JOURNAL OF VIROLOGY, Jan. 1992, p. 554–561
0022-538X/92/010554-08$02.00/0

Vol. 66, No. 1

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Herpes Simplex Virus Envelopment and Maturation Studied by Fracture Label

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Received 27 March 1991/Accepted 30 September 1991

Herpes simplex virus envelopment and maturation were investigated by thin-section fracture label. The distribution of glycoproteins B and D was analyzed by labeling with antibodies; the precursor and mature forms of the glycoproteins were differentiated by labeling with the lectins concanavalin A (ConA) and wheat germ agglutinin (WGA), respectively. We report that the two glycoproteins were readily detected in the intracellular virion, whether located between the inner and outer nuclear membranes or within cytoplasmic membrane-bound vesicles and in the inner and outer nuclear membranes themselves. The enveloped virion between the inner and outer nuclear membranes labeled with ConA but not with WGA. During the transit to the extracellular space the reactivity of the virion membranes with ConA decreased and that with WGA ensued. The results document that herpes simplex viruses acquire at the inner nuclear membrane an envelope carrying the immature forms of the glycoproteins and that during the transit to the extracellular space the envelope glycoproteins become of the fully processed type.

Herpes simplex virus type 1 (HSV-1) and HSV-2 acquire their envelope at the inner nuclear membrane (11, 21–23, 28). It has been firmly established by numerous investigations that the envelope of the extracellular virion contains the mature glycoproteins, whereas the infected cell membranes contain both the glycoprotein precursors, which are synthesized in rough endoplasmic reticulum (RER), and the mature forms processed by the Golgi enzymes (6, 8, 15, 16, 29, 31, 36). In order to understand the mechanism of HSV envelopment and maturation it is crucial to define the glycoprotein composition of the nuclear membrane patches at which envelopment takes place, specifically if the inner nuclear membranes contain a mixture of precursor and mature viral glycoproteins, reflecting what is found in the cytoplasm, or if they contain primarily the glycoprotein precursors. If only the precursors are present, it would follow that the virus acquires fully processed glycoproteins as it transits to the extracellular space. The data pertinent to this issue consist solely of the observations that in cells with specific defects in the Golgi glycosyltransferases (4, 30) or in cells exposed to monensin (17) or to other late glycosylation inhibitors (18), the egress of virus from the cell is hindered, and concomitantly virus carrying partially processed glycoproteins accumulates within the cytoplasm. While these observations were interpreted as evidence that the envelope glycoproteins become fully processed as the virus transits to the extracellular space and suggested an interaction of the virion particle with the Golgi (4, 17), the possibility exists that the transport mechanisms were nonfunctional because of absence of fully glycosylated cellular proteins. In this paper we analyzed directly the nuclear membranes of HSV-1-infected cells for the presence of viral glycoproteins and of precursors versus mature forms of the glycoproteins. We report that the envelope acquired by HSV at the inner nuclear membrane and the inner nuclear membrane itself contain the glycoprotein precursors and that during the transit to the extracellular space the virion glycoproteins become of the mature type. The study was performed by the thin-section fracture label technique (27), which combines cytochemical and immunochemical analyses to electron microscopy. It exposes freeze-fractured membranes to the labeling compounds and hence results in a high yield of labeling and gives full access to the labeling of large areas of the inner nuclear membranes, when the outer membrane is removed by the process of fracture. This technique provides the same preservation of the cell ultrastructure as conventional thin-section electron microscopy. The HSV glycoproteins B (gB) and D (gD) were labeled with antibodies. The precursor and mature forms of the glycoproteins were differentiated by labeling with the lectins concanavalin A (ConA) and wheat germ agglutinin (WGA).

Baby hamster kidney (BHK) cells were infected with HSV-1(F) (10) (10 PFU per cell) or mock infected. Freeze-fracture labeling was performed as previously described (27). Briefly, at 14 h after infection the cells were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 30 min, impregnated with 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 labeled with the antibodies or with the lectins, which was followed by colloidal gold conjugates as detailed in the figure legends, postfixed with osmium tetroxide, stained with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 812. Thin sections were

