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The Herpes Simplex Virus U_L20 Protein Compensates for the Differential Disruption of Exocytosis of Virions and Viral Membrane Glycoproteins Associated with Fragmentation of the Golgi Apparatus

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The Golgi apparatus is fragmented and dispersed in Vero cells but not in human 143TK$^-$ cells infected with wild-type herpes simplex virus 1. Moreover, a recombinant virus lacking the gene encoding the membrane protein U_L20 (U_L20^- virus) accumulates in the space between the inner and outer nuclear membranes of Vero cells but is exported and spreads from cell to cell in 143TK$^-$ cell cultures. Here we report that in Vero cells infected with U_L20^- virus, the virion envelope glycoproteins were of the immature type, whereas the viral glycoproteins associated with cell membranes were fully processed up to the addition of sialic acid, a *trans*-Golgi function. Moreover, the amounts of viral glycoproteins accumulating in the plasma membranes were considerably smaller than those detected on the surface of Vero cells infected with wild-type virus. In contrast, the amounts of viral glycoproteins present on the plasma membranes of 143TK$^-$ cells infected with wild-type or U_L20^- virus were nearly identical. We conclude that (i) in Vero cells infected with U_L20^- virus the block in the export of virions is at the entry into the exocytic pathway, and a second block in the exocytosis of viral glycoproteins associated with cytoplasmic membranes is due to an impairment of transport beyond Golgi fragments containing *trans*-Golgi enzymes and not to a failure of the Golgi oligosaccharide-processing functions; (ii) these defects are manifested in cells in which the Golgi apparatus is fragmented; and (iii) the U_L20 protein compensates for these defects by enabling transport to and from the fragmented Golgi apparatus.

Herpes simplex virus 1 (HSV-1) encodes numerous glycoproteins and nonglycosylated membrane proteins which function in virus entry, virion envelopment, sorting, and egress from infected cells. The specific issues addressed by this report involve viral maturation and other events which take place concurrently. Specifically, viral capsids are enveloped at the inner nuclear membrane, encased in transport vesicles, and transported through the cytoplasm to the extracellular space. Concurrently, viral glycoproteins associated with cellular membranes transit to the plasma membrane (reviewed in reference 34). In the course of the transit through the cytoplasm, the oligosaccharide chains on both virion glycoproteins and the glycoproteins associated with cellular membranes are processed by Golgi enzymes from high-mannose to mature forms (4, 43, 46).

The nucleotide sequence of the U_L20 open reading frame predicted a highly hydrophobic protein (29), and indeed the U_L20 protein possesses the properties of an intrinsic membrane protein (1) and is associated with virions and a variety of cellular membranes (47). The phenotype of an HSV-1 recombinant lacking the U_L20 gene (U_L20^- virus R7225) is of particular interest. The U_L20^- virus forms small syncytial plaques in 143TK$^-$ cells and in rabbit skin cells. In Vero and HEp-2 cells infectious virus is produced and remains cell associated, but no plaques are formed in these cell lines (1). The lack of production of plaques in Vero cells by a U_L20^- recombinant may have lead MacLean et al. (22) to conclude that the U_L20 gene is essential for HSV-1 replication in cell culture. Electron microscopic studies indicate that enveloped capsids accumulate between the inner and outer nuclear membranes and that virus particles are not present on the plasma membrane or in the space between adjacent infected cells (1). Moreover, notwithstanding the block in virion transport to the extracellular space, the majority of viral glycoproteins extracted from the U_L20^--infected cells appeared to be fully processed as assessed by their electrophoretic mobilities. Replacement of the deleted sequences fully restores the wild-type phenotype of the virus (1).

In the course of these studies, we noted that in infected Vero, HEp-2, and, to a lesser extent, BHK cells, the Golgi apparatus is fragmented and dispersed throughout the cytoplasm, whereas intact Golgi organelles are readily demonstrable in 143TK$^-$ cells (2). Moreover, in infected Vero cells the proteins associated with the Golgi apparatus and related compartments (e.g., β-COP and galactosyl transferase, etc.) appear to be both increased in amount and redistributed throughout the cytoplasm.

The initial question which led to these studies was whether in U_L20^- virus-infected Vero cells the Golgi enzymes were redistributed such that even the virion glycoproteins located between the inner and outer nuclear membranes were processed to mature forms. We report the remarkable observation that in Vero cells infected with the U_L20^- virus, the viral glycoproteins associated with virions sequestered in the space between the inner and outer nuclear membranes are of the immature type, whereas the viral glycoproteins associated with

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membranes contain complex-type oligosaccharides with terminal sialic acid but are transported to the plasma membranes only in small amounts. These observations reinforce the conclusion that the transport of virions to the extracellular space differs from that of glycoproteins from the rough endoplasmic reticulum through the Golgi apparatus, and they suggest that U_L20 protein is required for the encasement of virions by the outer nuclear membranes into transport vesicles which carry the virion through the Golgi apparatus to the extracellular space and for the transport of membrane-associated viral glycoproteins from a *trans*-Golgi-equivalent compartment to the plasma membrane.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human 143TK$^-$ cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum. HSV-1(F) (10) and the U_L20^- recombinant R7225 (1) were described previously. R7032 contains a deletion in the gE gene (24).

