Structural Rearrangement within an Enveloped Virus upon Binding to the Host Cell^{∇†}

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Received 12 June 2008/Accepted 11 August 2008

We have made the surprising discovery that the interactions of herpes simplex virus with its initial cell attachment receptor induce a rapid and highly efficient structural change in the tegument, the region of the virion situated between the membrane and the capsid. It has been known for nearly a decade that viruses can trigger host signaling pathways when they bind to receptors on the cell surface; however, until now there has been no evidence that a signal can be sent in reverse—from the “outside in”—across a viral membrane. Evidence for this signaling event was found during studies of UL16, a tegument protein that is conserved among all the herpesviruses. Previous work has demonstrated that UL16 is bound to capsids isolated from the cytoplasm of infected cells, but this interaction is destabilized during subsequent egress steps, leading to release of the extracellular virion. Pretreatment with *N*-ethylmaleimide, a small, membrane-permeating compound that covalently modifies free cysteines, restabilizes the interaction, thereby permitting the capsid-UL16 complex to be isolated following disruption of virions with NP-40. In the experiments described here, we found that the natural signal for release of UL16 from capsids is sent when virions merely bind to cells at 4°C. The internal change was also observed upon binding to immobilized heparin in a manner that requires viral glycoprotein C. This represents the first example of signaling across a viral envelope following receptor binding.

It is well established that viruses can trigger cellular signaling pathways when they bind to their receptors on the plasma membrane (reviewed in reference 26). For example, binding can induce virion movement across the cell surface, trigger polymerization of cortical actin filaments needed for endocytic uptake, and modify host gene expression to make the cellular environment more conducive for replication. It is also quite clear that binding triggers changes in the virion proteins that directly interact with the receptor, namely, the capsids of non-enveloped viruses (44) and the glycoproteins found on the surfaces of enveloped viruses (10). In contrast, there has been no evidence to suggest that signals are transmitted in reverse—across the membrane to the interior of any enveloped virus—upon receptor binding. Here we report that the interaction of herpes simplex virus (HSV) with its initial cell attachment protein (heparan sulfate) induces a rapid and highly efficient structural change in the tegument, the large proteinaceous region situated between the viral membrane and the DNA-containing capsid.

Our evidence for receptor-induced changes in the tegument was found during studies of UL16, a tegument protein that is conserved among all herpesviruses (47). The function of this protein is poorly understood, but based on its stable association with cytoplasmic capsids (27, 35) and its ability to interact with a membrane-bound tegument protein named UL11 (24),

we proposed that UL16 might provide a “bridging” function during virion budding events at the *trans*-Golgi network (24). More recently we found that the UL16-capsid interaction changes during virion egress from the cell. Specifically, very little of the UL16 present in extracellular virions was found to be capsid associated, even though the detergent-lysis conditions were the same as those used to isolate capsids from the cytoplasm (27).

Although the previous study would seem to suggest that UL16 is free from capsids in extracellular virions, there are two observations that made us suspect otherwise (27). First, studies of other herpesviruses have revealed that their homologs of UL16 are associated with capsids in detergent-disrupted, extracellular virions (21, 47, 52). Second, we found that UL16 is almost entirely capsid associated when extracellular virions are pretreated with either *N*-ethylmaleimide (NEM) or low pH, conditions that block the reactivity of “free” cysteine residues (27). Thus, it seems more likely (but still not proven) that treatments as different as pH and NEM would preserve an existing capsid interaction rather than inducing UL16 into a particular conformation that just happens to reenact capsid binding.

In the experiments described below, we show that following the binding of HSV to host cells at 4°C, the interaction of UL16 with the capsid was no longer detected when NEM was added. This change in the interior of the virus was also observed upon binding to immobilized attachment receptors (i.e., heparin, a surrogate for heparan sulfate) and required the viral glycoprotein C (gC). Taken together, the data presented here represent the first evidence for the transmission of a signal across a virus envelope as a result of glycoprotein-receptor interactions.

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† Supplemental material for this article may be found at <http://jvi.asm.org/>.

[∇] Published ahead of print on 20 August 2008.

MATERIALS AND METHODS

Virus and cells. Vero and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum, 5% bovine calf serum, penicillin, and streptomycin. Virus stocks were prepared by infecting confluent monolayers of Vero cells with the KOS strain of HSV type 1 or the Becker strain of pseudorabies virus (PRV) at a multiplicity of infection of 0.01 for 1 h. Following infection, cells were incubated in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum, 25 mM HEPES buffer, glutamine (0.3 μ g/ml), penicillin, and streptomycin until a complete cytopathic effect was visualized (~3 days). Cells were then scraped into the medium, frozen (-80°C) and thawed (37°C) three times, and then cup sonicated (4°C) at moderate power for three 1-min pulses. Cell debris was removed by centrifugation, and aliquots were then frozen at -80°C until use.

