the heel (H1), the sole (H2) and the toe (H3) with gL forming the buckle (Figure 7).
Other factors that influence entry

It is clear that other envelope proteins such as UL20, UL24, UL45, gM and gK (11, 12, 49, 72, 112) are involved in the regulation of membrane fusion. However, the study of fusion has been largely centered on the core glycoproteins essential for cell-cell fusion. Dissecting the natural roles of all the envelope proteins is difficult due to the number of possible regulatory interactions. There may be essential regulatory steps that lead to fusion in vivo that are short cut by attempting to minimize the fusion machinery to the minimum glycoproteins necessary for fusion.
Figure 4. gB structure

A) Domain arrangement of HSV-KOS gB, Domains of gB are indicated by Roman numerals and color coding. Disulphide bonds are indicated by blue bars joining paired cysteines. TM is the transmembrane region. Linker regions between domains II and III are indicated in purple.

B) A ribbon rendering of the crystal structure of gB. A monomer of the trimer is shown. Color-coding and numeration are as in A. Regions disordered in the structure are shown as dots.

C) A ribbon rendering of the gB trimer. One protomer of the trimer is colored as in A. The remaining protomers are grey.

D) A surface rendering of C.

(Image reproduced with permission from the publisher (78))
Figure 5. Structural homology of VSV-G and HSV-gB

Trimeric structures of prefusion (A) and postfusion (low pH) (B) proteins of herpes simplex virus gB and VSV G. The protein backbone is shown in ribbon representation. One protomer of each trimer is shown in color. Homologous protein domains are the same color. The residue (aa 303) that is altered in an H126 MAb resistant (mar) mutant is denoted in magenta spheres (CPK representation). Domain (D) designations in the table are as originally assigned. Ribbon structures were prepared with PyMOL (44) after (78, 142, 144).
Table 2. Summary of monoclonal antibodies to gB used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Domain</th>
<th>Conformation</th>
<th>Neutralizing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-dependent</td>
<td></td>
</tr>
<tr>
<td>DL16</td>
<td>nd</td>
<td>Y (Oligomer</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>specific)</td>
<td></td>
</tr>
<tr>
<td>H126</td>
<td>I</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>H1359</td>
<td>II-III</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>H1817</td>
<td>VI</td>
<td>N</td>
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<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>SS118</td>
<td>I</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>SS55</td>
<td>I</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
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<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>SS144</td>
<td>V</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

\(a, b,\) and \(d\) summarize data as previously described (19, 20, 94, 134, 146). \(b\) Determined by reactivity with HSV-1 gB under native and denaturing PAGE conditions. \(c\) Detects a small amount of gB after denaturing as described. \(d\) Defined as reducing HSV-1 infectivity. \(e\) gB epitope reactivity was measured by dot blot as in Materials and Methods. \(nd\), not determined.
Figure 6. gD

A) Domain arrangement of HSV-1 Patton strain gD. In green, yellow and red are the regions that form the HVEM binding domain, the Ig-like core, and the C-terminal region respectively. Disulphide bonded cysteines are indicated by numeration and are adjoined by black lines. N-linked oligosaccharides are indicated as lollipops. TM is the transmembrane region.

B) Ribbon representation of the gD(23–306) subunit. The structural elements are labeled and color coded as in A.

C) Ribbon representation of a proposed gD dimer.

D) Surface rendering of a gD dimmer showing how inaccessible the profusion domain is prior to binding. The right protomer is color coded as in A, the left promoter is shaded gray for clarity.

(Image adapted with permission from the publisher (99))
Table 3. Antibodies to specific regions of gD

<table>
<thead>
<tr>
<th>Ab</th>
<th>Epitope type</th>
<th>Binding region</th>
<th>Neutralizing</th>
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<td>1103</td>
<td>Continuous</td>
<td>1-23</td>
<td>yes</td>
</tr>
<tr>
<td>DL11</td>
<td>Discontinuous</td>
<td>1-223</td>
<td>yes</td>
</tr>
<tr>
<td>DL2</td>
<td>Discontinuous</td>
<td>21-226</td>
<td>no</td>
</tr>
<tr>
<td>DL6</td>
<td>Continuous</td>
<td>272-279</td>
<td>yes</td>
</tr>
<tr>
<td>R7</td>
<td>Polyclonal</td>
<td>N/A</td>
<td>yes</td>
</tr>
</tbody>
</table>

Data from references (37, 55, 85, 115, 121).
Figure 7. HSV-2 gH-gL

A) Domain arrangement of gH and gL. gH domains are colored green (H1), yellow (H2) and red (H3). gL is colored blue. Structurally un-resolved regions are indicated as dotted lines. Disulphide bonded cysteines (designated C) are numbered and joined by black lines.

B) A ribbon rendering of the structure colored as in A.

C) Surface rendering of the gH-gL structure. Heel, sole, and toe domains are indicated with the corresponding domains.

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Virus induced membrane fusion.

Viral fusion glycoproteins drive membrane fusion through conformational changes. Conformational changes are triggered by host cell cues (185). Triggers are proteolysis, receptor interaction, and the most common of all, low pH. Triggering can be a multi step process such as via sequential interaction with receptor and then co-receptor (HIV). Receptor binding followed by encountering low pH or proteolysis may also occur (137, 185).

The energy for membrane fusion is provided by conformational changes (Figure 8). Upon encountering their trigger, fusion glycoproteins undergo conformational changes that enable insertion of part of the fusion peptide into the host membrane. These regions are usually apolar and contain several bulky hydrophobic residues. Insertion causes deformation of the host membrane. There then follows collapse of the fusion intermediate into a postfusion conformation. This brings the membranes into proximity. The two membranes fuse and a pore forms. This pore expands until the viral cargo is released into the cytoplasm (53, 180, 185).

There are several distinct classes of fusion glycoprotein. Class I and II differ in the composition of their secondary structure, and in their prefusion oligomeric makeup. Class III fusion glycoproteins are similar to Class I in that they have large coiled coil regions and are trimeric in the prefusion conformation. Class III fusion glycoproteins have large β-sheet motifs including fusion loops reminiscent of those found in Class II fusion proteins. In addition, Class III fusion glycoproteins differ from both other classes in that they are able to reverse conformational changes once triggered (53, 180, 185).
Figure 8. Model of viral fusion glycoproteins fusion inducing conformational changes

A) Glycoprotein is in a metastable prefusion conformation.

B) Upon encountering their trigger (low pH is indicated), fusion glycoproteins undergo conformational changes that enable insertion of part of the fusion peptide into the host membrane. An extended intermediate conformation is cartooned.

C) There then follows collapse of the fusion intermediate into a postfusion conformation. The two membranes fuse and a pore forms. The viral cargo is released into the cytoplasm
The aim of this study

Much progress has been made over the last several years in understanding HSV entry into the host cell. However, the precise triggers of membrane fusion during virus entry remain to be determined.

The overall goal of this study is to define the role of low pH during the endocytic entry of HSV. In the first section, studies were designed to define precisely where low pH is acting in HSV entry. Having identified HSV gB as being triggered for fusion by low pH, the second section further characterizes conformational changes in gB. Studies are also designed to give insight into the mechanism of fusion. The third section examines conformations of gB throughout synthesis, processing and incorporation into the virion.
II. Materials and methods

Cells and viruses

Vero cells (American Type Culture Collection; ATCC; Rockville, Md.) and Cos-7 cells (provided by Shirley Taylor, Virginia Commonwealth University) were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, Calif.). CHO-nectin-1 cells were propagated in Ham's F12 medium (Life Technologies, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS; Life Technologies), 150 µg of puromycin (Sigma, St. Louis, Mo.)/ml, and 250 µg of Geneticin (Life Technologies)/ml. Cells were subcultured in nonselective medium prior to use in all experiments.

HSV-1 strain KOS (provided by Priscilla Schaffer, Harvard University) and HSV-2 strain 333 (provided by Stephen Straus, National Institutes of Health) were propagated and titered on Vero cells.

Antibodies

The biochemical characteristics of the antibodies used in this study are summarized in Tables 1 and 2 from (19, 20, 37, 55, 85, 94, 115, 121, 134, 146). In brief, the DL series of antibodies was raised against BHK cell extracts infected with HSV 1 and 2. The H series were isolated from mice immunized with HSV-1 14012 strain. R series were generated from rabbits immunized with purified HSV glycoproteins. SS and RC were generated by challenging mice with gB from HSV 1 and 2 that had been
purified from cell extracts (DL16 column), portions of which were native in conformation, alkylated or reduced.

**Purified proteins**

The following are a list of the purified proteins used throughout with explanation of their acquisition and relevant construction details.

**Ovalbumin**, purified grade-V (obtained from Sigma A5503), (Gallus gallus ovalbumin) contains 3 tryptophans and is 386 amino acids in length. This is purified from chicken hen egg whites.

**gC**, was gc1(457t) described in (171). gC from HSV-1 KOS is truncated at amino acid 457, 18 amino acids before its transmembrane region. This is expressed from recombinant baculoviruses in Sf9 cells. This construct has 8 tryptophans.

**gD-2t**, was obtained from Stephen Straus (41). This is gD from HSV-2 333 that has only its transmembrane region deleted and retains its C-terminal tail. The exact amino acid sequence is unclear in the literature. It was reported to be “constructed in a fashion similar to that reported by Stuve (87, 168). gD-2t was expressed in CHO cells. This is predicted to contain 6 tryptophans.

**gD(Δ290-299t)**. First described in Nicola 1996 (128), gD-1(290-299t) has amino acids 290 to 299 deleted, R replacing I at residue 290, and amino acids KIFL inserted after R. It has 6 tryptophans and is 389 amino acids in length. This is expressed from recombinant baculoviruses in Sf9 cells.

**s-gB** (obtained from Stephen Straus). The gB2ΔTM construct contains the following amino acids of gB from HSV-2 strain 333 (numbered per (168)
Ala23 to Asn725 fused to Gln799 to Leu904 (Ala726 to Leu798 are deleted). The deletion includes the transmembrane region based on Chou and Fasman (Ala745 to Leu798) (189). It is 809 amino acids in length. This was expressed from a recombinant construct in CHO cells.

**gB730** (obtained from Roselyn Eisenberg, Gary Cohen). Described in (20), this contains amino acids 31 to 730 of HSV KOS gB. It is truncated prior to the transmembrane and tail region. gB730 is 700 amino acids long and contains 7 tryptophans. This is expressed from recombinant baculoviruses in Sf9 insect cells.

**HVEMt**, was originally described in (182) as HVEM(200t). HVEMt contains amino acids 39-200 of HVEM. The first 38 amino acids are a signal sequence that is cleaved during synthesis. Amino acid 200 is just before the transmembrane region and was truncated to increase solubility of the construct. This is expressed from recombinant baculoviruses in Sf9 insect cells.

**Nectin-1t**, was originally described in (98) as HveCt. It contains amino acids 31-346 of Nectin. The first 30 amino acids are a signal sequence that is cleaved during synthesis. Amino acid 346 is just before the transmembrane region and was truncated to increase solubility of the construct. This is expressed from recombinant baculoviruses in Sf9 insect cells.

**Luciferase reporter assay for cell-cell fusion**

Cos-7 (effector) cells were grown in 6-well plates and transfected with plasmids encoding T7 RNA polymerase (pCAGT7), gB (pPEP98), gD (pPEP99), gH (pPEP100), and gL (pPEP101) (135) obtained from G. Cohen and R. Eisenberg. For experiments
with multiple glycoproteins, 0.8 µg of each glycoprotein-encoding plasmid was transfected per well. For experiments with single glycoproteins 3.0 µg of each glycoprotein encoding plasmid or empty vector was transfected with 0.8 µg of pCAGT7. Transfections were performed using Lipofectamine 2000 (Invitrogen). To prepare target cells, Vero cells in six-well plates were transfected with 2.0 µg per well of plasmid encoding the firefly luciferase gene under control of the T7 promoter (pT7EMCLuc). Cells were trypsinized, pelleted and resuspended in culture medium. Target and effector cells were mixed, plated in 24-well plates and incubated at 37°C overnight. Cells were then washed with PBS, incubated for 2 min in buffered pH medium (5 mM (each) HEPES (Life Technologies), 2-(N-morpholino)ethanesulfonic acid (MES; Sigma), and sodium succinate (Sigma), washed in PBS and incubated at 37°C. A second 2 min pH treatment was repeated at 3 hr. At 7 hr post-pH treatment, cells were washed with PBS and lysed (Luciferase Assay System; Promega). Lysates were mixed with substrate (Promega) and immediately assayed for light output using a Tropix TR717 microplate luminometer (Perkin Elmer). Assays were performed in triplicate and repeated at least three times for each experiment.

