AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

Cysteines and N-Glycosylation Sites Conserved among All Alphaherpesviruses Regulate Membrane Fusion in Herpes Simplex Virus 1 Infection

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ABSTRACT Neurotropism is a defining characteristic of alphaherpesvirus pathogenicity. Glycoprotein K (gK) is a conserved virion glycoprotein of all alphaherpesviruses that is not found in other herpesvirus subfamilies. The extracellular amino terminus of gK has been shown to be important to the ability of the prototypic alphaherpesvirus herpes simplex virus 1 (HSV-1) to enter neurons via axonal termini. Here, we determined the role of the two conserved N-linked glycosylation (N48 and N58) sites of gK in virus-induced cell fusion and replication. We found that N-linked glycosylation is important to the regulation of HSV-1-induced membrane fusion since mutating N58 to alanine (N58A) caused extensive virus-induced cell fusion. Due to the known contributions of N-linked glycosylation to protein processing and correct disulfide bond formation, we investigated whether the conserved extracellular cysteine residues within the amino terminus of gK contributed to the regulation of HSV-1-induced membrane fusion. We found that mutation of C37 and C114 residues led to a gK-null phenotype characterized by very small plaque formation and drastic reduction in infectious virus production, while mutation of C82 and C243 caused extensive virus-induced cell fusion. Comparison of N-linked glycosylation and cysteine mutant replication kinetics identified disparate effects on infectious virion egress from infected cells. Specifically, cysteine mutations caused defects in the accumulation of infectious virus in both the cellular and supernatant fractions, while glycosylation site mutants did not adversely affect virion egress from infected cells. These results demonstrate a critical role for the N glycosylation sites and cysteines for the structure and function of the amino terminus of gK.

IMPORTANCE We have previously identified important entry and neurotropic determinants in the amino terminus of HSV-1 glycoprotein K (gK). Alphaherpesvirus-mediated membrane fusion is a complex and highly regulated process that is not clearly understood. gK and UL20, which are highly conserved across all alphaherpesviruses, play important roles in the regulation of HSV-1 fusion in the context of infection. A greater understanding of mechanisms governing alphaherpesvirus membrane fusion is expected to inform the rational design of therapeutic and prevention strategies to combat herpesviral infection and pathogenesis. This work adds to the growing reports regarding the importance of gK to alphaherpesvirus pathogenesis and details important structural features of gK that are involved in gK-mediated regulation of virus-induced membrane fusion.

KEYWORDS N-glycosylation, conservation, cysteines, disulfide bonds, gK, glycoprotein B, glycoprotein K, herpes simplex virus, membrane fusion three-dimensional structure

Received 27 May 2017 **Accepted** 14 August 2017

Accepted manuscript posted online 23 August 2017

Citation Rider PJF, Naderi M, Bergeron S, Chouljenko VN, Brylinski M, Kousoulas KG. 2017. Cysteines and N-glycosylation sites conserved among all alphaherpesviruses regulate membrane fusion in herpes simplex virus 1 infection. J Virol 91:e00873-17. https://doi.org/10.1128/JVI.00873-17.

Editor Richard M. Longnecker, Northwestern University

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Membrane fusion is an essential step for entry of enveloped viruses into host cells (1–3). The process of viral fusion involves the conversion of the viral fusion protein from a prefusion to a postfusion conformation (2). This conformational change ultimately results in fusion of the virus envelope with host membranes and deposition of the virion capsid into the cytoplasm of infected cells. Proper control of virus-induced membrane fusion is essential for efficient virus replication and spread. Mechanisms of membrane fusion regulation include receptor binding, proteolytic processing, and pH dependence (1). In the simplest system, as with vesicular stomatitis virus (VSV), the fusion protein is the only protein on the viral envelope and mediates both receptor binding and subsequent fusion (4). Other viruses, such as herpesviruses, require protein complexes made up of multiple viral proteins to mediate fusion (5).

Herpesvirus fusion is a complex, highly coordinated process. All members of the herpesvirus family require a heterodimer of glycoprotein H and glycoprotein L (gH/gL), as well as glycoprotein B (gB), the only fusogenic glycoprotein, to mediate membrane fusion (5–8). Additionally, some species of herpesvirus such as herpes simplex virus 1 (HSV-1) and HSV-2 require the receptor binding glycoprotein D (gD). To facilitate membrane fusion in transient systems, it is sufficient to coexpress gD, gH/gL, and gB (9). However, the roles played by individual fusion complex members in mediating membrane fusion are poorly understood. Current evidence suggests that gD binds a receptor and transfers, presumably via a conformational change, an activation signal through gH/gL to gB, which then undergoes a conformational change from the prefusion to postfusion state (10).

Regulation of herpesvirus fusion is incompletely understood, and findings from transient systems may be misleading. It is clear that in the context of viral infection there are more viral proteins than the minimal fusion complex involved in the fusion process. This is most apparent in HSV-1 mutants that are found to exhibit dysregulated fusion resulting in the formation of syncytia. Mutations causing significant syncytial (syn) phenotypes have been found predominantly in HSV-1 gB, gK, and UL20 genes (11–15). gB is the conserved and only fusogen of all herpesviruses, whereas gK and UL20 are conserved only in neurotropic alphaherpesvirus and are not part of the minimal fusion complex (16). Neither gK nor UL20 possesses intrinsic fusogenic activity. The identification of syncytial virus strains which possess mutations in either gK or UL20 suggests a role for these proteins in the process of alphaherpesvirus fusion. Indeed, early studies demonstrated that transient expression of gK and UL20 with the minimal fusion complex resulted in a decrease in fusion mediated transiently, while syncytial mutations in gK did not increase cell-to-cell fusion (17).

gK is a multiple-transmembrane domain-containing glycoprotein that is found in the envelope of viral particles (18, 19). We along with others have shown that gK and UL20 form a complex in multiple alphaherpesvirus species (20–23). Further, we have demonstrated that the gK/UL20 complex interacts with fusion complex members gB and gH/gL (24). Specifically, we showed a direct interaction between the gK amino terminus and gB (24, 25). Importantly, we have shown a role for the amino terminus of gK in HSV-1 neurotropism as an HSV-1 with a deletion of amino acids 31 to 68 of the amino terminus of gK [gK(d31–68)] is unable to enter neurons via the axonal termini both *in vitro* and *in vivo* (19, 26, 27). A virus with this mutation in gK is currently in development as VC2, an HSV-1 vaccine that has been shown to be protective against both HSV-1 and HSV-2 infection in multiple animal models (26; S. Stanfield, P. J. F. Rider, J. Caskey, F. Del Piero, and K. G. Kousoulas, submitted for publication). VC2 has also been demonstrated to be highly immunogenic in monkey and mouse models (28, 29).

