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Effects of Deletions in the Carboxy-Terminal Hydrophobic Region of Herpes Simplex Virus Glycoprotein gB on Intracellular Transport and Membrane Anchoring

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The gB glycoprotein of herpes simplex virus type 1 is involved in viral entry and fusion and contains a predicted membrane-anchoring sequence of 69 hydrophobic amino acids, which can span the membrane three times, near the carboxy terminus. To define the membrane-anchoring sequence and the role of this hydrophobic stretch, we have constructed deletion mutants of gB-1, lacking one, two, or three predicted membrane-spanning segments within the 69 amino acids. Expression of the wild-type and mutant glycoproteins in COS-1 cells show that the mutant glycoproteins lacking segment 3 (amino acids 774 to 795 of the gB-1 protein) were secreted from the cells. Protease digestion and alkaline extraction of microsomes containing labeled mutant proteins further showed that segment 3 was sufficient for stable membrane anchoring of the glycoproteins, indicating that this segment may specify the transmembrane domain of the gB glycoprotein. Also, the mutant glycoproteins containing segment 3 were localized in the nuclear envelope, which is the site of virus budding. Deletion of any of the hydrophobic segments, however, affected the intracellular transport and processing of the mutant glycoproteins. The mutant glycoproteins, although localized in the nuclear envelope, failed to complement the gB-null virus (K082). These results suggest that the carboxy-terminal hydrophobic region contains essential structural determinants of the functional gB glycoprotein.

Viral membrane glycoproteins have been used extensively as model systems to study the mechanism of intracellular protein transport and sorting (24, 56, 60, 65, 66). Herpes simplex virus (HSV) is known to assemble and bud from the inner nuclear membrane of infected cells into the periplasmic space (19), and a number of HSV type 1 (HSV-1) glycoproteins are associated with the nuclei of infected cells (16). The membrane glycoproteins of HSV may, therefore, be used as model systems to study the transport of proteins to the nuclear membrane. The genome of HSV-1 is known to code for at least 10 glycoproteins (27, 33, 34, 44, 55, 65). Glycoprotein gB-1 is particularly interesting for two reasons: (i) gB-1 is essential for viral infectivity (42, 58) and is known to be involved in important biological functions, such as virus entry and cell fusion (10, 42, 46, 58, 65), and (ii) 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 (7, 8, 50). We have previously expressed the HSV gB-1 glycoprotein gene in COS-1 cells with cloned DNA. The expressed gB-1 protein is fully glycosylated, properly processed, and localized both in the nuclear envelope and in the plasma membrane. gB-1 is also biologically active and induced cell fusion at acidic pH in the absence of other HSV glycoproteins (3, 6). Recent studies using immunoelectron microscopy have demonstrated the localization of gB in the inner membrane of the nuclear envelope in HSV-1-infected cells (26, 67).

Studies using mutagenesis of hydrophobic amino acids located at the carboxy termini of a number of membrane glycoproteins suggest that these sequences of hydrophobic amino acids act not only as a membrane anchor but also play a role in intracellular transport (1, 12, 20, 41, 45, 51, 56, 60, 62, 64, 66). By assuming that a stretch of 20 to 22 hydrophobic amino acids can act as a membrane-anchoring sequence (22, 40, 43), it was predicted that the segment of 69 amino acids located near the carboxy terminus of the gB glycoprotein can span the membrane three times (50). However, calculation of the average hydrophobic indices of the three segments of 20 to 22 amino acids present within the stretch of 69 amino acids by using the hydropathy values of Kyte and Doolittle (40) showed that the three segments containing residues 727 to 746 (segment 1), 752 to 772 (segment 2), and 775 to 795 (segment 3) have hydrophobicities of 0.8, 1.7, and 2.4, respectively, suggesting that only two segments can serve as transmembrane anchors. Protection from proteolytic digestion of in vitro- and in vivo-synthesized gB-1 glycoprotein by insertion into membranes (15) as well as secretion of truncated gB proteins of various lengths (3, 11) established a transmembrane orientation of the gB protein with the NH_2 and COOH termini on opposite sides of the membrane but did not provide any information on the number of membrane-spanning segments present in the gB glycoprotein.

HSV-1 gB glycoprotein has been shown to exist as a homodimer (2, 14, 31, 59, 65, 69). By using the temperature-sensitive isolate *ts*B5, it was shown that the gB glycoprotein failed to form dimers at the nonpermissive temperature, suggesting that oligomerization is necessary for the production of infectious virus (13, 30). Studies with gB-1 mutants have suggested two regions critical for oligomerization, namely, residues 93 to 282 and residues 596 to 711 (31). Since the mutations in *ts*B5 lie outside these two regions (9), proper folding of the glycoprotein is also essential for oligomerization.

In this study, we have attempted to define the membrane-anchoring sequence of gB and to analyze the role of the hydrophobic amino acids in the COOH-terminal region of gB

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by constructing a series of mutant glycoproteins from which various lengths of hydrophobic amino acid sequences have been deleted by mutagenesis. The effects of deletion of the hydrophobic sequences from the gB glycoprotein on the interaction of the mutant proteins with membranes, intracellular transport and localization, oligomerization, and virus infectivity were studied. The results indicate that the segment containing amino acid residues 774 to 794 of the gB protein is sufficient for stable transmembrane anchoring and that deletion of amino acids from the hydrophobic carboxy-terminal sequence resulted in impaired intracellular transport. However, mutant gB glycoproteins containing residues 774 to 794 were localized in the nuclear envelope. Complementation experiments using a defective HSV (K082) lacking gB glycoprotein showed that the mutant glycoproteins, although localized in the nuclear envelope, could not produce infectious virus particles (11, 11a). These results suggest that the carboxy-terminal hydrophobic region of gB is not only involved in membrane anchoring but is also required for intracellular transport and localization.

MATERIALS AND METHODS

Materials. Restriction and DNA-modifying enzymes were purchased from Bethesda Research Laboratories or Pharmacia Canada. Endoglycosidase H (endo H) was from ICN-Immunobiologicals. Glucose oxidase and lactoperoxidase were from Boehringer Mannheim Canada. Anti-HSV-1 antiserum and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) were from Dako Corporation. Monoclonal antibodies to gB glycoprotein of HSV-1 were from M. Zweig (61) and P. Spear (14). A polyclonal anti-gB antibody was prepared by inoculating rabbits with a recombinant adenovirus containing the gB glycoprotein gene of HSV-1 (35). The gB-transformed Vero cell line designated D6 and the HSV-1 mutant K082 constructed by Cai et al. (11a) were obtained from J. Glorioso and M. Levine. The pKBXX plasmid was obtained from S. Person (11a). *Escherichia coli* BW 313 (*dut ung*) cells (39) 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.

