

The Amino Terminus of Herpes Simplex Virus 1 Glycoprotein K Is Required for Virion Entry via the Paired Immunoglobulin-Like Type-2 Receptor Alpha

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The herpes simplex virus 1 (HSV-1) glycoprotein K (gK)/UL20 protein complex is incorporated into virion envelopes and cellular membranes and functions during virus entry and cell-to-cell spread. To investigate the role of gK/UL20 in the context of a highly neurovirulent virus strain, the HSV-1 (McKrae) genome was cloned into a bacterial artificial chromosome plasmid (McKbac) and utilized to construct the mutant virus McK(gK Δ 31-68), carrying a 37-amino-acid deletion within the gK amino terminus. The McKbac virus entered efficiently into Chinese hamster ovary (CHO) cells constitutively expressing HSV-1 human receptors, nectin-1, herpesvirus entry mediator (HVEM), or paired immunoglobulin-like type-2 receptor alpha (PILR α). In contrast, the McK(gK Δ 31-68) virus failed to enter into CHO-PILR α cells, while it entered CHO cells expressing HVEM and nectin-1 more efficiently than the McKbac virus. Both McKbac and McK(gK Δ 31-68) viruses entered all CHO cells expressing HSV-1 receptors via a pH-independent pathway. The HSV-1(F) gB Δ 28syn mutant virus, encoding a carboxyl-terminal truncated gB, causes extensive cell fusion. Previously, we showed that the gK Δ 31-68 amino acid deletion abrogated gB Δ 28syn virus-induced cell fusion, indicating that the amino terminus of gK is required for gB-mediated virus-induced cell fusion (V. N. Chouljenko, A. V. Iyer, S. Chowdhury, D. V. Chouljenko, and K. G. J. Kousoulas, *Virology* 83:12301–12313, 2009). Surprisingly, the gK Δ 31-68/gB Δ 28syn virus caused extensive fusion of CHO-nectin-1 cells but limited cell fusion of CHO-PILR α cells. Coimmunoprecipitation experiments revealed that both gK and PILR α bound gB in infected cells. Collectively, these results indicate that the amino terminus of gK is functionally and physically associated with the gB-PILR α protein complex and regulates membrane fusion of the viral envelope with cellular membranes during virus entry as well as virus-induced cell-to-cell fusion.

The herpes simplex virus 1 (HSV-1) entry mechanism is both complex and unique among enveloped viruses, involving multiple glycoproteins for attachment, binding, and membrane fusion (1). Viral glycoproteins interact with different cellular receptors to facilitate virus entry. Initial attachment of the virus to cellular membranes is mediated by interaction of glycoproteins gB and gC with glycosaminoglycan (GAG) moieties of cell surface proteoglycans (2, 3). Attachment of virions to cellular membranes facilitates subsequent binding of gD to cellular receptors, including the herpesvirus entry mediator (HVEM, also called HveA), nectin-1 (HveC), and 3-O-sulfated heparan sulfate (4–6). Apparently, gB can also bind to additional cellular receptors, including paired immunoglobulin-like type 2 receptor alpha (PILR α), non-muscle myosin heavy chain IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG) (7–9). Sequential binding of gD and then gB to their respective cellular receptors during virus entry and virus-induced cell-to-cell fusion is thought to alter gB's conformation, resulting in gB-mediated membrane fusion (1, 10–12).

HSV-1 can enter into cells by utilizing different cell-dependent pathways: (i) virus entry into Vero and HEp-2 cells is predominantly mediated via pH-independent fusion of the viral envelope with the host cell membrane (13); (ii) virus entry into HeLa and Chinese hamster ovary (CHO) cells expressing the nectin-1 gD receptor is predominantly achieved via receptor-mediated endocytosis, followed by pH-dependent fusion of the viral envelope with endocytic membranes (14); and (iii) virus entry into C10 murine melanoma cells predominantly occurs via pH-independent endocytosis (13). Recently, it has been suggested that gB-specific receptors, such as PILR α , or other cellular plasma mem-

brane factors determine whether virions enter predominantly via fusion at the plasma membrane or via receptor-mediated endocytosis (pH dependent or pH independent), followed by fusion of the viral envelope with endosomal membranes (15).

HSV-1 gK has been shown to be involved in neurovirulence and immunomodulation (16, 17). We have shown that HSV-1 gK and UL20 functionally and physically interact, and this interaction is mandatory for their coordinated intracellular transport, cell surface expression, and functions in virion egress, virus-induced cell fusion, and virus entry (18–21). Recently, we showed that the amino terminus of gK interacts with gB and gH and can complement gB-mediated cell fusion (22, 23). Virions lacking the entire gK or the amino terminus of gK (amino acids [aa] 31 to 68) enter susceptible cells substantially slower than the wild-type virus (20, 21). Importantly, gK-null virions cannot infect neurons via their neuronal axons (24), an entry route known to involve fusion of the viral envelope with synaptic membranes of axonal neuronal termini (25). Overall, these results have strongly suggested that the gK/UL20 protein complex can regulate virus entry and virus-induced cell fusion via modulating gB and gH membrane fusion functions.

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Here, we show that the gK amino terminus is necessary for efficient virus entry via the gB-specific receptor PILR α but not for entry via the gD-specific receptors nectin-1 and HVEM. Moreover, we show that PILR α forms a multiprotein complex with gB and gK but not gD. In contrast to previous reports (14), we show that HSV-1(McKrae) entry occurs via a pH-independent mechanism. These results suggest a functional and physical association of gK/UL20 with gB and gB-specific receptors, such as PILR α , during virus entry and virus-induced cell fusion.