Confocal microscopy of virion gB during viral entry

CHO-nectin-1 cells were mock-treated or treated with 25 nM bafilomycin A1 for 15 min. HSV-1 KOS (MOI of 20) was bound to cells at 4°C for 1 hr. Cultures were shifted to 37°C for 1 hr in the constant presence of BFLA or vehicle control, and 0.5 mM cycloheximide. Cells were fixed with 3% paraformaldehyde (Thomas Scientific) and permeabilized with 0.2% Triton X-100 (Fisher Scientific). Virion gB was detected with 2
mg/ml MAb H126 or 0.5 mg/ml MAb H1817 (Virusys) followed by Alexa 488-labeled goat anti-mouse antibody. Samples were visualized with a Zeiss LSM 510 Meta microscope equipped with a 63x oil immersion objective lens. 50 to 70 cells are shown per panel.

Confocal microscopy of gB in the infected cell

Vero cells were infected with HSV-1 KOS at an MOI of 5. At 1 hours post infection cells were treated or mock treated with 25nM Bafilomycin-A1. At 6.5 hr p.i. cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100.

Pulse chase

Vero cells were infected with HSV-1 KOS (MOI 5) for 6 hrs. Cells were washed in PBS, then medium was replaced with DMEM without cysteine or methionine (Invitrogen). After 20 minutes medium was replaced with DMEM containing 400 µCi of radiolabeled cysteine and methionine (MP Biomedicals) for a 100 second pulse. To chase samples, DMEM with 20 mM HEPES, 5 mM L-cysteine, 5 mM L-methionine and 1 mM cycloheximide was added, then washed, and replaced a final time. To stop, samples were moved to ice and media was replaced with ice-cold PBS containing 20 mM N-ethylmaleimide (NEM). Samples were lysed with ice-cold 2% CHAPS in HBS containing 20 mM NEM and protease inhibitors (Roche). Samples were then immunoprecipitated over night using Pro-A agarose with an excess of the indicated antibody. gB was visualized using SDS-PAGE and autoradiography.
Antigenic conformation of virion gB vs. gB in infected cells

HSV gB from virions or from cell lysates was immunoprecipitated with specific anti-gB antibodies, MAbs H1817, SS10 and H126 are shown. Samples were analyzed by SDS-PAGE and Western blotting.

Triton X-114 partitioning

To increase the association of hydrophobic gB with the amphiphilic (pellet) phase, Triton X-114 (Fisher Scientific) was preconditioned to reduce the amount of the most hydrophilic Triton X-114 molecules and to enrich for the amphiphilic molecules. To precondition, the hydrophilic phases from each of three overnight condensation steps was discarded (24). Condensation was performed at 30°C. 200 ng of s-gB, gB730, gD-2t, gD-1(290-290t), or 20 µg of BSA were incubated with preconditioned 2% Triton X-114 in fusion medium buffered (MES, Succinate and HEPES as previously mentioned) to various pHs on ice for 10 min. Samples were incubated at 37°C for 10 min, and then pelleted at 25°C for 3 min at 300 x g. Aqueous and pellet phases were collected and either immunoprecipitated with MAb H1359 (gBs), DL6 (gDs) or precipitated with trichloroacetic acid (BSA).

Sucrose density centrifugation

Extracellular virions were lysed in 1% Triton X-100 for 30 min at room temperature and layered onto a step gradient of 8%-60% (wt/wt) sucrose in 20 mM each of MES, sodium succinate and HEPES, 175 mM NaCl, 2.5 mM EDTA, 2.5 mM
EGTA, and 1% Triton X-100. Indicated pHs were adjusted by adding HCl. Samples were sedimented at 40,000 rpm for 16 h at 4°C in a Beckman SW41 rotor. Fractions were collected, immunoprecipitated with MAb H1817 (Virusys), and analyzed by SDS-PAGE followed by immunoblotting with MAb H1359 to gB (Virusys).

**Dot blot analysis**

s-gB, gB730 or cell-free preparations of extracellular HSV-1 KOS were diluted in serum-free, bicarbonate-free DMEM with 0.2% bovine serum albumin (BSA) and 5 mM (each) HEPES (Life Technologies), 2-(N-morpholino)ethanesulfonic acid (MES; Sigma), and sodium succinate (Sigma) to achieve final pHs ranging from 7.4 to 4.8. Samples were incubated at 37°C for 10 min. Samples either were blotted directly to nitrocellulose with a Minifold dot blot system (Whatman) or were first neutralized by addition of pretitrated amounts of 0.05 N NaOH. For detection of gB, membranes were blocked and incubated with anti-gB monoclonal antibody H126 (94), H1359, H1817 (134) (Virusys), DL16, SS10, SS55 (20), SS106, SS144 (19) or R69 polyclonal antibody to gB provided by G. Cohen and R. Eisenberg. For detection of gD, anti-gD antibodies R7, DL2, DL6 1103 and DL11 (37, 55, 85, 115, 121) were incubated with membranes. After incubation with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescent substrate (Pierce) was added and membranes were exposed to X-ray film (Kodak).
Analysis of gB oligomeric structure by PAGE

HSV-1 KOS virions, HSV-2 333 virions, infected cell lysates, or a recombinant soluble form of gB (s-gB) was diluted in medium as described above for dot blotting. s-gB has the transmembrane domain deleted (gB-2t) (189) and was obtained from Stephen Straus. Samples were adjusted to the indicated pHs with pretitrated amounts of 0.05 N HCl and incubated at 37°C for 10 min. SDS (1%) was added, or samples remained untreated. Polyacrylamide gel electrophoresis (PAGE) sample buffer containing 0.2% sodium dodecyl sulfate (SDS) and no reducing agent was added ("native" conditions), and proteins were resolved by PAGE (37). After transfer to nitrocellulose, membranes were blocked and incubated with rabbit polyclonal antibodies specific for gB, gC, gD, or gH-gL (provided by G. Cohen and R. Eisenberg). After incubation with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescent substrate (Pierce) was added, and membranes were exposed to X-ray film (Kodak).

Clustal W alignment

The sequences of HSV KOS gB, gB730, HSV-333 gB, and s-gB were compared using Multiple Sequences Alignment tool “Clustal W” from EMBL. A full alignment was performed using the default settings.
Intrinsic fluorescence

300-600 ng of purified protein were diluted in 30 µL in Hypure H₂O (Fisher) containing 137 mM NaCl, 5 mM (each) HEPES (Life Technologies), MES (Sigma), and sodium succinate (Sigma) to achieve final pHs ranging from 7.3 to 5.1. The minimal detectable concentration of each protein varied slightly due to the number and environment of fluorescent amino acids present. Proteins were incubated at 37°C for 2 min and fluorescence was measured at room temperature using a Shimadzu RF-5301PC Spectrofluorophotometer (thanks to John Hackett, Darrell Peterson, VCU Department of Biochemistry for use of equipment). Excitation was performed at 280 nm. A 10 nm spectral bandwidth was used with a 0.5 s response time. Emission was recorded over 310-400 nm. Data was processed using Microsoft Excel. To test the reversibility of conformational changes, samples were prepared at pH 5.1, incubated for 2 minutes, and then neutralized with a pre-determined amount of NaOH. Samples were then incubated at 37°C for 30 seconds and fluorescence was measured. Non-neutralized samples had H₂O added in place of NaOH although this did not alter the shift in peak emission observed.

All proteins except for gD-2t (explained in results) had similar patterns of low pH induced emission shift at higher and lower concentrations.
Soluble gD binding ELISA

Soluble receptors HVEMt (15 µg/ml) or nectin-1t (10 µg/ml) in PBS were bound to microtitre plates overnight at 4 °C. Plates were incubated with 1% BSA and 1% chicken egg albumin (Sigma) in PBS (blocking buffer) for 1 hr at room temperature. Dilutions of gD(Δ290-299t) that had been treated with a buffer of pH 4.7 for 10 min at 37 °C, heated to 80°C for 10 min, or mock treated, were prepared in blocking buffer and were added for 3 hr at room temperature. Anti-gD MAb DL6 in blocking buffer was added for 1 hr at room temperature. Protein A-peroxidase (Roche Diagnostic) in blocking buffer was added for 1 hr at room temperature. 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid (Pierce) substrate was added.

HSV gB fusion loop mutations

Our collaborators at the laboratory of David Johnson kindly provided cell-associated virus preparations of fusion loop mutant viruses.

In brief, the mutant viruses were generated as follows (74, 179, 192); Mutant gB constructs were made in plasmid pPEP98 using a QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA). Primers were designed to mutate the gB gene to encode the specified amino acid sequences (sequence not detailed in literature). Mutations were performed by amplifying pPEP98 DNA with mutant generating PCR primers in PCR reactions. Sequencing of the entire gene confirmed the desired mutations. Insertion of mutant and wild type genes into the HSV-1 strain F BAC was done via galK recombination. For BAC mutagenesis, gB from mutant
or parental plasmids were excised with XhoI and BglII, and purified (QIAquick gel extraction kit (Qiagen, MD). DNA was transformed into SW102 bacteria containing the HSV-1 F strain BAC designated BAC-gBgalK/gH. BAC-gBgalK/gH has a kanamycin cassette in place of the glycoprotein H gene, and galK encoding sequence replacing gB. Temperature-sensitive lambda prophage recombination proteins induced recombination. Homologous regions between the mutant gB DNA and the flanking regions in the BAC sequence were predicted to undergo recombination. Selection was performed on M63 agar plates containing glycerol (0.2%; Fisher), d-biotin (1 mg/l), L-leucine (45 mg/l), 2-deoxy-galactose (0.2%; Ferro Pfanstiehl) and chloramphenicol (12.5 µg/ml). Bacteria containing BACs that retain the galK sequences metabolize 2-deoxy-galactose and accumulate a toxic build up of 2-deoxy-galactose-1-phosphate (Nucleic Acids Res. 2005 Feb 24;33(4):e36). Colonies that grew were believed to have replaced galK with the desired gB sequence were screened by sequencing. Clones that contained the desired sequences were grown and had DNA prepared via plasmid maxikit (Qiagen, MD). BAC DNA was then transfected into Vero derived F6 cells that express gH. Transfection was via the lipid reagent, TransFectin (Bio-Rad, Hercules, CA). The resulting HSV viruses were propagated and tittered in F6 cells. Cell associated virus preps were made by infecting Vero (wt) or gH complimenting F6 cells (mutants with ΔgH) for 18hrs. Cells were then scraped into tubes and freeze thawed three times. Large cell debris was removed by pelleting twice. gB assays were performed for viruses from the BAC-gBgalK/gH background with no gB (w.t.gB/gH-), with KOS gB (gBgalK/gH-), with the fusion loop 1 mutation Y179K (Y179K) and a fusion loop 2 mutation (A261D).
**DEPC treatment of HSV**

Virions of HSV KOS were concentrated and resuspended in PBS. 50ug of virus was treated with the DEPC concentration indicated for ten minutes at room temperature. Infectivity was then analyzed in the indicated cell types via β-galactosidase reporter assay (CHO derived cells) or plaque assay (Vero and HaCaT). DEPC concentration was determined from the increase in absorbance at wavelength 230 due to conversion of imidazole to N-carbethoxy imidazole and its molar extinction coefficient 3200.

**β-galactosidase reporter assay**

Confluent cell monolayers grown in 96-well dishes were infected with HSV (strain KOS) at an MOI of 5. Infected cells were incubated at 37°C for 6 h. Cells were lysed in 0.5% Nonidet P-40, chlorophenol red-β-D-galactopyranoside (Roche Diagnostics) was added, and β-galactosidase activity was determined by absorbance at 560 nm using a microtiter plate reader (Dynatech, Chantilly, Va.). Mean results were calculated for four replicate samples. The β-galactosidase activity from infected but mock-treated cells was defined as 100%.

**HSV plaque assay**

Confluent cell monolayers grown in 24-well plates were infected with serially diluted HSV-1 KOS. Infected cells were incubated at 37°C for 18 h and fixed in methanol. Visualization of plaques was aided by antibody detection and HRP
conversion of 4-chloro-1-naphthol to a blue/black color on the monolayer. Mean plaque number calculated from 4 wells is shown.
III. Identifying a role for pH in the endocytic entry of HSV

Mildly acidic pH promotes cell fusion mediated by HSV glycoproteins

Intracellular low pH is required for the cell entry of several herpesviruses, often in a cell type specific manner. The precise role of low pH in herpesviral entry is not clear. A luciferase reporter assay of cell-cell fusion was employed to investigate a direct, activating role of low pH in fusion mediated by HSV glycoproteins. Cells that transiently express the glycoproteins gB, gD, gH and gL fuse with target cells (135, 176) (Figure 9A). Thus, fusion mediated by glycoproteins expressed on the cell surface can occur at pH 7.4. When effector and target cells were mixed, allowed to interact, and then pulsed twice with culture medium of pH 6.0, there was an increase in cell-cell fusion mediated by gB, gD, and gH-gL (Figure 9A). This suggests that low pH has a stimulatory effect on the fusogenic activity of one or more HSV glycoproteins. Experiments where an individual glycoprotein was substituted with vector (vector, gD and gH-gL is shown) indicate that for optimal fusion in a low-pH environment, gB, gD and gH-gL are essential.

