Herpesvirus gB-Induced Fusion between the Virion Envelope and Outer Nuclear Membrane during Virus Egress Is Regulated by the Viral US3 Kinase[∇]

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Herpesvirus capsids collect along the inner surface of the nuclear envelope and bud into the perinuclear space. Enveloped virions then fuse with the outer nuclear membrane (NM). We previously showed that herpes simplex virus (HSV) glycoproteins gB and gH act in a redundant fashion to promote fusion between the virion envelope and the outer NM. HSV mutants lacking both gB and gH accumulate enveloped virions in herniations, vesicles that bulge into the nucleoplasm. Earlier studies had shown that HSV mutants lacking the viral serine/threonine kinase US3 also accumulate herniations. Here, we demonstrate that HSV gB is phosphorylated in a US3-dependent manner in HSV-infected cells, especially in a crude nuclear fraction. Moreover, US3 directly phosphorylated the gB cytoplasmic (CT) domain in *in vitro* assays. Deletion of gB in the context of a US3-null virus did not add substantially to defects in nuclear egress. The majority of the US3-dependent phosphorylation of gB involved the CT domain and amino acid T887, a residue present in a motif similar to that recognized by US3 in other proteins. HSV recombinants lacking gH and expressing either gB substitution mutation T887A or a gB truncated at residue 886 displayed substantial defects in nuclear egress. We concluded that phosphorylation of the gB CT domain is important for gB-mediated fusion with the outer NM. This suggested a model in which the US3 kinase is incorporated into the tegument layer (between the capsid and envelope) in HSV virions present in the perinuclear space. By this packaging, US3 might be brought close to the gB CT tail, leading to phosphorylation and triggering fusion between the virion envelope and the outer NM.

Most enveloped viruses assemble their capsids in the cytoplasm and then become enveloped by budding from the plasma membrane or into cytoplasmic (CT) membranes. Viruses that assemble capsids in the nucleus face the fundamental problem of transporting capsids across the nuclear envelope (NE). Non-enveloped viruses, such as polyomaviruses, that are relatively small can move through nuclear pores, and larger nonenveloped viruses, such as adenoviruses (Ad), disrupt the NE (12, 55). Herpesvirus capsids are too large to move through nuclear pores (18), and, instead, herpesviruses encode machinery which promotes envelopment at the inner nuclear membrane (NM) followed by fusion with the outer NM (29, 52). There appears to be very rapid fusion at the NE because few enveloped herpesvirus particles are normally observed within the perinuclear space. Once present in the cytoplasm, nonenveloped capsids acquire a secondary envelope, producing virions that bud into the Golgi apparatus or trans-Golgi network (13, 29, 52). These mature virions are then secreted from cells.

Some aspects of the envelopment of herpesviruses at the inner NM are known. Here, the viruses encounter a major obstacle, the nuclear lamina, a rigid network of lamin proteins

lining the inner NM. Herpesviruses disrupt the nuclear lamina in order to assemble along the inner surface of the NM (24, 31–33, 49). The alphaherpesviruses herpes simplex virus (HSV) and pseudorabies virus (PRV) disrupt the lamina by expressing two viral proteins, UL31 and UL34, that form a complex colocalizing to the inner NM (4, 44, 45, 50, 51). It appears that UL31 and UL34 promote virus assembly and egress by several related mechanisms, including (i) affecting maturation of viral replication intermediates so that capsids assemble adjacent to the NE (50, 51), (ii) causing displacement of and conformational changes in lamins A/C and B (4, 44, 50), and (iii) mislocalizing or otherwise altering integral membrane lamin receptors, such as the lamin B receptor and emerin, which tether lamins to the inner NE (24, 30, 32, 49).

During mitosis the nuclear lamina is disrupted through the action of cellular kinases, e.g., protein kinase C and cdc2, which phosphorylate lamins and lamin receptors (9, 11, 39). Alpha- and betaherpesvirus UL31 and UL34 homologues also attract protein kinase C, and possibly other cellular kinases, to the NE in a process that contributes to the disruption of the lamina (24, 33, 36). Alphaherpesviruses also express a viral serine/threonine protein kinase, US3, that phosphorylates lamins, lamin receptors, and UL31 and UL34 (21, 24, 31, 41–43, 45). HSV US3 recognizes a consensus motif similar to that recognized by cyclic AMP-dependent protein kinase A (PKA) (3, 25, 40, 42). HSV UL31 and UL34 both contain PKA/US3 consensus motifs and are phosphorylated in a US3-

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dependent fashion both in infected cells and in vitro (21, 31, 42, 48). All these results predict that the US3 kinase functions primarily in the first step of nuclear egress, assembly along the inner NM and envelopment. Nevertheless, HSV and PRV US3⁻ mutants accumulate large numbers of enveloped virions in and around the NE (45, 48, 57), consistent with defects in the second step of nuclear egress, deenvelopment or fusion with the outer NM.

Much less is known about the deenvelopment step of herpesvirus nuclear egress. Recently, we showed that HSV mutants lacking two membrane glycoproteins, gB and gH, were defective in this fusion (14). Large numbers of enveloped virions accumulated in the perinuclear space as well as in structures that we termed "herniations," membrane vesicles containing virions that bleb from the inner NM into the nucleoplasm. Importantly, either gB or gH/gL can suffice for deenvelopment (14), so it appears that HSV gB and gH/gL have redundant or overlapping roles in fusion with the outer NM. HSV gB and gH, which form a heterodimer with a second glycoprotein, gL, are membrane fusion proteins that promote virus entry into cells (6, 15, 46). There is evidence that gB and gH/gL are both fusion proteins capable of functioning to mix virion envelope and cellular lipids during membrane fusion (54). All other herpesviruses express gB and gH/gL homologues, suggesting that this fundamentally important step in reaching the cell surface may be shared across this family of viruses. Consistent with this notion, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, and PRV gB-null mutants all exhibit defects in nuclear egress (23, 26, 37). However, a recent study involving a PRV gB⁻ gH⁻ double mutant did not detect defects in nuclear egress (22). There may also be other herpesvirus membrane proteins involved in this egress fusion, because some HSV particles still reach the surfaces of cells infected with gB⁻ gH⁻ mutants, though there is also extensive accumulation in herniations (14).

