

Mutations in the Carboxyl-Terminal Hydrophobic Sequence of Human Cytomegalovirus Glycoprotein B Alter Transport and Protein Chaperone Binding

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Received 7 May 1996/Accepted 23 July 1996

Human cytomegalovirus glycoprotein B (gB) plays a role in the fusion of the virion envelope with the host cell membrane and in syncytium formation in infected cells. Hydrophobic sequences at the carboxyl terminus, amino acids (aa) 714 to 771, anchor gB in the lipid bilayer, but the unusual length of this domain suggests that it may serve another role in gB structure. To explore the function(s) of this region, we deleted aa 717 to 747 (gB Δ I mutation), aa 751 to 771 (gB Δ II mutation), and aa 717 to 772 (gB Δ I-II mutation) and constructed a substitution mutation, Lys-748 to Val (Lys748Val)-Asn749Ala-Pro750Ile (gB KNP^m). Mutated forms of gB were expressed in U373 glioblastoma cells and subjected to analysis by flow cytometry, confocal microscopy, and immunoprecipitation. Mutations gB Δ I-II and gB Δ II alone caused secretion of gB into the medium, confirming that aa 751 to 771 function as a membrane anchor. In contrast, mutations gB Δ I and gB KNP^m blocked cell surface expression and arrested gB transport in the endoplasmic reticulum (ER). Detailed examination of gB Δ I and gB KNP^m with a panel of monoclonal antibodies showed that the mutated forms were indistinguishable from wild-type gB in conformation and formed oligomers; however, they remained sensitive to endoglycosidase H and did not undergo endoproteolytic cleavage. Analysis of protein complexes formed by gB and molecular chaperones in the ER showed that calnexin and calreticulin, lectin-like chaperones, bound equal amounts of uncleaved wild-type gB, gB Δ I, and gB KNP^m, but the glucose-regulated proteins 78 (BiP) and 94 formed stable complexes only with the mutated forms, causing their retention in the ER. Our studies show that aa 714 to 750 are key residues in the architecture of gB molecules and that the ER chaperones, which facilitate gB folding and monitor the quality of glycoproteins, detect subtle changes in folding intermediates that are conferred by mutations in this region.

Human cytomegalovirus (HCMV) is a widespread infectious agent that causes severe morbidity and mortality in congenitally and perinatally infected newborns (34, 35, 52), immunosuppressed patients, including organ transplant recipients (27), and patients with AIDS (13). HCMV infection of the retina affects 20 to 30% of patients with AIDS (14, 15, 21). Glycoprotein B (gB) is the major target of neutralizing antibodies in HCMV-infected patients (4, 6, 39). Analysis of the nucleotide sequence of HCMV strain AD169 indicates that open reading frame UL55 encodes the homolog of herpes simplex virus type 1 (HSV-1) gB (8). gB is the most highly conserved of the herpesvirus glycoproteins, is required for virion infectivity (reviewed in reference 37), and functions in virion entry into cells by promoting fusion of the virion envelope with the plasma membrane (30, 56, 57).

HCMV strain AD169 gB consists of a signal peptide (amino acids [aa] 1 to 24), an ectodomain (aa 25 to 713), a hydrophobic carboxyl-terminal domain (aa 714 to 771), and an intracellular charged carboxyl tail (aa 772 to 906). The ectodomain is endoproteolytically processed between Arg-459 and Ser-460, which is mediated by furin in a post-endoplasmic reticulum (post-ER) compartment (51, 58). Following cleavage, the N- and C-terminal products remain associated by disulfide linkages (50). The long hydrophobic domain near the carboxyl

terminus, consisting of two hydrophobic segments separated by 3 aa, functions as a membrane anchor (51). Amino acid sequence analysis showed that this region is conserved among gBs of the α , β , and γ herpesviruses (see Fig. 1A). We and others have reported that truncation of the entire carboxyl terminus and the hydrophobic region results in secretion of HCMV gB into the culture medium (42, 51). Analysis of the secondary structure of HSV-1 gB predicted that the corresponding hydrophobic carboxyl region crosses the membrane three times (36); however, it was experimentally shown that hydrophobic segment II (aa 751 to 771 [see Fig. 1B]) is sufficient for membrane anchoring of HSV-1 gB (45) and HCMV gB (47). Thus, aa 714 to 750 may serve a role in the architecture of gB that is distinct from membrane anchoring.