MATERIALS AND METHODS

Cells and plasmids. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown and propagated in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics (22). VK302 cells permanently expressing gK were originally obtained from David Johnson (Oregon Health Sciences University, Portland, OR) and maintained in growth medium. CHO-nectin-1(human) cells were a gift from Richard Longnecker (Northwestern University, Chicago, IL) and were propagated in Ham's F12 growth medium supplemented with 10% FBS and 200 μ g/ml G418. The CHO-PILR α (human) cells were also obtained from R. Longnecker and were grown in Ham's F12 medium supplemented with 10% FBS and 700 μ g/ml G418. The human CHO-HVEM cells were created by using a PiggyBac Transposon system (System Biosciences) as described earlier (26). All cells were cultured in nonselective medium prior to use in the virus entry assay. 293 PEAK TM Rapid cells (a gift from R. Longnecker) were grown in DMEM supplemented with 10% FBS and antibiotics. Plasmids expressing the soluble human PILR α -IgG Fc hybrid protein (pME18S-PILR α -Ig) and CD200-Ig Fc fusion protein were kind gifts from Hishashi Arase (Osaka University, Osaka, Japan) and Y. Kawaguchi (University of Tokyo, Tokyo, Japan), respectively. Plasmids expressing the Ig fusion proteins were constructed as described previously (8).

Viruses. The clinical ocular, neuroinvasive HSV-1(McKrae) strain was obtained from J. M. Hill (Louisiana State University Health Sciences Center, New Orleans, LA). The McKrae genome was cloned into the bacterial artificial chromosome (BAC) vector, which also contains an excisable enhanced green fluorescent protein (EGFP) expression cassette. This BAC was used to recover the McKrae-EGFP-BAC (McKbac) virus. The recombinant mutant virus HSV-1(F) YE102-VC1 specifies gK and UL20 tagged with V5 and 3 \times FLAG antigenic epitopes, respectively. GFP-tagged vesicular stomatitis virus (VSV-GFP) was a kind gift from John Rose (Yale University, New Haven, CT). The mutant viruses gK Δ 31-68, gB Δ 28syn (deletion of 28 aa from the carboxyl terminus of gB), and gK Δ 31-68/gB Δ 28syn were described earlier (22).

Construction of mutant viruses. The HSV-1 McKrae (wild-type) strain was cloned into the BAC plasmid pBeloBAC11 (NEB) essentially as described previously (27), with the following modifications. Specifically, a gene construct was created wherein the LoxP recombination site was fused together with DNA fragments of approximately 1 kb of McKrae DNA sequence, encompassing portions of the UL3(L-left)-UL4(R-right) targeted genomic region by PCR-based overlap extension. This gene construct was subsequently cloned into the pCR2.1-TOPO plasmid vector (Invitrogen, Grand Island, NY). Similarly, another gene expression cassette containing the EGFP gene flanked by FLP recombination target (FRT) sites (enabling removal of the EGFP gene cassette) was constructed and cloned into pEF6/V5-His TOPO plasmid vector (Invitrogen) under the control of the EF1 α promoter. This gene cassette was excised from the vector by using EcoRI endonuclease and blunt ended by T4 polymerase. Subsequently, it was cloned into the blunt-ended BamHI site of pBeloBAC11. Cre recombinase enzyme (New England BioLabs, Ipswich, MA) was used for *in vitro* reaction to fuse together the L-LoxP-R ampicillin-resistant PCR 2.1-based plasmid and LoxP-EGFP chloramphenicol-resistant pBeloBAC-based plasmid. The plasmid DNA created after fusion with Cre recombinase was used to transfect Vero cells using Lipo-

fectamine 2000 (Invitrogen). The transfected cells were infected with HSV-1(McKrae) at 6 h posttransfection. Fluorescence microscopy was used to select fluorescent viral plaques that contained the inserted McKbac genome in the BAC plasmid (McKbac). The presence of the BAC plasmid was verified by PCR-assisted DNA sequencing. The recovered McKbac virus was utilized for the construction of gK mutant viruses. The mutant viruses gK Δ 31-68 (encoding gK carrying an in-frame deletion of aa 31 to 68) and gK Δ 31-117 (encoding gK carrying an in-frame deletion of aa 31 to 117) were created as described earlier for the HSV-1(F) genome cloned into BAC plasmid pYEbac102 (28).

Replication kinetics, plaque morphologies of McKbac and gK mutant viruses, and electron microscopy. Viral plaques were visualized by immunohistochemistry as we have previously described (29–32). Analysis of one-step growth kinetics was performed as described earlier (20, 22, 33). Confluent Vero cell monolayers were infected with each virus at 4°C for 1 h at a multiplicity of infection (MOI) of either 0.1 or 2. Thereafter, plates were incubated at 37°C and 5% CO₂ and virus was allowed to penetrate for 1 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0), and cells were subsequently incubated at 37°C and 5% CO₂. Viral titers at different times postinfection were obtained on the VK302 cell line that expresses the HSV-1(KOS) gK gene. The ultrastructural morphology of virions within infected cells was examined by transmission electron microscopy as described previously (30, 32, 34–36). All infected cells were processed at 18 h postinfection (hpi) and visualized by transmission electron microscopy.

Virus entry assay. Confluent monolayers of CHO-neo (not shown), CHO-HVEM, CHO-PILR α , CHO-nectin-1, and Vero cells were infected with McKbac, McK(gK Δ 31-68), and McK(gK Δ 31-117) at an MOI of 1 for 1 h at 34°C. The virus inoculum was subsequently removed, and the cultures were shifted to 37°C. Eight to 12 hpi, the cells were fixed, stained with anti-ICP4 antibody (Ab) (Virusys, Inc., Taneytown, MD) and Alexa Fluor 647 goat anti-mouse IgG1 (Life Technologies, Grand Island, NY), and analyzed by flow cytometry (fluorescent-activated cell sorting [FACS]). The relative efficiency of virus entry was calculated as the percentage of cells expressing ICP4 and normalized to CHO-neo entry values. Entry experiments were performed in triplicate, and error bars represent standard errors of the means. Comparison of groups was performed by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 5) (21). A *P* value of ≤ 0.05 was considered significant.

Treatments with lysosomotropic agents. Stock solutions of ammonium chloride (1.5 M) were prepared in distilled water immediately prior to use. Monensin (75 mM; Sigma, St. Louis, MO) and bafilomycin A1 (100 mM; Sigma) were dissolved in ethanol and dimethyl sulfoxide, respectively, and stored at -20°C . Growth medium was removed from cells and replaced by medium containing inhibitors, and the plate was incubated for 1 h at 37°C. After incubation, virus was added in the presence of the inhibitor for 1 h at 34°C. Subsequently, cells were incubated again in the presence of inhibitor until they were processed for flow cytometry.