Freeze-fracture and labeling with antibodies and lectins. Vero cells were infected with R7225 or HSV-1(F) (3 to 5 PFU per cell) or mock infected. At 24 h after infection with HSV-1(F) and 72 h after infection with R7225, cells were fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C, washed in PBS, impregnated in 30% glycerol in PBS, and frozen in Freon 22 cooled by liquid nitrogen. Frozen cells were fractured in liquid nitrogen by repeated crushing with a glass pestle and gradually deglycerinated. Fractured cells were incubated with monoclonal antibody H170 to gD (6) or with polyclonal antibody to gB (both diluted 1:5) for 1 h at 25°C and then labeled for 3 h at 4°C with colloidal gold (prepared by the citrate method) conjugated with protein A (46). Control experiments were performed each time by omitting the antibodies from the labeling procedure. Alternatively, fractured cells were incubated for 1 h at 37°C in a solution of 0.25 mg of wheat germ agglutinin (WGA) (Sigma Chemical Co., St. Louis, Mo.) per ml in 0.1 M Sorensen's phosphate buffer–4% polyvinylpyrrolidone, pH 7.4, for 1 h at 37°C and labeled with ovomucoid-coated colloidal gold for 3 h at 4°C. Control samples were preincubated in 0.4 M *N*-acetyl-D-glucosamine for 15 min at 37°C and then treated with WGA in the presence of the competitor sugar for 1 h at 37°C and labeled with ovomucoid-coated colloidal gold as described above (30).

Processing for electron microscopy. Fracture-labeled cells were postfixed with 1% osmium tetroxide, en bloc stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections unstained or poststained with uranyl acetate and lead hydroxide were examined.

Virion purification. Vero cells grown in roller bottles were infected with HSV-1(F) or R7225 (5 PFU per cell) and labeled with a mixture of [^{35}S]methionine and cysteine (Trans-label; 1,000 Ci/mmol; ICN Biomedicals) (20 μCi/ml) from 5 to 24 h after infection. Virions were partially purified from the cytoplasm in dextran T-10 gradients, as described previously (44). Virions were lysed in immunoprecipitation buffer, consisting of 1% Nonidet P-40 and 1% sodium deoxycholate in PBS, containing the protease inhibitors tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) and α-tolylsulfonyl chloromethyl ketone (TLCK) (Sigma Chemical Co.) at 10^{-5} M each. Glycoproteins C and D were immunoprecipitated from the virion lysates with monoclonal antibodies HC1 (28) and no. 30, a monoclonal antibody to gD (12), and harvested with protein A-Sepharose beads as previously described (3). Glycoproteins were electrophoretically separated in sodium dodecyl sulfate (SDS)–8.5% polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide. The gels were soaked in Amplify and exposed to Kodak X-Omat films for fluorography.

Cell surface iodination. Monolayer cultures in 35-mm-diameter dishes were infected with HSV-1(F) or R7225 (3 to 5 PFU per cell). At 21 or 24 h after infection, the monolayers were washed twice with PBS containing Ca and Mg, detached with PBS containing EDTA, labeled with ^{125}I (500 μCi per dish) for 30 min at room temperature with Jodo-Gen (Pierce) as described elsewhere (13), rinsed three times with PBS, and lysed in immunoprecipitation buffer.

Glycosidase digestion. Vero cells in 35-mm-diameter dishes infected with R7225 and HSV-1(F) or R7032 (5 PFU per cell) were labeled with a mixture of [^{35}S]methionine and cysteine (100 μCi/ml of medium containing 1/40 the usual concentration of unlabeled methionine and cysteine) between 5 and 18 h or 5 and 24 h after infection for neuraminidase or endo-β-*N*-acetylglucosaminidase H (endo H) and peptide N-glycosidase F (N-glycosidase F) digestions, respectively. Radioactive medium was removed, cells were rinsed three times, and, for neuraminidase digestion, cells were chased for 3 h in a medium containing the usual concentration of methionine and cysteine to allow incorporation of labeled precursors preferentially in the mature forms of the glycoproteins. Cells were lysed in immunoprecipitation buffer, and the lysates were reacted with monoclonal antibodies HC1 and no. 30 to gC and gD, respectively. The immune complexes were first harvested with protein A-Sepharose (Sigma Chemical Co.). For endo H and N-glycosidase F digestions, the glycoproteins were eluted in 0.8% SDS in 10 mM phosphate buffer, pH 7.2, for 20 min at 37°C and 0.5 min at 100°C and then further diluted in the appropriate buffers at 1:10 or 1:5 for endo H or N-glycosidase F, respectively. Endo H digestions were carried out overnight at 37°C with 25 mU of enzyme (Seikagaku Kogyo Co., Tokyo, Japan) in 50 mM sodium acetate buffer, pH 5.5. N-glycosidase F digestions were carried out overnight at 37°C with 5 U of enzyme (Boehringer, Mannheim, Germany) in 100 mM sodium phosphate buffer (pH 7.5)–50 mM EDTA–0.1% β-mercaptoethanol–0.5% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}. For neuraminidase digestion, the glycoproteins were eluted from the protein A-Sepharose by boiling for 3 min in a solution containing 150 mM NaCl and 0.2% Triton X-100, addition of an equal volume of 50 mM Na-acetate buffer, pH 5.5, and digestion overnight at 37°C with 100 mU of neuraminidase from *Vibrio cholerae* (1 U/ml; Behring, Behringwerke AG, Marburg, Germany). In each case, the controls were replicate samples incubated in parallel in the same buffers in the absence of the glycosidases. Digested glycoproteins were separated by electrophoresis, which was followed by fluorography, as described above.

RESULTS

Localization of viral glycoproteins D and B in freeze-fractured cells labeled with antibodies to glycoproteins or WGA lectin. In cells infected with wild-type virus, enveloped virions accumulate in the perinuclear space, in cytoplasmic vesicles, and in the extracellular space (33, 36, 46). In contrast, in Vero cells infected with the R7225 recombinant from which the U_L20 gene has been deleted, enveloped virions accumulate in the space between the inner and outer nuclear membranes (1). Only rarely are virions seen in the extracellular space or in cytoplasmic vesicles of infected cells. The phenotype is specific for the deletion in the U_L20 gene, inasmuch as restoration of the gene restores the wild-type phenotype (1). To analyze the envelope composition of the U_L20^- virions formed in Vero

cells, we examined freeze-fractured R7225-infected Vero cells with antibodies to the viral glycoproteins or with lectins.