In this study, we explored the effect of changes in the carboxyl-terminal hydrophobic region of HCMV gB by analyzing the processing and transport of gB with site-specific mutations in this region. We showed that mutated forms lacking this region are secreted into the medium and confirmed that hydrophobic segment II anchors gB in the plasma membrane. Our studies revealed that hydrophobic segment I and the intervening amino acids KNP play a central role in promoting the transport of gB through the exocytic pathway. Mutations in segment I and KNP arrested the transport of gB in the ER, prevented cleavage, and promoted the formation of stable complexes between gB and the ER chaperones glucose-regulated protein 78 (GRP78) and GRP94. Our results indicate that hydrophobic segment I and KNP are pivotal in the configuration of gB and hence in its transport.

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MATERIALS AND METHODS

Site-directed mutagenesis of HCMV gB and vector construction. The isolation and cloning of the coding sequence for HCMV gB (strain AD169) as a 3.1-kb *EagI* fragment were reported previously (42). This sequence was cloned into pBluescript (Stratagene) at the *NotI* site. For the convenience of site-directed mutagenesis and complete DNA sequencing, a 443-bp *EcoRI-NsiI* fragment containing the gB hydrophobic region was subcloned in pBluescript after the appropriate modifications of multiple cloning sites (an *NsiI* linker was inserted at the *KpnI* cloning site). Site-directed mutagenesis was done by the Kunkel method (26a). The uracil-containing single-stranded DNA was isolated from *Escherichia coli* CJ236 after superinfection of M13KO7 helper phage (Bio-Rad). The oligonucleotides were synthesized by the University of California San Francisco Bio-molecular Resource Facility. T7 DNA polymerase was used to synthesize the complementary DNA strand. After mutagenesis, the whole gB subclone was sequenced by the dideoxy-chain termination method (49).

For in-frame deletion of the hydrophobic segments of gB, oligonucleotides were designed to introduce *NruI* sites at codons 716, 747, 751, and 772. The oligonucleotide sequences and expected codon changes by this substitution mutagenesis were as follows (the *NruI* sites are underlined, and the substituted sequences are in lowercase letters): 5'-CGCCCAgTcgcgaCATGAGGTC-3' changed codon G-717 to R; 5'-GGGGTTTcgcgaGAAGGTGGC-3' changed codons L-747-K-748 to SR; 5'-GAAGGCTCgcgAGGGGTTTTTG-3' changed codons F-751-G-752 to SR; and 5'-CGCTGTCCgcaATAGATCAAATAA-3' changed codon T-772 to S. The deletion of gB hydrophobic segments by the introduction of two *NruI* sites and subsequent ligation resulted in an in-frame downstream coding sequence. For site-specific mutagenesis of aa 748 to 750, oligonucleotide 5'-GGTCCGAAtattgcacGAGGAAGGTG-3' (the introduced *SspI* site is underlined) was designed to replace amino acids K-748-N-749-P-50 with VAI (resulting in the mutant KNP^{mut}). The mutated gB coding sequences in pBluescript were removed as an *EagI* fragment to a eukaryotic expression vector pRc/CMV (Invitrogen) at the *NotI* site under the control of the HCMV immediate-early gene promoter. For transient expression of mutant gB protein in the T7 RNA promoter/T7 RNA polymerase system (28), an *NcoI* site was introduced at the gB initial codon by oligonucleotide 5'-GGATTCCATGgTCGTCGCGG-3' (the *NcoI* site is underlined). Then, the gB coding sequence was cloned in the pTMI vector at *NcoI-SpeI* sites.

Cell cultures and stock viruses. U373MG, a glioblastoma cell line, was obtained from the American Type Culture Collection (ATCC HTB17) and was grown in Dulbecco's minimum essential medium (DMEM)-high-glucose containing 10% fetal calf serum (FCS) (Hyclone) and antibiotics. Recombinant vaccinia virus vTF7-3 (28) was obtained from the American Type Culture Collection, and viral stock was prepared and titrated in Vero cells.

DNA transfection and vaccinia virus infection. Polycation polymer polybrene (Sigma) was used as an efficient transfection agent for glioblastoma cells. For optimal transfection efficiency, 13 μ g of polybrene, 6.5 μ g of CsCl gradient-purified DNA, and 5×10^6 PFU of recombinant vaccinia virus vTF7-3 were mixed in 1 ml of culture medium and incubated with a confluent cell sheet (5×10^5) in a 35-mm-diameter well for 12 h. Then, the cells were treated for 2 min with medium containing 30% dimethyl sulfoxide.