We examined the effect of a range of pH on glycoprotein-mediated fusion (Figure 9B). There was an increase in cell-cell fusion when cells were treated at decreasing pHs. Maximal fusion was reproducibly detected at pH 6.0 to 5.7. Intravesicular pH along the lysosome terminal endocytic pathway pH ranges from ~ 6.2 in early endosomes to ~ 4.8 in lysosomes (113). Thus, the pH optimum of cell-cell fusion is
within the range of pH found in endosomes. Control treatment of cells with pHs 7.4 to 4.7 did not affect luciferase production when both reporter components had been transfected into the same cell types (data not shown).

The effect of acidic pH on fusion mediated by the individual glycoproteins was assessed. Treatment with pH 6.0 enhanced the fusogenic activity of gB on its own (Figure 9C and 9D). The extent of fusion depended on the amount of gB plasmid used for transfection. There was little increase in fusion mediated by gD or gH-gL alone (Figure 9C). Fusion mediated by the four glycoproteins at pH 7.4 measured in relative light units was reproducibly greater than the pH-triggered fusion by gB alone (data not shown), consistent with the requirement of all four glycoproteins for optimal fusion and entry. Cell monolayers transfected with gB or gD have been reported previously to undergo pH-dependent cell fusion as detected by light microscopy (2, 26). We cannot rule out the possibility of pH effects on the structures and functions of gD and gH-gL. Since mildly acidic pH had the greatest effect on gB function, we pursued the effect of pH on gB in greater detail.
Figure 9. Effect of Low pH Treatment on HSV Glycoprotein-Induced Cell-Cell Fusion.

Cos-7 effector cells were grown and transfected with plasmids encoding T7 RNA polymerase and glycoproteins. Target Vero cells were transfected with a plasmid encoding the firefly luciferase gene under control of the T7 promoter (pT7EMCLuc). After 5 h target and effector cells were mixed, plated in 24-well plates and incubated at 37°C overnight. Cells were pulsed twice with pH media buffered and incubated for 9 hrs to allow luciferase expression.

(A) Cos7 cells expressing gB, gD, gH, gL or vector as indicated were mixed with Vero cells and treated with the indicated pHs. Luciferase activity measured in Relative Light Units (RLU) and expressed as a percent of luciferase activity from samples with gB, gD, and gH-gL at pH 7.4.

(B) Fusion mediated by gB, gD, and gH-gL over a range of pH is shown

(C) Fusion induced by the individual HSV glycoproteins at pH 7.4 and 6.0 is shown. More than three times the DNA was used for transfection relative to gB, gD, gH-gL (A).

(D) Fusion mediated by gB over a range of pH is shown.
Low-pH treatment alters the exposure of epitopes in the fusion domain of virion gB

Viral glycoprotein-mediated fusion is accompanied by conformational changes that result in domain rearrangement and exposure of hydrophobic fusion peptide sequences. The hydrophobic peptide then interacts with lipids of the target membrane, which is an essential destabilizing step in the fusion process (185).

To begin to determine whether intracellular low pH directly causes conformational change in gB associated with fusion and entry, we measured the reactivity of mouse monoclonal antibody (MAb) H126. H126 has complement-independent, virus-neutralizing activity and recognizes a linear epitope in the putative fusion domain of gB (domain I) (78, 94, 132, 146). In addition, a fusion-from-without form of virion gB with enhanced fusogenicity was shown to have an H126 epitope with altered accessibility (146).

HSV virions were exposed to pHs ranging from 7.4 to 4.8 and were immediately blotted to nitrocellulose membranes. Antibody binding was then assessed at pH 7.4. MAb H126 displayed diminished binding to virions that had been treated at pH <6.2 (Figure 10). Approximately 50% of H126 reactivity was lost in virions exposed to pH 5.9. Similar results were obtained with an additional MAb to domain I, SS55 (Figure 10; Table 4). This suggests a specific change in the antigenic structure of the fusion domain. As a control, MAb H1817 to N-terminal residues 31 to 43 (domain VI) of gB displayed unaltered binding to acid-treated virions (Figure 10). Similarly, MAbs H1359
and SS10, directed to domains III and IV, respectively, and gB-specific polyclonal antibody (PAb) R69, which also bound well to low pH-treated HSV (Figure 10; Table 4), suggest that the structure of gB is not globally altered and that the detected changes in reactivity are specific to domains I and V. Domains I and V are in close proximity and together form a functional region (19). Interestingly, MAbs to domain V, SS106 and SS144, had reduced binding to virions that had been treated with mildly acidic pH (Figure 10; Table 4), similar to the domain I-specific antibodies. Together, the results suggest that exposure of HSV to a pH of less than or equal to 6.2 changes the antigenic conformation of the gB functional region 1 that contains fusion loops (74).
Figure 10. Antibody reactivity of low pH-treated virions

Extracellular HSV-1 KOS virions (10^5 PFU) were treated for 10 min at 37°C with medium buffered to the indicated pHs and were blotted immediately to a nitrocellulose membrane. Blots were probed at neutral pH with the indicated gB-specific antibodies, followed by horseradish peroxidase-conjugated goat secondary antibody. The exposure shown for MAb H126 highlights the pH threshold of conformational change.
Table 4. Summary of monoclonal antibodies to gB used in this study

<table>
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<tr>
<th>Antibody</th>
<th>Domain</th>
<th>Conformation -dependent&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralizing&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reacts with low pH-treated gB&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>HSV-1 KOS</td>
<td>s-gB</td>
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<tr>
<td>DL16</td>
<td>nd</td>
<td>Y (Oligomer specific)</td>
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<td>Y&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>R69</td>
<td>polyclonal</td>
<td>na</td>
<td>+</td>
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Data in the first three columns have been previously described (19, 20, 94, 134, 146).

<sup>a</sup>Y, reactive with HSV-1 gB under native PAGE conditions but not under denaturing conditions. N, reactive under denaturing conditions only. <sup>b</sup>Reactive with native gB and with a small amount of gB under denaturing conditions (19). <sup>c</sup>Defined as reducing HSV-1 entry or plaque formation. <sup>d</sup>gB epitope reactivity was measured by dot blot as in Materials and Methods. nd, not determined; na, not applicable.
Mildly acidic pH alters the oligomeric structure of gB

Viral fusion proteins are oligomers (185). HSV gB is oligomeric, and an ectodomain form of HSV-1 gB has a trimeric structure (35, 78). Low pH-triggered membrane fusion can be accompanied by rearrangement of viral glycoprotein oligomer subunits (3, 178). Three approaches were used to determine the effect of acid pH on the quaternary structure of gB. First, HSV virions were solubilized with the nonionic detergent Triton X-100 and subjected to sucrose gradient centrifugation at pH 7.4 or pH 5.1. Fractions were analyzed by immunoprecipitation, SDS-PAGE, and immunoblotting for gB. At pH 7.4, gB was detected in fractions that corresponded to >181 kDa in molecular mass. This is consistent with gB oligomers and is similar to previous reports (81). At pH 7.4, a peak in fraction 6 and a broad peak from fractions 8 to 11 were observed (Figure 11A). In contrast, at pH 5.1, gB sedimented predominantly as a single population at fractions 6 and 7, suggesting that low pH caused gB to shift from a higher oligomeric form to a lower-density oligomer. Quantitation confirmed that the sedimentation profile of virion gB at pH 5.1 had shifted to a single, less dense peak (Figure 11A bottom).

Second, a monoclonal antibody specific for oligomeric gB, DL16 (7), was employed. HSV-1 KOS virions were treated with either pH 7.2 or 5.5 and blotted to membranes, and the reactivity of MAb DL16 was evaluated at neutral pH. After exposure to pH 5.5, there was a decrease in DL16 reactivity (Figure 11B). As a control, rabbit polyclonal antibody to gB (R69) detected both virion samples to a similar extent.

Lastly, these results were extended by a direct analysis of virion gB by PAGE.
Oligomers of HSV gB are comprised of noncovalently associated monomers. gB oligomers are disrupted experimentally by a combination of SDS and heat (35), resulting in monomeric gB that migrates at ~116 kDa after SDS-PAGE. We tested whether low pH had an effect on the detergent stability of the gB oligomeric structure. Virions were adjusted to pHs ranging from 7.4 to 5.1 and prepared for "native" PAGE in sample buffer containing 0.2% SDS without reducing agent and without heating. Under these conditions, regardless of pH treatment, gB migrated as a range of oligomeric species of >181 kDa (Figure 11C, lanes 1 and 2). Treatment of HSV with pH 7.4 followed by 1% SDS yielded gB species of similar high molecular weight (Figure 11C, lane 3), indicating that 1% SDS alone had no detectable effect on the gB oligomer. In contrast, pretreatment with pH <6.4 followed by 1% SDS reduced the number of gB species detected (Figure 2C, lanes 4 to 7). The highest-molecular-weight forms seemed to disappear, leaving only a single detectable oligomeric species of lower molecular weight (Figure 11C, lanes 6 and 7). This suggests that low pH alters the oligomeric structure of gB, making it more sensitive to disruption by SDS. With decreasing pH, there was an apparent decrease in detection of gB-reactive species. One explanation is that monomers are detected only weakly relative to oligomers under standard native PAGE analysis (data not shown). Alternately, upon activation by pH, gB may become part of a larger complex that does not enter the native gel. For example, during fusion, gB forms higher-molecular-weight complexes with gD and gH-gL (8, 19). Notably, the total amount of gB detected by dot blot does not change upon exposure to mildly acidic pH (Figure 10). Together, results from the three approaches (Figure 11A, B, and C) suggest that low pH alters the oligomeric conformation of virion gB, resulting in a lower-
order oligomer.

To address whether the >181 kilodalton gB-reactive bands shown in Figure 11C were indeed oligomers of gB, we tested whether other HSV entry glycoproteins comigrated with the gB-containing complexes. Virion gC, gD, gH, and gL at pH 7.4 each migrated independently of gB under native PAGE conditions (Figure 11D, lanes without 1% SDS). gB may associate with other proteins. However, since a purified form of the recombinant gB oligomer has a molecular weight similar to that of virion gB (see Figure 13A), we favor the interpretation that the high-molecular-weight species shown in Figure 11C represent gB homo-oligomers.

Treatment of virions with pH 5.1 did not affect the migration of gC, gD, gH or gL relative to pH 7.4 on native polyacrylamide gels (Figure 11D, lanes without 1% SDS). In addition, low pH did not reproducibly affect the detection of these glycoproteins, including the gH-gL hetero-oligomer (Figure 11D, braces). Virion gC, gD, gH, or gL was also not affected when subjected to the conditions that demonstrated the effect of low pH on the detergent stability of gB (Figure 11D, lanes with 1% SDS). Based on these analyses, gB may be the entry glycoprotein principally affected by pH.
Figure 11. Effect of low-pH treatment on the oligomeric state of gB.

(A, top panel) Virions were solubilized with 1% Triton X-100 and subjected to sedimentation through sucrose (8 to 60%) buffered to pH 7.4 or 5.1. gB was immunoprecipitated from each collected fraction with MAb H1817 prior to SDS-PAGE and immunoblotting with MAb H1359 for detection of gB. In parallel experiments, protein standards were employed to approximate the range of molecular weights of proteins in each fraction. (A, bottom panel) Results were quantitated by densitometry. (B) Binding of gB oligomer-specific monoclonal antibody DL16 to low pH-treated virions. As described in the legend to panel A, HSV-1 KOS virions were treated with pH 7.2 or 5.5, and then 2-fold dilutions were blotted to a membrane. (C) Virions were treated with the indicated pH, adjusted to 1% SDS where indicated, and then analyzed by PAGE and immunoblotting with R69 to detect gB. (D) HSV-1 KOS virions were treated at pH 7.4 or 5.1, solubilized with 1% SDS where indicated, and then analyzed by native PAGE and immunoblotting with polyclonal antibodies specific for gB, gC, gD, or gH-gL. Glycoprotein-specific bands are indicated with the name of the protein. Nonspecific bands that were detected in mock-infected, Vero cell-conditioned medium (not shown) are indicated by asterisks. Protein molecular weight standards are indicated on the left. α, anti.
**pH-triggered conformational changes are reversible**

Conformational changes in class I and class II fusion proteins are irreversible (185). As an example, the prefusion form of hemagglutinin (HA) (class I) in the influenza viral envelope exists in a metastable state. If it is triggered by low pH in the absence of a target membrane, HA is irreversibly converted to the postfusion form and can no longer mediate fusion with a subsequently presented membrane (184). In contrast, conformational changes in the class III fusion protein VSV-G are reversible, with prefusion and postfusion forms existing in a thermodynamic equilibrium (67, 141). The equilibrium is shifted toward the postfusion state at low pH. It is believed that class III fusion glycoproteins undergo reversible conformational changes to allow them to remain fusogenic as they encounter the low-pH of the trans-Golgi network. To investigate the reversibility of changes in gB, we again assayed the effect of 1% SDS on oligomer stability using native PAGE (Figure 11C). KOS virions were incubated at pH 5.1, re-neutralized to pH 7.4, and then 1% SDS was added (Figure 11C, lane 8). Oligomeric forms of gB were detected (Figure 11C, lane 8), indistinguishable from those of gB that had been kept at neutral pH (lane 3). This suggests that low pH-induced changes in the oligomeric structure of gB are reversible.

