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Herpesvirus entry into host cells mediated by endosomal low pH

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Abstract

Herpesviral pathogenesis stems from infection of multiple cell types including the site of latency and cells that support lytic replication. Herpesviruses utilize distinct cellular pathways, including low pH endocytic pathways, to enter different pathophysiologically relevant target cells. This review details the impact of the mildly acidic mileu of endosomes on the entry of herpesviruses, with particular emphasis on herpes simplex virus 1 (HSV-1). Epithelial cells, the portal of primary HSV-1 infection, support entry via low pH endocytosis mechanisms. Mildly acidic pH triggers reversible conformational changes in the HSV-1 class III fusion protein glycoprotein B (gB). In vitro treatment of herpes simplex virions with a similar pH range inactivates infectivity, likely by prematurely activating the viral entry machinery in the absence of a target membrane. How a given herpesvirus mediates both low pH and pH-independent entry events is a key unresolved question.

Keywords

Viral entry; herpesviruses; endosomes; low pH; herpes simplex viruses; endocytosis; gB

Introduction

The fundamental cellular activity of endocytosis is commandeered by the majority of both enveloped and non-enveloped animal virus families to introduce their genetic material to the cell interior (1). Endocytic processes offer the incoming virus the acidic environment of endosomal compartments, which is often needed to mediate penetration into the cytosol. Entry of enveloped viruses into cells requires the fusion of viral and cellular membranes. Membrane fusion is driven by conformational changes in viral fusion proteins, most commonly triggered by endosomal low pH, although sometimes by receptor binding alone (2).

Herpesviruses

Herpesviruses are large enveloped DNA viruses that cause characteristic lifelong latent infections. Upon successful entry into a host cell, herpesviral genomes are deposited to the nucleus. Depending on the cell type, herpesviruses produce progeny virions (lytic infection)

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and/or establishment of a latent infection. A given herpesvirus can enter via more than a single cellular pathway, also dependent on the target cell. Low pH-dependent herpesviral entry is the subject of this review with a focus on herpes simplex virus 1 (HSV-1), the prototype alphaherpesvirus. HSVs remain major pathogens worldwide causing cold sores, ocular and genital infections, and rare but often fatal encephalitis (3). In cell culture, HSV-1 can enter myriad cell lines derived from multiple species and tissues. In the immune-competent host, HSV infection is typically confined to the mucosal epithelia and neurons of the peripheral nervous system. However, in cases of disseminated infection of the immune-naive and immune-compromised HSV can infect diverse organ systems including the respiratory tract, gastrointestinal tract, and the central nervous system. This broad tropism may be explained in part by HSV's ability to utilize multiple entry routes, including low pH pathways.

Mildly acidic pH of vesicular compartments

Intracellular low pH is generated and maintained by large, membrane-associated multi-subunit vacuolar H^+-ATPases (V-ATPases) (4). A rotary mechanism powered by ATP hydrolysis transports protons from the cytosol to the interior of intracellular compartments. Regulation of V-ATPase activity, and subsequently intravesicular pH, occurs in several ways. For example, increased assembly of the V1 and V0 domains of the V-ATPase correlates with a decrease in pH (5). V-ATPase phosphorylation and membrane trafficking also regulate pump activity (6). Intracellular acidification is critical for many cellular processes. Early endosome acidification triggers the dissociation of internalized ligands (e.g., low density lipoprotein or transferrin) from their receptors and is also required for endosome maturation. Acid-activated, hydrolytic enzymes resident in lysosomes are required for degradation of proteins, lipids and nucleic acids. For the majority of both enveloped and non-enveloped viruses, a decrease in pH triggers viral surface proteins to initiate viral penetration of an internal host cell membrane (7). A drop in pH may serve as a cue for the virus to escape the endocytic pathway prior to arrival in the degradative lysosome.

pH-altering lysosomotropic agents

Viral entry via a low pH route is suggested when treatment of cells with a lysosomotropic agent (Table 1) blocks infection as demonstrated for Semliki Forest virus in the seminal paper by Helenius et al. (8). In the extracellular space, ammonium chloride exists predominantly in a protonated form that is membrane-impermeable. However, the non-protonated fraction enters the cell. Once inside a low pH compartment, ammonium chloride becomes protonated in a manner inversely proportional to pH. Ammonium chloride becomes trapped and concentrated in the compartment, elevating the luminal pH. Other weak bases such as chloroquine and methylamine operate in a similar manner (9). Bafilomycin A1 is a macrolide antibiotic from *Streptomyces griseus* that affects intracellular pH by inhibiting the function of all the vacuolar ATPases (4, 10). It is less effective at inhibiting E1E2 ATPases and is ineffective against F1F0 ATPases found in bacteria and mitochondria (11). Bafilomycin binds to the membrane-bound V0 domain, which mediates proton translocation (12).