The HSV gB and gH/gL fusion proteins appear to be triggered for virus entry into cells through the action of glycoprotein gD, which binds cellular receptors, e.g., nectin-1, and bridges gB and gH/gL into a larger complex that causes membrane fusion (1, 2). However, the triggering of gB and gH/gL for fusion with the outer NM is not understood. It would seem highly likely that these HSV glycoproteins, which are found in most cytoplasmic membranes as well as NMs, must be carefully regulated in terms of their fusion activity in order to preserve cellular architecture. The common phenotypes of HSV US3⁻ and gB⁻ gH⁻ mutants suggested that the US3 kinase might be involved in regulating deenvelopment fusion. Indeed, US3 is packaged into perinuclear virions (45) and, as such, might come into close proximity with gB and gH. To test this hypothesis, we characterized phosphorylation of gB and gH. The CT domain of HSV gB was found to be phosphorylated in a US3-dependent manner in HSV-infected cells, especially in a crude nuclear fraction. Moreover, gB was directly phosphorylated by US3 in an in vitro assay. gH was not substantially phosphorylated. An HSV mutant with a substitution reducing gB phosphorylation and lacking gH accumulated herniations filled with virions. We concluded that gB is phosphorylated by US3 and that this modification is important for gB-mediated fusion of the virion envelope and the outer NM.

MATERIALS AND METHODS

Cells and viruses. HaCaT cells are human keratinocytes (5) that mimic the cells HSV infects in vivo, and these cells produce abundant quantities of HSV particles (60). HaCaT and Vero cells were maintained in Dulbecco's modified Eagle medium (Gibco/Invitrogen) containing 8% fetal bovine serum (FBS) (Mediatech). HSV-1 strain F, F-derived US3⁻ mutant vRR1202, vRR1202rep (US3-R; a repaired version of vRR1202), and the US3 kinase-inactive mutant vRR1205 (48) were all propagated and titered using Vero cells. HSV recombinant YK551, encoding a mutant form of gB (gBT887A), and a repaired version of this virus, YK553, were recently described (20). HSV gB⁻ mutants were all propagated in VB38 cells that express gB, and HSV gB⁻ gH⁻ mutants were propagated in Vero-derived F6/gB12 cells, which express both gB and gH (14).

Construction of nonreplicating Ad vectors. To construct plasmids containing two stop codons resulting in truncation of gB at residue 800 or 825, plasmid pep98 (38) containing HSV-1 strain KOS gB gene sequences (a gift from Pat Spear, Northwestern Medical School) was used as a template in a site-directed mutagenesis protocol (Stratagene; QuikChange kit). For gB800stop the PCR oligonucleotide pair was CGTTACGTCATGCGGTGATAACTGCAGAGCAA CCCC and AAAGGCCAAGAAGGCCGCC, and for gB825stop the oligonucleotides were CCAACCCGGACGCGTGATAATCCGGGGAGGGCG and TGGGGTTCTTGAGCTCCT. Nonreplicating (E1⁻) Ad vectors expressing the mutant gB proteins were constructed with the AdMax-IQ system (<http://www.microbix.com>) as described previously (28). Briefly, gB800stop and gB825stop coding sequences were excised from plasmid pep98 with BglII and EcoRI and inserted into plasmid pDC316(io). The resulting plasmids were cotransfected with plasmid pBHGloxDE1,3Cre into 293 IQ cells (Microbix). 293 IQ cells express the lacZ repressor that reduces expression of the transgene (gB gene) during Ad vector replication in 293 cells. Ad vectors produced after 6 to 8 days were used to produce virus stocks using 293 IQ cells and titered on these cells.

Construction of recombinant HSV. Plasmids containing gB coding sequences with stop codons replacing codons for residues 800 and 801 (encoding gB800stop) and 825 and 826 (encoding gB825stop) were described above. Another plasmid in which stop codons replaced codons for 887 and 888 (encoding gB887stop) was similarly constructed by oligonucleotide-directed mutagenesis as described previously (60). A plasmid containing coding sequences for gB with substitution mutation T887A was described previously (20). To construct HSV recombinants expressing truncated gB molecules or gB with the T887A substitution, a two step mutagenesis protocol was used. First, wild-type gB gene sequences present in F-BAC or F-BACΔgH (which lacks the gH gene) (14) were replaced with *galK* sequences by lambda Red recombination (58). *galK* was directed to the gB gene by flanking *galK* sequences with sequences 50 bp downstream of the gB gene start codon and 50 bp upstream of the stop codon, producing BAC-gB/*galK*/gH-. In the second step, *galK* sequences in BAC-gB/*galK*/gH- were replaced by recombination between 50-nucleotide gB gene sequences downstream of the start codon and upstream of the stop codon and by substitutions of two stop codons in place of codons for residues 800 and 801 (gB800stop), 825 and 826 (gB825stop), or 887 and 888 (gB887stop) or by substitution of the sequence encoding gB with the T887A substitution. In this case, bacterial artificial chromosomes (BAC) containing mutant gB gene sequences were selected by selecting against *galK* expression. Mutations in the BAC were confirmed by PCR amplification of the gB gene and sequencing. BACΔgH plasmids containing mutant forms of the gB gene (the gB gene replaced with *galK* and gB800stop, gB825stop, gB887stop, and gBT887A genes) were transfected into complementing (expressing gB and gH) F6/gB12 cells (14) to produce recombinant HSV. To construct an HSV lacking both the gB and US3 genes, VB38 cells were coinfecting with the gB⁻ mutant F-BACgB- (14) and vRR1202, in which US3 sequences were replaced with red fluorescent protein sequences (48). Viruses were harvested and screened for red fluorescent protein fluorescence (indicating loss of the US3 gene) and an inability to express gB (plaques produced in VB38 cells but not in Vero cells). A recombinant HSV denoted vRR1202/gB- was plaque purified on VB38 cells and characterized for loss of gB and US3 (not shown).

Antibodies and immunoprecipitation of proteins. Immunoprecipitation of gB involved a mixture of monoclonal antibodies (MAb) 15βB2 (17) and I-144 (35). gH was immunoprecipitated with a mixture of MAb LP11, 52S, and 53S, which were described previously (16). Anti-gE MAb 3114 was also described previously (19). Immunoprecipitation of HSV glycoproteins from detergent extracts of virus-infected cells was performed as described previously (60).