Immunohistochemistry. Nearly confluent CHO-neo, CHO-nectin-1, and CHO-PILR α cell monolayers were infected at an MOI of 1 with mutant viruses gK Δ 31-68, gB Δ 28syn, and gK Δ 31-68/gB Δ 28syn [all mutants were constructed in the HSV-1(F) genetic background], and HSV-1(F). Fourteen hpi, monolayers were washed with Tris-buffered saline (TBS)-Ca-Mg and fixed with ice-cold 100% methanol for 10 min. Cell fusion was visualized by immunostaining with anti-HSV-1 polyclonal antibody as described previously (22).

Preparation of purified Ig fusion proteins and immunoprecipitation assay. 293 PEAK Rapid cells were transfected with the plasmid pME18S-PILR α -Ig and a control plasmid expressing human CD200-Ig Fc fusion protein with 293 Fectin (Invitrogen, Grand Island, NY). Supernatants from transfected cells were collected at 48 and 72 h after transfection and pooled. Ig fusion proteins were purified by protein A affinity chromatography and were subsequently used for immunoprecipitation. Vero cells infected with the YE102-VC1 virus were lysed with NP-40 lysis buffer (Invitrogen). Subsequently, lysates were immunoprecipitated with

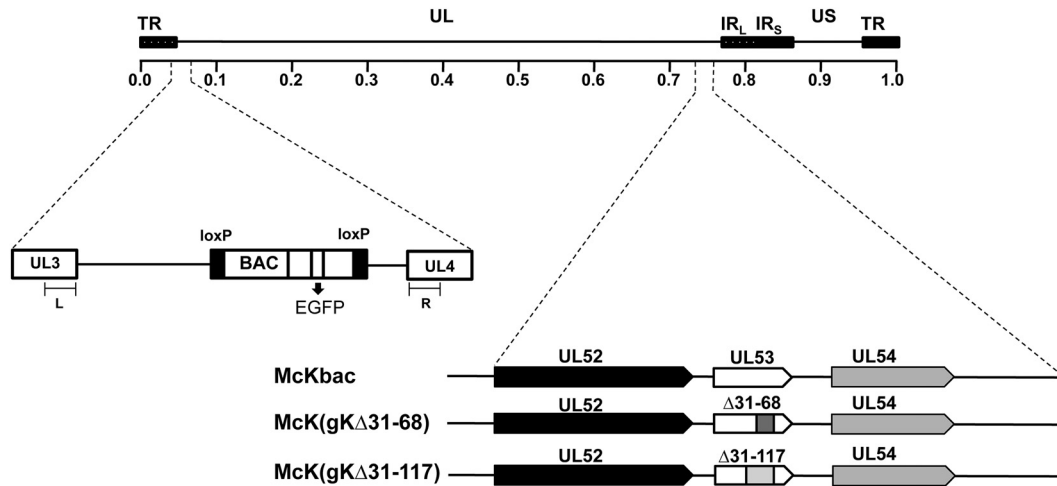


FIG 1 Cloning of the HSV-1(McKrae) genome into a BAC and schematic representation of mutant viruses. Shown is a linear representation of the HSV-1 genome structure, with two unique regions (long-UL and short-US), each flanked by a pair of terminal and inverted repeats (TR and IR, respectively). An expanded region of the HSV-1 genome in the intergenic region between UL3 and UL4 is shown. L and R represent DNA fragments derived from the UL3 and UL4 genes, respectively, used for recombination purposes. The BAC containing an EGFP sequence was inserted between the intergenic region of UL3 and UL4 to create a recombinant virus McKrae BAC (McKbac). The McKbac virus was utilized for the construction of gK mutant viruses gK Δ 31-68 (encoding gK carrying an in-frame deletion of aa 31 to 68) and gK Δ 31-117 (encoding gK carrying an in-frame deletion of aa 31 to 117). An expanded genomic region of the UL53 gene which encodes glycoprotein K (gK), flanked by UL52 and UL54 genes, is shown.

PILR α -Ig or CD200-Ig (control) using protein A Dynabeads (Invitrogen). Immunoprecipitates were separated on 5 to 20% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA), which were blotted with anti-gB, anti-gD, anti-FLAG, or anti-V5 Ab (21). Ig fusion proteins used for immunoprecipitation were detected by anti-human IgG Ab (Amersham Biosciences).

RESULTS

Construction and characterization of recombinant HSV-1(McKrae) viruses. To facilitate the construction of recombinant viruses carrying specific mutations in gK and other genes in the context of a highly neurovirulent virus strain, the HSV-1(McKrae) genome was cloned into a bacterial artificial chromosome essentially as described previously (27). Specifically, the BAC plasmid was inserted within the intergenic region between the UL3 and UL4 genes. The BAC plasmid contained a gene cassette encoding the enhanced green fluorescence protein (EGFP) under the elongation factor 1 alpha (EF1a) (Fig. 1). The cloned McKrae genome (McKbac) was subsequently utilized for the construction of recombinant viruses carrying deletions of 37 (gK Δ 31-68) and 86 (gK Δ 31-117) amino acids using the two-step markerless red recombination mutagenesis system (28) (Fig. 1), as previously described for the construction of similar mutant viruses in the HSV-1(F) genomic background (22).

The McKbac virus produced viral plaques on Vero cells that were similar to those of the HSV-1(McKrae) wild-type virus (not shown). The McK(gK Δ 31-117) recombinant virus produced very small viral plaques containing an average of 2 to 7 cells per plaque. In contrast, the McK(gK Δ 31-68) virus produced viral plaques that were much larger than those of the McK(gK Δ 31-117) virus, approaching approximately half the size of the McKbac wild-type-like plaques. Infection of VK302 cells that express the HSV-1(KOS) gK gene under the gD promoter control (37) resulted in efficient complementation for plaque morphology and virus spread for the McK(gK Δ 31-68) virus but not for the McK(gK Δ 31-117) virus (Fig. 2I). These plaque phenotypes were consistent with similar plaque morphologies and cellular spread defects exhibited

