Membrane Anchoring Domain of Herpes Simplex Virus Glycoprotein gB Is Sufficient for Nuclear Envelope Localization

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We have used the glycoprotein gB of herpes simplex virus type 1 (gB-1), which buds from the inner nuclear membrane, as a model protein to study localization of membrane proteins in the nuclear envelope. To determine whether specific domains of gB-1 glycoprotein are involved in localization in the nuclear envelope, we have used deletion mutants of gB-1 protein as well as chimeric proteins constructed by replacing the domains of the cell surface glycoprotein G of vesicular stomatitis virus with the corresponding domains of gB. Mutant and chimeric proteins expressed in COS cells were localized by immunoelectron microscopy. A chimeric protein (gB-G) containing the ectodomain of gB and the transmembrane and cytoplasmic domains of G did not localize in the nuclear envelope. When the ectodomain of G was fused to the transmembrane and cytoplasmic domains of gB, however, the resulting chimeric protein (G-gB) was localized in the nuclear envelope. Substitution of the transmembrane domain of G with the 69 hydrophobic amino acids containing the membrane anchoring domain of gB allowed the hybrid protein (G-tmgB) to be localized in the nuclear envelope, suggesting that residues 721 to 795 of gB can promote retention of proteins in the nuclear envelope. Deletion mutations in the hydrophobic region further showed that a transmembrane segment of 21 hydrophobic amino acids, residues 774 to 795 of gB, was sufficient for localization in the nuclear envelope. Since wild-type gB and the mutant and chimeric proteins that were localized in the nuclear envelope were also retained in the endoplasmic reticulum, the membrane spanning segment of gB could also influence retention in the endoplasmic reticulum.

The mechanism of sorting and transport of proteins to specific subcellular organelle membranes in eukaryotes has been of major recent interest. Glycoproteins of enveloped animal viruses have been extensively used as models to study the sorting of integral membrane proteins to cell surface, endoplasmic reticulum (ER), and Golgi complex membranes (16, 17, 19, 45, 51). In eukaryotic cells the nuclear envelope (NE) physically separates the nucleus from the cytoplasm and has a major role in nuclear import and export of macromolecules as well as in the organization of the nuclear architecture (5, 14, 15, 18). The nuclear envelope has a complex organization consisting of an outer and an inner nuclear membrane, nuclear lamina, and the nuclear pore complex (5, 14, 15, 18). Although excellent progress has been made in understanding the import of proteins into the nucleus (5, 15), information on the biogenesis of the NE, and in particular the mechanism of targeting of integral membrane proteins to the inner nuclear membrane, remains to be clarified. Recently the NE localization determinants of two integral membrane proteins, the nuclear pore complex protein gp210 (63) and the lamin B receptor (LBR) (53, 55), have been studied by using indirect immunofluorescence of transfected cells. In the case of gp210, the transmembrane and cytoplasmic domains could target a cell surface protein to the NE (63). However, in the case of the LBR, different results were reported; thus, in one case the first of the eight membrane anchoring segments of the LBR was

sufficient for localization to the NE (53), while the other study showed the requirement of the amino-terminal nucleoplasmic tail of the LBR for NE localization (55).

We have used the envelope glycoprotein of herpes simplex virus type 1 (HSV-1) as a model to study the targeting of integral membrane proteins to the nuclear membrane (2, 20, 46, 47). Since HSV acquires its envelope by budding through the inner membrane of the nuclear envelope (12, 21, 37, 39, 56, 59, 60), it may be assumed that the HSV envelope glycoproteins are targeted to and localized in the NE. The glycoprotein gB-1 of HSV-1 is essential for viral infectivity (33) and is known to be involved in virus entry and cell fusion (7, 9, 56). From the deduced amino acid sequence of gB-1, the presence of a large cytoplasmic domain of 109 amino acids and a hydrophobic stretch of 69 amino acids which could serve as the membrane anchor domain can be predicted (8, 44). We have previously expressed the gB-1 gene in COS-1 cells with cloned DNA. The expressed glycoprotein was localized both in the NE and in the plasma membrane (2). Recent studies using immunoelectron microscopy of HSV-1-infected cells confirmed that gB-1 was indeed localized in the inner membrane of the nucleus (21, 60). Earlier, using indirect immunofluorescence and biochemical cell fractionation, we showed that the deletion of the cytoplasmic domain of gB-1 did not affect its NE localization (47). Recently, using mutants with deletions in the predicted membrane anchoring sequence of 69 hydrophobic amino acids, we have shown that the segment containing amino acids 774 to 795 of gB-1 specifies the membrane anchoring domain and that mutants containing this segment of hydrophobic amino acids were localized in the NE (46).

In this study we have, as an extension of our previous work (46, 47), attempted to define the NE localization determinant(s) of gB-1 by immunoelectron microscopic localization of

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mutant gB-1 proteins containing shortened cytoplasmic tails or deletions in the predicted hydrophobic membrane anchoring domain as well as chimeric proteins constructed by swapping the domains of the cell surface protein G of vesicular stomatitis virus (VSV) with corresponding domains of gB-1 proteins. The results obtained with deletion mutants as well as with the chimeras show that the 69-amino-acid hydrophobic segment of gB-1 specifies NE localization and that the transmembrane segment encoding residues 774 to 795 of gB-1 was sufficient for localization of proteins in the NE. The results also indicated that proteins that were localized in the NE were also retained in the ER, suggesting that the membrane anchoring domain of gB-1 may also influence retention in the ER.

MATERIALS AND METHODS

Materials. Restriction and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Pharmacia Canada, or New England Biolabs. Endoglycosidase H (endo H) was from ICN-Immunobiologicals. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) was from Dako Corporation. *Escherichia coli* BW 313 (*dut ung*) cells (31) were obtained from N. Skipper, Allelix Laboratories, Mississauga, Ontario, Canada. Mutagenic oligonucleotides were synthesized on an Applied Biosystems model 381-A DNA synthesizer with β -cyanoethyl phosphoramidites or purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. COS-1 cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum. Recombinant adenoviruses expressing either glycoprotein B of HSV-1 (AdgB-1) (36) or glycoprotein G of VSV (AdG12) (52) were obtained from D. Johnson and L. Prevec, respectively.

Antibody preparation. Rabbits were injected subcutaneously three times at weekly intervals with 0.5 ml of 10^8 PFU of AdgB-1 or AdG12 diluted in phosphate-buffered saline (PBS) containing 2% rabbit serum. One week after the last injection, the rabbits were killed and bled, and the IgG fraction of the serum was isolated by ammonium sulfate precipitation as described previously (24).

Anti-gB antibodies were affinity purified by using a column made with a mixture of glycoproteins of HSV-1-infected cells. The glycoproteins were isolated from BHK cells infected with HSV-1 by using a lentil lectin-Sepharose 4B column as described by Pachl et al. (42). The glycoproteins were coupled to CNBr-activated Sepharose 4B (Sigma), and the beads were used for affinity purification of anti-gB antibody as described previously (24). Anti-G antibodies were affinity purified by using a column of G protein coupled to Sepharose 4B beads. G protein was isolated from gradient-purified VSV Indiana serotype as described previously (30).