To determine the contribution of the extracellular N-linked glycosylation sites located at residues 48 and 58 to the function of HSV-1 gK, we constructed three mutant viruses in which each asparagine residue was mutated to alanine independently as well as a double N-linked glycosylation site mutant. We found that virus lacking the N58 or lacking both sites was severely defective for regulation of viral fusion. We extended our analysis to include the amino terminal cysteines of gK. Our results demonstrate that glycosylation of HSV-1 gK is important to the regulation of fusion and that similarly the

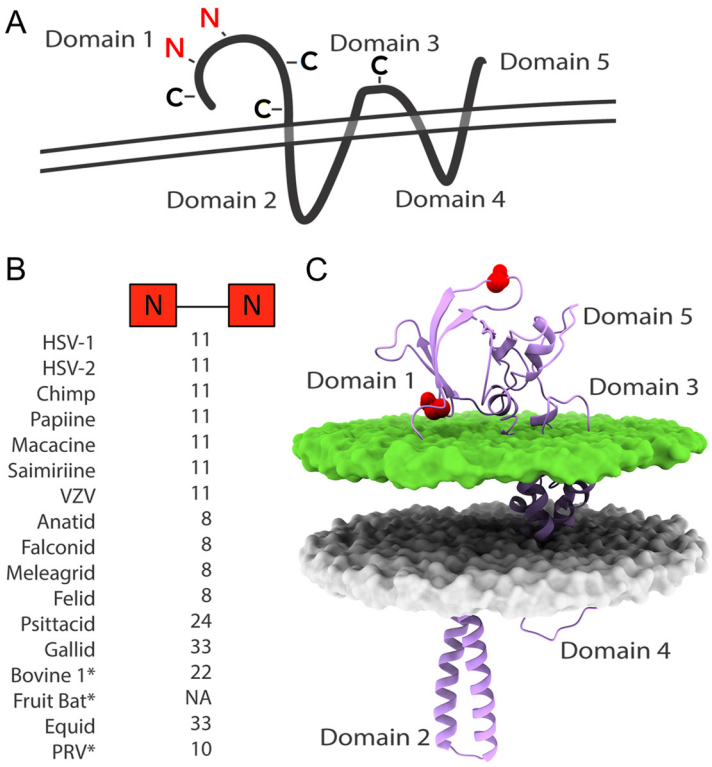


FIG 1 Conservation of alphaherpesvirus gK N-linked glycosylation sites. (A) Experimentally determined topology of HSV-1 gK illustrating location of N-linked glycosylation sites (red) and conserved cysteine residues. (B) Analysis of N-linked glycosylation site spacing (number of amino acids) across 17 distinct alphaherpesvirus gKs. (C) Ribbon diagram of the HSV-1 gK model placed in a hypothetical membrane. N-linked glycosylation sites are indicated by red spheres. Surface representations of extra- and intracellular phospholipid head groups are shown in green and gray, respectively. *, sequences that are divergent from other sequences with respect to conserved N glycosylation and cysteine residues across all alphaherpesviruses examined. NA, sequence has only one canonical N-glycosylation site.

extracellular cysteine residues of gK are critical for gK's role in regulation of HSV-1 fusion.

RESULTS

Analysis of conserved structural elements in the amino terminus of alphaherpesvirus gKs. HSV-1 gK is a four-transmembrane domain-containing glycoprotein with three extracellular and two intracellular domains (Fig. 1A). Comparative analysis of the primary structures for 17 alphaherpesvirus gKs from Table 1 reveals two conserved N-linked glycosylation sites in all species, except for the fruit bat gK, which possesses only one N-linked glycosylation site (Fig. 1B). Interestingly, the distance between the N-linked glycosylation sites appears to be well conserved among herpesvirus species which infect similar hosts. This is most evident among the primate and nonhuman primate alphaherpesviruses in which the N-linked glycosylation sites are all separated by 11 amino acids (which includes the asparagine residues), with the most striking comparison being that between simplex and varicella alphaherpesvirus species. Furthermore, with the exception of gallid herpesvirus, in the avian alphaherpesviruses, N-linked glycosylation sites are separated by 8 amino acids. In our previously predicted three-dimensional (3D) structure of gK (19), the N-linked glycosylation sites bracket the second predicted beta-pleated sheet (Fig. 1C). This structural position of the N-linked glycosylation sites bracketing a beta strand is conserved in several alphaherpesvirus gKs for which we have predicted structures (data not shown).

Construction and characterization of HSV-1 N-linked glycosylation mutants. To determine the role of gK N-linked glycosylation on infectious virus production and virus-induced membrane fusion, we generated three recombinant viruses, two in which

TABLE 1 Accession numbers for sequences used in this study

gK sequence	GenBank accession no.
Anatid herpesvirus 1	YP_003084366.1
Bovine herpesvirus 1	NP_045305.1
Cercopithecine herpesvirus 2	YP_164497.1
Chimpanzee alpha-1 herpesvirus	YP_009011041.1
Equid herpesvirus 1	YP_053051.1
Falconid herpesvirus 1	YP_009046554.1
Felid herpesvirus 1	YP_003331525.1
Fruit bat alphaherpesvirus 1	YP_009042116.1
Gallid herpesvirus 1	YP_182337.1
Human herpesvirus 2	YP_009137206.1
Human herpesvirus 3	NP_040128.1
Macacine herpesvirus 1	AIA09548.1
Meleagrid herpesvirus 1	NP_073348.1
Papiine herpesvirus 2	YP_443901.1
Psittacid herpesvirus 1	NP_944381.1
Saimiriine herpesvirus 1	YP_003933787.1
Suid herpesvirus 1	YP_068321.1