In this procedure cells are fractured, exposed to labeling reagents, embedded, and sectioned. The plasma membranes are accessible to the labeling reagents at all times. The intracellular membranes may react with the labeling reagents only if the plane of the fracture exposes the membrane and makes it accessible to the labeling reagents. The procedure allows the comparison of label distribution on the plasma membrane with that on fracture-exposed intracellular membranes. Furthermore, a global image of the cell may be obtained on the occasions when the fracture along the nuclear membrane is incomplete. For cells infected with wild-type HSV-1, it has been demonstrated previously by this approach that the immature forms of the glycoproteins are present on the nuclear membranes and on the virions located in the perinuclear space and that mature glycoproteins are present in virions enclosed in cytoplasmic vesicles and in extracellular virions, as well as in Golgi and plasma membranes (46).

Gold immunolabeling of U_L20^- virus-infected Vero cells with monoclonal antibody H170, specific for gD, appeared highly dense over the outer and inner nuclear membranes (Fig. 1) and on the enveloped virions accumulated in the perinuclear space (Fig. 1a, b, and c). Surprisingly, in all cells, gold particles were virtually absent or very rare on the unfractured plasma membranes (Fig. 1a and c). A nearly identical pattern of labeling was obtained with polyclonal antibodies to gB (data not shown). The failure to detect gD or gB in the plasma membranes of cells infected with the U_L20^- virus contrasted sharply with the results of studies on freeze-fractured Vero cells infected with the wild-type HSV-1(F). In this instance we observed a very high level of labeling with the anti-gD monoclonal antibody H170 (Fig. 1d and e) or with anti-gB polyclonal antibody (not shown) over the unfractured cell surfaces and on the extracellular virions. gD and gB were also present on outer and inner nuclear membranes, and on perinuclear intracellular virions (not shown), as could be expected from previous studies on BHK cells infected with wild-type virus (46).

In parallel experiments, infected cells were stained with WGA, a lectin that binds with high affinity to terminal sialic acid residues of glycoconjugates (21). Lectin labeling does not distinguish in what type of glycoconjugate—glycoprotein or glycolipid—the label is located or whether the label is located on viral or cellular glycoconjugates. Therefore, a positive WGA labeling is indicative of oligosaccharide processing, and a negative WGA labeling is indicative of unprocessed oligosaccharides. In freeze-fractured U_L20^- virus-infected Vero cells, WGA labeled neither nuclear membranes nor intracellular enveloped virions accumulating in the perinuclear space (Fig. 2b), whereas dense gold labeling was present on the unfractured plasma membranes (Fig. 2); this was due, at least in part, to binding to membrane glycolipids. Inasmuch as WGA binds to fully processed oligosaccharide chains, these results rule out the possibility that oligosaccharide chains on viral glycoproteins present in the nuclear membranes are fully processed; i.e., they are either of the high-mannose type or at an intermediate stage of processing. To differentiate between these two possibilities, freeze-fractured samples were stained with Helix pomatia lectin, which binds unsubstituted GalNAc (41). GalNAc is the first sugar in the assembly of O-linked oligosaccharides and is added very early in the posttranslational processing of HSV glycoproteins (7). No labeling of virions was observed with Helix pomatia (data not shown), suggesting that partially processed forms of the viral glycoproteins were not present in virions accumulating in U_L20^- virus-infected Vero cells.

U_L20^- virions contain predominantly unprocessed glycoproteins. To define further the extent of processing of viral glycoproteins present in the virions accumulating in U_L20^- virus-infected cells, intracellular virions were purified from the cytoplasm of Vero cells infected with the U_L20^- recombinant or with HSV-1(F) and labeled with a mixture of [^{35}S]methionine and cysteine. Glycoproteins C and D were simultaneously immunoprecipitated from the lysates of the purified virions and were electrophoretically separated in denaturing polyacrylamide gels. This procedure separates fast-migrating precursor forms from the slowly migrating mature forms of the glycoproteins. Extensive studies on HSV glycoproteins from our laboratories as well as from numerous other laboratories indicate that the fast-migrating precursor forms of viral glycoproteins contain endo H-sensitive high-mannose oligosaccharides, whereas the slowly migrating forms contain endo H-resistant complex-type oligosaccharides (4, 9, 38, 43, 49). The results (Fig. 3, two rightmost lanes) indicate that the precursor forms pgC and pgD, i.e., forms that carry high-mannose N-linked oligosaccharides, were present in partially purified virions from U_L20^- virus-infected cells, whereas a mixture of precursor and slowly migrating forms of both gC and gD was present in the partially purified virions from cells infected with wild-type virus [HSV-1(F)].

The infectivity of R7225 deserves a comment. In R7225 infectivity is maintained despite the concurrent deletion of the U_L20 gene and the virtual absence of mature glycoproteins. The latter feature is not surprising, since it has been repeatedly observed that HSV infectivity does not require fully processed glycoproteins and that partially processed glycoproteins suffice for this function. This was inferred from studies with mutant cell lines defective in Golgi enzymes or with cells treated with Golgi inhibitors, such as monensin and ammonium chloride (4). Tunicamycin and brefeldin A abolished infectivity. Tunicamycin induces the most drastic changes, since it completely abolishes N-linked glycosylation. Brefeldin A causes a reduction in the yield of infectious progeny (5, 50); however, it is not clear if this reflects a low specific infectivity or a reduction in the number of regularly assembled virion particles.

