

## Intracellular Trafficking of Two Major Epstein-Barr Virus Glycoproteins, gp350/220 and gp110

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The processing and intracellular localization of the two predominant Epstein-Barr virus glycoproteins expressed in late lytic infection were investigated. Immune light or electron microscopy of frozen fixed sections revealed that gp110 colocalized to the endoplasmic reticulum and to the nuclear membrane with the endoplasmic reticulum-resident protein, heavy-chain-binding protein (BiP), while gp350/220 accumulated in low abundance in the endoplasmic reticulum and was present in higher abundance in cytoplasmic structures presumed to be Golgi and in plasma membranes. Consistent with endoplasmic reticulum and nuclear membrane localization, the bulk of gp110 was sensitive to endoglycosidase H, indicating high-mannose, pre-Golgi, N-linked glycosylation; while consistent with Golgi and plasma membrane localization, gp350/220 was mostly resistant to endoglycosidase H because of complex N- and O-linked glycosylation. gp350/220 was as abundant in extracellular enveloped virus as in the plasma membrane but was much less abundant or undetected in internal cytoplasmic or nuclear membranes. In contrast, gp110-specific antibodies did not label extracellular or intracellular virus. These data indicate that the major antigenic components of gp110 are not incorporated into or are occluded in virions and that gp350/220 is added to virus in cytoplasmic transit through a process of de-envelopment and re-envelopment at the plasma membrane or at post-Golgi vesicles. Consistent with cytoplasmic de-envelopment and re-envelopment at the plasma membrane was the finding of some free nucleocapsids in the cytoplasm of cells with intact nuclear membranes and nucleocapsids which appeared to bud through the plasma membrane.

Epstein-Barr virus (EBV) is a human herpesvirus which has a markedly restricted host range, particularly in vitro. EBV establishes latent infection in human B lymphocytes. A fraction of the latently infected cells can be induced to permit virus replication. Such permissively infected cells undergo the characteristic cytopathic changes of herpesvirus infection, including cell chromatin margination, nucleocapsid assembly in the nucleus, envelopment by nucleocapsid budding through the modified inner nuclear membrane, and enveloped virus egress through cytoplasmic membranous channels or vesicles (4, 5). Mature enveloped herpesviruses have glycoproteins on their surface which have complex N- or O-linked oligosaccharides indicative of Golgi processing (for a review, see reference 20). Little is known about whether these viral glycoproteins are added at the time of initial envelopment and undergo subsequent Golgi level processing during virus egress or about how cytoplasmic enveloped virus transits through the plasma membrane.

EBV is unusual among herpesviruses in that it has a single predominant glycoprotein, gp350/220. gp350/220 is the ligand for CR2, a B-lymphocyte-specific surface protein which mediates EBV adsorption and endocytosis (6, 18, 22). The 907-amino-acid gp350/220 primary sequence includes 36 potential signals for N-linked glycosylation, a single hydrophobic transmembrane domain, and a short carboxy-terminal domain. gp350/220 is synthesized in the endoplasmic reticulum (ER), undergoes rapid cotranslational N-linked glycosylation, and is transported to the Golgi, where high-mannose N-linked oligosaccharides are trimmed and complex N- and O-linked oligosaccharides are added, as expected for a glycoprotein which undergoes Golgi level processing (3, 19, 19a, 21, 22). gp350/220 is a prominent constituent of the

infected-cell plasma membrane. Another abundant EBV-encoded late-replicative-cycle glycoprotein, gp110, is a primary sequence of 857 amino acids with nine potential signals for N-linked glycosylation, three potential membrane-spanning domains, and an unusual carboxy-terminal domain characterized by an extensive run of basic amino acids. Like gp350/220, gp110 also undergoes cotranslational N-linked glycosylation (7). In contrast to gp350/220, however, gp110 does not accumulate in the cell plasma membrane or in the virus envelope but is readily identified in cytoplasmic membranes and in the inner and outer nuclear membranes of permissively infected lymphocytes. Thus, gp110 is one of the few glycoproteins known to traffic to the inner nuclear membrane. Since virus acquires its envelope by budding through the inner nuclear membrane, the failure to find gp110 in virus is surprising. Moreover, since gp350/220 is the most abundant glycoprotein on the virus, both of these proteins would be expected to be found in similar abundance on the inner nuclear membrane and on virus if inner nuclear membrane envelopment confers upon the virion its definitive glycoprotein composition.

The objective of these investigations was to further examine gp350/220 and gp110 processing into infected-cell membranes and virus. Our initial working hypothesis was that gp110 and gp350/220 are incorporated into virus at the inner nuclear membrane and that failure to detect gp110 in mature virus was due to modification of immunoreactive determinants. We were surprised to find major differences between gp350/220 and gp110 in posttranslational cytoplasmic membrane trafficking.

### MATERIALS AND METHODS

**Cell culture.** Lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% newborn calf

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serum. EBV replication was induced in B95-8 and P3HR-1 cultures to 25 to 40% of the cells by adding 12-*O*-tetradecanoyl phorbol-13-acetate (20 ng/ml) and 0.5 mM *n*-butyrate to the cultures 3 days before harvesting.

**Cryoultramicrotomy.** Lymphocytes were processed by the Tokuyasu method (23). Briefly, cell pellets were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.1% glutaraldehyde for 1 h at 4°C. Fixed cells were embedded in 3% gelatin, refixed, and infused with 2.3 M sucrose for 17 h at 4°C. Infused cells were mounted, frozen, and stored in liquid nitrogen. Frozen thin sections 0.35  $\mu$ m thick were prepared by using a Cryo-ultramicrotome apparatus (Reichert) at -90°C. For immunofluorescence studies, sections were collected and placed onto microwell slides at 22°C for immunofluorescence processing. For immunoelectron microscopy studies, 60-nm frozen sections were collected and placed on Formvar-carbon-coated grids at 22°C for immunolabeling and embedding.

**Immunofluorescence.** All incubations of cryosections on microwell slides were performed at 22°C. To quench autofluorescence and deactivate any remaining free aldehyde groups, cryosections were incubated with 0.05 mg of sodium borohydride per ml, followed by 80 mM ammonium chloride, for 10 min each. Slides were then washed in PBS containing 10 mM glycine and 0.1% bovine serum albumin (BSA), incubated with PBS containing 20% goat serum, followed by diluted antiserum, for 30 min each. After being washed in PBS, the wells were incubated with fluorescein isothiocyanate- or Texas Red-conjugated goat anti-rabbit, -mouse, or -rat immunoglobulin G (Jackson Laboratory) diluted 1:1,000. For double-labeling experiments, both primary antibodies or both secondary antibodies were incubated with sections at the same time.

All immunofluorescence slides were preserved with 90% glycerol containing 0.1% *p*-phenylenediamine (11), and the cells were observed by using a Zeiss photomicroscope equipped with epifluorescence.

**Immunoelectron microscopy. (i) Surface whole cells.** Live cells were incubated on ice with antibodies for 45 min, washed with cold PBS containing 2% goat serum, and incubated with 5-nm-diameter colloidal gold-conjugated goat anti-mouse antibodies diluted 1:5 for 45 min. Antibodies were diluted in PBS containing 10% goat serum. After being washed, the cells were fixed in Karnovsky fixative (14) for 17 h at 4°C and treated with 1% osmium tetroxide. Finally, the cells were embedded in Epon, sectioned, stained, and viewed on a JEOL 100-CX II electron microscope.