Virus mutants. The gC-null mutant and revertant (gC-R) used in these studies were propagated in Vero cells as described above (kindly provided by Harvey Friedman [13]). The gB-null mutants (KO82 and F-BAC gB-) and complementing cells (VB38) used for their growth were generously supplied by David Johnson (12). To generate virions lacking gB, progeny of the gB-null viruses were obtained following infection of noncomplementing Vero cells. The Δ UL16 mutant used in the supporting studies (see the supplemental material) was a gift from Joel Baines and has been described previously (4).

NEM treatment of herpesviruses. Extracellular virions harvested at 18 to 24 h postinfection were treated with 10 mM NEM for 30 min at 37°C , either before or after removal of the envelope with NP-40 (final concentration, 0.5%; I3021; Sigma). Previous work revealed that this concentration and time of treatment efficiently modify free cysteines (5), and in our system they also maintain the UL16-capsid interaction in virions (27). Following NEM treatment, virions and capsids were pelleted at $83,500 \times g$ for 1 h and the samples were separated in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and electrotransferred to nitrocellulose membranes. The enhanced chemiluminescence method of immunoblot analysis was performed according to the manufacturer's instructions (Amersham). Rabbit anti-HSV UL16 and anti-VP5 sera were used at dilutions of 1:6,000 and 1:7,500, respectively. PRV UL16-specific antibodies were kindly provided by Thomas Mettenleiter and used at a dilution of 1:15,000.

Virus-cell binding assay. Confluent monolayers of cells on 100-mm plates were incubated with 1 ml of virus stock ($\sim 5 \times 10^8$ PFU) at 4°C with rocking for 45 min to allow virus binding. NEM was added to the virus either prior to or after incubation with the cells for 30 min at 4°C . Unbound virus and residual NEM were washed off the cells with 5 ml of phosphate-buffered saline (PBS). Viral membranes that remained bound to cells were solubilized with 4 ml of NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 8.0]) for 15 min at 37°C . The lysates were removed from the dish by pipetting, and insoluble material and cellular debris were removed by centrifugation for 10 min at $3,829 \times g$. Capsids in the supernatant were pelleted through a 700- μ l 30%-sucrose cushion (wt/vol in TNE [20 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1 mM EDTA]) in a SW55Ti rotor at $83,500 \times g$ for 1 h. Pellets were dissolved in SDS sample buffer (2% SDS, 62.5 mM Tris-HCl, 10% glycerol, 0.025% bromophenyl blue, 50 mM dithiothreitol [DTT], and 5% β -mercaptoethanol [BME]), boiled, separated by SDS-PAGE in 10% gels, and analyzed by immunoblotting using antibodies specific for VP5 and UL16. The amount of UL16 was determined by densitometry and normalized for VP5 levels.

Virus-heparin binding. Virus stocks ($\sim 1 \times 10^8$) were incubated with NEM for 30 min at 37°C either before or after a 30-min incubation with 40 μ l of heparin agarose slurry (20207; Pierce). Following incubations, the virus-coated beads were pelleted by centrifugation for 30 s in a microcentrifuge, washed in 1 ml of PBS, and resuspended in 1 ml of NP-40 lysis buffer for 15 min at 37°C . Beads and insoluble material were pelleted for 4 min at $18,000 \times g$. Capsids remaining in the supernatant were pelleted through a 150- μ l 30%-sucrose cushion for 1 h in a TLA 100.3 rotor at $83,500 \times g$ for 1 h. Pellets were dissolved in sample buffer, boiled, separated by SDS-PAGE in 10% gels, and analyzed by immunoblotting using antibodies specific for VP5 and UL16. In some experiments, soluble heparin or heparan sulfate (Sigma) was used at concentrations of 10 to 20 μ g/ml or 10 to 1,000 μ g/ml, respectively, prior to isolation of capsids as described above.

Analysis of capsids under nonreducing conditions. To analyze disulfide-bonded proteins, capsids harvested as described above were dissolved in sample buffer lacking reducing agents and split into two equal portions, and reducing agents (BME and DTT) were added to one. Both samples (reduced and nonreduced) were then boiled for 5 min at 95°C , separated by SDS-PAGE in 10% gels, and analyzed by immunoblotting.

FIG. 1. Sensitivity to NEM and NP-40 is conserved among alpha-herpesviruses. Extracellular HSV and PRV were harvested from infected Vero cells and treated with NEM or vehicle for 30 min at 37°C . Virions were then treated with NP-40 (+) or left untreated (-) to solubilize membranes. Intact virions or released capsids were pelleted through a 30%-sucrose cushion. Pellets and infected cells were dissolved in sample buffer and analyzed by immunoblotting with anti-VP5 and anti-UL16 serum.

