JOURNAL OF VIROLOGY, Sept. 1995, p. 5401–5413 Vol. 69, No. 9
0022-538X/95/$04.00+0

Herpes Simplex Virus Glycoprotein K Promotes Egress of Virus Particles

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Received 2 March 1995/Accepted 9 June 1995

Herpes simplex virus (HSV) glycoprotein K (gK) is thought to be intimately involved in the process by which infected cells fuse because HSV syncytial mutations frequently alter the gK (UL53) gene. Previously, we characterized gK produced in cells infected with wild-type HSV or syncytial HSV mutants and found that the glycoprotein was localized to nuclear and endoplasmic reticulum membranes and did not reach the cell surface (L. Hutchinson, C. Roop, and D. C. Johnson, J. Virol. 69:4556–4563, 1995). In this study, we have characterized a mutant HSV type 1, denoted F-gKβ, in which a *lacZ* gene cassette was inserted into the gK coding sequences. Since gK was found to be essential for virus replication, F-gKβ was propagated on complementing cells which can express gK. F-gKβ produced normal plaques bounded by nonfused cells when plated on complementing cells, although syncytia were observed when the cells produced smaller amounts of gK. In contrast, F-gKβ produced only microscopic plaques on Vero cells and normal human fibroblasts (which do not express gK) and these plaques were reduced by 10^2 to 10^6 in number. Further, large numbers of nonenveloped capsids accumulated in the cytoplasm of F-gKβ-infected Vero cells, virus particles did not reach the cell surface, and the few enveloped particles that were produced exhibited a reduced capacity to enter cells and initiate an infection of complementing cells. Overexpression of gK in HSV-infected cells also caused defects in virus egress, although particles accumulated in the perinuclear space and large multilamellar membranous structures juxtaposed with the nuclear envelope were observed. Together, these results demonstrate that gK regulates or facilitates egress of HSV from cells. How this property is connected to cell fusion is not clear. In this regard, gK may alter cell surface transport of viral particles or other viral components directly involved in the fusion process.

The fusion of cellular and viral membranes induced by herpes simplex virus (HSV) is essential for virus penetration into host cells, nucleocapsid envelopment, virus egress, and transfer of virus from infected to uninfected cells (10, 16, 40, 46, 57). These processes are complex and not well understood, primarily because a large number of HSV proteins (many of unknown function) have been implicated in membrane fusion and because cellular factors involved in the processes have largely not been described.

During production of plaques on monolayers of cultured cells, HSV can spread by direct cell-to-cell transmission, presumably across cell contacts or junctions (reviewed in reference 19). By a similar, but perhaps distinct, process, HSV can cause cultured cells to fuse at neutral pH. Wild-type strains of HSV usually promote aggregation of cultured cells, although fusion of cultured cells is observed to a limited degree and polykaryocytes have been observed in herpetic lesions of infected individuals (51, 65). By contrast, syncytial mutants of HSV, which can arise on passage of virus in cultured cells, cause cells to fuse with uninfected cells, producing massive syncytia. It is thought that the syncytial mutations affect viral proteins which play a direct role in the membrane fusion events, e.g., glycoprotein B (gB), or which regulate the fusion process in some unknown manner (reviewed in reference 65).

Syncytial mutations can arise in at least four different viral genes: the UL20 gene, which encodes a membrane protein necessary for viral egress (4, 43); the UL24 gene, which encodes a cytosolic protein of unknown function (35, 60); the UL27 (gB) gene (8, 59); and the UL53 gene (7, 53, 59), which encodes glycoprotein K (gK) (32). Deletion mutants lacking either the UL20 or UL24 coding sequences form syncytial plaques, implying that these proteins are not required for fusion and that loss of either protein induces fusion by deregulating some aspect of virus replication. By contrast, syncytial mutations in gB primarily involve amino acid substitutions or truncations (2, 10, 23, 68). Other point mutations in gB alter the rate of HSV entry (8, 23), and gB null mutants cannot enter cells and are blocked in virus penetration and cell fusion (10, 11, 46). Therefore, it appears likely that gB plays a direct role in membrane fusion events. Mutations in the UL20, UL24, and UL27 (gB) genes are infrequently observed, at least in some strains of HSV type 1 (HSV-1), and mutations in the UL53 (gK) gene predominate (6, 7, 41, 56, 58). The mutations in gK described to date have been restricted to amino acid substitutions (17, 20, 54), and it has been suggested that gK somehow regulates the fusion of cell surface membranes (reviewed in reference 34). Nonetheless, recent observations indicate that gK does not reach the cell surface and cannot be detected in virus particles (34), suggesting that gK is not directly involved in membrane fusion.

There is also evidence, primarily from studies of virus mutants, demonstrating that gB, gD, and gH/gL are required for cell fusion as well as for virus entry into cells (10, 11, 16, 22, 40, 58). In addition, HSV membrane proteins, not absolutely required for virus penetration, have also been implicated in cell fusion. Cell fusion was not observed with HSV-1 mutants carrying a syncytial form of gB and unable to express gE, gI, or gM or a membrane protein encoded by the UL45 gene (5, 16, 28). The role of the gE/gI heterooligomer in cell fusion is consistent

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with the observation that gE/gI fosters efficient cell-to-cell transmission of HSV across cell junctions (5, 19). However, others have reported that syncytial mutants lacking gE (47) or gI (19, 36) retain the syncytial phenotype, implying that these deletion mutants possess syncytial mutations in a gene other than UL27 (gB), e.g., that encoding gK. In addition, a variety of cell factors, including glycosaminoglycans, can influence fusion of infected cells (9, 62, 63).

In order to characterize the role of gK in membrane fusion and in HSV replication, we constructed and characterized an HSV-1 mutant, F-gKβ, unable to express gK. F-gKβ formed extremely rare microscopic plaques in the absence of gK, indicating that gK was required for HSV replication. Enveloped and unenveloped virus particles accumulated in the cytoplasm of cells in the absence of gK, and virus particles were not observed on the cell surface. When gK was overexpressed in the cells, there were similar disruptions in virus egress, although in this case the virus accumulated in the perinuclear space.

MATERIALS AND METHODS

Cells and viruses. Vero cells and normal human fibroblasts were grown in alpha-minimal essential medium (alpha-MEM) supplemented with 7 to 10% fetal calf serum (FCS), 1% penicillin-streptomycin (P/S; GIBCO Laboratories, Burlington, Ontario, Canada), and 0.3% L-glutamine (GIBCO). VK243, VK295, VK302, VK308, and gK-9 cells (33) were maintained in Dulbecco's modified Eagle medium lacking histidine (DUL-his) supplemented with 0.5 mM histidinol (Sigma Chemical Co., St. Louis, Mo.), 7% FCS, 1% P/S, and 0.3% L-glutamine. Prior to infection, VK243, VK295, VK302, VK308, and gK-9 cells were passaged once in alpha-MEM containing 7% FCS. HSV-1 wild-type strains F (obtained from P. G. Spear, University of Chicago, Chicago, Ill.) and KOS (obtained from J. Smiley, McMaster University, Hamilton, Ontario, Canada) and HSV-2 strain 333 (obtained from P. G. Spear, University of Chicago) were propagated on and had their titers determined on Vero cells. The gK-defective mutant strain, F-gKβ-308, was grown and had its titers determined on VK302 cells.

Plasmids. Plasmid pSV2HISsyn (33) includes a 3.4-kb *Eco*RI-*Bam*HI fragment from pSG28 (25) encoding the UL53 gene as well as a selectable marker, histidinol dehydrogenase from pSV2HIS (30). Plasmid pUC19syn (33) contains the UL53 open reading frame (ORF) within a 1.3-kb *Kpn*I-*Bam*HI fragment derived from pSV2HISsyn (33) inserted into the *Kpn*I and *Bam*HI sites of pUC19. Plasmid pD6p (a generous gift of S. Weller, University of Connecticut) contains a 4.3-kb ICP6::*lacZ* gene cassette bounded by *Bam*HI sites (26), and this fragment was excised, end repaired with T4 polymerase, and ligated into the unique *Hpa*I site (within the UL53 ORF) of pUC19syn so that the *lacZ* and UL53 genes were in the same direction, creating plasmid p19SZ. A 1.0-kb *Nae*I fragment, containing the UL53 ORF, was removed from pUC19syn and inserted in the *Sma*I site of pRIT2T (Pharmacia Chemicals, Dorval, Quebec, Canada), yielding pRIT2Tsyn, a protein A-gK fusion expression vector. Plasmid pCMVsyn (33) was constructed by blunt-end ligation of the 1.0-kb *Nae*I fragment from pUC19syn (33) into end-repaired *Bst*XI sites of pRc/CMV (Invitrogen Corporation, San Diego, Calif.), placing the UL53 ORF under control of the cytomegalovirus (CMV) immediate-early (IE) promoter and flanked by the bovine growth hormone polyadenylation site. Plasmid pSV2HDK (33) carries a 1.9-kb *Hin*dIII-*Eco*RI fragment comprising the UL53 ORF and the bovine growth hormone polyadenylation site from pCMVsyn (33) downstream of the HSV gD promoter in pSV2HISgD (40). A 2-kb *Bgl*II-*Xba*I fragment derived from pCMVsyn and containing a CMV IE promoter::UL53 gene cassette was inserted into the multiple cloning site within the US7-gI gene of pSS17L (21, 40) to create pSS17syn. Oligonucleotide AB924 (5′-GCT GCA GGG AAC GGA CGG CGA GCA T-3′), which hybridizes at the 5′ end of the UL53 ORF (purchased from The Institute for Molecular Biology and Biotechnology, McMaster University), was used for Southern blot analysis.