Orthophosphate labeling of HSV-infected and Ad vector-transduced cells. HaCaT cells were infected with HSV at 15 PFU/cell in medium containing 1% FBS for 2 h, and then the virus was removed and medium containing 1% FBS was added for four additional hours. The cells were washed twice in medium

lacking PO_4 and containing 0.5% dialyzed FBS and 10 mM HEPES, incubated in this medium for 45 min, and then incubated for 6 h in this medium containing 500 to 700 $\mu\text{Ci}/\text{ml}$ of $^{32}\text{PO}_4$. Other dishes of cells infected for 6 h were washed with minimal essential medium lacking cysteine and methionine and containing 10 mM HEPES buffer, pH 7.35, and 0.5% dialyzed FBS; cells were incubated in this medium for 45 min and then incubated in this medium containing 100 to 150 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine-cysteine for 6 h. In some experiments, cells were washed and in some cases subjected to fractionation. The cells were incubated in hypotonic buffer (10 mM NaCl, 10 mM Tris, pH 7.4, 1.5 mM MgCl_2 , 2 mM sodium vanadate, 5 mM sodium fluoride, 10 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride), scraped into the buffer, and incubated for a total of 10 min on ice. The cells were then subjected to Dounce homogenization until the majority of cells were disrupted but nuclei remained intact (10 strokes with a loose A pestle and 20 to 30 strokes with a tight B pestle). Samples were centrifuged at $750 \times g$ for 5 min, and the pellets were washed once in hypotonic buffer. Supernatants from the low-speed centrifugation were centrifuged at $85,000$ to $110,000 \times g$ in a Beckman Ti70.1 rotor for 30 min. The low-speed and high-speed pellets were then resuspended in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% deoxycholate, 2 mM sodium vanadate, 5 mM sodium fluoride, 10 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). HaCaT cells were prepared for transduction with Ad vectors by first treating the cells for 5 min with EDTA to disrupt cell junctions. Cells were washed in medium lacking serum and incubated with Ad vectors expressing wild-type or mutant gB (150 PFU/cell, defined using 293 cells) for 2 h in medium containing 1% FBS. The virus was replaced with medium containing 5% FBS for 36 h. The cells were then infected with F-BACgB- (14) for 6 h before being labeled with $^{32}\text{PO}_4$ or [^{35}S]methionine-cysteine for 6 h as described above. Detergent lysates were frozen at -70°C , thawed the next day, and centrifuged at $85,000$ to $110,000 \times g$, and supernatants were subjected to immunoprecipitation.

Preparation of GST fusion proteins. The construction of recombinant baculoviruses expressing glutathione *S*-transferase (GST)-US3 and US3 (K220A) was described previously (31). GST-US3 proteins were purified from baculovirus-infected insect cells with GST-Sepharose and eluted with glutathione as described previously (31). The GST-gB cytoplasmic (CT) domain fusion protein includes residues 796 to 904 of the gB CT domain, which was fused to the C terminus of GST and was constructed using plasmid pGEX-2T and oligonucleotides TCGGATCCCGTACGTCATGCGG and 3'CGGAATTCTCACAGGT CGTCCTC5' to amplify gB gene sequences from plasmid pep98. Other GST fusion proteins included GST-gE-CT, containing 105 residues of the HSV gE CT domain (amino acids [aa] 445 to 550) fused onto the C terminus of GST (encoded by plasmid pGEX-2T; a gift from Colin Crump and Gary Thomas, Vollum Institute, Oregon Health and Science University); GST-ICP47, containing the entire HSV-1 ICP47 protein (56) (encoded by pGX-2T); GST-US11, containing the entire 215-aa human cytomegalovirus (HCMV) US11 protein fused to the C terminus of GST (encoded by pGX-2T); and GST-US9C, containing the 24 C-terminal residues of HCMV US9 fused to the C terminus of GST (encoded by pGEX-2T). Bacteria containing these plasmids were grown to log phase, and expression of GST proteins was induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 2 h. Then bacteria were pelleted and washed in cold STE buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA) before addition of 100 $\mu\text{g}/\text{ml}$ lysozyme and 5 mM dithiothreitol (DTT), followed by 1.5% Sarkosyl. Bacterial extracts were sonicated and centrifuged at $3,000 \times g$ for 10 min, and then Triton X-100 was added to the supernatants to a concentration of 2.5%. Glutathione-Sepharose was added, and samples were incubated for 14 to 18 h at 4°C , washed with phosphate-buffered saline containing 5 mM DTT, and then washed and stored in 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM DTT, and 10% glycerol. A fraction of the proteins bound on the beads was eluted by boiling in 2% sodium dodecyl sulfate (SDS) and characterized by electrophoresis and staining with Coomassie brilliant blue (CBB).

In vitro US3 kinase assays. Approximately, 2 to 2.5 μg of GST or GST fusion proteins bound to glutathione-Sepharose was added to 40 μl of kinase buffer (50 mM Tris, pH 9, 20 mM MgCl_2 , 0.1% NP-40, 1 mM DTT) as described previously (21, 31). To this 0.1 μg of GST-US3 or 0.2 μg of GST-US3 (K220A) was added. Cold ATP was added to 10 μM , and 10 μCi of [γ - ^{32}P]ATP was added. Samples were incubated at 30°C for 30 min and placed on ice, and 100 μl of TNE (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA) was added. Twenty microliters of TNE-washed glutathione-Sepharose was added, and samples were then washed four times with TNE. The phosphorylated proteins were eluted by boiling the samples in buffer containing 2% SDS and subjected to electrophoresis using a 12% polyacrylamide gel. The gel was stained with CBB to verify the presence of substrates before being dried and exposed to film.