Mutagenesis. The 2.1-kb *Sal*I-*Eco*RI fragment of p9gB (p91023 [B] containing full-length wild-type gB-1 gene [3]) was cloned into *Sal*I-*Eco*RI-digested M13mp18. Recombinant M13mp18-gB was used for mutagenesis by the method of Kunkel et al. (39). Uridine-containing template DNA was prepared by infecting *E. coli* BW 313 (*dut ung*) cells with M13mp18 gB in the presence of 0.5 μg uridine per ml. The oligonucleotides used for mutagenesis were CACGCCGA CGCCGCGGTCGGCAA, CGCGCGGTCGGCAAGAACCC CTTTGGGGCGCT, TCCTTCATGTCCAACCGTTACGT CATGCGG, GTCATCCACGCCGACAACCCCTTTGGGG CGCT, CGCGCGGTCGGCAAGCGTTACGTCATGCGG, and GTCATCCACGCCGACCGTTACGTCATGCGG for constructing mutants Δ1, Δ2, Δ3, Δ1,2, Δ2,3, and Δ1,2,3, respectively. The Δ1,3 mutant was constructed by using primers for Δ1 and Δ3 mutants. The mutagenic oligonucleotide was annealed to the template DNA, and second-strand synthesis was performed with Klenow DNA polymerase. The reaction mixtures were used to transform competent *E. coli* JM 109 cells. The plaques were screened by nucleotide sequence analysis of the junction regions of the mutants (57). The replicative-form DNA from the mutant bacteriophage was digested with *Sal*I and *Eco*RI, and the fragment was ligated to the 6.8-kb fragment obtained from *Eco*RI and *Sal*I digestion of pXMgB DNA (pXM [68] containing the full-length gB-1 gene), regenerating pXMgB harboring the desired mutation in the gB gene. The pXM plasmid (68) was used as the expression vector rather than p91023 B (3), since transfection with pXMgB resulted in a larger number of COS cells expressing gB glycoproteins. The mutants were also cloned into pKBXX (11a) with *Sna*BI and *Kpn*I in order to perform the complementation studies.

Transfection, labeling, and immunoprecipitation. Subconfluent monolayers of COS-1 cells were transfected by the $Ca_3(PO_4)_2$ precipitation protocol (28, 54). The transfected cells were labeled with [^{35}S]methionine at 40 to 45 h posttransfection and processed for immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described earlier (54).

Intracellular transport. Transfected COS-1 cells were fixed either with 2% paraformaldehyde for surface immunofluorescence or with acetone at −20°C for internal immunofluorescence (53) at 40 to 45 h posttransfection and were reacted with anti-HSV-1 antiserum (1:20 dilution) or monoclonal anti-gB 3S antibody (61) and stained with FITC-conjugated goat anti-rabbit IgG as described previously (54). Cell surface labeling using lactoperoxidase-catalyzed iodination was carried out as described previously (29, 54). Transport of the mutant gB proteins to the Golgi apparatus was determined by the acquisition of endo H resistance (37) by the procedure described previously (54). Fractionation of transfected cells labeled with [^{35}S]methionine into nuclear and postnuclear fractions was done as described before (54).

Membrane anchoring of mutant gB proteins. The insertion of gB and mutant gB glycoproteins into membranes was monitored by proteolytic digestion of microsomes isolated from COS-1 cells expressing the wild-type and mutant gB glycoproteins by a procedure previously described (15) with some modifications. Transfected COS-1 cells were labeled for 30 min with [^{35}S]methionine at 40 to 45 h posttransfection and were then suspended in a hypotonic buffer (10 mM Tris-HCl [pH 7.4], 10 mM KCl, 5 mM $MgCl_2$) and disrupted in a Dounce homogenizer. The cell lysate was adjusted to a NaCl concentration of 150 mM and centrifuged at 5,000 × *g* for 5 min. The supernatant fraction was centrifuged at 150,000 × *g* for 30 min at 4°C. The pellet containing membranes was suspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM $MgCl_2$. Aliquots of the membrane suspension were digested at 4°C with 50 μg of trypsin per ml in the absence or presence of 1% Triton X-100. Proteolysis was stopped by adding 5 times more trypsin inhibitor (Sigma), 40 μg of phenylmethylsulfonyl fluoride per ml, and 100 U of Trasylol per ml. The reaction mixture was put in a solution containing 0.5% Nonidet P-40 (NP-40), 0.25% Na deoxycholate, 66 mM Na_2EDTA, and 25 mM Tris-HCl, pH 7.4. The labeled glycoproteins were immunoprecipitated with the polyclonal anti-gB antibody (35) and analyzed on SDS-polyacrylamide gels (3).

The anchoring of the gB and mutant glycoproteins into membranes was also determined by extraction of the membranes containing labeled glycoproteins under alkaline conditions (23). Membranes containing [^{35}S]methionine-labeled gB and mutant glycoproteins were isolated from transfected COS-1 cells as described above. The pelleted membranes were suspended in 0.1 M Na_2CO_3 (pH 11.5) and incubated

on ice for 30 min. The extracted membranes were centrifuged at 150,000 × g for 30 min at 4°C. The pellet containing integral membrane proteins anchored to the membranes was dissolved in a solution consisting of 0.5% NP-40, 0.25% Na deoxycholate, 66 mM Na_2EDTA, and 25 mM Tris-HCl, pH 7.4. The extracted nonanchored glycoproteins were recovered from the supernatant fraction by precipitation with 10% trichloroacetic acid and dissolved in a buffer containing 0.5% NP-40, 0.25% Na deoxycholate, 66 mM Na_2EDTA, and 25 mM Tris-HCl, pH 7.4. The gB-related proteins were immunoprecipitated with rabbit anti-gB antibody and analyzed on an SDS–7.5% polyacrylamide gel.

Oligomerization assay. The oligomeric state of the gB-1 glycoprotein was determined by two different methods: (i) SDS-PAGE analysis and (ii) sucrose gradient sedimentation.

(i) SDS-PAGE analysis. The heat-dissociable, detergent-stable oligomers of gB-1 glycoproteins were analyzed on an SDS-polyacrylamide gel as described by Claesson-Welsh and Spear (14) and Sarmiento and Spear (59). COS-1 cells transfected with plasmids containing gB-1 or mutant genes were labeled with [^{35}S]methionine at 40 to 45 h posttransfection, and the cells were lysed in a buffer containing 0.5% NP-40, 0.5% Na deoxycholate, 0.15 M NaCl, 1 mM phenylmethylsulfunoyl fluoride, 100 U of aprotinin per ml, and 10 mM Tris-HCl (pH 7.4). The postnuclear supernatant factions were immunoprecipitated with monoclonal anti-gB 3S (61) and II-105 (14) antibodies. The immunoprecipitates were extracted with sample buffer containing 50 mM Tris-HCl (pH 6.8), 0.3% SDS, and 0.1% 2-mercaptoethanol without heating and analyzed on a 7.5% polyacrylamide gel (14, 59).

(ii) Sucrose gradient sedimentation. The oligomeric forms of gB-1 glycoprotein were also analyzed by sucrose density gradient sedimentation as described previously (18, 20). Transfected COS-1 cells labeled at 40 to 45 h posttransfection were extracted in a buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), and 100 mM NaCl. The postnuclear fractions were analyzed on a 12-ml continuous 5 to 20% (wt/vol) sucrose gradient in a solution consisting of 0.1% Triton X-100, 50 mM Tris-HCl (pH 7.4), and 100 mM NaCl. The gradients were centrifuged at 41,000 rpm at 4°C for 18 h in a Beckman SW41 rotor. Fractions were collected from the bottom of the gradient, and odd-numbered fractions were immunoprecipitated with a polyclonal anti-gB antibody (35) after the addition of 0.5 ml of a detergent solution containing 1% NP-40, 0.4% Na deoxycholate, 0.3% SDS, 12.5 mM Na_2EDTA, and 50 mM Tris-HCl (pH 7.4). The immunoprecipitates were analyzed on an SDS-polyacrylamide gel (3).

