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Herpes Simplex Virus Glycoprotein K Is Known To Influence Fusion of Infected Cells, yet Is Not on the Cell Surface

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Syncytial mutants of herpes simplex virus (HSV) cause extensive fusion of cultured cells, whereas wild-type HSV primarily causes cell rounding and aggregation. A large fraction of syncytial viruses contain mutations in the UL53 gene, which encodes glycoprotein K (gK). Previously, we demonstrated that wild-type and syncytial forms of gK are expressed at similar levels and possess identical electrophoretic mobilities. Using immunofluorescence, we show that gK is not transported to the surfaces of cells infected with either wild-type or syncytial HSV. Instead, gK accumulates in the perinuclear and nuclear membranes of cells. This finding is in contrast to the behavior of all other HSV glycoproteins described to date, which reach the cell surface. When gK was expressed in the absence of other HSV proteins, using a recombinant adenovirus vector, a similar perinuclear and nuclear pattern was observed. In addition, gK remained sensitive to endoglycosidase H, consistent with the hypothesis that gK does not reach the Golgi apparatus and is retained in the endoplasmic reticulum and nuclear envelope. Therefore, although gK mutations promote fusion between the surface membranes of HSV-infected cells, the glycoprotein does not reach the plasma membrane and, thus, must influence fusion indirectly.

Entry of herpes simplex virus (HSV) into host cells is thought to involve fusion of the virion envelope with the plasma membrane (reviewed in reference 52). By what is thought to be an analogous process, newly synthesized viral glycoproteins appearing in the plasma membranes of infected cells can cause infected cells to fuse. Wild-type HSV type 1 (HSV-1) fuses cells rarely, whereas syncytial (syn) mutants of HSV-1 cause extensive cell fusion (14, 22, 40). In at least some virus strains, the majority of syn mutations map to the UL53 gene (3, 41, 44, 46, 48), which encodes the hydrophobic, multiple membrane-spanning glycoprotein gK (24, 43). Of the mutant UL53 genes that have been sequenced, all contained amino acid substitutions at residue 40 within the N-terminal extracellular domain of gK (12, 42), and we refer to these proteins as syncytial forms of gK. Apart from a few observations, little is known about how gK functions during virus replication or in membrane fusion. HSV-1-infected cells express relatively low levels of gK (24), and transfected cells or cells infected with recombinant adenovirus vectors expressing wild-type gK resist fusion induced by infection with syncytial mutants of HSV gK (25). The importance of gK in the replication of other herpesviruses is underscored by the observation that other members of the alphaherpesvirus family all share homologs of the protein (10, 11, 33, 39, 54, 57). HSV-1 expresses at least 10 other membrane glycoproteins, as well as additional membrane proteins which are not glycosylated (1, 2, 23, 24, 37, 51), and a subset of these proteins, gB, gD, and the gH-gL complex, are known to be essential for virus entry into cells and for cell fusion (6, 7, 15, 18, 36, 46).

The other HSV-1 glycoproteins described to date all appear in infected cells as two protein species, differing in N-linked and O-linked oligosaccharides, so that the immature form of the protein is processed to the mature form during transit through the Golgi apparatus (23, 32, 51, 52). In contrast, gK exists as a single 40-kDa protein species, which is modified with N-linked oligosaccharides, in cells infected with both wild-type and syncytial HSV-1 (24). Given this difference and the apparent role of gK in cell fusion, it was of interest to determine whether gK reaches the Golgi apparatus and the cell surface. In this report, we demonstrate that gK remains in an endoglycosidase H (endo H)-sensitive form and does not reach the surfaces of infected cells.

gK is retained in the perinuclear and nuclear membranes of HSV-1-infected cells. Immunofluorescence microscopy was used to evaluate the subcellular localization of gK and to compare this with that of gD, which is found in the plasma membrane and Golgi apparatus as well as the endoplasmic reticulum (ER) and nuclear membranes (8, 9, 29, 50). Immunofluorescence experiments were performed as previously described (21, 56), with the following modifications. Vero cells were cultured on glass coverslips and infected with HSV-1 (20 or 30 PFU per cell) for 9 to 14 h or left uninfected. All cells were washed with phosphate-buffered saline (PBS) (pH 7.2) containing 1 mM $MgCl_2$ (PBS-Mg), fixed with 2 or 4% paraformaldehyde for 10 min, and rinsed with PBS-Mg. To examine internal distribution of the proteins, the cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min (56), or for surface immunofluorescence, the cells were not permeabilized. Excess aldehyde groups were inactivated with 0.2 M ethanolamine, pH 7.5, for 1.5 h; the cells were washed with PBS-Mg containing 2% bovine serum albumin (BSA) (PBS-BSA) and incubated with PBS-BSA containing 2% goat serum for 1 to 3 h. Rabbit anti-gD serum (4) was diluted 1:500 or 1:1,000, or individual or pooled gK-specific antipeptide sera were diluted 1:200 in PBS-BSA and added to cells for 1 to 2 h. The gK-specific antipeptide antibodies are directed to hydrophilic domains in gK and were characterized in an earlier study (24). Anti-UL53-1 reacts with residues 31 to 46 of gK, anti-UL53-3 recognizes residues 273 to 289, and anti-UL53-4 recognizes residues 89 to 104. All primary antisera were extensively preadsorbed against fixed, permeabilized, uninfected