by the HSV-1(F) gK Δ 31-117 and gK Δ 31-68 mutant viruses with the following exceptions: (i) the McK(gK Δ 31-68) virus produced partially syncytial plaques in VK302 cells (Fig. 2I, panel E), unlike the HSV-1(F) gK Δ 31-68 virus characterized previously (22), and (ii) the McK(gK Δ 31-117) virus was not efficiently complemented in VK302 cells (Fig. 2I, panel F), unlike the HSV-1(F) gK Δ 31-117 virus (22). The apparent lack of complementation of the McK(gK Δ 31-117) virus by VK302 cells was not due to potential mutations elsewhere in the viral genome, since a recombinant virus produced by rescuing the gK Δ 31-117 deletion with homologous HSV-1(McKrae) gK sequence produced wild-type-like plaques (not shown). The McK(gK Δ 31-68) mutant virus replicated efficiently in Vero cells, achieving viral titers of approximately 1 log less than the parental McKbac virus. In contrast, the McK(gK Δ 31-117) virus replicated inefficiently, producing viral titers that were more than 3 logs less than that of the McKbac virus at both low MOI (0.1) and relatively high MOI (2) (Fig. 2II).

Ultrastructural characterization of McKbac and gK mutant viruses. The ultrastructural phenotypes of all mutant viruses relative to the wild-type parental virus were investigated at 18 hpi by visually examining 50 randomly selected virus-infected Vero cells using transmission electron microscopy. The McKbac virus did not exhibit any apparent defects in cytoplasmic virion envelopment and egress. Fully enveloped virion particles were observed in both intracellular compartments as well as in extracellular spaces (Fig. 3). Similarly, mutant virus McK(gK Δ 31-68) did not show any apparent defect in envelopment, and fully enveloped virion particles were excreted out of infected cells. Ultrastructural visualization of Vero cells infected with mutant virus McK(gK Δ 31-117) showed severe defects in virion envelopment and egress, characterized by the presence of numerous unenveloped capsids in the cytoplasm. These results are in agreement with the ultrastructural morphologies of mutant viruses carrying the same gK mutations in the HSV-1(F) genetic background (22).

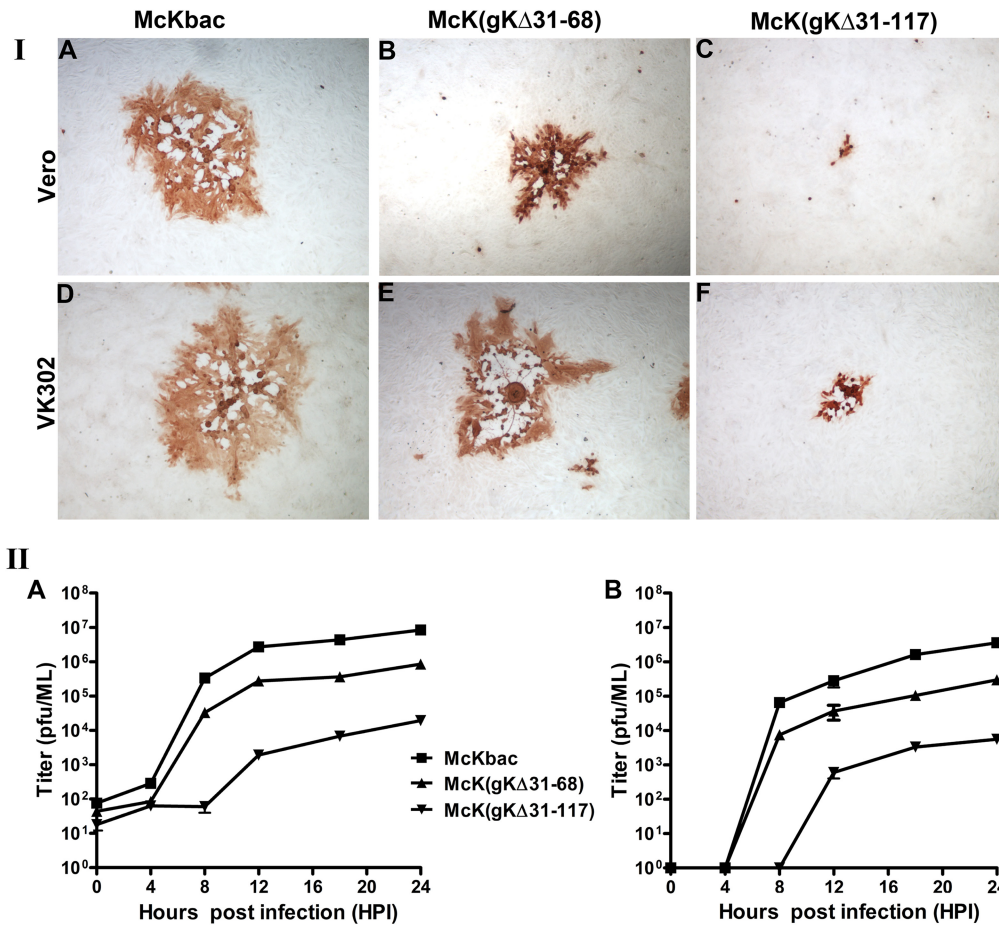


FIG 2 Plaque morphology and replication kinetics of the McKbac and gK mutant viruses. (I) Plaque morphology. Vero cells were infected with each virus at an MOI of 0.001, and at 48 hpi viral plaques were fixed with methanol and stained with anti-HSV antibodies as described in Materials and Methods. Representative viral plaques of all gK mutant viruses and the McKbac virus are shown on both Vero (A to C) and VK302 (D to F) cells. (II) Replication kinetics. Vero cells were infected with each virus at either a low MOI (0.1) or a high MOI (2), and the numbers of infectious viruses produced were determined on VK302 cells at different times postinfection. Viral titers after high-MOI infection are shown in panel A, and low-MOI infections are shown in panel B. All experiments were performed in triplicate. Error bars represent standard errors of the means.