either the first asparagine (N48A) or the second asparagine (N58A) of the predicted N-linked glycosylation sites was replaced with alanine and one recombinant virus having both asparagines replaced with alanines (N48A N58A) (Fig. 1). Recombinant viruses were generated using the VC1-F-BAC virus. The VC1-F-BAC virus is a modified HSV-1(F) strain cloned as a bacterial artificial chromosome (BAC) that possesses a V5 epitope-tagged gK and a Flag-tagged UL20 that have been described previously (30). BAC-derived virus was reconstituted on the gK-complementing cell line VK302. Virus titers were determined, and the virus was used to infect either Vero cells or the gK-complementing cell line VK302. At 48 h postinfection (hpi) cells were fixed, and immunohistochemistry (IHC) was performed to identify HSV-positive plaques (Fig. 2). Readily apparent was the extensive syncytial formation in Vero cells infected with the mutant virus N58A, in which the second N-linked glycosylation (N58-to-A58 change) was mutagenized, as well as with the virus N48A N58A having both N-linked glycosylation sites replaced with alanines. In contrast, plaques from virus in which the first N-linked glycosylation (N48A) was mutagenized were indistinguishable from wild type-plaques (Fig. 2).

To determine the effect of glycosylation mutagenesis on the gK protein, we performed a Western blot analysis on protein extracted from cells infected with the parental VC1 virus or with the glycosylation mutant viruses. Vero cells were infected at a multiplicity of infection (MOI) of 5 for 20 h and lysed, and Western blot analysis was performed. Infection of Vero cells with the parental virus yielded a single band of approximately 37 kDa, as previously described (31, 32) (Fig. 3A). However, gK from cells infected with either the N48A or N58A virus appeared as two bands, migrating with apparent molecular masses of 33 and 30 kDa. N58A gK was produced consistently at lower levels than parental or N48A gKs. The double glycosylation mutant was not detectable via Western blotting of protein lysates. However, after immunoprecipitation using the V5 tag, all gKs were detected, including the gK specified by the double glycosylation mutant, which was now visible as a faint protein species migrating with an apparent molecular mass of 29 kDa (Fig. 3B).

To determine the effect of gK N-linked glycosylation on virus production, the replication kinetics of mutant viruses were examined. Vero cells were infected at an MOI of 0.1, and supernatants were separated from monolayers at 0, 4, 12, 24, and 36 h postinfection. Samples were frozen, and titers were determined via plaque assay. All glycosylation site mutant viruses reached titers similar to the titer of the parental VC1 virus in either the supernatant or monolayers (Fig. 4). However, the N58A virus and the double glycosylation mutant virus displayed approximately 1-log-lower titers in both supernatant and cell pellet fractions at 24 h, suggesting a possible delay in assembly, envelopment, or egress of this virus.

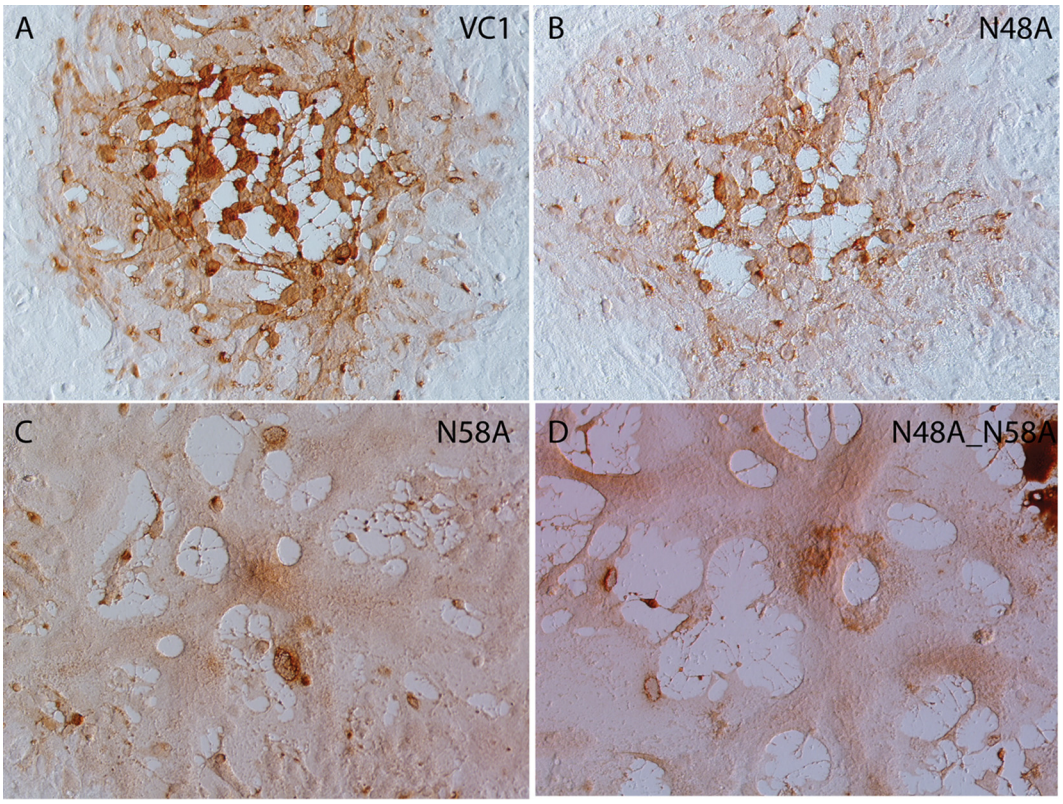


FIG 2 Plaque phenotypes of HSV-1 gK N-linked glycosylation mutants. Vero cells infected with the indicated virus for 48 h were fixed, and immunohistochemistry was performed with the parental virus (VC1) and N-linked glycosylation mutants N48A, N58A, and N48A N58A, as indicated.