Antibodies. Synthetic peptide UL53-4 and rabbit anti-UL53-4 peptide sera have been described previously (32). Monoclonal antibody (MAb) 15βB2, which recognizes HSV-1 and HSV-2 gB (37), was a gift of S. Bacchetti (McMaster University). MAb LP2, which reacts with HSV-1 and HSV-2 gD (44), was a gift of T. Minson (Cambridge University). MAb 53S directed towards HSV gH was obtained as a hybridoma from the American Type Culture Collection. Rabbit anti-thymidine kinase (TK) is directed to HSV TK produced in *Escherichia coli* and was a generous gift from W. Summers (Yale University).

Construction of Vero cells expressing gK and the gK-negative HSV-1, F-gKβ. Vero cells were transfected with plasmid pSV2HDK or pSV2HISsyn, and transformants were selected by using medium lacking histidine and containing 0.15 to 1 mM histidinol, as previously described (33). Isolated colonies of cells were cloned by using cloning cylinders, and the cells were screened initially by dot blot Southern analysis with oligonucleotide AB924. Cells found to contain more than five copies of the UL53 (gK) gene were characterized further for the inducible expression of gK after infection with HSV-2, and then cells were labelled with [^{35}S]methionine and immunoprecipitated with the anti-UL53-4 serum, which does not recognize HSV-2 gK (32). Three cell lines capable of expressing gK, VK243, VK295, and VK308, were cotransfected with plasmid p19SZ DNA and infectious HSV-1 (F) genomic DNA extracted from cytoplasmic HSV nucleocapsids (64) by the $CaPO_4$ method (27) followed by a glycerol shock (40). Transfected cells were incubated for 2 to 4 days at 37°C and then harvested and sonicated after extensive virus-induced cytopathic effect was observed. Virus preparations were diluted and plated on the progenitor cell line, and recombinant viruses which expressed β-galactosidase were detected by using an overlay of alpha-MEM containing 2% FCS, 0.5% low-melting-temperature agarose, and 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim Canada Ltd., Dorval, Quebec, Canada) per ml. Blue plaques were picked, subjected to three additional rounds of plaque purification on the appropriate cell line, and finally cloned by limiting dilution.

Production of infectious virus, plaque-forming efficiency, and plaque morphology of F-gKβ on different cell types. The replication and plaque-forming efficiencies of wild-type F and F-gKβ were assessed by infecting Vero or VK302 cell monolayers with 2 PFU per cell and harvesting the cells in medium (the cells were resuspended in 1/10 the volume of cell culture supernatant) after 24 h at 37°C. The cells were disrupted by one round of freeze-thawing followed by sonication, and then titers of viruses were determined on monolayers of normal human fibroblasts or Vero or VK302 cells. After 1.5 to 2 h at 37°C, the virus inoculum was removed and cells were incubated in alpha-MEM containing 1% FCS and 0.1% human gamma globulin for 96 h and then stained with crystal violet. Plaques were photographed with a Zeiss inverted microscope.

Radiolabelling of proteins, immunoprecipitation, and gel electrophoresis. Vero cells or Vero cell transformants were infected with HSV-1 or HSV-2 by using 2.5, 5, or 30 PFU per cell. Two to 4 h after infection, the cells were washed three times with medium 199 lacking methionine and cysteine and containing 1% dialyzed FCS (labelling medium) and then incubated for 2 to 5 h with labelling medium containing [^{35}S]methionine and [^{35}S]cysteine (50 to 200 μCi of each per ml). Pulse-chase experiments were performed by washing cells three times with labelling medium 3 h after infection and incubating cells for 20 min with [^{35}S]methionine and [^{35}S]cysteine (100 μCi of each per ml) in labelling medium. Cell extracts were made, by using a solution of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate (NP-40/DOC buffer) containing 2 mg of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5 mM phenanthroline, and 10 μg of aprotinin (Sigma) per ml, immediately (pulse) or after the cells had been washed with alpha-MEM containing 1% FCS and incubated an additional 90 or 210 min (chases). Insoluble material was removed by centrifugation at high speed (86,000 × *g*) for 60 min, and in some instances the extracts were precleared by incubation with *Staphylococcus aureus* Cowan and rabbit anti-gE/gI serum, as previously described (29, 31). Extracts derived from 3.5×10^5 to 5.5×10^5 cells were incubated with mouse or rabbit antibodies (1 to 10 μl of serum or ascites fluid) at 4°C for 90 min and then with protein A-Sepharose (50 to 100 μl; Pharmacia Chemicals). In some experiments, anti-UL53-4 peptide serum (5 or 10 μl) was preincubated with 50 or 100 μg of synthetic UL53-4 peptide for 60 min at 4°C. Antigen-antibody complexes were washed three times with NP-40/DOC buffer or under more stringent conditions, as described previously (31), and then precipitated proteins were eluted by adding 50 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, bromophenol blue, and 2% β-mercaptoethanol and the samples were heated at 37°C for 30 min (gK samples) or at 100°C for 5 min. Proteins were subjected to electrophoresis as described previously (31), and the gels were dried and analyzed by using a Molecular Dynamics Image Quant phosphoimager or, alternatively, infused with Enhance (Dupont, Montreal, Quebec, Canada), dried, and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.).

Electron microscopy. Vero or VK302 cells were infected with wild-type HSV-1 (F) or F-gKβ by using 5 PFU per cell, and then the cells were incubated at 37°C for 20 to 24 h. The cells were washed with phosphate-buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 15 min. The cells were scraped from the dishes, collected by centrifugation at 800 × *g* for 5 min, resuspended in cacodylate buffer, and processed for electron microscopy as described elsewhere (38).

RESULTS

Construction of a mutant HSV-1 unable to express gK. The UL52 and UL53 genes partially overlap, and the UL52 gene is essential for viral DNA synthesis (26). Therefore, to construct an HSV-1 UL53 mutant, it was important not to interrupt the overlapping UL52 gene. On the basis of this consideration, we constructed a plasmid, p19SZ, in which an ICP6 promoter::*lacZ* cassette (26) was inserted into an *Hpa*I site 336 nucleotides downstream of the UL53 start codon (Fig. 1), allowing expression of about one-third of the gK polypeptide (17, 54).

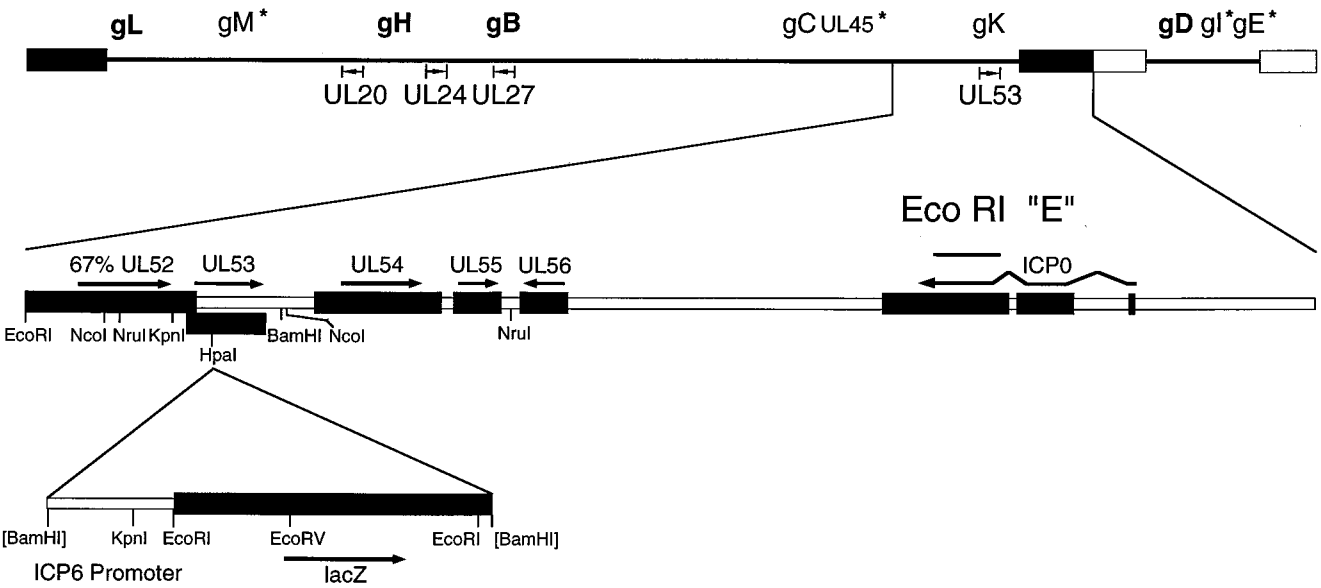

FIG. 1. Schematic representations of the HSV-1 genome, genes involved in cell fusion, and the genomic structure of the gK mutant virus. The HSV-1 genome is represented as unique long (U_L) and short (U_S) segments flanked by inverted repeats (filled and open boxes). The positions of genes encoding proteins which govern HSV-induced cell fusion are indicated along the genomic map. Mutations in the UL20, UL24, UL27-gB, and UL53-gK genes (indicated below the map) can produce the syncytial phenotype. The UL1-gL, UL22-gH, UL27-gB, and US6-gD glycoprotein genes (boldface type) are essential for HSV-1 replication in cultured cells, cell fusion, and virus entry. Virus-induced cell fusion associated with a syncytial mutation in gB requires HSV genes encoding UL10-gM, US7-gI, US8-gE, and UL45-membrane protein (asterisks). The *Eco*RI fragment of HSV-1 genomic DNA, containing 67% of the UL52 gene and the UL53, UL54, UL55, UL56, and ICP0 genes, is shown magnified to indicate the insertion of an ICP6::*lacZ* gene cassette into the UL53 gene at an *Hpa*I site (located 336 nucleotides into the UL53 ORF). Brackets around the *Bam*HI sites indicate that these sites were lost when the ICP6::*lacZ* cassette was cloned into the *Hpa*I site in UL53.