Inhibition of virus infection by these pH-altering agents may suggest an endocytosis mode of entry, but this should be demonstrated directly and independently by microscopic approaches coupled with perturbation of specific endocytosis pathways. Importantly, lysosomotropic agents also alter the pH of secretory compartments such as trans-Golgi network vesicles. Thus, when ascribing the importance of a low pH environment to viral entry it is important to distinguish between effects on endosomal compartments during entry and downstream effects such as viral protein maturation and processing. This can be achieved experimentally by specifically assaying entry events at appropriately early times post-infection. Another indirect effect of modifying intravesicular pH on virus entry might be the disruption of the normal cellular distribution of host receptors.

HSV-1 entry

The promiscuous entry activity of HSV may be explained at least partly by HSV's ability to use multiple entry pathways, including low pH-dependent and-independent routes. Recent excellent reviews describe the current understanding of HSV receptor binding and membrane fusion in depth (13–15). Entry of HSV-1 can be initiated by attachment of virions to cell surface glycosaminoglycans, principally heparan sulfate (16). Attachment is not essential for subsequent fusion and entry. It is mediated by HSV-1 gC and to a lesser extent gB. This is followed by a requisite binding to one of several host cell entry receptors mediated by gD, including nectin-1, a calcium-independent, immunoglobulin-like cellular adhesion molecule (17–19) and HVEM, a member of the tumor necrosis factor receptor family (20). HSV-1s with individually deleted gB, gD, gH or gL fail to fuse with target cells and are incompetent for entry (21–25). Therefore, gB, gD, and gH/gL are required for virus-cell fusion and entry, but it has not been formally shown whether they are sufficient. The viral envelope contains > 10 additional viral proteins that may have roles in entry. Cell-to-cell spread of HSV is additionally facilitated by gE and gI (26). In the case of cell-cell fusion assays, gB, gD, and gH/gL expressed on the transfected cell surface in the absence of other viral gene products are both necessary and sufficient for fusion (27).

Early studies of HSV-1 entry routes

It is increasingly appreciated that herpesviruses utilize diverse entry pathways, including low pH endocytic routes to enter their pathophysiologically relevant cell targets. However, for decades HSV-1 along with HIV was considered a prototype virus that enters via fusion with the plasma membrane in a pH-independent manner (28–34). This conclusion was based on early electron microscopic images of direct entry of HSV-1 capsids at the cell surface (28, 29) and functional studies of a small subset of susceptible cells, including the African green monkey kidney cell line Vero. For many years Vero has been a workhorse for HSV researchers. Vero cells are easily propagated in culture and yield high HSV titers. HSV entry does indeed proceed by direct pH-independent penetration at the Vero plasma membrane. Virus-neutralizing antibodies block HSV-1 fusion with the surface of Vero cells (30), and HSV-1 entry into Vero and HEp-2 cells is not blocked by lysosomotropic agents (31, 32). In addition to Vero cells, human neurons, human foreskin fibroblasts, HaCaT and rat-kangaroo kidney (PtK2) cells are proposed to support HSV-1 entry by plasma membrane fusion (35–38).

Low pH-dependent, endocytic entry of HSV-1

In 2003, HeLa cells and CHO cells expressing human receptors that bind to HSV-1 gD, were shown to support HSV-1 and HSV-2 entry via a low pH, endocytic pathway (34). Over a short period of time the notion that herpesviruses utilize acid-dependent endocytosis in a cell-type dependent manner became a conventional idea. This was due to analysis of HSV entry into additional cell types, increased characterization of the pathway, and identification of similar low pH routes for other herpesviruses including Kaposi's sarcoma-associated herpesvirus (39), human cytomegalovirus (40), varicella zoster virus (41), and equine herpesvirus 1 (42). Table 2 documents the low pH-dependent entry of different herpesvirus-cell combinations.