Complementation assay. Subconfluent monolayers of Vero cells were transfected by the DEAE-dextran protocol (11). Briefly, 0.5 to 3 μg of pKBXX plasmid (11, 11a) encoding the deletion mutants was added to 1 ml of serum-free growth medium containing 100 μg of DEAE-dextran per ml. This mixture was incubated at room temperature for 30 min, while the Vero cells in 60-cm-diameter dishes were washed twice with serum-free medium. Medium containing DNA and DEAE-dextran was added to the cells and incubated at 37°C for 2 to 4 h, then the medium was removed, cells were washed twice with serum-free medium and finally 5 ml of regular medium was added and cells were incubated at 37°C. At 17 h posttransfection, each plate was infected with 10^6 PFU of K082 virus (11, 11a) in 0.2 ml serum-free medium and incubated at 37°C for 2 h. Virions remaining outside the cell were removed by treatment with glycine buffer (8 g of NaCl, 0.38 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 7.5 g of glycine [all in 1 liter]; pH 3.0) followed by two washings with phosphate-buffered saline. The cultures were incubated at 37°C for 24 h and then harvested, and viral stocks were prepared and titers were determined on D6 (11, 11a) and Vero cells.

RESULTS

Rationale for construction of deletion mutants. The sequence of the hydrophobic region corresponding to amino acid residues 727 to 795 of HSV-1 glycoprotein gB-1 (7) is shown in Fig. 1A. The hydrophobic segments of 20 to 22 amino acids corresponding to residues 727 to 746, 752 to 772, and 775 to 795 have been designated segments 1, 2, and 3, respectively. Segment 1 is adjacent to the ectodomain, while segment 3 precedes the cytoplasmic domain. The hydrophobicities (40) of segments 1, 2, and 3 were calculated to be 0.8, 1.7, and 2.4, respectively. To investigate the functional significance of these three segments, oligonucleotide-directed mutagenesis was used to delete specific segments from the hydrophobic region, as depicted in Fig. 1B. The resulting seven mutant proteins (Fig. 1B) were studied to determine the roles that the segments play in transmembrane anchoring, intracellular transport and localization, oligomerization, and virus infectivity.

Expression and glycosylation of mutant gB-1 proteins. To determine whether the mutant glycoproteins are synthesized and glycosylated, COS-1 cells were transfected with pXM encoding the deletion mutants and the wild-type gB glycoprotein. The transfected cells were metabolically labeled with [^{35}S]methionine, and the proteins from the cell lysate immunoprecipitated with anti-HSV-1 antiserum were analyzed on SDS-polyacrylamide gels. It can be seen from Fig. 2A that all the mutant gB-1 constructs expressed proteins which immunoprecipitated specifically with anti-HSV antiserum and had slightly smaller molecular sizes than the wild-type gB glycoprotein. The mutant glycoproteins were also immunoprecipitated by a gB-1-specific 3S monoclonal antibody (data not shown). The multiple bands observed for both wild-type and mutant gB-1 proteins represent proteins with various degrees of glycosylation and processing. To examine whether deletion of segments of the potential transmembrane domain influenced the glycosylation of gB-1 protein, we further analyzed the proteins expressed in the presence of tunicamycin. Results presented in Fig. 2A show that the mutant gB-1 proteins produced in the absence of tunicamycin have a larger molecular size than those produced in the presence of tunicamycin, indicating that the mutant gB-1 proteins were glycosylated. The multiple bands observed in the tunicamycin-treated samples could be caused by proteolysis of the nonglycosylated proteins.

Hydrophobic segment 3 is sufficient for transmembrane anchoring of gB-1 glycoprotein. To analyze the role of the three hydrophobic segments in membrane integration, we used three different approaches: (i) secretion of glycoproteins by transfected COS-1 cells into the medium, (ii) susceptibility to protease digestion of the proteins present in microsomal vesicles, and (iii) alkaline extraction of the microsomes containing labeled proteins.

It has previously been shown that removal of the hydrophobic and cytoplasmic domains from gB-1 glycoprotein resulted in secretion of the truncated glycoprotein into the medium (3, 11, 48). Removal of the hydrophobic domain of other transmembrane proteins also resulted in their secretion (56, 60, 66). Results presented in Fig. 2B show that the mutant gB-1 glycoproteins Δ1,3, Δ2,3, Δ3, and Δ1,2,3 were secreted into the medium, whereas the others were present only intracellularly. The secretion of the mutant glycopro-

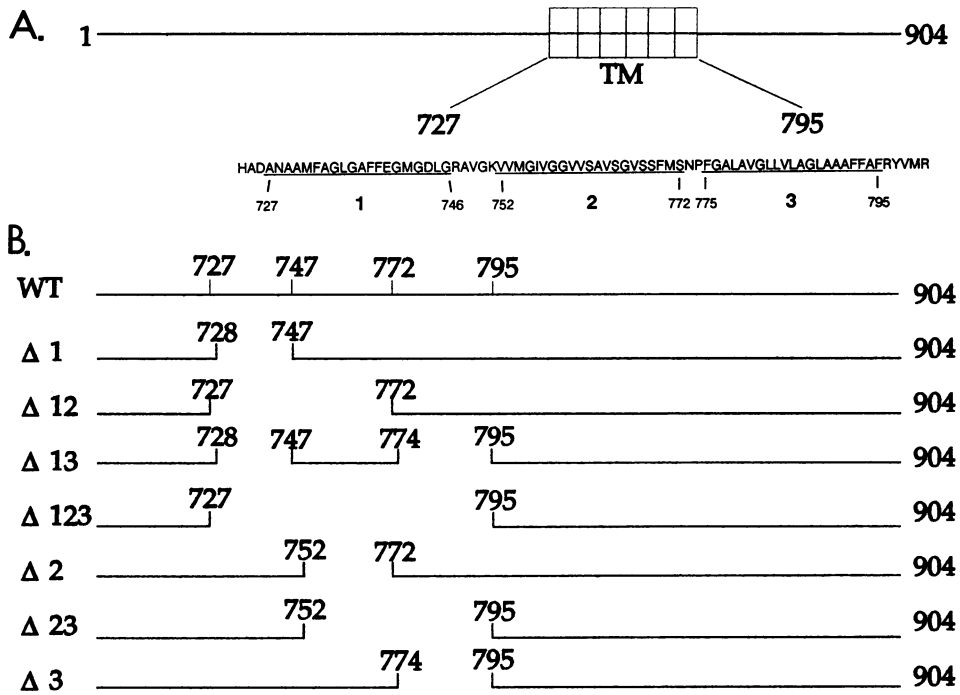

FIG. 1. (A) Linear representation of HSV-1 gB glycoprotein. The numbers correspond to the amino acid residue numbers of the HSV-1 gB glycoprotein deduced from nucleotide sequence data (7, 8). The three proposed hydrophobic segments are indicated (TM). The amino acid sequence of the hydrophobic domain is shown underlined, with the amino acid and segment numbers as indicated. (B) Schematic representation of the deletion mutants in the hydrophobic region of HSV-1 gB gene. Plasmid pXM containing the mutant gB-1 genes are designated Δ1, Δ1,2, Δ1,3, Δ2, Δ2,3, Δ3, and Δ1,2,3. The gaps show the amino acids deleted, and the numbers indicate the first and the last residues of the peptide that has been deleted in the mutant. WT, wild type.

teins was dependent upon glycosylation, since no secretion was observed when tunicamycin was present. The kinetics of secretion of the mutant gB-1 glycoproteins was determined by pulse-chase experiments. Transfected COS-1 cells were labeled for 30 min and then chased with medium containing excess methionine for 30, 90, 180, and 300 min. The immunoprecipitates from the media and the cell lysates were analyzed by gel electrophoresis (Fig. 3). The amounts of Δ2,3 and Δ1,2,3 mutant glycoproteins secreted after a 30-min chase was considerably more than the amounts secreted in the same time from cells transfected with Δ3 and Δ1,3 mutants. Densitometric analyses of the fluorograms showed that for Δ3, Δ1,3, Δ2,3, and Δ1,2,3 gB, the proportions of the protein secreted into the medium after 180-min chase were 29, 36, 45, and 57%, respectively.