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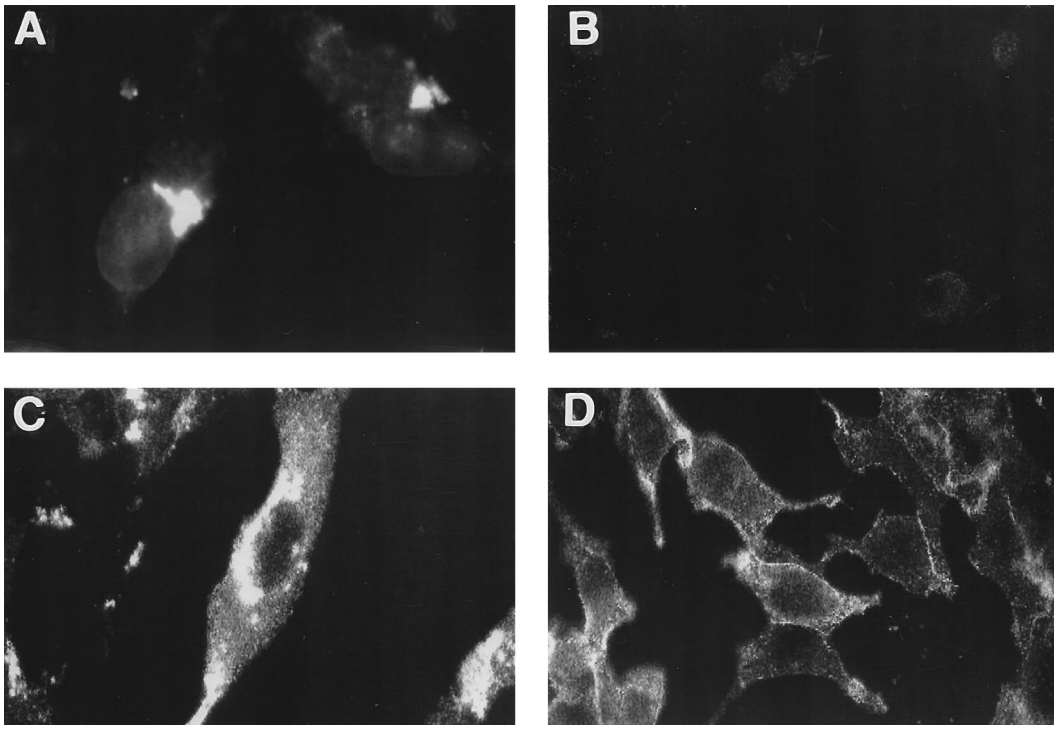

FIG. 1. gK is not transported to the surfaces of HSV-1-infected cells. Vero cell monolayers growing on glass coverslips were infected with HSV-1 (KOS) at 20 PFU per cell. After 12 h, all cells were fixed with 4% paraformaldehyde for 10 min and then either permeabilized with 0.2% Triton X-100 (A and C) or not permeabilized (B and D). The cells were incubated with a mixture of anti-UL53-1 and anti-UL53-4 antibodies (A and B) or rabbit anti-gD antibody (C and D) and subsequently washed with PBS-BSA before incubation with fluorescein-conjugated goat anti-rabbit IgG antibody. The coverslips were washed with PBS-BSA and mounted on glass slides. Photography was done using a Reichert fluorescence microscope.

Vero cells and then centrifuged at 18,000 × *g* for 1 h and filtered through a 0.2-μm-pore-size filter before use. After incubation with these primary antibodies, the cells were washed with PBS-BSA and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) serum (immunoaffinity purified; Jackson Immuno Research Laboratories, West Grove, Pa.) that had been diluted 1:60 in PBS-BSA. Coverslips were mounted on microscope slides, using 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) (21) or Vecta Shield fluorescence mounting medium (Vector Laboratories Inc., Burlingame, Calif.).