RESULTS

The capsids of herpesviruses are stable in nonionic detergents, but proteins in the viral membrane and many of the proteins in the tegument are released by such treatments. We recently showed that most of the UL16 molecules present in extracellular HSV do not pellet with the capsid following NP-40 treatment; however, the opposite is found when free cysteines are covalently modified with the small, membrane-permeating compound NEM (resulting in a shift in gel mobility) prior to envelope disruption (Fig. 1, HSV) (27). In contrast, the UL16 homologs of beta- and gammaherpesviruses have been found to be mostly capsid associated even in the absence of NEM in similar types of experiments (21, 47, 52). To ascertain whether sensitivity to NP-40 and NEM might be a property that is specific to alphaherpesviruses, PRV was examined, and its UL16 homolog indeed behaved similarly to that of HSV (Fig. 1). On average, ~85% of the PRV molecules were released by NP-40 treatment, but ~80% pelleted with capsids when the virions were pretreated with NEM. Although the HSV and PRV homologs differ dramatically in their total numbers of cysteines (20 versus 11, respectively), 8 of these appear to be conserved based on alignments of UL16-related sequences from all herpesviruses (see the supplemental material), and thus, it is likely that one or more of these are targets for NEM.

FIG. 2. Binding of viruses to cells triggers release of UL16 from capsids. (A) Virus was mock (–) or NEM treated either prior to (P) or after (A) binding to confluent monolayers of cells for 45 min at 4°C. Following virus binding or NEM treatments, viral membranes were solubilized with NP-40 lysis buffer for 15 min. Capsids were then pelleted through a 30%-sucrose cushion, dissolved in sample buffer, and analyzed by immunoblotting. The bottom panel is an overexposed (OE) version of the middle one. (B) In a variation of the experiment, NEM was added to bound (A_b) and free (A_f) virions from the same culture dish prior to the addition of NP-40 and the isolation of capsids. As controls, NEM was not added (–) or was added to virions prior (P) to cell binding.

UL16 is released from capsids upon cell binding. Although NP-40-containing buffers are widely used in studies of protein interactions (e.g., in the discovery of UL16 as a binding partner of UL11 [24]), there are other examples of interactions being disrupted by NP-40 treatment. One that had a profound influence on our studies is the interaction between the SU and TM proteins of murine leukemia virus (MLV). In brief, these proteins are found on the exterior of MLV, where they mediate binding and fusion with the host cell during the initial steps of infection. Although they are linked by a single disulfide bond, this bond is broken when the virus is exposed to NP-40 but preserved if the virus is first treated with NEM (36). The free cysteine important for this activity is located in SU, just two amino acids away from the cysteine that is disulfide bonded to TM. NP-40 artificially triggers a disulfide-exchange reaction in which the SU-TM disulfide bond is broken and transferred to the CXXC motif within SU; however, the natural trigger for SU-TM separation is binding of MLV to its receptor (36, 45).

Upon closer inspection of the conserved cysteines of UL16, we noticed a CXXC motif (see the supplemental material). Although UL16 is not present on the surface of HSV (27), we were nevertheless compelled to test the possibility that cell binding might somehow send a signal inward to trigger the capsid release mechanism. Accordingly, cells were exposed to either untreated or NEM-treated virions at 4°C, a temperature that allows binding but prevents fusion from occurring. After virus attachment, NEM was added to the monolayer that had received untreated virions, and 30 min later, unbound virions were washed away. The remaining virions were then stripped of their membranes with NP-40, and their capsids were pelleted through a sucrose cushion. Thus, in both cases, the virions were exposed to NEM prior to NP-40 treatment; only the

timing of addition differed. Capsids and their associated proteins were separated by SDS-PAGE and detected by immunoblot analyses.

If cell binding is the natural trigger for the release of UL16 from capsids, then little or no UL16 would be expected to be present in the material pelleted from the cell lysate unless NEM had been added prior to binding, and this was found to be the case (Fig. 2A). As expected, NP-40 efficiently removed the viral envelope regardless of NEM treatment, since viral gC and the outer tegument protein UL11 did not pellet with capsids (data not shown). Data from five independent experiments revealed that only 5% ($\pm 3\%$) of the UL16 molecules were capsid bound after cell binding compared to results for the pretreated control. Overexposure of the film confirmed that NEM had been added to the culture following virion binding, because some of the UL16 remaining on the capsids was shifted to the expected size (Fig. 2A, bottom panel). Higher-molecular-weight bands were detected with the anti-UL16 serum (see the supplemental material), but these appear to be irrelevant because they were also observed in experiments using a virus that lacks the UL16-coding sequence and in experiments with PRV using HSV-specific UL16 antibodies (see the supplemental material).

Although secretory pathways should be inhibited at 4°C, we considered the possibility that a factor in the growth medium (rather than direct physical contact with a component on the cell surface) was responsible for the release of UL16. For this, cells were incubated with HSV, and capsids from virions that did not bind were examined (Fig. 2B). UL16 remained capsid associated in these virions even though it was released from those that had bound to the cells or did not receive NEM (Fig. 2B, compare A_f to A_b and minus-NEM samples). Again, UL16 was detected with capsids if NEM was added to virions prior to cell binding (Fig. 2B, P sample).

To determine whether cysteines of host proteins are required for the release mechanism, cells were treated with NEM and then washed prior to the addition of virus. This protocol did not prevent virus binding or release of UL16 from capsids (see the supplemental material). Moreover, release was observed in experiments with HeLa cells (see the supplemental material), where the virus enters by endocytosis rather than by direct fusion at the plasma membrane (32). Thus, the mechanism does not appear to be cell type specific or to require fusion.