Glycoproteins C and D are present in reduced amounts in the plasma membranes of U_L20^- virus-infected Vero cells. The unexpected observation that plasma membranes of U_L20^- virus-infected Vero cells did not contain detectable amounts of gB or gD contrasted sharply with earlier observations that the viral glycoproteins made in U_L20^- virus-infected Vero cells have the slow electrophoretic mobility characteristic of mature glycoproteins made in cells infected with wild-type virus. These two results taken together suggest that while the HSV-1 glycoproteins made in U_L20^- virus-infected Vero cells may be fully processed, they are not transported to the plasma membrane—an observation without precedent.

To verify the observation that viral glycoproteins fail to be transported to the plasma membrane, Vero cells infected with U_L20^- virus or with HSV-1(F) were surface labeled with ^{125}I at 21 h after infection. Replicate cultures were also labeled with [^{35}S]methionine and cysteine. Glycoproteins C and D were each precipitated at the same time from each culture and were separated by denaturing polyacrylamide gel electrophoresis. The results show that gC and gD were readily available for iodination at the surface of cells infected with wild-type virus, whereas only a small amount of gC and almost no gD were available for iodination at the surface of U_L20^- virus-infected cells (Fig. 3, middle lanes), although nearly equivalent amounts of gC and gD were labeled with ^{35}S-amino acids in these cells (Fig. 3, leftmost lanes). Moreover, the ^{35}S-labeled glycoproteins displayed for the most part the slow electrophoretic

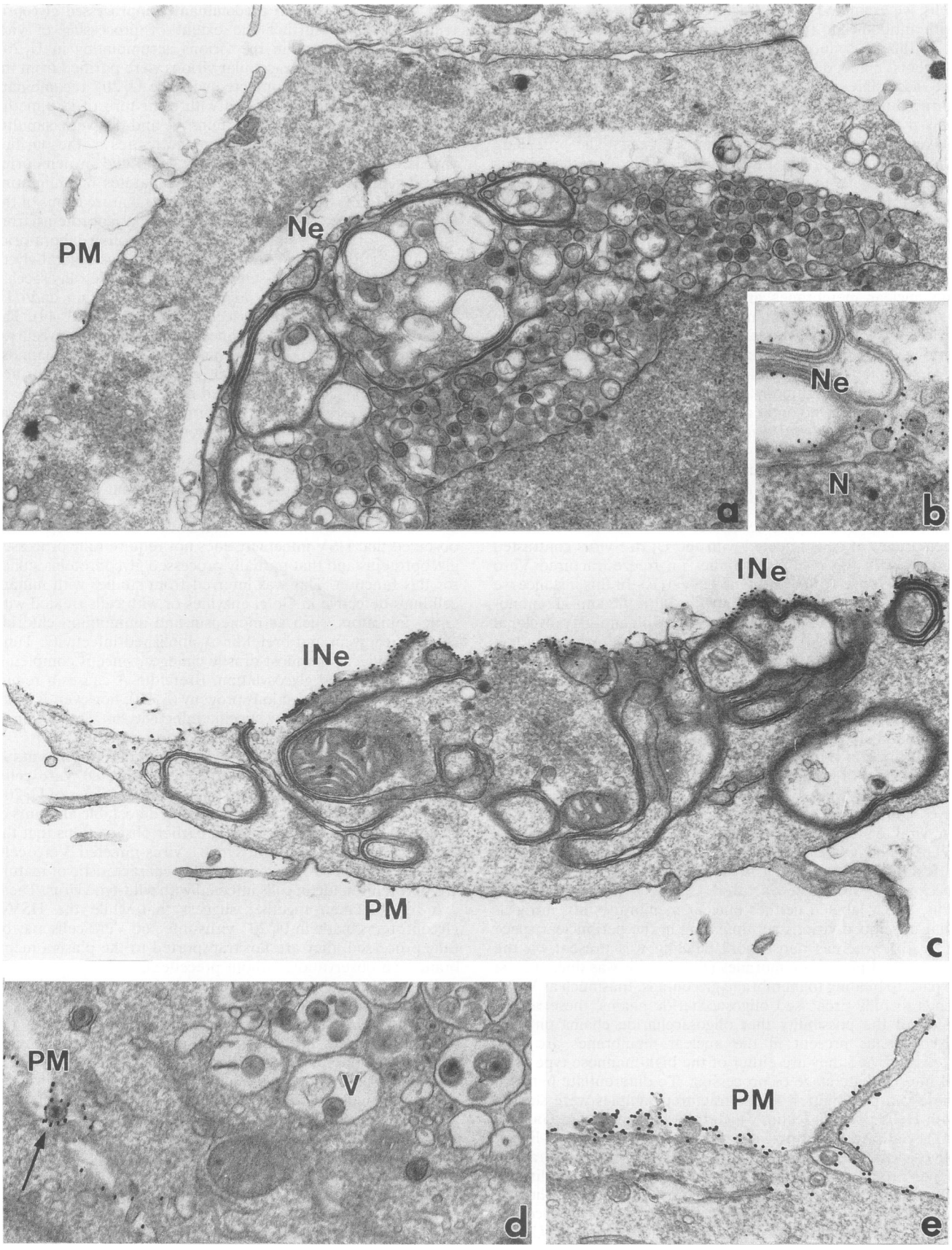
PM
Ne
a
Ne
N
b
INe
INe
PM
c
PM
V
d
PM
e

FIG. 1. Gold immunolabel with monoclonal antibody H170 to gD of freeze-fractured Vero cells infected with R7225 (a to c) or HSV-1(F) (d and e). Numerous enveloped virions are seen accumulated in a large space between the nuclear membranes, whereas virions are not observed in the cytoplasmic area and in the extracellular space (a to c). The immunolabeling is dense over the freeze-fractured outer and inner nuclear membranes (a and c), as well as on the enveloped virions accumulated in the space between the nuclear membranes (b). The unfractured plasma membranes appear unlabeled (a and c). Enveloped virions are visible in intracellular vacuoles and in the extracellular space (arrow) of HSV-1(F)-infected Vero cells (d); dense labeling is present on the unfractured plasma membranes (e). PM, plasma membrane; Ne, nuclear membranes; INe, inner nuclear envelope; V, vacuoles. Magnifications: (a) ×18,000; (b) ×24,000; (c) ×21,000; (d and e) ×32,000.