HSV-1 entry via a low pH endocytic pathway is rapid. During an infection synchronized by first binding to the cell surface at 4 C, HSV-1 is internalized from the plasma membrane with a $t_{½}$ of 8–9 min. Trafficking of the enveloped virus followed by fusion with an acidic compartment occurs by 30 minutes post-infection (25). The intracellular site of penetration is not known. Its identification is complicated given that many entering particles visualized by fluorescence microscopy are non-infectious. The pH threshold of HSV-1 gB conformational changes is ~ 6.2 to 6.4 (43), which broadly corresponds to early endosomal pH.

Despite ample evidence of herpesviral inhibition by lysosomotropic inhibitors, direct activation of membrane fusion by acidic pH remains to be demonstrated. The application of mildly acid pH to cell-bound HSV-1 does not promote membrane fusion with the plasma membrane, as it does for other viruses (44) (45–47). Cells transfected with HSV-1 gB, gD, and gH/gL will fuse with target cells without a requirement for low pH (27). However, the transfected or infected cell plasma membranes are quite different effectors of fusion than actual viral particles involved in entry. All herpesviruses acquire their envelopes from interior membranes. Thus, the viral envelope may differ from infected and transfected plasma membranes in ways that render it capable of both pH-dependent and pH-independent fusion. Entry into cells that support HSV low pH entry requires gB, gD, and gH/gL, as does entry via direct penetration (25). The envelope proteins gG, gE, gI, gJ, gM, UL45, and Us9 are dispensable for low pH entry (48, 49). Proteins that specifically function in low pH fusion and entry of HSV-1 have not been identified.

Low pH-independent entry pathways of herpesviruses

The focus of this article is the contribution of mildly acidic intracellular pH to herpesviral entry, but it is important to note that bona fide herpesvirus entry routes involve pH-independent mechanisms. These routes include endocytosis followed by pH-independent membrane fusion and fusion with the plasma membrane. When herpesvirus entry into a given cell type is only modestly inhibited by lysosomotropic agents, this may indicate that both pH-dependent and pH-independent pathways are being utilized. Also, the same cell type maintained in different laboratories can yield differential sensitivity to lysosomotropic agents (34, 36, 38, 50–52).

A low pH requirement has not been reported for Epstein Barr virus (EBV) entry into any cell type. EBV enters B cells by pH-independent endocytosis and epithelial cells by direct penetration (53, 54). Human cytomegalovirus enters fibroblasts and dendritic cells by a pH-independent mechanism (40, 55–58). Many studies using several cell types report HSV-1 entry that is resistant to inhibition by lysosomotropic agents (36, 38, 51, 52, 59, 60). Polyethylene glycol-induced fusion of HSV-1 with the plasma membrane of wild type CHO or B78 cells results in viral entry (44), suggesting that these cells are capable of mediating HSV-1 entry via a non-endocytic, pH-independent route.

Conformational changes in herpesviral fusion protein gB triggered by mildly acidic pH

Glycoprotein gB is conserved among all herpesviruses and is considered the primary fusion protein (13–15). By definition, the pre-fusion conformation of gB is present in infectious virions. Pre-fusion HSV-1 gB is altered in direct response to low pH (43, 61–63). I propose that this provides part of a molecular explanation for why herpesviruses require endosomal pH for entry. A mildly acidic pH of < 6.4 causes specific antigenic changes in virion gB. These include changes in Domains I and V, which constitute the functional region of gB containing the hydrophobic, bipartite fusion loops. Conformation is not globally altered by low pH as Domains II, III and IV are not affected (Fig. 1). Importantly, gB conformational change is also detected during viral entry by endocytosis, when the incoming virus arrives in an acidic compartment (43). Low pH-dependent antigenic changes in gB from murid herpesvirus 4 have also been detected during viral entry (64). Low pH-triggered changes in other herpesvirus gBs have not yet been reported. Mildly acidic pH causes a change in the oligomeric structure of HSV-1 gB, as demonstrated by several approaches. Following density centrifugation, gB from low pH-treated virions sediments at a lower density than gB from untreated virions. Virions previously treated with low pH and blotted to nitrocellulose have reduced reactivity with oligomer-specific monoclonal antibody DL16 (43). Analysis of gB by different iterations of mildly denaturing PAGE also detects an alteration in oligomer structure (43, 61–63, 65). The nature and significance of the change in gB quaternary structure remains to be determined.