Binding to heparin beads is sufficient for release. Although virus fusion does not occur at 4°C, the cell surface is a complex environment, and it seemed possible (though not likely) that a host factor might insert through the viral membrane to alter the tegument in a manner that does not directly involve cell receptors. To address this, we sought a cell-free system for binding virions to their receptors. Because the efficiency of UL16 release was enormous even at low temperatures, we hypothesized that the earliest virus attachment factors would be involved. These are provided by the heparan sulfate moieties of proteoglycans (42), which are abundantly scattered over the cell surface (37). It is well known that herpesviruses can efficiently interact with heparin-coated beads in a manner that mimics virus attachment (49), and therefore, we treated HSV with NEM either before or after binding to such beads. The amount of UL16 that was capsid associated in the col-

FIG. 3. Binding to immobilized heparin is sufficient to initiate UL16 release. HSV (A) or PRV (B) was incubated with or without (–) NEM treatment either prior to (P) or after (A) binding to heparin beads. The samples were pelleted, washed with PBS, and dissolved in NP-40 lysis buffer. Insoluble material was removed by centrifugation, and capsids were pelleted through a 30%-sucrose cushion, dissolved in sample buffer, and analyzed by immunoblotting. (C) HSV was incubated with increasing amounts of soluble heparan sulfate (HS) or heparin for 30 min and then treated with NEM. Virions were lysed with NP-40, and released capsids were pelleted through sucrose and analyzed by immunoblotting. (D) As a control, a portion (20%) of the heparin-treated virus from panel C was incubated with heparin agarose for 30 min. Virions bound to beads were pelleted, dissolved in sample buffer, and analyzed by immunoblotting with anti-VP5 serum.

lected virions was measured following solubilization with NP-40. The results clearly showed that engagement of the virus with immobilized heparin was sufficient to trigger the efficient release of UL16 from capsids (Fig. 3A). On average (three experiments), only 12% ($\pm 11\%$) of the UL16 molecules were capsid associated after binding compared to results for the pretreated control. Similar results were obtained using PRV (Fig. 3B), where equal amounts of capsids were analyzed (as measured by Ponceau S staining; data not shown).

Soluble heparin blocks the adsorption of HSV to host cells (20), and therefore, we considered the possibility that it might also trigger release of UL16 from capsids. Virions were incubated for 30 min with increasing amounts of soluble heparin, and then NEM was added. Soluble heparan sulfate was also tested. Neither compound was able to trigger the release of UL16 (Fig. 3C) despite being able to inhibit the binding of virions to the heparin-coated beads (Fig. 3D). This shows that the release mechanism requires heparin (or heparan sulfate) to be immobilized, possibly to enable receptor-glycoprotein clustering on the exterior of the viral envelope (see Discussion).

FIG. 4. gC is required for heparin bead- but not cell-binding-triggered release. (A) Wild-type (WT) or mutant (ΔgB , gC-R, or ΔgC) viruses were pelleted through 30%-sucrose cushions and analyzed by immunoblotting for the presence of gB and gC. VP5-specific antibodies were used to confirm the presence of virus particles. (B) Virus stocks tested in panel A were incubated with or without (–) NEM treatment either prior to (P) or after (A) binding to heparin beads. Capsids were pelleted through a 30%-sucrose cushion, dissolved in sample buffer, and analyzed by immunoblotting. (C) Virus was mock (–) or NEM treated either prior to (P) or after (A) binding to cells for 45 min at 4°C. Following virus binding or NEM treatments, viral membranes were solubilized with lysis buffer, and capsids were pelleted and analyzed as described above.

gC is required for heparin-triggered release. It has been known for some time that the viral glycoproteins that interact with heparan sulfate are gB and gC (42). gC is thought to be the predominant heparan sulfate binding protein; however, not all gC-null strains are defective in virus attachment to cells (23, 25). Additionally, in its absence, HSV can still associate with heparan sulfate through gB (23). To test the involvement of these glycoproteins in the release mechanism, mutant viruses that lack gC or gB (Fig. 4A) were analyzed in the heparin-coated bead binding assay (6, 12, 13). In the absence of gB, virus particles efficiently bound to immobilized heparin and UL16 was readily released from the capsids (Fig. 4B). Identical results were obtained with a different gB-null virus (data not shown) (12). In contrast, virus particles lacking gC that bound to beads still had capsid-associated UL16 regardless of the timing of NEM addition (Fig. 4B). The wild-type revertant of this virus (gC-R) regained the ability to trigger release of UL16, although not as efficiently as the wild-type HSV KOS strain (78% versus 88%, respectively). This difference may be due to strain variation, since the gC-null and gC-R constructs were derived from the NS strain (13). Intriguingly, when the gC-null virus was incubated with cells at 4°C, the release mechanism was efficiently triggered (Fig. 4C), suggesting that other, downstream glycoprotein-receptor interactions are capable of sending the signal (see Discussion).