mobility typical of fully processed forms. Altogether the results strongly indicate that the viral glycoproteins C and D are present in highly reduced amounts in the plasma membranes of U_L20^- virus-infected Vero cells compared with cells infected with wild-type virus and imply a block in the viral glycoprotein transport along the exocytic pathway.

Extent of processing of viral glycoproteins C and D associated with cellular membranes in U_L20^- virus-infected Vero cells. The presence in the cytoplasm of U_L20^- virus-infected cells of glycoproteins with an electrophoretic mobility characteristic of wild-type virus mature glycoproteins and the near absence of glycoproteins from the plasma membranes suggest either that the slowly migrating forms of the glycoproteins are not fully glycosylated or that a block in glycoprotein transport occurs after completion of the oligosaccharide chains and prior to their appearance at the plasma membrane. To discriminate between these two possibilities, we analyzed the extent of viral glycoprotein processing by glycosidase digestions. Since the sequential and coordinate steps in oligosaccharide processing of the glycoproteins take place concomitantly with the movement of the glycoprotein, this measure also gives an account of the compartment reached by the glycoprotein along the exocytic pathway.

First, we ruled out that the slowly migrating forms of the viral glycoproteins C and D made in U_L20^--infected Vero cells contained unprocessed oligosaccharides by digestion with endo H, an endoglycosidase that specifically cleaves high-mannose oligosaccharides. As shown in Fig. 4A, the electrophoretic mobilities of the slowly migrating forms of [^{35}S]methionine- and cysteine-labeled gC and gD were not altered following endo H digestion. The results were indistinguishable for glycoproteins immunoprecipitated from cells infected with either R7225 or wild-type virus. Susceptibility to cleavage of the precursors pgC and pgD accounts for the effectiveness of endo H digestion. Digestion of portions of the same samples with N-glycosidase F, an enzyme that cleaves all types of N-linked glycans, resulted in a drastic increase in the electrophoretic mobilities of the slowly migrating forms of gC and gD (Fig. 4B). Together, N-glycosidase F sensitivity and endo H insensitivity indicate that the slowly migrating forms of gC and gD carry complex-type oligosaccharides. This was true for the glycoproteins immunoprecipitated from cells infected with either U_L20^- or wild-type virus. Digestion with N-glycosidase F did not appear to be exhaustive. This may be explained by the unfavorable assay conditions, particularly the inactivation of the enzyme by SDS, as pointed out by the manufacturer.

To better define the extent of processing of the viral glycoproteins and further dissect the transport along the exocytic pathway, we determined whether the glycoproteins made in U_L20^- virus-infected cells contained sialic acid residues, which are the terminal sugars in N- and, at least in part, O-linked oligosaccharides of HSV glycoproteins (7, 18, 38–40). Glycoproteins C and D were immunoprecipitated from lysates of [^{35}S]methionine- and cysteine-labeled Vero cells infected with U_L20^- virus and, for comparison, with wild-type HSV-1(F) or with the R7032 virus containing U_L20 but lacking glycoprotein E (24) and then were subjected to neuraminidase digestion and electrophoretic separation in a denaturing polyacrylamide gel. Whereas removal of sialic acid generally causes an increase in the electrophoretic mobility of glycoproteins, for glycoproteins rich in clustered O-linked oligosaccharides, this treatment causes a decrease rather than an increase in electrophoretic mobility (14, 25, 40). Figure 5 shows that desialylated gC from both U_L20^- and U_L20^+ virus-infected cells had a lower electrophoretic mobility than its sialylated counterpart, whereas desialylated gD migrated faster than its sialylated counterpart. Figure 5 also shows that the electrophoretic mobilities of the precursors pgC and pgD, which carry the high-mannose oligosaccharides, were unaffected by neuraminidase treatment, indicating that the effect on the mature form of the glycoproteins was not the consequence of nonspecific oligosaccharide degradation by the neuraminidase treatment.