**(ii) Cryosections.** Cryosections on grids were processed in drops placed on Parafilm. The grids were incubated with 80 mM ammonium chloride for 10 min, followed by PBS containing 10 mM glycine and 0.1% bovine serum albumin, PBS with 20% goat serum, and diluted antibody, for 30 min each. All washes and antibody dilutions were in PBS with 10 mM glycine and 0.1% bovine serum albumin. After being washed, the grids were incubated with colloidal gold-conjugated goat anti-mouse or -rabbit immunoglobulin G diluted 1:20. For double-labeling experiments, both primary antibodies or both secondary antibodies were incubated with sections at the same time. After being immunolabeled, the grids were washed with PBS and water, stained with 2% uranyl acetate, and embedded in a thin layer of 2% methylcellulose as described by Tokuyasu (23). Specimens were viewed with a JEOL 100-CX II electron microscope.

**Metabolic labeling.** Cells were incubated with 1 mCi of [<sup>35</sup>S]-Trans label (ICN Pharmaceuticals Inc.) per ml in 90% methionine-free RPMI 1640 medium supplemented with 10%

dialyzed fetal bovine serum. From pulse labeling, cells were preincubated in methionine-free RPMI 1640 medium supplemented with dialyzed fetal bovine serum for 30 min.

**Immunoprecipitations.** Cells were washed in cold PBS with 0.2 mM phenylmethylsulfonyl fluoride and lysed with PBS containing 1% Nonidet P-40 and the following protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5 mg of leupeptin per ml, and 0.7 mg of pepstatin per ml. The crude soluble extract was divided into aliquots, and antibody or serum was added and incubated for 1 h at 4°C. A 25- $\mu$ l volume of protein A-Sepharose CL-4B (Sigma Chemical Co.) was added and incubated for 30 min at 4°C. The immunoprecipitate was washed five times with PBS containing 0.5% Nonidet P-40, once with 50 mM Tris hydrochloride (pH 7.5)-500 mM sodium chloride-0.5% Nonidet P-40, and once with 20 mM Tris hydrochloride (pH 7.5)-0.5% Nonidet P-40 and then boiled in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For endoglycosidase H (endo H) treatment, immunoprecipitates were boiled in endo H buffer (50 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride) and 3 mU of endo H (Boehringer Mannheim Biochemicals) was added for 17 h at 37°C. The endo H-treated immunoprecipitates were then boiled in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The gels were stained with 0.03% Coomassie brilliant blue G in 25% (vol/vol) isopropanol-10% (vol/vol) glacial acetic acid, destained in 10% (vol/vol) isopropanol-10% (vol/vol) glacial acetic acid, and treated with Amplify (Amersham Corp.) for 30 min before gel drying. The dried gel was exposed to XAR-5 film at -80°C.

## RESULTS

**Synthesis and processing of gp110 and gp350/220.** After high-mannose N-linked oligosaccharide addition in the ER lumen, many glycoproteins are transported to the Golgi, where high-mannose oligosaccharides are trimmed and complex N- and O-linked oligosaccharides are added (for a review, see reference 19). Since endo H specifically cleaves high-mannose N-linked oligosaccharides and not complex oligosaccharides, resistance to endo H treatment indicates that a glycoprotein has been processed through the Golgi. Because previous studies indicated that herpesvirus glycoproteins, including gp350/220, undergo complex N-linked oligosaccharide addition (3, 19a, 20-22), we expected that mature gp350/220 and gp110 would be endo H resistant. The extents of Golgi processing of gp110 and gp350/220 were determined by their endo H sensitivities. For these studies, we used two gp110-specific monoclonal antibodies which had been previously identified by their reactivity with a 125-kilodalton (kDa) late virus replication-associated cytoplasmic antigen (15). These monoclonal antibodies immunoprecipitated gp110 from gp110-transfected NIH 3T3 cells but not from control-vector-transfected NIH 3T3 cells (Fig. 1). These monoclonal antibodies or monoclonal antibodies specific for gp350/220 were then used to monitor the posttranslational processing of gp350/220 and gp110 in infected lymphocytes. Induced EBV-infected lymphocytes pulse-labeled with [<sup>35</sup>S]methionine for 4 h were immunoprecipitated with monoclonal antibodies, and the immunoprecipitated proteins were analyzed for complex sugar conversion by comparison of their sizes before and after endo H treatment (Fig. 2). With 4 h of pulse-labeling, anti-gp110 monoclonal antibodies detected full-size gp110 and a small amount of a diffuse

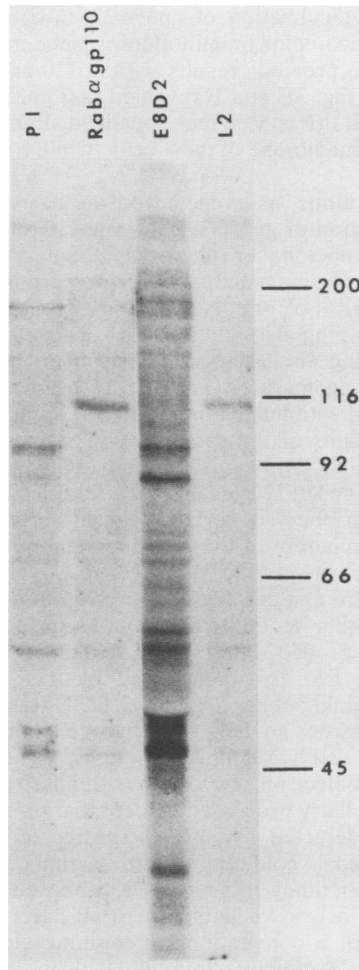


FIG. 1. Recognition of the BALF4 110-kDa protein by monoclonal antibody L2. pZ110-5-transfected  $\psi$  am 22b cells expressing gp110 (7) were metabolically labeled with [ $^{35}$ S]methionine for 16 h, and the extracts were immunoprecipitated with preimmune rabbit serum (PI), TO16-specific rabbit serum (Rab $\alpha$ gp110), a monoclonal antibody specific for an EBV 105-kDa early protein (E8D2) (1), and a monoclonal antibody specific for an EBV viral capsid-associated antigen (L2) (15). Only TO16-specific serum and L2 recognized 110-kDa protein gp110. pZIP-NEO-SV(X)1-transfected cells were similarly processed, and no specific proteins were detected. The positions of the molecular weight standards (in thousands) are indicated on the right.

larger protein of approximately 125 kDa. Both gp110 forms were endo H sensitive; the 125-kDa proteins were slightly reduced to 120 kDa, while the predominant gp110 band was reduced to 93-kDa, the size of nascent gp110 (7). gp350/220-specific monoclonal antibody 72A1 (9), 2L10, or MA17 immunoprecipitated proteins of 350 and 220 kDa and a small amount of a 145-kDa protein similar in size to nascent gp350. As expected on the basis of previous studies, full-size gp350/220 was unaffected by endo H treatment, since gp350/220 contains complex N- and O-linked sugars. Some 200- to 220-kDa gp350 precursor molecules were immunoprecipitated by all three monoclonal antibodies and were reduced by endo H to 145 kDa, the size of the gp350 nascent protein (10). Overnight labeling and immunoprecipitation experiments with either of the two gp110-specific monoclonal antibodies yielded a distribution of gp110 identical to that