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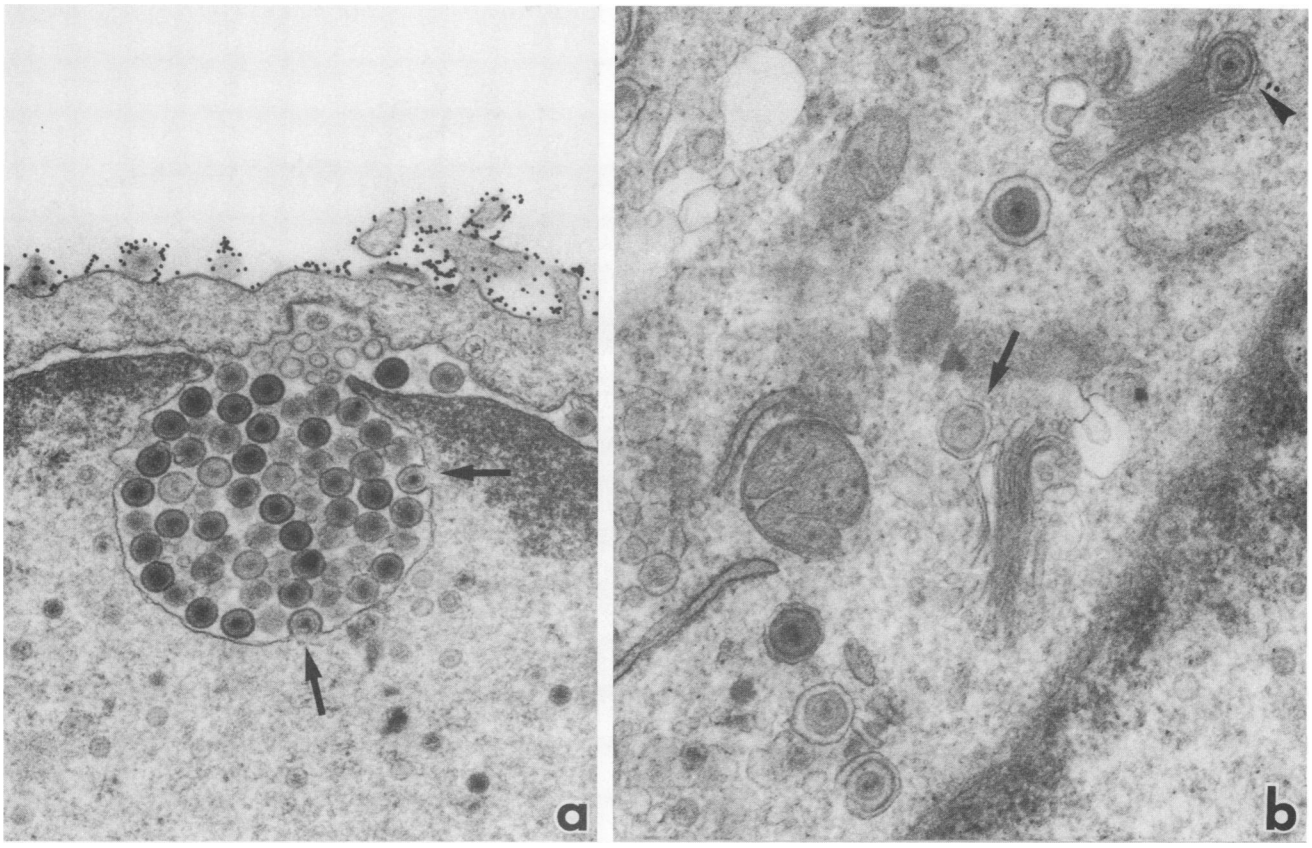

FIG. 1. HSV envelopment (a) and transit through the cytoplasm (b) in HSV-1(F)-infected BHK cells. (a) Intranuclear capsids, budding figures at the inner nuclear membrane (arrows) and enveloped virions accumulating in the space between the inner and the outer nuclear membranes are visible. (b) In the cytoplasm, enveloped virions are frequently observed within membrane-bound vesicles, in the proximity of (arrow) or strictly associated with (arrowhead) Golgi cisternae. Plasma membranes and an extracellular virion are labeled with WGA. To label with WGA, cells were incubated for 1 h at 37°C in a solution of 0.25 mg of WGA (Sigma Chemical Co., St. Louis, Mo.) per ml in 0.1 M Sorensen's phosphate buffer–4% polyvinylpyrrolidone, pH 7.4, for 30 min at 37°C and labeled with ovomucoid-coated colloidal gold for 1 h at 25°C (27). To test for the specificity of label, replicate 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. Magnifications, ×35,000 (a) and ×41,000 (b).

examined unstained and poststained with uranyl acetate and lead hydroxide.

Preliminarily, HSV envelopment and transit through the cytoplasm were morphologically examined in unfractured cells. Virions budding at the inner nuclear membrane and enveloped virions accumulating in the space between the inner and the outer nuclear membranes were frequently observed (Fig. 1a). Enveloped virions in transit from the nucleus to the extracellular compartment were detected in large amounts within membrane-bound vesicles (Fig. 1b) and to a lesser extent within larger vacuoles. They were prominent in proximity of the Golgi apparatus, and in some cases they could be detected strictly associated with or within the Golgi cisternae (Fig. 1b). Unenveloped capsids were observed in the cytoplasm, most often juxtaposed to membrane-bound structures (Fig. 1b). Enveloped extracellular virions were present in large amounts at this time of infection (Fig. 1a); however, nucleocapsids budding at the plasma membrane were never observed.