It is known that N-linked glycosylation plays a major role in the proper folding and processing of glycoproteins (33, 34). Specifically, N-linked glycosylation has been demonstrated to facilitate proper disulfide bond formation (35). In nearly all alphaherpesvirus gKs, there are four extracellular cysteines: three in domain 1 and one in domain 3 (Fig. 5A). Alignment of gK primary structures similar to the one shown in Fig. 1B demonstrates the conservation of spacing between cysteine residues and N-linked glycosylation sites across all human and nonhuman primate simplex viruses (Fig. 5B). Remarkably, the spacing between the second and third cysteine residues of domain 1 is 33 amino acids in all but one alphaherpesvirus species that possess three or more cysteine residues in domain 1. Further, in our predicted structure for gK, all four

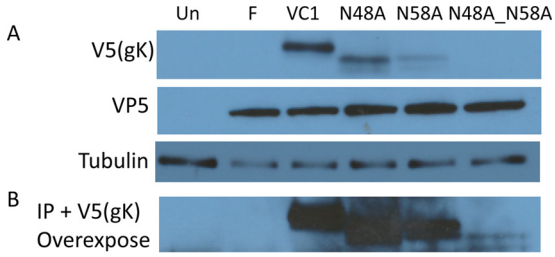


FIG 3 Mutation of N-linked glycosylation sites leads to increased mobility of HSV-1 gK protein. Uninfected Vero cells (Un) are compared to Vero cells infected with the wild-type HSV-1(F), with the parental strain (VC1) with V5-tagged gK, and with VC1-derived single (N48A and N58A) and double (N48A N58A) N-linked glycosylation mutants. (A) Cells were infected at a multiplicity of infection of 5 and lysed at 20 h postinfection. Lysates were separated via SDS-PAGE and analyzed by Western blotting. VP5 (UL19) is the major capsid protein of HSV-1. VP5 and tubulin served as loading controls. (B) Lysates were immunoprecipitated (IP) with V5 antibody, and precipitates were separated via SDS-PAGE and analyzed by Western blotting.

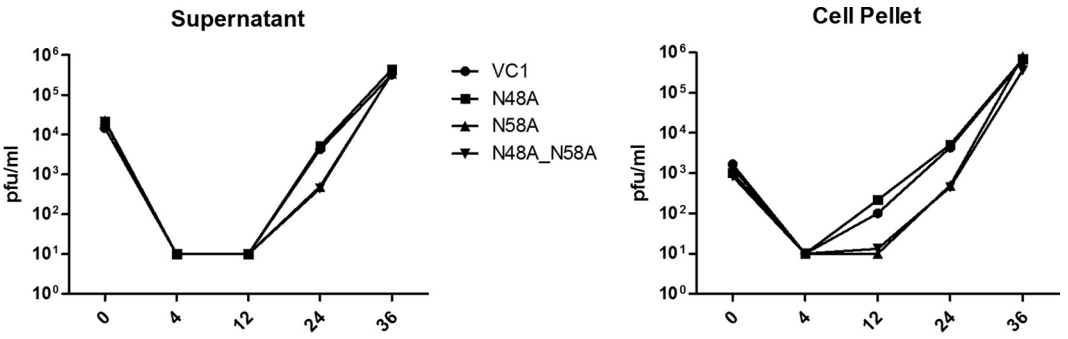
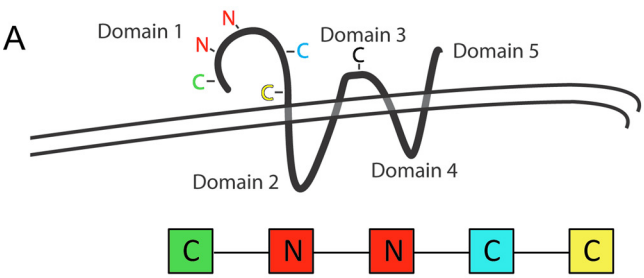


FIG 4 Mutation of N-linked glycosylation sites does not affect peak viral titers. Vero cells were infected with either the parental strain (VC1), VC1-derived single N-linked glycosylation mutants (N48A and N58A), or a double mutant (N48A N58A). Supernatants and cell pellets, as indicated, were separated at specified hours postinfection, and plaque assays were performed to determine viral titers.

extracellular cysteines are predicted to be near enough to one another to potentially form intramolecular disulfide bonds (Fig. 5C).

To address the role of the extracellular cysteine residues of HSV-1 gK, we constructed four mutant viruses, each with a deletion of a single cysteine, denoted as dC37, dC82, dC114, and dC243. Viruses were recovered on the complementing cell line VK302, and their plaque morphologies were characterized on both Vero and VK302 cells. The dC37 and dC114 viruses formed very small plaques on Vero cells, similar to those of the gK-null virus plaques, while the viruses with mutations in either C82 or



B

HSV-1	12	11	25	33
HSV-2	12	11	25	33
Chimp	12	11	25	33
Papiine	12	11	24	33
Macacine	12	11	24	33
Saimiriine	12	11	34	33
VZV	19	11	16	33
Anatid	10	8	30	33
Falconid	16	8	26	33
Meleagrid	15	8	24	33
Felid	15	8	27	33
Psittacid	20	24	26	33
Gallid	29	33	36	24
Bovine 1*	8(10)(13)	22	10	33
Fruit Bat*	12	NA	NA	33
Equid	22	33	27	NA
PRV*	NA	10	23	NA

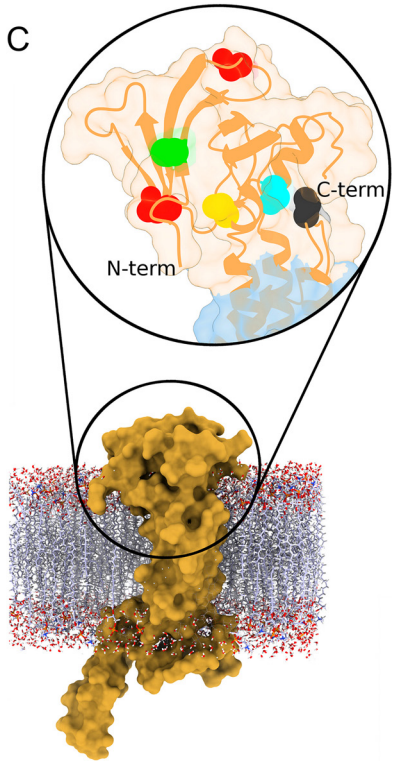


FIG 5 Conservation of cysteine residues in the amino terminus of alphaherpesvirus gKs. (A) Experimentally determined topology of HSV-1 gK illustrating location of cysteine residues in HSV-1 gK. (B) Analysis of cysteine residue spacing (number of amino acids) for the amino terminus of 17 alphaherpesvirus gKs. (C) Surface representation of the predicted 3D model of HSV-1 gK placed in a hypothetical lipid bilayer. A close-up view of the cysteine and asparagine residues in the extracellular domains is featured in a transparent surface representation of the gK model. Colors in panels B and C match those in panel A. *, sequences that are divergent from other sequences with respect to conserved N glycosylation and cysteine residues across all alphaherpesviruses examined. NA, number could not be calculated due to differences in conserved residues.