Plasmid construction and mutagenesis. The 3.4-kb *Bgl*II-*Eco*RI fragment of p9gB (2) and the 1.7-kb fragment of pSVGL (48) were cloned into the vector pXM (64) for expression of wild-type gB and G proteins, respectively. Oligonucleotide-directed mutagenesis by the method of Kunkel et al. (31) was used to construct gB mutants gBCT799, gB Δ 12, and gB Δ 23 as described previously (46, 47). Chimera gB-G was constructed by joining a *Bgl*II-*Tth*111I fragment of gB with the *Taq*I-*Eco*RI fragment of G containing the transmembrane and cytoplasmic domains. To construct the G-gB hybrid, an *Xho*I site was introduced at nucleotide position 1416 of G (32), and a *Tth*111I-*Eco*RI fragment containing the transmembrane and cytoplasmic domains of gB was cloned in the *Xho*I site of G. The chimera G-tmgB was constructed by fusing a *Bgl*II-*Mlu*I fragment of G-gB into the *Bgl*II-*Xho*I site of pXMG and then

deleting the ectodomain of gB and the transmembrane domain of G by using the primer 5'-GCCTTCTTCGCCTTTCGAG TTGGTATCCATCTT-3'. The two hybrids G-tmgB Δ 1 and G-tmgB Δ 12 were constructed from G-tmgB by using primers used for gB Δ 1 and gB Δ 12 mutants, respectively (46).

Transfection, labeling, and immunoprecipitation. Subconfluent monolayers of COS-1 cells were transfected by the $\text{Ca}_3(\text{PO}_4)_2$ precipitation protocol (22, 46). The transfected cells were labeled with [^{35}S]methionine at 24 to 40 h posttransfection and processed for immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described earlier (46).

Intracellular transport. Transfected COS-1 cells were fixed with 2% paraformaldehyde for surface immunofluorescence. For internal immunofluorescence cells were fixed with 2% paraformaldehyde, treated with 1% Triton X-100, and reacted with anti-gB or anti-G antibody and stained with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as described previously (46). Transport of the mutant gB proteins to the Golgi complex was determined by the acquisition of endo H resistance (29) as described previously (46).

Immunoelectron microscopy. COS-1 cells at 24 to 40 h posttransfection were fixed for 2 h at room temperature with 2% glutaraldehyde in 0.03 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 7.4). The cells were then washed with 0.03 M PIPES (pH 7.4), scraped with a razor blade, centrifuged, and embedded in 2% Noble agar. The specimens were cut into small cubes (1 mm³), dehydrated with ethanol, and embedded in Lowicryl K4M (Polysciences) at -20°C (21, 50). Ultrathin sections on nickel grids coated with a Formvar film were labeled by incubation with the affinity-purified antibodies at room temperature. Before the antibody incubation, the grids were washed with PBS and preincubated with PBS containing 1% bovine serum albumin and 5% goat serum (buffer A). The grids were incubated with anti-gB or anti-G diluted in buffer A for 1 h and then with a goat anti-rabbit antibody coupled to 1-nm gold (Amersham International) diluted in buffer A for 30 min. The grids were washed with PBS and distilled water and dried. The 1-nm gold was visualized by the silver enhancement method of Danscherg and Nørsgaard (11). The specimens were observed with a JEOL JEM 1200EX electron microscope, using an acceleration potential of 80 kV.

For statistical analysis, random pictures of cells were taken. For each cell, the average number of silver grains per micrometer of NE, ER, or plasma membrane was determined. The data were analyzed as described earlier (21, 62).

RESULTS

We have previously expressed the HSV-1 glycoprotein gB-1 gene in COS cells by using cloned DNA. The expressed gB-1 protein is transported to both the nuclear membrane and the cell surface (2). Truncation of the 109-amino-acid cytoplasmic tail showed that this domain did not affect the nuclear localization (47). Deletions in the predicted membrane anchoring sequence of the carboxy-terminal 69 hydrophobic amino acids of gB-1 (residues 727 to 795) showed that a hydrophobic sequence present in segment 3 (amino acids 774 to 795 of gB-1 protein) was sufficient for stable membrane anchoring and thus may specify the transmembrane domain of gB-1 glycoprotein. Also, all mutants containing segment 3 were localized in the NE (46). To examine whether a specific domain of gB-1 glycoprotein is required for localization in the NE, we have used a number of deletion mutants (46) as well as chimeric proteins. The cytoplasmic tail mutant CT799 was generated by creating a chain-terminating codon at amino acid residue 799

FIG. 1. Schematic diagram of gB mutants and chimeras used. (a) Amino acid sequences of the hydrophobic carboxy-terminal region of gB-1 and the transmembrane domain of G protein. (b) Numbers above and below the boxes are amino acid residues, starting from the N terminus, for gB (solid boxes) and G (open boxes), respectively. Deletions are shown by gaps. The larger box in the middle represents the predicted transmembrane domain. EC, ectodomain; TM, transmembrane anchor; CT, cytoplasmic domain.

(47); thus this mutant contained only 3 amino acids in the cytoplasmic tail, compared with 106 residues present in the wild-type gB-1 proteins (8, 44). The mutants gBΔ12 and gBΔ3 were constructed by deleting amino acid residues 727 to 772 and 774 to 795, respectively, from the 69-hydrophobic-amino-acid segment of gB-1 (46). The chimeric proteins were constructed by swapping domains of gB-1 protein with the corresponding domains of a reporter protein G of VSV. Glycoprotein G is a type I membrane protein containing a transmembrane domain of 20 hydrophobic amino acids and a cytoplasmic tail of 29 amino acids (49a) and has been used extensively as a model for cell surface protein as well as a reporter protein to study intracellular protein trafficking (1, 13, 16, 17, 19, 49, 51, 57). A schematic diagram of the mutants and the chimeric proteins is shown in Fig. 1. The chimeras G-gB and gB-G were constructed by replacing the ectodomains of the gB-1 and G proteins with the ectodomains of G and gB-1, respectively. The chimera G-tmgB was constructed by replacing the transmembrane domain of G protein with the predicted membrane anchoring domain of the carboxy-terminal 69 hydrophobic amino acids of gB-1. The chimeras G-tmgBΔ1 and G-tmgBΔ12 were obtained by replacing the transmembrane domain of G protein with the hydrophobic amino acids 747 to 795 and 773 to 795 of gB-1, respectively. The mutant genes were expressed transiently, as before (2, 20, 46, 47), in COS cells by using the high-efficiency vector pXM (27, 64), and the

FIG. 2. Expression of wild-type and mutant proteins in COS-1 cells. Untransfected cells or cells transfected with gB, G, CT799, gB-G, G-gB, G-tmgB, G-tmgBΔ12, or G-tmgBΔ1 were labeled with [³⁵S]methionine at 24 to 40 h posttransfection for 2 h and immunoprecipitated with anti-gB or anti-G antibodies. The proteins were then analyzed by SDS-PAGE followed by fluorography (46). Numbers at left are molecular weights (K, thousands).

intracellular transport and localization of the expressed glycoproteins were studied. Intracellular localization was determined by indirect immunofluorescence as well as at the ultrastructural level by immunoelectron microscopy.