The transmembrane anchoring of the mutant proteins was analyzed by protease sensitivity of microsomal vesicles containing labeled proteins. Proteins anchored to the membrane vesicles would show partial protection caused by proteolytic removal of the exposed cytoplasmic domain. In contrast, proteins lacking a membrane anchor domain would be translocated into the lumen of the vesicle and thus be completely protected from protease digestion. COS-1 cells transfected with the wild type and deletion mutants of gB were labeled for 30 min when most of the proteins synthesized will be in the endoplasmic reticulum. Microsomal membrane vesicles containing the labeled proteins were isolated and digested with trypsin under conditions which protect the integrity of the membrane vesicles. The results are summarized in Fig. 4. Deletions of the first (Δ1), second (Δ2), and both first and second (Δ1,2) hydrophobic segments (Fig. 4) neither prevented membrane anchoring nor changed the orientation of the protein in the membrane, as shown by the proteolytic removal of about a 10,000-Da peptide from each of the mutants. A similar decrease of 10,000 Da in the size of the wild-type gB glycoprotein present in the microsomal vesicles from COS-1 cells transfected with the gB gene (Fig. 4) or from cells infected with HSV-1 (15) further showed that mutant glycoproteins Δ1, Δ2, and Δ1,2 have the same membrane orientation as the wild-type gB protein. Analysis of mutants lacking the first and third (Δ1,3), second and third (Δ2,3), and all three hydrophobic segments (Δ1,2,3) showed that the mutant proteins were totally protected from protease digestion, indicating that these proteins must be completely translocated into the lumen of the microsomal vesicles. These results suggest that the translocation of the proteins lacking segment 3 into the lumen of the vesicles could be caused by the requirement of segment 3 for stable membrane anchoring. When, however, microsomes containing mutant gB protein lacking segment 3 (Δ3) were treated with protease, a predominant band of the same size as the Δ3 gB protein not treated with protease and another minor band of 10,000 Da smaller in size were observed. This result suggested that for the mutant gB protein containing the first and second hydrophobic segments, the association with the membrane may not be very stable. A small fraction of the mutant Δ3 glycoprotein was anchored in the microsomes as a transmembrane protein, while the majority of the Δ3 protein was translocated into the lumen. One could also note that for mutant Δ1,3, which contained only segment 2, a very small amount of the mutant protein was partially digested, indicating that hydrophobic segment 2 could also act as a membrane anchor, but this interaction was less stable than that observed with segment 3.

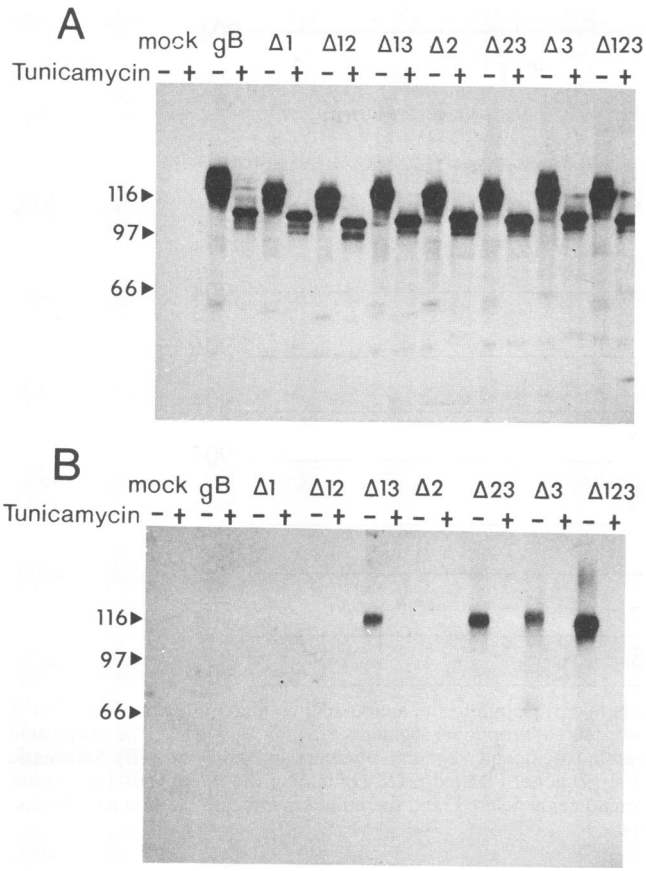

FIG. 2. Expression, glycosylation, and secretion of mutant gB glycoproteins. Subconfluent monolayers of COS-1 cells were transfected with the mutant gB constructs. At 40 h posttransfection, the cells were starved in methionine-deficient medium for 1 h in the absence (−) or presence (+) of tunicamycin (2 μg/ml) and labeled with [^{35}S] methionine for 2 h. Cell lysate (A) and medium (B) were immunoprecipitated with anti-HSV-1 antiserum, and the immunoprecipitates were subjected to electrophoresis on SDS–10% polyacrylamide gels. The gels were fluorgraphed, dried, and exposed at −70°C. The numbers to the left of the gels show the sizes (in kilodaltons) of molecular mass markers.

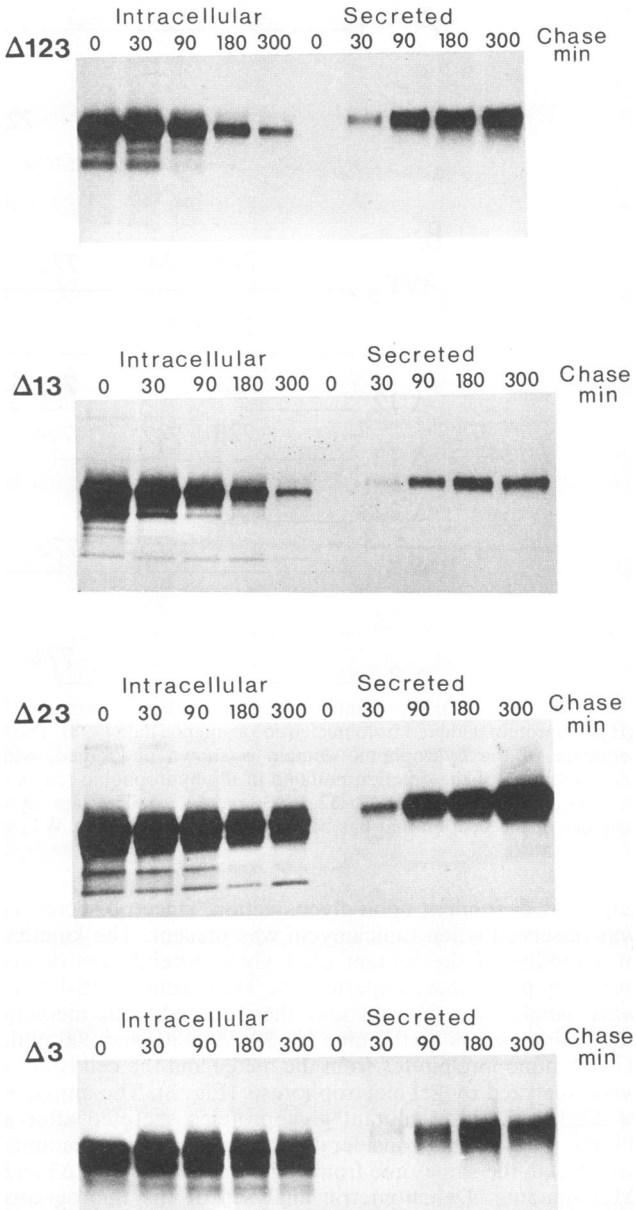

FIG. 3. Kinetics of secretion of mutant gB glycoproteins. Subconfluent monolayers of COS-1 cells were transfected with the secreted gB mutant constructs Δ1,2,3, Δ1,3, Δ2,3, and Δ3. At 40 h posttransfection, the cells were starved for 1 h in medium lacking methionine, pulse-labeled with [^{35}S]methionine for 30 min, then chased with methionine-rich medium for 0, 30, 90, 180, and 300 min. Extracts from the cell lysate (Intracellular) and medium (Secreted) were immunoprecipitated with anti-HSV-1 antiserum and run on SDS-polyacrylamide gels. For densitometric quantitation, exposures in the linear range of the film were used.