Cotransfection of Vero cells with HSV-1 (F) DNA and p19SZ yielded recombinant viruses which produced blue plaques under overlays containing X-Gal. However, Southern blot analysis revealed that the entire p19SZ plasmid had been introduced into the HSV-1 genome, regenerating the wild-type UL53 gene, probably through a single recombination crossover event (data not shown). MacLean et al. (43) previously reported unsuccessful efforts to mutate the UL53 gene; thus, it appeared that gK was essential for replication in these cultured cells.

To complement gK mutants, we constructed cell lines which could express gK. Previous attempts to increase gK synthesis by introducing a human CMV promoter-driven UL53 gene cassette into the HSV-1 genome (unpublished data) suggested that altering the temporal expression or abundance of gK interfered with virus replication. Efforts to produce cell lines which overexpress gK confirmed this hypothesis and also suggested that gK was toxic to cells when expressed even at low levels (33). Consequently, we endeavored to establish cell lines which produced gK in response to HSV infection yet expressed little or no gK before infection. Vero cells were transfected with either of two plasmids: pSV2HISsyn, which has the UL53 gene coupled to its own promoter, or pSV2HDK, which contains the UL53 ORF under control of the HSV-1 gD promoter (33). Both plasmids carry the selectable marker histidinol dehydrogenase (30, 40).

Histidinol-resistant transformants were screened initially for plasmid copy number by Southern dot blot analysis. Those cells which had over five copies of the plasmid were subsequently screened for gK expression by infecting the cells with HSV-2 and immunoprecipitating HSV-1 gK with an antipeptide serum, anti-UL53-4, which does not recognize HSV-2 gK (32). Among the 302 pSV2HDK transformants that were characterized, cell lines VK243, VK295, and VK308 represent 3 of 19 clones that produced detectable amounts of gK and a fourth cell transformant, VK302, produced substantially higher levels of gK (Fig. 2). Certain of the 210 cell lines derived from pSV2HISsyn expressed low but detectable levels of gK (data not shown) and were not characterized further. As with other cell lines carrying gD promoter constructs (22, 40), VK243, VK295, VK308, and VK302 cells did not express detectable amounts of gK prior to infection (data not shown). The prominent bands ranging from 60 to 90 kDa in HSV-1 (KOS)-infected cells were derived from HSV immunoglobulin G Fc receptor proteins gE and gI (32, 36).

To obtain an HSV-1 gK mutant, VK243, VK295, and VK308 cells were cotransfected with infectious HSV-1 (F) DNA and plasmid p19SZ. Viruses derived from the tranfections of individual cell lines were screened for blue plaques and subjected to four additional rounds of plaque purification with the same cells. Three mutants, F-gKβ-243, F-gKβ-295, and F-gKβ-308, derived independently from cell lines VK243, VK295, and VK308, respectively, were examined by Southern blot analysis using oligonucleotide AB924, which hybridizes to the first 25 nucleotides of the UL53 ORF (Fig. 1). The presence of 6.1-kb *Nco*I and 9.2-kb *Nru*I fragments and the absence of the wild-type 2.1-kb *Nco*I and 5.0-kb *Nru*I fragments indicated that F-gKβ-243, F-gKβ-295, and F-gKβ-308 contained only the disrupted form of the UL53 gene (Fig. 3). Other Southern blot analyses verified that plasmid sequences were not introduced into the mutant viruses (data not shown). All three gK-negative viruses exhibited a syncytial phenotype when plated on VK243, VK295, and VK308 cells and other cell lines that expressed detectable quantities of gK (data not shown), probably because all of these cells produce lower levels of gK than are observed in cells infected with wild-type HSV-1 (Fig. 2). In contrast, all three viruses produced nonsyncytial plaques when plated on VK302 cells (F-gKβ-308 is shown in Fig. 5), which express gK at levels more similar to those in HSV-infected cells. Therefore, subsequent experiments employed the VK302

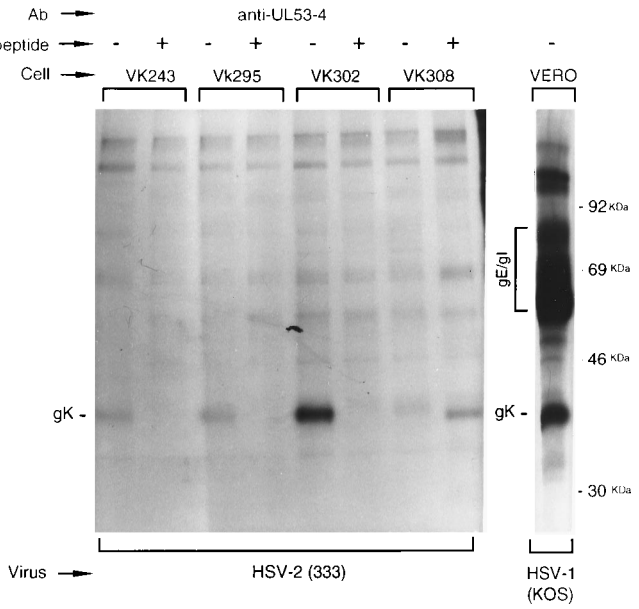

FIG. 2. Expression of HSV-1 gK in Vero cell transformants after infection with HSV-2. Transfected cell lines VK243, VK295, VK302, and VK308 were infected with HSV-2, and for comparison, Vero cells were infected with HSV-1 (KOS). Infected cells were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine for 5 h beginning 4.5 h after infection. Extracts of the cells were made and then incubated with rabbit anti-UL53-4 peptide sera, which had been preincubated with (+) or without (−) UL53-4 peptide. Anti-UL53-4 recognizes HSV-1 gK but not HSV-2 gK (32). Antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions (see Materials and Methods). Precipitated proteins were eluted at 37°C for 30 min, and electrophoresis was performed with 12% polyacrylamide gels. The positions of gK, HSV-1 immunoglobulin G Fc receptor proteins gE and gI, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated. Ab, antibody.

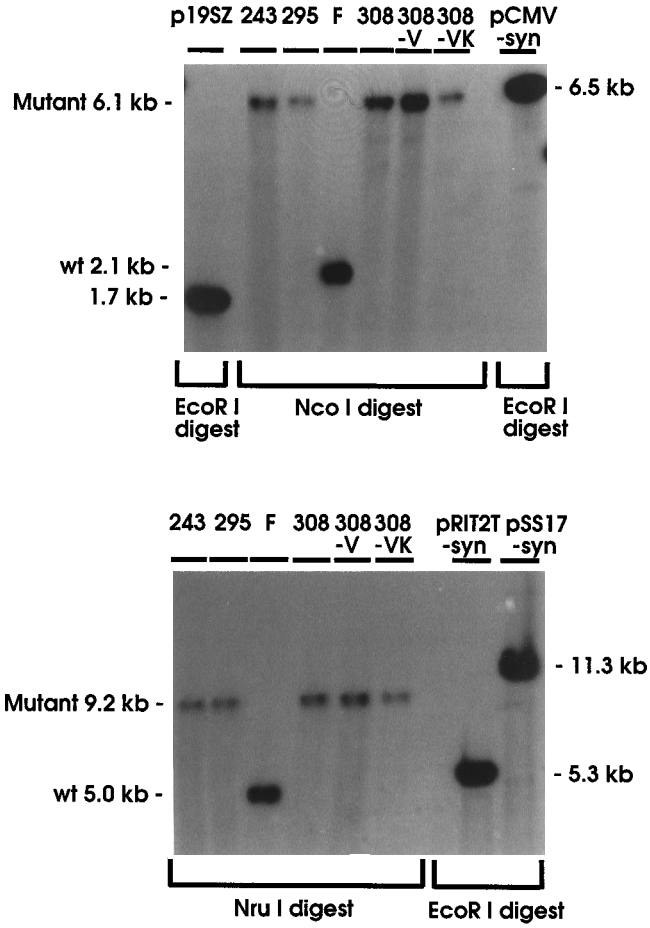

FIG. 3. Southern blot analysis of mutant (F-gKβ) viral DNA. Viral DNA was extracted from Vero cells infected with mutant viruses or wild-type HSV-1 (F) and then digested with *Nco*I (top panel) or *Nru*I (bottom panel). As markers, plasmids p19SZ, pCMVsyn, pRIT2Tsyn, and pSS17syn were digested with *Eco*RI yielding 1.7-, 6.5-, 5.3-, and 11.3-kb fragments. DNA fragments were separated on a 1% agarose gel, transferred to nylon membranes, and probed with a ^{32}P-labelled oligonucleotide, AB924, which hybridizes with the first 25 nucleotides of the UL53 ORF. Lanes: 243 (F-gKβ-243), a mutant isolated after transfection and plaque purification on VK243 cells; 295 (F-gKβ-295), a mutant isolated after transfection and plaque purification on VK295 cells; F, the wild-type HSV-1 strain; 308 (F-gKβ-308), a mutant isolated after transfection and plaque purification on VK308 cells. Lanes 308-V (F-gKβ-308V) and 308-VK (F-gKβ-308-VK) indicate virus preparations after additional rounds of plaque purification.

cell line and the F-gKβ-308 isolate, which will henceforth be designated F-gKβ. All three of the initial isolates failed to produce plaques on Vero cells and exhibited defects similar to those of F-gKβ in other experiments.