Both the cellular distribution (this study) and the relative abundance (24) of gK differed from those of gD. In permeabilized, HSV-1-infected cells, gD was found in nuclear and perinuclear regions of the cells, as well as distributed throughout cytoplasmic and surface membranes, and was associated with cellular processes (Fig. 1C). When the cells were not permeabilized there was also strong, granular, surface fluorescence (Fig. 1D). By contrast, gK was relatively localized almost exclusively to the perinuclear and nuclear membranes, and low levels of gK-specific fluorescence were detected in permeabilized cells (Fig. 1A); fluorescence was not detected on the cell surface when the cells were not permeabilized (Fig. 1B). The low levels of gK-specific fluorescence associated with permeabilized cells were consistent with previous observations that gK is expressed at low levels in HSV-infected cells (24). A small fraction of nonpermeabilized cells exhibited weak fluorescence after staining with anti-gK antibodies; however, the pattern of fluorescence was similar to that of permeabilized cells, i.e., a perinuclear pattern was observed, indicating that membrane damage had been caused, probably by HSV infection (not shown).

The results shown in Fig. 1 were obtained with a mixture of two antipeptide sera, anti-UL53-1 and anti-UL53-4. It was formally possible that only a single antipeptide antibody actually reacted with gK in these experiments and that the epitope for this antibody was masked as the protein matured. This might cause the antibodies to react with only the immature form of gK present in the ER and nuclear envelope but not with gK on the plasma membrane. Therefore, we examined whether each of the individual anti-gK antibodies could react individually with gK in HSV-infected cells. In addition, we considered the possibility that the fluorescence pattern obtained with the anti-gK antibodies was affected by binding of the Fc domain of rabbit antibodies to the HSV-1 Fc receptor. Therefore, we used the HSV-1 gE-negative mutant, F-gEβ, which does not have detectable Fc receptor activity (13). When HSV-1-infected Vero cells were fixed, permeabilized, and then stained with either anti-UL53-1, anti-UL53-3, or anti-UL53-4 serum, a pattern of fluorescence similar to that observed in Fig. 1 was obtained (not shown). However, the fluorescence produced by individual antisera was lower than that with pooled sera, making photography difficult. Increasing the concentration of individual antisera had little effect, suggesting that each antibody contributed to the fluorescent signal in an additive fashion.

To provide evidence that all three antipeptide antibodies individually reacted with gK and to rule out the possibility that a small fraction of gK was on the cell surface, we used a Vero cell transformant (gK-9) which contains ≈200 copies of the UL53-gK gene and expresses 10-fold more gK after infection with HSV-1 than is observed in comparably infected Vero cells (25). gK-9 cells were infected with F-gEβ, fixed, and then permeabilized and stained with either anti-UL53-1, anti-

UL53-3, or anti-UL53-4 antibody. All three anti-gK antibodies produced a pattern characterized by a perinuclear ring extending into the cytoplasm from the nuclear envelope, and there was an absence of staining of the plasma membrane and cell processes (Fig. 2A, C, and E). There was little or no fluorescence when nonpermeabilized, F-gEβ-infected gK-9 cells were stained with individual anti-gK antibodies (Fig. 2B, D, and F), and the low level of fluorescence that could occasionally be detected was not significantly different than that observed with preimmune sera (Fig. 2J). By contrast, gD was associated with the plasma membrane and cellular processes as well as being localized to perinuclear and nuclear membranes of HSV-infected gK-9 cells (Fig. 2G and H).

Since the anti-UL53-1 and anti-UL53-4 peptide sera are directed toward sequences in gK flanking the N-linked glycosylation signals (24, 43), we would expect that the antibodies would detect gK on the cell surface. Moreover, glycosylation does not interfere with the ability of these antisera to react with gK (24). Similar results were obtained when cells were stained with anti-gK antibodies prior to fixation (results not shown). Therefore it appears highly unlikely that the epitopes for all three antipeptide sera are lost during maturation of gK; this was supported by pulse-chase experiments in which anti-UL53-4 antibody (see Fig. 5) and anti-UL53-1 and anti-UL53-3 antibodies (not shown) reacted with gK produced after a relatively long chase period. With permeabilized gK-9 cells, the intensity of fluorescence obtained with individual and pooled anti-gK antipeptide sera was similar to that observed with the anti-gD antibodies: both antibodies produced intense fluorescence. Therefore, we would have expected to easily detect gK on the cell surface, if a significant fraction of the protein was present there.

