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An Endoplasmic Reticulum-Retained Herpes Simplex Virus Glycoprotein H Is Absent from Secreted Virions: Evidence for Reenvelopment during Egress

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Although it is generally accepted that one of the first steps of herpesvirus egress is the acquisition of an envelope by nucleocapsids budding into the inner nuclear membrane, later events in the pathway are not well understood. We tested the hypothesis that the virus then undergoes de-envelopment, followed by reenvelopment at membranes outside the endoplasmic reticulum (ER), by constructing a recombinant virus in which the expression of an essential glycoprotein, gH, is restricted to the inner nuclear membrane-ER by means of the ER retention motif, KKXX. This targeting signal conferred the predicted ER localization properties on gH in recombinant virus-infected cells, and gH and gL polypeptides failed to become processed to their mature forms. Cells infected with the recombinant virus released particles with 100-fold less infectivity than those released by cells infected with the wild-type parent virus, yet the number of enveloped virus particles released into the medium was unaltered. These particles contained normal amounts of gD and VP16 but did not contain detectable amounts of gH, and these data are consistent with a model of virus exit whereby naked nucleocapsids in the cytoplasm acquire their final envelope from a subcellular compartment other than the ER-inner nuclear membrane.

The egress of herpesvirus particles from an infected cell is believed to initiate with the envelopment of nucleocapsids at the inner lamella of the nuclear membrane (12), where virions can be observed budding through the inner nuclear membrane into the perinuclear space. However, subsequent routes taken by these particles as they travel out of the cell are less well understood and remain a contentious issue. One view is that enveloped virions move via the secretory pathway by vesicular transport from the endoplasmic reticulum (ER) to the Golgi complex and *trans*-Golgi network, ultimately reaching the plasma membrane where they are released. This mechanism of egress implies that the virion acquires a full complement of transmembrane glycoproteins at the inner nuclear membrane and that these immature glycoproteins are processed in situ during egress through the Golgi compartment. Evidence in favor of this model was reported by Johnson and Spear (20), who showed that ionophores which disrupt the budding of vesicles from the Golgi, such as monensin, inhibit the transport of herpes simplex virus (HSV) progeny virions to the cell surface and lead to an accumulation of enveloped particles in what are believed to be Golgi-derived vacuoles. The view that herpesvirus egress involves a single envelopment process has the intuitive virtue of economy, and this model finds favor in most standard texts (26). An alternative model for virus egress involves the fusion of enveloped virus particles in the perinuclear cisterna or the ER lumen with the outer nuclear membrane, thus releasing nucleocapsids into the cytoplasm; capsids are often observed in herpesvirus-infected cells adjacent to membrane-bound vesicular structures, and it has been suggested that these represent virions in the process of reenvelopment by Golgi-derived vacuoles. Data supporting this pathway (initially proposed by Stackpole [31]) have been described for varicella-zoster virus (15, 37) and for pseudorabies virus (35), and studies of HSV trafficking in a neuronal-epidermal cell culture system have highlighted the prevalence of unenveloped nucleocapsids in terminal axons (25). However, arguments against this pathway include analysis of a mutation in HSV type 1 (HSV-1) glycoprotein D (gD) which results in the accumulation of large numbers of unenveloped capsids in the cytoplasm. This mutation also causes reduced yields of extracellular virus, and it has, therefore, been proposed that naked cytoplasmic nucleocapsids represent a dead end rather than a stage in the route of virus egress (9).

One of the limitations of the data supporting these two models of egress is that they rely heavily on the interpretation of electron microscopic images and on the identification of subcellular membranes and organelles. We have taken a genetic approach to investigate this problem by constructing recombinant HSVs in which an essential glycoprotein (gH) is retained in the membranes of the ER by means of the ER-targeting signal, KKXX (18). KKXX motifs (such as the sequence KKSL, used in this study) are thought to act as retrieval signals, bringing membrane proteins from a post-ER sorting compartment back to the ER (19). We also made a control virus with a KKSLAL motif at the carboxy terminus of gH, since the addition of two amino acids to the KKXX sequence is known to override the retention signal, enabling proteins to traffic out to the cell surface. We reasoned that if the virus undergoes a de-envelopment and reenvelopment route of exit, then restriction of gH to the ER would lead to reduced infectivity of secreted virus particles (since these virions would not contain gH) without affecting the number of virus particles released. If, on the other hand, the virus retains the envelope acquired at the nuclear membrane, restriction of gH to this

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compartment should have no effect on infectious particle secretion.