Characterization of McKbac and gK mutant viral entry into Vero and CHO cells expressing different HSV-1 cellular receptors. We have shown previously that HSV-1(F) recombinant viruses gKΔ31-68 and gKΔ31-117 exhibit slower kinetics of entry

into Vero cells than their parental wild-type virus (21). To ascertain the relative efficiencies of virus entry into cells expressing different viral receptors, viruses were allowed to enter into cells for an hour at 34°C, and virus entry was detected by monitoring the

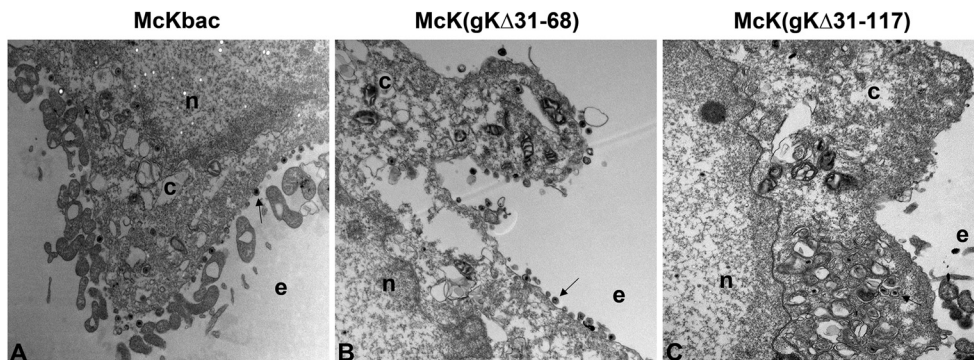


FIG 3 Ultrastructural morphology of McKbac and gK mutant viruses. Electron micrographs of Vero cells infected at an MOI of 2 with McKbac (A), McK(gKΔ31-68) (B), and McK(gKΔ31-117) (C) and processed for electron microscopy at 18 hpi are shown. Nucleus (n), cytoplasm (c), and extracellular space (e) are marked. Arrows indicate virion particles.

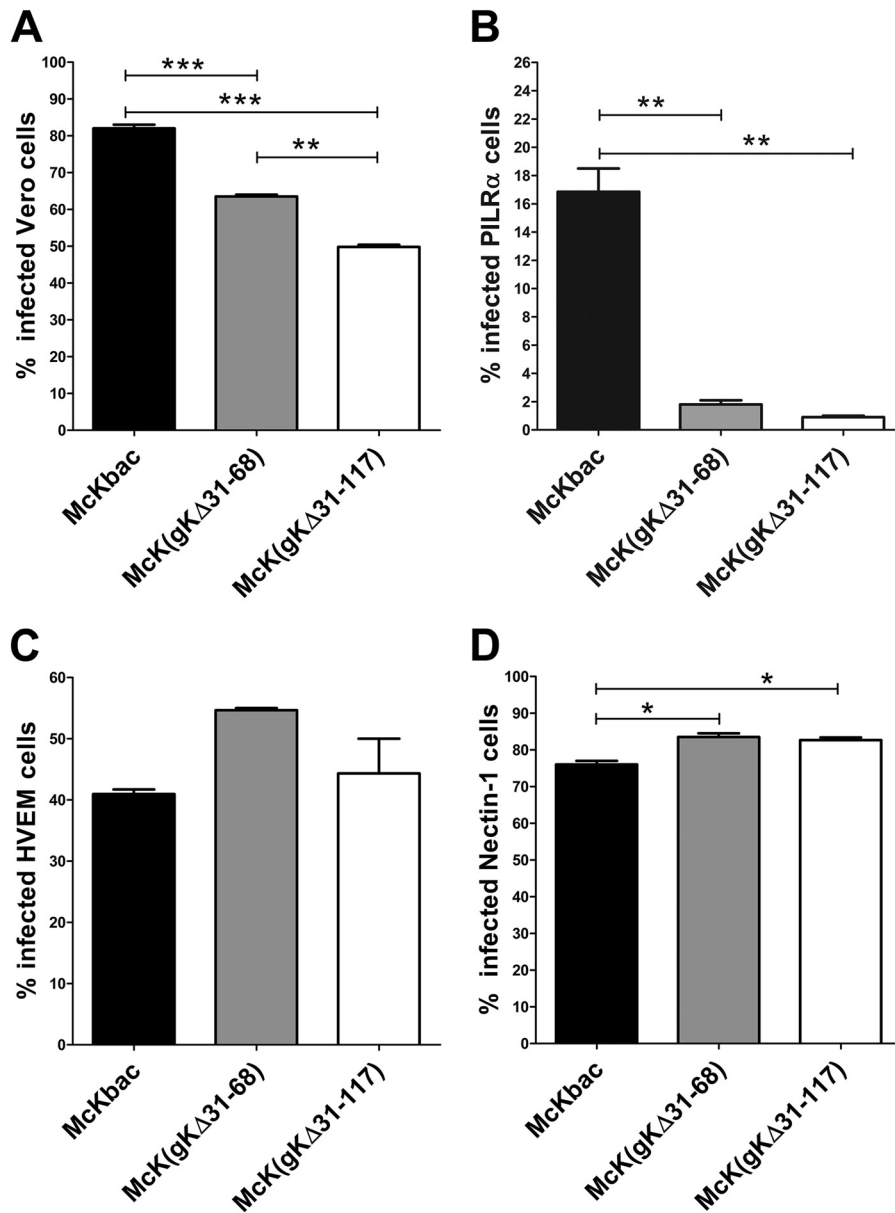


FIG 4 Comparison of entry efficiencies of McKbac and gK mutant viruses. (A) Entry into Vero cells. (B) Entry into CHO-PILR α cells. (C) Entry into CHO-HVEM cells. (D) Entry into CHO-nectin-1 cells. All cells were infected with McKbac and the gK mutant viruses gK Δ 31-68 and gK Δ 31-117 at an MOI of 1. At 8 to 12 hpi, the cells were stained with anti-ICP4 antibody and analyzed by flow cytometry to determine the percentage of infected cells. Comparison of groups was performed by one-way ANOVA (see Materials and Methods). $P \leq 0.05$ is considered significant. *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

level of ICP4 protein expression at 8 to 12 hpi as described previously (21) (also see Materials and Methods). Both gK mutant viruses entered into Vero cells, albeit with decreased efficiencies compared to the McKbac virus (Fig. 4A). These entry defects were particularly pronounced in PILR α -expressing CHO cells, in which both gK mutant viruses entered up to 8-fold less efficiently than the McKbac virus (Fig. 4B). In contrast, all viruses entered efficiently into both CHO-nectin-1 and CHO-HVEM cells, while gK mutant viruses appeared to enter more efficiently than the McKbac virus (Fig. 4C and D).