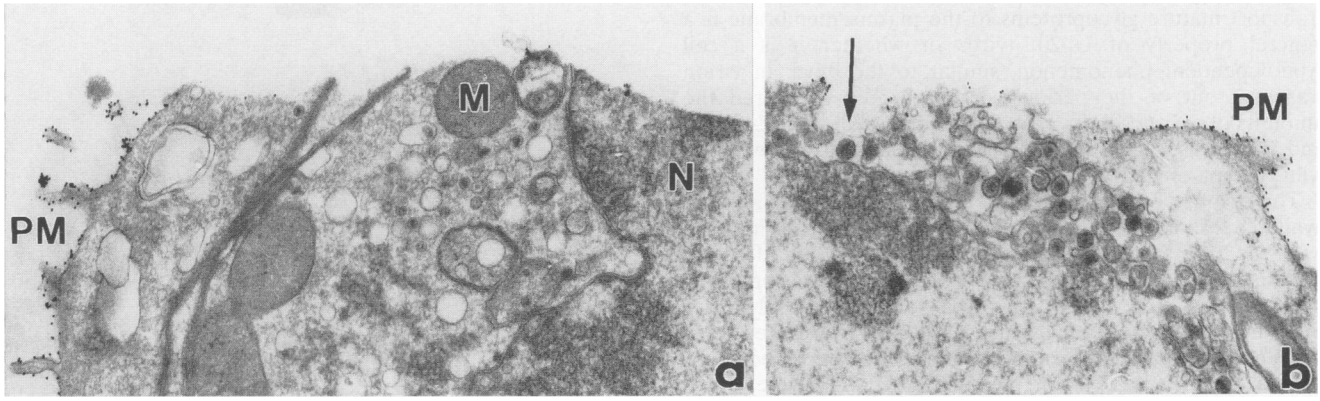

FIG. 2. WGA label of freeze-fractured Vero cells infected with R7225. The enveloped virions that accumulated in the space between the nuclear membranes are unlabeled (arrow in panel b). Freeze-fractured nuclear membranes are unlabeled (b), whereas the unfractured plasma membranes are densely labeled (a and b). N, nucleus; M, mitochondrion; PM, plasma membrane. Magnification, ×16,000.

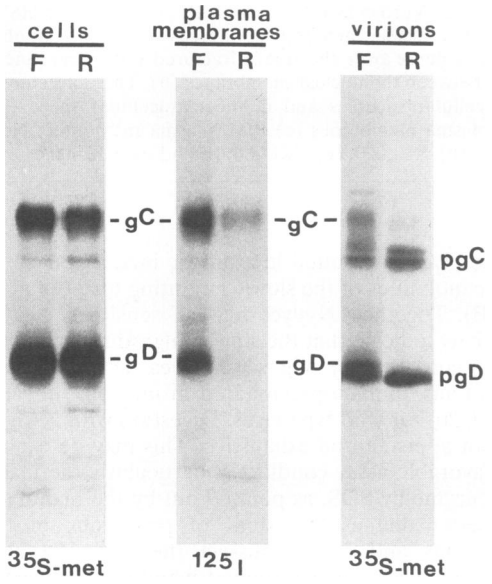

FIG. 3. Fluorographic image of electrophoretically separated glycoproteins C and D immunoprecipitated from Vero cells infected with HSV-1(F) (F) or R7225 (R) or from purified virions. For the two lanes on the left, cells were labeled with a mixture of [^{35}S]methionine and cysteine from 5 to 20 h after infection. For the middle lanes, cells were labeled with ^{125}I at 21 h after infection. For the two lanes on the right, virions were partially purified from the cytoplasm of infected Vero cells labeled between 5 and 24 h after infection. Glycoproteins C and D were simultaneously immunoprecipitated from the lysates and separated by denaturing polyacrylamide gel electrophoresis.

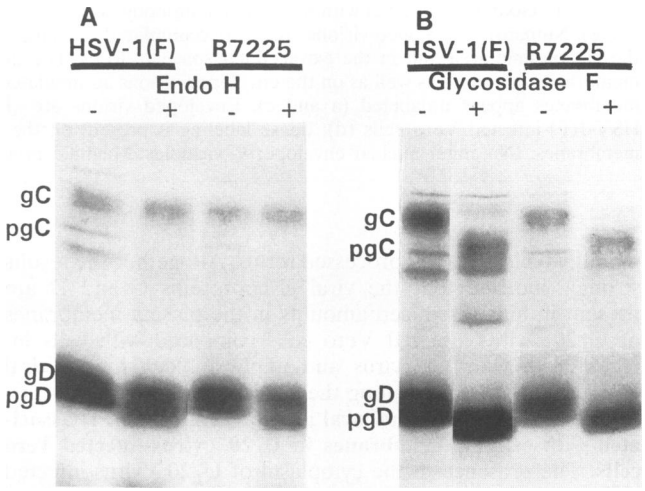

FIG. 4. Fluorographic image of electrophoretically separated glycoproteins C and D immunoprecipitated from Vero cells infected with HSV-1(F) or the U_L20^- virus R7225 and subjected to endo H (A) or N-glycosidase F (B) digestion. The glycoproteins, harvested with monoclonal antibody HC1 or no. 30 on protein A-Sepharose beads, were released from the beads and subjected to the glycosidase digestion (+). Replicate control samples (−) were processed in the same manner except that the enzymes were omitted during the digestion. The fluorographic images shown are of the same gel.

With respect to the experiments described in this study, the phenotype of the gE^- virus (R7032) is identical to that of the wild-type parent, HSV-1(F) (not shown). The relevant results of this experiment are that the electrophoretic mobilities of gC and gD immunoprecipitated from lysates of U_L20^- virus-infected Vero cells were virtually identical to those for the U_L20^+ virus and indicate that in cells infected with the U_L20^- recombinant, gC and gD contained the terminal sialic acid and therefore were fully processed.

Glycoproteins C and D are present in similar amounts on the surfaces of 143TK$^-$ cells infected with U_L20^- virus and its wild-type parent. As indicated above, the phenotype of U_L20^- virus is different in Vero and 143TK$^-$ cells. The purpose of this series of experiments was to determine whether the failure to transport mature glycoproteins to the plasma membrane is a general property of U_L20^- virus or whether it is a cell type-dependent phenomenon, similar to the block in virion transport out of the perinuclear space. We compared the amounts of gC and gD ^{125}I labeled on the surfaces of 143TK$^-$ and Vero cells 24 h after infection with U_L20^- virus or with the wild-type parent HSV-1(F).