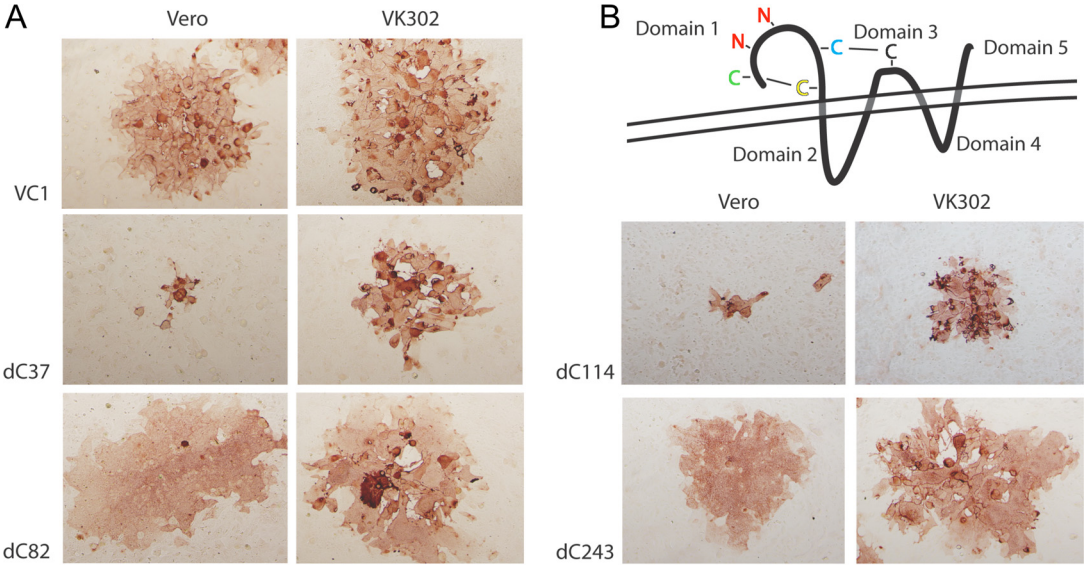


FIG 6 Plaque phenotypes for HSV-1 gK cysteine mutants. (A) Immunohistochemistry of Vero cells and gK-complementing cells (VK302) infected for 48 h with parental virus (VC1) or with cysteine deletion mutant viruses. (B) Topology of HSV-1 gK illustrating putative disulfide bonding between conserved cysteine residues, indicated by solid lines.

C243 exhibited a strong syncytial (syn) phenotype (Fig. 6). Importantly, both dC37 and dC114 viruses were efficiently complemented on VK302 cells. gK syn viruses have been reported to be complemented (reverted) to a wild-type plaque phenotype on VK302 cells (36). However, both dC82 and dC243 retained their syncytial phenotypes even in VK302 cells, suggesting that the cysteine mutations result in syncytial phenotypes that are dominant.

To determine the effect of the cysteine mutations on the level of gK production, Western blot analysis was performed as described in Materials and Methods. Loss of any of the cysteine residues did not alter the apparent molecular mass of gK in comparison to that of the parental VC1 virus gK (Fig. 7). Overall protein levels of gK were found to be reduced by the absence of individual cysteines.

To determine the effect of cysteine mutation on virus growth, we conducted growth analysis of all mutants in both Vero cells and the gK-complementing cell line VK302. Supernatants and cell pellets were assayed separately. dC37 and dC114 exhibited the greatest defects in growth (Fig. 8). dC37 and dC114 virus titers in the supernatant at 36 hpi were 4 logs lower than the parental VC1 virus titers. dC82 and dC243 viruses had higher peak titers in the supernatant, but the differences between the titers of these viruses and those of dC37 and dC114 were not significant. A similar pattern was seen in cell pellet titers, with dC37 and dC114 achieving approximately 3-log-lower peak titers than those of the VC1 parental virus. Titers of dC82 and dC243 viruses obtained from cell pellets were significantly lower, but the viruses reached higher peak titers

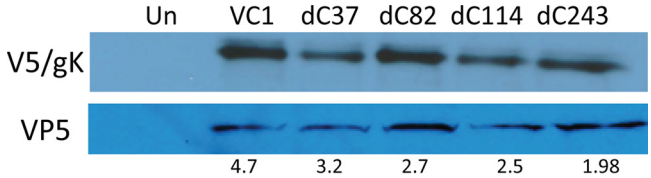


FIG 7 Mutation of HSV-1 gK cysteine residues has a limited effect on gK protein levels in infected cells. Uninfected Vero cells (Un) are compared to Vero cells infected with the parental strain (VC1) with V5-tagged gK or VC1-derived cysteine mutants. Cells infected with the indicated viruses at an MOI of 5 were lysed at 20 h postinfection. Lysates were separated via SDS-PAGE and analyzed by Western blotting. VP5 (UL19) is the major capsid protein of HSV-1 and served as a loading control. Numbers underneath the blot are ratios of intensity of V5/gK to VP5 for each lane.