Virus mutant F-gKβ does not express gK. We next examined the ability of mutant viruses to express gK. Vero or VK302 cells were infected with F-gKβ or wild-type HSV-1 (F) labelled with [^{35}S]methionine and [^{35}S]cysteine, and cell extracts were mixed with anti-UL53-4 peptide serum, which recognizes gK residues 89 to 104 (32). The anti-UL53-4 antibodies precipitated the 40-kDa gK protein from extracts of wild-type-strain F-infected Vero cells and also from F-gKβ-infected VK302 cells (Fig. 4). The full-length 40-kDa gK protein was not detected in extracts of F-gKβ-infected Vero cells; instead, a protein of 28 kDa was detected. This 28-kDa protein was also expressed in F-gKβ-infected VK302 cells, in addition to the 40-kDa gK polypeptide (Fig. 4). The 40-kDa gK protein and the novel 28-kDa protein were not observed when anti-UL53-4 serum was preincubated with the UL53-4 peptide (Fig. 4). Identical results were obtained with the anti-UL53-1 peptide serum (data not shown), which reacts with gK residues 31 to 46 (32). Examination of DNA sequences at the insertion site of the ICP6::*lacZ* cassette into the UL53 gene indicated that the first 112 residues of gK (including the signal peptide and N-linked glycosylation sites but none of the transmembrane domains) were fused to 26 residues derived from the ICP6::*lacZ* cassette. The addition of N-linked oligosaccharides would be expected to alter the electrophoretic mobility of the F-gKβ fusion protein from the predicted value of 15 kDa to the apparent size of approximately 28 kDa. This is based on the observation that the apparent molecular mass of wild-type gK increases from 29 kDa to approximately 40 kDa on glycosylation (32). The finding that this protein is recognized by two gK-specific antipeptide sera strongly supports the view that the 28-kDa protein is derived from the N terminus of gK.

Plaque production by F-gKβ and marker rescue experiments. F-gKβ formed plaques on VK302 cells, although these plaques were reduced in size compared with those produced by wild-type HSV-1 (F) (Fig. 5), suggesting that complementation was incomplete. Wild-type HSV-1 produced normal plaques on VK-302 cells, and equal quantities of infectious viruses were produced by wild-type HSV-1 on VK-302 and Vero cells (data not shown). By contrast, F-gKβ failed to form normal plaques on Vero cells (Fig. 5) and on human R970 cells (data not shown). At very low frequencies ($<10^{-5}$ relative to the number of plaques formed on VK302 cells), microscopic plaques (consisting of three to six infected cells) were observed on Vero cell

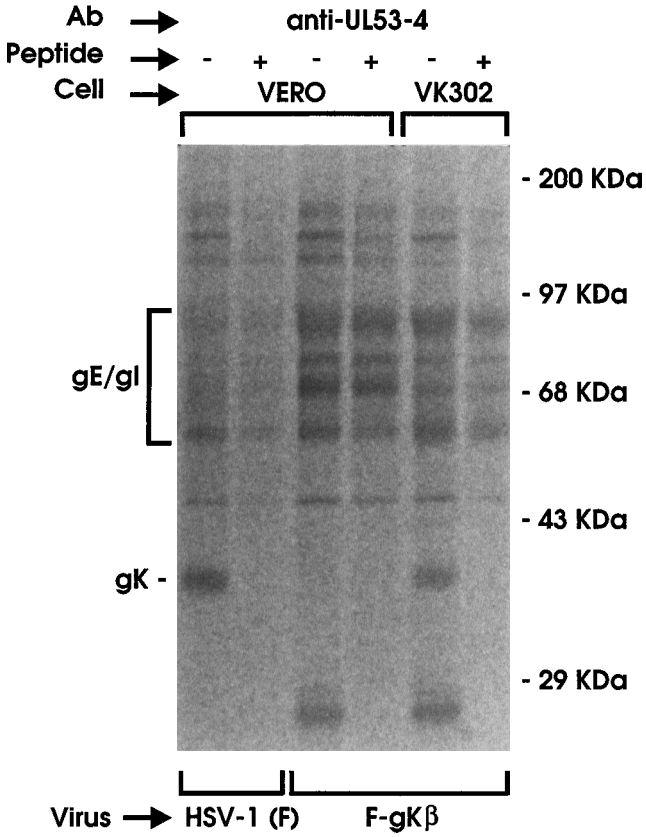

FIG. 4. Expression of gK in Vero and VK302 cells infected with F-gKβ. Vero cells and VK302 cells were infected with F-gKβ, and for comparison, Vero cells were infected with wild-type HSV-1 (F). Infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine, and extracts were mixed with anti-UL53-4 serum (−) or with anti-UL53-4 serum preincubated with peptide UL53-4 (+). Antigen-antibody complexes were eluted from protein A-Sepharose at 37°C for 30 min, subjected to electrophoresis on 12% polyacrylamide gels, dried, and analyzed with a Molecular Dynamics Image Quant phosphoimager. The positions of the 40-kDa form of HSV-1 gK, the HSV immunoglobulin G Fc receptor proteins gE and gI, and marker proteins of 200, 97, 68, 43, and 29 kDa are indicated. Ab, antibody.

monolayers infected with approximately 1 PFU of F-gKβ per cell, and the plaques were detected only after extended periods (72 to 96 h) (Fig. 5; Table 1). F-gKβ also formed microscopic plaques on normal human fibroblasts; however, the reduction in plaque-forming efficiency was less striking than with Vero cells (Fig. 5; Table 1), possibly because small HSV-1 plaques are more easily detected on fibroblast monolayers. It is highly unlikely that these microscopic F-gKβ plaques were caused by wild-type revertants, which can contaminate HSV-1 preparations derived from complementing cells. These revertants apparently derive from recombination events between viral and cellular copies of the gene (11, 58); however, this type of recombination is unlikely because VK302 cells lack the HSV-1 sequences flanking the UL53 ORF. In addition, we have not detected wild-type HSV-1, even at low levels, in the F-gKβ stocks.

To verify that the defects in virus replication were related to the mutation in the UL53 gene and not defects in other genes, a marker rescue experiment was performed. Vero cells were cotransfected with F-gKβ and either plasmid pUC19syn, which contains the wild-type UL53 gene, or pUC19. No wild-type plaques were observed with pUC19, whereas hundreds of plaques were observed on monolayers transfected with pUC19 syn and F-gKβ DNA. Several recombinants derived from the pUC19syn transfection were plaque purified and found to replicate normally on Vero cells (data not shown). Therefore, we conclude that gK is required for production of HSV plaques on monkey Vero cells and human fibroblasts and R970 cells.

Markedly less infectious virus is produced in the absence of gK. To evaluate the effects the gK mutation on production of infectious viruses, we infected Vero cells or VK302 cells with F-gKβ or wild-type F, using 2 PFU per cell, and harvested the cells after 24 h. Under these conditions, over 95% of the cells expressed viral antigens and were thus infected by the input virus (data not shown), so that this can be considered a single-step growth analysis. Infectious viruses were assayed by plaque titration with VK302 cells to determine if virions produced in the absence of gK were capable of initiating an infection of complementing cells. The titers of wild type HSV-1 (F) derived from Vero cells and F-gKβ derived from complementing VK302 cells were virtually identical, demonstrating good complementation in this single-step infection (Table 1). F-gKβ virions produced on Vero cells (lacking gK) formed plaques on VK302 cells; however, these were reduced in number by about 100-fold relative to numbers obtained with F-gKβ preparations derived from VK302 cells. Further, Vero cells infected with F-gKβ at 2 PFU per cell yielded only approximately 0.5 PFU per cell when this virus was plated on VK302 cells, consistent with the notion that gK is required for HSV replication. The data shown in Table 1 involve infectious virus derived from cells which were suspended in the culture media and then disrupted by sonication. However, we also analyzed infectious virus present in the culture media from F-gKβ-infected Vero cells and found a 300- to 1,000-fold reduction in the amount of virus produced relative to the amount produced by F-gKβ-infected VK-302 cells. Therefore, in the absence of gK, markedly less infectious virus was produced.

To further evaluate the infectivity of virus particles lacking gK, we examined expression of early viral proteins in cells infected with F-gKβ. VK302 or Vero cells were infected with Vero-derived HSV-1 (F), Vero-derived F-gKβ, or VK302-derived F-gKβ, and then the cells were labeled with [^{35}S]methionine and [^{35}S]cysteine and HSV-1 early proteins, TK, or gD was immunoprecipitated. Since approximately equal amounts of infectious HSV-1 (F) and F-gKβ were produced on Vero and VK302, respectively (Table 1), cells were infected with equal quantities of all three virus stocks, which corresponded to approximately 2 PFU per cell for Vero-derived F and VK302-derived F-gKβ. Under these conditions, Vero-derived F-gKβ (lacking gK) expressed 50-fold less TK and gD in both Vero and VK302 than was observed in cells infected with wild-type HSV-1 or with VK302-derived F-gKβ (Fig. 6). Similar results were observed when two HSV-1 immediate-early proteins, ICP4 and ICP6, were immunoprecipitated (data not shown). When the input of Vero-derived F-gKβ was increased about 100-fold, so that cells were infected with 2.5 PFU per cell (PFU here refers to plaques formed on VK302 cells), the expression of TK increased to 20% of that observed with HSV-1 (F) (data not shown). These results show clearly that HSV-1 virions derived from cells in which gK is not expressed display a reduced capacity to enter cells and initiate early stages of virus replication.