Low pH triggers gB to become more hydrophobic as measured by Triton X-114 partitioning (43, 63). This may suggest that membrane-interacting regions are revealed. Fusion-from-without (FFWO) is the rapid induction of cell fusion by the contact of virions with target cells. The combination of two amino acid mutations in gB, one in the ectodomain (V553A) and one in the cytoplasmic tail (A855V), confers FFWO activity to wild type HSV (66, 67). FFWO gB differs from wild type gB in that it has reduced reactivity with monoclonal antibodies DL16 and H126. Interestingly, low pH-treated wild type gB has reduced reactivity with these same antibodies (68), suggesting that a highly fusogenic form of gB resembles gB that has undergone low pH-triggered conformation changes. However, a more direct link between pH-triggered changes in gB and fusion activity has yet to be established. Mildly acidic pH has no detectable effect on gD antigenic structure, nor does it affect gD binding to its nectin-1 or HVEM receptors (63). Low pH affects the antigenic structure of gH/gL and some of the detected changes are irreversible (62). Whether these alterations

influence viral entry is of importance and unknown. HSV-2 gB, which can substitute for HSV-1 gB in cell-cell fusion (69), also undergoes pH-triggered changes (43, 70).

Low pH alters the conformation of soluble forms of HSV gB

Mildly acidic pH alters the conformation of recombinant, purified forms of gB, indicating that low pH directly affects gB structure in absence of other virion components (43, 62, 63, 65). A membrane-truncated, ectodomain construct of gB (gB730), for which the x-ray structure is known and which currently represents the post-fusion conformation of gB (71), binds to liposomes at neutral pH via its exposed fusion loops (72). However, HSV-1 particles do not bind to liposomes at neutral pH likely because the pre-fusion form of gB present in virions has hidden fusion loops. A soluble form of gB containing the membrane-proximal region, missing from gB730, fails to bind to liposomes (73, 74). In the presence of the soluble gD-receptor HVEM and mildly acidic pH, HSV-1 associates with liposomes (75). Soluble nectin-1 receptor does not enable virus association with membranes under the same conditions for reasons that are not clear. Soluble gH/gL does not associate with liposomes at neutral pH. At low pH, it does so only in the presence of gB730 with functional fusion loops, suggesting a pH-induced association of gB and gH/gL (62). Fusion loop 2 in the gB730 x-ray structure at pH 5.5 is flipped outward relative to the neutral pH structure (65). As compared to full-length, pre-fusion gB present in the viral envelope, gB730 undergoes only limited conformational changes in response to mildly acidic pH, consistent with gB730 representing a post-fusion conformation. Another soluble form of gB undergoes conformational change(s) at pH 5.1 as measured by intrinsic fluorescence spectroscopy, a biophysical technique independent of antibody binding or detergent treatment of gB (63). In addition to virion and soluble gBs, gB from transfected cells and HSV-infected cells also undergo conformational changes in response to low pH (63, 70).

Virus fusion mechanisms are diverse, yet all involve large-scale conformational changes in fusion proteins (2). During fusion, there may be a large-scale rearrangement of gB folded domains such as proposed for another class III fusion protein, vesicular stomatitis virus G (76). However, the extent of gB conformational change(s) is an unresolved issue, as a pre-fusion structure is not yet available. The structure of a full-length, membrane-bound form of gB is distinct from the post-fusion conformation (77). Whether this structure resembles pre-fusion gB or an intermediate conformation remains to be determined. In addition to mildly acidic pH, the full range of conformational changes in gB during endocytic entry into epithelial cells may require gD, gH/gL and additional host factors, including a receptor that binds to gD.

If the detected conformational changes in gB indeed mediate membrane fusion during low pH entry, how might pH-independent entry proceed? One possibility is that surface receptor binding may functionally substitute for endosomal low pH in cases of entry via penetration at the plasma membrane. Neutral pH conformational changes in gB are expected to occur during plasma membrane fusion. They have not been identified but are predicted to be similar to those induced by pH. In the case of HSV-1, several cellular molecules bind to gB including paired immunoglobulin-like type 2 receptor alpha, non-muscle myosin IIA, and

myelin-associated glycoprotein (78–80). It remains to be seen whether they trigger pH-independent conformational changes.