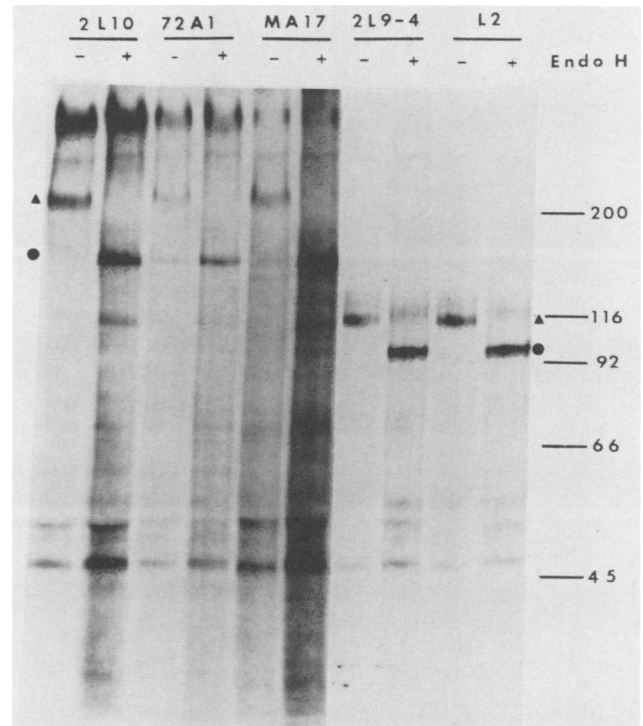


FIG. 2. Endo H treatment of gp110 and gp350/220 immunoprecipitates. Induced B95-8 cells were labeled with [ $^{35}$ S]methionine for 4 h, and the cell extracts were immunoprecipitated with gp350/220-specific monoclonal antibodies (2L10, 72A1, and MA17) and gp110-specific monoclonal antibodies (2L9-4 and L2). The immunoprecipitates were incubated with (+) or without (-) endo H and subjected to 6% polyacrylamide gel electrophoresis. The positions of the endo H-sensitive forms of gp350 and gp110 before ( $\blacktriangle$ ) and after ( $\bullet$ ) endo H treatment and the molecular weight standards (in thousands) are indicated. All three gp350/220-specific monoclonal antibodies recognized 350-, 220-, and 145-kDa proteins. After endo H treatment, the 220-kDa protein was reduced to 145 kDa. Both gp110-specific monoclonal antibodies recognized 125- and 110-kDa proteins. After endo H treatment, the predominant 110-kDa protein was reduced to 93 kDa and the 125-kDa protein was reduced to 120 kDa.

shown in Fig. 2, as previously described (15). In contrast, pulse-chase labeling and immunoprecipitation with gp350/220-specific monoclonal antibodies revealed that endo H-sensitive (high-mannose) gp350/220 completely disappeared with a half-life of approximately 30 min (22). These data indicate that most gp110 molecules are processed solely to high-mannose N-linked glycosylation, characteristic of endoplasmic reticular compartments, while most gp350/220 molecules are processed to contain complex N- and O-linked oligosaccharides, characteristic of Golgi modification.

**Intracellular location of gp110 and gp350/220.** Previous studies localized gp110 to the nuclear membranes and ER after permeabilization with organic fixatives which may not have assured equal antibody access to all cell compartments. To assure access to all cell compartments, cells were first fixed and frozen, and then 0.35- $\mu$ m sections were incubated with gp110-, gp350/220- or BiP-specific antibodies. BiP, immunoglobulin heavy-chain-binding protein, was used as a marker for an ER luminal resident protein (2, 8, 16). Sites of antibody binding were detected with fluorochrome (Texas Red or fluorescein isothiocyanate)-conjugated secondary

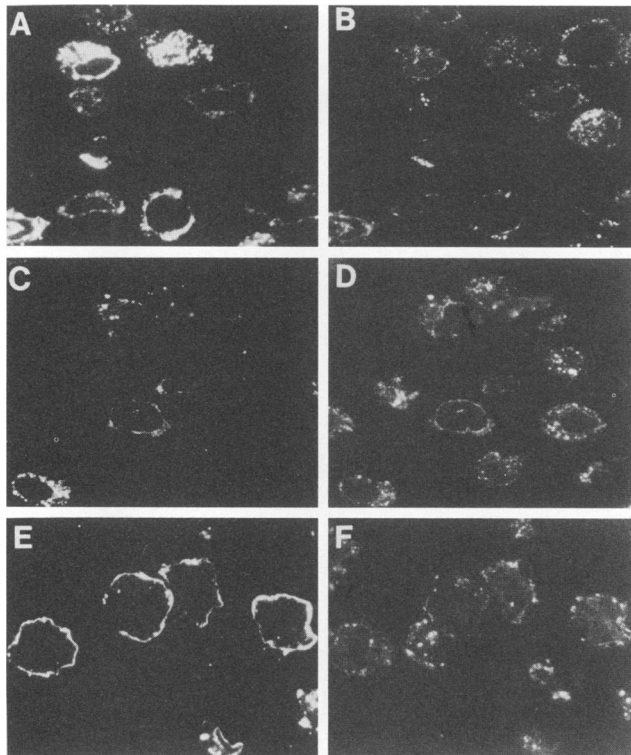


FIG. 3. Colocalization of gp110, but not gp350/220, with the ER-resident protein immunoglobulin heavy-chain-binding protein (BiP). Induced B95-8 cells were fixed in PBS with 2% paraformaldehyde and 0.1% glutaraldehyde and processed for cryoultramicrotomy by the Tokuyasu method (23). Cryosections 0.35  $\mu$ m thick were incubated with antibodies for double immunolabeling. (A to D) Double labeling with affinity-purified TO16-specific (gp110-specific) rabbit antibodies (diluted 1:5) (A and C) and a rat monoclonal antibody specific for BiP (diluted 1:1) (B and D). (E and F) Double labeling with rabbit anti-gp350/220 (diluted 1:100) (E) and the rat anti-BiP monoclonal antibody (F). Texas Red-conjugated goat anti-rabbit immunoglobulin G and fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin G antibodies were coincubated for visualization. Sections were viewed on a fluorescence microscope equipped with specific filter barriers for distinct detection of each fluorochrome. Panels A and B are photomicrographs of the same field, and panels C and D are photomicrographs of another field. Superimpositions of panel A on panel B and panel C on panel D revealed that gp110 colocalized to nuclear membranes and the ER with BiP and not to the plasma membrane. Panels E and F are photomicrographs of a third field. Superimposition of gp350/220 (E) on BiP (F) revealed that gp350/220 localized to the plasma membrane, peripheral to BiP localization.

antibodies. The two fluorochromes could be distinguished by epifluorescence with appropriate specific barrier filters.

The ER resident protein marker, BiP (16), localized diffusely throughout the cytoplasm and in the nuclear membrane but not in the plasma membrane (Fig. 3B, D, and F). Figure 3A and B shows photomicrographs of the same field depicting the localization of gp110 (3A) and BiP (3B) identified by two-color immunofluorescence microscopy. Superimposition of Fig. 3A and B revealed that gp110 colocalized with BiP, indicating that gp110 is also an ER-resident protein. Similarly, Fig. 3C and D is another pair of photomicrographs showing gp110 and BiP colocalization. While BiP stained every cell, gp110 was detected in approximately 40% of the cells, reflecting the percentage of cells replicating EBV. Figure 3E and F shows photomicrographs of a field

depicting the localization of gp350/220 (3E) and BiP (3F) identified by two-color immunofluorescence microscopy. In contrast to the previous results with gp110 and BiP, superimposition of Fig. 3E and F revealed that gp350/220 did not colocalize with BiP and, in fact, localized almost exclusively to the plasma membrane of most cells, while BiP localized to the ER.

In other sections, as expected on the basis of the differential localization of gp350/220 from gp110 and BiP, gp350/220 mouse monoclonal or rabbit polyclonal antibodies heavily stained the plasma membrane of cells permissive for virus replication (40% of the cells). Some cells with plasma membrane staining also had a single localized area of cytoplasmic staining similar to what has been previously observed with Golgi markers (Fig. 4B). Little, if any, gp350/220 was associated with nuclear membranes. Superimposition of photomicrographs of the same section stained with gp350/220-specific (Fig. 4B) or gp110-specific (Fig. 4A) antibodies revealed that gp350/220 localized to the plasma membrane around the periphery of gp110 localization and that gp110 localized prominently to the nuclear membrane. No gp110-specific plasma membrane fluorescence was detected. Similar results were evident on superimposition of photomicrographs of another section stained with gp350/220 (Fig. 4D) and gp110 (Fig. 4C). Thus, the distribution of gp110 was similar to an ER distribution and distinct from that of gp350/220. Staining of gp110 and gp350/220 was not observed in either Louckes, an EBV-negative cell line, or IB4, an EBV-infected cell line nonpermissive for virus replication.

