Low pH-Induced Conformational Change in Herpes Simplex Virus Glycoprotein B

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Herpesviruses can enter host cells by pH-dependent endocytosis pathways in a cell-specific manner. Envelope glycoprotein B (gB) is conserved among all herpesviruses and is a critical component of the complex that mediates membrane fusion and entry.

Here we demonstrate that mildly acidic low pH triggers specific conformational changes in herpes simplex virus (HSV) gB. The antigenic structure of gB was specifically altered by exposure to low pH both \textit{in vitro} and during entry into host cells. The oligomeric conformation of gB was altered at a similar pH range. Exposure to acid pH appeared to convert virion gB to a lower order oligomer. The detected conformational changes were reversible, similar to other Class III fusion proteins. Exposure of purified, recombinant gB to mildly acidic pH resulted in similar changes in conformation and caused gB to become more hydrophobic, suggesting that low pH directly affects gB. We propose that intracellular low pH induces alterations in gB conformation that, together with additional triggers such as receptor-binding, are essential for virion-cell fusion during herpesviral entry by endocytosis.
Herpes simplex virus (HSV) is an important human pathogen, causing significant morbidity and mortality worldwide. HSV enters host cells by fusion of the viral envelope with either an endosomal membrane (38) or the plasma membrane (63). The entry pathway taken is thought to be determined by both virus (17, 45) and host cell factors (4, 17, 35, 39, 45). Based on experiments with lysosomotropic agents, which elevate the normally low pH of endosomes, acidic pH has been implicated in the endocytic entry of HSV into several cell types including human epithelial cells (37). Low pH has also recently been implicated in cell infection by several other human and veterinary herpesviruses (1, 21, 26, 47). The mechanistic role of endosomal pH in herpesvirus entry into cells is not known.

Herpesviruses are a paradigm for membrane fusion mediated by a complex of several glycoproteins. We have proposed that HSV likely encodes machinery to mediate both pH-dependent and pH-independent membrane fusion reactions. Envelope glycoproteins gB, gD, and the heterodimer gH-gL are required for both pH-independent and pH-dependent entry pathways (11, 22, 30, 39, 46). Interaction of gD with one of its cognate receptors is an essential trigger for membrane fusion and entry (13, 52), regardless of cellular pathway. However, engagement of a gD-receptor is not sufficient for fusion, and at least one additional unknown trigger involving gB, or gH-gL is likely necessary. gB is conserved among all herpesviruses and in all cases studied to date, it plays roles in viral entry including receptor-binding and membrane fusion. The crystal structure of an ectodomain fragment of HSV-1 gB is an elongated, rod-like structure containing hydrophobic internal fusion loops (28). This structure bears striking
architectural homology to the low pH, post-fusion form of G from vesicular stomatitis
virus (43). Both gB and G structures have features of Class I and Class II fusion proteins
and are thus designated as Class III proteins (57).

During entry of the majority of virus families, low pH directly acts on
glycoproteins to induce membrane fusion (60). In some cases, the low pH trigger is not
sufficient, and it plays an indirect role. For example, host cell proteases such as
cathepsins D and L, require intravesicular low pH to cleave Ebola virus and SARS
glycoproteins to trigger fusion (14, 51).

We investigated the role of low pH in the molecular mechanism of herpesviral
entry. The results suggest that mildly acidic pH, similar to that found within endosomes,
triggers a conformational change in gB. We propose that together with other cellular
cues such as receptor interaction, intracellular low pH can play a direct activating role in
HSV membrane fusion and entry.

MATERIALS AND METHODS

Cells and viruses. Vero cells (American Type Culture Collection; ATCC; Rockville,
Md.) 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.). 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.

Dot blot analysis. Cell-free preparations of extracellular HSV-1 KOS were diluted in
serum-free, bicarbonate-free DMEM with 0.2% BSA and 5 mM each of 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 were either blotted directly to nitrocellulose with a Mini Fold dot blot system (Whatman) or were first neutralized by addition of pre-titrated amounts of 0.05 N NaOH. Membranes were blocked and incubated with anti-gB monoclonal antibody H126 (32), H1359, H1817 (Virusys), DL16, SS10, SS55 (8), SS106, SS144 (7) or R69 polyclonal antibody to gB provided by G. Cohen and R. Eisenberg, University of Pennsylvania. After incubation with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescent substrate (Pierce) was added, and blots were exposed to X-ray film (Kodak).