MATERIALS AND METHODS

Cells and viruses. BHK-21 and CR1 cells were grown in Glasgow's modified Eagle medium supplemented with 10% newborn calf serum and tryptose phosphate broth. CR1 cells are a stably transformed Vero cell line which express HSV-1 gH under the control of the HSV-1 gD promoter (provided by Cantab Pharmaceuticals, Cambridge, United Kingdom). This cell line is analogous to the helper cell line F6 (14) but contains HSV-1 gH coding sequences from which all flanking sequences have been removed. The construction of this cell line will be described elsewhere. The HSV strain SC16 was grown, and titers were determined in BHK cells. Recombinant viruses containing modified gH sequences were grown in CR1 cells, and titers were determined on CR1 cells.

Antibodies. gH expression was detected with monoclonal antibodies LP11 (6) and 52S and 53S (29) and with polyclonal serum against a trpE-gH fusion protein (13). VP16 and gD were detected with monoclonal antibodies LP1 and LP14, respectively (23, 24). gL expression was detected with polyclonal rabbit serum raised against a β-galactosidase fusion protein derived from a pEX vector construct (32) containing gL coding sequence from nucleotide 9,629 to 10,011 according to the numbering of the HSV genome described by McGeoch et al. (22).

Plasmids and mutagenesis. Plasmid pgHLmpSC11 (5) was digested to release the HFEM gH gene as a 3.5-kb *Bgl*II fragment. This DNA was end repaired with the Klenow fragment of DNA polymerase and ligated with end-repaired *Eco*RI-digested plasmid SMH3 to yield pSMH3gH1 (36). Fragments containing modified 3′ gH sequences were generated with pSMH3gH1 as the template DNA and the oligonucleotide primer 5′GCCGCAATTGCGCCCGGG3′ (a perfect match to the region of gH which includes the *Xma*I site at position 43,974) and a mutagenic primer. The primer used to append a KKXX motif to the C terminus of gH was 5′GATCATGCATCTATTAGAGGCTCTTCTTACTTTCGCGTCT CCAAAAAAACG3′, which modifies the carboxy terminus to encode the four additional residues KKSL, and that used to add the residues KKSLAL was 5′G CGCATGCATGTCTAGAGAATTCCTATTAGAGAGCGAGGCTCTTC TTACTTTCGCGTCTCCAAAAAAACGG3′. PCR mixtures contained 20 pmol of each primer, 20 ng of pSMHgH1 template DNA, 1 mM (each) deoxynucleoside triphosphate, and 0.5 U of *Taq* DNA polymerase (Cetus) in the manufacturer's recommended buffer in a final volume of 25 μl. Reaction mixtures were overlaid with mineral oil, subjected to 25 cycles of 94°C for 0.5 min, 55°C for 1 min, and 72°C for 0.5 min, and chased for 5 min at 72°C. The PCR fragments were digested with *Xma*I and *Nsi*I (for KKSL) or with *Xma*I and *Xba*I (for KKSLAL) and ligated with *Xma*I-*Nsi*I- or *Xma*I-*Xba*I-digested pGEMHO (36). This manipulation reconstructs modified 3′ portions of the gH gene from the *Eco*RV site at position 44,247 to the mutagenic primer sequence. The modified sequences were subsequently isolated as *Bst*XI-*Nsi*I fragments (for KKXX) or a *Bst*XI-*Xba*I fragments for KKX4 and ligated to *Bst*XI-*Xba*I-digested pSMHgH1 following appropriate repair of noncompatible ends.

The shuttle vector used to generate the recombinant viruses described in this report is derived from pIMB52 provided by M. Boursnell, Cantab Pharmaceuticals. This plasmid contains HSV-1 sequences which flank the gH gene, with an *Hpa*I restriction site in place of the gH coding region. The gH flanking sequences in pIMB52 contain HSV DNA from position 47,056 to 46,386 (5′ flank) and from position 43,875 to 43,298 (3′ flank). Modified gH genes were inserted in pIMB52 and were named pIMPL KKXX and pIMPL KKX4.

pIMB52iegal is the plasmid used to generate a β-galactosidase-expressing virus which lacks the entire gH coding region. This plasmid contains a human cytomegalovirus immediate early promoter-*lacZ* gene cassette (derived as an end-repaired *Hin*dIII fragment from pMV10 [14]) ligated at the *Hpa*I site of pIMB52.