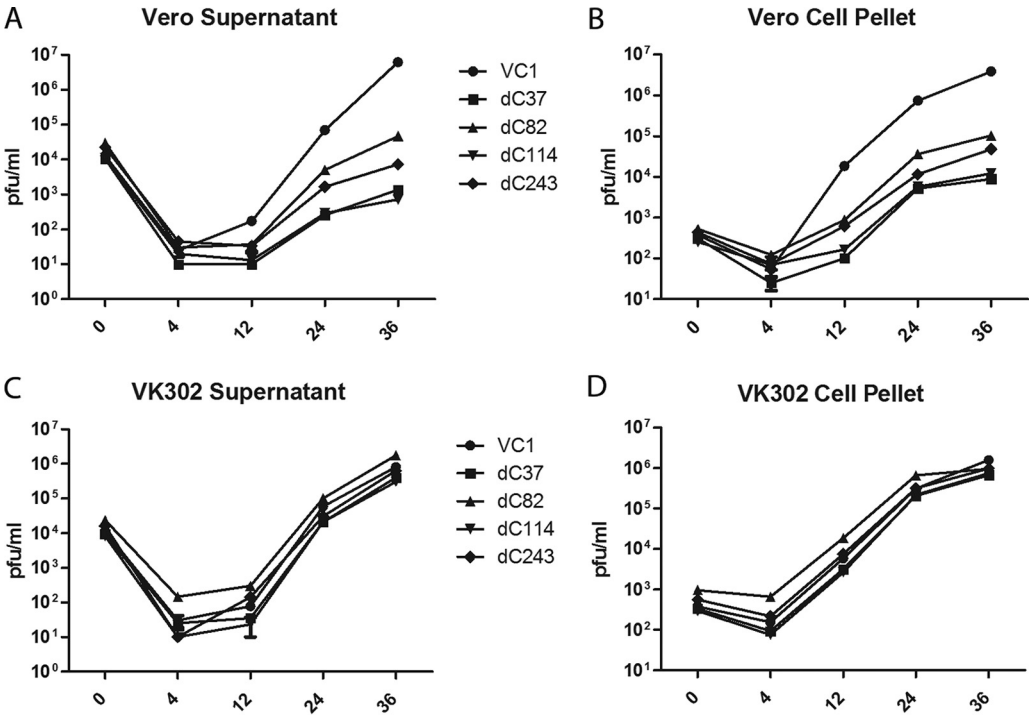


FIG 8 Cysteine mutants are defective for growth in both cell pellet and supernatant fractions. Vero cells (A and B) and gK-complementing (VK302) cells (C and D) were infected with parental virus (VC1) and the indicated cysteine mutant viruses. Supernatants and cell pellets, as indicated, were separated at various times postinfection, and plaque assays were performed to determine viral titers.

than the dC37 and dC114 viruses. Importantly, all viruses tested were complemented for growth on VK302 cells, and titers were indistinguishable from those of the parental VC1 virus at all time points assayed, both in supernatants and cell pellets.

DISCUSSION

Wild-type HSV-1 strains cause a limited amount of cell-to-cell fusion, while mutations in three genes coding for gB, gK, and UL20 cause extensive syncytium formation, defining these genes as key players in virus-induced membrane fusion phenomena. All three proteins are membrane proteins found in mature virion particles. gB is the sole herpesviral fusogen. UL20 is a nonglycosylated four-transmembrane domain envelope protein, which forms a complex with gK in all alphaherpesvirus species tested and interacts with carboxyl terminus of gB (20, 22–24, 37, 38). gK is the only HSV-1 protein in which most syncytial mutations map to the extracellular portion of the protein and specifically its amino terminus, which is also known to interact with the amino terminus of gB. In this regard, the gK/UL20 protein complex appears to regulate gB's fusogenicity through direct binding to gB at both its extracellular and intracellular domains. Here, we have explored the role of gK's amino-terminal N glycosylation and the conserved cysteine residues in infectious virus production and membrane fusion by generating single and double mutant viruses. The results indicated that at least one of the two N glycosylation sites and two of the four conserved cysteine residues play crucial roles in the function of gK in infectious virus production and membrane fusion.

Glycoproteins embedded in viral envelopes serve important roles for entry, assembly, immune evasion, signaling, and pathogenesis (39). Specifically, N-linked glycosylation of the influenza virus hemagglutinin and neuraminidase has been shown to influence receptor binding and virulence (40–44). A major focus of work in glycobiology is the role of N-linked glycosylation in protein processing (34, 45–47). N-linked glycosylation takes place in the endoplasmic reticulum, and further modifications to glycans

occur in the Golgi network. However, initial glycosylation in the endoplasmic reticulum occurs cotranslationally, which is thought to be critical for proper folding of nascent proteins. Seminal work of Hammond et al. demonstrated a critical role for N-linked glycosylation in viral glycoprotein folding (48), and it has since been shown that the positioning of glycosylation sites is important for optimal protein expression (33). N-linked glycans are critical for directing proper folding of viral and cellular proteins, and proper folding is important for correct disulfide bond formation (35, 45, 49, 50). Not surprisingly, due to the importance of glycosylation to protein processing, mutations in glycosylation have profound effects on virion assembly. N-linked glycosylation sites have been shown to be important for proper localization and subsequent incorporation of the Ebola virus envelope glycoprotein (GP) into pseudovirions and Lassa virus glycoprotein GP-C cleavage (51, 52). N-linked glycosylation of West Nile and Zika virus envelope proteins influences assembly and infectivity in a cell-type-specific manner (53–56). Mutations in VSV G protein that affect N-linked glycosylation sites lead to improper disulfide bonding affecting transport and maturation of the viral fusogen (57).

The role of N-glycosylation on infectious virus produced and virus-induced cell fusion. A striking feature of N-glycosylation sites of gK is the largely conserved spacing of these sites among most alphaherpesviruses, suggesting a conserved role in the overall structure and function of gK. N-glycosylation may contribute to preservation of the overall structure of the amino terminus of gK and facilitate interactions of gK with the amino terminus of gB or potentially with other viral and cellular proteins. Mutations in gK leading to syncytium formation support a role for gK in the negative regulation of fusion. Indeed, in transient-transfection assays expression of HSV-1 gB, gD, and gH/gL along with UL20 and gK led to a decrease in the amount of fusion, while the same transfection where wild-type gK was replaced by gK carrying a syncytial mutation did not have any effect on membrane fusion (17). It is possible that the N-glycosylation moieties facilitate interaction with gB and are involved in gB-mediated fusion. Our mutagenesis analysis indicates that the second glycosylation site at residue 58 plays a crucial role since when it is changed, the resultant virus causes extensive cell fusion, while the N-glycosylation site at residue 48 did not appear to produce a substantial effect in either virus production or membrane fusion. Therefore, the N48 glycosylation site appears to play a secondary role, potentially involved in other unknown functions of gK. The apparent conservation of the distance between N-linked glycosylation sites in the amino terminus of alphaherpesvirus gKs suggests that this domain bracketed by the N glycosylation sites may play a role in binding a host protein. Alternatively, the use of N-linked glycosylation to evade humoral immunity is well documented (58, 59). In addition, the VC2 live attenuated vaccine strain, which has both N glycosylation sites deleted, has been shown in guinea pigs and mice to generate robust immune responses that are superior to those produced by its parental wild-type virus HSV-1(F) (26, 28, 29; unpublished observations). Finally, mutant N58A and the double mutant N48A N58A produced significantly smaller amounts of gK as detected in Western immunoblots. We believe that this was primarily due to solubility problems since lack of glycosylation causes increased aggregation. However, it is also possible that smaller amounts of gK may have contributed to the generation of the observed syncytial phenotype.