For immunoelectron microscopy, thinner (0.06- $\mu$ m) sections were similarly processed, except that primary antibody binding was detected by species-specific large- or small-particle colloidal gold-conjugated secondary antibodies. While cryoembedding in sucrose, as opposed to polymers, assures equal access of antibody to all parts of a section, preservation of a conventional membrane electron microscopic image is difficult. Immunoelectron microscopy of sucrose-infused cryosections confirmed the UV light microscopic observations described above and the previous immunoelectron microscopic localization of gp110 (7). Double-labeling immunoelectron microscopy of cryosections confirmed that gp110 and BiP colocalized to nuclear and cytoplasmic membranes and not to the plasma membrane (Fig. 5). Moreover, neither gp110- nor BiP-specific antibodies localized to intracellular or extracellular enveloped virus. Occasionally a low level of gp110-specific immunogold labeling was evident near virions between the inner and outer nuclear membranes. As was previously demonstrated (7), gp110 did localize to the nuclear membrane and, to a lesser extent, to cytoplasmic membranes, which frequently contained virus, but not to the plasma membrane (Fig. 6 and 7A).

In similar sections of cells with viral capsids assembling in the nucleus and undergoing envelopment at the nuclear membrane, gp350/220 localized almost exclusively to the plasma membrane and to extracellular virus (Fig. 7B and 8). Similar high-density immunostaining was evident in the plasma membrane and the viral envelope. In contrast, hardly any gp350/220-specific immunostaining was evident at the nuclear membrane or at intracellular enveloped virus. Identical results were obtained with monoclonal antibodies 72A1 and 2L10, which recognize different epitopes on the outer surface of gp350/220, including endo H-sensitive forms of gp350/220. Double-labeling immunoelectron microscopy of cryosections revealed that gp350/220 labeled the plasma membrane (Fig. 7C) and extracellular virus (Fig. 8) but not

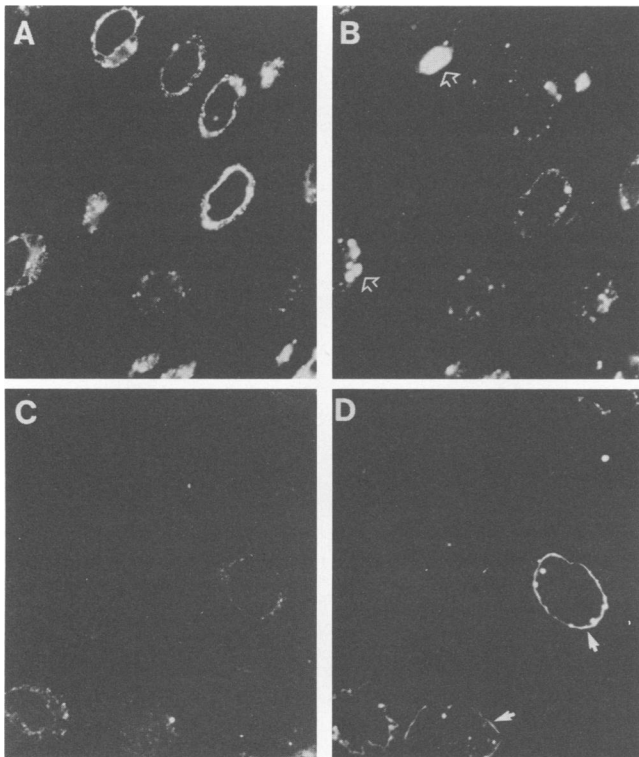


FIG. 4. Distinctness of the intracellular distribution of gp110 from that of gp350/220 in identical cryosections of EBV-infected lymphocytes. Sections 0.35  $\mu\text{m}$  thick were prepared as described in the legend to Fig. 3. Sections were coincubated with affinity-purified TO16-specific (gp110-specific) rabbit antibodies (1:5 dilution) and gp350/220 murine monoclonal antibodies (diluted either 1:100 [2L10 ascites] or 1:1 [72A1 tissue culture supernatant]). Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G and Texas Red-conjugated goat anti-mouse immunoglobulin G antibodies were coincubated for visualization. Panels: A and C, gp110 localization; B, gp350/220 (stained with 2L10) colocalization in the same field with gp110 in panel A; D, gp350/220 (stained with 72A1) colocalization in the same field with gp110 in panel C. gp350/220 localized primarily to the plasma membrane (solid arrows), with some cytoplasmic localization similar to that of characterized Golgi proteins (open arrows), but not to the nuclear membrane. In identical cells, gp110 localized to nuclear membranes and cytoplasmically but did not colocalize with gp350/220 cytoplasmically and was not detected in the plasma membrane. No staining was detected with any of the three antibodies when they were incubated with cryosections of IB4 (a latently infected EBV lymphoblastoid cell line) or cryosections of Louckes (an EBV-negative lymphoblastoid cell line). Also, preimmune rabbit and nonimmune mouse sera displayed no fluorescence.

the nuclear membrane, whereas in the same cells, gp110 localized to the nuclear membrane and cytoplasmic vesicles. Again, gp350/220 was not detected on the intracellular enveloped virus, either near the plasma membrane or between the inner and outer nuclear membranes (Fig. 8). Since the monoclonal antibodies detect all forms of gp350/220, the absence of immunoreactive gp350/220 from intracellular EBV and the similar high density on the plasma membrane and extracellular virus suggests that gp350/220 is incorporated into the viral envelope during movement of the virus through the plasma membrane. Alternatively, gp350/220 could be added to enveloped virus at a putative ephemeral (or rapidly transitory) late stage of virus passage through the cytoplasm. Although the possibility that the epitopes recog-