Syncytial forms of gK are not expressed on the surfaces of infected cells. Two independently isolated HSV-1 syncytial strains, MP (22) and syn-20 (44), possess mutations affecting a single amino acid in the UL53 open reading frame (12, 42) and express gK proteins which exhibit electrophoretic mobilities similar to that of wild-type gK (24). To determine if syncytial mutations alter the subcellular distribution of gK, Vero cells were infected with MP and immunofluorescence analysis was performed as described above. The subcellular distribution of gK was more difficult to discern in these experiments; however, gK-specific fluorescence was observed in a region surrounding the clumped nuclei of MP-infected cells when the cells were permeabilized (Fig. 3A). No fluorescence, above the background observed with preimmune serum (Fig. 3F), was observed when nonpermeabilized, MP-infected cells were stained with anti-gK antibodies (Fig. 3B). Again, gD was associated with both perinuclear and surface membranes (Fig. 3C), and nonpermeabilized cells displayed strong gD-specific fluorescence (Fig. 3D). Similar results to those shown for HSV-1 MP were obtained with HSV-1 syn-20 (not shown). Therefore, it appears that the mutations in MP and syn-20 gK do not grossly affect intracellular distribution of the protein, at least not sufficiently so as to promote their transport to the cell surface.

gK expressed in the absence of other HSV-1 proteins is perinuclear. Earlier studies with another HSV glycoprotein, gH, demonstrated that this protein must form a complex with a second glycoprotein, gL, in order to be transported to the cell surface (16, 23, 45). Therefore, it was of interest to determine whether the retention of gK in HSV-infected cells was related to the expression of other HSV polypeptides. HSV glycoproteins expressed by adenovirus vectors exhibit targeting and functional properties similar to glycoproteins expressed by HSV (20, 23, 25, 29). We used an adenovirus vector, AdgK (24, 25), to express gK in the absence of other HSV proteins. Vero cell monolayers were infected with AdgK (1,000 PFU per cell) for 27 h or with AddlE3, a control adenovirus lacking E3 sequences (19). Previously, we demonstrated that under these conditions of infection, AdgK produces approximately three times the quantity of gK normally expressed in Vero cells infected with HSV for 12 h (25). Twenty-seven hours after infection is relatively early in the replicative cycle of adenovirus, and cytopathic effects were not noted, though a relatively high multiplicity of infection (1,000 PFU per cell) of AdgK was used. Anti-gK antibodies stained the perinuclear and nuclear membranes of permeabilized, AdgK-infected cells (Fig. 4A); this was not observed in AddlE3-infected cells (Fig. 4C). There was no gK on the surfaces of AdgK-infected cells (Fig. 4B). Therefore, it appears that gK is localized to perinuclear and nuclear membranes without a requirement for expression of other HSV polypeptides.

N-linked oligosaccharides on gK are not processed. To investigate the intracellular transport of gK further, we characterized posttranslational processing of gK oligosaccharides by using endo H, which removes immature, high-mannose oligosaccharides but not mature, complex oligosaccharides produced after glycoproteins move into the Golgi apparatus (34). Radiolabelling of cells, immunoprecipitations, and gel electrophoresis have been described previously (23–25) and were performed with modifications described in the legend to Fig. 5. After cells were pulse-labelled for 20 min with [^{35}S]methionine and [^{35}S]cysteine, a major species of 40 kDa as well as a minor protein species of 38 kDa was immunoprecipitated from HSV-infected cells and these proteins were not observed when the anti-gK serum was preincubated with peptide (Fig. 5). A single form of gK with an electrophoretic mobility of 29 kDa was observed after extracts were digested with endo H, supporting the conclusion that both the 40- and 38-kDa proteins contained immature, high-mannose oligosaccharides. Of two potential glycosylation signals in gK, one includes an aspartic acid residue (12, 42) which often reduces the use of a site (34) and therefore, the 38-kDa protein is likely a partially modified form of gK. This conclusion is supported by in vitro translation experiments (24) and when gK was expressed using insect cells (17). The disappearance of the 38-kDa protein during the 90-min chase (Fig. 5) may be due to breakdown/proteolysis, which has perhaps been accelerated because of the underglycosylation. Nevertheless, the 40-kDa protein appears to be the predominant form of gK in infected cells (24–26).

Similarly, the 40-kDa protein was the predominant form observed in the chase samples (Fig. 5). Moreover, the 40-kDa protein remained sensitive to endo H in both chase samples (Fig. 5). In contrast, other HSV glycoproteins gD, gE, and gI became predominantly resistant to endo H after the 235-min chase (not shown). The upper part of the gel was not included because this region included intense bands derived from the HSV Fc receptor proteins gE and gI, which are commonly observed in such immunoprecipitations (27). However, a more complete study using these antibodies demonstrated that there are no gK species with electrophoretic mobilities slower than 40 kDa (24). The intensity of the gK band dropped moderately during the chase periods; however, this reduction is probably the result of protein turnover, since the intensity of the gE and gI bands declined in a proportionate fashion during the chase in this experiment. It is possible that a fraction of gK is converted to an endo H-resistant form and that this fraction is not recognized by the anti-UL53-4 antibody; however, this is unlikely because similar results were obtained with the anti-UL53-1 and anti-UL53-3 antibodies and we and others have found that these antibodies do not detect proteins other than 38 and 40 kDa on Western immunoblots (results not shown;