In a first series of experiments the distribution of gB and gD on the intracellular and plasma membranes as well as on extracellular and intracellular virions was compared by labeling with the monoclonal antibody H170, reacting with a continuous N-terminal epitope (residues 8 to 23) of gD (7), and rabbit polyclonal antibodies raised against an anchorless secreted form of gB (20). Fracture immunolabeling was performed on isolated cells to allow the simultaneous comparison of the pattern of labeling of the surface as well as of the fractured intracellular membranes (see Fig. 3b). In freeze-fractured cells the inner and the outer nuclear membranes were assigned as previously described (25, 30–32)

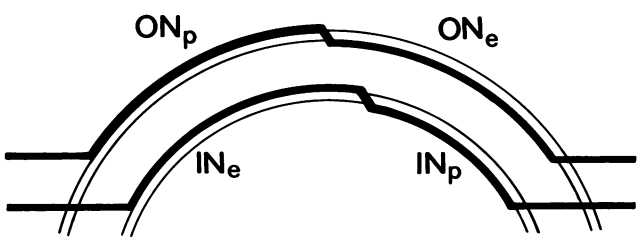

FIG. 2. Schematic representation of the freeze-fracture planes (solid lines) at the level of the inner and outer nuclear membranes and of the fracture faces that become exposed to the labels. IN_p and IN_e, protoplasmic and exoplasmic faces, respectively, of the inner nuclear membrane. ON_p and ON_e, protoplasmic and exoplasmic faces, respectively, of the outer nuclear membrane.