Mass spectrometric analyses for phosphopeptides. HaCaT cells ($\approx 10^8$ cells) were infected with HSV for 12 h. Then the cells were swollen in hypotonic buffer

and Dounce homogenized, and a low-speed pellet was obtained as described above. HSV gB was immunoprecipitated as described above and then digested with endoglycosidase F (New England Biolabs) by the manufacturer's instructions. Immunoprecipitated proteins were subjected to electrophoresis using 4 to 20% gradient polyacrylamide gels, gels were stained with CBB, and the band corresponding to gB was excised. Gel bands were cut into four pieces, washed with 100 mM $(\text{NH}_4)_2\text{CO}_3$ and then with acetonitrile, and dried, and proteins were reduced in 10 mM DTT and alkylated with 55 mM iodoacetic acid. Peptides were generated by treating gel plugs with 1 ng/ml trypsin (Promega) overnight at 35°C and then extracted with 1% formic acid. Liquid chromatography-mass spectrometry (LC-MS) was performed using an Eksigent Nano 2D LC instrument (Applied Biosystems) and a Astrosil C_{18} column (Stellar Phases, Langhorne, PA). A gradient of mobile phase A (water, propanol, formic acid [97.4:2.5:0.1, vol/vol/vol]) and phase B (acetonitrile, propan-2-ol, water, formic acid [80:10:9.9:0.1, vol/vol/vol/vol]) was used to elute peptides. Eluants were introduced into a QTRAP4000 mass spectrometer through a micro-ion spray head fitted with a Picotip emitter (FS-360 75 15 N). Three different types of analyses were performed: (i) positive mode LC-tandem MS (MS/MS) with data-dependent scans set to trigger on the eight most intense ions, (ii) negative mode LC-MS with data-dependent switching to positive mode for MS/MS analysis (59), and (iii) precursor ion scanning in the negative mode attempting to discern m/z 79 (PO_3^-) marker ions for phosphoserine-, phosphothreonine-, and phosphotyrosine-containing peptides (8, 62). MS/MS spectra were analyzed with the Mascot search engine (Matrix Science).

Electron microscopy. HaCaT cells were infected with HSV at 15 PFU/cell for 18 h, fixed while still on plastic dishes, and processed for electron microscopy as described previously (13).

RESULTS

US3-dependent phosphorylation of gB but not gH. To determine whether gB and gH were phosphorylated in a US3-dependent manner, HaCaT human keratinocytes were infected with the HSV US3-null mutant vRR1202 or repair virus vRR1202rep (US3-R), which expresses US3 (48). The cells were labeled with $^{32}\text{PO}_4$ or with [^{35}S]methionine-cysteine, in both cases for 6 h beginning 6 h after infection. Then the cells were subjected to Dounce homogenization, and a crude nuclear fraction (denoted P; centrifuged at $750 \times g$) was prepared. The membranes and organelles in the supernatant from centrifugation at $750 \times g$ were centrifuged at high speed, producing a fraction denoted S. HSV glycoproteins gB and gH were immunoprecipitated from detergent extracts of these preparations. In cells infected with HSV US3-R (which expresses US3) there was phosphorylation of gB in both fractions but there was more-extensive labeling in the P fraction (Fig. 1). [^{35}S]methionine-cysteine labeling of gB indicated that only $\approx 25\%$ of the total gB was present in the P fraction, so that the specific activity of the $^{32}\text{PO}_4$ label associated with gB in the P fraction was substantially higher. The amount of $^{32}\text{PO}_4$ incorporated into gB in the P fraction in cells infected with US3⁻ HSV was $\approx 20\%$ of the corresponding amount in US3-R-infected cells (Fig. 1). The P fraction contained most cellular nuclei but also contained other cellular membranes. As a result, there were both immature (faster-migrating) and mature (more slowly migrating) forms of gB and gH in both P and S fractions (Fig. 1). Previously, Compton and Courtney (10) showed that gB present in a nuclear fraction was enriched for the immature form. However, in their studies nuclei were stripped with mild detergent, removing the endoplasmic reticulum and other perinuclear membranes. When we performed a similar procedure, too little $^{32}\text{PO}_4$ remained in the samples for complete analyses (not shown). We observed little or no phosphorylation of gH in either the P or S fraction (Fig. 1).

To further characterize the specificity of gB phosphoryla-

FIG. 1. US3-dependent phosphorylation of gB but not gH. HaCaT keratinocytes were infected with HSV vRR1202 (US3⁻ mutant) or vRR1202rep- (US3-R), which is a repaired version of vRR1202, for 6 h. The cells were subsequently labeled for 6 h with either [³⁵S]methionine-cysteine (bottom) or ³²PO₄ (top). Cells were swollen in hypotonic buffer, Dounce homogenized, and fractionated into a low-speed (750 × g) pellet (P) and membranes centrifuged from supernatants (S). gB (left) and gH (right) were immunoprecipitated from detergent extracts of these membranes. The positions of mature and immature forms of both gB and gH are indicated. The amounts of [³⁵S]methionine-cysteine and ³²PO₄-labeled gB were determined by phosphorimager analysis and are indicated at the bottom of the left panels.

tion, HaCaT cells were infected with vRR1202 (US3⁻), US3-R, or a mutant expressing a kinase-dead US3 (K220A) (48). Cells were lysed in NP-40–deoxycholate buffer, and total gB, gH, or gE was immunoprecipitated. Again, phosphorylation of gB was largely dependent upon US3, and the kinase-dead US3 behaved similarly to the US3-null mutant (Fig. 2). There was no obvious phosphorylation of gH, with or without US3. HSV glycoprotein gE was extensively phosphorylated, but this did not depend upon US3 (Fig. 2). Previously, we demonstrated phosphorylation of HSV gE at casein kinase II consensus sites in the CT domain of gE (60). We concluded that gB was primarily phosphorylated in a US3-dependent fashion, although a fraction (20 to 33%) of gB was phosphorylated without US3.

Phosphorylation of gB in vitro. An in vitro assay was used to test whether US3 could phosphorylate gB directly. Because US3 is present in the nucleoplasm and cytoplasm and within enveloped virions (45), it appeared likely that the gB CT domain would be the substrate for US3. The entire gB CT domain (aa 796 to 903) was fused onto the C terminus of GST

FIG. 2. US3-dependent phosphorylation of gB depends upon the kinase activity of US3. HaCaT cells were infected with HSV vRR1205, which expresses kinase-inactive US3 (K220A), vRR1202 (US3⁻ mutant), or US3-R for 6 h. Subsequently, the cells were labeled for 6 h with either [³⁵S]methionine-cysteine (right) or ³²PO₄ (left). Cells were extracted with detergents and not fractionated as in Fig. 1. gB, gH, and gE were immunoprecipitated from detergent extracts of membranes.

and expressed in bacteria and purified as described previously (56). Wild-type US3 and a kinase-inactivated mutant US3 (K220A) were fused onto the C terminus of GST sequences, expressed by a baculovirus in insect cells, and purified with glutathione-Sepharose as described previously (31). In vitro kinase assays involving 2 μg of GST-gB or GST bound to glutathione beads and mixed with ≈0.1 μg of GST-US3 or 0.2 μg of GST-US3 (K220A) and [³²P]ATP for 30 min were performed (21, 31). An additional amount of glutathione beads was added (to capture autophosphorylated US3), the beads were pelleted, and proteins were eluted by boiling the beads in buffer containing 2% SDS and were subjected to electrophoresis. The gB CT domain was extensively phosphorylated by US3, and there was little phosphorylation of the gB CT domain with US3 (K220A) (Fig. 3). Much less phosphorylation was observed with GST (without gB sequences). There was also substantial phosphorylation of the US3 present in these assays, as previously described (21, 31). As specificity controls, we also