To extend the findings of reversibility, we tested whether pH-induced antigenic changes were reversible, using a modification of the dot blot approach on HSV virions. pH 5.5-treated HSV that was blotted to nitrocellulose displayed decreased reactivity with MAb H126, relative to pH 7.2 treatment (Figure 12). Although this is an overexposure, the reduction in H126 reactivity is consistent with the results shown in Figure 10. However, when virions were treated first at pH 5.5 and then adjusted back to pH 7.2
prior to being blotted, H126 reactivity was partly recovered. Similar results were obtained with the oligomer-specific MAb DL16 (Figure 12), supporting the notion of reversibility. Control polyclonal antibody to gB R69 reacted similarly with HSV that had been subjected to each of the different pH conditions (Figure 12). Immobilization of acid-treated virions on nitrocellulose membranes may limit the reversibility of alterations in gB. This would explain why several of the MAbs to gB have diminished reactivity with acid-treated HSV after it is bound to nitrocellulose (Figure 10 and Table 4).
Figure 12. Reversibility of pH-induced conformational changes in gB

Extracellular HSV-1 KOS virions were treated with medium buffered to pH 7.2 or 5.5. For the indicated samples, pH was neutralized back to 7.2 for 10 min at 37°C. Twofold dilutions were blotted immediately to nitrocellulose membranes. Membranes were probed at neutral pH with antibody H126, DL16, or R69, followed by the appropriate horseradish peroxidase-conjugated secondary antibody.
2-fold dilution of HSV-1

pH: 1.2  5.5  5.5→7.2  1.2  5.5  5.5→7.2  1.2  5.5  5.5→7.2

H126  DL16  R69
**Acid pH alters the conformation of a soluble, recombinant form of gB**

Thus far, pH-triggered changes have been detected in virion gB in the context of the HSV envelope, which includes the other glycoproteins. gB, gD, and gH-gL likely act together during membrane fusion. To address whether changes specific to gB could be observed in the absence of other virion components, purified gB in isolation was evaluated. We utilized a soluble form of gB (s-gB) from HSV-2 strain 333 (189). gB molecules from HSV-1 KOS and HSV-2 333 are 86% identical. In this form of gB, the transmembrane domain has been deleted, and the cytosolic tail is fused directly to the ectodomain. s-gB is secreted and purified from CHO cells (168, 189). It migrates as an oligomer on native PAGE (Figure 13A), binds to cell surface glycosaminoglycan receptors (189), and reacts with all monoclonal antibodies tested, including the oligomer-specific antibody DL16 (Figure 13A; Table 4).

Treatment of s-gB with mildly acidic pH followed by 1% SDS affected its oligomeric conformation (Figure 13B). A pH of 5.1 converted oligomeric s-gB into a monomer (Figure 13B). Conversion to a monomer began at pH <6.3 and was complete by pH 5.9 (Figure 13C). Thus, the quaternary structure of purified gB was altered by the same range of mildly acidic pH as that of gB that is present in the viral envelope (compare Figure 13C and 11C). Acid-induced changes in s-gB were reversible (Figure 13B, center). Low-pH treatment converted gB present in HSV-2 333 virions into a lower-molecular-weight oligomeric form (Figure 13B, right), similar to the result obtained with HSV-1 KOS gB (Figure 11C). Interestingly, this contrasts with the acid-dependent conversion of the s-gB oligomer into a monomer. The difference may be due to the absence of a membrane anchor or the absence of interaction with gD or gH-gL.
Nonetheless, low pH affects the oligomeric structures of both purified gB and virion gB. The reversibility of changes to both forms of gB is further demonstrated in Figure 13A. Samples that were treated with pH 5.4, re-neutralized to pH 7.2, and then analyzed by native PAGE migrated as oligomers, similar to the migration of samples that were not exposed to low pH (Figure 13A). The change induced by pH 5.4 that was noted using other approaches was likely reversed prior to sample loading. Lastly, s-gB that was treated with acidic pHs and was then bound to nitrocellulose had a pattern of reactivity with MAbs similar to that of pH-treated virion gB (Table 4), indicating that pH directly triggers specific changes in gB antigenic structure. In total, the results suggest that gB need not be present in a membrane, nor does gD, gH, gL, or any other viral component need to be present in order for the observed pH-dependent changes to take place. In the context of the virion interacting with the host cell membrane during fusion, additional changes in gB likely occur that are not apparent in the current experiments.
Figure 13. Effect of low-pH treatment on the conformation of purified gB

(A) HSV-1 KOS virions (10^5 PFU) or s-gB (150 ng) in serum-free medium with 0.2% BSA were kept at pH 7.2 or adjusted to pH 5.5 for 10 min at 37°C with 0.05 N HCl. The pH of acidified samples was re-neutralized to 7.2 with 0.05 N NaOH for 10 min at 37°C. Samples were analyzed by native PAGE and immunoblotting with gB-specific PAb R69 or MAb DL16. (B) Soluble gB derived from HSV-2 strain 333 (s-gB) was treated with pH 7.4 or 5.1 and then solubilized with 1% SDS either before or after neutralization of pH (as described in the legend to Figure 11C). HSV-2 strain 333 virions were also treated at pH 7.4 or 5.1 and solubilized with 1% SDS. (C) s-gB was treated with a range of pH, as indicated, and then solubilized with 1% SDS. Samples were analyzed by PAGE and immunoblot detection with R69 (gB).
Low pH increases the hydrophobicity of gB

Conformational change associated with fusion is often accompanied by transient exposure of hydrophobic regions, namely fusion peptides or fusion loops. To begin to address whether conformational changes have an activating effect on gB, we used the nonionic detergent Triton X-114 and s-gB. Upon centrifugation at room temperature, Triton X-114 can be separated into hydrophilic (aqueous, top) and amphiphilic (detergent, bottom) phases (24). Protein forms that are more hydrophobic associate with hydrophobic Triton X-114 molecules and partition with the amphiphilic phase. This approach was used to define pH-induced changes in alphavirus fusion glycoproteins (92).

Purified s-gB that was treated at pH 7.4 was mixed with Triton X-114 at 37°C. The mixture was centrifuged, and water-soluble gB was recovered from the aqueous phase (Figure 14), where hydrophilic proteins are expected to partition. Little to no gB was detected in the detergent phase, suggesting little hydrophobic interaction of gB with Triton X-114. In contrast, as a result of treatment with pH 5.9 or 5.1, a fraction of the total gB partitioned in the detergent phase (Figure 14), suggesting that low pH caused gB to become more hydrophobic. As a control, low-pH treatment of bovine serum albumin did not alter its hydrophobic character (Figure 14). The results are consistent with the hypothesis that pH results in exposure of hydrophobic regions in gB. This increase in hydrophobicity may correlate with changes in gB conformation, such as alterations in oligomeric structure, and with activation of entry.
Figure 14. Effect of low-pH treatment on the hydrophobicity of gB

Soluble gB or BSA was added to 2% Triton X-114 that had been adjusted to the indicated pH. Samples were incubated for 10 min at 37°C and were centrifuged at 300 x g for 3 min. The aqueous supernatant phase and detergent phase were collected and diluted 20-fold in PBS. s-gB samples were subjected to immunoprecipitation with antibody to gB, followed by SDS-PAGE and immunoblotting for gB. BSA samples were trichloroacetic acid (TCA) precipitated and analyzed by SDS-PAGE and Coomassie blue staining.
Intracellular low pH affects the H126 epitope of virion gB during viral entry

The kinetics of the distinct steps in the endocytic entry pathway of HSV have been delineated in CHO-nectin-1 cells (127). Endocytic internalization of HSV from the CHO cell surface has a half-life (t1/2) of ~9 min. Enveloped virions are trafficked through cellular compartments for up to ~30 min postinfection (p.i.). Penetration of >50% of infectious virions from a low-pH compartment (virus-cell membrane fusion) occurs by 60 min p.i (127). To probe the exposure of the H126 epitope on gB during endocytic entry of HSV, CHO-nectin-1 cells were infected with HSV-1 for 1 h, and input gB was monitored by immunofluorescence and confocal microscopy. The early time of infection analyzed and the addition of cycloheximide ensured that the signal detected was due to input gB and not to the newly synthesized gB. gB that was brought into the cell with entering virions was detected by MAb H126 as punctate staining (Figure 15). Bafilomycin A1 (BFLA), a vacuolar H+ ATPase inhibitor that prevents endosome acidification, inhibits HSV entry into CHO-nectin-1 cells (125). When infection proceeded in the presence of BFLA, there was a detectable and reproducible increase in the H126-reactive gB detected (Figure 15). As a control, MAb H1817 recognized input intracellular gB to a similar extent in the absence or presence of BFLA (Figure 15). This suggests that the N terminus of gB is accessible to antibody, even after exposure to Intravesicular low pH. In contrast, the H126 epitope in domain I appears to be altered during viral entry upon exposure to acidic pH. This change may be detected because all virion gB molecules are exposed to endosomal low pH, regardless of whether they are directly involved in viral entry.
Figure 15. Effect of bafilomycin A1 on the conformation of HSV gB during viral entry

CHO-nectin-1 cells were mock treated (left) or treated with 25 nM bafilomycin A1 (right, +BFLA) for 15 min. HSV-1 KOS (MOI of 20) was bound to cells at 4°C for 1 h. Cultures were shifted to 37°C for 1 h in the constant presence of BFLA and 0.5 mM cycloheximide. Virion gB was visualized with MAb H126 or MAb H1817 followed by Alexa 488-labeled goat anti-mouse antibody. Samples were visualized by confocal microscopy at 63x magnification. A total of 50 to 70 cells are shown per panel.
Discussion

We demonstrate that conformational change in the fusogenic herpesviral glycoprotein gB is triggered by the mildly acidic pH that is typically encountered in the endosomal network (pH 5.0 to 6.0). Low pH specifically alters the antigenic structure and oligomeric conformation of gB. Both conformational change and an increase in the hydrophobic character of gB occur at a similar pH range. This work describes a critical feature of the complex molecular mechanism of herpesviral entry by pH-dependent endocytosis, a pathway that is employed by HSV in biologically relevant cell types, including epithelial cells.

MAb H126 has HSV-neutralizing activity (94). The H126 epitope in the fusion domain of gB becomes less accessible upon exposure of gB to low pH both in vitro and during viral entry. Thus, H126 may neutralize virus infection by blocking conformational change in gB or by preventing contact of fusion loops with the target membrane. Interestingly, H126 neutralizes HSV entry to a similar extent in cells, regardless of whether the cells support pH-dependent or pH-independent entry (49, 146) HSV-1 strain ANG path has a highly fusogenic form of gB that is responsible for fusion-from-without (FFWO) activity (149), (157). Two distinct mutations in gB are responsible for FFWO. Notably, gB from ANG path has reduced reactivity with MAbs DL16 and H126 (146), (48). Thus, the antigenic conformation of a mutant gB with enhanced fusion activity is similar to the conformation of low pH-treated wild-type gB, supporting the notion that acid pH triggers the fusion activity of gB.

As gB is the most-conserved herpesviral glycoprotein, similar low-pH activation of gB from other herpesviruses may also occur. Cell monolayers transfected with gB or gD
alone have been reported previously to undergo pH-dependent cell fusion (2, 26). Low pH has little to no detectable effect on gD's antigenic structure or on its ability to bind to receptors (125) (our unpublished data). The present data suggest that gB is the principle glycoprotein affected by endosomal pH. However, acid effects on other glycoproteins such as gH, which contains a putative fusion peptide (69), remain to be investigated further.

Host cell triggers that can cause conformational changes in glycoproteins leading to fusion include the low-pH milieu of the endosome, binding receptor, and cleavage by endosomal proteases (185). HSV likely requires more than a single cellular trigger to mediate membrane fusion and entry. HSV gD binding to one of its cognate receptors causes a displacement of the C terminus of gD (99). This change in gD conformation is thought to initiate the fusion process mediated by gB and gH-gL (8, 65, 169). In the case of HSV entry into cells by acid-dependent endocytosis, we propose that the direct action of endosomal pH on gB is required in addition to engagement of a gD receptor. While we currently have no evidence for the involvement of pH-activated cellular proteases in HSV entry (our unpublished data), other indispensable cellular triggers may be necessary to complete the fusion process.