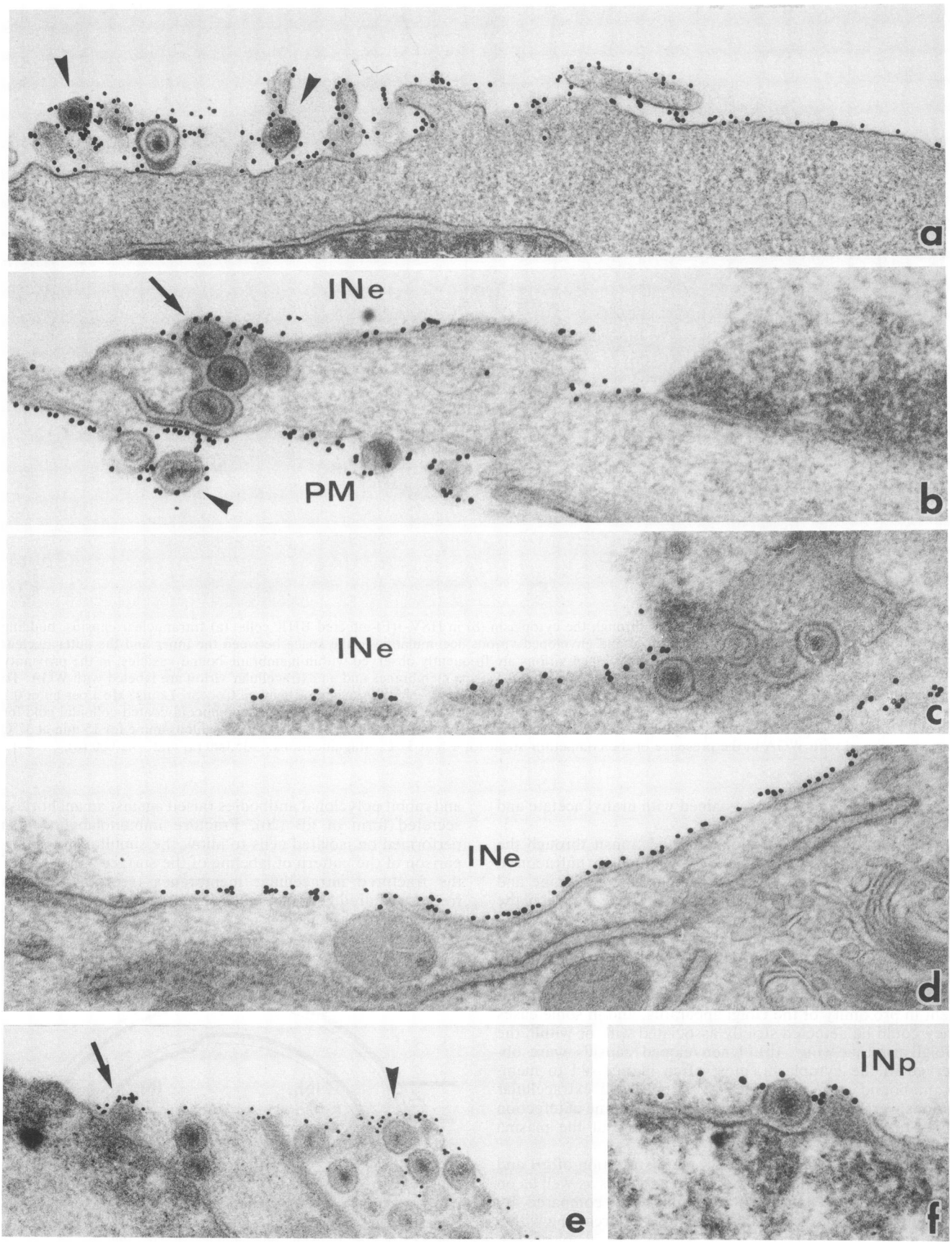
a
INe
PM
b
INe
c
INe
d
e
INp
f

TABLE 1. Quantitative comparison of gB and gD immunolabeling on unfractured plasma membranes and extracellular virions and on freeze-fractured inner and outer nuclear membranes in HSV-1(F)-infected BHK cells

Antibody	Gold particles/μm of membrane measured[a]			
	Plasma	Inner nuclear	Outer nuclear	Extracellular virions
gB	8.85 ± 2.88 (8)[b]	13.43 ± 1.12 (7)	7.08 ± 0.64 (9)	10.65 ± 0.46 (20)
gD	7.85 ± 3.05 (10)	6.93 ± 4.25 (9)	6.46 ± 0.57 (9)	9.50 ± 0.31 (20)

[a] Immunolabeling was performed with the antibodies indicated in the text, followed by protein A-colloidal gold, as described in the legend to Fig. 3. The evaluation of the labeling density, determined as gold particles per micrometer of freeze-fractured membrane, and statistical analysis (independent *t* test) were performed with the Sigma Scan Measurement System (Sigma Scan-Jandel Scientific). The length of the membranes measured were 28.07, 25.32, and 16.56 μm for the plasma membranes and inner nuclear and outer nuclear membranes, respectively, in the case of the gB-specific labeling and 77.26, 27.45, and 27.18 μm for the plasma membranes and inner nuclear and outer nuclear membranes, respectively, in the case of the gD-specific labeling. Figures represent the average number of gold particles per micrometer of membrane measured ± standard error of the mean. In parentheses are given the numbers of micrographs counted.

(Fig. 2 [modified from reference 26]). In particular, the inner nuclear membranes were identified when the corresponding unfractured outer nuclear membranes were visible beneath the fracture plane. The identification was frequently confirmed by the presence of cross-fractured chromatin following the plane of the fracture. In contrast, the outer nuclear membranes were identified by the presence of the corresponding unfractured inner nuclear membranes beneath the fracture plane. As previously described (1, 35), immunolabeling of protoplasmic faces corresponds to transmembrane proteins that are dragged during fracture across the outer leaflet of the bilayer; labeling of exoplasmic faces results from postfracture reorganization events occurring during thawing, when interrupted bilayer fragments are reconstituted (for a schematic representation, see Fig. 2). The gB- and the gD-specific label localized to the envelope of the intracellular virions, whether located in the space between the inner and outer nuclear membranes (Fig. 3b, e, and f) or within membrane-bound cytoplasmic vesicles. The gB- and gD-specific label localized also to the membranes of the virion-surrounding vesicles. Fracture faces of both inner and outer nuclear membranes were themselves heavily and uniformly labeled by the antibodies to gB and to gD (Fig. 3b through f). As expected, both antibodies labeled heavily and uniformly the mature virion in the extracellular space and the infected cell plasma membranes (Fig. 3a and e). A quantitative comparison of the gold particle density on unfractured cell surfaces and extracellular virions and on freeze-fractured inner and outer nuclear membranes showed a very similar density for the gD-specific labeling (Table 1). By contrast, the gB-specific labeling displayed a higher density on the inner nuclear membranes than on the other membranes (Table 1). As expected, freeze-fractured Golgi cisternae appeared to be densely labeled by the two antibodies (data not shown). Capsids exposed to the labeling on cross-fractured cytoplasms and on cross-fractured chromatin were virtually unlabeled, as were the fractured membranes of uninfected cells observed in control experiments (data not shown).

A few points regarding these results should be made. (i) The new findings are the presence of gB and gD in the envelope of virions located between the inner and outer nuclear membranes and at the inner nuclear membranes. The latter result was not obvious, inasmuch as the two nuclear membranes have long been considered to differ. Whereas the outer nuclear membrane is contiguous with RER and may itself be the site of glycoprotein biosynthesis, the inner membrane is a distinct membrane, connected to the outer membrane through the nuclear pores (13). Nuclei isolated from HSV-infected cells were previously reported to contain the viral glycoproteins, but the inner and outer nuclear membranes were not differentiated (9). The presence of viral glycoproteins at the inner nuclear membranes is not a feature typical of herpesvirus-infected cells, since even in other viral systems the transmembrane proteins reach the inner nuclear membrane, probably by diffusion from the outer nuclear membrane (32, 34). (ii) The higher density of gB at the inner nuclear membrane versus the outer nuclear membrane and the plasma membranes may be interpreted in light of other peculiar features of this glycoprotein. In pulse-chase studies the rate of conversion of gB from precursor to mature form was found to be much higher than that of other HSV glycoproteins, and the gB precursor was detectable in significant amounts even after a 6-h chase (3). gB is known to bind to the RER resident protein grp76 (24), also named BiP. In addition, gB carries in the cytoplasmic C terminus the consensus sequence Lys-Lys-Arg-Lys, known to target proteins to the nucleus. It is tempting to speculate that the accumulation of gB at the inner nuclear membrane and its low maturation rate are related. It remains to be elucidated whether the relative retention of gB at the inner nuclear membrane is affected by its ability to bind grp76, by the consensus sequence for nuclear import, or by a yet unknown mechanism. (iii) The presence of HSV glycopro-