**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. The indicated pHs were obtained with 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, and analyzed by SDS-PAGE followed by immunoblotting with MAb H1359 to gB. Data were quantitated by densitometry using ImageJ 1.38x (NIH).

**Analysis of gB oligomeric structure by PAGE.** HSV-1 KOS, HSV-2 333 or recombinant s-gB was diluted in medium as described above for dot blot. s-gB has the transmembrane domain deleted (gB2ΔTM) (53) and was obtained from Stephen Straus, NIAID. Samples were adjusted to indicated pHs with pre-titrated amounts of 0.05 N HCl and incubated at 37°C for 10 min. 1% SDS 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 (16). 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).

**Triton X-114 partitioning.** To increase the association of hydrophobic gB with the amphiphilic (detergent) 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 (9). The hydrophilic phases from each of three condensation steps was discarded. 200 ng of s-gB or 20 µg of BSA were incubated with preconditioned 2% Triton X-114 in fusion medium buffered to various pHs on ice for 10 min. Samples were incubated at 37°C for 10 min, and then centrifuged at 300 x g for 3 min at 25°C. Aqueous and detergent phases were collected and either immunoprecipitated with MAb H1359 to gB or precipitated with trichloroacetic acid. Precipitates were analyzed by SDS-PAGE followed either by Western blotting with MAb H1359 for gB or Coomassie blue staining for BSA.

**Confocal microscopy of virion gB during viral entry.** CHO-nectin-1 cells were mock-treated or treated with 25 nM bafilomycin A1 (BFLA) 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) in PBS and permeabilized with
0.2% Triton X-100 (Fisher Scientific). Virion gB was detected with 2 µg/ml MAb H126 or 0.5 µg/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.

RESULTS

Low pH Treatment Alters the Exposure of Epitopes in the Fusion Domain of Virion gB. Intracellular 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. 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 (60).

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) (28, 32, 40, 45). In addition, a fusion-from-without form of virion gB with enhanced fusogenicity was shown to have an H126 epitope with altered accessibility (45).

HSV virions were exposed to pHs ranging from 7.4 to 4.8 and were immediately blotted to nitrocellulose membrane. Antibody binding was then assessed at pH 7.4. MAb H126 displayed diminished binding to virions that had been treated at pH < 6.2
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 (Fig. 1, Table 1). 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 (Fig. 1). Similarly, MAbs H1359 and SS10, directed to Domains III and IV, respectively, and gB-specific polyclonal Ab R69, also bound well to low pH-treated HSV (Fig. 1, Table 1), suggesting that the structure of gB is not globally altered and that the detected changes in reactivity are specific. Domains I and V are in close proximity and together form a functional region (7). Interestingly, MAbs to Domain V, SS106 and SS144, had reduced binding to virions that had been treated with mildly acidic pH (Fig. 1, Table 1), 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 that contains fusion loops.

**Mildly Acidic pH Alters the Oligomeric Structure of gB.** Viral fusion proteins are oligomers (60). HSV gB is oligomeric, and an ectodomain form of HSV-1 gB has a trimeric structure (15, 28). Low pH-triggered membrane fusion can be accompanied by rearrangement of viral glycoprotein oligomer subunits (3, 56). Three approaches were used to determine the effect of acid pH on the quaternary structure of gB. First, HSV was 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 weight. This is consistent with gB oligomers...
and is similar to previous reports (29). At pH 7.4, a peak in fraction 6 and a broad peak in fractions 8 to 11 were observed (Fig. 2A). 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 (Fig. 2A, lower panel).