Single-step growth analysis. Dishes (5-cm diameter) containing 3×10^6 BHK cells were infected at 10 PFU per cell for 1 h at 37°C, and then were washed in phosphate-buffered saline (PBS), treated for 2 min with 135 mM NaCl–10 mM KCl–40 mM citric acid, pH 3, to inactivate unpenetrated virions (36), and returned to neutral pH by washing twice with medium and overlaying with 5 ml of modified Eagle medium-newborn calf serum. At various times after infection, supernatant and infected cells were harvested and infected cells were sonicated and stored at −70°C. All samples were assayed for infectious virus by titration on CR1 cell monolayers.

Neutralization assays. A total of 2×10^4 PFU of HSV-1 was mixed with twofold serial dilutions of the anti-gH monoclonal antibody LP11 (as hybridoma supernatant), and after incubation for 1 h at room temperature the residual infectivity was assayed on CR1 cells. For cases in which the neutralization characteristics of viruses carrying wild-type or mutant gH molecules were compared, the wild-type virus contained a *lacZ* cassette inserted in the Us5 gene (3). The two viruses were mixed and neutralized in single reactions, and the surviving progeny were distinguished by staining for β-galactosidase.

Immunofluorescence. BHK cells seeded on glass coverslips were infected at a multiplicity of infection of 10 for 18 h with recombinant viruses. The infected cells were fixed in 2% formaldehyde in PBS for 5 min at room temperature and washed three times with PBS containing 1% fetal calf serum (FCS). The cells were permeabilized by incubation in PBS containing 1% Triton X-100, 10% sucrose, and 1% FCS for 5 min and then were washed 3 times in PBS–1% FCS. All subsequent incubations and washes were in PBS containing 1% FCS. Coverslips were incubated for 1 h in antibody (culture supernatant diluted 1:2), washed three times, incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (DAKO) at a dilution of 1:50 for 45 min, and washed three times, and immunofluorescence was visualized by confocal microscopy.

Western blotting (immunoblotting). Extracts of infected BHK cells (harvested 18 h after infection at 10 PFU per cell) or pelleted virions were boiled in Laemmli buffer and subjected to denaturing electrophoresis in polyacrylamide gels. Proteins were transferred to nitrocellulose membranes which were incubated with the appropriate antibodies and were detected with enhanced chemiluminescence reagent (Amersham, Amersham, United Kingdom) as previously described (36).

Particle counting. Virions suspended in 1 mM Tris-HCl, pH 7.5, were mixed with an equal volume of 254-nm diameter latex particles suspended at a concentration of 10^{10} particles per ml in bovine serum albumin (2 mg/ml) and an equal volume of 2% phosphotungstic acid, pH 7.0. The mixture was sonicated for 30 s, and specimens were prepared by the loop-drop method described by Watson et al. (34).

RESULTS

Construction of recombinant viruses. (i) Construction of a gH-negative virus. An HSV-1 SC16-derived parental virus lacking the entire gH coding region was constructed by cotransfection of CR1 cells with 10 μg of SC16-infected cell DNA and with 2.5 μg of pIMB52iegal by using the modified calcium phosphate preparation method of Chen and Okayama (10). Cotransfection progeny were harvested and replated on CR1 monolayers, and blue-staining plaques were picked after the cells were overlaid with agar containing X-Gal (5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside) as previously described (14). Recombinant progeny were subsequently plaque-purified three times, and the correct insertion of the cytomegalovirus immediate early promoter-*lacZ* cassette at the gH locus was established by Southern hybridization (data not shown). This virus is called SCZgH$^-$pA.

(ii) Construction of recombinant viruses containing modified gH genes. CR1 cells were cotransfected with 10 μg of SCZgH$^-$pA-infected cell DNA and 2.5 μg of either pIMPL KKXX or pIMPL KKX4 DNA. Cotransfection progeny were plated onto CR1 monolayers with an agarose overlay containing X-Gal, and white plaques were picked and purified by limiting dilution on CR1 cells. Two independent isolates of each virus were purified, and the presence of the targeting signal was confirmed by nucleotide sequencing. These viruses were named SC16-gHKKXX and SC16-gHKKX4.