FIG. 3. Fracture immunolabel of HSV-1(F)-infected BHK cells. (a, b, and e) The cell plasma membranes and extracellular virions (arrowheads) are densely labeled by monoclonal antibody H170 (a and b) and by the anti-gB polyclonal antibodies (e). Exoplasmic faces of freeze-fractured inner nuclear membranes are also labeled by both H170 monoclonal antibody (b and c) and anti-gB polyclonal antibodies (e) (arrow). IN_e, exoplasmic face of freeze-fractured inner nuclear membrane; IN_p, protoplasmic face of freeze-fractured inner nuclear membrane; PM, unfractured plasma membrane. Immunolabeling was performed by using monoclonal antibody H170 (1:5 in PBS for 60 min at 25°C) or anti-gB polyclonal antibodies (1:5 in PBS for 60 min at 25°C) and labeled for 3 h at 4°C with colloidal gold (prepared by the citrate method) conjugated with protein A (Pharmacia, Uppsala, Sweden) as previously described (33). The inner and outer nuclear membranes were identified by the presence of the corresponding unfractured outer and inner membranes visible underneath the fracture plane; the identification of the inner nuclear membranes was often confirmed by the presence of cross-fractured chromatin following the fractured membranes. Magnifications, ×42,000 (a), ×49,000 (b), ×59,000 (c), ×53,000 (d), ×39,000 (e), and ×61,000 (f).