Expression and intracellular transport of chimeric proteins. COS-1 cells were transfected with pXM, encoding the chimeras and the wild-type gB and G glycoproteins. The transfected cells were metabolically labeled with [³⁵S]methionine, and the proteins from the cell lysate immunoprecipitated with rabbit polyclonal anti-gB or anti-G antiserum were analyzed on SDS-polyacrylamide gels. It can be seen from Fig. 2 that the hybrid gB-G was immunoprecipitated specifically with anti-gB antibody, while the chimeras G-gB, G-tmgB, G-tmgBΔ1, and G-tmgBΔ12 were recognized only by anti-G antibody. The protein G-gB was also immunoprecipitated with an antipeptide antibody (anti-gB CT) raised against the carboxy-terminal 16 amino acids of gB-1 (data not shown).

Acquisition of endo H-resistant oligosaccharides by glycoproteins has been used as a measure of the rate of transport of the protein from the ER to the Golgi complex (29). To examine the rate of intracellular transport of the hybrid glycoproteins compared with that of wild-type gB-1 or G, the immunoprecipitates from a pulse-chase experiment were digested with endo H and analyzed on SDS-polyacrylamide gels. Previously it was shown that a fraction of gB-1 protein remained endo H sensitive even after a long period of chase (10, 26). In agreement, we have found that the wild-type gB becomes almost totally endo H resistant after a 1-h chase period, while the mutants gBCT799, gBΔ12, and gBΔ3 were only partially resistant to endo H even after a 3-h chase period (46, 47). A calculation of the rates of loss of endo H sensitivity showed that while the wild-type gB-1 and the mutant gBΔ3 had half-lives ($t_{1/2}$) of 27 and 135 min, respectively, for transport to the Golgi complex, the mutants gBCT799 and gBΔ12 had $t_{1/2}$ of more than 3 h (46, 47) and were thus not transported from ER to Golgi complex efficiently. Figure 3 shows that the maturation of the oligosaccharides of the hybrid glycoproteins occurred at different rates. Following a 15-min pulse, all of the hybrid proteins were completely digested with endo H, indicating that they are glycosylated with N-linked oligosaccha-

FIG. 3. Intracellular transport of mutant gB-1 glycoproteins as determined by endo H digestion. COS-1 cells transfected with the mutant constructs were pulse-labeled with [35 S]methionine at 24 or 40 h posttransfection for 15 min and then chased with methionine-rich medium for 0 min, 30 min, 1 h, or 2 h. Cell lysates were immunoprecipitated with anti-gB or anti-G antiserum and resuspended in sodium phosphate buffer, and an aliquot was subjected to digestion with endo H as described in the text. Only the results for the chimeras are presented. The quantitation of the bands by densitometry was done by using exposures in the linear range of the films.

rides. The rate of transport of the hybrid gB-G from the ER to the Golgi complex was slower ($t_{1/2}$ of 52 min) than that of the wild-type gB. The hybrid G-gB protein, in contrast, was transported extremely slowly and remained endo H sensitive after a chase of 2 h. In agreement with the published data (48), wild-type G protein showed a rapid rate of transport from ER to Golgi complex ($t_{1/2}$ of 22 min). The chimeras G-tmgB and G-tmgB Δ 12 showed rates of acquisition of endo H resistance ($t_{1/2}$ of 33 and 27 min, respectively) comparable to that of gB-1. In contrast, the hybrid G-tmgB Δ 1 was very slowly transported from the ER to the Golgi complex. Moreover, the amount of the endo H-resistant form of G-tmgB decreased with increasing chase time, and very little G-tmgB was observed after a 2-h chase, suggesting degradation of the chimera at the cell surface. Similar to the case with gB and gB-G, a fraction of G-tmgB was sensitive to endo H even after 2 h of chase. It appears that when the ectodomain of gB is replaced with the ectodomain of G, the hybrid protein is not transported from the ER to the Golgi complex, but replacement of the transmembrane domain of G with either the 69 hydrophobic residues (G-tmgB) or the 21-residue membrane anchoring segment (G-tmgB Δ 12) of gB-1 restores intracellular transport. Surprisingly, mutant G-tmgB Δ 1, which contains the transmembrane domain, including segments 2 and 3 (residues 745 to 795), was retained in the ER.

Intracellular localization of mutant gB-1 glycoproteins by immunofluorescence staining. The gB-1 glycoprotein has been shown to be localized in the ER, Golgi complex, and cell surface as well as in the NE by cell fractionation, immunofluorescence, or immunoelectron microscopy (2, 10, 21, 28, 46, 47, 59, 60). G protein, on the other hand, is localized in the cell surface, ER, and Golgi complex (1, 13, 16, 17, 45, 48, 49, 51, 57). COS cells transfected with the wild-type gB-1 or G gene and the mutant gB-1 as well as the chimeric genes were analyzed for intracellular localization of the proteins by indi-

rect immunofluorescence. Cells fixed with paraformaldehyde were treated with Triton X-100 and were reacted sequentially with anti-gB-1 or anti-G rabbit IgG and fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Fluorescence microscopic examination showed that the wild-type gB was located in intracellular structures which appear to be ER and NE as well as in the perinuclear region (Fig. 4D), which is in agreement with earlier reports (2, 28). As shown earlier (48), glycoprotein G was localized predominantly in a compact juxtanuclear region consistent with the Golgi complex and perinuclear structures. No staining was observed in the NE rim (Fig. 4E). The internal staining pattern observed with the gB-G chimera was very similar to that observed with G (Fig. 4F). No bright nuclear rim staining was observed. In contrast, the chimeras G-gB, G-tmgB, G-tmgB Δ 12, and G-tmgB Δ 1 all showed internal immunofluorescence patterns similar to that observed in the case of gB-1 protein (Fig. 4J, K, L, and N, respectively). All four of the chimeric proteins containing the membrane anchoring domains of gB-1 showed strong nuclear rim staining as well as perinuclear labeling, indicating NE and ER localization. As reported earlier (46, 47), the staining patterns for the mutants gBCT799 and gB Δ 12 were similar to that of wild-type gB-1, while the mutant gB Δ 3 showed weak staining of the nuclear rim. However, when cells fixed with paraformaldehyde were examined for cell surface immunofluorescence, both wild-type gB-1 and G (Fig. 4A and B, respectively) and chimeras gB-G, G-tmgB, and G-tmgB Δ 12 (Fig. 4C, H, and I, respectively) showed cell surface labeling. As reported previously, mutant gBCT799 showed very faint surface labeling (47), while mutant gB Δ 3 showed cell surface labeling similar to that of gB (46). Hybrids G-gB and G-tmgB Δ 1 (Fig. 4G and M, respectively), similar to the mutant gB Δ 12 (46), failed to show any surface labeling. The patterns of the surface immunofluorescence of the two hybrids G-tmgB (Fig. 4H) and G-tmgB Δ 12 (Fig. 4I) were strikingly different. The surface fluorescence of G-tmgB Δ 12 was similar to that observed with wild-type gB (Fig. 4A) and G (Fig. 4B). In contrast, G-tmgB showed a punctate pattern of distribution on the plasma membrane. The punctate distribution of G-tmgB on cell surface could be due to its degradation in the cell surface. The decrease in the amount of the endo H-resistant form of G-tmgB with increasing chase time (Fig. 3) is in agreement with this possibility.