Effects of lysosomotropic agents on entry of McKbac and gK mutant viruses into different cell types. It has been reported that HSV-1 enters into HeLa and CHO-nectin-1 cells via pH-depen-

dent endocytosis (14). Lysosomotropic inhibitors NH $_4$ Cl, monensin, and bafilomycin inhibit the acidification of endosomes. The effect of these inhibitors on virus entry was investigated and compared to virus entry of vesicular stomatitis virus (VSV), which is known to enter via pH-sensitive endocytosis (38). NH $_4$ Cl and monensin inhibited VSV entry into CHO-nectin-1 cells in a dose-dependent manner (Fig. 5C and D). However, the two inhibitors inhibited neither McKbac nor gK mutant virus entry (Fig. 5A and B). Similarly, NH $_4$ Cl, monensin, and bafilomycin did not inhibit McKbac virus entry into CHO-PILR α cells (Fig. 5E, F, and G).

Effects of gK mutations on the ability of gB to cause virus-induced cell fusion in CHO cells expressing gB- or gD-specific receptors. To investigate the relationship between gB-induced

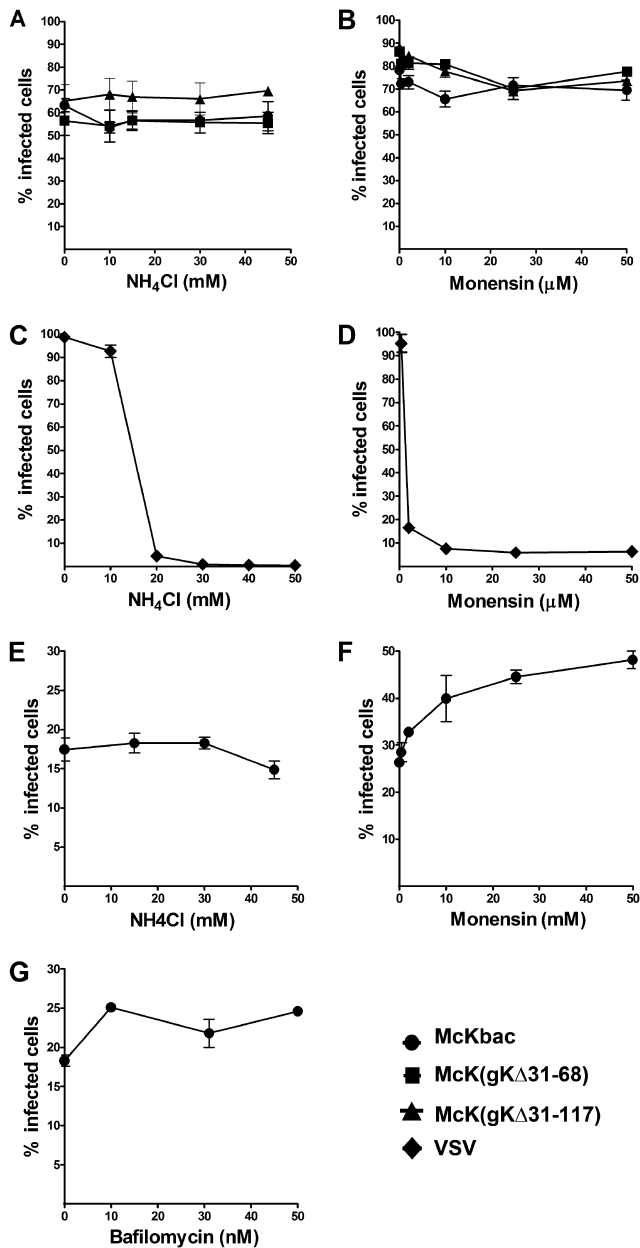


FIG 5 Effect of lysosomotropic agents on McKbac and gK mutant virus entry. Cells (CHO-nectin-1) were pretreated with different concentrations of ammonium chloride (A and C) and monensin (B and D) and infected with McKbac and McKbac gK mutants (A and B) or VSV (C and D) for 1 h at 34°C in the presence of the inhibitor. One hour after infection, the inoculum was removed and the cells were incubated in the presence of each inhibitor at 37°C until the cells were processed. The percentages of infected cells were determined after staining with anti-ICP4 antibody and analyzed by flow cytometry. Six hours after VSV infection, the percentage of GFP-positive cells were measured by flow cytometry. CHO-PILRα cells were pretreated with different concentrations of ammonium chloride (E), monensin (F), and bafilomycin (G), infected with McKbac, and processed for flow cytometry as described above.

cell fusion and specific viral receptors, the previously characterized set of viruses, HSV-1(F)gBΔ28syn, HSV-1(F)gKΔ31-68, and HSV-1(F)gKΔ31-68/gBΔ28syn, was utilized (22). Cells expressing different viral receptors were infected with these viruses, and the relative extent of cell-to-cell fusion was compared to that of the

HSV-1(F) wild-type virus (Fig. 6). The gBΔ28syn virus caused extensive fusion in Vero (22), CHO-nectin-1, CHO-HVEM (not shown), and CHO-PILRα cells but not in CHO-neo cells. In contrast, gKΔ31-68/gBΔ28syn caused extensive cell fusion in both CHO-HVEM (not shown) and CHO-nectin-1 cells, limited fusion in CHO-PILRα cells (Fig. 6), and no fusion in Vero cells (reference 22 and data not shown).