To test the nature of the association of the mutant gB proteins with the membrane, microsomal vesicles containing labeled proteins were extracted with alkali (23). Under these conditions, peripheral membrane proteins as well as proteins lacking any membrane anchor domain will be extracted into the supernatant fraction, whereas integral membrane proteins will be retained in the pellet. As expected, wild-type gB protein was retained in the membranous pellet fraction after extraction with 0.1 M Na_2CO_3. The mutant gB protein lacking the entire hydrophobic membrane anchor region (Δ1,2,3) was extracted mostly into the supernatant fraction. Mutant gB proteins lacking the first (Δ1), second (Δ2), and both first and second (Δ1,2) hydrophobic segments were not extracted by alkali but were present in the pellet fractions, whereas mutants retaining the first (Δ2,3) and second (Δ1,3) hydrophobic segments behaved similar to the mutant Δ1,2,3 and were mostly extracted into the supernatant fraction. Mutant Δ3, containing both the first and second hydrophobic segments, however, was present both in the pellet and in the supernatant fraction, indicating that this mutant was able to integrate into the membrane, but the interaction was not as stable as other integral membrane proteins (data not shown). Taken together, these results indicate that segment 3 may be the membrane anchor domain.

Deletion of hydrophobic sequences affects intracellular transport. Acquisition of endo H-resistant oligosaccharides by glycoproteins has been generally used as a measure of the rate of transport of the protein from the endoplasmic reticulum (ER) to the Golgi complex (37). To examine the rate of

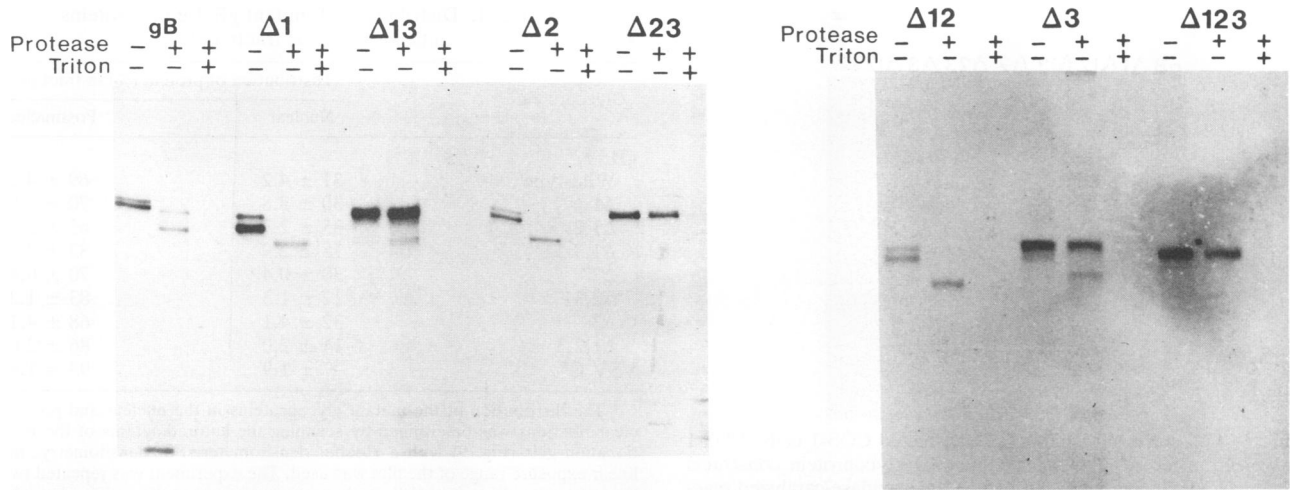

FIG. 4. Protease sensitivity of membrane-inserted wild-type and mutant gB proteins. Microsomal membranes containing labeled gB and mutant glycoproteins were isolated from COS-1 cells transfected with expression plasmids containing wild-type and mutant gB genes. At 40 h posttransfection, the proteins were labeled for 30 min and the microsomal membranes were pelleted as described in Materials and Methods. The suspended membrane vesicles were treated with 100 μg of trypsin per ml in the absence (−) or presence (+) of 1% Triton X-100 for 30 min on ice. The samples were immunoprecipitated with rabbit polyclonal anti-gB antibody and analyzed on an SDS–7.5% polyacrylamide gel and visualized by autoradiography.

intracellular transport of these mutant gB-1 glycoproteins compared with that of wild-type gB-1, the immunoprecipitates from a pulse-chase experiment were digested with endo H and analyzed on SDS-polyacrylamide gels. Figure 5 shows that the maturation of the oligosaccharides of the mutant gB-1 glycoproteins occurred at a much slower rate. For the sake of brevity, only the results with the wild type and Δ3, Δ2,3, and Δ1,2,3 mutants of gB are shown. The results for mutants Δ1, Δ1,2, Δ1,3, and Δ2 were similar to those for Δ2,3 and Δ1,2,3, that is, these mutants were only partially resistant to endo H, even after a 3-h chase (data not shown). It was observed that whereas the wild-type gB-1 became almost totally resistant to endo H after a 1-h chase period, the mutant glycoproteins were only partially resistant to endo H, even after a 3-h chase period. When the rates of endo H sensitivity of the various gB proteins were calculated, it was found that wild-type gB-1 had a half-life ($t_{1/2}$) of 27 min for transport to the Golgi complex, while the $t_{1/2}$ of Δ3 gB was 135 min, and mutants Δ1, Δ1,2, Δ1,3, Δ2, Δ2,3, and Δ1,2,3 had $t_{1/2}$s of more than 3 h.

Intracellular localization of mutant gB-1 glycoproteins by immunofluorescence staining. The gB glycoprotein has been shown to be localized in the ER, Golgi complex, and cell surface as well as in the nuclear envelope (3, 26, 65, 67) by immunofluorescence or immunoelectron microscopy. To determine the intracellular localization of mutant gB-1 proteins, COS-1 cells transfected with appropriate DNA were fixed with cold acetone and were reacted sequentially with anti-HSV-1 rabbit IgG and FITC-labeled goat anti-rabbit IgG. Fluorescence microscopic examination revealed that some of the mutant gB-1 constructs showed perinuclear fluorescence similar to wild-type gB-1. The staining patterns for mutants Δ1,3, Δ2,3, and Δ1,2,3 were somewhat different than the pattern observed with wild-type gB proteins. However, when the cells fixed with paraformaldehyde were examined for cell surface immunofluorescence, the wild-type gB-1 and mutant Δ3 gB showed cell surface labeling. However, mutants Δ1, Δ1,2, Δ1,3, Δ2, Δ2,3, and Δ1,2,3 failed to show any surface labeling (data not shown).