Ultrastructural localization of membrane glycoproteins. Immunofluorescence analyses of cells expressing wild-type gB and proteins containing the membrane spanning domain of gB showed localization in the NE as well as in the ER. However, the immunofluorescence data are not precise because of overlapping of membrane organelles in the perinuclear region and cannot be quantitated easily (23). We therefore used immunoelectron microscopy to determine the localization of the proteins at a more detailed resolution at the ultrastructure level of the intracellular membranes. Griffiths et al. (23) have recently critically assessed the use of immunofluorescence microscopy to study localization of intracellular membrane compartments in cells and emphasized that a complete and accurate localization of the membrane compartments requires analyses using both immunofluorescence and electron microscopy.

To study the distribution of wild-type gB and G as well as of the mutants of gB and the chimeras in the intracellular membrane locations at the ultrastructure level, transfected COS cells were fixed with glutaraldehyde and embedded at low temperature in Lowicryl K4M. Ultrathin sections were then incubated sequentially with the affinity-purified rabbit anti-gB or anti-G IgG and goat anti-rabbit IgG coupled to 1-nm gold. The gold complex was visualized by silver enhancement. Using

FIG. 4. Cell surface and intracellular localization of wild-type and mutant proteins by immunofluorescence. COS-1 cells transfected with gB (A and D), G (B and E), gB-G (C and F), G-gB (G and J), G-tmgB (H and K), G-tmgB Δ 12 (I and L), or G-tmgB Δ 1 (M and N) were fixed with paraformaldehyde and processed for cell surface (A, B, C, G, H, I, and M) or internal (D, E, F, J, K, L, and N) immunofluorescence. For internal immunofluorescence, the cells were fixed with paraformaldehyde and then treated with 1% Triton to permeabilize the cells. Bars, 60 μ m for panels A, B, C, G, H, I, L, M, and N and 20 μ m for panels D, E, F, J, and K. Small arrowheads indicate NE rim.

this postembedding immunolabeling technique, we previously confirmed that in HSV-1-infected cells gB-1 is localized in the inner membrane of the NE (21). Similar results were also obtained by using freeze-fracture labeling (60). Strong labeling on the NE of COS cells transfected with gB plasmids (Fig. 5A) indicates that the localization of gB protein on the NE did not require the expression of other HSV genes. The absence of silver grains in the sections of COS cells not expressing gB-1 and treated with anti-gB antibody (Fig. 5B) or in sections of cells expressing gB-1 and reacted with a nonspecific antibody, namely, purified anti-G antibody (Fig. 5C), established the specificity of the labeling method. Also, no labeling was

observed when sections of nontransfected cells were incubated with affinity-purified anti-G antibody or when sections of cells expressing G were reacted with anti-gB-1 antibody (data not shown). The ER of cells transfected with gB-1 was also intensely labeled. Expressed gB-1 protein was also localized in the plasma membrane and in cytoplasmic vesicles of various sizes (Fig. 5A). In contrast, when COS cells expressing G protein were analyzed, the plasma membrane was intensely labeled and small cytoplasmic vesicles and Golgi-like structures were labeled with anti-G antibody, but only a few silver grains were observed on the ER, while the NE showed very few grains of silver (Fig. 5D). The distribution of grains on the

FIG. 5. Glycoprotein gB but not G is localized in the NE. COS-1 cells transfected with gB (A and C) or with G (D) or not transfected (B) were processed for electron microscopy and labeled with anti-gB (A and B) or anti-G (C and D) antibodies as described in the text. Arrowheads in panels B and C correspond to nonspecific labeling. Go, Golgi complex; NU, nucleus; PM, plasma membrane. Bars, 1 μ m.

TABLE 1. Labeling density of silver particles on intracellular membranes of COS cells transfected with gB mutants and chimeras

Transfecting plasmid	No. of silver particles (mean $\pm$ SEM)/ μ m of membrane measured			No. of cells counted
	NE	ER	Plasma membrane	
None	0.15 $\pm$ 0.05	0.08 $\pm$ 0.047	0.13 $\pm$ 0.04	11
gB	6.0 $\pm$ 0.77	7.0 $\pm$ 0.9	3.8 $\pm$ 0.50	33
G	1.4 $\pm$ 0.2	2.2 $\pm$ 0.25	8.4 $\pm$ 0.9	24
gB-G	1.1 $\pm$ 0.2	1.9 $\pm$ 0.25	6.8 $\pm$ 0.99	24
G-gB	11 $\pm$ 1.1	16 $\pm$ 2.2	0.18 $\pm$ 0.03	15
G-tmgB	7.4 $\pm$ 0.9	6.9 $\pm$ 1.1	2.7 $\pm$ 0.7	24
gBCT799	3.3 $\pm$ 0.3	4.1 $\pm$ 0.6	1.4 $\pm$ 0.2	19
gB Δ 12	5.9 $\pm$ 0.9	6.8 $\pm$ 1.0	0.15 $\pm$ 0.03	13
gB Δ 3	2.1 $\pm$ 0.3	2.7 $\pm$ 0.4	2.5 $\pm$ 0.5	15
G-tmgB Δ 12	10.1 $\pm$ 1.1	6.2 $\pm$ 0.9	7.1 $\pm$ 1.1	15

plasma membrane was fairly uniform. The labeling intensity of the NE of cells expressing G was higher than the background (Table 1), which indicates that a small fraction of G protein was localized in the NE. A similar distribution of G protein in this compartment was also observed previously in VSV-infected cells (4). Quantitation of the intracellular distribution of silver grains showed that only 6% of the total number of silver grains were localized in the NE of cells expressing G, compared with 25% in cells expressing gB-1 (Table 2). The outer and the inner membranes of the NE were not well separated in most of the cases, and therefore no attempts were made to distinguish the localizations of proteins in these two membranes. However, in a few specimens the outer and inner membranes were separated. The distributions of silver grains in the outer and inner membranes in these cases were quite similar (data not shown). The labeling density (grains per micrometer of membrane) of gB-1 on the NE was also about four times higher than that observed with G (Table 1). Taken together, these data suggest that gB-1 was specifically localized in the NE. The density of labeling of gB-1 in the ER membrane was similar to that observed for the NE, showing that a fraction of the gB-1 protein was retained in the ER. The labeling density of G in the plasma membrane was very high and was two times higher than that observed with the gB-1 protein (Table 1). When the ratios of the labeling densities in the plasma membrane and in the NE were compared, values of 0.5 and 4 were observed for the gB-1 and G proteins, respectively. These