UL16 is not disulfide bonded to capsids. To further investigate the signaling mechanism, the disulfide-bonded state of UL16 was examined before and after binding of virions to heparin beads (Fig. 5). In these experiments, intact virions were analyzed to visualize the entire population of UL16 inside

FIG. 5. UL16 is not disulfide bound to the capsid or other proteins. HSV or PRV was incubated with heparin beads for 30 min along with NEM treatment either prior to binding (P), after binding (A), in the sample buffer (SB), or not at all (-). Virus-bound beads were pelleted, washed with PBS, and dissolved in sample buffer lacking reducing agents. Samples were split, with half receiving BME and DTT (reducing) and the other half receiving neither (non-reducing) prior to immunoblot analysis.

the envelope (both capsid bound and released). The results obtained from reducing (control) gels were as expected, with all of the UL16 molecules running as slower migrating, modified monomers regardless of the timing of the NEM treatments, confirming that NEM had equal access to UL16 within virions under all conditions. The results from nonreducing gels showed that UL16 is also monomeric after virions bind to beads, whether NEM was added for 30 min before or after this step. However, when NEM was omitted, most of the UL16 molecules were not visible at any position in the gel. This is likely due to the spontaneous formation of irrelevant disulfide bonds during virion disruption. Similar results were found for PRV, except that its UL16 protein did not form intermolecular disulfide bonds in the absence of NEM, presumably because it contains fewer cysteines. However, these conditions did produce a striking increase in mobility for UL16 relative to the reduced form (compare the leftmost lanes in the bottom two panels: minus-NEM samples), which is indicative of an efficient creation of irrelevant intramolecular disulfide bonds during virion disruption in the absence of NEM (48). A small amount of such a fast-migrating species was also seen for HSV when gels were overexposed or loaded with larger amounts of virions (data not shown). Collectively, these data suggest that receptor binding does not trigger UL16 to form static, intermolecular disulfide bonds following release from the capsid. However, because we can detect only the beginning and final states of UL16 in these assays, it is possible that disulfide-bonded intermediates form in the release pathway.

DISCUSSION

The experiments described here have produced the first evidence for “outside-in” signaling across the membrane of an enveloped virus. Specifically, we discovered that binding of

FIG. 6. Probable players in the UL16-capsid release mechanism. (A) UL16 is capsid associated in the extracellular virion (red circles) and contains numerous free cysteines (emanating lines). The observed rearrangement requires clustering of one or more viral glycoproteins (blue), which must transmit a signal either directly to UL16 or to one of their binding partners (yellow). (B) gC and gB bind heparan sulfate (HS) and also interact with other glycoproteins (dotted lines). UL11 (yellow), a known binding partner of UL16, may facilitate release of UL16 through its interaction with the cytoplasmic tails of gE and gD.

HSV to its initial cell receptor triggers a rapid and efficient change within the tegument, leading to destabilization of the UL16-capsid interaction (Fig. 6A). While this finding was quite unexpected, it is reassuring that “signaling from within” has been reported from studies of retroviruses. For example, the fusion activity needed for MLV entry is absent until the viral protease cleaves the C terminus of the TM protein on the inside of the virus (1, 38). And in the case of human immunodeficiency virus, fusion activity is absent until the Gag protein on the inside is cleaved to create the mature virus (31, 50). Thus, it seems reasonable to expect that alterations on the outside of an enveloped virus might be of great importance to functions located inside.

How is the signal transmitted into HSV to release UL16 from capsids? There are many interesting clues (Fig. 6B). Starting on the outside surface of the virus, it is well known that the viral glycoproteins gB and gC interact with heparan sulfate (42) while gD can bind to 3-*O*-sulfated heparan sulfate (41). Our data reveal that gC is needed for the release of UL16 in the heparin-bead assay but not in the cell-binding assay, where other virus-receptor interactions are available. In this regard, it is interesting that gC has been reported to be in close proximity to gD (18), the viral protein that recognizes the cell receptor needed for entry (of which there are three alternatives: HVEM, nectin 1, or 3-*O*-sulfated heparan sulfate) and works in cooperation with gB, gH, and gL to mediate fusion (42). Moreover, binding to entry receptors leads to conforma-

tional alterations in gD (15, 22, 43) and changes in its association with gB, gH, and gL (3, 7). This complexity has only increased with the recent discovery of an HSV coreceptor, PILR α , which binds to gB and appears to be vital for entry into certain cell types in conjunction with gD-receptor interactions (40). In light of the multitude of dynamic interactions that occur on the virus surface, it is easy to imagine that these might alter the inside of the virion, and thus, it is possible that one or more of these four glycoproteins is capable of triggering the release of UL16, even in the absence of heparan sulfate (Fig. 6B). This is clearly supported by the efficient release of UL16 in the gC-null virus following cell binding.