Cell surface iodination of transfected COS-1 cells. To determine the levels of the mutant gB-1 glycoproteins present on the cell surface in a more quantitative way, transfected COS-1 cells were radioiodinated in a lactoperoxidase-catalyzed reaction. The lysates of iodinated cells were immunoprecipitated with anti-HSV-1 antiserum and analyzed by SDS-PAGE. It is clear from Fig. 6 that no labeled protein could be detected in cells transfected with any of the gB mutants except Δ3. Cells transfected with wild-type gB-1

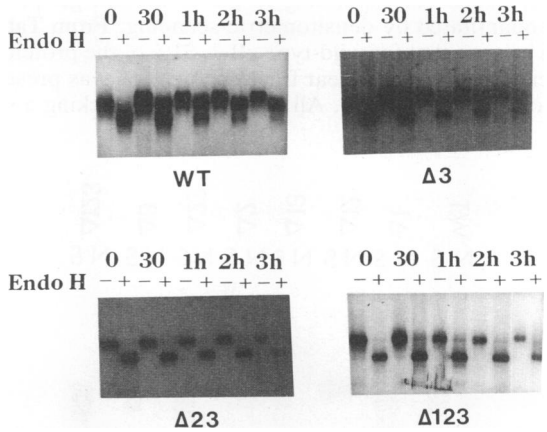

FIG. 5. Intracellular transport of mutant gB-1 glycoproteins, as determined by endo H digestion. COS-1 cells transfected with the mutant gB-1 constructs were pulse-labeled with [^{35}S]methionine 40 h posttransfection for 15 min and then chased with methionine-rich medium for 0 or 30 min or 1, 2, or 3 h. Cell lysates were immunoprecipitated with anti-HSV-1 antiserum and resuspended in sodium phosphate buffer, and an aliquot subjected to digestion with endoglycosidase H as described. Only the results of wild-type and gB-1 mutants Δ3, Δ2,3, and Δ1,2,3 are presented. Results obtained from the rest of the deletion mutants are indicated in the text. The quantitation of the bands by densitometry was done with exposures in the linear range of the films.

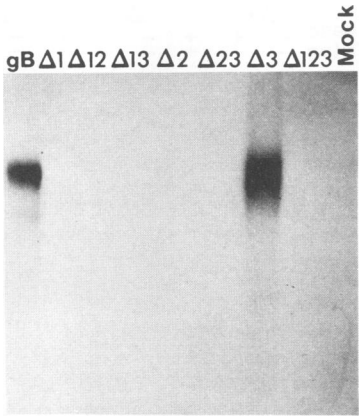

FIG. 6. Cell surface iodination of transfected COS-1 cells. COS-1 cells were transfected with the mutant gB-1 glycoprotein constructs and were radioiodinated with ^{125}I in a lactoperoxidase-catalyzed reaction 40 h posttransfection as described previously (54). Cell lysates were immunoprecipitated and analyzed by SDS-PAGE.

and mutant Δ3 showed strong surface labeling. These results agree with the immunofluorescence results described earlier which also showed that only wild-type gB and mutant Δ3 gB protein showed immunofluorescence staining of the cell surface.

Intracellular localization of mutant gB-1 glycoproteins by cell fractionation. It has been earlier shown by biochemical fractionation that glycoprotein gB is associated with the nuclear fraction of cells infected with HSV-1 (16) or transfected with gB-expressing plasmids (3, 54). We therefore determined the intracellular distribution of the mutant gB-1 glycoproteins by biochemical fractionation of the transfected cell extracts into nuclear and postnuclear fractions. The anti-HSV-1 antiserum immunoprecipitate from each fraction was analyzed on SDS-polyacrylamide gels (Fig. 7), and the protein bands corresponding to gB or mutant gB proteins were quantitated by densitometric scanning. From Table 1, it can be seen that for wild-type gB-1, 31% of the protein was associated with the nuclear fraction and 69% was present in the postnuclear fraction. All of the mutants lacking a single

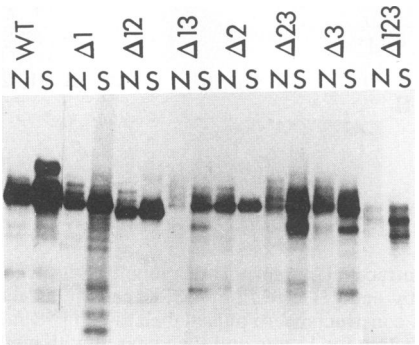

FIG. 7. Intracellular localization of mutant gB-1 glycoproteins by cellular fractionation. COS-1 cells were transfected with the mutant gB-1 glycoprotein constructs and 40 h later were labeled with [^{35}S]methionine and fractionated into the nuclear fraction (N) and postnuclear fraction (S). Each fraction was subjected to immunoprecipitation with anti-HSV-1 antiserum and analyzed by SDS-PAGE. The bands were quantitated by densitometry, and the results are summarized in Table 1.

TABLE 1. Distribution of mutant gB-1 glycoproteins into intracellular fractions[a]

Glycoprotein	Distribution of protein (%) in fraction:	
	Nuclear	Postnuclear
gB		
Wild-type[b]	31 ± 4.2	69 ± 4.2
Δ1	30 ± 3.8	70 ± 3.8
Δ1,2	35 ± 2.7	65 ± 2.7
Δ1,3	18 ± 2.9	82 ± 2.9
Δ2[b]	30 ± 0.4	70 ± 0.4
Δ2,3	17 ± 1.3	83 ± 1.3
Δ3	32 ± 4.1	68 ± 4.1
Δ1,2,3	14 ± 2.0	86 ± 2.0
VSV G[b]	7 ± 1.9	93 ± 1.9

[a] The distribution of the mutant glycoproteins in the nuclear and postnuclear fractions was determined by scanning the autoradiograms of the fractionation gels (Fig. 9) with a Hoeffer densitometer. For densitometry, the linear exposure range of the film was used. The experiment was repeated two times for each mutant, and the values shown represent the averaged results from four independent scans of each lane, with the error expressed as the standard deviations of the averaged values.

[b] For wild-type gB and vesicular stomatitis virus (VSV) glycoprotein G, four independent fractionations were performed, while for Δ2, the values were obtained from only one fractionation. The vesicular stomatitis virus glycoprotein G was expressed by using expression plasmid pSVGL (29).

segment and mutant Δ1,2 had about 30 to 35% of the total gB-1 glycoprotein in the nuclear fraction. The amount of mutant Δ1,3, Δ2,3, and Δ1,2,3 glycoproteins present in the nuclear fraction was reduced to 14 to 18%. For a control, we also determined the localization of vesicular stomatitis virus glycoprotein G which is expressed at the cell surface (1, 56). Only about 7% of the total G protein expressed in COS-1 cells was present in the nuclear fraction.

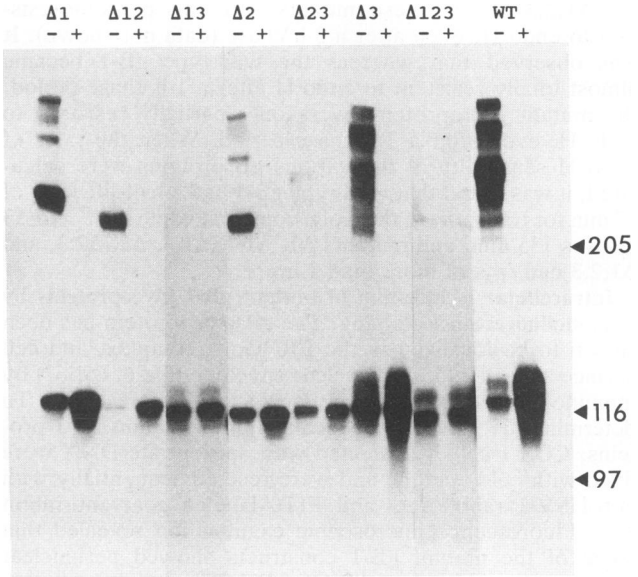

FIG. 8. Analysis of oligomer formation of mutant gB-1 glycoproteins by SDS-PAGE. COS-1 cells transfected with wild-type (WT)- and mutant-gB-containing plasmids were labeled with [^{35}S]methionine at 40 h posttransfection, and cell extracts were obtained under nondenaturing conditions. A portion of the sample was heated and the unheated (−) and heated (+) samples were analyzed on a polyacrylamide gel containing 0.3% SDS. The numbers to the right of the gel show the sizes (in kilodaltons) of molecular mass markers.