Reversibility of gB conformational changes

The pH-triggered conformational changes in HSV gB detected to date are reversible. These include changes in antigenic reactivity (43, 61, 63), oligomeric conformation (43, 61–63, 65), and intrinsic fluorescence (63). Reversible changes induced by pH have been described for the class III fusion proteins VSV G (76, 81) and baculovirus gp64 (82). Unlike class I and II fusion proteins, which undergo irreversible conformational changes, pre-fusion and post-fusion forms of class III proteins are proposed to exist in a pH equilibrium (83, 84). The equilibrium is shifted toward the post-fusion state at low pH. HSV-1 and pseudorabies virus travel through acidic compartments during egress (85, 86). Reversibility may allow fusion proteins to avoid nonspecific activation during assembly and egress through the low pH environment of the secretory pathway. While reversibility is a hallmark of class III proteins, its molecular determinants and its relationship to fusion activity are not clear. The energy released during a reversible conformational change may be limited relative to the irreversible changes of class I fusion proteins, suggesting important ramifications for fusion and entry, such as the minimum number important ramifications for fusion and entry, such as the minimum number of trimers required for fusion (87).

Inactivation of HSV-1 infectivity by low pH

Inactivation of virions by pretreatment with mildly acidic pH is a trait common to viruses that enter via an acid-activated mechanism. In the absence of a target membrane, low pH prematurely activates the fusion protein, so when the pH-treated virion is added to cells, it is incompetent for fusion and entry (88, 89). Viruses that enter exclusively via a pH-independent mechanism, such as Sendai virus (90) and HIV (91), are typically not inactivated by low pH pre-treatment.

The infectivity of isolated HSV particles exposed to low pH in vitro is rapidly and irreversibly reduced in a temperature-dependent manner (34). Inactivation begins at pH ~ 6.0. The part(s) of the virion that are inactivated is not known. The pH-dependent changes in gB conformation detected thus far are likely not responsible for inactivation since they are reversible. FFWO strains of HSV-1 with highly fusogenic forms of gB are sensitive to low pH inactivation similar to wild type strains (61). HSV-1 mutants individually deleted for envelope proteins gG, gE, gI, gJ, gM, UL45, and Us9 are acid-inactivated similar to wild type HSV (48, 49), suggesting that none of these proteins is responsible for low pH inactivation. The target of inactivation may be one or more components of the required entry machinery, gD, gH/gL, a region of gB, or one of the remaining envelope proteins yet to be analyzed. Infectivity of varicella-zoster virions is also diminished by pretreatment with mildly acidic pH (41). Identification of the virion target of herpesvirus inactivation by mildly acidic pH and the mechanism of inactivation may reveal key features of the low pH entry process.

Penetration of cell-bound herpesvirions is irreversibly blocked by exposure to a highly acidic glycine or sodium citrate buffer of pH 3.0 (92, 93). Inactivation of non-penetrated, non-internalized attached herpesvirions by pH 3.0 buffer is often employed as an experimental step in determination of the kinetics of viral uptake from the cell surface. It remains to be seen whether inactivation by pH 3.0 and the more physiologically relevant mildly acidic pH share a similar mechanism.

Summary and future efforts

It has become increasingly clear that herpesviruses utilize endosomal acidic pH entry pathways in a cell-specific manner. The precise activating role that pH plays is a key unanswered question. The full complement of herpesviral and host factors required for cell entry has remained elusive. Endosomal pH is likely one of several cues for herpesviral entry, including cellular receptors that bind either to the conserved herpesviral gB or gH or to subfamily-specific envelope proteins. Two or more of these cellular triggers may be needed to complete the entry process. The sequence of these virus-host interactions and where endosomal pH fits in is an outstanding question. If fusion can proceed in acid-dependent and independent manners, how similar is the execution of fusion? The acidic compartment that serves as the site of intracellular fusion following endocytic uptake also remains to be identified. Low pH-dependent conformational changes in the core fusion protein gB have been identified, but their specific role in the fusion mechanism is another critical unresolved issue.