TABLE 2. Distribution of silver grains in intracellular membranes of COS cells transfected with gB mutants and chimeras

Transfecting plasmid	% ($\pm$ SEM) of silver grains associated with:			No. of cells examined	Total no. of grains counted
	NE	Cytoplasmic membrane ^a	Plasma membrane		
gB	25 $\pm$ 2.9	45 $\pm$ 3.0	30 $\pm$ 2.4	14	4,893
G	6.3 $\pm$ 0.87	31 $\pm$ 5.6	62 $\pm$ 5.6	11	3,917
gBCT799	25 $\pm$ 3.2	61 $\pm$ 4.7	13 $\pm$ 3.2	15	3,802
gB-G	7.1 $\pm$ 0.99	53 $\pm$ 5.0	40 $\pm$ 4.8	15	4,122
G-gB	33 $\pm$ 3.8	67 $\pm$ 4.3	0.87 $\pm$ 0.23	11	3,902
G-tmgB	24 $\pm$ 4.7	67 $\pm$ 4.2	7.7 $\pm$ 2.9	13	6,558
gB Δ 12	38 $\pm$ 5.2	60 $\pm$ 5.4	2.0 $\pm$ 0.53	8	2,446
gB Δ 3	16 $\pm$ 3.3	64 $\pm$ 5.7	20 $\pm$ 4.7	11	2,829
G-tmgB Δ 12	33 $\pm$ 4.5	46 $\pm$ 5.2	21 $\pm$ 5.1	8	3,617

^a Cytoplasmic membrane includes the ER, Golgi, and other membrane vesicles.

data suggest that G protein is preferentially transported to the plasma membrane and hence can be used as a reporter protein to study NE localization. G protein has also been used as a reporter protein to determine the Golgi retention signal (35, 58).

The ectodomain of gB-1 does not specify NE localization. To determine the role of the ectodomain of gB-1 in NE localization, two chimeras, gB-G and G-gB, were constructed by switching the membrane anchoring domain and the cytoplasmic tail or the ectodomain of gB with the corresponding domains of G protein. The two hybrids showed clearly distinct patterns of labeling. Cells expressing gB-G were strongly labeled on the plasma membrane and on cytoplasmic vesicles, while very few silver grains were located on the NE and on the ER (Fig. 6A and Table 1). The distribution of gB-G was very similar to the distribution of G, and only 7% of gB-G was associated with the NE (Table 2). These data and results from the immunofluorescence analysis suggest that the ectodomain of gB-1 is not sufficient for NE localization. When cells expressing G-gB were processed for immunoelectron microscopy with anti-G antibody, both the NE and the ER showed intense labeling (Fig. 6B and C and Table 1), but the plasma membrane and Golgi-like vesicles were not labeled. About 33% of the labeling in cells expressing G-gB was associated with the NE (Table 2). These results show that the ectodomain of gB-1 cannot direct the chimera gB-G to the NE, while the carboxy-terminal transmembrane and cytoplasmic domains of gB-1 can target the chimera G-gB to the NE, suggesting that the carboxy-terminal region of gB may specify NE localization.

The transmembrane domain of gB specifies NE localization. Results obtained from the two chimeras gB-G and G-gB suggest that the determinants for NE localization of gB must be present either in the transmembrane domain or in the cytoplasmic tail or in both. Our previous studies using gB-1 mutants with shortened cytoplasmic tails have shown that the cytoplasmic tail may not be required for NE localization (47). However, those results were obtained with immunofluorescence and biochemical cell fractionation studies. To obtain more definitive results, we studied the intracellular localization of the cytoplasmic tail mutant gBCT799 by immunoelectron microscopy. The results presented in Fig. 7A show labeling of the NE and the ER of cells expressing gBCT799. About 25% of the labeling was associated with the NE (Table 2), suggesting that the deletion of the cytoplasmic tail did not affect the NE localization. In agreement with the immunofluorescence data (47), the plasma membrane was labeled poorly (Fig. 7A and Table 1). The amount of labeling for cells expressing gBCT799 was generally lower than that observed for cells expressing gB-1. This could be due to the fact that expression vector p91023 was used for expressing gBCT799 (47), while the vector pXM was used to express the gB-1 gene. To prove directly that the predicted transmembrane domain of the carboxy-terminal 69 hydrophobic amino acids of gB-1 were sufficient for NE localization, the chimera G-tmgB, in which the transmembrane segment of G was replaced with the 69-amino-acid predicted membrane anchoring segment of gB-1, was constructed. COS cells transfected with G-tmgB plasmid were analyzed by postlabeling immunoelectron microscopy. Cells expressing G-tmgB showed strong labeling of the NE and the ER (Fig. 7B and Table 1). About 24% of the silver grains were localized in the NE of cells expressing G-tmgB (Table 2), indicating that the predicted membrane anchoring sequence of gB-1 was sufficient to specify NE localization. The labeling density of G-tmgB in the NE was similar to that of gB-1 and was about four- to fivefold higher than that observed in the cases of G and gB-G (Table 1).

FIG. 6. The cytoplasmic tail and transmembrane region of gB are sufficient for NE localization. COS-1 cells transfected with gB-G (A) or G-gB (B and C) were processed for electron microscopy and labeled with anti-gB (A) or anti-G (B and C) antibodies as described in the text. An extension of the G-gB-transfected cell (B) is shown in panel C. Bars, 1 μ m. Abbreviations are as described in the legend to Fig. 5.

FIG. 7. The hydrophobic domain of gB is sufficient to specify NE localization. COS-1 cells transfected with gBCT799 (A) or G-tmgB (B) were processed for electron microscopy and labeled with anti-gB and anti-G antibody, respectively. Bars, 1 μ m. Abbreviations are as described in the legend to Fig. 5.

Plasma membrane labeling of cells expressing G-tmgB was strong, but the grains were not uniformly distributed (Fig. 7B); rather, they were present in patches of the plasma membrane. This was in agreement with the punctuate surface immunofluorescence labeling pattern observed with G-tmgB.

Hydrophobic segment 3 is sufficient for NE localization of gB-1 protein. Using deletion mutants lacking one, two, or three

segments of hydrophobic amino acids in the predicted membrane spanning domains of 69 residues in gB-1, we previously showed that segment 3 represents the membrane anchoring domain of gB-1 (46). To determine whether segment 3 was sufficient for NE localization of gB-1, we used the deletion mutants gB Δ 12 and gB Δ 3. Earlier (46) we showed that the protein gB Δ 12, which contains only segment 3, can stably

FIG. 8. The hydrophobic membrane anchoring segment 3 (residues 774 to 795) is sufficient to specify NE localization. COS-1 cells transfected with mutant gB Δ 12, gB Δ 3, G-tmgB Δ 1, or G-tmgB Δ 12 (A, B, C, and D, respectively) were processed for electron microscopy. Cells transfected with gB Δ 12 or gB Δ 3 were labeled with anti-gB antibody, while cells transfected with G-tmgB Δ 1 or G-tmgB Δ 12 were labeled with anti-G antibody. Bars, 1 μ m. The magnifications for G-tmgB Δ 1 and G-tmgB Δ 12 were $\times 17,000$ and $\times 16,000$, respectively. Abbreviations are as described in the legend to Fig. 5.