Analysis of expression of gH by recombinant viruses. Two approaches were taken to examine the ability of the KKXX ER retention signal to confer the predicted localization properties on gH and the ability of the KKX4 motif to reverse these properties. Firstly, cells were infected with recombinant SC16-gHKKXX or SC16-gHKKX4 and gH localization was examined by immunofluorescence. Secondly, the electrophoretic mobility of gH was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to find whether, as predicted, gHKKXX would be synthesized only as an immature glycoprotein. Since gL forms a complex with gH (16), the retention of gH in the ER should result in the retention of gL also. Therefore, we examined the electrophoretic mobility of gL by immunoblotting. Figure 1 shows the results of immunofluorescence experiments. Cells infected with SC16-gHKKX4 exhibited cell surface and internal gH staining and were indistinguishable from cells infected with the wild-type parent virus. No gH could be detected on the surface of cells infected with SC16-gH KKXX, but permeabilized cells gave a perinuclear staining pattern typical of ER proteins.

Figure 2 shows the electrophoretic mobility of gH and gL

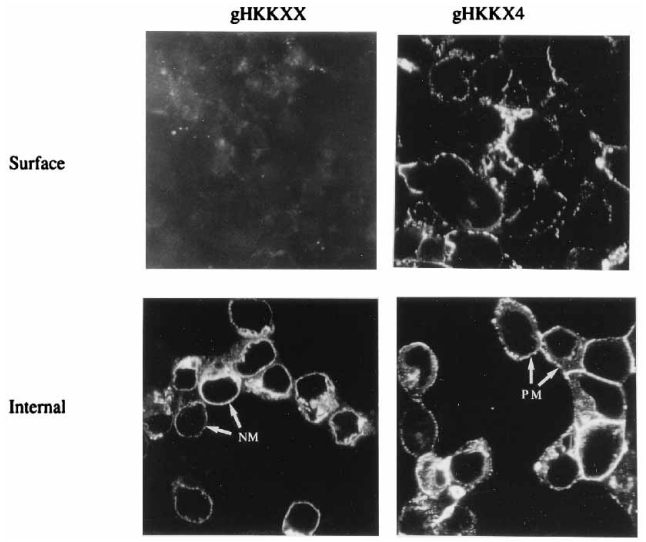

FIG. 1. Localization of gH in BHK cells infected with recombinant viruses. Infected cells were stained with a pool of monoclonal antibodies (LP11, 52S, and 53S) to gH. Internal staining was detected after cells were permeabilized with Triton X-100, and fluorescence was visualized by confocal microscopy. Nuclear membrane staining in permeabilized gHKKXX-infected cells (NM) and the predominantly plasma membrane localization of gH in gHKKX4-infected cells (PM) are indicated by arrows.

polypeptides synthesized by cells infected with recombinant viruses SC16-gHKKXX or SC16-gHKKX4. Two independently constructed isolates of each recombinant were used in these experiments. Cells infected with SC16-gHKKX4 produce mature 110-kDa gH and mature 40-kDa gL (13, 16), whereas cells infected with SC16-gHKKXX produce only precursor forms of both proteins. The results of immunofluorescence and electrophoretic studies are entirely consistent with the predicted effects of the KKXX and KKX4 motifs engineered into the cytoplasmic C terminus of the gH molecule.

Growth characteristics of recombinant viruses. Preliminary experiments established that two independently derived SC16-gHKKX4 recombinants formed plaques with equal efficiency on CR1 and Vero cell monolayers and that the plaques were indistinguishable from those formed by the parental wild-type virus. In contrast, recombinant SC16-gHKKXX failed to form plaques on Vero cells, but microscopic examination of infected monolayers after 4 days revealed small foci of cytopathic effect.

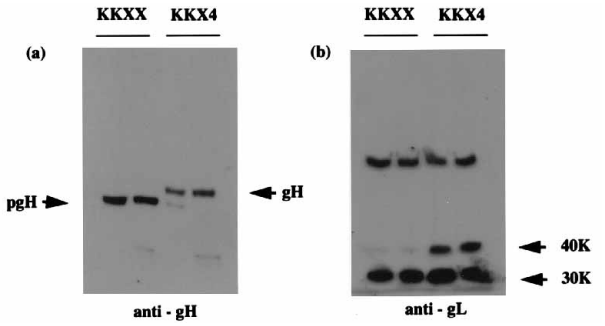

FIG. 2. Analysis of gH and gL polypeptides in lysates of cells infected with recombinant viruses. Immunoblots of infected cell proteins were incubated with either anti-gH polyclonal rabbit serum (a) or with anti-gL polyclonal rabbit serum (b). Each track contains protein extracts from 10^5 cells harvested 18 h after infection at 10 PFU/cell. The high-molecular-weight species detected by the anti-gL serum is present in lysates of uninfected cells. pgH, precursor form of gH.