FIG. 3. US3 phosphorylates gB in vitro. GST fusion proteins in which the following viral proteins or peptides were fused onto the C terminus of GST (encoded by plasmid pGEX-2T) were constructed: the HCMV US9 24-residue CT domain, the entire HCMV US11 protein, the HSV gE CT domain, the HSV gB CT domain, and the entire HSV ICP47 protein. These fusion proteins and GST alone were bound onto glutathione-Sepharose and then mixed with recombinant GST-US3 or GST-US3 (K220A) (kinase inactive) purified from insect cells in the presence of [γ - 32 P]ATP for 30 min at 30°C. Additional glutathione-Sepharose was added to capture the phosphorylated US3, and the Sepharose beads were washed. The proteins were eluted by boiling the beads in buffer containing 2% SDS and characterized by electrophoresis. The star in each individual lane indicates the position of the GST fusion protein, and the position of US3 is indicated at the left. Carb, carbonic anhydrase, 30 kDa.

characterized the phosphorylation of several other viral proteins by US3. The HCMV US11 protein fused at the C terminus of GST was poorly phosphorylated by US3 (Fig. 3). By contrast, the 24 C-terminal residues of HCMV US9 (CT domain), the 105-residue CT domain of HSV glycoprotein gE, and the entire HSV ICP47 protein, all fused onto the C terminus of GST, were extensively phosphorylated by US3 in vitro (Fig. 3). The gE CT domain (RASGKG, RYSQA) and ICP47 (RRTGG) contain US3 consensus phosphorylation motifs which likely act as PKA/US3 consensus sites for phosphorylation in vitro. However, these results differed from what was found in HSV-infected cells, where US3 did not contribute to phosphorylation of gE (Fig. 2). Nevertheless, the salient point here is that gB is phosphorylated in HSV-infected cells in a US3-dependent fashion, and, in these in vitro assays, US3 can directly phosphorylate gB.

Mass spectrometric analyses of gB phosphopeptides. Protein phosphorylation can be investigated by MS (8, 62). Increases of 79 or 80 Da (depending upon whether negatively or positively charged ions are analyzed) in the masses of peptides indicate phosphorylation of specific amino acids within those peptides. However, there can also be dephosphorylation during protein purification, and phosphopeptides may not ionize or fragment well (53, 61). Thus, these analyses do not accurately quantify the molar ratios of phosphorylated versus unphosphorylated peptides. To overcome problems in detecting phosphorylated peptides, three separate types of analyses were performed on peptides derived from gB and separated by LC, as described in Materials and Methods. HSV gB was immunoprecipitated from HSV-infected cells subjected to gel electrophoresis and digested with trypsin, and then peptides were analyzed by mass spectroscopy. Three threonine residues and one serine residue in the gB CT domain, T814, T821, S873, and T877, were identified as phosphoamino acids by these analyses (Fig. 4). Each of these four phosphorylated residues in the gB CT domain was found to be phosphorylated by more than one analytical technique, but in a majority of analyses the residues

FIG. 4. Mass spectrophotometric analyses for phosphopeptides in HSV gB. HSV gB was immunoprecipitated from HaCaT cells infected with wild-type HSV, digested with endoglycosidase F, and purified by gel electrophoresis. Gel bands containing gB were reduced, alkylated, digested with trypsin, and then analyzed by MS as described in Materials and Methods. A cartoon describes the gB CT domain and the sites of potential phosphorylation: T814, T821, S873, T877, and T887. Also shown are the positions corresponding to stop codons, which were placed in mutant forms of the gB gene, and a T→A substitution constructed at residue T887.

FIG. 5. gB is phosphorylated in uninfected cells but more extensively in HSV-infected cells. Recombinant Ad vectors were used to express wild-type gB (gBwt), gB800stop, which contains only 4 residues of the large gB CT domain, and gB825stop, which contains the 29 N-terminal residues of the gB CT domain in HaCaT cells for 36 h. Other cells were not transduced with any Ad vectors (UN). The cells were either left uninfected (no HSV) or infected with F-BACgB-, an HSV gB-null mutant (HSV) for 6 h and then radiolabeled for six additional hours with [35 S]methionine-cysteine (left) or 32 PO $_4$ (right). The cells were fractionated as for Fig. 1, the low-speed pellet fraction was extracted with detergents, and gB was immunoprecipitated from detergent extracts.

were found to be phosphorylated by a single technique. There was no evidence of phosphotyrosine in the gB-derived peptides. There was a fifth potential site for phosphorylation in the gB CT domain at residue T887, which is embedded in a sequence (RRNT₈₈₇NY) representing a consensus motif for US3 and PKA (41). However, T887 was not observed in any phosphorylated peptide in the mass spectroscopic studies, perhaps related to dephosphorylation or an inability to detect this phosphopeptide. Thus, these five sites represented a starting point for a mutational analyses of gB.

Mutational analysis of gB CT domain residues that are phosphorylated in HSV-infected cells. In order to begin to characterize phosphorylation of the gB CT domain, we constructed two truncated forms of gB. For gB800stop, codons 800 and 801 are stop codons, removing all but four residues of the large gB CT domain (Fig. 4). gB825stop is truncated so that three of the potential phosphoamino acids, S873, T877, and T887, were removed. These truncated gB molecules, as well as wild-type gB, were expressed using nonreplicating Ad vectors. HaCaT cells were transduced for 42 h with Ad vectors to produce levels of expression of gB similar to that in HSV-infected cells. Some dishes of these transduced cells were infected with an HSV gB-null mutant, F-BAC-gB- (14) for 6 h to cause expression of HSV proteins, including US3. Other dishes of cells were not infected with HSV. The cells were then labeled with either [35 S]methionine-cysteine or 32 PO $_4$ for 6 h. The truncated versions of gB, gB800stop and gB825stop, labeled with [35 S]methionine-cysteine, migrated faster than wild-type gB, as expected. There was extensive phosphorylation of wild-type gB expressed in cells infected with the gB-null HSV (Fig. 5). By contrast, there was little phosphorylation of gB800stop and gB825stop expressed in HSV-infected cells. When wild-type gB was expressed without HSV infection, there was also phosphorylation of gB, although at levels of $\approx$ 20 to 30% of those observed with other HSV proteins. gB800stop and gB835stop were again poorly phosphorylated in uninfected cells. Together, these results suggested that the majority of phosphorylation of gB occurs in the CT domain between residues 826 and the C terminus and that this phosphorylation can also occur less efficiently by the action of cellular kinases.