PILRα interacts with gB, gK, and UL20p in virus-infected cells. gB interacts with the amino terminus of PILRα (8). Moreover, we have shown that the amino terminus of gK, as a free peptide of 82 amino acids, interacted with the amino terminus of gB and complemented gB-mediated cell fusion (23). To investigate whether PILRα interacted simultaneously with both gB and gK, the purified PILRα-Ig fusion protein was mixed with cellular extracts obtained from infected cells and immunoprecipitated using magnetic beads coated with protein A (see Materials and Methods). Immunoprecipitates were subsequently tested for the presence of gB, gD, gK, and UL20 using specific antibodies for each protein in Western immunoblotting. Detection of gK and UL20 was accomplished after infection of Vero cells with the YE102-VC1 virus, which expresses gK tagged with the V5 epitope and UL20 tagged with the 3× FLAG epitope (21). Virus-infected cell lysates were immunoprecipitated with soluble PILRα (Fig. 7A, C, E, G, and I) and control IgG fusion protein CD200 IgG (Fig. 7B, D, F, H, and J). PILRα-Ig fusion protein immunoprecipitated gB (Fig. 7C, lane 2), UL20p (Fig. 7E, lane 2), and glycoprotein K (Fig. 7G, lane 2) but not gD (Fig. 7A, lane 2). The control CD200 IgG fusion protein did not react with any of the viral proteins (Fig. 7).

DISCUSSION

Glycoprotein K and UL20p are highly conserved among alphaherpesviruses, suggesting that they serve important functions that have been evolutionarily conserved (39). Both HSV-1 proteins have been intimately associated with membrane fusion phenomena, since mutations in either gK or UL20p cause extensive virus-induced cell fusion (31, 37, 40–45). Moreover, both gK and UL20p are structural components of the virion particle and function during virus entry (20, 21). Recent evidence suggests that gK and UL20p modulate these membrane fusion phenomena by direct interactions with viral gB (22, 23). Here, we have utilized CHO cells expressing individual viral receptors to demonstrate that gK is functionally and physically associated with the gB-PILRα interacting protein complex.

The McKrae viral strain was isolated from an ocular clinical sample of a patient suffering from acute infection (46). This viral strain is particularly virulent in mice and rabbits compared to other laboratory and clinical strains (47). Initial investigations suggested that the McKrae viral strain is able to utilize the PILRα receptor more efficiently than other viral strains (26). Therefore, the McKrae genome was cloned into a BAC plasmid to facilitate the construction of additional recombinant viruses with defined mutations in gK and UL20p. The McKbac virus appeared to have characteristics similar to those of the McKrae wild-type virus with regard to viral growth and plaque formation. Moreover, initial results suggest that insertion of the BAC plasmid did not substantially affect the neurovirulent characteristics of the parental McKrae virus in mice infected via the ocular route (not shown).

Recombinant McKbac viruses containing the gKΔ31-68 and gKΔ31-117 mutations appeared to replicate in a manner similar to that previously reported for identical mutations in the HSV-1(F)

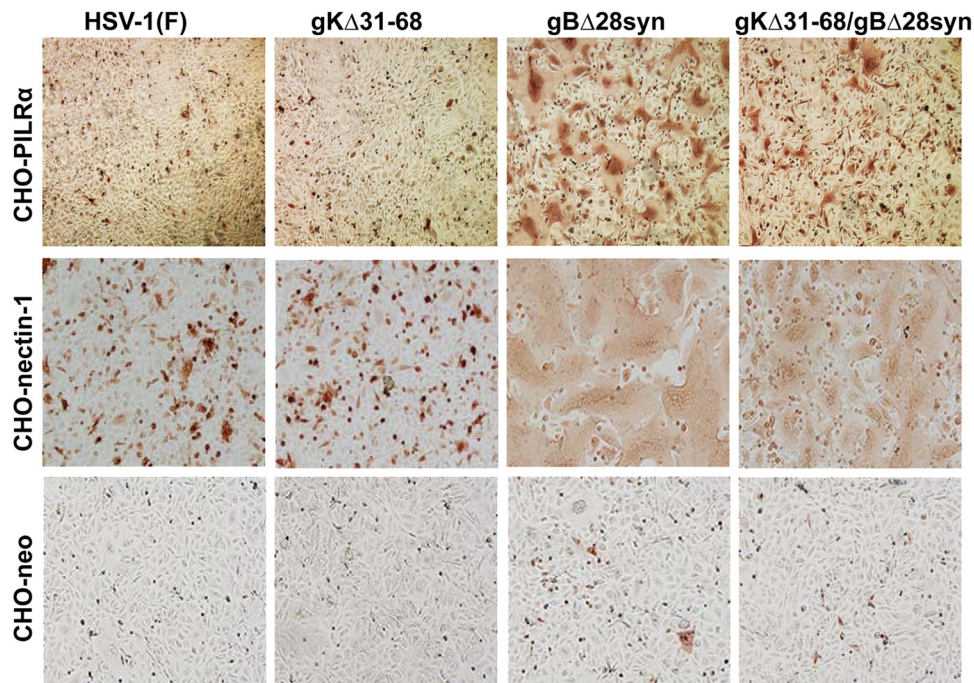


FIG 6 Visualization of relative extent of virus-induced cell fusion in CHO cells expressing different cellular receptors. CHO-nectin-1, CHO-PILR α , and CHO-neo cells were infected with HSV-1(F), gK Δ 31-68, gB Δ 28syn, and gK Δ 31-68/gB Δ 28syn. Fourteen hpi the cells were fixed with methanol and stained with anti-HSV antibody.