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, blotted to membrane, and the reactivity of MAb DL16 was evaluated at neutral pH. After exposure to pH 5.5, there was a decrease in DL16 reactivity (Fig. 2B). 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 non-covalently associated monomers. gB oligomers are disrupted experimentally by a combination of SDS and heat (15), resulting in monomeric gB that migrates at ~ 116 kilodaltons 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 from 7.4 to 5.1 and prepared for “native” PAGE by making samples to 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 kilodaltons (Fig. 2C, lanes 1 and 2). Treatment of HSV with pH 7.4 followed by 1% SDS yielded gB species of similar high molecular weight (Fig. 2C, lane 3), indicating that 1% SDS alone had no detectable effect on the gB oligomer. In contrast, pre-treatment with pHs < 6.4 followed by 1% SDS reduced the number of gB species
detected (Fig. 2C, lanes 4-7). The highest molecular weight forms seemed to disappear, leaving only a single detectable oligomeric species of lower molecular weight (Fig. 2C, 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 monomer is detected only weakly relative to oligomer 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 (5, 6). Notably, the total amount of gB detected by dot blot does not change upon exposure to mildly acidic pH (Fig. 1). Together, results from the three approaches (Fig. 2A, 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 in Figure 2C were indeed oligomers of gB, we tested whether other HSV entry glycoproteins co-migrated with the gB-containing complexes. Virion gC, gD, gH, and gL at pH 7.4 each migrated independently of gB under native PAGE conditions (Fig. 2D, -1% SDS lanes). gB may associate with other proteins. However, since a purified form of recombinant gB oligomer has a similar molecular weight as virion gB (Fig. 4A), we favor the interpretation that the high molecular weight species in Figure 2C 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 (Fig. 2D, -1% SDS lanes). In addition, low pH did not reproducibly affect the detection of these glycoproteins, including the gH-
gL hetero-oligomer (Fig. 2D, braces). Virion gC, gD, gH or gL were also not affected when subjected to the conditions that demonstrated the effect of low pH on the detergent stability of gB (Fig. 2D, + 1% SDS lanes). Based on these analyses, gB may be the entry glycoprotein principally affected by pH.

**pH-Triggered Conformational Changes are Reversible.** Conformational changes in Class I and Class II fusion proteins are irreversible (60). As an example, the pre-fusion form of hemagluttinin (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 post-fusion form and can no longer mediate fusion with a subsequently presented membrane (59). In contrast, conformational changes in the Class III fusion protein VSV G are reversible, with pre-fusion and post-fusion forms existing in a thermodynamic equilibrium (24, 42). The equilibrium is shifted toward the post-fusion state at low pH. To investigate reversibility of changes in gB, we again assayed the effect of 1% SDS on oligomer stability using native PAGE (Fig. 2C). KOS virions were incubated at pH 5.1, re-neutralized to pH 7.4, and then 1% SDS was added (Fig. 2C, lane 8). Oligomeric forms of gB were detected (lane 8), indistinguishable from gB that had been kept at neutral pH (Fig. 2C, 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. pH 5.5-treated HSV that was blotted to nitrocellulose displayed decreased reactivity with MAb H126 relative to pH 7.2 treatment (Fig. 3). Although this is an overexposure, the reduction in H126 reactivity is consistent with results in Figure 1. However, when virions were
treated first at pH 5.5 and then adjusted back to 7.2 prior to blotting, H126 reactivity was partly recovered. Similar results were obtained with the oligomer-specific MAb DL16 (Fig. 3), 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 (Fig. 3). Immobilization of acid-treated virions on nitrocellulose membrane 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 (Fig. 1 and Table 1).

**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 (53). gB molecules from HSV-1 KOS and HSV-2 333 are 86% identical. In this form of gB, the 57 amino acid transmembrane domain has been deleted and the cytosolic tail is fused directly to the ectodomain. s-gB is secreted and purified from CHO cells (53). It migrates as an oligomer on native PAGE (Fig. 4A), binds to cell surface glycosaminoglycan receptors (61), and reacts with all monoclonal antibodies tested, including the oligomer-specific antibody DL16 (Fig. 4A, Table 1).