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Synopsis

Herpesviridae comprise a large family of enveloped DNA viruses that utilize several cellular entry pathways in a cell type dependent manner. This review focuses primarily on our understanding of low pH entry mechanisms, with emphasis on the prototype alphaherpesvirus, herpes simplex virus 1 (HSV-1). In our model of infection, HSV-1 enters epithelial cells by a mildly acidic endosomal pathway, and HSV-1 entry into neurons, the site of latency, occurs by direct penetration at the cell surface. Low pH-triggered conformational changes in envelope glycoproteins, particularly the main fusion protein gB, are also described.

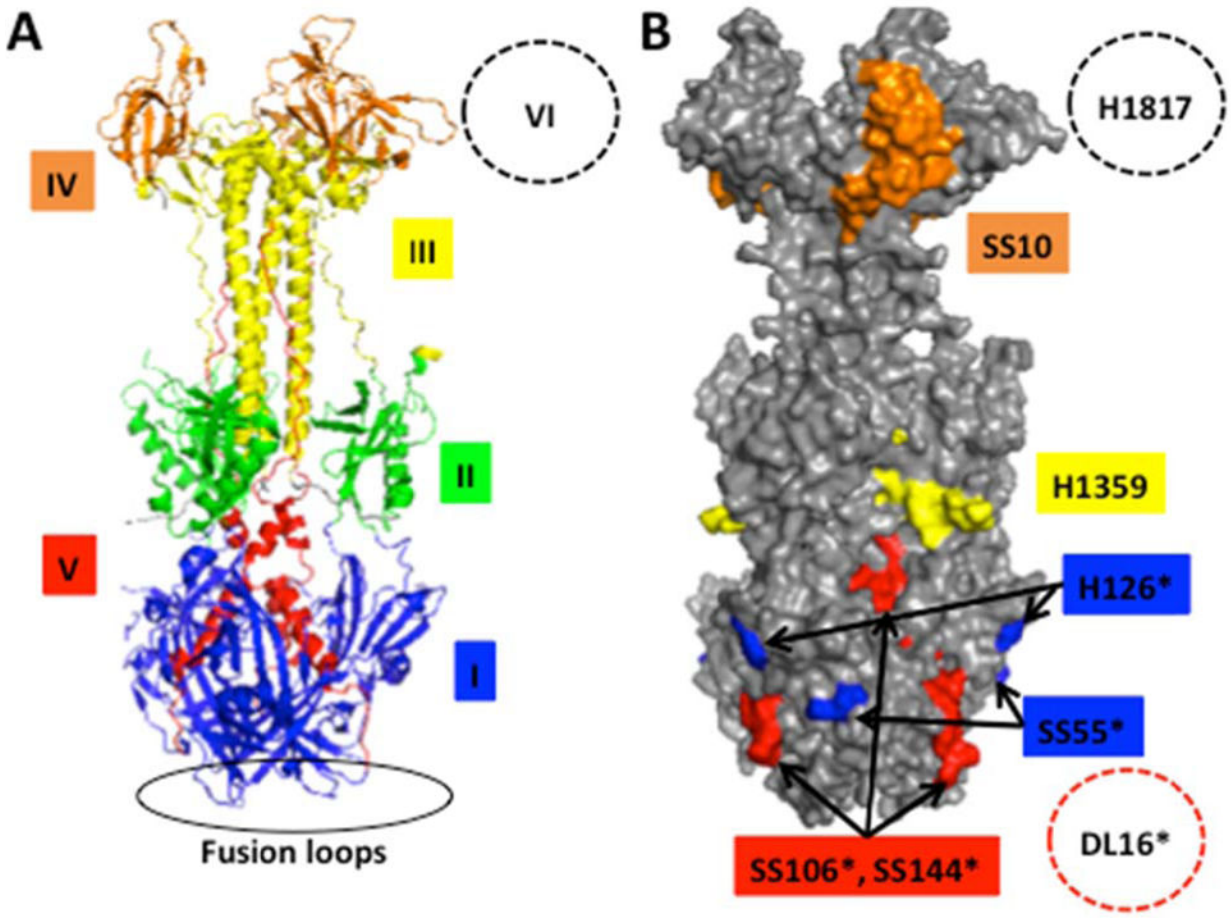

Figure 1.