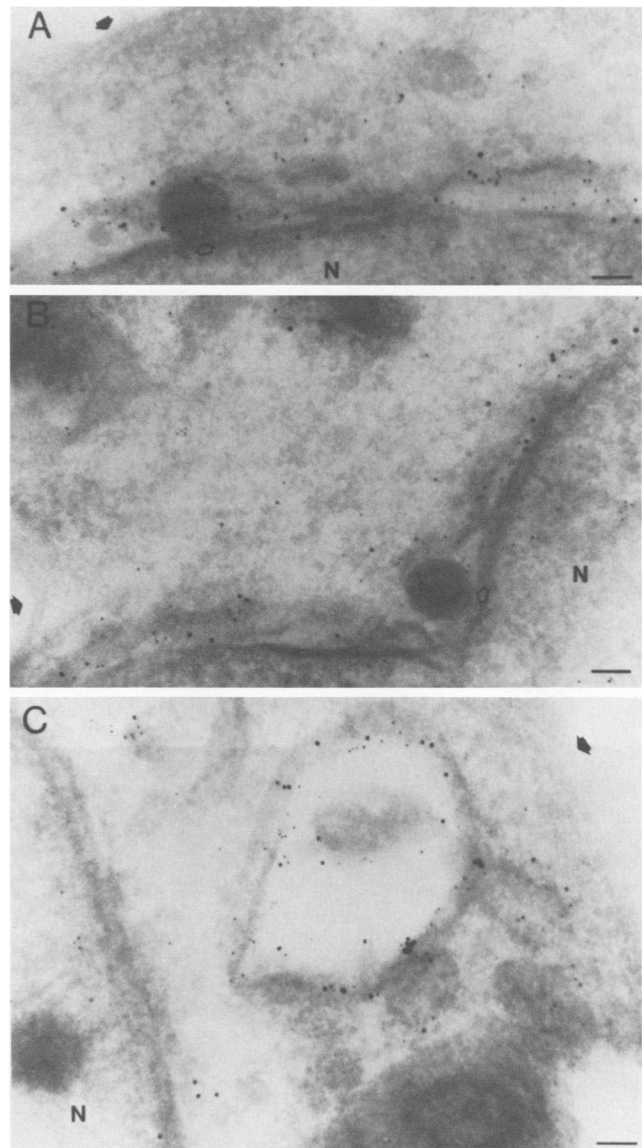


FIG. 5. Ultrastructural colocalization of BiP and gp110 in cryosections. Induced B95-8 cells were prepared as described in the legend to Fig. 3. (A to C) Cryosections 60 nm thick were coincubated with affinity-purified TO16-specific (gp110-specific) rabbit antibodies diluted 1:5 and a rat monoclonal antibody specific for BiP diluted 1:1. Colloidal gold (10-nm particle diameter)-conjugated goat anti-rabbit immunoglobulin G and colloidal gold (5-nm particle diameter)-conjugated goat anti-rat immunoglobulin G antibodies were coincubated for visualization. gp110 (large gold particles) colocalized with BiP (smaller gold particles) in the nuclear membrane (A and B) and cytoplasmically and also in cytoplasmic vesicles (C), but neither localized to the plasma membrane. The plasma membrane (filled arrow), enveloped virus between the inner and outer nuclear membranes (open arrow), and nucleus (N) are indicated. Bars, 100 nm.

nized by the gp350/220- or gp110-specific monoclonal antibodies are occluded or absent at specific intracellular locations cannot be completely dismissed, cryoultramicrotomy of sucrose-embedded cells should result in similar antibody access to the various cellular compartments. Furthermore, similar results were obtained with polyvalent rabbit serum in light microscopic staining of slightly thicker sections.



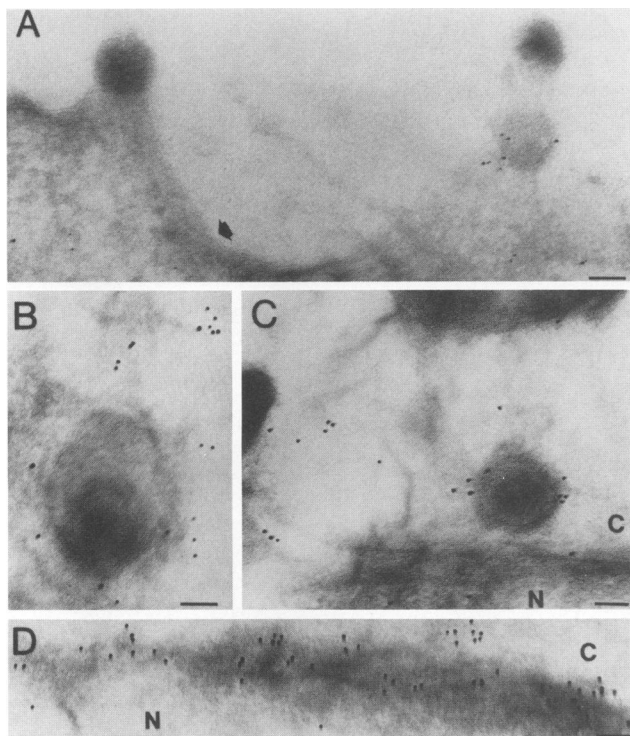


FIG. 6. Ultrastructural localization of gp110 in cryosections. Induced B95-8 cells were processed as described in the legend to Fig. 3. Cryosections 60 nm thick were incubated with affinity-purified TO16-specific (gp110-specific) rabbit antibodies diluted 1:5, followed by 5-nm-diameter (A) or 10-nm-diameter (B and C) colloidal gold particle-conjugated goat anti-rabbit immunoglobulin G. gp110 localized to nuclear membranes (D) and cytoplasmic vesicles (B and C) but not to plasma membranes (A). (A) Two virions (upper left and upper right) in the process of budding through the plasma membrane. The unusual finding of a gp110-positive peripheral cytoplasmic vesicle, containing virus, is also shown. (C) Vesicle (250-nm diameter) containing intracellular enveloped EBV (180-nm diameter). gp110 localized to the vesicle but not to the virus. The plasma membrane (filled arrow), nucleus (N), and cytoplasm (C) are indicated. Bars, 100 nm.

**Electron microscopy of viral egress.** Since the gp350/220 density in the plasma membrane is similar to that in enveloped virus and there is little, if any, gp350/220 in the nuclear membrane, gp350/220 is likely to be added to enveloped virus during cytoplasmic egress or at the plasma membrane. A morphologic study of EBV egress was undertaken to search for evidence of virus envelopment at the plasma membrane. Conventional fixation-embedding electron microscopic analysis was undertaken to facilitate the identification of intracellular structures. To identify cells permissive for virus replication, cells were surface labeled with 72A1, a gp350/220-specific monoclonal antibody, followed by 5-nm-diameter colloidal gold particle-conjugated goat anti-mouse immunoglobulin G. The stained cells were then fixed, embedded in Epon, and processed for electron microscopy. gp350/220 was readily detected on the plasma membrane of cells replicating EBV, as well as on the surface of extracellular virus in close proximity to the cell membrane (Fig. 9). As noted in the above-described studies of cells which were immunolabeled after fixation, gp350/220 was as abundant on the cell plasma membrane as on the extracellular virus. gp350/220 labeling was associated with spikes up to 50 nm

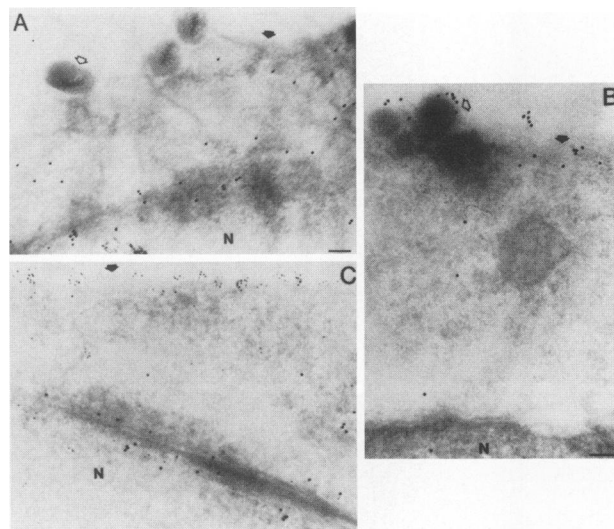


FIG. 7. Ultrastructural colocalization of gp110 and gp350/220 in cryosections. Induced B95-8 cells were prepared as described in the legend to Fig. 3. Sections 60 nm thick were incubated with affinity-purified TO16-specific (gp110-specific) rabbit antibodies diluted 1:5, followed by 10-nm-diameter colloidal gold particle-conjugated goat anti-rabbit immunoglobulin G (A), a gp350/220-specific mouse monoclonal antibody 72A1 supernatant diluted 1:1, and 10-nm-diameter colloidal gold particle-conjugated goat anti-mouse immunoglobulin G (B). gp110 localized to nuclear membranes but not to plasma membranes or extracellular virus (A). gp350/220 localized primarily to plasma membranes and extracellular virus with similar density but not to the nuclear membrane (B). Double labeling of a similar section of cells using gp110-specific rabbit antibodies, followed by 10-nm-diameter colloidal gold particle-conjugated goat anti-rabbit immunoglobulin G and 72A1, followed by 5-nm-diameter colloidal gold particle-conjugated goat anti-mouse immunoglobulin G (C). gp110 localized to the nuclear membrane (large gold particles), as in panel A, with gp350/220 localizing to the plasma membrane (smaller gold particles) in the same cell (C). The plasma membrane (filled arrow), virus (open arrow), and nucleus (N) are indicated. Bars, 100 nm.

long extending from the plasma membrane or virus envelopes. Intracellular virus was examined only in cells with a morphologically intact nuclear membrane so as to avoid studying late secondary cytocidal effects on viral egress. Very little cytoplasmic virus was evident in such cells. Enveloped virus was detected in large amounts on the cell surface and to a much lesser extent in cytoplasmic vesicles (Fig. 10C and D). Naked nucleocapsids were abundant in the nucleus but were also occasionally detected in the cytoplasm (Fig. 10A, B, and E). Some nucleocapsids were near or appeared to bud from the plasma membrane (Fig. 11). The finding of naked nucleocapsids in the cytoplasm in close juxtaposition to the plasma membrane is consistent with the possibility that virus could undergo a process of de-envelopment in the cytoplasm and re-envelopment at the plasma membrane.