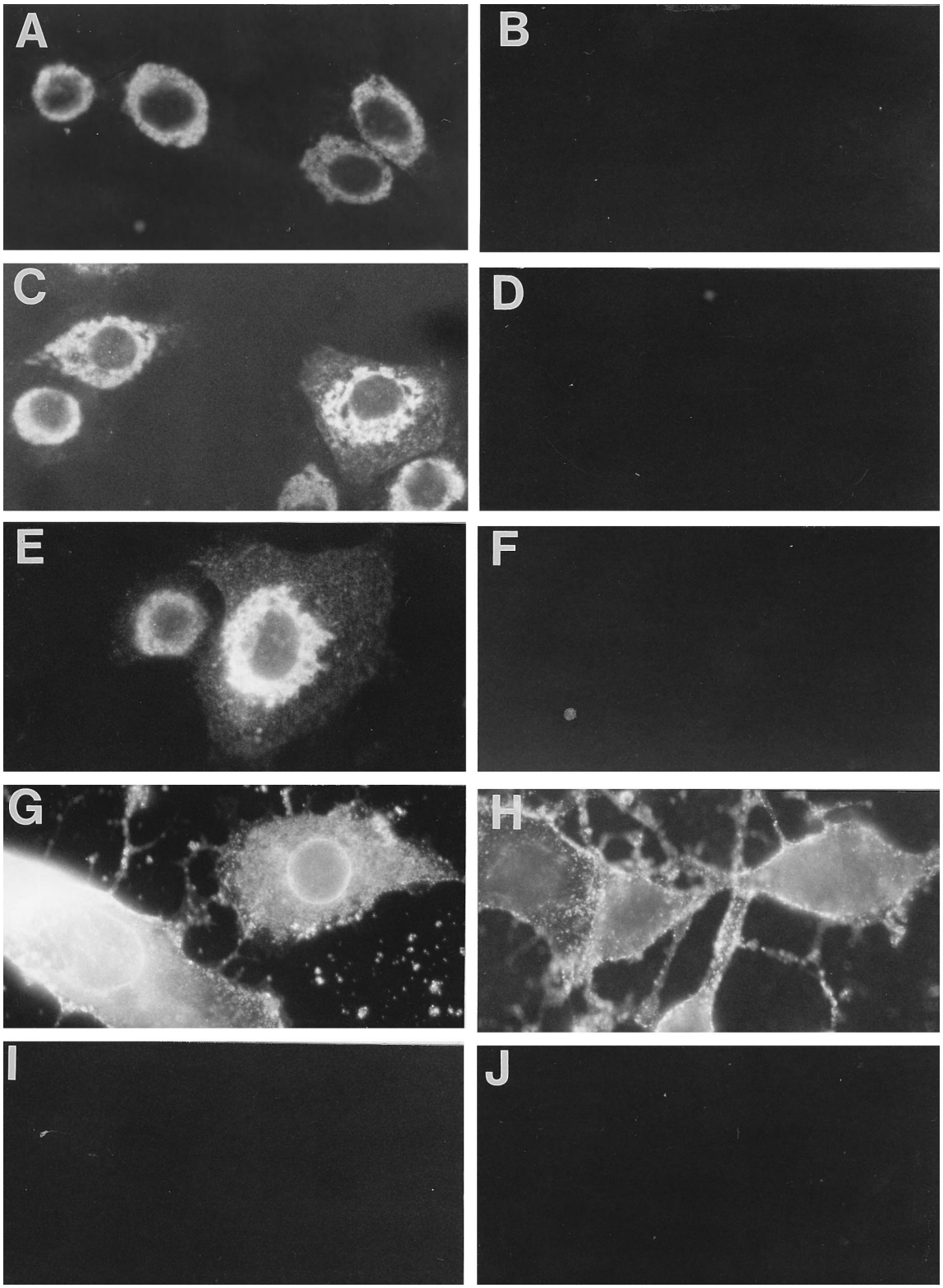

FIG. 2. Immunofluorescence staining of gK in HSV-1-infected gK-9 cells. gK-9 cells were infected with HSV-1 (F-gEβ) at 30 PFU per cell, and after 12 h the cells were fixed with 2% paraformaldehyde and then some monolayers were permeabilized with 0.2% Triton X-100 for 5 min (A, C, E, G, and I), while others were not permeabilized (B, D, F, H, and J). The cells were incubated with individual antisera directed against the UL53-1 peptide (A and B), the UL53-3 peptide (C and D), or the UL53-4 peptide (E and F) or with anti-gD antiserum (G and H) or preimmune serum (I and J). The cells were washed, incubated with fluorescein-conjugated goat anti-rabbit IgG antibodies, washed again, and then mounted on glass slides.