Subcellular distribution of the virus particles in cells lacking gK. F-gKβ-infected Vero or VK302 cells and HSV-1 (F)-infected Vero cells were examined by electron microscopy to determine if there were defects in virus assembly or egress in the absence of gK. Similar quantities of virus particles were observed in Vero cells infected with F and with F-gKβ and in F-gKβ-infected VK302 cells; however, the distribution of the

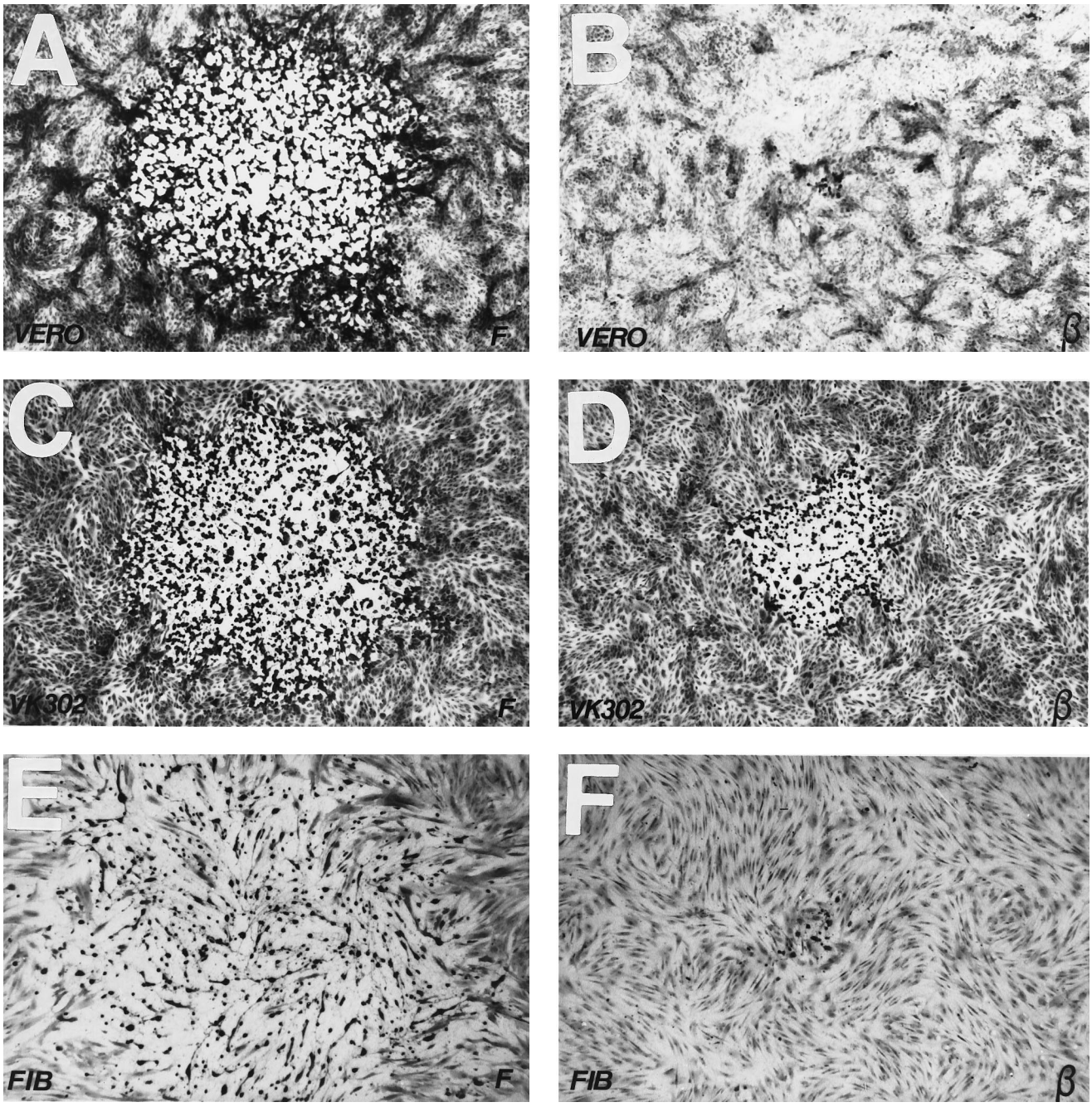

FIG. 5. Morphology of F-gKβ plaques produced on normal human fibroblasts and Vero and VK302 cells. Monolayers of Vero (A and B) or VK302 cells (C and D) or human fibroblasts (FIB) (E and F) were infected with wild-type HSV-1 (F) (A, C, and E) or the gK-negative mutant, F-gKβ (B, D, and F), overlaid with medium containing 0.1% human gamma globulin, and incubated at 37°C. After 74 h, the cells were fixed and stained with crystal violet. Plaques were photographed with a Zeiss inverted microscope. Magnification, ×92.

particles and the ratio of enveloped to nonenveloped virus particles differed quite dramatically (Fig. 7; Table 2). Virus particles were rarely observed on the surfaces of F-gKβ-infected Vero cells, whereas enveloped virions were frequent and numerous components of the surfaces of F-infected Vero cells and of F-gKβ-infected VK-302 cells (Fig. 7). The few virions which were occasionally observed near the surface of F-gKβ-infected Vero cells were mostly unenveloped particles and were often surrounded by cell debris, indicating that the particles originated from damaged cells. The lack of virus particles on the surfaces of F-gKβ-infected Vero cells was consistent with our observation that there was a 300- to 1,000-fold decrease in the number of infectious virus particles which accumulated in the cell culture supernatant (see above). Large numbers of unenveloped capsids accumulated in the cytoplasm of F-gKβ-infected Vero cells and, to a lesser extent, enveloped virions were found within membrane vesicles (Fig. 7; Table 2). Moreover, enveloped virions in the cytoplasm of F-gKβ-infected Vero cells were frequently morphologically aberrant, e.g., there were several capsids within a single envelope or

TABLE 1. Production of infectious virus and plaque formation by F-gKβ on noncomplementing cells

Virus	Cell type grown on[a]	Genotype	Phenotype	Cell type on which titers determined[b]	Titer (PFU/ml)	Titer relative to VK302[c]
F	Vero	gK^+	gK^+	Vero	8.6×10^8	1.02
				VK302	8.4×10^8	1.00
				Fibroblasts	6.0×10^8	0.71
F-gKβ	Vero	gK^-	gK^-	Vero	$<10^{2}$[d]	$<10^{-5}$
				VK302	8.8×10^6	1.00
				Fibroblasts	4.8×10^{5}[d]	0.054
F-gKβ	VK302	gK^-	gK^+	Vero	$<10^{3}$[d]	$<10^{-6}$
				VK302	8.2×10^8	1.00
				Fibroblasts	5.5×10^{7}[d]	0.067

[a] The indicated cells were infected with 2 PFU per cell and incubated at 37°C for 24 h. Infected cells were harvested in cell culture media and disrupted by sonication.
[b] Serial dilutions were plated on the indicated cells and then incubated at 37°C for 96 h.
[c] The virus titer on VK302 cells was defined as 1.00, and the relative titers on fibroblasts and Vero cells are indicated.
[d] Microscopic plaques were composed of 3 to 6 cells, whereas plaques caused by wild-type HSV-1 were composed of 100 to 200 cells.

partially enveloped structures (Fig. 7, panel 3). By contrast, in wild-type-F-infected Vero cells and F-gKβ-infected VK-302 cells, there were fewer unenveloped particles in the cytoplasm and the majority of enveloped particles that accumulated inside cells were found in the space between the inner and outer nuclear membranes (perinuclear space). In the absence of gK, there were fivefold fewer enveloped particles present in the perinuclear space (Table 2). For all three samples, F-gKβ-infected Vero cells, F-infected Vero cells, and F-gKβ-infected VK302 cells, over 3,500 virus particles in association with at least 15 randomly selected cells were counted; thus, the observations should be considered highly significant. These results demonstrated that virus particles produced in the absence of gK seldom reach the cell surface and extracellular space and accumulate predominantly in the cytoplasm as unenveloped particles and morphologically aberrant particles. It should also be noted that although there were four- to eightfold fewer enveloped particles produced in F-gKβ-infected Vero cells (Table 2), these enveloped particles were of very low specific infectivity because infectious viruses were reduced 100-fold (Table 1).