## DISCUSSION

The data reported here and previously published studies of EBV envelopment and egress need to be considered in the context of glycoprotein trafficking and herpesvirus morphogenesis. Two major late EBV glycoproteins, gp110 and gp350/220, differ substantially in post-ER processing, intracellular trafficking, and virion association. Both gp110 and

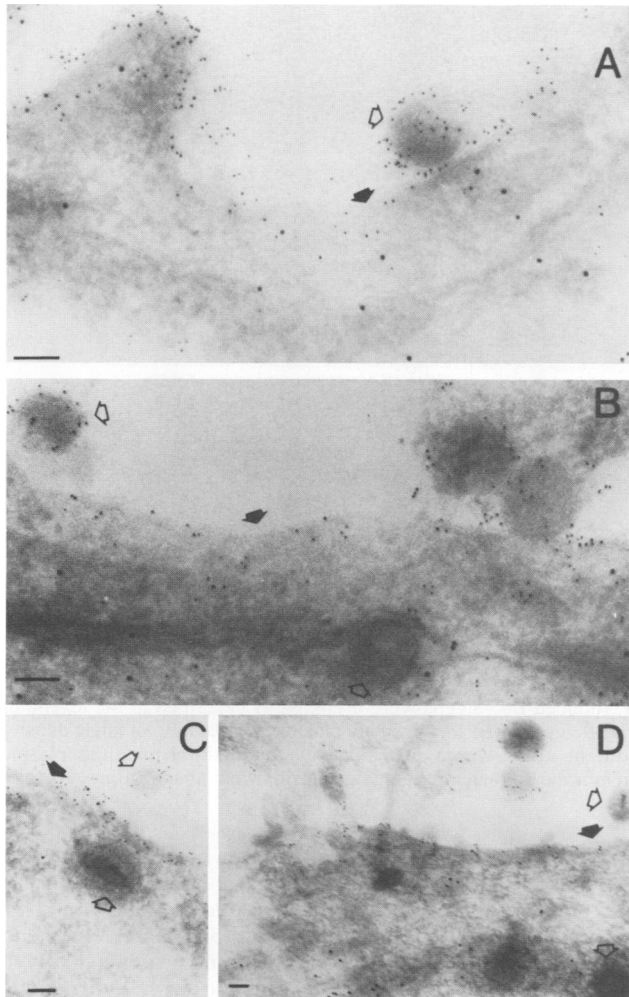


FIG. 8. Ultrastructural localization of gp350/220 in cryosections double labeled with gp110. Induced B95-8 cells were prepared as described in the legend to Fig. 3. Cryosections 60 nm thick were incubated with gp350/220-specific mouse monoclonal antibodies 72A1 diluted 1:1 (A and B) and 2L10 diluted 1:100 (C and D) and affinity purified TO16-specific (gp110-specific) rabbit antibodies (A to D). Colloidal gold particle (5-nm diameter)-conjugated goat anti-mouse immunoglobulin G and colloidal gold particle (10-nm diameter)-conjugated goat anti-rabbit immunoglobulin G antibodies were coincubated for visualization. gp350/220 (smaller gold particles) localized to plasma membranes and extracellular virus displaying similar densities of staining (A to D) but not to the nuclear membrane (B and D). gp110 (large gold particles) localized primarily to nuclear membranes and the cytoplasm but not the plasma membrane. gp350/220 or gp110 did not localize to intracellular enveloped virus either between the inner and outer nuclear membranes (B and D, lower open arrows) or near the plasma membrane (C, lower open arrow). The plasma membrane (filled arrow) and virus (open arrow) are indicated. Bars, 100 nm.

gp350/220 undergo rapid posttranslational N-linked glycosylation. gp110 largely remains confined to the ER and, as expected for an ER-resident protein, it is in the nuclear membrane. The predominant immunoreactive form of gp110 is 110 kDa, as demonstrated by immunoblots and long-term labeling and immunoprecipitation experiments with polyvalent rabbit serum immune to procaryotic synthesized gp110. The abundant stable 110-kDa gp110 form has only endo H-sensitive high-mannose N-linked oligosaccharides. This

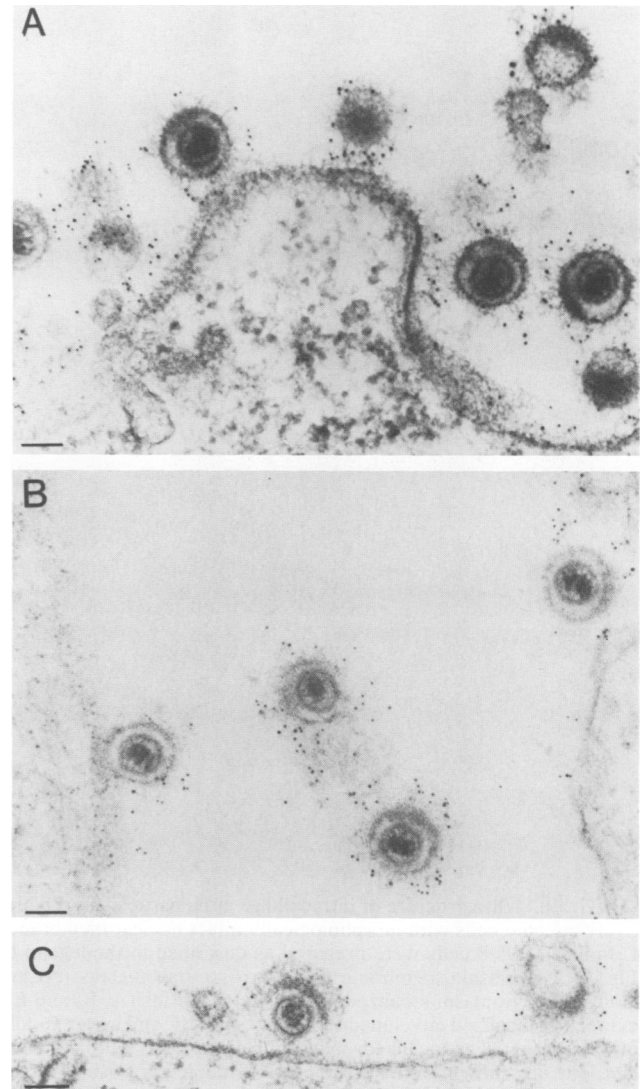


FIG. 9. Ultrastructure of mature gp350/220 in live, surface-labeled, productively EBV-infected lymphocytes. Induced B95-8 cells were incubated with gp350/220-specific mouse monoclonal antibody 72A1 diluted 1:1, followed by 5-nm-diameter colloidal gold particle-conjugated goat anti-mouse immunoglobulin G antibodies. Stained cells were fixed in Karnovsky fixative (14), embedded in Epon, and processed for electron microscopy. gp350/220 localized to both the plasma membrane and extracellular virus. Bars, 100 nm.

indicates that the abundant stable gp110 product has not trafficked through the Golgi. Thus, gp110 must lack a necessary signal for Golgi transport or possess a signal for ER retention or nuclear membrane localization.