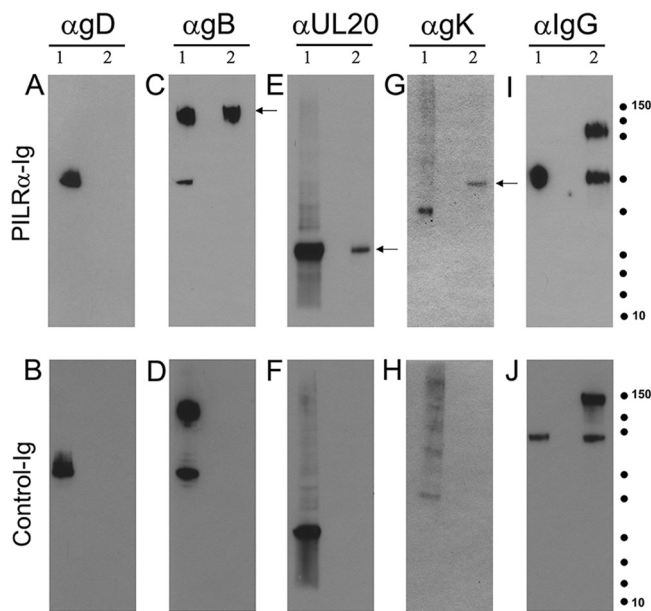


FIG 7 Soluble PILR α interacts with gB, gK, and UL20p in virus-infected cells. Western blot analysis of immunoprecipitates of PILR α ligand from HSV-1-infected cells is shown. Vero cells were infected with a double-tagged HSV-1 virus (YE102-VC1). (A to H) Infected lysates were immunoprecipitated with PILR α -Ig or CD200-Ig (control) and the immunoprecipitates were separated by SDS-PAGE, followed by blotting with anti-gB, anti-gD, anti-V5 (gK), and anti-FLAG (UL20) Ab. (I and J) The Ig fusion proteins used for immunoprecipitation were detected by anti-human IgG Ab. Molecular mass standards are shown as dots (150, 100, 75, 50, 37.25, 20, 15, and 10 kDa; Precision Plus protein standards; Bio-Rad). Lane 1, lysate from infected cells; lane 2, lysates immunoprecipitated with PILR α -Ig or CD200-Ig. Arrows indicate protein species of interest.

pYEbac102 genetic background. Plaque phenotypes, replication kinetics, and ultrastructure morphologies were generally similar irrespective of the viral genetic background (22), with the following differences. The McK(gK Δ 31-68) virus caused cell fusion in the gK-complementing cell line VK302, while the HSV-1(F) gK Δ 31-68 virus did not (22). Moreover, the HSV-1(F) gK Δ 31-117 virus was efficiently complemented by VK302 cells (22), while the McK(gK Δ 31-117) virus was not. Recently, we sequenced all major HSV-1(McKrae) genes encoding membrane proteins and glycoproteins (gB, gC, gD, gH, gL, gK, and UL20p) involved in membrane fusion and compared their primary structures to those of other viral strains. These results showed that the McKrae and F viral strains have identical gK and UL20 amino acid sequences; however, there are specific amino acid differences between their respective gB glycoproteins, predominantly located in their amino-terminal portions. Similarly, there are two amino acid differences between the gK expressed in VK302 cells (KOS genetic origin) and the gK amino acid sequences encoded by the McKrae and F strains at amino acid positions 101 (KOS-Thr to McKrae-Met) and 309 (KOS-Met to McKrae-Val) (26, 42). Therefore, these gB and gK amino acid differences may alter the known interactions of gK with gB (22, 23), resulting in the observed differential complementation of McK(gK Δ 31-117) and F(gK Δ 31-117) mutant viruses in VK302 cells.

Viral glycoproteins gB, gD, gH, and gL play important roles in mediating fusion of the viral envelope with cellular membranes and fusion of adjacent infected cells (1, 10). Historically, gK and UL20 have been intimately associated with virus-induced cell fusion because mutations in either gK or UL20 cause extensive cell fusion in a variety of cell types. A largely unexplained phenomenon is that gK and UL20 are absolutely required for virus-induced cell fusion even in the presence of highly fusogenic mutations in

gB (22). Moreover, gK syncytial mutations cause extensive cell fusion of many cell types, unlike mutations in gB and UL20 that do not (48). In addition, mutants lacking gK or lacking a portion of the amino terminus of gK cause delayed entry kinetics in Vero cells, suggesting that gK and UL20 function similarly during cell-to-cell fusion and virus envelope-to-cell fusion. In support of these findings, we show here for the first time that gK functions in both virus entry and virus-induced cell fusion in conjunction with the PILR α receptor. Specifically, gK mutants were unable to enter into CHO-PILR α cells, while they efficiently entered CHO-nectin-1 and CHO-HVEM cells. This functional association between gK and the gB-specific receptor PILR α was also demonstrated by the inability of the gK Δ 31-68/gB Δ 28Syn virus to cause extensive fusion of CHO-PILR α cells, while both CHO-nectin-1 and CHO-HVEM cells were efficiently fused. Similarly, the previously reported inability of the gK Δ 31-68/gB Δ 28Syn virus to cause fusion in Vero cells (22) may be due to other gB-specific receptors, since Vero cells do not express PILR α (S. Chowdhury and K. G. Kousoulas, unpublished data). Vero cells are known to express the nonmuscle myosin heavy chain IIA (NMHC-IIA) gB-specific receptor (7), which may function in a manner similar to that of PILR α , requiring gK for optimum gB-mediated membrane fusion.

HSV-1 virions can enter cells by fusion at the plasma membrane or by fusion with endosomal membranes in acidic or neutral endosomes after endocytosis (14). Previous work has indicated that HSV-1 enters via a pH-dependent endocytic pathway into CHO-nectin-1 cells (14). However, testing a variety of lysosomotropic inhibitors that prevent acidification of endosomes revealed that the McKbac virus entered into all cells in the presence of increasing concentrations of inhibitors, i.e., through a pH-independent entry mechanism. In contrast with our results, it has been suggested that HSV-1 enters into CHO-nectin-1 cells via a pH-dependent mechanism (14). We have found that the viability of cells treated with the various inhibitors should be carefully monitored to ensure that cell death does not affect virus entry experiments. Clearly, HSV-1 McKbac and gK mutant viruses enter into all cells via a pH-independent mechanism, unlike VSV. Additional studies are required to discern whether the different receptors influence virus entry via either fusion at the plasma membrane or fusion with endosomal membranes.

The amino terminus of gK was shown to physically and functionally interact with the amino terminus of gB (23). Here, we show that soluble PILR α was able to precipitate gB, gK, and UL20, suggesting that PILR α , gB, gK, and UL20 form a functional protein complex in infected cells. Based on the fact that PILR α binds to the amino terminus of gB, it is possible that gB binds both PILR α and the gK/UL20 protein complex independently of each other, utilizing distinct gB domains. PILR α may also bind gK/UL20 in the presence or absence of gB. These possibilities will be discerned in future experiments. The observed functional and physical association between gK/UL20 and the gB-specific receptor PILR α and potentially other gB-specific receptors, such as NMHCIIA, suggest that gK/UL20 is required for optimum regulation of the fusogenic properties of gB. Finally, our recent findings that gK-null virions do not infect neuronal axons (24, 49) suggest the potential involvement of gB-specific receptors in neuronal infections.

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