FIG. 9. Mutant G protein retained in the ER is not localized in the NE. COS-1 cells transfected with mutant GH6 were processed for immunofluorescence and immunoelectron microscopy with anti-G antibody. (A) Surface immunofluorescence; (B) internal immunofluorescence; (C) immunoelectron microscopy of cells labeled with anti-G antibody. Bars, 60, 20, and 1 μ m for panels A, B, and C, respectively. Abbreviations are as described in the legend to Fig. 5.

anchor to the membrane. In the case of the mutant gB Δ 3, most of the protein was secreted, but a small fraction was anchored to intracellular membranes (46). When cells expressing the gB Δ 12 or gB Δ 3 mutant were processed for immunoelectron microscopy, the NE and ER of cells expressing gB Δ 12 were strongly labeled (Fig. 8A). The overall distribution of gB Δ 12 in the cell was similar to that of G-gB; 38% of the total number of grains were present in the NE (Table 2). The mutant gB Δ 3 showed labeling of the NE, ER, and plasma membrane (Fig. 8B and Table 1), but the distribution of silver grains in the NE was weaker (16%, compared with 25 and 38% for gB and gB Δ 12, respectively) (Table 2). In agreement with the immunofluorescence data (46), the immunoelectron microscopy results showed that gB Δ 3 was also localized in the cell surface, while mutant gB Δ 12 failed to be transported to the plasma membrane (Fig. 8B and A, respectively). Thus, segment 3 may be sufficient for NE localization of gB but is not essential for NE retention, because NE localization at a reduced efficiency was observed with the mutant gB protein lacking segment 3 (gB Δ 3) (Fig. 8B and Table 2). The labeling density of gB Δ 3 was essentially similar in the NE, ER, and plasma membrane (Table 1).

To test whether segment 3 alone was sufficient for NE localization, chimeric glycoproteins G-tmgB Δ 1 and G-tmgB Δ 12 were constructed. In chimera G-tmgB Δ 1, the transmembrane segment of G protein was replaced with segments 2 and 3 of gB-1, while the hybrid G-tmgB Δ 12 contained segment 3 of gB-1 in place of the transmembrane segment of G. Results of immunoelectron microscopic analyses of cells expressing G-tmgB Δ 1 and G-tmgB Δ 12 show that the NE and the ER were strongly labeled in both cases (Fig. 8C and D, respectively). However, only the plasma membrane of the cells expressing G-tmgB Δ 12 showed strong labeling (Fig. 8D). The two mutants G-tmgB Δ 1 and G-tmgB Δ 12, however, showed a marked

difference in the distribution of the proteins (Fig. 8C and D). Thus, deletion of segment 1 leads to an increased accumulation in the cytoplasmic membranes (Fig. 8C), while deletion of segments 1 and 2 causes increased localization in the plasma membrane (Fig. 8D).

It may be noted that gB as well as mutants of gB or chimeras that were localized in the NE also accumulated in the ER. The labeling densities of proteins in the NE and ER of cells expressing these proteins were also similar (Table 1), suggesting that the membrane anchoring segment of gB-1 not only can promote localization of proteins in the NE but also may influence the retention of proteins in the ER.

The possibility that localization of proteins in the NE could be due to retention in the ER was tested by using a mutant of G protein. We have previously shown that the mutant GH6, constructed by insertion of three amino acids at position 244 of G protein, did not acquire endo H resistance and failed to reach the cell surface (32) and thus was retained in the ER. Immunofluorescence analyses of cells expressing GH6 showed staining of perinuclear regions similar to that seen with G or gB-G (Fig. 9B). No staining of the cell surface was observed (Fig. 9A). When cells expressing GH6 were examined by immunoelectron microscopy, the labeling was associated almost exclusively with a cytoplasmic membrane structure resembling the ER network. No labeling was observed in the cell surface, and very few grains were associated with the NE (Fig. 9C). These results suggest that retention in the ER alone may not be sufficient for localization of the protein in the NE.

DISCUSSION

The results presented in this report show that the carboxy-terminal sequence of 69 hydrophobic residues of gB-1 glycoprotein can specify its localization in the NE. The fact that

mutant gB Δ 12 and the chimera G-tmgB Δ 12, both containing only segment 3 as the membrane spanning sequence, were present in the NE also suggests that segment 3 is sufficient for NE localization. Mutant gB Δ 3 lacking segment 3 was also located in the NE, but the labeling in the NE in the case of gB Δ 3 was much less than that observed with gB mutants or chimeras containing segment 3. It may also be noted that the absence of segments 1 and 2 in the mutant gB Δ 12 and in the chimera G-tmgB Δ 12 resulted in increased accumulation in the NE. Although they showed very similar NE localizations, gB Δ 12 and G-tmgB Δ 12 had markedly different distributions in the cytoplasmic and plasma membranes. The absence of segment 1 (G-tmgB Δ 1) resulted in increased accumulation in the cytoplasmic membrane, whereas the absence of segments 1 and 2 (G-tmgB Δ 12) resulted in enhanced localization in the plasma membrane. Taken together, these results suggest that in the case of gB-1 protein, regions other than segment 3 may also be contributing, albeit weakly, to NE localization. Thus, although segment 3 is sufficient, segments 1 and 2 and possibly the cytoplasmic domain may augment localization of gB-1 protein in the NE. Studies with chimeric proteins, however, showed that the cytoplasmic domain and segments 1 and 2 of the transmembrane domain of gB-1 protein were not necessary for NE localization, but their presence in the hybrid protein resulted in a higher retention in the cytoplasmic compartments (67% for both G-gB and G-tmgB compared with 46% for G-tmgB Δ 12). Furthermore, deletion of segments 1 and 2 from G-tmgB resulted in a threefold increase in the localization of G-tmgB Δ 12 in the plasma membrane. The sequence present in segment 3 is very hydrophobic and lacks uncharged polar amino acids but shows a high degree of sequence conservation with similar regions of glycoproteins of other herpes viruses which are homologous to gB of HSV. In fact, within the α -herpesvirus family, segment 3 of gB homologs is almost totally conserved. In all of these cases, segment 3 contains an invariant proline preceding the first hydrophobic residue and thus could be part of a β -turn that links segments 2 and 3 (44). Studies with mutants with deletions in the hydrophobic region further showed that these sequences also played an important role in intracellular transport of the glycoprotein and in virus infectivity (46). The highly conserved segment 3 may therefore not only function in membrane anchoring and viral entry but also promote localization in the NE.