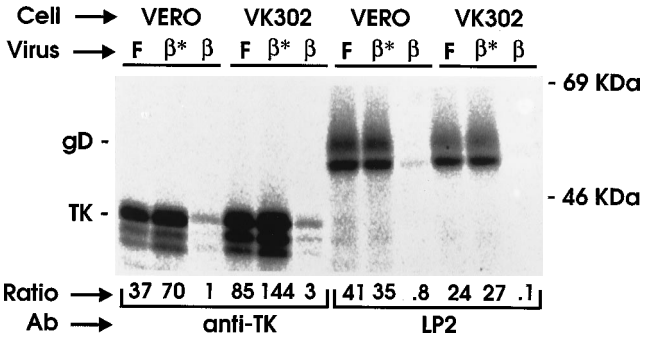

FIG. 6. Expression of early viral proteins in cells infected with wild-type HSV-1 (F) or the gK-negative mutant F-gKβ. Preparations of wild-type HSV-1 (F) or F-gKβ were produced with Vero cells (F and β) or with complementing VK302 cells for F-gKβ (β*) by infecting the cells with 2.0 PFU per cell. The virus preparations were made in parallel, and equal amounts of the two preparations were used to infect Vero or VK302 cells [2.5 PFU per cell for HSV-1 (F) and F-gKβ (β*)]. After 2 h, the cells were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine for 2 h. Cell extracts were mixed with MAb LP2, specific for gD, or rabbit serum specific for TK (anti-TK) and subsequently with protein A-Sepharose. The precipitated proteins were separated by electrophoresis in 8.5% polyacrylamide gels. The gels were dried, and the relative amount of each HSV-1 protein was quantified with a phosphoimager (Ratio). HSV-1 proteins gD and TK and molecular mass markers of 69 and 46 kDa are indicated. Ab, antibody.

Processing and transport of viral glycoproteins in F-gKβ-infected Vero cells. Previous studies have suggested that inhibition of HSV glycoprotein glycosylation or processing can result in the intracytoplasmic accumulation of virus particles and inhibition of virus egress (13, 28, 49, 52, 59). Since egress of HSV-1 appears to be impaired in viruses lacking gK, we investigated whether the maturation of HSV-1 glycoproteins was altered in F-gKβ-infected Vero cells. Vero and VK302 cells were infected with wild-type F or F-gKβ and radiolabelled by using a pulse-chase protocol, and gB, gD, gH, or TK was immunoprecipitated from cell extracts. Since F-gKβ was derived from complementing cells, similar quantities of gB, gD, and gH were observed in wild-type-F- and F-gKβ-infected cells, but in the F-gKβ-infected Vero cells we would not expect expression of gK. On the basis of their reduced electrophoretic mobilities after a chase period, the HSV glycoproteins were converted to mature glycoproteins with similar kinetics in cells infected with mutant and wild-type viruses (Fig. 8). Proteolytic degradation of a fraction of gB and TK was observed, a common phenomenon in extracts from Vero cells (50). These results indicated that, although virus egress appears to be inhibited in the absence of gK, the bulk of the HSV-1 glycoproteins were processed to the mature forms in the medial Golgi and *trans*-Golgi apparatus and probably reach the cell surface. Because the enveloped virus particles which accumulated in F-gKβ-infected cells were distributed throughout the cytoplasm as well as in the perinuclear space, it is likely that the particles contained a mixture of processed and unprocessed forms of the glycoproteins, though analysis of the total glycoprotein expressed in cells does not necessarily address this point (4).

In studies employing a UL20$^-$ HSV-1, which also exhibits an egress defect in Vero cells, evidence was presented that HSV gD and gC were present in reduced amounts on the cell surface (1). Consequently, we used confocal immunofluorescence microscopy to compare subcellular localization and cell surface expression of gD in Vero cells infected with wild-type HSV-1 (F) and F-gKβ. Although these results were not absolutely quantitative, the comparison showed clearly that there were no large differences in the cellular distribution or abundance of gD in F-gKβ-infected Vero cells relative to wild-type-F-infected Vero cells (data not shown). Therefore, the transport of

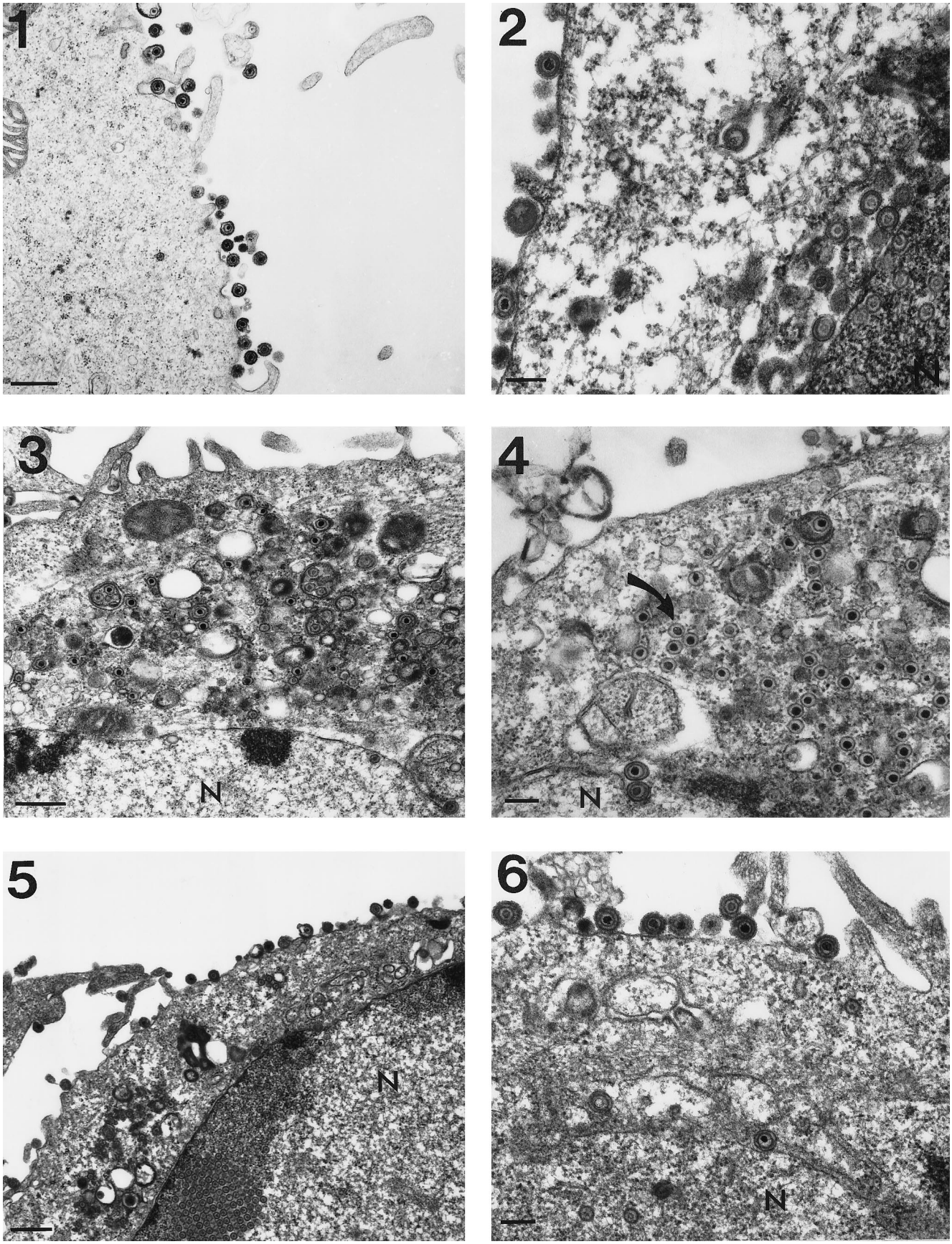

FIG. 7. Electron micrographs of cells infected with HSV-1 (F) or mutant F-gKβ. Vero cells were infected with wild-type HSV-1 (F) (1 and 2) or F-gKβ (3 and 4), and VK302 cells were infected with F-gKβ (5 and 6) by using 5 PFU/cell and then the cells were incubated at 37°C for 20 to 24 h. The cells were fixed with 2% glutaraldehyde and prepared for electron microscopy. The arrow indicates cytoplasmic unenveloped nucleocapsids which accumulate in F-gKβ-infected Vero cells. N, nucleus. Bars, 500 nm in panels 1, 3, and 5 and 200 nm in panels 2, 4, and 6.

TABLE 2. Distribution of virions in Vero cells infected with HSV-1 (F) or F-gKβ

Subcellular locale	Virion structure	Avg no. (%) of particles/cell[a]	
		HSV-1 (F)	F-gKβ
Nucleus	Nonenveloped	34 (14)	36 (15)
Perinuclear space	Enveloped	99 (39)	18 (7.3)
Cytoplasm	Enveloped	22 (8.8)	38 (15)
	Nonenveloped	13 (5.2)	120 (49)
Cell Surface	Enveloped	80 (32)	8 (3.2)[b]
	Nonenveloped	3 (1.2)	27 (11)[b]
Total (all locations)		251 (100)	247 (100)

[a] Virus particles present in the nucleus, perinuclear space, and cytoplasm and on the cell surface were counted in electron micrographs of at least 15 randomly sampled Vero cells infected with HSV-1 (F) or F-gKβ. The numbers represent the average number of particles per cell, and the numbers in parentheses indicate the percentage of particles per cell.

[b] The virus particles were loosely associated with the cell surface and appeared to originate from cells that were damaged.

the bulk of the HSV glycoproteins was not dramatically affected in the absence of gK.