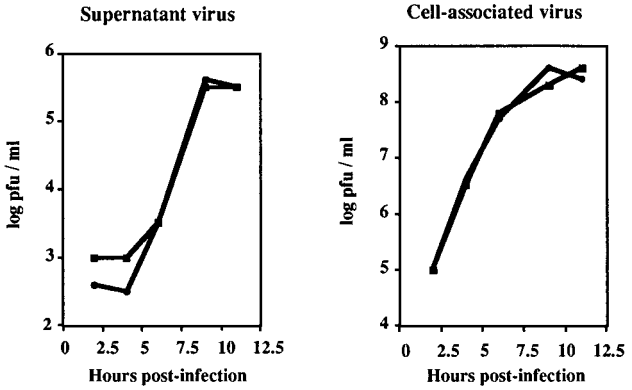

FIG. 3. Single-step growth kinetics of SC16 (●) and SC16-gHKKX4 (■) in BHK cells. Yields of cell-associated and released infectious virus were determined by plaque assay following the infection of 3×10^6 BHK cells at 10 PFU per cell. Supernatant titers represent one-fifth of the total yield, and cell-associated titers represent total cell-associated yield. All values are the means of duplicate treatments.

When the monolayers were harvested and sonicated and infectivity was assayed on CR1 cells, it was apparent that, despite the failure to form plaques, significant growth of the recombinant virus in Vero cells had occurred. The growth characteristics of the recombinant viruses following high-multiplicity infection were therefore studied. Initially we compared the one-step growth characteristics of the parent virus HSV-1 SC16 with those of the recombinant SC16-gHKKX4. Figure 3 indicates that SC16-gHKKX4 is indistinguishable from wild-type virus in the kinetics of synthesis of secreted and cell-associated infectivity. Subsequent experiments, therefore, compared the two recombinant viruses only.

Monolayers of 3×10^6 BHK cells were infected at a multiplicity of infection of 10 with the recombinant viruses, and after 12 h the yields of secreted and cell-associated virus were assayed. The results in Table 1 show that recombinant SC16-gHKKXX secretes particles with approximately 100-fold less infectivity than does recombinant SC16-gHKKX4. The yield of cell-associated SC16-gHKKXX is reduced by a similar factor, but the measurement of cell-associated virus does not distinguish between true intracellular infectivity and infectious particles that have exited the cell but are not yet released into the medium. In order to measure intracellular infectivity alone,

TABLE 1. Yields of secreted and cell-associated infectivity[a]

Sample	Time post-infection (h)	Infectivity (PFU/3×10^6 cells)	
		KKXX	KKX4
Supernatant	2	0	0
	12	1.8×10^3	2.5×10^5
Cell associated	2	2×10^3	3×10^3
	12	4×10^6	2×10^8
Cell associated (after citrate wash)	12	1.3×10^6	1.2×10^7

[a] BHK cells (3×10^6) were infected at 5 PFU per cell with SC16-gHKKXX or SC16-gHKKX4, and residual inoculum was inactivated by a pH 3 citrate wash. At 12 h after infection, supernatants were harvested, cells were scraped off and harvested, and one set of infected cells was treated with citrate buffer, pH 3, for 2 min and washed twice with modified Eagle medium. Samples were also taken at 2 h postinfection to determine the efficacy of the citrate wash. All samples were sonicated and assayed for infectious virus on CR1 monolayers. The values represent total numbers of PFU from 3×10^6 cells and are the means of duplicate treatments.

TABLE 2. Enveloped particles and infectivities of released recombinant virions[a]

Recombinant virus	Total no. of PFU	Total no. of enveloped particles (SEM)
KKXX	2×10^7	6.2×10^{10} (0.9×10^{10})
KKX4	3×10^9	5×10^{10} (0.7×10^{10})

[a] Total yields of infectious virus and of enveloped virus particles released into the supernatants of 2×10^8 recombinant virus-infected cells. Infectivity assays were performed in duplicate, and particle counts represent values obtained from six independent counts in which groups of 20 latex particles were scored against numbers of enveloped virus particles.

parallel cultures were washed briefly with citrate buffer, pH 3.0, as described in Materials and Methods, to inactivate extracellular virus prior to sonication and assay. The results (Table 1) show that about 95% of the cell-associated SC16-gHKKX4 is in fact extracellular. In contrast treatment at pH 3.0 reduced the yield of cell-associated SC16-gHKKXX by a factor of 2 to 3. If we accept that treatment of infected cells at pH 3.0 allows a correct measurement of intracellular infectivity, then the results in Table 1 can be summarized as follows: the effect of the ER retention signal in gH is to reduce the yield of extracellular infectious virus by about 100-fold and the yield of intracellular infectious virus by about 10-fold.