Three ER luminal proteins, BiP, disulfide isomerase, and grp94, contain a tetrapeptide ER-targeting signal sequence, lysine-aspartic acid-glutamic acid-leucine (KDEL) at the carboxy terminus. There is no KDEL sequence predicted for gp110 (or gp350/220). Alterations in the KDEL sequence or in its position changes known ER-resident proteins to secretory proteins (17). Thus, Golgi processing or secretion is presumed to be a default designation (19) and gp110, which lacks the KDEL sequence, should traffic to the Golgi. In fact, a small amount of Golgi processing may be indicated by the immunoprecipitation of the largely endo H-sensitive

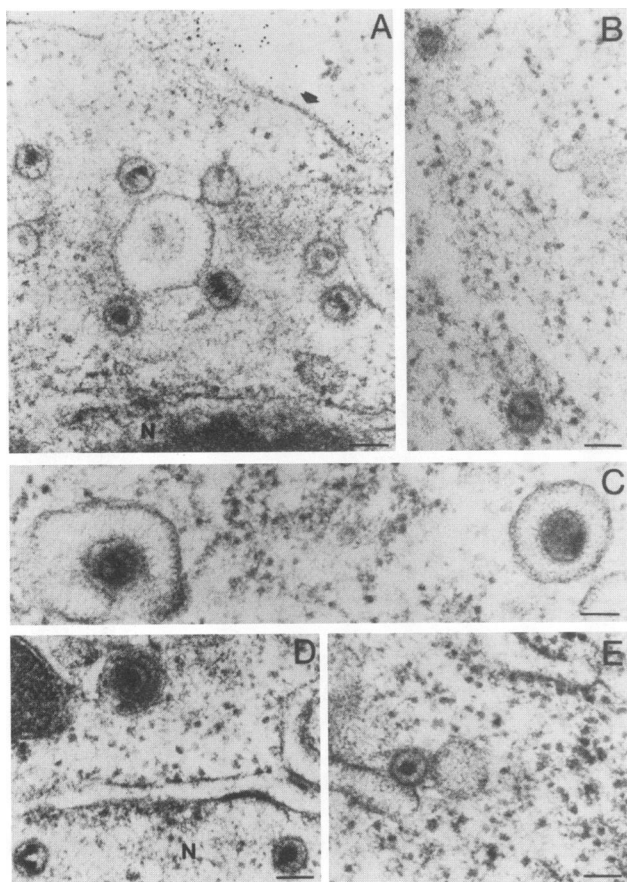


FIG. 10. Ultrastructure of intracellular virus particles in productively infected cells with morphologically intact nuclear membranes I. Induced B95-8 cells were prepared as described in the legend to Fig. 9. The plasma membrane (filled arrow) and nucleus (N) are indicated. Cytoplasm-localized naked nucleocapsids (A, B, and E), cytoplasm-localized enveloped virus in vesicles (C and upper middle of D), and nucleus-localized naked nucleocapsids (D, lower right and left) are shown. Panel A (upper left) depicts a portion of the plasma membrane with gp350/220 localization. Bars, 100 nm.

120-kDa gp110-cross-reactive protein seen as a minor species in some immunoprecipitations with anti-gp110 sera. However, since the bulk of gp110 is ER and nuclear membrane targeted, gp110 may have a different ER localization signal or gp110 may interact with a designated ER-resident protein and this interaction may prevent gp110 from proceeding to the Golgi. In preliminary experiments, immunoprecipitation of BiP from EBV-infected cells resulted in more effective coimmunoprecipitation of gp110 than gp350/220. However, conditions for reproducible selective coimmunoprecipitations of gp110 without gp350/220 were not established.

The inner and outer nuclear membranes are extensions of the ER. gp110 is the only glycoprotein which has been demonstrated to inhabit the inner nuclear membrane. Interestingly, the predicted carboxy-terminal gp110 domain, which should reside on the cytoplasmic or nucleoplasmic side of the membrane, includes an Arg-Lys-Arg-Arg sequence. Similar basic oligopeptides characterize nuclear protein localization signals (13) and could anchor the carboxy-terminal gp110 domain in the nucleus. In preliminary experiments using gp110-specific antisera on immunoblots of nonionic detergent extracts or residues of cells induced to

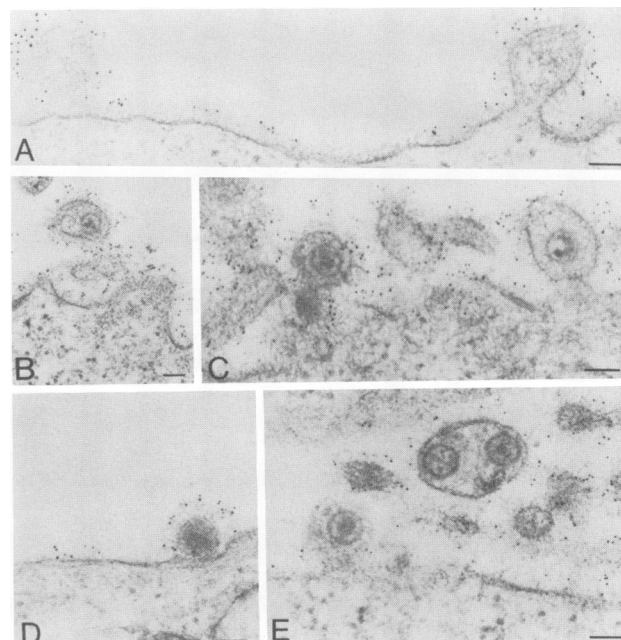


FIG. 11. Ultrastructure of intracellular virus particles in productively infected cells with morphologically intact nuclear membranes II. Induced B95-8 cells were prepared as described in the legend to Fig. 9. gp350/220 localized to plasma membranes at high density both in areas where nucleocapsids budded through the plasma membrane and where the nucleocapsids were absent. Bars, 100 nm.

replicate EBV, gp110 was detected in both the detergent-extractable and -insoluble fractions. Detergent-insoluble gp110 was persistently insoluble (data not shown). This suggests that gp110 associates with cytoskeletal elements in either the nucleus or the cytoplasm. Some proteins involved in viral egress may need to associate with cytoskeletal elements to permit specific transport mechanisms. The basic carboxy terminus of gp110, or the cytoskeletal association of gp110 could be a factor in gp110 ER retention versus Golgi transport.

EBV, like other herpesviruses (20), acquires an envelope when it buds through the inner nuclear membrane. Enveloped virus between the inner and outer nuclear membranes has spikes on its outer surface which are likely to be glycoproteins, since they are morphologically similar to the glycoprotein spikes on mature extracellular virus. It is therefore surprising that gp110, which is in the inner nuclear membrane, is not detected on virus. Our results indicate that failure to detect gp110 on virus is not an artifact of poor penetration of antibody into the nuclear membrane lumen. We cannot, however, exclude the possibility that our polyclonal or monoclonal antibodies detected a limited number of epitopes not present on a part of gp110 which may be incorporated into the envelope or occluded on the gp110 incorporated into the envelope. If full-length gp110 is incorporated into the virus envelope, it is unlikely to be part of the mature virus, since the antibodies work well on immunoblots and immunoprecipitations but do not detect gp110 in extracellular-virus preparations. Thus, the role of gp110 in EBV morphogenesis and egress remains uncertain, and as has previously been hypothesized, gp110 may have a primary role in membrane modification for viral egress.

gp350/220 is similarly synthesized in the ER but undergoes rapid transport to the Golgi, complex N- and O-linked