Treatment of s-gB with mildly acid pH followed by 1% SDS affected its oligomeric conformation (Fig. 4B). A pH of 5.1 converted oligomeric s-gB to monomer (Fig. 4B). Conversion to monomer began at pH < 6.3 and was complete by pH 5.9 (Fig. 4B).
Thus, the quaternary structure of purified gB was altered by the same range of mildly acidic pH as gB that is present in the viral envelope (compare Figs. 4C and 2C). Acid-induced changes in s-gB were reversible (Fig. 4B, center panel). Low pH treatment converted gB present in HSV-2 333 to a lower molecular weight oligomeric form (Fig. 4B, right panel), similar to the result obtained with HSV-1 KOS gB (Fig. 2C). Interestingly, this contrasts with the acid-dependent conversion of s-gB oligomer to 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 structure of both purified gB and virion gB. The reversibility of changes to both forms of gB is further demonstrated in Figure 4A. Samples that were treated with pH 5.4, re-neutralized to pH 7.2, and then analyzed by native PAGE, migrated as oligomer similar to samples that were not exposed to low pH (Fig. 4A). Lastly, s-gB that was treated with acidic pHs and was then bound to nitrocellulose had a similar pattern of reactivity with MAbs as pH-treated virion gB (Table 1), 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 do gD, gH, gL or any other viral components 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.

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 non-ionic 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 (9). 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 (31).

Purified s-gB that was treated at pH 7.4 was mixed with Triton X-114 at 30°C. The mixture was centrifuged, and water-soluble gB was recovered from the aqueous phase (Fig. 5), where hydrophilic proteins are expected to partition. Little to no gB was detected in the detergent phase, suggesting little hydrophobic interaction of gB with the 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 (Fig. 5), 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 (Fig. 5). The results are consistent with the notion 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.

**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 (39). Endocytic internalization of HSV from the CHO cell surface has a t₁/₂ of ~ 9 min. Enveloped virions are trafficked through cellular compartments for up to ~ 30 min p.i. Penetration of > 50% of infectious virions from a low pH compartment (virus-cell membrane fusion) occurs by 60 min p.i (39). 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 presence of cycloheximide ensured that the signal detected was due to input gB and not from newly synthesized gB. gB that was brought into the cell with entering virions was detected by MAb H126 as punctate staining (Fig. 6). Bafilomycin A1 (BFLA), a vacuolar H⁺ ATPase inhibitor that prevents endosome acidification, inhibits HSV entry into CHO-nectin-1 cells (38). When infection proceeded in the presence of BFLA there was a detectable and reproducible increase in the H126-reactive gB detected (Fig. 6). As a control, MAb H1817 recognized input intracellular gB to a similar extent in the absence or presence of BFLA (Fig. 6). 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.

**DISCUSSION**

In this paper, we demonstrate that conformational change in the fusogenic herpesviral glycoprotein gB is triggered by 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. Conformational change and an increase in the hydrophobic character of gB both 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 (32). 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 cells support pH-dependent or pH-independent entry (18, 45). HSV-1 strain ANG path has a highly fusogenic form of gB that is responsible for fusion-from-without activity (48). Two distinct mutations in gB are responsible for FFWO. Notably, gB from ANG path has reduced reactivity with MAbs DL16 and H126 (45).

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, 10). Low pH has little to no detectable effect on gD’s antigenic structure or on its ability to bind to receptors (38); unpublished data). The present data suggest that gB is a principal target of endosomal pH. However, acid effects on other glycoproteins such as gH, which contains a putative fusion peptide (25), remain to be investigated further.

Host cell triggers that can cause conformational changes in glycoproteins leading to fusion include the low pH milieu of an endosome, binding to receptors, and cleavage by endosomal proteases (60). 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 (33). This change in gD conformation is thought to initiate the fusion process mediated by gB and gH-gL (5, 23, 54). 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 (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 (11). The recently identified gB receptor PILRalpha may trigger direct penetration of HSV with the cell surface (4, 50). However, fusion probably cannot occur without a gD receptor and gD, even when PILRalpha has a role (20, 50). 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 (18, 45). It is tempting to speculate that in cells that support pH-independent entry, binding to a gB-specific receptor such as PILRalpha 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 (55). In this surrogate assay, transfected cells that express gB, gD, gH and gL on the cell surface are mixed with untransfected target cells. Comparisons of cell-cell fusion with virus-cell fusion must be
drawn cautiously. Results from cell fusion and viral entry assays do not always agree (12, 36, 62). Herpesviral envelopes are derived from internal cellular membranes, not the plasma membrane. Glycoproteins displayed on the plasma membrane of transfected cells may have distinct roles in fusion (i.e., are activated differently) than glycoproteins that are actually incorporated into virions. We are currently evaluating the effect of pH on glycoprotein-induced cell-cell fusion.