The results (Fig. 6) show that the amounts of gC and gD available for iodination on the surface of 143TK$^-$ cells infected with U_L20^- or with HSV-1(F) were nearly identical. In contrast, the amounts of gC and gD detected on the surface of Vero cells infected with U_L20^- virus were considerably lower than those labeled on the surface of HSV-1(F)-infected cells. We should note that the amounts of glycoproteins labeled on the surface of Vero cells infected with HSV-1(F) were reproducibly higher than those on 143TK$^-$ cells. With high reproducibility, we also detected a small amount of glycoprotein precursors at the cell surfaces. This finding was unexpected but not surprising. Reexamination of published data indicates that both plasma membranes and extracellular virions may carry small amounts of glycoproteins with electrophoretic mobilities compatible with those of precursor forms (see, for example, references 16, 20, 23, and 42). In addition, particularly for gC, it has been documented that conditions that hamper the processing of the glycoprotein (e.g., exposure to tunicamycin

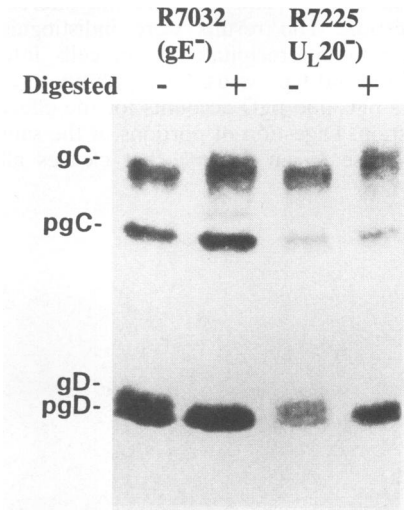

FIG. 5. Fluorographic image of electrophoretically separated glycoproteins C and D immunoprecipitated from Vero cells infected with R7032 or U_L20^- virus (R7225) and subjected to neuraminidase digestion. The glycoproteins, harvested with monoclonal antibody HC1 or no. 30 on protein A-Sepharose beads, were released from the beads and subjected to neuraminidase digestion (+). Replicate control samples (−) were processed in the same manner except that neuraminidase was omitted during the digestion.

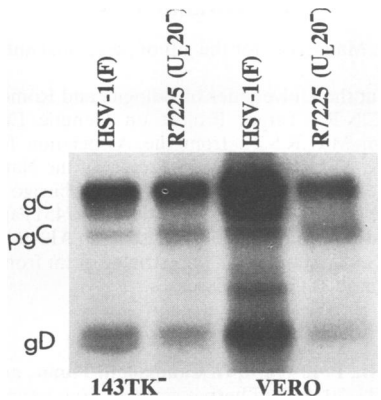

FIG. 6. Autoradiographic image of electrophoretically separated glycoproteins C and D from human 143TK$^-$ and Vero cells infected with HSV-1(F) or U_L20^- virus and surface labeled at 24 h after infection with ^{125}I. The cells were washed, solubilized with Nonidet P-40, and reacted with monoclonal antibody HC1 or no. 30 to gC and gD, respectively, and the immune complexes were harvested with protein A-Sepharose beads and electrophoretically separated on denaturing polyacrylamide gels. The bands containing the mature forms of gC and gD and the precursor pgC are marked.

or 2-deoxyglucose) do not prevent the appearance of the glycoprotein precursor at the cell surface, implying that partially processed forms of the glycoprotein are able to traverse the exocytic pathway (16, 27). The phenomenon that we and others have observed may reflect inefficiency in processing late in infection rather than a complete breakdown of the exocytic pathway.

DISCUSSION

In this and previous reports (1, 2) we have described two contrasting systems for exocytosis of enveloped HSV virions and of viral glycoproteins associated with cellular membranes.

In the first system, Vero cells infected with U_L20^- virus, we observed previously that the virions are restricted to the space between the inner and outer nuclear membranes. Here we report that the virion envelope glycoproteins are of the immature type as determined by their electrophoretic mobilities and by the failure to react with the Helix pomatia lectin or WGA. In contrast, the glycoproteins associated with the cellular membranes are fully processed, as inferred by N-glycosidase F sensitivity coupled to endo H insensitivity and by susceptibility to neuraminidase, which removes terminal sialic acids. Sialyl transferases are typical Golgi enzymes, normally located in the *trans*-Golgi compartment. Therefore, our studies indicate that in U_L20^- virus-infected Vero cells the membrane-associated viral glycoproteins reach a compartment very distal in the exocytic pathway. A striking feature of the current results is that the amounts of viral glycoproteins present in the plasma membranes were a small fraction of the amounts present in Vero cells infected with wild-type virus. We conclude that in the absence of U_L20, in Vero cells, the process involving exocytosis of virions differs from that of the glycoproteins associated with cellular membranes. The exocytosis of virions is blocked early, at the encasement of enveloped virions into transport vesicles by the outer nuclear membrane. The exocytosis of glycoproteins associated with cellular membranes is blocked very late in the exocytic pathway, likely at the egress from Golgi fragments containing *trans*-Golgi enzymes.

The second system involves a comparison of Vero and 143TK$^-$ cells infected with U_L20^- virus. In contrast to the blocks in exocytosis in Vero cells described above, in infected 143TK$^-$ cells virions are exported to the extracellular space, although the yields of infectious progeny are lower than that of wild-type virus, and the amounts of viral glycoproteins exported to the plasma membranes of cells infected with the wild-type parent or with U_L20^- virus are similar.