The role of cysteine residues within the amino terminus of gK in infectious virus production and virus-induced cell fusion. Syncytial cysteine mutants were defective for growth in cell pellet fractions, and little infectious virus was recovered from the supernatant fraction. Therefore, we hypothesize that these cysteine residues are involved in the overall structure of the amino terminus of gK through disulfide bond formation. This allows the consideration that mutagenesis of the N58 glycosylation site that produces a syncytial phenotype may displace formation of the appropriate disulfide bond involving the cysteine residues that appear to also be involved in regulation of membrane fusion.

The phenotypes of the cysteine mutants suggest disulfide bonding between C37 and C114 as well as between C82 and C243. These biological data support our predicted structure for gK as these amino acid pairings are found to be in close

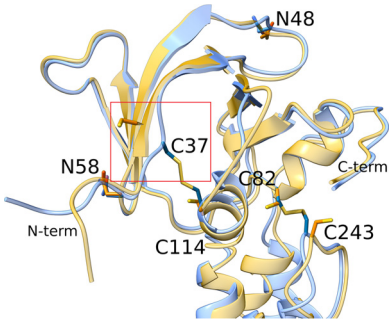


FIG 9 Refinement to gK protein model. Remodeled gK protein incorporates disulfide bond formation between C37 and C114 and between C82 and C243. Extracellular domains of the early and the refined gK protein structures are superimposed and shown in gold and blue, respectively. The red box highlights the predicted shortening of the first beta strand due to the introduction of disulfide bonding between C37 and C114. The initial gK models had an LGscore and MaxSub scores of 1.977 and 0.160, respectively. Including the disulfide bonds improved both scores (LGscore, 2.058; Maxsub score, 0.166). Asparagine and cysteine residues are shown as sticks.

proximity in the model. We have refined our model to include these new constraints (Fig. 9). Including the disulfide bonds improved the quality of the model, as indicated by both LGscore and MaxSub score. Including the disulfide bonding between residues C82 and C114 resulted in little change to the predicted structure. However, including disulfide bonding between residues C37 and C114 resulted in a dramatic shortening of the first predicted beta sheet in the amino terminus of gK.

We have reported previously that virus with a deletion in amino acids 31 to 47 of HSV-1 gK, which includes the first cysteine, exhibits a gK-null phenotype (25). A virus in which the entire amino terminus of gK was deleted was similarly defective. However, virus with a deletion spanning amino acids 31 to 68 of HSV-1 gK, which includes the first cysteine as well as both N-linked glycosylation sites, exhibits only a 1-log defect in peak titers and is not syncytial. These results suggest a potentially important relationship between glycosylation and disulfide bond formation in the amino terminus of gK. We hypothesize that C38 and C115 residues are critical to achieving proper structural conformation of the amino terminus of gK. The inability to achieve the proper structure may interfere with gK's transit or function in some manner that results in a null phenotype. Interestingly mutation of the glycosylation site N58 leads to a syncytial phenotype, while deletion of the region of amino acids 31 to 68 that contains both N-glycosylation sites does not. It is possible that the gK domain that is bracketed and includes both glycosylation sites may be dispensable for infectious virus production but necessary for gK regulation of gB-mediated cell fusion since syncytial mutations in gB fail to cause fusion in the presence of the gK(d31–68) mutant (25). Our syncytial gK mutants appear to be dominant negative since syncytial mutants in this study produced syncytial plaques on the gK-complementing cell line VK302. These gK mutants may act in a dominant negative manner by retaining their ability to bind gB while being unable to regulate its activity. This ability to bind gB may prevent wild-type gK from binding and thus restoring the proper regulation of fusion.

An intriguing possibility suggested by the role of disulfide bond reduction in the conversion of many viral fusion proteins from a pre- to postfusion conformation is that the probable disulfide linkage between cysteines 82 and 243 of gK acts as a molecular switch for gB-mediated fusion. The generation of free thiols by reduction of disulfide bonds has been shown to be important for fusion protein activity in multiple virus families (60). Protein disulfide isomerases (PDI) are cellular enzymes that catalyze the breaking and formation of disulfide bonds (61). For Newcastle disease virus (NDV) overexpression of PDI increased conversion of F protein to the postfusion conformation and enhanced fusogenicity of NDV (62). Additionally, entry of HIV-1 requires disulfide exchange (63), and inhibition of thiol-reactive proteins prevents entry of HIV (64, 65). Similarly, thiol-reactive proteins may be involved in HSV-1 membrane fusion. The high

TABLE 2 Oligonucleotides used in this study[a]