To further characterize phosphorylation of gB in the context

of HSV-infected cells, we constructed HSVs expressing gB800stop or gB825stop. Since gB is essential for HSV replication and the gB CT is required for function (47), these HSVs were propagated on complementing cells that express gB but were analyzed for expression of gB and phosphorylation on noncomplementing cells. gB825stop displayed a smaller gB polypeptide labeled with [35 S]Met-Cys, as expected (Fig. 6, left). However, expression of gB800stop was problematic; virus stocks produced with gB-complementing cells were of low titer, and in multiple experiments, when these virus stocks were used to infect HaCaT cells, expression of gB was low. Apparently, gB800stop acts as a dominant negative protein given that gB functions as a dimer or trimer, poisoning HSV replication even in complementing cells expressing wild-type gB. Wild-type HSV was extensively phosphorylated, whereas gB825stop was poorly phosphorylated (Fig. 6, right). Again, this was consis-

FIG. 6. Phosphorylation of truncated or substitution mutants of gB expressed by HSV. HaCaT cells were infected with wild-type (W.t.) HSV; HSV recombinants that express gB800stop or gB825stop; YK551, which expresses gB in which T887 was converted to alanine; or YK553, a repaired version of YK551. As described for Fig. 1, the cells were infected for 6 h, radiolabeled with either [35 S]methionine-cysteine (35 S) or 32 PO $_4$ (32 P) for 6 h, and then fractionated to derive a low-speed pellet. Anti-gB MAb were used to immunoprecipitate gB from detergent extracts of the low-speed pellet. The numbers below the lanes represent quantification of the signals in gB bands obtained with a phosphorimager.

FIG. 7. Electron microscopic analyses of cells infected with HSV mutants lacking US3 or both gB and US3. HaCaT cells were infected with (A) vRR1202 (US3⁻) or (B) vRR1202/gB⁻ (US3⁻ gB⁻) for 18 h. The cells were fixed and processed for electron microscopy as described previously (13). Thin sections of the cells were viewed and photographed by transmission electron microscopy. The nuclei of cells are indicated.

tent with phosphorylation of the C-terminal end of the gB CT domain.

As noted above, there is a tyrosine residue, T887, present within protein residues 884 to 889 that is predicted to be a PKA/US3 phosphorylation site. In studies carried out in parallel with our observation that there is US3-dependent phosphorylation of gB, one of our laboratories (Y.K.) recently produced a recombinant HSV, denoted YK551, which expresses a mutant form of gB with a Thr→Ala substitution, as well as a repaired version of YK551, denoted YK553 (20). YK551, which expresses gB T887A, was not recognized by an antibody that recognizes PKA/US3 substrate motifs that are phosphorylated and does not recognize substrates without phosphorylation (20). However, these studies did not include radiolabeling of gB with ³²PO₄ and did not characterize whether there was phosphorylation at other gB CT domain residues. In order to determine to what extent T887 contributed to the phosphorylation of gB and whether residues such as 873 and 877 (identified in phosphopeptides by MS) might also be phosphorylated, we radiolabeled YK551-infected cells with ³²PO₄ or [³⁵S]methionine-cysteine. A comparison of gB produced by YK551 to that produced by YK553 and wild-type HSV showed that phosphorylation was reduced by 70 to 80% (Fig. 6). However, a comparison of YK551 gB with gB825stop (reduced by 98%) indicated that there appeared to be some phosphorylation of residues other than T887A in the C-terminal gB CT domain (Fig. 6). Several other experiments also showed 70 to 80% reductions in phosphorylation of YK551 gB, and an independently constructed HSV recombinant expressing a gB encoded by a gene for which codons 887 and 888 were stop codons displayed reduced phosphorylation of gB (20 to

25%) compared with wild-type gB (not shown). We concluded that the majority of the phosphorylation of gB occurs at amino acid T887.

Characterization of an HSV gB⁻ US3⁻ double mutant. HSV US3⁻ and gB⁻ gH⁻ mutants accumulate substantial quantities of enveloped virions in herniations or in the perinuclear space. However, both mutants also display virions on cell surfaces, albeit in reduced quantities (14, 48). As noted before (14), there appear to be other mechanisms for nuclear egress, either other viral fusion proteins or other processes that allow virions to cross the NE, although gB and gH are important in this process. It was of interest to determine whether the defects in nuclear egress observed with the US3⁻ mutant might be exacerbated by further deletion of gB. In one sense this tests whether gB and US3 act in the same pathway or completely different pathways. An HSV recombinant, denoted vRR1202/gB⁻, lacking both US3 and gB was constructed. HaCaT keratinocytes were infected with vRR1202 (lacking US3), vRR1202/gB⁻ (lacking both US3 and gB), or vRR1202rep⁻ (a repaired version of vRR1202 that expresses US3), and then sections of the cells were characterized by electron microscopy. Numerous herniations were observed in vRR1202-infected HaCaT cells as described previously (48) (Fig. 7A) and seldom observed with vRR1202rep⁻ (not shown). There were also numerous herniations, with vRR1202/gB⁻ (Fig. 7B). Note that in the image shown vRR1202 (US3⁻) displayed several herniations with outward invaginations and these were also observed with vRR1202/gB⁻ (US3⁻ gB⁻), although fewer in the specific image shown. With both vRR1202 and vRR1202/gB⁻ we observed cell surface virions in some images (although not those shown) and these were reduced in numbers compared with

those for US3-R. The assembly of nucleocapsids in the nucleus was not altered with either US3⁻ or gB⁻ gH⁻ viruses, as was described previously (14, 34). We counted over 1,000 enveloped virions associated with each of the vRR1202rep-, vRR1202-, and vRR1202/gB-infected cells, thereby specifically quantifying enveloped particles found in the perinuclear space and herniations and on cell surfaces. As a measure of defects in nuclear egress, we determined the ratio of enveloped virions in the perinuclear space or herniations to cell surface enveloped virions. Cells infected with the repaired HSV vRR1202rep displayed a ratio of nuclear to cell surface virions of 0.05, i.e., most enveloped virions were present on the cell surface. By contrast, vRR1202- and vRR1202/gB-infected cells displayed a nuclear/cell surface enveloped virion ratios of 4.5 and 6.5, respectively. It should be noted that it is impossible to compare numbers of enveloped particles on a per cell basis, because there is large variability in the numbers of particles produced by individual cells. We concluded that the loss of gB did not dramatically increase the defects in nuclear egress associated with the US3⁻ mutant.