The most striking difference between infected Vero and 143TK$^-$ cells is the fragmentation and dispersal of the Golgi apparatus in the former cell line. The fragmentation of the Golgi apparatus in Vero cells is independent of the presence and expression of the U_L20 gene. The key observation is that the differential blocks in the exocytosis of virions and of glycoproteins associated with the cellular membranes occur in cells in which the Golgi apparatus became fragmented and dispersed during infection and only in the absence of the U_L20 gene product. This correlation is reinforced by the observation that the phenotype of U_L20^- virus-infected HEp-2 cells is very similar to that of Vero cells infected with the same virus. Specifically, in HEp-2 cells the Golgi apparatus is also fragmented (2), and the U_L20^- virus does not form plaques (1). In addition, we have observed that U_L20^- virions are blocked at the perinuclear space and that glycoproteins C and D, associated with the cellular membranes, have electrophoretic mobilities typical of mature glycoproteins and are transported to the plasma membranes in very small amounts (data not shown).

Relevant to an understanding of the results reported here are several key observations regarding exocytosis of HSV virions and of viral glycoproteins associated with cellular membranes in cells in which the Golgi apparatus is fragmented.

(i) HSV virions could be viewed as secreted macromolecules which differ from the ordinary secreted proteins in two respects. Unlike secreted proteins, which are translocated into the lumen of the endoplasmic reticulum, virions are generated in the space between the inner and outer nuclear membranes by envelopment of capsids by the inner nuclear membrane. In cells infected with wild-type virus, it has been observed that the virions enter branched tubular structures similar to the connections between the outer nuclear membrane and rough endoplasmic reticulum and ultimately appear in transport vesicles (19, 36). Virion envelope glycoproteins are processed by Golgi enzymes. At the end of the exocytic pathway, the transport vesicles fuse with the plasma membranes, releasing virions into extracellular space. Evidence suggesting that a scanning mechanism discriminates against and for the presence of specific viral gene products in virion envelopes has been published. Thus, in cells infected with a *ts* glycoprotein H mutant and incubated at the nonpermissive temperature, virions without gH were transported into the extracellular space but were not infectious, whereas infectious virions containing gH were retained in the infected cells (8). Our results suggest that the formation of vesicles of the type used for virion transport in Vero cells involves a specific cellular function which may be complemented by one (U_L20) or more viral genes and does not arise by a default mechanism, i.e., by the mere presence of material between the inner and outer nuclear membranes.

(ii) HSV glycoproteins expressed in cells infected with wild-type virus are membrane bound and transported to the plasma membrane and hence are not likely to contain Golgi retention signals. With the exception of gH (11, 17, 32), HSV glycoproteins expressed in uninfected cells are transported to the plasma membranes (see, e.g., references 20, 35, and 37), suggesting that their transport does not require the presence of U_L20 protein.

(iii) The few conditions which lead to the fragmentation of the Golgi apparatus affect glycoprotein transport differently. Thus, the processing and transport of glycoproteins to the plasma membrane is not affected in cells infected with Uukuniemi virus, even though the Golgi apparatus is fragmented (15). In marked contrast, in mitotic cells the glycoproteins remain in the endoplasmic reticulum and are not processed by the Golgi apparatus, although the enzymatic capacity remains intact (48). In cells exposed to the sponge metabolite ilimaquinone, the Golgi apparatus is fragmented and transport from the endoplasmic reticulum to Golgi is unaffected, but the transport beyond *cis*-Golgi is blocked (45).

Our results, consistent with these observations, lead us to the following conclusions.

(i) The observation that in U_L20^- virus-infected Vero cells viral glycoproteins associated with cytoplasmic membranes are fully processed but not transported beyond fragments containing *trans*-Golgi enzymes indicates that the block is due to an impairment in transport beyond Golgi fragments and not in the oligosaccharide-processing functions of this cellular organelle. Normally the *trans*-Golgi connects with the extracellular space by way of the *trans*-Golgi network, a tubulo-vesicular structure which mediates sorting of proteins and lipids destined to lysosomes, endosomes, plasma membranes, and apical or basal domains of polarized cells. It may also retain molecules subjected to a regulated release. In principle, therefore, intra-Golgi transport may be differentiated from that between the *trans*-Golgi and the plasma membrane. Our data suggest that under conditions which result in the fragmentation of the Golgi apparatus, the transport from the Golgi fragments containing *trans*-Golgi enzymes is impaired and that the function of the U_L20 protein is to supply the function missing from the infected cell.

(ii) The exocytic pathway normally initiates from the rough endoplasmic reticulum through its interactions with the Golgi apparatus. The outer nuclear membrane, which is continuous with the endoplasmic reticulum, is itself a site of membrane glycoprotein synthesis and transport (26, 31). It is not known whether the exocytosis of glycoproteins from the outer nuclear membrane requires a flow to the endoplasmic reticulum. It is conceivable that the nuclear membrane is a dynamic structure which may acquire tubulo-vesicular structures which facilitate exocytosis. If this is the case, we may speculate that HSV virions avail themselves of these structures for encasement into transport vesicles for exocytosis from the infected cell and that the formation of these structures requires the integrity of framework which maintains the Golgi apparatus intact. Again, U_L20 protein complements this function in cells in which the Golgi apparatus is fragmented.

The function of U_L20 protein may be similar in the exocytosis of both virions and the viral glycoproteins associated with cellular membranes. For example, U_L20 protein could maintain the transport network even though the Golgi apparatus is fragmented in order to enable the exocytosis of virions to the fragmented Golgi apparatus and of viral membrane glycoproteins to the plasma membrane. A nonexclusive alternative is that U_L20 causes an increase in the formation of tubulo-vesicular structures in both the outer nuclear membrane and the Golgi apparatus, thereby ensuring the vesicular flow. The mechanism by which U_L20 corrects the defects associated with the fragmentation and dispersal of the Golgi apparatus remains to be elucidated. A major virtue of the U_L20 protein is that it may be an excellent probe of cellular vesicular transport.

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