Effect of low pH on the antigenic structure of HSV-1 gB (A) gB ectodomain trimer representing a post-fusion conformation. (B) Location of monoclonal antibody binding sites. Monoclonal antibody-resistant mutations in Domain I, which contains bipartite hydrophobic fusion loops, map to amino acid residue 303 for H126 and residues 203, 335 and 199 for SS55 (108, 109). MAb H1359 to Domain III maps approximately to 487–505 (110). The MAb SS10 epitope in Domain IV maps to 640–670 based on binding to fragments and peptides (111). MAbs SS106 and SS144 to Domain V both bind to a 697–725 peptide (71). The oligomer-specific MAb DL16 binds to Domain V as determined by electron microscopy (109). The MAb H1817 epitope in Domain VI (not resolved in the structure) maps to 31–43 based on binding to fragments and peptides (111). MAbs that exhibit reduced reactivity with pre-fusion gB that has been pre-treated with pH <6.2 are marked with (*). The reactivity of unmarked MAbs is unchanged by low pH pre-treatment (43, 61, 63).

Table 1

Toolbox of lysosomotropic agents that inhibit low pH viral entry.

Agent	Class	Target	Inhibitory range
Ammonium chloride	weak base	Lumen of acidic compartment	mM
Bafilomycin A1	macrolide	V0 domain of V-ATPases	nM
Chloroquine	weak base	Lumen of acidic compartment	mM
Methylamine	weak base	Lumen of acidic compartment	mM
Monensin	polyether	Monovalent cations	μM

Table 2

Herpesviral entry into host cells inhibited by lysosomotropic agents.

Herpesvirus (strain, isolate)	Cell type	Agent	References
Alphaherpesvirus subfamily			
HSV-1 (KOS, F)	CHO-nectin-1, CHO-HVEM cells	A, B, C, M	(34, 50, 94)
HSV-1 (KOS, F)	CHO-HVEM cells	A, B, M	(34, 50)
HSV-1 (KOSrid1)	CHO-nectin-1, CHO-nectin-2 cells, HeLa	A, M	(34, 95)
HSV-1 (KOS, F)	HeLa	A, B, M	(34, 50)
HSV-1 (MP)	CHO-nectin-1 cells	A, M	(34)
HSV-1 (F)	J-nectin-1-EGFR1, J-nectin1-GPI cells	A, B	(50)
HSV-1	HaCaT	A, M	(36, 38)
HSV-1 (KOS)	Normal human epidermal keratinocytes	A, M	(36, 38)
HSV-1 (KOS)	Human squamous cell carcinoma (SCC13) keratinocytes	B, M	(36)
HSV-1 (KOS)	Primary human corneal fibroblasts	A, B, C	(94)
HSV-1 (KOS)	Human retinal pigment epithelial cells	A, B, C	(96)
HSV-1 (KOS)	Human corneal epithelial, conjunctival epithelial cells	B, C	(97, 98)
HSV-1 (ANG, ANGpath)	CHO-nectin-1, CHO-HVEM cells	A, M	(68, 95)
HSV-1 (F)	293T-integrin αVβ3, J-nectin-1-integrin αVβ3	B	(99)
HSV-2 (G)	CHO-nectin-1 cells, CHO-HVEM cells	A, M	(34)
VZV (POka)	CHO-K1	A	(41)
EHV-1 (RacL11)	CHO-K1	B	(42)
EHV-1 (HH1)	E. Derm cells	A, B	(100)
Betaherpesvirus subfamily			
HCMV (TR)	Retinal pigment epithelial cell line ARPE-19	A, B, C	(40)
HCMV (TR)	Transformed human umbilical vein endothelial cells	A, B, C	(40)
HCMV (*fibro*BADrUL131)	ARPE-19	A, B	(101)
HCMV (*fibro*BFX *wt*)	ARPE-19	A, B	(101)
MCMV (m74-null)	NIH 3T3 cells	A, B	(102)
Gammaherpesvirus subfamily			
KSHV	Human foreskin fibroblasts	A, B	(39)
KSHV	293T (T1H6) cells	A, B, M	(103)
KSHV	Human umbilical vein endothelial cells	A, B, M	(104, 105)
KSHV	Human dermal microvascular endothelial cells	A, B	(104)
KSHV	Human THP-1 monocytes	A, B	(106)
RRV (26–95)	Rhesus fibroblasts	A, B, M	(107)
MuHV-4 (G2.4)	Mouse mammary (NMuMG) epithelial cells	B, Con	(64)

A, ammonium chloride

B, bafilomycin A1

C, chloroquine

Con, concanamycin A

M, monensin