Properties of secreted virions. Since the effect of the KKXX retention signal in gH is to reduce the production of secreted infectious progeny by approximately 100-fold, we asked whether this was due to a reduction in virion secretion or to a reduction in the specific infectivity of the secreted virions. BHK cells (2×10^8) were infected at a multiplicity of infection of 5 with either SC16-gHKKXX or SC16-gHKKX4. After 24 h, the medium was harvested and subjected to low-speed centrifugation (5,000 $\times$ *g* for 10 min) to remove cells and debris and infectivity was assayed on CR1 cells. Virions were then pelleted from 100 ml of medium by centrifugation at 40,000 $\times$ *g* for 1 h and resuspended in 1 ml of 1 mM Tris-HCl, pH 7.5, and the yield of enveloped virus particles was determined by electron microscopy as described in Materials and Methods. Table 2 shows that the yields of secreted particles from the recombinants are not significantly different from one another and indicates that the loss of infectivity which results from the insertion of an ER retention signal in gH is due to a reduction in the specific infectivity of the secreted virions.

Samples containing 10^{10} enveloped virions were then subjected to PAGE, and the blots were probed with antibodies against gH, gD, or the tegument protein VP16. Wild-type virus particles (10^{10}) were included for comparison. Figure 4 shows

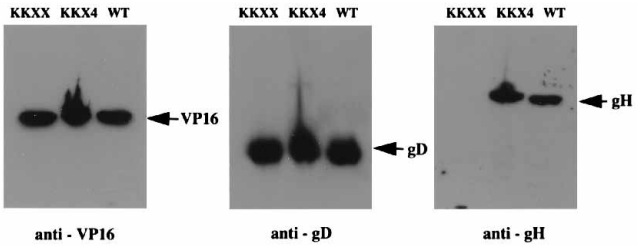

FIG. 4. Western blotting of released SC16-gHKKXX and SC16-gHKKX4 virions with antibodies to VP16, gD, and gH. Lysates corresponding to approximately 10^{10} particles of recombinant or parental (WT) viruses were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with monoclonal antibodies to VP16 and glycoprotein D or with polyclonal antiserum to gH.

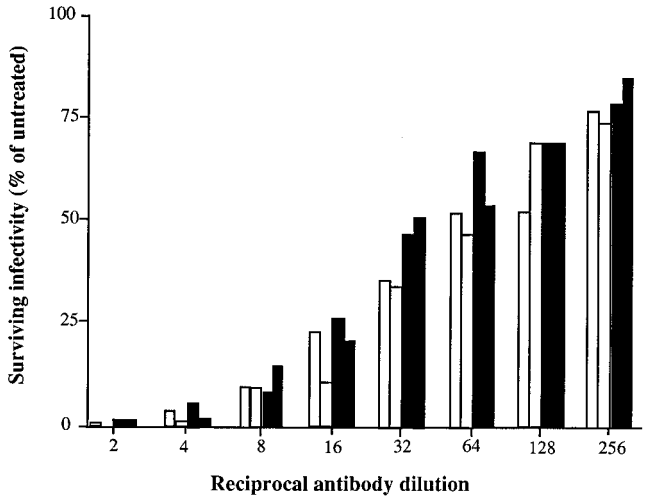

FIG. 5. Effect of LP11 on residual gHKKXX infectivity. Equivalent amounts of SC16-gHKKXX (open bars) and wild-type cell-associated infectious virus (solid bars), harvested from infected Vero cells, were incubated with dilutions of monoclonal antibody LP11. Surviving infectivity is expressed as a percentage of untreated samples, and the results are presented as pairs of duplicates.

that virions from cells infected with SC16-gHKKX4 or SC16-gHKKXX contain approximately similar amounts of gD and VP16 and are indistinguishable from the wild-type virus in this respect. SC16-gHKKXX virions, however, contained no detectable gH, whereas SC16-gHKKX4 virions contain wild-type levels. We conclude that the loss of infectivity in secreted SC16-gHKKXX virions is due to the absence of gH.

The nature of residual gHKKXX infectivity. The effect of restricting gH expression to the membranes of the ER was a significant reduction in the yield of infectious virus. This phenotype is equally consistent with the views that (i) a proportion of virions produced by gHKKXX-infected cells contain normal amounts of gH while the majority are noninfectious because of its absence, (ii) all particles produced by gHKKXX incorporate fewer molecules of gH per virion than does the wild-type virus, and (iii) a proportion of gH-independent infectivity is present in gHKKXX-infected cells.