Overexpression of gK inhibits HSV egress. Previously, we described and characterized a Vero cell transformant, gK-9, which contains several hundred copies of the UL53 (gK) gene and expresses approximately 15-fold more gK after infection with HSV-1 than is observed to be present in comparably infected Vero cells (33). HSV-1 forms plaques inefficiently on gK-9 cells, such that the numbers of plaques were reduced by approximately 200-fold relative to the number produced on Vero cells (33). It appeared possible that overexpression of the gK protein might also inhibit virus egress. gK-9 cells were infected with wild-type HSV-1 (F), and then after 20 h the cells were prepared for electron microscopy. As with F-gKβ-infected Vero cells, virus particles were rarely observed on the surfaces of infected gK-9 cells (Fig. 9). However, in contrast to the observations with F-gKβ-infected Vero cells, virtually all of the enveloped virus particles found in infected gK-9 cells accumulated in the perinuclear space and cytoplasmic nucleocapsids were not detected. Moreover, we frequently observed large multilamellar membranous structures juxtaposed with the nuclear membranes of infected gK-9 cells (Fig. 9, panel 4, arrow). Since gK-9 cells do not exhibit defects in HSV protein synthesis, glycosylation, or protein transport (33, 34), it would appear that overexpression of gK causes defects in virus egress, just as there are defects in this process when gK is not expressed.

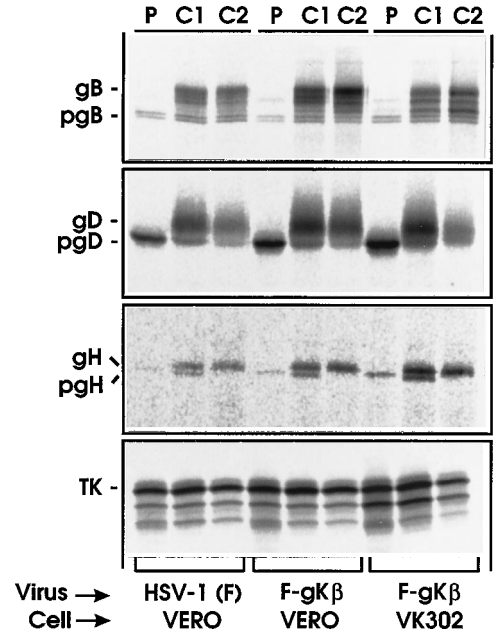

FIG. 8. Pulse-chase analysis of HSV-1 glycoproteins in cells infected with wild-type HSV-1 (F) or gK-negative F-gKβ. Vero cells were infected with HSV-1 (F) or F-gKβ, and VK302 cells were infected with F-gKβ (derived from VK302 cells). After 3 h, infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min and then cell extracts were made immediately (P) or the cells were washed with medium containing methionine and cysteine and incubated in this medium for 90 min (C1) or 210 min (C2) before cell extracts were made. Cell extracts were mixed with MAb 15βB2, specific for gB; LP2, specific for gD; 53S, specific for gH; or rabbit anti-TK serum, and precipitated proteins were eluted from protein-A Sepharose and analyzed on 12% acrylamide gels. The positions of HSV-1 proteins pgB, gB, pgD, gD, pgH, gH, and TK are indicated.

DISCUSSION

In this report, we describe an HSV-1 mutant, F-gKβ, which is unable to express gK and demonstrate that gK is essential for virus replication in cultured monkey Vero cells, human R970 cells, and normal human fibroblasts. The primary defect in replication of this gK-negative mutant appears to lie in virus egress from the nucleus of infected cells to the cell surface. In Vero cells infected with F-gKβ, virus particles were rarely observed on the cell surface, whereas in cells infected with wild-type HSV-1, the surface membranes were frequently encrusted with virus particles late in the infection. Moreover, large numbers of unenveloped cytoplasmic particles accumulated in the cytoplasm of F-gKβ-infected Vero cells. Those enveloped virus particles that were present in the cytoplasm of F-gKβ-infected Vero cells were of low specific infectivity, and the particles entered host cells or initiated expression of early proteins poorly. For example, expression of TK was reduced by 70-fold when virus particles were produced in cells lacking gK. gK$^-$ viruses, produced in Vero cells, displayed a 100-fold reduction in plaque formation on complementing cells, even though the number of enveloped capsids observed in F-gKβ-infected Vero cells was only 2- to 4-fold lower than was observed in F-infected Vero or F-gKβ-infected VK-302 cells. Therefore, only a small fraction of the enveloped particles produced in the absence of gK were actually able to initiate an infection. The low infectivity of these viruses was amplified during production of virus plaques, so that plaques were extremely rare, microscopic, and detected only after extended periods.

In order to avoid disrupting the UL52 gene, which is required for HSV DNA replication (26), we inserted a *lacZ* gene cassette into a region of the UL53 ORF which allowed expression of 112 residues of gK (approximately one-third of the protein) fused to 26 residues derived from the ICP6::*lacZ* gene cassette. Because this protein contains the gK signal peptide, it is probable that the protein passes into the endoplasmic reticulum (ER), where the signal peptide is cleaved, since the signal peptide is removed from a truncated gK protein translated in vitro (55). Without the membrane-spanning domains of gK or a means of localizing itself to membranes, it is highly doubtful that the truncated protein could function like gK in any fashion. It is conceivable that the truncated protein was somehow

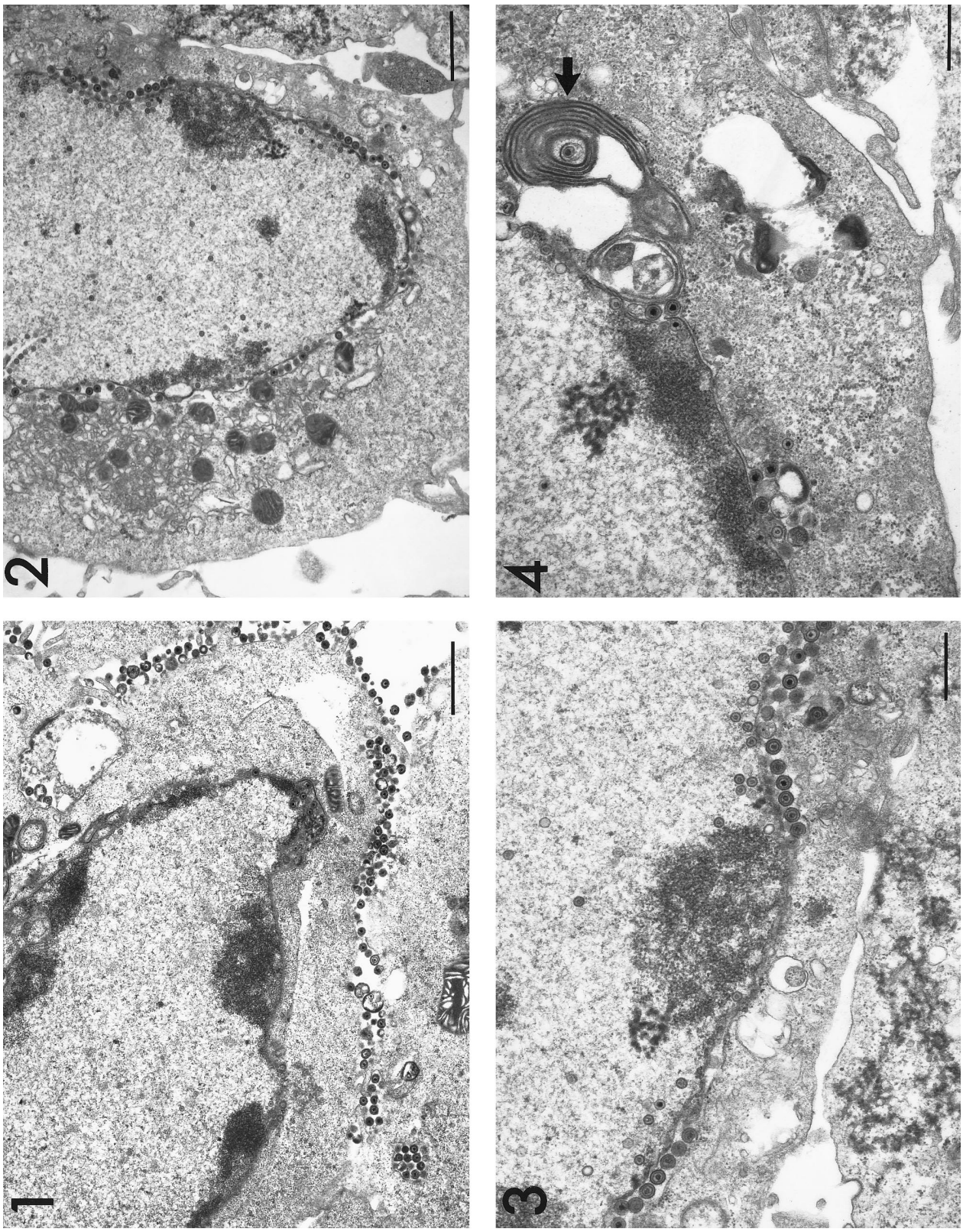

FIG. 9. Electron micrographs of HSV-infected Vero or gK-9 cells, which overexpress gK. Monolayers of Vero cells (1) or gK-9 cells (2, 3, and 4) were infected with HSV-1 (F) by using 5 PFU per cell and then the cells were incubated at 37°C for 22 h, fixed with 2% glutaraldehyde, and processed for electron micrographs. The arrow indicates a multilamellar vesicle juxtaposed with the nuclear envelope. Bars, 1 μm in panels 1 and 2 and 500 nm in panels 3 and 4.

poisonous to HSV replication; however, this did not occur, because the truncated protein was expressed in F-gKβ-infected VK-302 cells, where replication was normal. In addition, we would argue that the phenotype of F-gKβ, which shows defects in virus egress from the nuclear envelope to cytoplasmic membranes, is consistent with previous observations that gK is localized to nuclear and ER membranes (34). Moreover, to our knowledge there are no precedents for negative effects stemming from such truncated proteins.