Regardless of the mechanism used for transducing the signal through the membrane, there must be a means for getting that information to capsid-associated UL16. Many tegument proteins have been found to interact with the tails of viral glycoproteins and also with one another (8, 11, 14, 17, 34, 53). Of particular interest in this regard is the UL11 protein (Fig. 6B), a direct binding partner of UL16 (24, 51) that is also capable of interacting with the tails of gD and gE (11). Moreover, the UL11-UL16 interaction is blocked by NEM (51). Thus, binding of HSV to cell receptors might induce conformational changes in viral glycoproteins, which in turn affect UL11-UL16-capsid interactions. Due to the short cytoplasmic tail of gC, it is unlikely that the signal is sent directly through this glycoprotein. A more attractive model is that clustering of gC alters interactions between other glycoproteins on the surface, which then transmit the signal to UL16 through UL11. In support of this hypothesis, there is evidence for potential interactions of gC with gB, gD, and gL on the virion surface (18). On the other hand, the structure of the tegument is very poorly understood, and therefore, it is difficult to have confidence in making these predictions. For example, it is unknown whether UL16 is actually bound to UL11 within the virion, either before or after receptor binding.

What features of UL16 are important for the capsid release mechanism? At the moment, all that can be said is that conserved, free cysteines must be involved. This is based on the ability of NEM to block release and shift the mobility of UL16 from both HSV and PRV. The presence of CXXC among the conserved residues is intriguing because this motif is found in the active sites of many enzymes, such as those that mediate disulfide-exchange reactions (2) and those that modify proteins with palmitate (28). However, the thiol group of cysteine is the most reactive of all side chains (9), and single cysteines in combinations with other amino acids constitute many active sites, such as those of the cysteine proteases (29). Moreover, free cysteines are found in metal-binding domains (e.g., zinc fingers), which play numerous structural roles (16). Given the large number of free cysteines that appear to be present in UL16, it is possible that they serve multiple roles, and at this point it is difficult to rule any mechanism out. For example, although we found no evidence to suggest that UL16 is disulfide bound to any other protein before or after receptor binding, it is possible that transient disulfide bonds are formed as the release mechanism proceeds. Future investigations of the role of the conserved cysteine residues are warranted; however, in doing so, it is important to keep in mind that free cysteines in other tegument proteins (rather than UL16) may have critical roles in the release mechanism.

What might be the purpose of receptor-induced rearrangements of the tegument? There are two possibilities that spring to mind. In the simplest of these, inward signaling triggers the earliest steps of uncoating in preparation for postfusion events (e.g., targeting the capsid to the nucleus of the infected cell). The other possibility is that rearrangements in the tegument serve to regulate the fusion activity of viral glycoproteins in a manner analogous to that found in retroviruses (see above). In this model, fusion activity would be inhibited when tegument proteins are bound to the tails of viral glycoproteins, but interactions with proteoglycans or other receptors would trigger rearrangements that allow the subsequent events of fusion to proceed. In support of this model, mutations in the cytoplasmic tails of gB and gH have been reported to enhance or abolish fusion activity in cell-cell and virus-cell fusion assays (19, 33, 39, 46). Therefore, it seems likely that the interaction of tegument proteins with the tails of glycoproteins could also influence fusion activity. The two proposed models are not mutually exclusive, and given the complexity of the tegument, other roles for the rearrangements may be envisioned as more is learned about its organization. Of particular interest in this regard is the ability of NEM to completely eliminate virion infectivity (27) even though it does not prevent virion binding to the host cells (this study).

In summary, our recent studies of UL16 have shown that the structure of the tegument changes in ways that no one anticipated. During virus assembly and egress, the interaction of UL16 with the capsid is initially quite stable in NP-40, but by the time the virion is released from the cell, that interaction has been altered such that free cysteines have to be modified to prevent NP-40- or receptor-induced release (27). And while it has long been hypothesized that fusion of the virus with the host cell leads to phosphorylation of tegument proteins to destabilize their interactions during uncoating (30), our data show that the structure of the tegument is altered when the virus merely binds to receptors. Clearly, there is a tremendous amount of work to be done in elucidating the dynamic interactions of tegument proteins.

ACKNOWLEDGMENTS

We extend thanks to our PSU colleagues Jacob Marsh, Michael Murphy, Nicolas Baird, and Kevin O'Regan for helpful discussions. Special thanks go to Todd Wisner (David Johnson's laboratory) and Elizabeth Wills (Joel Baines' laboratory) for providing technical help on growing mutant viruses used in these studies. We also thank Gary Cohen and Rebecca Craven for careful review of the manuscript and Pat Spear for useful suggestions.

This work was supported by an NIH grant to J.W.W. (AI071286). D.G.M. was supported by a training grant from the NIH (CA60395).

REFERENCES

1. Aguilar, H. C., W. F. Anderson, and P. M. Cannon. 2003. Cytoplasmic tail of Moloney murine leukemia virus envelope protein influences the conformation of the extracellular domain: implications for mechanism of action of the R peptide. *J. Virol.* 77:1281–1291.
2. Appenzeller-Herzog, C., and L. Ellgaard. 2008. In vivo reduction-oxidation state of protein disulfide isomerase: the two active sites independently occur in the reduced and oxidized forms. *Antioxid. Redox Signal.* 10:55–64.
3. Atanasiu, D., J. C. Whitbeck, T. M. Cairns, B. Reilly, G. H. Cohen, and R. J. Eisenberg. 2007. Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. *Proc. Natl. Acad. Sci. USA* 104:18718–18723.
4. Baines, J. D., and B. Roizman. 1991. The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *J. Virol.* 65:938–944.