We attempted to discriminate between these three likely scenarios by examining the sensitivity of residual gHKKXX infectivity to neutralization by a monoclonal antibody directed against gH. Equivalent numbers of PFUs from wild-type and gHKKXX-infected cells were incubated with a series of twofold dilutions of LP11 antibody, and the neutralization profiles of these two populations of infectious virus are shown in Fig. 5. LP11 neutralized gHKKXX infectivity with the same efficiency as it neutralized wild-type virus, suggesting that the residual infectivity in gHKKXX-infected cells is composed of virus particles which contain wild-type amounts of gH.

Effect of the ER retention signal in gH of a syncytial virus. Cells infected by syncytial HSV mutants exhibit uncontrolled fusion with their neighbors, and it is conceivable that such mutants might also differ in their intracellular transport characteristics. We therefore introduced the KKXX motif into the cytoplasmic tail of gH of the HSV-1 mutant SC16 gB^{ANG}, a derivative of SC16 which contains a syncytial mutation in the gB gene corresponding to that found in the ANG strain (3). SC16 gB^{ANG}-gHKKXX exhibited the same properties as its nonsyncytial homolog; namely, it failed to plaque in Vero cells and it secreted normal numbers of noninfectious particles deficient in gH (data not shown).

DISCUSSION

The addition of the sequence KKSL to the C-terminal cytoplasmic tail of HSV-1 gH resulted in a pattern of intracellular expression consistent with the identification of the KKXX motif as an ER retention signal (18); no gH could be detected on the surface of infected cells by immunofluorescence, and permeabilized cells exhibited prominent perinuclear staining. The addition of a further two residues (yielding KKSLAL) reversed this phenotype. The consequence of retaining gH in the ER is that progeny virions secreted into the medium contain no detectable gH and are noninfectious. The obvious implication is that the envelope of each of these virions is acquired from a post-ER compartment—the Golgi apparatus, a post-Golgi vesicle, or the plasma membrane. This interpretation is consistent with recent data on the envelopment of varicella-zoster virus and pseudorabies virus (15, 35, 37), with the effects of brefeldin A on HSV maturation (11), and with the observation of naked nucleocapsids in the terminal axons of neurones infected with HSV (25). Our data are difficult to reconcile with a model of egress in which the envelope acquired by budding through the inner nuclear membrane is retained by the virus during exit via vesicular transport.

A trivial explanation of our results is that the C-terminal addition of the KKSL creates an assembly problem such that gH is excluded from the envelope during budding, thus accounting for the absence of gH in progeny virions and for their very low levels of specific infectivity. Two lines of reasoning make this explanation implausible. First, it seems very unlikely that the addition of the KKSL peptide would exclude gH from the assembly process, whereas the addition of two further residues, as in KKSLAL, would restore gH assembly into virions to wild-type levels. Second, if gHKKXX were inefficiently incorporated into virions we would predict that the residual infectivity might be distinguishable from the wild-type virus in its sensitivity to neutralization by a gH-specific antibody. In fact, the residual infectivity in cells infected with SC16-gHKKXX is neutralized normally by an anti-gH antibody. The simplest interpretation is that this infectivity represents a fraction of virions that contain normal amounts of gH and is consistent with the idea that these virions are present in a specific cellular compartment.

Cells infected with recombinant SC16-gHKKXX yield about 1/10 of the intracellular infectivity of cells infected with wild-type virus (Table 1). These virions contain gH and must, therefore, have acquired their membranes from the ER compartment. The simplest explanation is that these virions reside in the lumen of the ER and represent particles that have budded at the inner nuclear membrane. Our results are therefore consistent with a two-stage egress route, as illustrated in Fig. 6, in which the two potential routes of egress and the predicted effects of restricting gH expression to the ER on infectious gH-positive virus particle output are depicted. We favor a model (depicted as route A in Fig. 6) in which infectious virions appear first in the ER lumen, having acquired a full complement of glycoproteins during budding at the inner nuclear membrane; the argument for this step is supported by the studies of Torrisi et al. (33), who reported the presence of gB and gD on virus particles in the perinuclear space and on the inner nuclear membrane. This step is unaffected by an ER retention signal in gH. These enveloped particles then fuse with the ER membrane to release naked nucleocapsids into the cytoplasm, where they acquire their final envelope by budding into the Golgi apparatus or into a post-Golgi membrane.