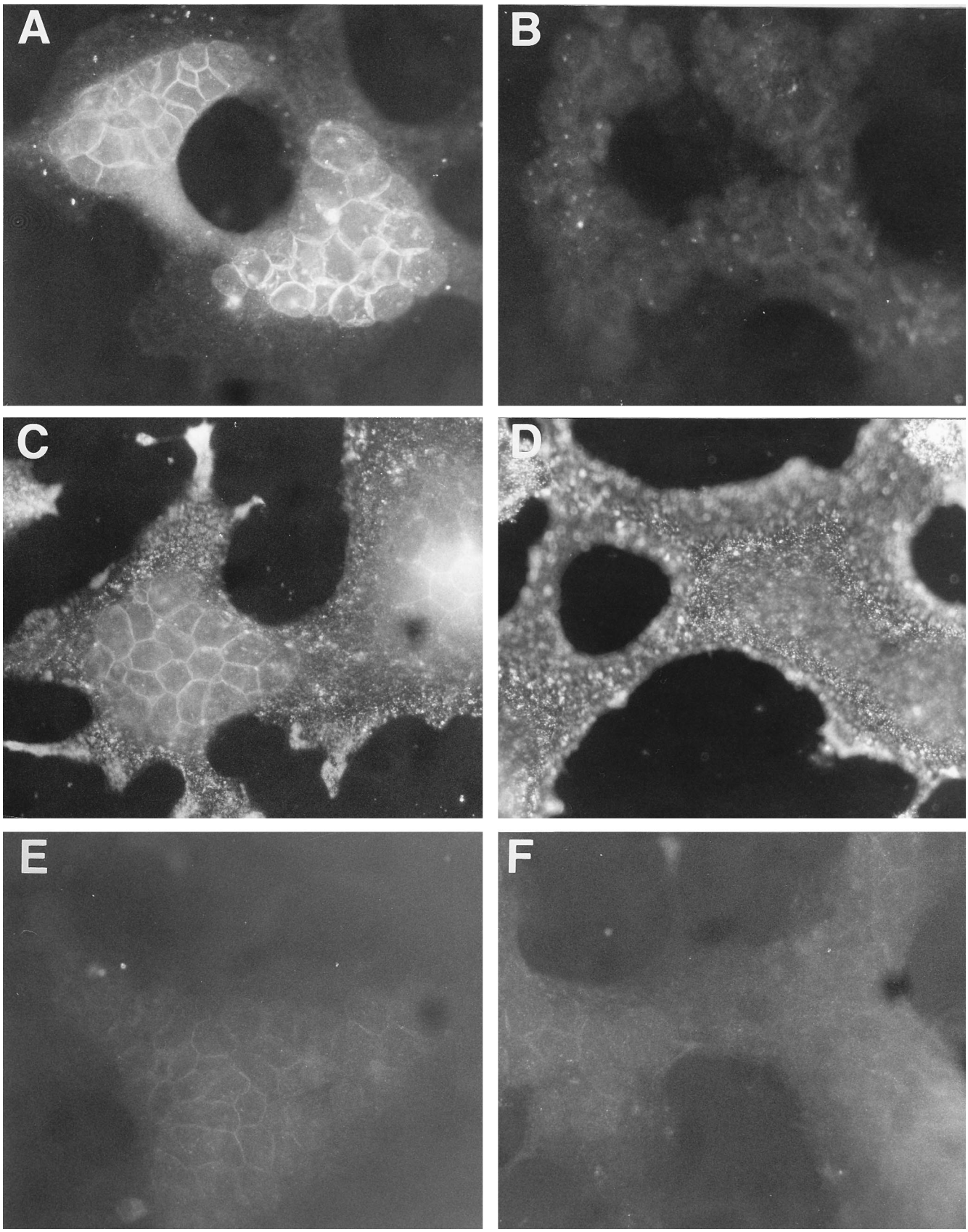

FIG. 3. gK is restricted to the perinuclear region of cells infected with HSV-1 syncytial mutants. Vero cell monolayers growing on glass coverslips were infected with HSV-1 strain MP at 30 PFU per cell, and 13 h later the cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.2% Triton X-100 (A, C, and E) or were not permeabilized (B, D, and F). The cells were incubated with a mixture of rabbit anti-UL53-1 and anti-UL53-4 sera (A and B), rabbit anti-gD serum (C and D), or preimmune sera (E and F) and then washed and incubated with fluorescein-conjugated goat anti-rabbit IgG. Coverslips were washed, mounted on slides, and fluorescence photographed.