5. Bolen, J. B., and M. A. Israel. 1983. Inhibition of polyoma virus middle T antigen-associated tyrosyl kinase activity by N-ethylmaleimide. *J. Biol. Chem.* **258**:15135–15140.
6. Cai, W. Z., S. Person, C. DebRoy, and B. H. Gu. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. *J. Mol. Biol.* **201**:575–588.
7. Campadelli-Fiume, G., M. Amasio, E. Avitabile, A. Cerretani, C. Forghieri, T. Gianni, and L. Menotti. 2007. The multipartite system that mediates entry of herpes simplex virus into the cell. *Rev. Med. Virol.* **17**:313–326.
8. Chi, J. H., C. A. Harley, A. Mukhopadhyay, and D. W. Wilson. 2005. The cytoplasmic tail of herpes simplex virus envelope glycoprotein D binds to the tegument protein VP22 and to capsids. *J. Gen. Virol.* **86**:253–261.
9. Creighton, T. E. 1993. Proteins: structures and molecular properties. W. H. Freeman & Co., New York, NY.
10. Dimitrov, D. S. 2004. Virus entry: molecular mechanisms and biomedical applications. *Nat. Rev. Microbiol.* **2**:109–122.
11. Farnsworth, A., T. W. Wisner, and D. C. Johnson. 2007. Cytoplasmic residues of herpes simplex virus glycoprotein gE required for secondary envelopment and binding of tegument proteins VP22 and UL11 to gE and gD. *J. Virol.* **81**:319–331.
12. Farnsworth, A., T. W. Wisner, M. Webb, R. Roller, G. Cohen, R. Eisenberg, and D. C. Johnson. 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane. *Proc. Natl. Acad. Sci. USA* **104**:10187–10192.
13. Friedman, H. M., L. Wang, N. O. Fishman, J. D. Lambris, R. J. Eisenberg, G. H. Cohen, and J. Lubinski. 1996. Immune evasion properties of herpes simplex virus type 1 glycoprotein gC. *J. Virol.* **70**:4253–4260.
14. Fuchs, W., B. G. Klupp, H. Granzow, C. Hengartner, A. Brack, A. Mundt, L. W. Enquist, and T. C. Mettenleiter. 2002. Physical interaction between envelope glycoproteins E and M of pseudorabies virus and the major tegument protein UL49. *J. Virol.* **76**:8208–8217.
15. Fusco, D., C. Forghieri, and G. Campadelli-Fiume. 2005. The pro-fusion domain of herpes simplex virus glycoprotein D (gD) interacts with the gD N terminus and is displaced by soluble forms of viral receptors. *Proc. Natl. Acad. Sci. USA* **102**:9323–9328.
16. Gamsjaeger, R., C. K. Liew, F. E. Loughlin, M. Crossley, and J. P. Mackay. 2007. Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends Biochem. Sci.* **32**:63–70.
17. Gross, S. T., C. A. Harley, and D. W. Wilson. 2003. The cytoplasmic tail of herpes simplex virus glycoprotein H binds to the tegument protein VP16 in vitro and in vivo. *Virology* **317**:1–12.
18. Handler, C. G., R. J. Eisenberg, and G. H. Cohen. 1996. Oligomeric structure of glycoproteins in herpes simplex virus type 1. *J. Virol.* **70**:6067–6070.
19. Harman, A., H. Browne, and T. Minson. 2002. The transmembrane domain and cytoplasmic tail of herpes simplex virus type 1 glycoprotein H play a role in membrane fusion. *J. Virol.* **76**:10708–10716.
20. Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* **65**:1090–1098.
21. Johannsen, E., M. Luftig, M. R. Chase, S. Weicksel, E. Cahir-McFarland, D. Illanes, D. Sarracino, and E. Kieff. 2004. Proteins of purified Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* **101**:16286–16291.
22. Krummenacher, C., V. M. Supek, J. C. Whitbeck, E. Lazear, S. A. Connolly, R. J. Eisenberg, G. H. Cohen, D. C. Wiley, and A. Carfi. 2005. Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. *EMBO J.* **24**:4144–4153.
23. Laquerre, S., R. Argnani, D. B. Anderson, S. Zucchini, R. Manservigi, and J. C. Glorioso. 1998. Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. *J. Virol.* **72**:6119–6130.
24. Loomis, J. S., R. J. Courtney, and J. W. Wills. 2003. Binding partners for the UL11 tegument protein of herpes simplex virus type 1. *J. Virol.* **77**:11417–11424.
25. Lubinski, J. M., L. Wang, A. M. Soulika, R. Burger, R. A. Wetsel, H. Colten, G. H. Cohen, R. J. Eisenberg, J. D. Lambris, and H. M. Friedman. 1998. Herpes simplex virus type 1 glycoprotein gC mediates immune evasion in vivo. *J. Virol.* **72**:8257–8263.
26. Marsh, M., and A. Helenius. 2006. Virus entry: open sesame. *Cell* **124**:729–740.
27. Meckes, D. G., Jr., and J. W. Wills. 2007. Dynamic interactions of the UL16 tegument protein with the capsid of herpes simplex virus. *J. Virol.* **81**:13028–13036.