Transmembrane domains of a number of integral membrane proteins targeted to intracellular membranes have recently been shown to specify retention or sorting signals (3, 6, 35, 38, 41, 43, 53, 63). Membrane spanning segments of the spike glycoprotein M of infectious bronchitis virus, which buds in the Golgi complex, was shown to contain a Golgi retention signal (35, 58). Membrane anchoring sequences of a number of enzymes localized in the Golgi membrane were also sufficient for targeting reporter proteins to the Golgi complex (3, 6, 38, 40, 41, 43). The transmembrane domain of the nuclear pore complex protein gp210 was also sufficient for sorting to the pore membrane of the NE (63). Similarly, the first of the eight transmembrane segments of the LBR was shown to be sufficient for targeting to the inner nuclear membrane (53). Our results thus provide further evidence that membrane anchoring domains of proteins may contain information required for localization of the proteins in organelle membranes such as the Golgi complex or NE. However, no obvious homology between the transmembrane domains of gp210, the LBR, and gB-1 glycoprotein was observed.

The three distinct NE domains, i.e., the inner nuclear membrane, outer nuclear membrane, and nuclear pore complex, are in continuity with the ER membrane (14, 15, 18), and

therefore protein synthesized in the ER or in the outer nuclear membrane may reach all of these domains of the NE by lateral diffusion. However, unique integral membrane proteins have been identified in the inner nuclear membrane and in the nuclear pore complex, suggesting the existence of specific NE targeting signals (14, 15, 18). The NE targeting signal may specifically retain proteins synthesized in the outer nuclear membrane following lateral diffusion along the continuous membrane to the inner membrane or the pore complex. Such a mechanism was proposed to explain NE targeting signals of the LBR (53) and the inner membrane protein gp210 (63). HSV glycoproteins such as gB-1 that are targeted to the inner nuclear membrane for virus assembly may also follow the same pathway. Alternatively, gB-1 may be targeted to the NE by yet-uncharacterized transport vesicles originating from the ER or by a specific sorting receptor which transports it to the inner membrane. Interactions of the membrane spanning domain of gB-1 with the specific transport vesicle or with the transmembrane domain of the sorting receptor may mediate the NE sorting process. The transmembrane domain of gB-1 may also interact with the membrane spanning regions of proteins resident in the inner nuclear membrane to form oligomeric structures which are retained in the NE. Such a mechanism involving "kin recognition" mediated by the transmembrane domains of Golgi resident proteins has been proposed as a mechanism for retention of proteins in the Golgi membrane (35, 41). In HSV-infected cells, viral envelope glycoproteins may also form hetero-oligomers by interacting with each other via the membrane anchoring domain and be retained in the inner nuclear membrane where virus budding occurs (45).

The fact that gB-1 as well as mutants of gB and chimeras that are localized in the NE also accumulate in the ER suggests that the transmembrane domain of gB may contain both ER retention and NE localization signals. However, studies with ER transmembrane proteins have shown that a cytoplasmic signal consisting of the C-terminal sequence Lys-Lys-X-X or Lys-X-Lys-X-X (where X can correspond to any amino acid) is required for retention or retrieval of proteins in the ER (25, 34, 43). The observed accumulation in the ER could be due to overexpression of the protein in the HSV-infected cells (54) or in cells transfected with a high-level expression vector, such as the pXM vector used in this study (27, 64). Indeed, overexpression has been shown to cause retention in the ER of proteins targeted to the Golgi complex (38, 40, 41), nuclear pore complex (63), or inner nuclear membrane (53). Our results with the mutant GH6, which is retained in the ER but is not localized in the NE, also suggest that accumulation in the ER alone may not be sufficient for localization in the NE. Mutagenesis of the transmembrane segment 3 of gB-1, which is in progress, may help to further elucidate the mechanism of how this domain functions as an NE localization signal.

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REFERENCES

1. Adams, G. A., and J. K. Rose. 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell* 41:1007-1015.
2. Ali, M. A., M. Butcher, and H. P. Ghosh. 1987. Expression and nuclear envelope localization of biologically active fusion glycoprotein gB of herpes simplex virus in mammalian cells using