In addition to endosomal pH, one or more cell factors may serve the redundant function of triggering conformational change in gB. In a similar vein, distinct cell receptors serve a partly redundant receptor-binding role for gD. gB is required for pH-neutral fusion of HSV with the plasma membrane of a subset of cell types, such as Vero cells (27). The recently identified gB receptor PILRα may trigger direct penetration of HSV with the cell surface (6, 153). However, fusion probably cannot occur without a gD
receptor and gD, even when PILRα has a role (58, 153). All gB-specific neutralizing antibodies tested have a similar inhibitory effect on HSV entry, regardless of whether entry into the target cell type is dependent on intracellular low pH (50, 146). It is tempting to speculate that in cells that support pH-independent entry, binding to a gB-specific receptor such as PILRα may functionally substitute for endosomal pH and induce a conformational change in gB that leads to pH-independent entry.

Unlike glycoproteins from viruses that mediate fusion exclusively at low pH, HSV cell-cell fusion can occur at physiologic pH (176). Shown here, low pH enhances membrane fusion mediated by gB, gD, gH and gL. Acid-activated gB alone causes cell-cell fusion, indicating that the fusogenic potential of gB can be triggered by acid. Interestingly, gB or gH independently appear to be sufficient to trigger fusion with the outer nuclear membrane during HSV egress (59). However, the quartet of gB, gD, gH and gL is necessary to mediate optimal fusion in the cell-cell fusion assay at neutral (176) and low pHs, and likely during virus-cell fusion during entry.

Full-length gB from HSV-2 lacking only the transmembrane region (s-gB) was not sufficiently hydrophobic to associate detectably with micelles of Triton X-114. Only upon treatment with mildly acidic pH did s-gB associate with the detergent phase, suggesting an increase in hydrophobic character. Interestingly, low-pH treatment of virions increases their hydrophobic nature, as measured by binding to liposomes in the presence of soluble receptor (183). gB is a likely candidate for mediating pH-triggered association of virions with membrane. The similar effects of low pH on s-gB and on virion gB suggest that the transmembrane-deleted gB used in this study may resemble the prefusion form found in the virion envelope.
The structure of a truncated form of HSV-1 gB (called gB730) is thought to be the postfusion form (78, 103). Unlike s-gB, which becomes hydrophobic upon exposure to low pH (Figure 5), gB730 is sufficiently hydrophobic at neutral pH to bind to liposomes (73). The fusion loops are surface exposed in the gB730 structure and are responsible for liposome binding. The absence of N-terminal or C-terminal residues from gB730 may drive it irreversibly to the postfusion conformation, regardless of pH.

HSV gB undergoes a reversible change in structure in response to pH, similar to the change in other class III fusion proteins, VSV-G (50) and baculovirus gp64 (194). Thus, reversibility of conformational change may be a general feature of class III fusion proteins. Interestingly, low-pH treatment of virions irreversibly inactivates viral entry (125). This seeming paradox may be explained by irreversible pH-induced changes in gB, gD, or gH-gL that have yet to be identified.

Our results suggest that low pH may cause a destabilization of the oligomeric conformation of s-gB and virion gB. Similar disruption of soluble gB from HSV-1 was reported previously (152). The observation that the functional region of gB that contains the hydrophobic fusion loops is altered by mildly acidic pH suggests that low pH may facilitate the proper gB-target membrane contact necessary for entry. In the case of VSV, low pH causes a tighter association of G subunits, making them more stable (50). In response to low pH, the bipartite fusion loops of each G monomer are thought to pack as a trimer and contact the target membrane (142). In contrast, virion gB appears to become a lower-order oligomer upon pH activation. This may be a difference in the fusion mechanisms between these two class III fusion proteins. This distinction might reflect the necessity of other HSV glycoproteins to complete the fusion process.
IV. Structure-function analysis of HSV glycoproteins at low pH.

Monoclonal antibodies detect low pH-induced conformational changes in s-gB but not gB730

Conformational changes have been observed in the prefusion form of virion gBs and a soluble form of gB that has only its transmembrane region (TM in Figure 4) deleted (809 aa length, denoted s-gB) (Figures 10, 13, 14; Table 4; ). In contrast, an ectodomain fragment of gB (700 aa length, denoted gB730) that has its membrane proximal region, transmembrane region and cytoplasmic tail region deleted (Figure 4) was reported to have essentially the same structure at both neutral and low pH (21, 78). A later analysis confirmed that low pH did in fact effect functional region 1 of gB730, but the effect was modest (163). gB730 may not be in the appropriate conformation to test low-pH induced conformational changes. Both neutral and low pH structures still maintain an overall postfusion (low pH) like structure (78, 141). It was therefore of interest to directly compare low pH induced conformational changes in s-gB, which undergoes conformational changes similar to virion gB, and gB730.

In order to compare conformational changes in the two forms of gB, we measured reactivity to representative mouse monoclonal antibodies as in Figure 10. s-gB and gB730 were exposed to pHs ranging from 7.4 to 4.8 and were immediately blotted to nitrocellulose. 40 ng of each gB was added to each dot reaction. Polyclonal antibody R69 reacted equally to similar amounts of s-gB or gB730. R69 also reacted equally to gB that had been treated with the range of indicated pHs (Figure 16). Thus, gB binding to nitrocellulose is unaffected by pH treatment. H126 that binds to an
epitope in domain 1 of gB, displayed diminished detection to s-gB that had been treated at lower pHs, as seen in Figure 10. In contrast, H126 detected gB730 to an equivalent extent across the range of pHs, indicating that gB730 is not able to undergo the same low pH induced conformational changes that s-gB can (Figure 16). SS106, which detects conformational changes in domain V of gB, and DL16 which detects conformational changes and is oligomer specific, were also used in this assay. SS106 detected low pH induced conformational changes in s-gB but not gB730 (Figure 16). DL16 also detected conformation change in s-gB but only minor changes if any were detected in gB730. SS10 epitope remained easily detected in all the conditions tested, further indicating that gB730 does not undergo different conformational changes to s-gB (Figure 16). Together, results indicate that gB730 most closely resembles a postfusion form of gB that is less susceptible to pH relative to s-gB and the form of gB found in the virion. It is possible that s-gB and gB730 undergo slightly different conformational changes and that slight structural differences may account for this result.
Figure 16. Antibody reactivity of purified recombinant gBs

40 ng of s-gB or gB730 were treated for 10 min at 37°C with medium buffered to the indicated pHs and were blotted immediately to a nitrocellulose membrane. Blots were probed at neutral pH with the indicated gB-specific antibodies, followed by horseradish peroxidase-conjugated goat secondary antibody.
Effect of pH on the hydrophobicity of gB730 and s-gB

Triton X114 partitioning was used to further probe how low pH affects the hydrophobicity of HSV glycoproteins (as shown for s-gB in Figure 14) (24). As in Figure 14, there was increased detection of s-gB in the detergent phase at pH 5.1, suggesting s-gB was triggered to become more hydrophobic at low pH. In contrast there was little change in the partitioning of gB730 in response to pH 5.1 (Figure 17). This suggests that gB730 is not undergoing low pH induced changes that make it more hydrophobic. Detection of gB730 in the detergent phase at neutral pH indicates that gB730 is hydrophobic at neutral pH. This is consistent with the ability of gB730 to bind with liposomes (73). In addition, the effect of pH on the detergent partitioning of gD was investigated. Two soluble forms gD, gD-2t and gD(Δ290-299t) were studied. Detection of gD in the aqueous phase and detergent phases was similar at pH 7.4 and 5.1. This indicated that gD does not become hydrophobic in response to pH 5.1. Both forms of gD appeared largely hydrophilic at both pHs. An alternative explanation for the increase in s-gBs hydrophobicity at low pH may concern the protein expression system used. Proteins expressed in mammalian cells (s-gB) may become hydrophobic at low-pH whereas those expressed in insect cell do not (gB730). One explanation would be differential glycosylation between expression systems. As gD-2t is also expressed in mammalian cells this alternative explanation is may not be likely.
Figure 17. Effect of low-pH treatment on the hydrophobicity of s-gB, gB730, gD-2t and gD(Δ290-299t).

Proteins were added to 2% Triton X-114 that had been adjusted to the indicated pH. Samples were incubated for 10 min at 37°C and were centrifuged at 300 x g for 3 min. The aqueous supernatant phase and detergent phase were collected and diluted 20-fold in PBS. Samples were subjected to immunoprecipitation (H1817 for gB, DL6 for gD) followed by SDS-PAGE and immunoblotting (H1359 gB, DL6 for gD).
pH: 7.4  \hspace{1cm} 5.1

- aqueous \hspace{0.5cm} detergent
- aqueous \hspace{0.5cm} detergent

s-gB

kB730

gD-2t

gD(Δ290-299t)
**Temperature dependence of conformational changes in gB**

Like many biological processes, viral entry is slowed at temperatures below 37°C. The triggering of conformational changes in fusion glycoproteins can be temperature dependent (147, 166, 181, 184). To test whether conformational changes in HSV gB are sensitive to temperature, HSV-1 KOS virions were subjected to a range of low pH at 4, 25, 37 or 50°C for 10 min and then analyzed by dot blot as described in Figure 10. SS10 detected gB to an equivalent extent over the range of pHs and temperatures tested (Figure 18). Thus, gB bound to nitrocellulose and was not degraded under these conditions. A decease in H126 reactivity indicates low pH induced conformational changes are occurring in Domain 1 of gB, as has been shown with virions incubated at 37°C. When virions were treated with the range of pHs at 4°C or 25°C, there was little to no conformational change detected by H126 even at the lowest pH of 5.1. At 50°C there was diminished detection at pHs higher that those seen at 37°C. This suggests that low pH-induced conformational changes in gB may be enhanced by treatment at 50°C. Together these results suggest that warm temperatures enhance conformational changes and colder temperatures inhibit them. Although not directly compared hear, virus entry is inhibited by temperature. This is consistent with a biologically relevant role for the conformational changes.
Figure 18. Antibody reactivity of HSV KOS virion gB over a range of pH and temperature

Extracellular HSV-1 KOS virions (10⁵ PFU) were treated for 10 min at the indicated temperatures with medium buffered to the indicated pHs. Samples were then cooled to 4°C and blotted immediately to a nitrocellulose membrane. Blots were probed at neutral pH with the indicated gB-specific antibodies, followed by horseradish peroxidase-conjugated goat secondary antibody.
Effect of low pH on the antigenic conformation of HSV gD and its receptor binding function

Analysis of gB, gD and gH-gL revealed that HSV gB may be the principal glycoprotein affected by low pH. Further analysis of low pH induced conformational changes in virion gD was performed with monoclonal antibodies. HSV virions were treated with a range of pHs from 7.4 to 5.2 and were probed with a panel of anti-gD monoclonal antibodies that bind to distinct regions across gD (Table 3) (37, 55, 85, 115, 121). Monoclonal antibodies to the C-terminal region (DL6), the Ig-like core (DL11, DL2) and the N-terminus (1103) reacted to virion gD to an equivalent extent regardless of the pH of treatment. R7 a polyclonal antibody to gD was used as a control and detected gD to an equivalent extent under all the conditions tested (Figure 19). For comparison, the same virion preparation was probed with anti-gB monoclonal antibodies H126 and H1817, indicating that low pH conformational changes in HSV glycoproteins are detectable under these conditions. It cannot be ruled out that gD is undergoing low pH induced changes that we did not detect.

Having shown previously that low pH impairs HSV entry function, we determined whether low pH affects the ability of gD to bind to the gD receptors (127). The ability of acid-treated, soluble gD to bind to HVEMt or nectin-1t was evaluated by a binding ELISA (98, 182). Soluble gD that was treated with pH 4.7 bound to immobilised HVEMt in a dose-dependent manner similar to that of untreated gD(Δ290-299t) (Figure 20A). Control, heat-treated gD(Δ290-299t), however, had severely diminished binding activity (Figure 20A). Acid-treated and untreated gD(Δ290-299t) also bound comparably to
immobilised nectin-1t (Figure 20C). Taken together the results indicate that low pH pre-treatment had no effect on the interaction of gD with its receptors, supporting the notion that the conformation of gD responsible for receptor binding is not affected by pH. Also, pH triggered inactivation of HSV occurs at a required, post-gD binding step in entry.
Figure 19. Antibody reactivity of low pH-treated virions

Extracellular HSV-1 KOS virions (10^5 PFU) were treated for 10 min at 37°C with medium buffered to the indicated pHs and were blotted immediately to a nitrocellulose membrane. Blots were probed at neutral pH with the indicated gB or gD-specific antibodies, followed by horseradish peroxidase-conjugated goat secondary antibody.
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Figure 20. Effect of low pH pre-treatment on binding to HSV entry receptors

A) and (B) Low pH-treated soluble gD binds to receptors. Microtitre plates coated with (A) HVEMt or (B) nectin-1t were incubated with increasing concentrations of gD(Δ290-299t) that had been heated to 80°C, treated at pH 4.7, or left untreated. Bound gD was detected with MAb DL6 followed by peroxidase-conjugated Protein A and substrate. Absorbance was read at 410 nm. Each value represents the average of duplicate wells.
Effect of pH on the intrinsic fluorescence of purified HSV glycoproteins

The intrinsic fluorescence emission spectra of purified glycoproteins s-gB, gB730, gD-2t, gD(Δ290-299t), gC and chicken ovalbumin were recorded at pH 5.1 and 7.3 (Figure 21A). Intrinsic fluorescence is a common way to study conformational changes in proteins, including viral entry glycoproteins (Influenza-HA, VSV-G) (29, 96, 139). Tryptophans are the dominantly fluorescent amino acid of proteins and are excited with photons of 280 nm wavelength. The degree of excitation depends on the environment of the tryptophan. Hence, when tryptophans return to ground electronic state, a photon is released at a wavelength determined by its environment (101). If a proteins peak emission wavelength shifts from a starting point towards the red end of the spectrum, this indicates exposure of tryptophans to the solvent. A blue shift in the emission spectrum indicates internalization of tryptophans within the protein (101). Both s-gB and gB730 have 7 tryptophans in highly conserved regions of gB (Figure 22), this makes direct comparison by this approach more powerful.