28. Mitchell, D. A., A. Vasudevan, M. E. Linder, and R. J. Deschenes. 2006. Protein palmitoylation by a family of DHHC protein S-acyltransferases. *J. Lipid Res.* **47**:1118–1127.
29. Mohamed, M. M., and B. F. Sloane. 2006. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat. Rev. Cancer* **6**:764–775.
30. Morrison, E. E., Y. F. Wang, and D. M. Meredith. 1998. Phosphorylation of structural components promotes dissociation of the herpes simplex virus type 1 tegument. *J. Virol.* **72**:7108–7114.
31. Murakami, T., S. Ablan, E. O. Freed, and Y. Tanaka. 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. *J. Virol.* **78**:1026–1031.
32. Nicola, A. V., A. M. McEvoy, and S. E. Straus. 2003. Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. *J. Virol.* **77**:5324–5332.
33. Nixdorf, R., B. G. Klupp, A. Karger, and T. C. Mettenleiter. 2000. Effects of truncation of the carboxy terminus of pseudorabies virus glycoprotein B on infectivity. *J. Virol.* **74**:7137–7145.
34. O'Regan, K. J., M. A. Bucks, M. A. Murphy, J. W. Wills, and R. J. Courtney. 2007. A conserved region of the herpes simplex virus type 1 tegument protein VP22 facilitates interaction with the cytoplasmic tail of glycoprotein E (gE). *Virology* **358**:192–200.
35. Oshima, S., T. Daikoku, S. Shibata, H. Yamada, F. Goshima, and Y. Nishiyama. 1998. Characterization of the UL16 gene product of herpes simplex virus type 2. *Arch. Virol.* **143**:863–880.
36. Pinter, A., R. Kopelman, Z. Li, S. C. Kayman, and D. A. Sanders. 1997. Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes. *J. Virol.* **71**:8073–8077.
37. Rabenstein, D. L. 2002. Heparin and heparan sulfate: structure and function. *Nat. Prod. Rep.* **19**:312–331.
38. Rein, A., J. Mirro, J. G. Haynes, S. M. Ernst, and K. Nagashima. 1994. Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. *J. Virol.* **68**:1773–1781.
39. Ruel, N., A. Zago, and P. G. Spear. 2006. Alanine substitution of conserved residues in the cytoplasmic tail of herpes simplex virus gB can enhance or abolish cell fusion activity and viral entry. *Virology* **346**:229–237.
40. Satoh, T., J. Arii, T. Suenaga, J. Wang, A. Kogure, J. Uehori, N. Arase, I. Shiratori, S. Tanaka, Y. Kawaguchi, P. G. Spear, L. L. Lanier, and H. Arase. 2008. PILR α is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. *Cell* **132**:935–944.
41. Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**:13–22.
42. Spear, P. G. 2004. Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol.* **6**:401–410.
43. Spear, P. G., S. Manoj, M. Yoon, C. R. Jogger, A. Zago, and D. Myscowski. 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.
44. Tsai, B. 2007. Penetration of nonenveloped viruses into the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **23**:23–43.
45. Wallin, M., M. Ekstrom, and H. Garoff. 2004. Isomerization of the intersubunit disulphide-bond in Env controls retrovirus fusion. *EMBO J.* **23**:54–65.
46. Wanas, E., S. Efler, K. Ghosh, and H. P. Ghosh. 1999. Mutations in the conserved carboxy-terminal hydrophobic region of glycoprotein gB affect infectivity of herpes simplex virus. *J. Gen. Virol.* **80**:3189–3198.
47. Wing, B. A., G. C. Lee, and E. S. Huang. 1996. The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J. Virol.* **70**:3339–3345.
48. Wootton, S. K., and D. Yoo. 2003. Homo-oligomerization of the porcine reproductive and respiratory syndrome virus nucleocapsid protein and the role of disulfide linkages. *J. Virol.* **77**:4546–4557.
49. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**:52–58.
50. Wyma, D. J., J. Jiang, J. Shi, J. Zhou, J. E. Lineberger, M. D. Miller, and C. Aiken. 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. *J. Virol.* **78**:3429–3435.
51. Yeh, P. C., D. G. Meckes, Jr., and J. W. Wills. 2008. Analysis of the interaction between the UL11 and UL16 tegument proteins of herpes simplex virus. *J. Virol.* **82**:10693–10700.
52. Zhu, F. X., J. M. Chong, L. Wu, and Y. Yuan. 2005. Virion proteins of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **79**:800–811.
53. Zhu, Q., and R. J. Courtney. 1994. Chemical cross-linking of virion envelope and tegument proteins of herpes simplex virus type 1. *Virology* **204**:590–599.