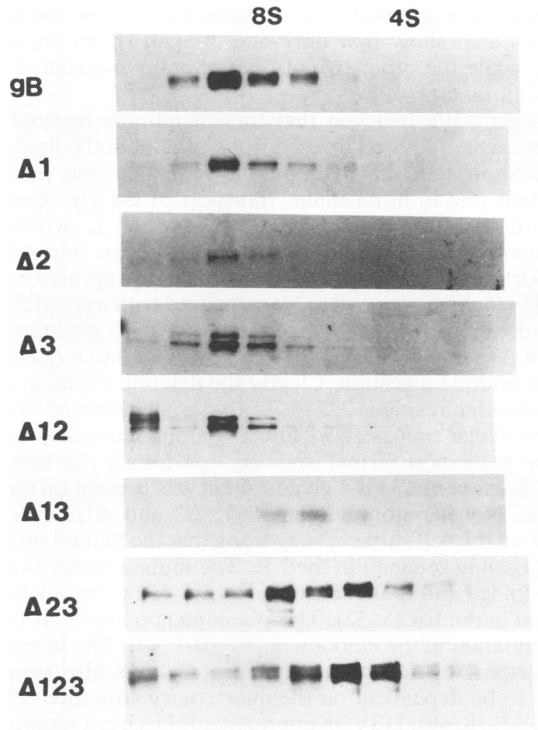

FIG. 9. Analysis of oligomer formation of mutant gB-1 glycoproteins by sucrose gradient sedimentation. Transfected COS-1 cells were labeled with [^{35}S]methionine 40 h posttransfection, and the [^{35}S]methionine-labeled gB proteins expressed were extracted under nondenaturing conditions and analyzed on a 5 to 20% sucrose gradient as described in Materials and Methods. Alternate fractions were immunoprecipitated with anti-gB antibody and analyzed by SDS-PAGE. Fraction 1 corresponded to the bottom of the gradient. The positions of 8S and 4S markers are indicated at the top of the figure.

Oligomerization of mutant gB-1 glycoproteins. The proper transport of a newly synthesized membrane glycoprotein from the ER to the cell surface depends on the acquisition of correct tertiary and quaternary structure (32, 38). Since gB glycoproteins of HSV-1 form dimers (2, 14, 31, 59, 65, 69), the oligomeric states of the mutant gB glycoproteins were also examined. Cells transfected with mutant constructs were pulse-labeled with [^{35}S]methionine for 15 min followed by a 2-h chase. Cell extracts were immunoprecipitated with monoclonal 3S anti-gB and extracted in the appropriate buffer. Samples were divided into two aliquots, and one aliquot was subjected to 2 min of boiling prior to SDS-PAGE. The results show that all mutants except for Δ1,3, Δ2,3, and Δ1,2,3 formed dimers which dissociated on heating, suggesting that segment 3 in some way may promote formation of gB dimers (Fig. 8). Multiple forms of oligomers of gB and mutants Δ3 and Δ1 were also observed. The presence of similar multiple oligomeric species of gB was also reported in HSV virions or infected cells (14). These results were confirmed by sucrose gradient sedimentation analysis of labeled cell extracts obtained from cells transfected with mutant gB constructs. Fractions collected from these gradients were immunoprecipitated with polyclonal anti-gB antibody, and the immunoprecipitates were analyzed by SDS-PAGE. The results presented in Fig. 9 also show that mutants lacking segment 3 and any other segment (that is, mutants Δ1,3, Δ2,3, and Δ1,2,3) were present in the regions of the gradient containing monomers only, while mutants containing segment 3 or segments 1 and 2 together formed dimers. The mutants that form dimers either have segment 3 present (mutants Δ1, Δ2, and Δ1,2) or both segments 1 and 2 present (mutant Δ3). It thus appears that if segment 3 is not present, both segments 1 and 2 must be present for dimerization. However, segment 3 alone is sufficient for dimers to form, since mutant Δ1,2 forms dimers.

Complementation of a gB-null virus by the gB-1 deletion mutants. To determine the abilities of the mutant gB proteins to complement the gB defective virus K082, a complementation assay using the mutant constructs was performed as described previously (11). Briefly, Vero cells were transfected with plasmid pKBXX (11, 11a) containing the mutant constructs. These cells were subsequently infected with the K082 virus which contains a nonsense mutation at residue 43 of gB (11, 11a). This virus can grow only on a gB-producing cell, such as D6 cells (11, 11a). The titers of virus stocks obtained from transfected Vero cells after infection with K082 virus were determined for both Vero and D6 cells. If the mutant gB construct had the ability to complement, then titers on the D6 cells would be at least 100-fold higher than titers on Vero cells (11). It was found that none of the mutant constructs were able to complement K082 (Table 2). However, when Vero cells were transfected with pKBXX plasmid containing wild-type gB gene followed by infection with K082 virus, the virus titer on D6 cells was about 150-fold

TABLE 2. Complementation of K082 virus by transfection with plasmids containing mutant gB constructs[a]

Transfecting plasmid containing gB construct	Virus titer (mean ± SEM) on cell line:		Plaquing efficiency in Vero cells[b]	Complementation efficiency (%)[c]
	Vero (10^4 PFU/ml)	D6 (PFU/ml)		
Wild type	2.3 ± 0.3	3.46 ± 0.62 × 10^6	150	100
Δ1	2.4 ± 0.2	2.8 ± 0.2 × 10^4	1.20	0.80
Δ12	2.8 ± 0.4	2.4 ± 0.3 × 10^4	0.86	0.57
Δ13	2.6 ± 0.4	2.2 ± 0.3 × 10^4	0.85	0.57
Δ2	2.7 ± 0.3	3.2 ± 0.4 × 10^4	1.20	0.80
Δ23	2.8 ± 0.5	2.7 ± 0.3 × 10^4	0.96	0.64
Δ3	2.5 ± 0.2	2.9 ± 0.4 × 10^4	1.20	0.80
Δ123	1.5 ± 0.2	1.4 ± 0.1 × 10^4	1.93	0.62
Control (no plasmid)	2.2 ± 0.1	2.7 ± 0.2 × 10^4	1.21	0.80

[a] Vero cells were transfected with pKBXX plasmid containing the wild-type gB or mutant gB constructs. Transfected cells were infected with K082 virus at a multiplicity of infection of 2 after 24 h. Virus stocks were isolated 24 h later and titered on both Vero and D6 cells. Titers are average of at least two sets of separate complementation experiments.

[b] Plaquing efficiency of complemented virus is expressed as the ratio of titer on D6 cells to the titer on Vero cells.

[c] Complementation efficiency is expressed as the percentage of plaquing efficiency of virus produced by transfection with wild-type gB plasmid pKBXX.

higher than the titer on Vero cells because of complementation by the wild-type gB glycoprotein.