Several models have been proposed to account for egress of herpesviruses from cells. There is consensus that viral nucleocapsids, formed in the nucleus, acquire a lipid envelope containing immature forms of the HSV glycoproteins at the inner nuclear envelope and that these particles transiently accumulate in the perinuclear space i.e., the region between the inner and outer nuclear membranes (15, 45, 61, 66, 69). However, there is controversy over the process by which these perinuclear particles reach the cell surface. One camp suggests that enveloped virus particles are transported to the cell surface in the interior of a series of membranous vesicles or tubules derived from the ER and Golgi apparatus (12, 38, 45, 61, 66). Others have argued that enveloped particles present in the perinuclear space can fuse with cellular membranes (e.g., the outer nuclear envelope) producing unenveloped cytoplasmic capsids. These unenveloped capsids are thought to subsequently acquire an envelope derived from cytoplasmic, e.g., Golgi-apparatus-derived, membranes modified with viral glycoproteins (24, 39, 48, 69). Much of the debate as to how herpesviruses exit cells surrounds observations of nucleocapsids juxtaposed with cytoplasmic membrane patches, presumably modified with viral glycoproteins. From these static electron microscopic images, it is by no means clear whether particles associated with cytosolic membranes are in the process of envelopment or deenvelopment. Similarly, studies using inhibitors of membrane transport processes do not discriminate between these models of virus egress (14, 38). Cytoplasmic nucleocapsids can be observed in HSV-infected cells; however, these capsids have been attributed to a dead-end pathway in which enveloped particles fuse with cytoplasmic vesicles (12). It is possible that the egress pathway used by HSV differs from that of other herpesviruses, especially in different cell types. Until further genetic and biochemical analyses of this problem are carried out, the question of how herpesviruses exit cells will remain open.

Virus mutants such as that characterized here, and others that are described below, offer some of the best opportunities for understanding herpesvirus egress. It appears unlikely that gK alters the initial envelopment of capsids at the inner nuclear envelope because normal or larger than normal amounts of virus particles reach the cytoplasm of F-gKβ-infected Vero cells. However, in the absence of gK, reduced numbers of enveloped particles were observed in the perinuclear space. Moreover, dramatically increased numbers of unenveloped capsids were observed in the cytoplasm of F-gKβ-infected Vero cells. These results demonstrate that gK facilitates transport of virus particles through the cytoplasm to the cell surface and plays a role in the earliest transport processes involving movement of enveloped particles from the perinuclear space.

On the basis of the two models for herpesvirus egress, gK could function in either of two ways. If enveloped virus particles transit through the cytoplasmic vesicular network, as described, e.g., by Johnson and Spear (38), then gK may prevent fusion between cellular and viral membranes, so that the particles do not lose their envelope during transport. By this model, cytosolic capsids would be considered a dead-end product (12). Further, gK may inhibit fusion between the virion envelope and the outer nuclear envelope, because in the absence of gK fewer enveloped virions accumulated in the perinuclear space. However, if the second model, described by Jones and Grose (39) and Whealy et al. (69), involving a series of envelopments and deenvelopments is correct, one could hypothesize that gK promotes envelopment of capsids on cytoplasmic membranes. gK is primarily localized to a perinuclear region as determined by immunofluorescence experiments, and gK oligosaccharides are not processed in the medial Golgi and *trans*-Golgi apparatus, consistent with localization in the nuclear envelope, the ER, and the *cis*-Golgi apparatus (34). Thus, if indeed gK functions to promote envelopment of cytosolic capsids, it should do so in the ER or *cis*-Golgi apparatus. This might also be considered unlikely because most of the electron microscopic studies propose reenvelopment in the *trans*-Golgi apparatus; in addition, gK has not been detected in the virus envelope (34). Also, a role for gK in promoting reenvelopment in the cytoplasm is difficult to rationalize with observations of decreased numbers of enveloped particles in the perinuclear space.

Consistent with the hypothesis that gK functions during the early stages of virus egress, i.e., in nuclear and ER membranes, overexpression of gK in HSV-infected gK-9 cells caused enveloped particles to accumulate in the perinuclear space. It is interesting that the absence of gK has the opposite effect, causing reduced numbers of enveloped particles to accumulate in the perinuclear space. Moreover, gK overexpression was associated with accumulation of large multilamellar vesicles, apparently derived from the outer nuclear envelope. If one hypothesizes that gK functions to inhibit membrane fusion events, overexpression of wild-type gK might also inhibit vesicular transport of viruses from the perinuclear space, since membrane fusion occurs as vesicles pinch off the ER and nuclear envelope. This could cause accumulation of virus particles in the perinuclear space and multilamellar vesicles next to the nuclear envelope. In the absence of gK, unrestricted fusion of enveloped particles with the outer nuclear envelope might reduce the numbers of particles present in the perinuclear space. In the deenvelopment-reenvelopment model of egress, by which it could be argued that gK facilitates envelopment of capsids at cytoplasmic sites, it is more difficult to understand how gK overexpression would promote accumulation of perinuclear particles or multilamellar vesicles. By this model, if one argues that gK possesses a property which promotes envelopment, overexpression of the glycoprotein might not be expected to cause particles to accumulate in the perinuclear space.

Considering all of this, we favor a model of HSV egress in which enveloped virus particles transit from the perinuclear space through the Golgi network in membrane vesicles in order to gain access to the cell surface. We propose that in this model, gK prevents fusion between virion and cellular membranes. In the absence of gK, there is unrestricted fusion between viral and cellular membranes (the outer nuclear envelope, the ER, and perhaps the Golgi network), so that capsids accumulate in the cytoplasm. Overexpression of gK apparently has the opposite effect, obstructing vesicular transport of virus particles out of the perinuclear space, a process which requires membrane fusion. However, it should be noted that we have not observed inhibition of HSV glycoprotein transport in HSV-infected gK-9 cells (33, 34).

A number of other HSV-1 mutants with defects in virus egress have been described. An HSV-1 mutant expressing a temperature-sensitive form of gH retains infectious particles containing gH in cells and secretes noninfectious virus particles lacking gH (18). It is not clear whether these extracellular

particles lose gH, e.g., by proteolysis during egress, or whether mutant gH is not incorporated into the envelope of those particles which ultimately escape cells. An HSV-1 mutant with a defect in the UL11 gene product, a myristylated membrane protein, displays reduced levels of cell surface and extracellular virus and increased quantities of unenveloped capsids in the cytoplasm (3, 42). The ratio of unenveloped/enveloped cytoplasmic nucleocapsids produced by the UL11 mutant was approximately 3:1, similar to that observed with our gK mutant. The UL11 mutant can produce plaques, although these are reduced in number. However, the UL11 defect appears much less severe than the defect with the gK$^-$ mutant described here. Notwithstanding this difference, the UL11 and gK mutations produce similar phenotypes, suggesting that the proteins have similar functions and may, perhaps, interact in some manner to promote passage of enveloped particles through the cytoplasm. The UL20 gene product has some properties similar to those of gK, since both proteins are multimembrane-spanning proteins and are predominately localized to the nuclear and perinuclear membranes, although UL20 is also localized to the Golgi apparatus (4, 17, 34, 43, 54, 67). However, in contrast to the gK mutation, which caused accumulation of capsids and enveloped particles in the cytoplasm, the UL20 mutation caused accumulation of enveloped particles in the perinuclear space of Vero cells (4). The phenotype of the UL20$^-$ mutant was more similar to that we observed when gK was overexpressed. This result again points to the complexity of the viral egress process.

It is not entirely clear how the results reported here relate to previous observations that point mutations in gK lead to fusion of infected cells. Since gK cannot be detected on the surfaces of infected cells or in virions (34), it is unlikely that gK is present in the fusion complex used during cell fusion or as virus enters cells. The most likely explanation for the syncytial phenotype of gK mutants, given the observations reported here, is that these point mutations alter transport of the fusion complex or some other viral component, e.g., the regulator of fusion to the cell surface. Consistent with this hypothesis are two observations. When gK was expressed at less than wild-type levels, e.g., in F-gKβ-infected VK243, VK295, or VK308 cells, syncytial plaques were produced; thus, underexpression of gK, an internal protein, promotes fusion between surface membranes. Secondly, we recently observed that infection of cells with syncytial HSV mutants leads to the accumulation of larger than normal quantities of cytoplasmic virus particles (33a). Furthermore, the UL20$^-$ mutant formed small, syncytial plaques on 143 cells, similar to those we observed when gK was expressed at reduced levels. These results suggest that production of syncytia by HSV mutants is linked to defects in egress of virus particles and add additional support to the hypothesis that gK regulates fusion at intracellular sites. One explanation of cell fusion would be that fewer virus particles or other viral components reach the surfaces of cells infected with syncytial mutants, yet viral fusion complexes e.g., gB, gD, and gH/gL, reach the surface and function in an unregulated manner. Clearly, the relationship between HSV egress and cell fusion is complex, and further characterization of gK and gK mutants will be necessary before this protein's role in virus egress can be adequately understood.

ACKNOWLEDGMENTS

We thank Stan Person, Jim Smiley, Craig Smibert, Steven Primorac, and Cindy Roop for encouragement during the long and difficult course of this work; Kim Goldsmith for excellent technical assistance; and Ernie Spitzer for assistance with electron microscopy.

Support for this research was provided by grants from the National Cancer Institute of Canada (NCIC) and the Medical Research Council of Canada (MRCC). L.H. was supported by an MRCC studentship. D.C.J. will be a Senior Scientist of the NCIC until the impending termination of this award program by the agency.

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