Characterization of HSV recombinants lacking gH and expressing mutant gB containing a substitution mutation (T887A) or truncated at residue 886. To evaluate how phosphorylation of T887, the major phosphorylation site in the gB CT domain, affected nuclear egress, it was necessary to construct an HSV recombinant with the T887A substitution and also lacking the gH gene. Using our HSV BAC, we constructed recombinants lacking the gH gene and either expressing the T887A substitution or containing stop codons in place of codons 887 and 888, so that the 18 C-terminal residues were removed (Fig. 4). The two viruses F-gBT887A/gH- and F-gB887stop/gH- were produced by transfecting BAC into F6/gB12 cells that express both gB and gH (14). Other studies confirmed that F-gBT887A/gH- and F-gB887stop/gH- displayed reduced phosphorylation of gB, in line with Fig. 6 (not shown). In order to investigate defects in nuclear egress, HaCaT cells were infected with F-gBT887A/gH-, F-gB887stop/gH-, wild-type HSV, or F-gB/galK/gH-, in which gB gene sequences were replaced with *galK* and gH gene sequences were lacking. Note that F-gB/galK/gH- is a newly constructed gB⁻ gH⁻ virus, different from the gB⁻ gH⁻ mutant denoted F-BAC gB-/gH-, described previously (14). Cells infected with wild-type HSV exhibited few perinuclear enveloped virions; instead, most enveloped virions were present on cell surfaces (Fig. 8A). As with F-BAC gB-/gH- (14), cells infected with F-gB/galK/gH- displayed numerous herniations (Fig. 8B). Cells infected with F-gBT887A/gH- or F-gB887stop/gH- also exhibited herniations (Fig. 8C, E, and F), as well as accumulations of perinuclear virions (Fig. 8D). To quantify the effects of these mutations, we counted enveloped virions present in the perinuclear space or herniations and cell surface virions (Table 1). Again, we derived the ratio of enveloped virions present in the perinuclear space or herniations to cell surface virions as a measure of defects in nuclear egress. Wild-type HSV exhibited an NE/cell surface virion ratio of 0.06 (Table 1), as had been described before (14). By contrast, F-gBT887A/gH- and F-gB887stop/gH- produced many more perinuclear virions and herniations and the NE/cell surface virion ratios were 1.9 and 1.7, respectively. This compared with a ratio of 2.9 for the HSV gB⁻ gH⁻ double mutant in this experiment. We con-

cluded that the T887A substitution in gB or truncation of gB at residue 886 markedly reduces gB-mediated fusion between the virion and the outer NM.

DISCUSSION

Observations that either of the two HSV fusion proteins gB and gH can mediate fusion between the virion envelope and the outer NM represented an important first glimpse of this novel viral membrane fusion process. Nevertheless, there is a great deal to learn about how herpesviruses traverse the NE. Especially interesting is how the fusion between the virion envelope and outer NM is triggered or regulated. When enveloped viruses enter cells by fusion with cellular membranes, viral fusion proteins are activated by binding to cellular receptors, which is coupled with conformation changes, associated with, e.g., the low pH of endosomes. HSV entry fusion involves the triggering of viral fusion proteins gB and gH/gL following the binding of a third glycoprotein, gD, to cellular receptors. A larger complex of gB/gD/gH/gL forms; this complex can promote fusion of the virion envelope with cellular membranes (1, 2). Both gB and gH/gL appear to be directly involved in membrane fusion, i.e., in mixing of lipids (54), and both glycoproteins are required for entry fusion. HSV fusion at the outer NM appears to be a different process in several respects. For example, either gB or gH can suffice for nuclear egress. It is not clear whether HSV gD is required for the fusion with NMs. All that can be said is that HSV gD⁻ mutants do not exhibit obvious accumulation of nuclear capsids or enveloped virions (27). gD receptors such as nectin-1 and HVEM are primarily cell surface proteins and may not be major components of NMs. The similar phenotypes of HSV US3⁻ and gB⁻ gH⁻ mutants and substantial accumulation of US3 in NMs and perinuclear virions prompted us to investigate whether US3 might activate either gB or gH to trigger their fusion with NMs.

Indeed, gB was phosphorylated in a US3-dependent manner in HSV-infected cells, while gH was not phosphorylated. The majority of the phosphorylated gB was found in a crude nuclear fraction. Although there was a clear dependence of the phosphorylation of gB on expression of US3, there were also significant amounts (≈ 20 to 33%) of gB phosphorylated with the US3⁻ mutant or when gB was expressed using an Ad vector without other HSV proteins. Thus, cellular kinases can also phosphorylate gB, though to a lesser extent. US3 is a functional homologue of cyclic AMP-dependent PKA (3). Thus, it is possible that PKA phosphorylates gB as well. Perhaps, PKA is attracted into assembling HSV virions, as is the case for protein kinase C (36). Notwithstanding this, most of the phosphorylation of gB in HSV-infected cells depended upon US3. Moreover, US3 could phosphorylate the gB CT domain in vitro, supporting direct phosphorylation of gB by US3 in HSV-infected cells.

To identify regions of the gB CT domain that are phosphorylated, we initially performed MS on peptides derived from gB. We focused on phosphopeptides present in the gB CT domain, given the assumption that US3 is present in the cytoplasm, nucleoplasm associated with the inner NM, and the tegument layer of perinuclear virions. Four phosphorylated residues were identified: T814, T821, S873, and T877. As discussed above, MS does not allow accurate determina-

FIG. 8. Electron micrographs of cells infected with HSV recominants expressing gBT887A or gB8887stop and lacking the gH gene. HaCaT cells were infected with (A) wild-type HSV-1, (B) F-BAC gB/galK/gH- (a mutant lacking gB and gH), (C and D) F-BAC gBT887A/gH-, or (E and F) F-BAC gB887stop/gH- for 18 h. The cells were fixed and processed for electron microscopy as described previously (13). Thin sections of the cells were viewed and photographed by transmission electron microscopy. The nuclei of cells are indicated.