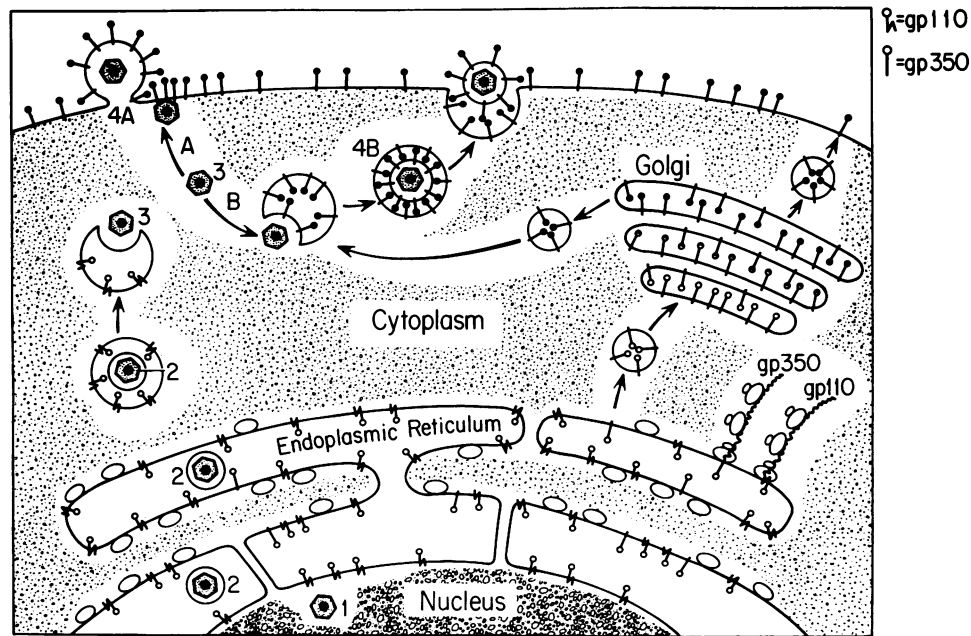


FIG. 12. Possible paths of EBV envelopment and egress. gp350/220 and gp110 are two EBV glycoproteins with different intracellular localizations and oligosaccharide processing. gp110 and gp350/220 are synthesized in the ER, where they undergo N-linked glycosylation. Most of gp110 remains localized to the ER compartment, including the inner and outer nuclear membranes. A small amount of gp110 may undergo Golgi transit and N- and O-linked oligosaccharide modification. Despite its presence in the nuclear membrane, gp110 is not present in the viral envelope. gp350/220 undergoes rapid transit to the Golgi and O- and complex N-linked glycosylation. Open circles indicate high-mannose-type oligosaccharides; half-filled circles indicate endo H-resistant, complex oligosaccharides; and closed circles indicate fully mature oligosaccharides. The proposed steps in virion morphogenesis are as follows. 1, Nucleocapsids (shaded hexagons) are assembled within the nucleus, acquire an initial envelope, and leave the nucleus by budding through the gp110-modified inner lamella of the nuclear membrane without acquiring detectable gp110 or gp350/220. 2, Enveloped virus is transported through the perinuclear, gp110-modified ER, which is continuous with the space between the inner and outer nuclear membranes. 3, vesicles release nucleocapsids into the cytoplasm. 4, The nucleocapsid acquires a definitive envelope either by budding through the plasma membrane containing Golgi-processed viral glycoproteins (4A) or by budding into post-Golgi derived vesicles containing gp350/220 and other viral glycoproteins, followed by exocytosis of the enveloped virion (4B).

oligosaccharide addition, and transit to the plasma membrane (3, 21, 22). Immunoelectron microscopy of cryosections confirmed that most of the gp350/220 in EBV-infected cells localizes to the plasma membrane and to the surface of extracellular virus. Although the gp350/220-specific monoclonal antibodies, 2L10 and 72A1, used in this study recognized ER- or Golgi-processed gp350/220 (Fig. 2), these monoclonal antibodies detected little gp350/220 in the nuclear envelope or in intracellular enveloped virions within or near the nuclear membrane. A persistent endo H-sensitive gp350/220 fraction was not detected in long-term pulse-chase studies, as would be expected if a gp350/220 fraction remained in the ER or nuclear membrane. Thus, gp350/220 appears to be efficiently transported out of the ER to the Golgi and may be added to the EBV envelope during EBV egress through the cytoplasm or at the plasma membrane. Similar observations have been made regarding varicella-zoster virus gpI, the distant homolog of gp350/220 (12). Since gpI is a major virion glycoprotein, a model for cytoplasmic envelopment for varicella-zoster virus has been proposed.

Several possibilities for EBV morphogenesis can be considered (Fig. 12). Enveloped EBV is found in cytoplasmic membranous structures which could be continuous with or fuse with vesicles of the cell secretory pathway, and gp350/220 could be added during this transit. This would require fusion of the virus envelope with the secretory vesicle membrane for gp350/220 to be transferred from the vesicle membrane to the virus envelope. Such fusion has not been

evident in electron micrographs nor is there significant gp350/220 labeling of intracytoplasmic virus. Alternatively, enveloped EBV may undergo fusion with the vesicle membrane, releasing nucleocapsids into the cytoplasm so that they can be re-enveloped at the plasma membrane. Several lines of evidence support this latter hypothesis. (i) gp350/220 is the major glycoprotein on the virus and was easily detected in immunoelectron microscopy and in immunoprecipitations or immunoblots of purified virus. In immunofluorescence or immunoelectron microscopy of cryosections, although gp110 was evident in the nuclear envelope and endoplasmic reticulum, gp350/220 localization was clearly distinct from that of gp110 and the pattern detected was consistent with plasma membrane and Golgi localization. Moreover, most of the cells stained for gp350/220 using either monoclonal mouse or polyclonal rabbit antibodies displayed only plasma membrane localization. In fact, gp350/220 could not be detected on enveloped virions observed between the inner and outer nuclear membranes. (ii) In surface immunolabeling of live infected cells, gp350/220 was readily detected on the plasma membrane and on the envelope of extracellular virus in equally high abundances. (iii) Naked nucleocapsids were observed in the cytoplasm, some of which appeared to bud through the plasma membrane. Similar observations have been recorded since the discovery of EBV (4, 5). Since this phenomenon was not frequently observed, we have to hypothesize that de-envelopment and re-envelopment occur very rapidly *in vivo*.

These cytoplasmic nucleocapsids are not likely to be a result of nuclear leakage, since we confined our analysis to cells which appeared to have intact nuclear membranes. Further, these nucleocapsids near the plasma membrane are not likely to result from re-entry of the virus into the cell, since the B95-8 virus-producing cell line does not express the cell receptor for EBV, CR2 (J. Tanner, unpublished data). Thus, EBV could only re-adsorb to the B95-8 cell membrane if another EBV receptor existed.

Recently, Torrisi et al. used freeze-fracture immunolabeling techniques to study nuclear membrane localization of gp350/220 (24). Monoclonal antibody 2L10 reacted with gp350/220 on the plasma membrane and extracellular virus but not with nuclear membranes, while MA17 reacted with both plasma and nuclear membranes. They concluded that gp350/220 was detected in the nuclear membrane with MA17 because MA17 recognized nuclear membrane (high-mannose) gp350/220, while 2L10 only recognized gp350/220 in the plasma membrane because it recognizes gp350/220 only after complex glycosylation (in the Golgi). However, 2L10 recognized high-mannose gp350/220, as well as complex N- and O-linked glycosylated gp350/220 (Fig. 2). Thus, 2L10 should have detected nuclear membrane, as well as plasma membrane, forms of gp350/220. Additionally, immunofluorescence or immunoelectron microscopy studies using MA17 were inconclusive because a high degree of nonspecific binding resulted (data not shown). gp350/220, like most glycoproteins, should transiently localize, in part, to the outer nuclear membrane. Our studies suggest that gp350/220 is rapidly transported from ER compartments (including the nuclear envelope) to the Golgi, whereas gp110 is detained.

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