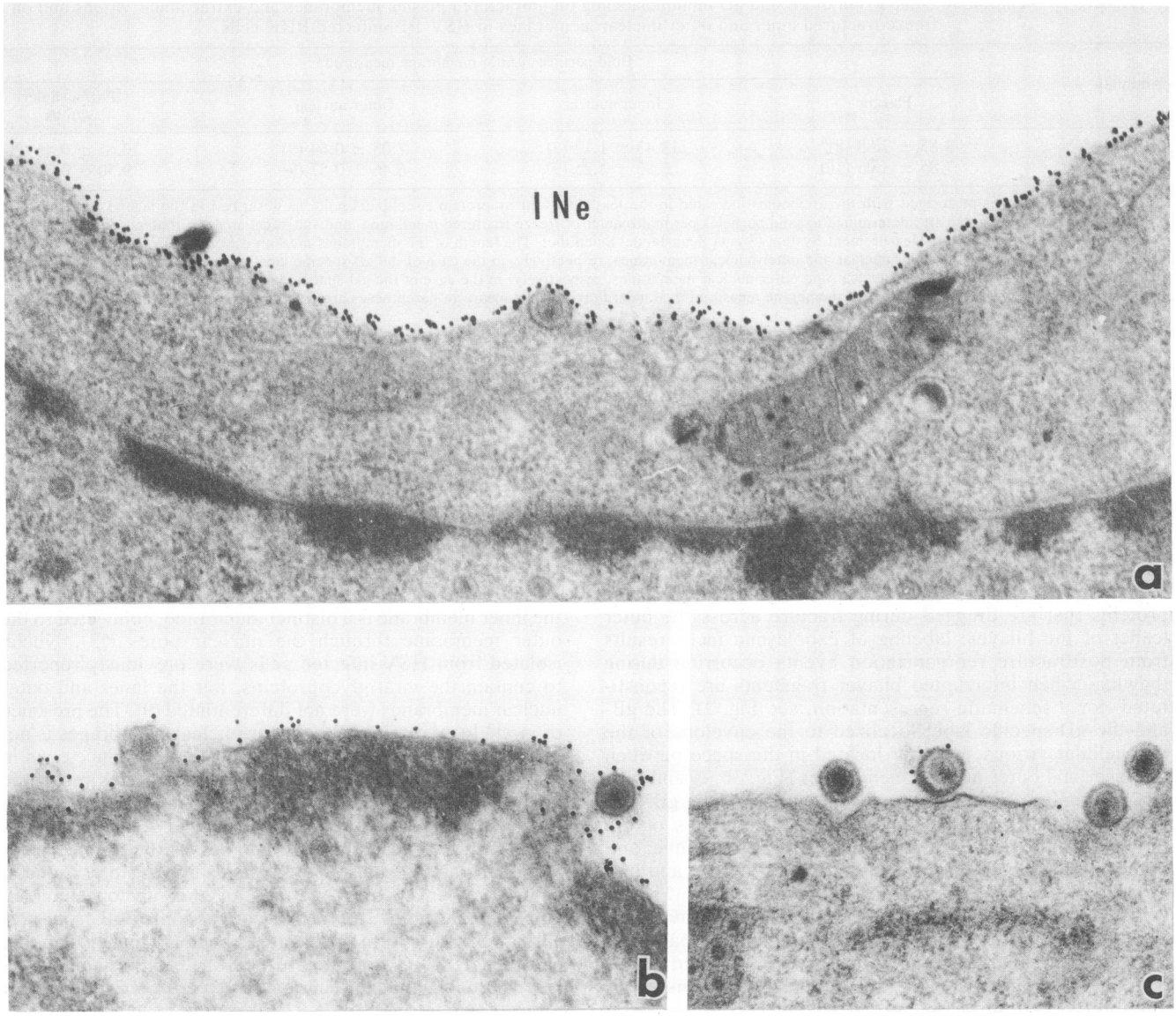

FIG. 4. ConA fracture label of HSV-1(F)-infected BHK cells. (a and b) The exoplasmic (a) and the protoplasmic (b) leaflets of a freeze-fractured inner nuclear membrane are labeled by ConA, as was the enveloped virion located between the inner and the outer nuclear membranes. (c) The cell surfaces and the extracellular virions are only weakly labeled. IN_e, exoplasmic face of freeze-fractured inner nuclear membrane; IN_p, protoplasmic face of inner nuclear membrane; PM, unfractured plasma membranes. Fractured cells were incubated with ConA (Sigma) (1 mg/ml in Sorensen's phosphate buffer) for 30 min at 37°C and labeled with horseradish peroxidase-coated colloidal gold for 1 h at 25°C (27). To test for the specificity of label, replicate samples were preincubated in 0.4 M methyl-α-D-mannopyranoside for 15 min at 37°C and then treated with ConA in the presence of the competitor sugar for 1 h at 37°C. Magnification, ×39,000.

teins in the membranes of the virion-surrounding vesicles is consistent with their origin from the outer nuclear membrane and is in keeping with previous studies showing the presence of viral glycoproteins in the pool of intracellular membranes (16).

In a second series of experiments precursor and mature forms of the glycoproteins were labeled with ConA and WGA, respectively. ConA binds with high-affinity high-mannose oligosaccharides typical of glycoprotein precursors, whereas WGA binds with high-affinity sialic acid and *N*-acetylglucosamine residues present in the processed N- and O-linked oligosaccharides (19). Figures 3 and 4 show that the label with ConA but not the label with WGA localized to the envelope of the virion in the space between the inner and the outer nuclear membranes. The enveloped virion within membrane-bound vesicles underwent a change in reactivity to the lectins and became densely labeled by WGA and weakly labeled by ConA (Fig. 4 and 5). The extracellular enveloped virions conformed to the latter pattern of reactivity (Fig. 4 and 5). The persistence of a low level of reactivity to ConA in the extracellular virion glycoproteins is consistent with the persistence of high-mannose oligosaccharides in the mature forms of some HSV glycoproteins, e.g., gB and gH (12, 29, 36), and of cellular glycoproteins as well.