At the neutral pH of 7.3 s-gB had a peak emission at 330.4 nm. Upon treatment at the pH of 5.1, the peak fluorescence of s-gB shifted 2.2 nm towards the red spectrum (Figure 21). This suggests that tryptophan residues are exposed as s-gB undergoes low pH induced conformational changes. Interestingly when gB730 was treated at low pH, a 1 nm blue shift in gB730 emission spectra occurred. This may indicate that gB730 undergoes a small conformational change that is different to s-gB. This is consistent with the antigenic and hydrophobicity analysis (Figures 16 and 17), which indicate that
the conformational change in gB730 is not as great as that in s-gB. We observed in Figure 12 that low pH induced conformational changes in gB are reversible upon neutralization. Reversibility of conformational change is a characteristic of class III fusion glycoproteins. The reversibility of gB conformation change was tested in parallel with the low pH spectra shift study (Figure 21. light gray solid line). Upon neutralization peak fluorescence of low pH, both s-gB and gB730 shifted back to within 0.2 nm of their fluorescence at neutral pH. This is further evidence that low pH induced conformational changes in HSV gB are reversible. This is antibody-independent, biophysical evidence that herpes virus gB undergoes reversible, low pH-induced conformational changes.

The fluorescence emission spectra of ovalbumin, gC, gD-2t and gD(Δ290-299t) were very similar at a pH of 7.3 or 5.1 (Figure 21). This indicates that under these conditions (see materials and methods) these proteins do not undergo detectable low pH induced conformational changes. At high (24 ng/µl) protein concentration the emission spectrum of gD-2t underwent a 4 nm blue shift. The shift in gD-2t was absent at lower concentration. Thus, the shift is likely attributable to concentration dependent protein aggregation and not biologically relevant conformational change. The absence of conformational changes in these other proteins further indicates that changes in s-gB are specific.
Figure 21. Low pH induced conformational change as determined by intrinsic fluorescence

A) The spectral profiles of the indicated proteins were determined at neutral and low pH. For gB neutralized samples are also shown. Excitation was performed at 280 nm. Emission was recorded over 310-400 nm. The peak fluorescence in percent is shown. Shifts in peak fluorescence indicate net exposure (nm increase) or internalization (lower nm) of tryptophans as conformational change occurs.

B) Comparison of size and nature of conformation change. Peak fluorescence at neutral pH was normalized to 0. Shape positions indicate how many nm in wavelength and in which direction each spectrum shifted from 0 upon encountering low pH.
A

Fluorescence (percent)

Wavelength (nm)

S-gB

pH 7.3

pH 5.1

pH 5.1*7.4

gD-2t

Ovalbumin

Fluorescence (percent)

Wavelength (nm)

gB730

Fluorescence (percent)

Wavelength (nm)

g(Δ290-299t)

gC

Fluorescence (percent)

Wavelength (nm)

B

Low pH-induced emission peak shift (nm)

Tryptophan buried ↔ Tryptophan exposed

Peak emission shift (nm)

○ Ovalbumin

● gC

▲ s-gB

■ gD-2t

□ gB730

■ gD(Δ290-299t)

□ gD(Δ290-299t)
Figure 22. Clustal W alignment of gBs used in this study.

A clustal W multiple sequence alignment of virion and soluble gBs. Alignment was performed using EMBLs clustal W data base. A full alignment was performed using the default settings. Tryptophans are highlighted with W. The sequences aligned are labeled as follows.

333 = HSV-2 strain 333
S = s-gB (cloned from HSV-2 strain 333)
KOS = HSV-1 KOS
730 = gB730 (from HSV-1 KOS)
CLUSTAL W (1.83) multiple sequence alignment

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Table 5. Statistics of gB clustal W alignment.

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Start of pairwise alignments

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- Sequences (1:3) Aligned, Score: 86
- Sequences (1:4) Aligned, Score: 86
- Sequences (2:3) Aligned, Score: 89
- Sequences (2:4) Aligned, Score: 89
- Sequences (3:4) Aligned, Score: 108

Guide tree file created: [/hmi/extern/clusterw-work/interactive/2010122117/clustalw-20101221-171039956.dnd]

Start of multiple alignment

There are 3 groups

Aligning:
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- Group 2: Sequences: 1  Score: 12288
- Group 3: Sequences: 1  Score: 6087

Alignment score 26907

CLUSTALW multiple alignment file created: [/hmi/extern/clusterw-work/interactive/2010122117/clustalw-20101221-171039956_a3a]
Fusion loop mutants of gB

To probe the relationship between the conformational changes and the fusion function of gB, we determined the effect of mildly acidic pH on gB bearing mutations in its fusion loops (Figure 23A) (73, 74, 170, 192). Substituting polar residues for hydrophobic residues in the fusion loops of gB diminished fusion activity in a cell-cell fusion assay (74). Surface expression of gB with fusion loop mutations was shown to be similar to KOS gB expressed from the same plasmid (74). It is hypothesized that hydrophobic residues in the bipartite fusion loops of gB are required for the insertion of gBs fusion domain into the host cell membrane (Figure 24) (74, 170). If fusion loop mutants are defective for insertion into the host membrane they will not cause fusion but may still undergo low pH induced conformational changes. Alternatively, mutations in the fusion loops may result in gB that undergoes either altered conformational changes or, no change at all. Because high titres of these mutant viruses are difficult to obtain, assays for the conformational change were performed on lysates of cells infected with mutant viruses. Viruses were initially made from the BAC-gBgalK/gH background. Cell-associated virus samples have either no gB (gBgalK/gH-), KOS gB (w.t.gB/gH-), gB with the fusion loop 1 mutation Y179K, or a fusion loop 2 mutation A261D (Figure 24) (192). All viruses lack gH but were propagated in gH-complementing F6 cells. A brief description of the construction of the fusion loop mutants is detailed in the Materials and Methods.

Amounts of cell-associated virus that reacted equally to MAbs prior to pH treatment were exposed to pHs ranging from 7.4 to 4.8. They were then immediately blotted to nitrocellulose membranes. Antibody binding was then assessed at pH 7.4.
Representative monoclonal antibodies to specific regions of gB were used to analyze low pH induced conformational changes as in Figure 10. Importantly, MAb H126 (domain I), SS106 (domain V), SS10 (domain III) and DL16 (oligomer specific) detected antigenic changes in cell-associated KOS gB to an extent equivalent to that seen in HSV KOS virions (Figure 24A). pH induced changes in domains I, V and oligomeric changes occur at a similar threshold (~pH 6) to virion gB. This indicates that cellular gB is capable of undergoing the same low pH induced conformational changes seen in virions. Analysis of gB with the fusion loop 1 mutation, fusion loop 2 mutation and gB rescued virus was performed. This revealed that all gBs are able to undergo specific antigenic changes in domain I (H126), domain V (SS106) and also oligomeric changes (DL16). Low pH induced changes in these regions are equivalent in nature to those seen in cell associated HSV-1 KOS gB, virion gB, and soluble gB. This suggests that fusion loop mutants of gB are defective for fusion for reasons other than impaired or altered conformational changes. The detergent stability of the fusion loop mutants was analysed with the native gel assay as in Figure 11C. Once again at around a pH of 6 the upper oligomeric band of each gB became unstable (Figure 24B). This indicated that all of the cell lysate gBs underwent similar changes in oligomeric stability as seen for virion gB (Figure 23). Cell-associated gB, and fusion loop mutant gBs appear to undergo low pH induced oligomeric changes.

The reversibility of conformational changes in cell-associated WT gB, gB A261D and gB Y179K was analysed. It was possible that a defect in reversibility was responsible for diminished fusion function of mutant gB. A MAb dot blot assay for reversibility was used as in Figure 12. In each of the gBs tested domain epitopes to
H126, SS106, and DI16 had diminished detection at pH 5.3 (Figure 25A). Detection was at least partially restored by neutralizing pH to 7.4 prior to binding. To further the analysis a native gel SDS assay to test reversibility of changes of an oligomeric nature was used as in Figure 11C. At the pH of 5.3 the upper oligomeric band of gB is no longer detected (Figure 25B). Neutralizing the sample prior to the addition of SDS made the upper band detectable again indicating that pH-induced changes of an oligomeric nature are reversible in all samples.

The dot blot and SDS assay both reveal that cell associated WT gB and gB with mutations in either fusion loop, undergo low pH induced conformational changes that are reversible in nature. These are similar to those seen in virion gB (Figures 11C and 12).
Figure 23. Mutations in the fusion loops of gB

HSV gB hydrophobic ridge is surrounded by charged residues on the surface of the molecule. A ribbon diagram of the HSV protomer (A) and molecular surface representation of the trimer (B) are shown. In each, one protomer is colored by secondary structure succession, using blue (domain I), green (domain II), yellow (domain III), orange (domain IV), and red (domain V). The box in panel A shows the primary amino acid sequences of the fusion loops. The box in panel B shows the base of the gB trimer, rotated 90°. For the boxes in both panels A and B, highlighted hydrophobic residues are colored in blue and charged residues are shown in red. All structural figures were generated, in part, using PyMOL Molecular Graphics System software.

(Reproduced with permission from the publisher (74))
Figure 24. Low pH induced changes in cell-associated wild type gB and fusion loop mutant gB

A) Dot blot analysis

The indicated infected Vero or derived F6 cell lysates were diluted in buffered media to achieve final pHs ranging from 7.4 to 5.2. Samples were incubated at 37 °C for 5 min. and blotted directly to nitrocellulose. Membranes were blocked and incubated at neutral pH with anti-gB monoclonal antibodies H126 (Domain I), SS10 (Domain III), SS106 (Domain V), DL16 (trimer-specific). After incubation with horseradish peroxidase-conjugated goat-anti-mouse antibody, enhanced chemiluminescent substrate (Pierce) was added, and blots were exposed to X-ray film (Kodak).

B) Assay for sensitivity of oligomeric gB to detergent

The indicated infected cell lysates were treated with medium adjusted to pHs ranging from 7.4 to 5.2 as described for panel A. Lysates were adjusted to 1% SDS and were then added to PAGE sample buffer containing 0.2% SDS and no reducing agent (“native” conditions). Samples were not heated and were resolved by PAGE. After transfer to nitrocellulose, membranes were blocked and incubated with rabbit polyclonal antibody specific for gB. After incubation with horseradish peroxidase-conjugated goat-anti-rabbit antibody, enhanced chemiluminescent substrate (Pierce) was added and membranes were exposed to X-ray film (Kodak).
Figure 25. Reversibility of conformational changes for fusion loop mutants

A) Reversibility of pH-induced conformational changes in gB from infected cell lysates.
Cell-associated gB preparations were treated with medium buffered to pH 7.2 or 5.5.
For the indicated samples, pH was neutralized back to 7.2 for 10 min at 37°C and
blotted immediately to nitrocellulose membranes. Membranes were probed at neutral
pH with the indicated antibodies, followed by horseradish peroxidase-conjugated
secondary antibody.