Name	Sequence
N48A forward	CGCTGCACCGATGTATTTACGCGGTACGCCCCACCGGCACCAACGCAGACACCGCCCTCG**CAACCAATTAACCAATTCTGATTAG**
N48A reverse	CGTCGGGGCCCCCAGAAACAATAGGGTCTGGTTCATTTTCATCCACACGAGGGCGGTGTCTGCGTTGGTGCCGGTGGGGCGTACCG **AGGATGACGACGATAAGTAGGG**
N58A forward	GTACGCCCCACCGGCACCAACAACGACACCGCCCTCGTGTGGATGAAAATGGCACAGACCCTATTG**CAACCAATTAACCAATT CTGATTAG**
N58A reverse	CGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCGTCGGGGCCCCCAGAAACAATAGGGTCTGTGCCATTTTC **AGGATGACGACGATAAGTAGGG**
Delta C37 forward	TCCACACGAGGGCGGTGTCGTTGTTGGTGCCGGTGGGGCGTACCGCGTAAATTCGGTGCAGCGGACTGGCACCGAAG **AGGATGACGACGATAAGTAGGG**
Delta C37 reverse	GGCGTACGGCCTCGTGCTCGTGTGGTACACCGTCTTCGGTGCCAGTCCGCTGCACCGAATTTACGCGGTACGCCCCACCGGCA **CAACCAATTAACCAATTCTGATTAG**
Delta C82 forward	CATGGCGTCGGGTGGGACCTGGAAGGGCACGACCCTACCCGCGATAAGATTGGCGTAGATATGGGCGTGGTTGCGCCAGC **AGGATGACGACGATAAGTAGGG**
Delta C82 reverse	TGGGGGCCCCGACGCACCCCCCCAACGGGGGCTGGCGCAACCACGCCCATATCTACGCCAATCTTATCGCGGGTAGGGTCGT **CAACCAATTAACCAATTCTGATTAG**
Delta C114 forward	GAACCACCCTACGACCACCAGACGCACCCGTGTGTACCATAGGGTCTCCAGTGCAGGATGACGACGATAAGTAGGG
Delta C114 reverse	GGTCCCACCCGACGCCATGAATCGTCGGATCATGAACGTCCACGAGGCAGTTAAC**GCA**CTGGAGACCCTATGGTACACACGGGTGC **CAACCAATTAACCAATTCTGATTAG**
Delta C243 forward	TCTGTCAGGCCGATGGTGGAGACAAAACACCAGGTGGTGATGGTCAGAAACAGGGGGTATGTGATCGC **AGGATGACGACGATAAGTAGGG**
Delta C243 reverse	CTTTGTGGCCGTGGGTCTCATCGTCGGCACCGCTTTCATATCCCGGGGGGCAGCGATCACATACCCCCTGTTTCT GACCATCACCACCTG**CAACCAATTAACCAATTCTGATTAG**
HSV-1 gK F	GACACCATAAGTACGTGGCAT
HSV-1 gK R	CTGGGTCCTCCTACAGCTAGT

[a]Boldface indicates homology to the pEPkan-S plasmid sequence.

degree of predicted structural conservation among all alphaherpesvirus-encoded gKs suggests that gK functions in a similar manner to facilitate virus entry especially into neuronal axons. This hypothesis is currently being tested in our laboratory for other alphaherpesviruses including varicella-zoster virus (VZV) and bovine herpesvirus-1 (BHV-1).

MATERIALS AND METHODS

Cells. African green monkey kidney cells (Vero cells) were obtained from the ATCC (Rockville, MD). The Vero cell-derived gK-complementing cell line VK302 was originally obtained from David Johnson (Oregon Health Sciences University, Portland, OR). Both Vero and VK302 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Immunohistochemistry. Vero or VK302 cells were infected with either VC1 or mutant virus for 48 h and fixed with 3.5% formalin overnight at room temperature. After cells were washed, they were incubated with anti-HSV-1 antibody (Agilent, Santa Clara, CA) for 1 h. Subsequently, polyclonal goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (HRP) (Agilent, Santa Clara, CA) were added for 30 min at room temperature. For detection, NovaRED peroxidase (HRP) substrate (Vector, Burlingame, CA) was applied until an appropriate level of staining was obtained. Images were obtained using an Olympus IX2 inverted microscope and cellSens software (Olympus, Waltham, MA).

Virus and BAC mutagenesis. Bacterial artificial chromosome (BAC) plasmid pYEbac102 carrying the HSV-1(F) genome (a gift from Y. Kawaguchi, University of Tokyo, Japan) was used to construct YE102-VC1 (VC1) as previously described (30). VC1 was used to construct all recombinant BACs described in this study. High-efficiency markerless DNA manipulation of VC1 was achieved using two-step red-mediated recombination (66). Oligonucleotides used in the construction of recombinant virus are presented in Table 2. Recombinant HSV-1 was recovered after BACs were transfected into Vero cells using Lipofectamine according to the manufacturer's protocol. DNA was extracted from viral stocks, and gK was sequenced to ensure the presence of the desired mutation.

Immunoprecipitation and Western blot assays. Uninfected and infected cells were lysed using NP-40. Lysate was immunoprecipitated with protein G magnetic Dynabeads (Thermo Fisher) bound to mouse V5 antibody (46-1157; Invitrogen) according to the manufacturer's instructions. The protein was eluted from the magnetic beads in 40 μl of elution buffer and used for immunoblot assays. Immunoblot assays were carried out using anti-VP5 (ab6508; Abcam), rabbit anti-beta tubulin (ab179513; Abcam), goat anti-mouse HRP (Abcam), and goat anti-rabbit HRP (Abcam). Intensity quantification of Western blots was carried out using ImageJ (67).

Molecular visualization. The structure of HSV-1 gK was predicted using assembly of separate domain models (19). Molecular visualization was performed with UCSF ChimeraX, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (UCSF), to generate a hybrid surface-cartoon representation of the protein. The N terminus and the third

domain, an extracellular loop, are shown in orange ribbon diagrams with a transparent surface, and the rest of the protein, including the transmembrane domain, is shown in blue. The gK protein model has been placed in a lipid bilayer using the PPM server (68). Based on PPM predictions, the thickness of the hydrophobic depth of the protein is estimated to be 17.0 ± 1.5 Å, while the ΔG of protein transfer into the membrane with a tilt angle of 35 ± 4° is estimated to be −20.4 kcal/mol. Model quality was determined via the LGscore and MaxSub score (69).

ACKNOWLEDGMENTS

K.G.K. and P.J.F.R. were funded by the Louisiana Board of Regents Governor's Biotechnology Initiative, by NIH grant P20GM103424, and by the Louisiana State University School of Veterinary Medicine (LSU/SVM) Division of Biotechnology & Molecular Medicine. M.B. and M.N. were supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R35GM119524.

We thank Rajiv Gandhi Govindaraj for his help with generating the protein structure molecular graphics.

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