The experimental design adopted in these studies com-

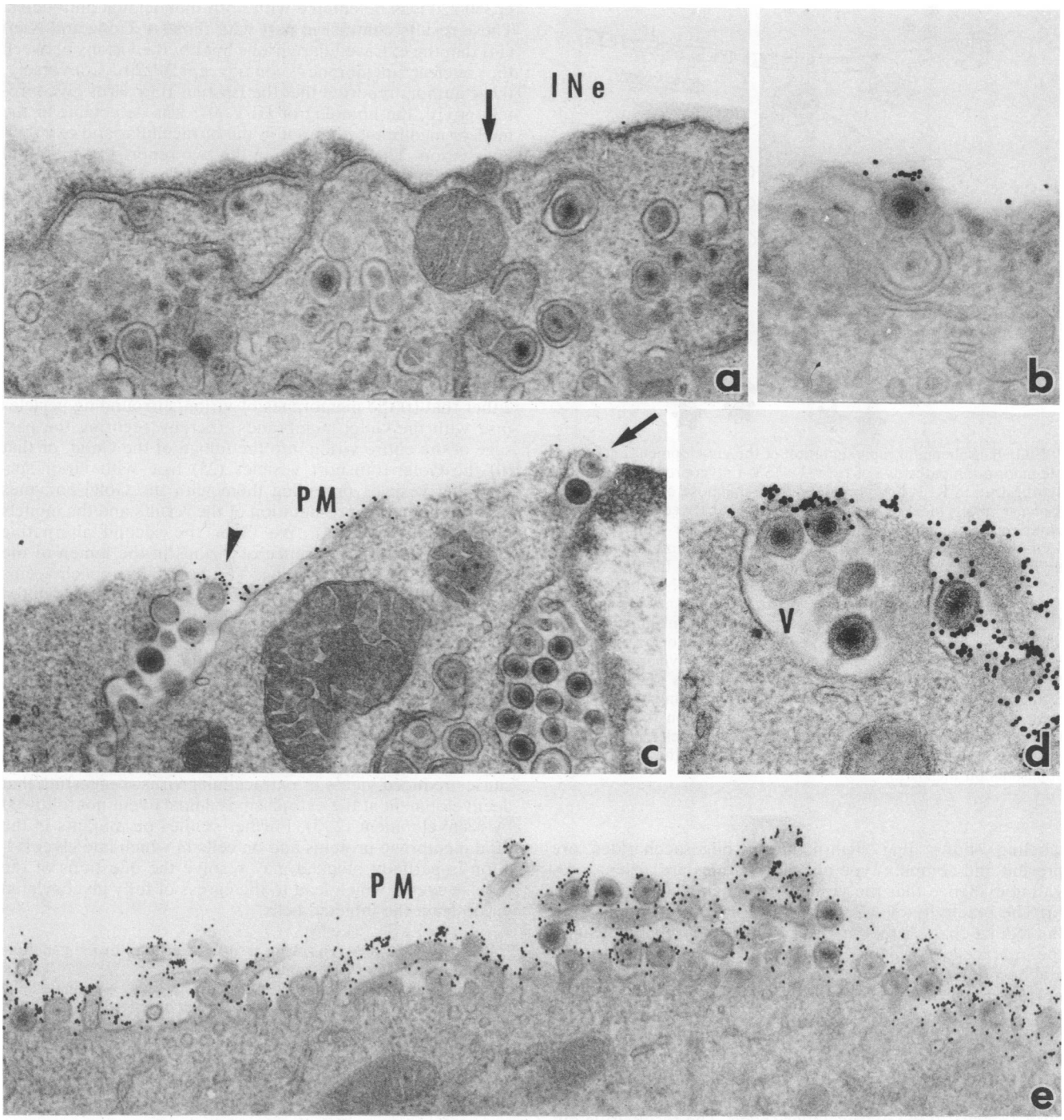

FIG. 5. WGA fracture label of HSV-1(F)-infected BHK cells. (a and c) Freeze-fractured inner nuclear membranes appear unlabeled as well as the enveloped virion located between the inner and outer nuclear membranes (arrows). The enveloped virions within intracytoplasmic vacuoles exposed to the labeling by the fracture plane (d) and the extracellular virion (c [arrowhead] and e) are densely labeled. The membrane of the cytoplasmic vesicle that surrounds enveloped virions (b) and the cell surfaces (e) are also heavily labeled. IN_e, exoplasmic face of freeze-fractured inner nuclear membrane; PM, unfractured plasma membranes; V, vesicle. Fractured cells were incubated with WGA, as detailed in the legend to Fig. 1. Magnifications, ×39,000 (a), ×56,000 (b), ×39,000 (c), ×56,000 (d), and ×28,000 (e).

bined the labeling of the viral glycoproteins with antibodies to the labeling of the oligosaccharide moieties with lectins. Notwithstanding the fact that the lectins bound both the viral glycoproteins and the cellular glycoconjugates, the following results are well documented. (i) The envelope of the virions located between the inner and outer nuclear membranes and the inner nuclear membrane itself contain the two viral glycoproteins analyzed, gB and gD. The pattern of lectin