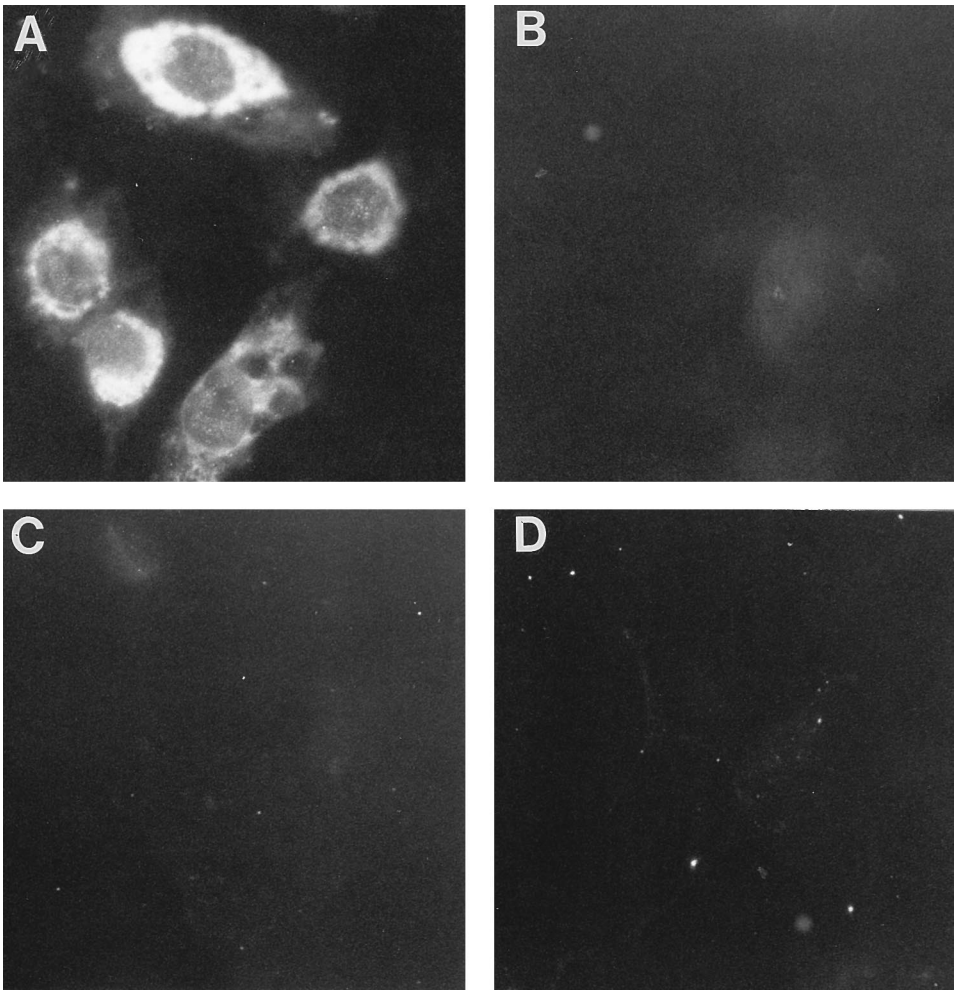

FIG. 4. Immunofluorescence staining of cells infected with AdgK, an adenovirus vector expressing gK. Vero cells growing on glass coverslips were infected with AdgK (A and B) or AddlE3 (C and D) at 1,000 PFU per cell. At 27 h after infection, the cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.2% Triton X-100 (A and C) or not permeabilized (B and D); then they were incubated with a mixture of anti-UL53-1 and anti-UL53-4 sera. The cells were washed and incubated with fluorescein-conjugated goat anti-rabbit IgG serum. The coverslips were washed and mounted on glass slides.

17). Similarly, gK expressed at much higher levels in gK-9 cells did not become endo H resistant and syncytial forms of gK remained endo H sensitive (not shown).

Conclusions. The results presented here support the conclusion that HSV-1 gK is retained in the nuclear envelope and ER in HSV-infected cells. Immunofluorescence staining with three different gK-specific antipeptide sera demonstrated that gK accumulates in perinuclear and nuclear membranes of HSV-infected Vero cells. Moreover, gK could not be detected on the surfaces of HSV-infected cells, a result which distinguishes gK from all other HSV glycoproteins studied to date (1, 16, 18, 20, 23, 27–31, 45, 53). gK expressed by using a recombinant adenovirus vector was restricted to perinuclear and nuclear membranes, supporting the conclusion that other HSV proteins are not required for intracellular retention of gK. In addition, gK produced by syncytial HSV-1 did not reach the cell surface.