DISCUSSION

The glycoprotein gB-1 is a transmembrane protein containing a hydrophobic stretch of 69 amino acids near the COOH terminus. It has been predicted that this hydrophobic domain could traverse the membrane three times. We have made deletion mutations encompassing segments of the hydrophobic region and tested the mutants for their membrane-anchoring ability by three types of assays: (i) protection from protease digestion of the fully translocated protein into microsomal vesicles, (ii) extraction of nonintegral membrane proteins with alkali, and (iii) secretion into the medium. The results presented here show that deletion of segments 1, 2, and 3 together produced a protein which did not behave like an integral membrane protein but behaved more like a secretory protein containing no membrane anchor domain. The results thus established that the hydrophobic stretch of 69 amino acids contained the membrane anchor sequence of gB. Deletion of segment 3 along with segment 2 or 1 produced mutant proteins which behaved like mutant Δ1,2,3. In contrast, mutant proteins lacking segment 1 or 2, either alone or in combination, led to the formation of proteins which behaved like the wild-type gB. Wild-type gB, as well as mutant Δ1, Δ2, and Δ1,2 proteins showed the typical characteristics of integral membrane proteins. It appears, therefore, that segment 3 was sufficient for anchoring of the gB protein in cell membranes. However, analysis of the mutant lacking only segment 3 showed that although a major fraction of the protein behaved like a secretory protein, a small fraction was sensitive to protease digestion and resistant to alkali extraction and was present in the cell surface. These data suggest that segments 1 and 2 could possess membrane-anchoring activity, but the interaction is weak, and thus most of the Δ3 mutant behaves like a secretory protein. A mutant lacking segment 1 and 3 also showed that a very small amount of protein was sensitive to proteolysis and resistant to alkaline extraction, suggesting that segment 2 may also possess a weak membrane anchor activity. That segment 1 did not have any membrane-anchoring potential on its own was shown by the fact that mutant Δ2,3 behaved like mutant Δ1,2,3 and produced a secretory protein. These results suggest that segment 3 is required for membrane anchoring of gB-1 glycoprotein. Segment 2, which has a hydrophobicity of 1.7 (compared with the hydrophobicity of 2.4 of segment 3) can also interact with the membrane, but the interaction is not strong enough for a stable membrane anchorage. It is possible that segment 2 is merely embedded on the surface of the membrane. That segment 3 of gB-1 glycoprotein may be the segment involved in stable interaction with the membrane was further supported by in vitro translocation studies using synthetic RNAs from the mutant glycoprotein genes. In an in vitro transcription-translation system, mutant glycoproteins synthesized in the presence of dog pancreas microsomes were completely translocated and hence, totally protected from proteolytic digestion when segment 3 was absent from the synthetic RNA (63). Taken together, these results suggest that in common with other viral glycoproteins (51), glycoprotein gB is anchored to the membrane by a single span of hydrophobic sequence including residues 775 to 795 (segment 3). The other two segments could be interacting with the membrane, but not in a transmembrane orientation. Studies with the rotavirus nonstructural protein NS28 which contains three putative hydrophobic membrane anchoring domains also show that only one domain spans the membrane, while the other two are peripherally associated with the membrane (4, 12).

It is generally believed that transmembrane hydrophobic sequences are required to anchor the protein in the lipid. Our results show that the hydrophobic sequence also plays an important role in intracellular transport of the glycoprotein. Deletion mutants such as Δ1, Δ2, and Δ1,2, which are anchored to the membrane, showed defective intracellular transport of the glycoprotein. The mutant glycoproteins were endo H sensitive, even after 3 h compared with a $t_{1/2}$ of 27 min for endo H digestion of the wild-type gB-1 glycoprotein. Mutant Δ3 which is also secreted, however, had a $t_{1/2}$ of 135 min for endo H digestion. Clearly the deletion of amino acids located within residues 727 to 795 drastically affected the rate of intracellular transport. Results of immunofluorescence and surface iodination further showed that for all the mutants, only a fraction of Δ3 gB-1 glycoprotein was present on the cell surface. Nonsecretory mutants Δ1, Δ2 and Δ1,2 failed to appear on the cell surface, suggesting that the mutant proteins were possibly retained in the ER. The mutant proteins could be misfolded and thus recognized by the ER protein BIP and retained in the ER (5, 32). This phenomenon has been shown for a mutation in the ectodomain of gB-1 (47). The intracellular transport of membrane glycoproteins has also been reported to be dependent on the quarternary structure (17, 20, 21, 25, 32, 38, 49). HSV glycoprotein gB has been reported to be a dimer (2, 14, 31, 59, 65, 69), and several groups have worked on identifying the sequences required for dimerization. Highlander et al. (31) have identified two regions essential for dimerization: (i) an upstream site composed of residues 93 to 282 and (ii) a downstream site composed of residues 596 to 711. Our studies could not detect any gB dimers with mutants Δ1,2,3, Δ2,3, and Δ1,3, all of which were not anchored to membranes, suggesting that stable anchoring to the membrane may be needed for dimerization. In contrast, Quadri et al. (52) have reported that membrane anchoring is not required for dimerization. It should be noted that mutant pTR690 lacked the entire 69-residue hydrophobic region as well as 84 residues of the cytoplasmic tail (52), whereas the deletion mutants used in the present study contained the entire cytoplasmic tail. The presence of the cytoplasmic tail in our mutants could affect the stability of the dimer, resulting in the observed absence of dimerization for mutants not anchored to membranes. The temperature-sensitive mutant tsB5 does not form dimers at the nonpermissive temperature (30, 58), and at permissive temperature, the dimer is unstable (13, 30). However, the two point mutations in tsB5 are located at amino acid positions 552 and 857 (9), both of which are outside the proposed regions involved in oligomerization (31). The mutations in these two positions could affect the stability of the dimer because of improper folding of the mutant glycoprotein.

We have earlier shown that gB-1 glycoprotein expressed in the absence of other HSV-1 proteins was localized in the nuclear envelope (3). Immunofluorescence studies (36) and biochemical fractionation studies (16) with HSV-1-infected cells have also shown the localization of gB-1 protein in the nucleus. Recent studies using immunoelectron microscopy have confirmed that gB-1 protein is localized in the inner membrane of HSV-1-infected cells (26, 67). Results presented here show that the mutant glycoproteins containing segment 3 are associated with the nuclei. Thus, although these mutants are defective in intracellular transport to the Golgi complex or the cell surface, they form dimers and are transported to the nuclear envelope in a manner similar to

that of the wild-type protein and could, therefore, be incorporated into the virion particles. The results of the complementation experiments showed that none of the mutants could produce infectious virion particles. These results agree with those of Cai et al. (11). In their experiments using linker insertion addition and chain termination mutations in gB, they found that addition mutations at residue 734, which is located in segment 1, abolished complementation. Chain termination mutations within the 69-amino-acid hydrophobic domain also had no complementation activity. Studies with temperature-sensitive mutants such as tsB5 (58) and tsJ12 (42) as well as the gB-null virus K082 (10, 11) showed that noninfectious virion particles lacking any gB protein can attach to cells but fail to penetrate them (10, 11, 42, 58). Experiments with a gB-null syncytial virus, *syn*082, established that gB is essential for HSV-1-induced cell fusion (10). Complementation studies with a number of gB mutants further showed a strong correlation between complementation and fusogenic property of gB (10). The failure of the deletion mutants to complement K082 could be explained as follows. (i) Mutants Δ1,3, Δ2,3, and Δ1,2,3 lacked a membrane-anchoring domain and thus failed to be incorporated in the virion particles. (ii) Mutants Δ1, Δ1,2, and Δ2 were stably anchored to the membrane as well as associated with the nuclei and thus could be incorporated into the virion particles. These particles were noninfectious, however, suggesting the possibility that the virion particles containing mutant gB proteins lacking segments of the hydrophobic sequences were incompetent for virus entry. Thus, the hydrophobic region may be involved in the structural determinants of gB required for penetration as well as cell fusion. (iii) Alternatively, mutants Δ1, Δ1,2, and Δ2, although associated with the nuclear membrane, could not be incorporated in the virion particles, suggesting that the hydrophobic stretch of gB is required for its assembly in the viral envelope.

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