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 (58). 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 pre-fusion form found in the virion envelope.

The structure of a truncated form of HSV-1 gB that comprises residues 31-730 (called gB730) is thought to be the post-fusion form (28, 34). Unlike s-gB, which becomes hydrophobic upon exposure to low pH (Fig. 5), gB730 is sufficiently hydrophobic at neutral pH to bind to liposomes (27). 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 post-fusion conformation, regardless of pH.
HSV gB undergoes a reversible change in structure in response to pH, similar to the other Class III fusion proteins VSV G (19, 44) and baculovirus gp64 (64). 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 (38). 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 (49). 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 (19). 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 (42). In contrast, virion gB appears to become a lower order oligomer upon pH-activation. This may be a difference in fusion mechanisms between these two Class III fusion proteins. This distinction might reflect the necessity of other HSV glycoproteins to complete the fusion process.

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**FIGURE LEGENDS**

**Figure 1. 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 nitrocellulose membrane. Blots were probed at neutral pH with the indicated gB-specific Abs followed by horseradish peroxidase conjugated goat secondary antibody. The exposure shown for MAb H126 highlights the pH threshold of conformational change.

**Figure 2. Effect of low pH treatment on the oligomeric state of gB.** (A) Virions were solubilized with 1% Triton X-100 and subjected to sedimentation through sucrose (8-60%) 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. (Lower panel) Results were quantitated by densitometry. (B) Binding of gB oligomer-specific monoclonal antibody DL16 to low pH-treated virions. As in Figure 1A, HSV-1 KOS virions were treated with pH 7.2 or 5.5, and then two-fold dilutions were blotted to membrane. (C) Virions were treated with the indicated pH, solubilized with 1% SDS where indicated, and then analyzed by PAGE and immunoblotted for detection of 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 by arrows. Nonspecific bands that were detected in mock-infected, Vero cell conditioned medium (not shown) are indicated by asterisks. Protein molecular weight standards in kilodaltons are indicated to the left.

Figure 3. 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. Two-fold dilutions were blotted immediately to nitrocellulose membrane. Membranes were probed at neutral pH with antibodies H126, DL16 or R69 followed by appropriate horseradish peroxidase conjugated secondary antibody. The exposures shown document the reversibility of reactivity.

Figure 4. 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 in Fig. 2C). 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 immunoblotting with R69 for detection of gB.

Figure 5. 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 30°C and were centrifuged at 300 x g for 3 min. The aqueous supernatant phase and detergent phase were collected and diluted twenty-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 TCA precipitated and analyzed by SDS-PAGE and Coomassie blue staining.

Figure 6. 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 hr. Cultures were shifted to 37°C for 1 hr 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. Nuclei were detected with DAPI (not shown). Samples were visualized by confocal microscopy at 63X magnification. 50 to 70 cells are shown per panel.
Table 1. Summary of monoclonal antibodies to gB used in this study

<table>
<thead>
<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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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HSV-1 KOS</td>
</tr>
<tr>
<td>DL16</td>
<td>nd</td>
<td>Y (Oligomer specific)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>H126</td>
<td>I</td>
<td>N</td>
<td>+</td>
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<tr>
<td>H1359</td>
<td>III</td>
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<tr>
<td>H1817</td>
<td>VI</td>
<td>N</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SS10</td>
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<td>Y&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
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<td>SS106</td>
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<td>N</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SS144</td>
<td>V</td>
<td>Y&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>-</td>
</tr>
<tr>
<td>R69</td>
<td>polyclonal</td>
<td>na</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data in the first three columns have been previously described (7, 8, 32, 41, 45).<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 (7). <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.