Supporting the hypothesis that gK does not leave the perinuclear compartment of cells, gK oligosaccharides remained entirely endo H sensitive. This result supports the immunofluorescence data and suggests that gK does not reach the medial Golgi. It should be noted that the mature form of HSV-1 gB also contains a fraction of N-linked oligosaccharides which remains sensitive to endo H, though other gB oligosaccharides acquire endo H resistance (32, 55) and the other HSV glycoproteins characterized to date become predominantly endo H resistant (1, 32, 45, 51). We know of no example of a herpesvirus glycoprotein which reaches the cell surface without oligosaccharide processing in the Golgi apparatus. gK does not possess obvious ER retention motifs previously identified in type I and type II membrane proteins or the KDEL/HDEL retention motif of ER luminal proteins (reviewed in reference 49).

There is ample evidence that mutations in gK produce striking effects in cells, causing extensive cell fusion within 4 to 6 h after HSV infection (40). These observations have suggested that gK plays a central role in regulating fusion of infected cells and, perhaps, fusion of the virion envelope with cellular membranes during the process of virus entry into cells. Several models have been put forward to explain the effects of gK mutations on infected cells, though none of these models have been confirmed experimentally. It has been proposed that gK (i) possesses an innate fusion-inducing activity which is triggered by mutations in the protein, (ii) catalyzes or indirectly influences a process which controls or regulates the functions of viral fusion proteins, (iii) interacts with components of the fusion complex in the plasma membrane and virion envelope

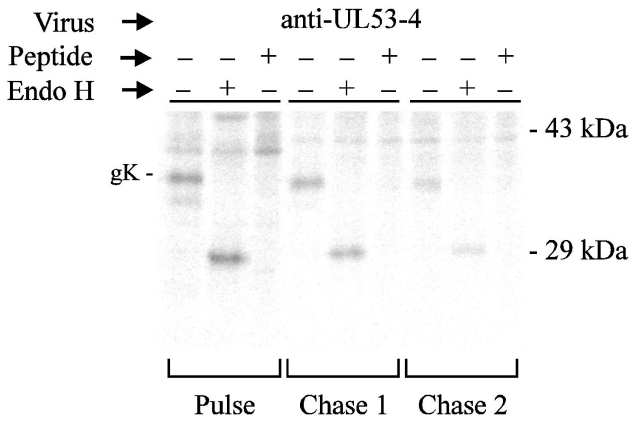

FIG. 5. gK oligosaccharides are not processed in HSV-1-infected cells. Vero cells were infected with HSV-1(F) at 20 PFU per cell. After 4 h, the cells were labelled with [^{35}S]methionine and [^{35}S]cysteine (100 μCi of each per ml) for 20 min (Pulse) and cell extracts were made immediately or the label was chased for 90 (Chase 1) or 235 (Chase 2) min before cell extracts were made. Cell extracts (from approximately 10^6 cells) were clarified by centrifugation and then mixed with 10 μl of anti-UL53-4 serum (−) or with 10 μl of anti-UL53-4 serum preincubated with 100 μg of UL53-4 peptide (+). Antigen-antibody complexes were washed and incubated in denaturing buffer (0.5% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol) at 37°C for 30 min, and then the mixture was diluted and proteins were digested with endo H (+) or mock digested (−) for 3 h at 37°C. Precipitated proteins were diluted in sample buffer, placed at 37°C for 30 min, and subjected to electrophoresis on SDS-polyacrylamide (14%) gels. The gels were dried and analyzed using a Molecular Dynamics Image Quant phosphoimager. The positions of gK and molecular mass markers of 43 and 29 kDa are indicated.

to regulate fusion, or (iv) influences cell surface transport of the HSV or host cell proteins which affect cell-cell fusion (5, 25, 26, 35, 38, 44, 47, 51, 52). The results presented here rule out a number of these possibilities because gK is not present in the plasma membrane and, therefore, it is unlikely that it plays a direct role in fusion of cells. Moreover, the small quantities of gK expressed in HSV-infected cells relative to other glycoproteins involved in cell-cell fusion is more consistent with a regulatory role for gK. It is also unlikely that gK is associated with mature virus particles at the cell surface, because we could not detect gK at the cell surface by immunofluorescence (Fig. 1). In addition, we have been unable to detect gK labelled with either [^{35}S]methionine and [^{35}S]cysteine or ^{125}I in virus particles purified from the medium of infected cells (not shown), although a previous communication (24) indicated incorrectly that we had done so. If gK is not part of the virus particle, it appears unlikely that it participates directly in fusion of the virion envelope during entry.

Therefore, gK is an unusual HSV glycoprotein that accumulates in cytoplasmic membranes, the ER, and the nuclear envelope and does not reach the cell surface. We have recently constructed an HSV-1 mutant unable to express gK, and this mutant is unable to produce infectious viruses; instead, viruses accumulate within intracellular membranes (26). The phenotype of the mutant is consistent with the internal localization of gK observed here, and the results demonstrate that gK is essential for virus replication.

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