While this model is consistent with our data and with recent reports on the egress of varicella-zoster virus and pseudorabies

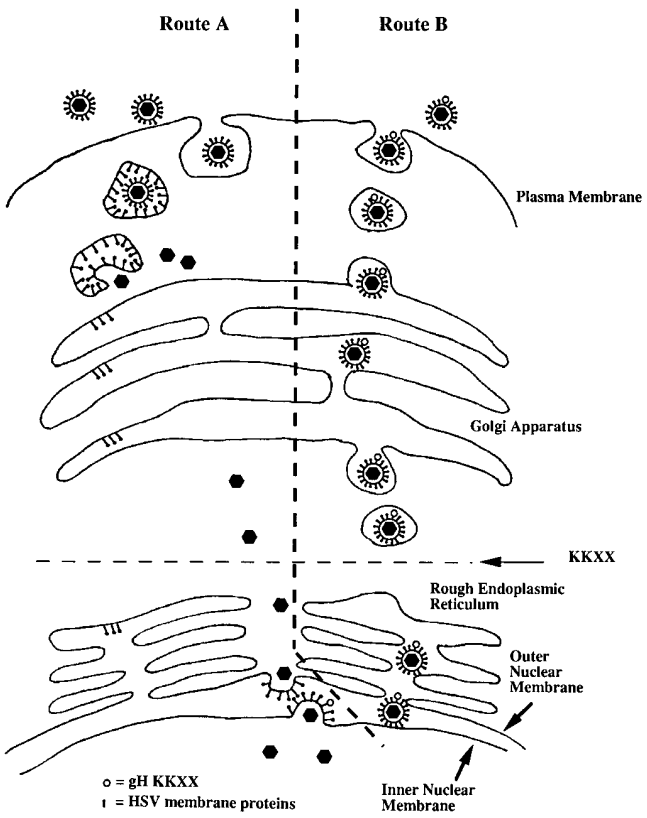

FIG. 6. Routes of HSV egress. Two alternative routes of HSV egress are illustrated in schematic form. (Route A) Virus particles lose their initial membrane by fusion at the outer nuclear membrane-ER and acquire their final membrane at the Golgi or a post-Golgi compartment. (Route B) Virus particles acquire an envelope by budding at the inner nuclear membrane and retain this envelope during exocytic vesicular transport. Route A is consistent with the data presented in this paper in that the addition of an ER retention signal to gH in recombinant SC16-gHKKXX eliminates gH from secreted particles.

virus, a number of significant uncertainties remain. First, the view that infectious particles containing viral glycoproteins are present in the ER lumen presupposes that HSV glycoproteins are present on the inner nuclear membrane, yet there are no described mechanisms for the transport of large transmembrane proteins from the ER to the inner nuclear membrane. If such transport mechanisms exist or are induced by HSV-1 infection, then we cannot exclude the possibility that the KKXX motif interferes with transport to the inner nuclear membrane and hence that gHKKXX is unavailable for assembly into enveloped virions in this compartment. Second, if enveloped particles in the ER lumen fuse with the ER membrane to release naked nucleocapsids into the cytoplasm, then it might be supposed that the likely fusion mechanism would be a virus-induced fusion analogous to that used by the virus for entry into the host cell. The latter process is thought to require HSV-1 gB, gD, gH and gL, yet mutants lacking each of these proteins have been constructed and appear to be competent in the synthesis of enveloped particles containing processed glycoproteins (7, 14, 21, 27). These essential glycoproteins cannot, therefore, be necessary for the processes of envelopment at the inner nuclear membrane, de-envelopment, and reenvelopment. It is, however, not unreasonable to suppose that fusion of the envelope with the inner surface of the ER membrane might differ in some respects from fusion between the envelope and plasma membrane. There is a large body of evidence

to suggest that HSV envelope-plasma membrane attachment and fusion is dependent upon the presence of cell surface heparan sulfate (28, 30). The assembly of these complex glycosamingoglycan chains is believed to occur in the Golgi, and there is no evidence that such proteoglycans exist in the perinuclear space. In addition, there are a growing number of gene products, such as gK (17), ICP34.5 (4), and the product of UL20 (2), which appear to affect the egress of enveloped perinuclear virions, at least in some cell lines.

Finally, we note that our interpretation of the data presented in this paper is based on current views of membranes and membrane protein trafficking in normal cells. Dramatic alterations in the organization of microtubules and Golgi apparatus in some cell types infected with HSV (1, 8) have, however, been observed, and we accept the fact that the modification of trafficking pathways may accompany these structural changes.

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