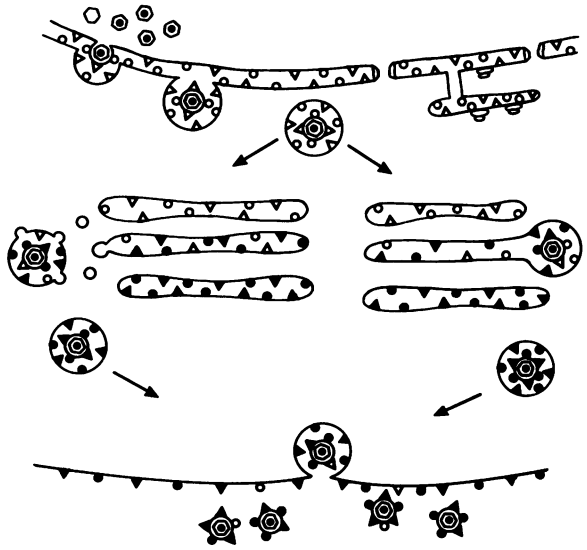

FIG. 6. Schematic representation of the envelopment and possible maturation pathways of HSV-1. HSV-1 glycoprotein precursors synthesized in RER and containing high-mannose oligosaccharides (open symbols) localize to the virions present between the inner and outer nuclear membranes and to the two nuclear membranes, in addition to the RER. The fully mature HSV glycoproteins processed by the Golgi enzymes (closed symbols) localize to the intracellular virions contained in the membrane-bound vesicles, to the extracellular virion, and to the Golgi apparatus and plasma membranes. HSVs acquire at the inner nuclear membrane an envelope containing the glycoprotein precursors and transit the cytoplasm enclosed in membrane-bound vesicles. Maturation of the envelope glycoproteins might occur as follows. The transport vesicles which pinch off from the Golgi stacks fuse with the virion-surrounding vesicles, thus providing the Golgi enzymes (Golgi apparatus on the left). The virion-surrounding vesicles fuse with the Golgi stacks, enabling the passage of the entire virion into the lumen of the Golgi (Golgi apparatus on the right).

labeling shows that high-mannose oligosaccharides are present and complex-type oligosaccharides are absent, providing evidence that the viral glycoproteins are precursors. (ii) The reactivity of the virion envelope glycoproteins with the lectins changes as the virus transits from the nucleus to the extracellular space. (iii) Within the limit of the two glycoproteins tested, the virions located between the two nuclear membranes contain the same glycoprotein species as the extracellular virions. The process of HSV envelopment and maturation documented by current results envisions that HSVs acquire at the inner nuclear membrane an envelope with the glycoprotein precursors and that the envelope glycoproteins become of the fully glycosylated type as the virus transits to the extracellular space. The results rule out two of the maturation models, namely, that HSVs acquire at the nuclear membrane an envelope containing the mature glycoproteins or that HSVs acquire at the nuclear membrane an envelope which differs from the final envelope in terms of glycoprotein species.

Current results parallel an earlier report from one of our laboratories that the major glycoprotein gp350/220 of Epstein-Barr virus is present in the virion located between the nuclear membranes and in the inner nuclear membranes as well as in the extracellular virion (33). At the nuclear membranes gp350/220 was only detectable with one of two monoclonal antibodies used, whereas the extracellular gp350/220 was detectable with both monoclonal antibodies. These results contrast in part with those of Gong and Kieff (14) that the extracellular virions but not the virions between the nuclear membranes contain gp350/220. Conversely, these authors reported that the Epstein-Barr virus glycoprotein gp110, the homolog of HSV gB, was detectable at the nuclear membranes but not in the intracellular and extracellular virion. The differences in the two reports stem both in the technique and in the antibodies. Whereas Torrisi et al. (33) used the fracture label, Gong and Kieff (14) used cryosection labeling, and only the monoclonal antibody that Torrisi et al. (33) reported to label the extracellular virions and the plasma membranes was common to the two studies. Both of the monoclonal antibodies used by Gong and Kieff (14) may have reacted only with the mature form of gp350/220.

The details of the interaction of HSV glycoprotein with the Golgi apparatus remain to be elucidated. Of the various possible modalities of the interaction, the most likely are either that (i) the membranes of virion-surrounding vesicles fuse with the Golgi membranes, thereby securing the passage of the entire virion into the lumen of the Golgi, or that (ii) the Golgi transport vesicles (25) fuse with virion-surrounding vesicles providing them with the Golgi enzymes (for a schematic representation of the results and the models proposed, see Fig. 6). We favor the second alternative simply because the presence of virions in the lumen of the Golgi stacks is a rare finding. The possibility which seems less likely is that nucleocapsids are sequentially engulfed into and released out of the Golgi membranes. Such a mechanism would result in the accumulation of unenveloped capsids in the cytoplasm in proximity of the Golgi. While unenveloped capsids are readily found in the cytoplasm, they are usually present juxtaposed to any cellular membrane, they frequently show signs of degradation, and a mutation in gD which favors the accumulation of large amounts of unenveloped capsids in the cytoplasm also causes reduced yields of extracellular virus, suggesting that deenvelopment at the cytoplasm is a final event not followed by reenvelopment (2, 5). Further studies on mutants in the viral membrane proteins and on cells in which late glycosylation is partially blocked may resolve the questions of the precise events which lead to the egress of fully glycosylated virion from the infected cells.

We thank R. Manservigi, University of Ferrara, for the generous gift of anti-gB rabbit polyclonal serum.

The studies were aided by grants from Consiglio Nazionale delle Ricerche (CNR); MURST; Target Project on Biotechnology and Bioinstrumentation, CNR; Target Project of Genetic Engineering, CNR (9100011A3); Associazione Italiana per la Ricerca sul Cancro AIRC; Istituto Superiore di Sanità-Progetto AIDS; and NIH grant AI-30873 from the National Institute of Allergy and Infectious Disease.

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