B) Cell-associated gB preparations were treated with the indicated pH, treated with 1% SDS, and then analyzed by PAGE and immunoblotting for detection of gB.
Modification of virion histidines by DEPC treatment preferentially inhibits pH-dependent endocytic entry

Histidine protonation is believed to be important for the low pH activation of several viral fusion glycoproteins (30, 95, 120, 164). Histidine is the only amino acid that has a pKa in the physiological pH range found within the lysosome terminal endocytic pathway (120). The pKa of histidine is 6.1 and conformational changes in gB occur at a pH of pH 5.9. Protonation of histidines may thusly trigger membrane-fusion-inducing conformational changes within gB, as HSV encounters the low pH of endosomes. Modification of the N-omega-2 nitrogen of the imidazole ring in histyl residues in proteins can be accomplished by reacting with diethylpyrocarbonate to produce N-carbethoxyhistidyl derivatives. DEPC has been used to inhibit the entry of several low pH-dependent viruses (164, 165). In order to study histidine modification, HSV-1 KOS was pelleted by ultra-centrifugation and virions were resuspended in PBS. Concentrated virus was treated with DEPC and diluted in an excess of media. Infectivity was then analyzed in the indicated cell types via β-galactosidase reporter assay (CHO-derived cells) or plaque assay (Vero and HaCaT cells) (Figure 26). At high concentration of DEPC treatment, infection by DEPC treated virus was undetectable suggesting that protonation of histidines is important for HSV entry. As shown in Figure 26, low concentration DEPC treatment of virus preferentially inhibited HSV entry into cells that support low pH endocytic entry of HSV (CHO-nectin-1 and HaCaT). Entry into cells that support pH-independent entry (Vero and C10) was inhibited to a lesser extent. This suggests that histidine residues may be more important in the low pH entry pathway of
HSV in which endosomes serve as a potential source of protonation.
Figure 26. Analysis of infectivity of DEPC treated virions

Virions of HSV-1 KOS were concentrated and resuspended in PBS. 50 μg of virus was treated with the DEPC concentration indicated for 10 min. Infectivity was then analyzed in the indicated cell types via β-galactosidase reporter assay (CHO-nectin-1 or C10 derived cells) or plaque assay (Vero or HaCaT).
Discussion

In nature class III fusion glycoproteins exist in a metastable equilibrium between conformations (141, 143). It is demonstrated that gB730 was constructed in such a way that restricts conformation change. Limiting gB conformational changes may have been beneficial in the crystallization of gB as crystals would have been more organized (78). In the future, experimental designs should be mindful of the restrictions imposed by using gB730. Several published experiments with gB730 now also have alternative explanations than those documented due to this finding (8, 9, 78, 163). Most importantly to my work, the recent publication of a low pH form of gB730 does not likely reveal the pre to postfusion transition of gB (163). gB730 should not be expected to undergo the full range of conformational changes observed during fusion because it represents a postfusion (low-pH) form that has already been activated.

Of note, the s-gBs and gB730 are different; s-gB has only the TM region missing while gB-730 is missing around 100 amino acids more than s-gB. Also, s-gB is from HSV-2 strain 333 and is produced in mammalian cells while gB730 is from HSV-1 strain KOS and is produced in insect cells (see Materials and Methods). The gBs are from different origins and there may be potential differences in triggering mechanisms for the two glycoproteins. However, low pH induced conformational changes seen in gB from KOS virions match those of s-gB. Therefore, if biologically relevant low pH induced conformational changes were occurring in gB730, they would likely be similar to those detected in KOS virion gB, and should have been detected. Several reasons why gB730
is unable to change conformation include, differences in glycosylation and processing, or different deleted regions that result in different conformations.

If that gB730 is in a single low-pH postfusion conformation, domain 1 and 5 reactivity to antibody could be expected to be diminished prior to pH treatment. It could be speculated that epitopes were not diminished in gB730 because the pre-fusion conformation of s-gB and the structure of gB730 are similar. Alternatively, we may be detecting an extended intermediate conformation of s-gB. Another cellular or viral cue may be needed, before that gB can complete conformational change and adopt a structure like that of gB730. Alternatively, gB730 may be aberrantly folded with these epitopes exposed. If conformational changes in gB are reversible, and gB730 was functional then crystallizing gB at neutral pH should have revealed the prefusion form of gB (78, 163). As conformational changes did not occur, regions of gB730 may be folded in a way that does not occur in the virus.

Interestingly, gB730 was recently shown to adopt head-to-head structures in which functional region 1 of two adjacent timers gB730 associate in response to low pH (163). With regards to the intrinsic tryptophan fluorescence experiments, such an arrangement may bury the tryptophan in fusion loop 1. This would account for a shift in wavelength without significant conformational changes occurring (101). Both gBs have 7 tryptophans in regions highly conserved between the two gBs, so it is unlikely differences in conformational changes are due to differences in the relative positions of tryptophan between gBs (see clustal alignment Figure 22; Table 5). A potential explanation for exposure of the tryptophans of s-gB is that protomers are becoming unstable and disassociating at low pH. Disassociation may not be a plausible
explanation as we show in Figure 13 that ionic detergent is needed to break hydrophobic interactions at s-gBs protomer interface. In addition, disassociation would likely result in the increased exposure of epitopes at low-pH. Together the intrinsic fluorescence data are consistent with low pH induced conformational changes in s-gB that are larger than those seen in gB730, especially given that trimers of gB730 may be associating (for comparison see Figure 21 B). Association of gB730 trimers at low pH (and not conformational change) would also explain why we do not see conformational changes in gB730 in our other data. Many of the analyses suggest that conformational changes in s-gB match those seen in virions during endocytic entry.

In general, numerous low-pH structures of several class III glycoproteins have been labeled “postfusion” (13, 78, 90). For class I and II fusion glycoproteins postfusion and low-pH conformations are the same, as conformational changes are final and non-reversible (185). Due to the reversible nature of the conformational changes in class III glycoproteins, it may be more accurate to say that the low pH structures are glycoproteins in a fusion active conformation. Following fusion (postfusion), the glycoproteins would encounter the neutral pH of the cytoplasm and undergo reversal of conformational change so the true pre- and postfusion conformations may be identical.

The temperature-dependence of conformational change in gB is characteristically similar to a number of other glycoproteins (147, 166, 181, 184). The inhibition of conformational change maybe due to a lower free energy of gB-ligand interaction, or for gB conformational change post ligand-binding. In addition, decreased membrane fluidity at low temperature may have a role (166, 184), although soluble gB in the absence of membrane can undergo conformational changes. Interestingly, HSV inactivation with
low pH is also inhibited by low temperature (125). This correlation opens up the possibility that irreversible conformational changes not yet detected in gB or other glycoproteins may be responsible for viral inactivation by low pH.

gD does not undergo detectable low pH induced conformational changes and is unaltered in receptor binding after treatment with low-pH. It is not clear at which point during endocytosis that receptor binding occurs (124, 125, 127). gD receptor interaction at low pH may still be affected by undetected changes in gD or low pH induced changes in the receptor itself. These data indicate that gD is not triggered for fusion by low pH. Future work will study if that gD bound to receptor undergoes conformational changes in response to low pH. We cannot rule out the possibility that gD is activated by low pH after receptor binding to perform another function during entry.

Until identification of conformational changes in gB it was unclear how the energy needed to drive two membranes together may be provided. It had been shown that gB730 could associate with liposomes by its fusion loops and a similar role in membrane fusion was implicated (74). It is suggested here that combination of conformational change and functional hydrophobic fusion loops are needed for fusion. Conformational changes in domains 1 and 5 of gB alone are not sufficient for fusion. Recent experiments show that gB730 when added in trans cannot cause membrane fusion in a cell-cell fusion assay (with gD and gH-gL) (9). This suggests that association of gB fusion loops with membranes alone is also not sufficient to cause membrane fusion. This is further evidence that the combination of conformational change in gB and insertion of gB fusion loops into host membrane are needed. I favor the explanation that
hydrophobic residues are needed for insertion of gB fusion loops into the host cell membrane enabling conformational changes in gB to drive the membranes together.

DEPC modification of histidines in HSV KOS, inhibits low pH dependent entry more than pH independent entry. This is consistent with histidines being important in the pH sensing mechanism of low pH induced membrane fusion (30, 95, 120, 164). In the case of pH independent entry, histidine modification of gB may cause steric hindrance thereby affecting fusion and entry. The importance of histidine modification may be masked by effects that DEPC is having either in gB, or other components of the virus. At higher concentrations DEPC likely modifies histidines in other motifs of several molecules that are essential to both entry pathways of HSV. Further work will study if that gB conformational change is inhibited at lower DEPC concentrations. As DEPC inhibition of endocytic entry over plasma membrane entry may have been expected to be larger, other amino acids may play a role in the pH sensing mechanism of gB activation. Amino acids such as arginine can have their pKa altered to physiological pH due to interactions with neighboring residues.
V. Analysis of gB structure during synthesis and maturation.

The antigenic conformation of virion gB is distinct from cell expressed gB

Several glycoproteins undergo steps in which their properties change throughout synthesis, processing and release from the cell (102, 119, 185). To investigate the possibility that gB may achieve distinct conformations during processing, immunoprecipitation studies were performed to compare virion gB to gB from the infected cell (Figure. 27). Of the panel of antibodies tested antigenic differences between cell and virion gB were seen for H126 and SS10. H126 and SS10 detected more gB in the virion than in the cell relative to H1817. This indicates that gB in the virion is conformationally distinct from cellular gB. Since we IP at neutral pH, and pH-induced conformational changes are reversible, it is not likely that we are detecting conformational changes due to low pH. In addition, other epitopes known to be sensitive to pH do not behave the same way as the H126 epitope in this experiment, and SS10 was shown to be unaffected by pH treatments and appears changed in this experiment. These data are consistent with virion gB having a conformation that is distinct from cellular gB.
Figure 27. Antigenic conformation of virion gB vs. gB in infected cells

HSV-1 KOS gB from virions or from cell lysates was immunoprecipitated (Pro A agarose) with specific anti-gB antibodies. Immunoprecipitation conditions were identical between samples. Cell lysates were prepared by infecting Vero cells with HSV-1 KOS (MOI 5) for 6 hrs. Cells were than detergent lysed (CHAPS). MAbs H1817, SS10 and H126 are shown. Samples were analyzed by SDS-PAGE and Western blotting.
<table>
<thead>
<tr>
<th>Epitope gB</th>
<th>Virion gB</th>
<th>Cell associated gB</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1817</td>
<td>SS10</td>
<td>H126</td>
</tr>
<tr>
<td>H1817</td>
<td>SS10</td>
<td>H126</td>
</tr>
</tbody>
</table>

The table shows the expression levels of gB epitopes in virion and cell-associated forms across different samples (H1817, SS10, H126).
Alteration of intracellular pH affects antigenic conformation of newly synthesized gB

It is believed that class III fusion glycoproteins undergo reversible conformational changes to allow them to remain fusogenic as they encounter the low-pH of the trans-Golgi network (77, 141-144). To test whether low pH is causing conformational change in newly synthesized gB, intracellular pH was elevated by treatment with the lysosomotropic agent Bafilomycin A1 (BFLA). LCSM revealed that treatment of cells with BFLA increased detection of gB with MAb H126 but not PAb R69 (Figure 28 top). ImageJ quantification of micrographs revealed that BFLA detection in panel D is 30-40% greater then C. This is consistent with gB undergoing specific conformational changes as it traverses a low pH compartment.

Cellular gB is found in antigenically distinct populations

It is known that gB performs several functions at different locations in the cell (17, 20, 153, 156, 176, 192) and, I have several lines of evidence to suggest that cellular gB can undergo conformational change (Figures 10, 11, 14 and 21). We investigated the possibility that gB adopts different antigenically distinct conformations in different locations within the cell. Infected Vero cells at 6.5 hrs post infection were analyzed by LSCM (Figure 28 lower panel). HSV gB-specific polyclonal antibody (PAb) R69 reacted with total cellular gB at the nuclear membrane, ER/Golgi compartments, punctate compartments throughout the cell and the plasma membrane (Figure 28A). In contrast, MAb DL21 reacted preferentially with punctate compartments reminiscent of acidic
endosomes/lysosome. SS118 preferentially recognized gB present at the nuclear membrane and plasma membrane, these are both known sites of membrane fusion and suggest that a common event may be responsible for the distinct antigenic conformation. Several MAbs such as H126 and SS55 preferentially detect gB in peri-nuclear compartments such as the Golgi. Together, these data suggest that there may be four or more different populations of gB found in the cell and virus.
Figure 28. Detecting pH-induced conformation change in newly synthesized gB

A-D) Top panel:
Vero cells were infected with HSV-1 KOS (MOI of 5). At 6.5 hr p.i. cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. B and D were treated from 1 hr PI with Bafilomycin A1. A and C were mock treated. HSV gB was detected in A and B with PAb-R69 and in C and D with MAb H126. Visualization was by LSCM at 63X magnification. Arrows indicate gB at the plasma membrane (PM), nuclear membrane (NM), Golgi-like perinuclear location (PN) or at punctate vesicular compartments (P).