- cloned DNA. *Proc. Natl. Acad. Sci. USA* **84**:5675–5679.
3. Aoki, D., N. Lee, N. Yamaguchi, C. Dubois, and P. M. Fukudo. 1992. Golgi retention of a trans-Golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within the membrane anchoring domain. *Proc. Natl. Acad. Sci. USA* **89**:4319–4323.
4. Bergmann, J. E., and S. J. Singer. 1983. Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected chinese hamster ovary cells. *J. Cell Biol.* **97**:1777–1787.
5. Burke, B. 1990. The nuclear envelope and nuclear transport. *Curr. Opin. Cell Biol.* **2**:514–520.
6. Burke, J., J. M. Pettitt, H. Schachter, M. Sarkar, and P. A. Gleeson. 1992. The transmembrane and flanking sequences of β 1,2-N-acetylglucosaminyltransferase I specify medial-Golgi localization. *J. Biol. Chem.* **267**:24433–24440.
7. Butcher, M., K. Raviprakash, and H. P. Ghosh. 1990. Acid pH induced fusion of cells by herpes simplex virus glycoproteins gB and gD. *J. Biol. Chem.* **265**:5862–5868.
8. Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence specifying the glycoprotein gene gB, of herpes simplex virus type 1. *Virology* **133**:301–314.
9. Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62**:2596–2604.
10. Compton, T., and R. J. Courtney. 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. *J. Virol.* **49**:594–597.
11. Danscherg, G., and R. Nørsgaard. 1983. Light microscopic visualization of colloidal gold on resin-embedded tissue. *J. Histochem. Cytochem.* **31**:1394–1398.
12. Darlington, R. W., and L. H. Moss. 1968. Herpesvirus envelopment. *J. Virol.* **2**:48–55.
13. Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology* **193**:545–562.
14. Dessev, G. N. 1992. Nuclear envelope structure. *Curr. Opin. Cell Biol.* **4**:430–435.
15. Dingwall, C., and R. Laskey. 1992. The nuclear membrane. *Science* **258**:942–947.
16. Einfeld, D., and E. Hunter. 1991. Transport of membrane proteins to cell surface. *Curr. Top. Microbiol. Immunol.* **170**:107–139.
17. Garoff, H. 1985. Using recombinant DNA techniques to study protein targeting in the eukaryotic cell. *Annu. Rev. Cell Biol.* **1**:403–445.
18. Gerace, L., and B. Burke. 1988. Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* **4**:335–374.
19. Ghosh, H. P. 1980. Synthesis and maturation of glycoproteins of enveloped animal viruses. *Rev. Infect. Dis.* **2**:26–39.
20. Ghosh-Choudhury, N., M. Butcher, and H. P. Ghosh. 1990. Expression from cloned DNA of biologically active glycoprotein C of herpes simplex virus type 1 in mammalian cells. *J. Gen. Virol.* **71**:689–699.
21. Gilbert, R., and H. P. Ghosh. 1993. Immunoelectron microscopic localization of herpes simplex virus glycoprotein gB in the nuclear envelope of infected cells. *Virus Res.* **28**:217–231.
22. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467.
23. Griffiths, G., R. C. Parton, J. Lucocq, B. van Deurs, D. Brown, J. W. Slot, and H. J. Geuze. 1993. The immunofluorescent era of membrane traffic. *Trends Cell Biol.* **3**:214–219.
24. Harlow, E. D., and D. Lane. 1988. *Antibodies, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9**:3153–3162.
26. Johnson, D. C., and P. G. Spear. 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. *Cell* **32**:987–997.
27. Kaufman, R. J. 1990. Vectors used for expression in mammalian cells. *Methods Enzymol.* **185**:487–511.
28. Koga, J., S. Chatterjee, and R. J. Whitley. 1986. Studies on herpes simplex virus type 1 glycoproteins using monoclonal antibodies. *Virology* **151**:385–389.
29. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**:631–664.
30. Kotwal, G. J., J. Capone, R. A. Irving, S. H. Rhee, P. Bilan, F. Toneyuzzo, T. Hofmann, and H. P. Ghosh. 1983. Viral membrane glycoproteins: comparison of the amino terminal amino acid sequences of the precursor and mature glycoproteins of three serotypes of vesicular stomatitis virus. *Virology* **129**:1–11.
31. Kunkel, T., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
32. Li, Y., C. Drone, E. Sat, and H. P. Ghosh. 1993. Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. *J. Virol.* **67**:4070–4077.
33. Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type-1. *Virology* **114**:149–160.
34. Luzio, J. P., and G. Banting. 1993. Eukaryotic membrane traffic: retrieval and retention mechanism to achieve organelle residence. *Trends Biochem. Sci.* **18**:395–398.
35. Machamer, C. 1991. Golgi retention signals: do membranes hold the key? *Trends Cell Biol.* **1**:141–144.
36. McDermott, M. R., F. L. Graham, T. Hanke, and D. C. Johnson. 1989. Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. *Virology* **169**:244–247.
37. Morgan, C., H. M. Rose, M. Holden, and E. P. Jones. 1959. Electron microscopic observation on the development of herpes simplex virus. *J. Exp. Med.* **110**:643–656.
38. Munro, S. 1991. Sequences within and adjacent to the transmembrane segment of α -2,6 sialyltransferase specify Golgi retention. *EMBO J.* **10**:3577–3588.
39. Nii, S., C. Morgan, and H. M. Rose. 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. *J. Virol.* **2**:517–536.
40. Nilsson, T., J. M. Cucocq, D. MacKay, and G. Warren. 1991. The membrane spanning domain of β -1,4-galactosyltransferase specifies trans Golgi localization. *EMBO J.* **10**:3567–3575.
41. Nilsson, T., P. Slusarewicz, M. H. Hoe, and G. Warren. 1993. Kin recognition: a model for the retention of Golgi enzymes. *FEBS Lett.* **330**:1–4.
42. Pachi, C., R. L. Burke, L. L. Stuve, L. Sanchez-Pescador, G. Van Nest, F. Masiarz, and D. Dina. 1987. Expression of cell-associated and secreted forms of herpes simplex virus type 1 glycoprotein gB in mammalian cells. *J. Virol.* **61**:315–325.
43. Pelham, H. R. B., and S. Munro. 1993. Sorting of membrane proteins in the secretory pathway. *Cell* **75**:603–605.
44. Pellet, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman. 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. *J. Virol.* **53**:243–253.
45. Pettersson, R. F. 1991. Protein localization and virus assembly at intracellular membranes. *Curr. Top. Microbiol. Immunol.* **170**:67–106.
46. Rasile, L., K. Ghosh, K. Raviprakash, and H. P. Ghosh. 1993. Effects of deletions in the carboxy-terminal hydrophobic region of herpes simplex virus glycoprotein gB on intracellular transport and membrane anchoring. *J. Virol.* **67**:4856–4866.
47. Raviprakash, K., L. Rasile, K. Ghosh, and H. P. Ghosh. 1990. Shortened cytoplasmic domain affects intracellular transport but not nuclear localization of a viral glycoprotein. *J. Biol. Chem.* **265**:1777–1782.
48. Rose, J. K., and J. E. Bergmann. 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell* **34**:513–534.
49. Rose, J. K., and R. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **4**:257–288.
- 49a. Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding

- regions. *J. Virol.* **39**:519–528.
50. **Roth, J., M. Bendayan, E. Carlemalm, W. Villiger, and M. Garavito.** 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J. Histochem. Cytochem.* **29**:663–671.
 51. **Schlesinger, M. J., and S. Schlesinger.** 1987. Domains of virus glycoproteins. *Adv. Virus Res.* **33**:1–44.
 52. **Schneider, M., F. L. Graham, and L. Prevec.** 1989. Expression of the glycoprotein of vesicular stomatitis virus by infectious adenovirus vectors. *J. Gen. Virol.* **70**:417–427.
 53. **Smith, S., and G. Blobel.** 1993. The first membrane spanning region of lamin B receptor is sufficient for sorting to the inner nuclear membrane. *J. Cell Biol.* **120**:631–637.
 54. **Sommer, M., and R. J. Courtney.** 1991. Differential rates of processing and transport of herpes simplex virus type 1 glycoproteins gB and gC. *J. Virol.* **65**:520–525.
 55. **Soullam, B., and H. J. Worman.** 1993. The amino terminal domain of the lamin B receptor is a nuclear envelope targeting signal. *J. Cell Biol.* **120**:1093–1100.
 56. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), *Viral fusion mechanisms*. CRC Press, Boca Raton, Fla.
 57. **Stephens, E. B., and R. W. Compans.** 1988. Assembly of animal viruses at cellular membranes. *Annu. Rev. Microbiol.* **42**:489–516.
 58. **Swift, A. M., and C. Machamer.** 1991. A Golgi retention signal in a membrane-spanning domain of corona virus E1 protein. *J. Cell Biol.* **115**:19–30.
 59. **Torrise, M. R., M. Cirone, A. Pavan, C. Zompetta, G. Barile, L. Frati, and A. Faggioni.** 1989. Localization of Epstein-Barr virus envelope glycoproteins on the inner nuclear membrane of virus-producing cells. *J. Virol.* **63**:828–832.
 60. **Torrise, M. R., C. DiLazzaro, A. Pavan, L. Pereira, and G. Campadelli-Fiume.** 1992. Herpes simplex virus envelopment and maturation studied by fracture label. *J. Virol.* **66**:554–561.
 61. **Torrise, M. R., L. V. Lotti, A. Pavan, G. Migliaccio, and S. Bonatti.** 1987. Free diffusion to and from the inner nuclear membrane of newly synthesized plasma membrane glycoproteins. *J. Cell Biol.* **104**:733–737.
 62. **Woolson, R. F.** 1987. *Statistical methods for the analysis of biomedical data*. John Wiley & Sons, New York.
 63. **Wozniak, R. W., and G. Blobel.** 1992. The single transmembrane segment of gp210 is sufficient for sorting to the pore membrane domain of the nuclear envelope. *J. Cell Biol.* **119**:1441–1449.
 64. **Yang, Y.-C., A. B. Ciarletta, P. A. Temple, M. P. Chung, S. Kovacic, J. S. Witek-Giannotti, A. C. Leary, R. Kriz, R. E. Donahue, G. G. Wong, and S. C. Clark.** 1986. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**:3–10.