TABLE 1. Enveloped virus particles produced by HSV recombinants expressing gBT887A or gB8887stop and lacking the gH gene

HSV	No. of:		Ratio ^c
	Enveloped perinuclear virions ^a	Cell surface virions ^b	
Wild type	104	1,861	0.06
F-gB/galK/gH-	1,639	559	2.9
F-gB887stop/gH-	1,121	590	1.9
F-gBT887A/gH-	1,251	736	1.7

^a Enveloped virus particles present in herniations or in the perinuclear space.^b Enveloped virus particles present on the surfaces of cells.^c The ratio of enveloped perinuclear virions to cell surface virions.

tion of molar ratios of phosphorylated versus nonphosphorylated residues. Residue T887, near the C terminus of the gB CT domain, which was predicted to be part of a US3 consensus motif, was not observed as part of any phosphopeptide in these analyses. Truncated forms of gB, gB800stop and gB825stop, were not substantially phosphorylated, demonstrating that most of the phosphorylation involved gB CT domain residues C terminal to residue 825. It should be kept in mind that truncation mutations can affect sequences distant from phosphorylation sites that can modify phosphorylation. Work done in parallel with our studies demonstrated that T887A was phosphorylated in HSV-infected cells, on the basis of detection of an epitope in gB recognized by a MAbs specific for PKA/US3 substrates that are phosphorylated (20). We extended these observations by showing that a substitution mutant, gBT887A, displayed reduced phosphorylation, by 70 to 80%, compared with wild-type gB. Therefore, the majority of phosphorylation of gB involves the CT domain and specifically residue T887. Since T887 forms part of a PKA/US3 consensus motif, this was further evidence that US3 phosphorylated gB directly. However, from a comparison of the phosphorylation of gB825stop with that of gBT887A (expressed by YK551), it appears that there may also be phosphorylation of other residues in the gB CT domain, e.g., S873 or T877, to a lesser extent.

HSV US3⁻ and gB⁻ gH⁻ mutants have similar phenotypes, i.e., accumulation of enveloped virions in the perinuclear space or herniations. We characterized a US3⁻ gB⁻ double mutant and found that the phenotype of the US3⁻ mutant was not exacerbated by deletion of gB. In evaluating these experiments it is important to recognize that the HSV gB⁻ gH⁻ and US3⁻ mutants display cell surface virions even though these mutants also accumulate substantial quantities of enveloped virions in herniations (often 20 to 80 times that seen with wild-type HSV). Thus, there appear to be other mechanisms for nuclear egress, beyond those involving gB and gH. However, deletion of gB from the US3⁻ mutant did not decrease the numbers of cell surface virions or increase herniations substantially. These observations, coupled with the US3-dependent phosphorylation of gB, were consistent with the notion that US3 and gB act in the same or related pathways to promote nuclear egress, although there are other interpretations of these data. However, it is also very likely that US3 has other functions during HSV nuclear egress, perhaps involving the phosphorylation of US31 and UL34 and other viral proteins required for nuclear

egress. Related to this, the HSV US3⁻ gB⁻ mutant expresses gH, which has the capacity to promote fusion with the outer NM (14). Again, this suggests that US3 participates in nuclear egress in other ways, so that the US3⁻ mutant has other defects. Moreover, gH was not appreciably phosphorylated and must be triggered for fusion at the outer NM by some other mechanism.

To address the role of US3-dependent gB phosphorylation more directly, we constructed HSV recombinants lacking gH and expressing the T887A substitution or truncated at residue 886. Both these mutants exhibited substantial defects in nuclear egress, accumulating numerous enveloped virions within the perinuclear space and in herniations. There were quantitatively fewer herniations and perinuclear virions with the F-gBT887A/gH- and F-gBT887stop/gH- mutants than with the gB⁻ gH⁻ double deletion mutant, F-gB/galK/gH- (Table 1). Nevertheless, the T887 mutants were substantially (30-fold) more inhibited for nuclear egress than wild-type HSV on the basis of a comparison of ratios of nuclear to cell surface virions. We concluded that phosphorylation of the gB US3 consensus motif at residue T887 contributes importantly to the fusion between the virion envelope and the outer NM.

These observations have important implications for our understanding of how gB functions to mediate fusion between the virion envelope and the outer NM and how this compares to the role gB plays in entry fusion. Recombinant HSV YK551, which expresses gBT887A and wild-type gH, can be propagated on Vero cells (which do not express gB), producing relatively normal titers, although differences in levels of cell surface expression of gB were observed (20). gB function is absolutely required for entry (6). Given that YK551 grows normally on Vero cells, gB containing the T887A substitution functions relatively normally for HSV entry into cells. By contrast, F-gBT887A/gH-, expressing the T887A substitution and lacking the gH gene, exhibited major defects in nuclear egress. Thus, entry fusion does not substantially depend upon phosphorylation of T887, yet gB-mediated fusion between the virus and the outer NM largely requires this phosphorylation. We also found that the majority of phosphorylated gB was present in a crude nuclear fraction of cells, again supporting the notion that this modification functions in nuclear egress and not in entry fusion. This mutation is the first to indicate the separation of these two different membrane fusion processes.

HSV US3 accumulates on the inner surface of the inner NM and is incorporated into the tegument layers of the virions that bud into the perinuclear space (45). Consistent with an important role in nuclear egress, the smaller form of the PRV US3 protein predominantly localizes to the nucleus (7). Assembly of US3 into the virion tegument layer (between the capsid and envelope) might bring US3 into close proximity with the gB CT domain. This is consistent with our hypothesis that US3-mediated phosphorylation of the CT domain of gB at residue 887 can activate gB, promoting fusion of the virion envelope with the outer NM. Activation of gB for fusion likely occurs rapidly, as perinuclear virions are rare in cells infected with wild-type HSV. To alter the fusogenic potential of gB that is present in perinuclear virions, phosphorylation of the gB CT domain must alter the oligomerization of gB or change the conformation of the extracellular domain of gB, the key domain in membrane fusion. The gB CT domain also plays an important

or essential role in cell-cell fusion and virus entry (reviewed in reference 47). These observations are significant in that this is the first example that we are aware of in which a viral fusion protein is triggered for membrane fusion by posttranslational modification of the CT domain of the protein.

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