Bottom panel:
Vero cells were infected with HSV-1 KOS (MOI of 5). At 6.5 hr p.i. cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. HSV gB was detected with indicated antibodies. Visualization was by LSCM at 63X magnification.
Pulse chase analysis of HSV gB reveals co-translational folding and trimerization of gB

To monitor the folding of gB as it is synthesized on the ribosome, gB epitopes were monitored over time using pulse-chase analysis (Figure 29) (102). A short pulse-label of 100s was used to define a population of gB. Assuming an elongation rate of around 3 amino acids per second, based on the average rate of translation in eukaryotes, a 100 second pulse would label less than half of the 904 amino acid gB (33, 123). Each of the 8 distinct epitopes tested were weakly detected directly following a 100 second pulse, indicating that gB begins folding prior to release from the ribosome and translocon. In addition, the oligomer-specific MAb DL16 also detected gB directly following the pulse. This indicates that gB is oligomerizing while still attached to the ribosome. The correct folding of gB must begin rapidly if it is recognized by a conformation-dependent oligomer-specific antibody. There was no reproducible difference in detection of conformation-dependent or independent epitopes. Following pulse, gB was chased over time. All epitopes appear to peak at 1 hour chase. This suggests that folding is continuing post-translationally. There are several epitopes that appear to have diminished detection at 2 hours chase. This suggests that following peak folding, additional events concerning the antigenic conformation of gB are occurring.
Figure 29. Pulse chase analysis of gB folding

Vero cells were infected with HSV-1 KOS at an MOI of 5. At 6 hr p.i. cells were pulse labeled with (400 µCi) $^{35}$S-labeled cysteine and methionine (MP biosciences) for 100 s. Wells were processed without chase (0) or chased for the indicated time in excess cold cysteine and methionine plus cycloheximide. Cells were lysed at 4°C with CHAPS in the presence of N-Ethylmaleimide. Samples were immunoprecipitated (IP) for 2 hr using Pro A agarose beads and specific anti-gB antibodies. Samples were boiled in non-reducing Laemmli buffer and analyzed with 6% SDS-PAGE and autoradiography. Quantitation was performed using the Measure function of NIH ImageJ 1.38X.
Discussion

Figure 15 demonstrates that gB undergoes conformational changes during viral entry. Here, it is demonstrated that gB can be found in several antigenic conformations in different places within the cell and virion (Figure 27 and 28). Firstly, it was established that total cell associated gB is antigenically distinct to virion gB. There are several interpretations of this; 1.) gB may have a binding partner that occludes epitopes in the cell, the binding partner may release upon virion maturation exposing the epitopes. 2.) These epitopes may not develop until gB is close to being incorporated into the virion, and may signify a maturational step for the glycoprotein. 3.) gB may undergo conformational changes within the cell that depletes these epitopes. 4.) gB with these epitopes may be preferentially incorporated into virions or degraded within the cell.

Class I and II glycoproteins undergo proteolytic maturation steps late in processing (1, 52, 53, 185). Maturation steps prevent premature triggering and fusion of viral fusion glycoproteins (185). This maybe the first evidence of a maturation step in class III fusion glycoprotein synthesis.

Interestingly, MAb SS10 maps to a region close to the putative receptor-binding region of gB (6, 58, 153). If these antigenic differences are not due to a fusion maturation step, then HSV may have evolved a way to shield the receptor-binding region of gB. This would prevent premature binding of receptor. Premature receptor binding represents a problem for numerous viruses and there are several known examples of how viruses circumvent this problem. The most attractive example to note is that of influenza neuraminidase. Neuraminidase cleaves sialic acids on glycans to which influenza is bound (reviewed in (66). The glycans would otherwise bind virions at
the cell surface indefinitely. The structurally designed anti influenza drugs *Relenza* and *Tamiflu* are both neuraminidase inhibitors. Further work may reveal this antigenic change is indicating an attractive drug target for HSV.

Several antigenically distinct populations of gB were seen within the cell via confocal microscopy. How these relate to function is not clear. Colocalization with cellular markers will help characterize compartments that contain gB. However, it must be remembered that cellular markers are rearranged in herpes virus infected cells (42, 76). From the corresponding light images (not shown) it is indicated that SS118 preferentially detects gB at the plasma membrane and the nuclear membrane. These are both sites of membrane fusion (25, 125, 192). Antigenically distinct gB was also seen in punctate compartments within the cell. It is unclear what compartments these are. They may represent secretory vesicles on the way to the cell surface, endosomes, recycling endosomes, lysosomes or a mixture of several of these. gB is known to recycle from the plasma membrane to recycling endosomes, and a mutant of gB is known to be retained at the cell surface (17, 18). It maybe of interest to examine structure-function correlations in recycling defective gB. Another antigenically distinct population of gB stains in a pattern reminiscent of the ER Golgi. This population may have epitopes that are detected more strongly during processing, or that rapidly leave the cell in virions. In all of these populations differential detection may be due to changes in conformation, or gB may alternatively release or bind to a binding partner that occludes the epitope at different sites within the cell. Pulse chase analysis of gB folding did not yield any relatable findings regarding the appearance or disappearance of these epitopes. Pulse labeling indicated that these epitopes of gB appear (and peak)
at around the same time, whether antigenic differences are the cause or effect of gBs arrival in these distinct populations also remains to be seen.

Pulse chase also revealed that a small population of gB trimerized on the ribosome. Whilst co-translational protein folding is now thought to be common for many epitopes of large proteins (22, 57, 63, 75, 86, 93, 123, 136, 150), detection of cotranslational oligomerization has only been demonstrated for a small number of proteins (38, 70). Notably examples of these include viral glycoproteins (70). This suggests a paralogously evolved functional reason for viral glycoprotein cotranslational oligomerization. Perhaps protomers are functioning to chaperone folding of their adjacent counterparts without being released as true chaperones are. This may obviate the need for the virus to encode separate chaperone proteins while aiding the folding of metastable trimers.

Class I and II glycoproteins avoid premature irreversible activation by having a maturation step that occurs after they encounter potential triggers. Here we show that gB is undergoing conformational changes in the secretory apparatus, acting like other Class III glycoproteins. The reversible nature of these changes means that gB is able to refold into a triggerable conformation post-neutralization. Pulse chase was used in an attempt to verify these data (not shown) but only a modest increase of the H126 epitope was seen in the presence of BFLA. We believe that the reversible nature of the conformational change meant that in the neutral pH of immunoprecipitation, conformational changes were reversed. In contrast, fixation of the epitopes prior to antibody binding allowed detection of conformational changes with LSCM. These data provide intriguing insights into the genesis and function of gB within the cell and virus.
Taken together, a possible order of events would be; gB cotranslationally folding and oligomerizing, then undergoing reversible changes as it traverses the secretory apparatus. gB may migrate to several sites in the cell where it has different binding partners or different conformations. One population of gB then undergoes a maturational step at a point close to incorporation into the virion. Infectious virions are released from the cell and the viral lifecycle continues. Figure 30 summarizes the antigenic data presented here for gB during virus entry and egress.
Figure 30. The antigenic conformation of gB throughout the HSV lifecycle

This summarizes the conformational changes that were identified throughout sections III, IV and V. Details regarding non gB related events that may be inferred from the figure have been previously described in Figure 3.

A) A ribbon cartoon of a monomer of gB. Domains are indicated by roman numerals and color-coding. Antibody binding regions are indicated as previously detailed in Table 2.

B) During the endocytic entry pathway of HSV, incoming virions encounter the low pH of an acidic compartment consistent with endosomal pH. The low pH then triggers conformational changes in domains I and IV of gB. pH induces changes are detected by epitopes prefixed by a star. DL16, which is oligomer specific, indicates oligomeric changes in gB are occurring in response to pH.

During viral replication, as viral egress is occurring, gB is preferentially detected in certain cellular locations. A pound prefixing an antibodies name indicates preferential detection. In addition, the H126 epitope on gB was shown to be sensitive to the low pH encountered during gB synthesis (pH sensitivity again indicated by the pound).

Black arrows indicate virion movement (entry and egress).
Blue arrows indicate capsid movement (egress).
Red arrows indicate glycoprotein movement in synthesis and maturation.
Green arrows point to the locations where gB epitopes are either pH sensitive or are preferentially detected.
Future directions

Several avenues of further study have been indicated throughout. Further work generating and analyzing mutations of gB is desirable. We have shown that fusion loop mutants can undergo conformational changes but not accomplish fusion. Further mutations are needed to delineate regions in gB that are necessary for conformational change. It would have to be predicted that mutants that are defective for conformational change, would be defective for fusion. It would also be interesting to see whether that gBs from other herpes viruses could undergo conformational changes using similar approaches. In comparing and contrasting the mechanisms that build up to membrane fusion, we may identify novel steps.

An antigenic analysis to monitor any low pH induced conformational changes in gH-gL is absent from this work and is also a future direction. In addition, although the low pH induced cell-cell fusion is reproducible it is still being perfected.

The cell-cell fusion assay (with and/or without pH) could also be extended to include many other HSV glycoproteins that have currently not been evaluated in their fusion function. There may be entire regulatory pathways that lead to fusion that are circumvented by leaving out so many membrane proteins. In addition, CMV has proteins that direct the entry pathway taken into certain cell types. There may well yet be an analogous protein or complex of proteins in HSV. A comprehensive analysis of HSV deletion mutant viruses may reveal proteins that direct the entry pathway taken.
Concluding remarks

Herpesviruses are a paradigm for viral fusion that requires multiple glycoprotein components. Herpesvirus fusion mechanisms have been refractory to study due to their complexity. This dissertation addresses a question of fundamental importance; why do herpesviruses require acidic pH for the initiation of infection? This work provides evidence that the core fusogenic protein, gB is triggered by the cellular cue, endosomal pH. Drugs that inhibit the identified conformational changes in gB, are of potential therapeutic benefit. Studying HSV glycoproteins is of paramount importance to vaccine development as the most successful vaccines to date have been glycoprotein subunit vaccines. The findings presented here have shaped our current understanding of herpesviral fusion in entry.
VI. Bibliography


7. **Atanasiu, D., W. T. Saw, G. H. Cohen, and R. J. Eisenberg.** Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J Virol **84**:12292-9.


regions of Herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion. J Virol 84:3825-34.


23. **Bjerke, S. L., and R. J. Roller.** 2006. Roles for herpes simplex virus type 1 UL34 and US3 proteins in disrupting the nuclear lamina during herpes simplex virus type 1 egress. Virology **347:**261-76.


25. **Browne, H., B. Bruun, and T. Minson.** 2001. Plasma membrane requirements for cell fusion induced by herpes simplex virus type 1 glycoproteins gB, gD, gH and gL. J Gen Virol **82:**1419-22.


58. **Fan, Q., E. Lin, T. Satoh, H. Arase, and P. G. Spear.** 2009. Differential effects on cell fusion activity of mutations in herpes simplex virus 1 glycoprotein B (gB) dependent on whether a gD receptor or a gB receptor is overexpressed. J Virol **83**:7384-90.


68. Gianni, T., M. Amasio, and G. Campadelli-Fiume. 2009. Herpes simplex virus gD forms distinct complexes with fusion executors gB and gH/gL in part through the C-terminal profusion domain. J Biol Chem 284:17370-82.

69. Gianni, T., P. L. Martelli, R. Casadio, and G. Campadelli-Fiume. 2005. The ectodomain of herpes simplex virus glycoprotein H contains a membrane alpha-


determination and genetic content of the short unique region in the genome of


enveloped virus upon binding to the host cell. J Virol 82:10429-35.

simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion,
while neither gB and gK nor gB and UL20p function redundantly in virion de-

113. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and

has multiple mechanisms for blocking virus-induced interferon production. J Virol
78:8411-20.

virus recombinant that exhibits a single-chain antibody to HER2/neu enters cells
through the mammary tumor receptor, independently of the gD receptors. J Virol
80:5531-9.

2005. Glycoprotein D receptor-dependent, low-pH-independent endocytic entry

simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF

microscopic observations on the development of herpes simplex virus. J Exp


130. **Park, R., and J. D. Baines.** 2006. Herpes simplex virus type 1 infection induces activation and recruitment of protein kinase C to the nuclear membrane and increased phosphorylation of lamin B. J Virol **80**:494-504.


135. **Pertel, P. E., A. Fridberg, M. L. Parish, and P. G. Spear.** 2001. Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. Virology **279**:313-24.


162. **Spear, P. G., S. Manoj, M. Yoon, C. R. Jogger, A. Zago, and D. Mysofski.** 2006. Different receptors binding to distinct interfaces on herpes simplex virus gD can trigger events leading to cell fusion and viral entry. Virology **344:**17-24.


membrane requires the fusogenic activity of herpes simplex virus gB. J Virol 83:11847-56.


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**PUBLICATIONS**


Dollery, S. J., K. D. Lane, M. G. Delboy, D. G. Roller and A. V. Nicola. 2010. Role of the UL45 Protein in Herpes Simplex Virus Entry Via Low pH-Dependent Endocytosis and its Relationship to the Conformation and Function of Glycoprotein B. Virus Res. 149:115-8


**ABSTRACTS**


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