Immune reagents. Murine monoclonal antibodies (MAbs) to HCMV gB were as described previously (30, 38, 42). Antibodies to protein chaperones (murine MAb to GRP78, rat MAb to GRP94, and rabbit polyclonal antibody to the carboxyl terminus of calnexin) were purchased from StressGen. Rabbit polyclonal antibody to calreticulin was purchased from Affinity BioReagents. Goat anti-mouse immunoglobulin G (IgG) conjugated with fluorescein isothiocyanate (FITC) or Texas red was purchased from Caltag. Goat anti-rat IgG conjugated with Texas red and LcH agglutinin from plant lentil (*Lens culinaris*) conjugated with FITC were purchased from E-Y Laboratories.

Immunofluorescence and confocal microscopy. Expression of HCMV gB was detected by indirect immunofluorescence assays using murine MAbs (30, 38, 42). At 24 h after transfection and infection with vaccinia virus vTF7-3, U373 cells on glass coverslips were fixed in 80% methanol at -20°C and reacted with the individual MAbs or a pool of MAbs to gB in phosphate-buffered saline (PBS) with 10% FCS (1:200) for 1 h at 37°C . Cells were washed in PBS three times, and goat anti-mouse IgG conjugated with FITC or Texas red, diluted (1:200) in PBS with 10% FCS, was added and incubated for 30 to 60 min at 37°C . Next, the cells were reacted with antisera to calnexin, which was used as an ER marker, and this was followed by reactions with Texas red-conjugated secondary goat anti-rat IgG and FITC-conjugated LcH agglutinin, which was used as a Golgi marker. Cells were examined for immunofluorescence with a Bio-Rad MRC600 confocal microscope.

Flow cytometry analysis. U373 cells stably transfected with the mutated gB constructs were selected as described previously (56). The cells were detached from the wells by using cell dissociation buffer (PBS based, enzyme free; GIBCO-BRL). The pooled MAbs to HCMV gB were added (1:200) in PBS with 10% FCS and incubated at 4°C for 1 h. The cells were washed in PBS, and FITC-conjugated goat anti-mouse IgG was added (1:200) with 10% FCS and incubated at 4°C for 30 min. Afterwards, the cells were washed in PBS, propidium iodide was added to a final concentration of 2 μ g/ml, and 10^4 cells per sample were subjected to analysis in a FACS analyzer IV (Becton Dickinson, Mountain View, Calif.).

Radiolabeling and immunoprecipitation of proteins. U373 cells were transfected using the polybrene method and infected with vTF7-3 as described above. Immediately after dimethyl sulfoxide shock, cells were labeled with [^{35}S]Trans-label (100 mCi/ml; specific activity, 10 μ Ci/ml; ICN) in methionine- and cysteine-free DMEM without serum for 12 to 16 h. For pulse-chase experiments, cells were incubated in DMEM supplemented with 10% FCS for 1.5 h to allow cells to express gB after dimethyl sulfoxide shock. Then, cells were washed twice with PBS and starved in methionine- and cysteine-free DMEM for 30 min at 37°C before they were labeled for 30 min. Following extensive washing, the monolayers were chased in DMEM containing 10% FCS and 10-fold concentrations of unlabeled methionine and cysteine. Both medium and cell fractions were suspended in PBS with 1% Nonidet P-40, 1% sodium deoxycholate, and 100 μ g of phenylmethylsulfonyl fluoride per ml. MAb CH177-3 was bound to protein A-Sepharose CL-4B beads (Sigma) and used to immunoprecipitate HCMV gB as described previously (38). Precipitated proteins were eluted in sample buffer containing 2% sodium dodecyl sulfate (SDS) and 2% β -mercaptoethanol by boiling for 5 min and were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (9% polyacrylamide) in gels cross-linked with diallyltartardiamide. For nonreducing gels, proteins were eluted at room temperature in 2% or 0.2% SDS without β -mercaptoethanol, with or without boiling for 3 min, and analyzed by SDS-PAGE (7% polyacrylamide). The gels were dried and autoradiographed on Kodak BioMAX film. The [^{14}C]-radiolabeled rainbow molecular mass markers (Amersham) used were myosin (220 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

For coimmunoprecipitation experiments, U373 cells were transfected and infected as described above and then labeled for 12 h immediately after dimethyl sulfoxide shock. In one mock-transfected sample, tunicamycin (1.5 μ g/ml) was added during transfection and radiolabeling. Cells were solubilized with 2% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate) in HBS (200 mM NaCl, 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.5]) for 30 min on ice, and then aprotinase (10 U/ml) and phenylmethylsulfonyl fluoride (100 μ g/ml) were added. Murine MAb to GRP78, rat MAb to GRP94, and rabbit polyclonal antisera to calnexin and calreticulin were diluted 1:200 for immunoprecipitation. Binding reactions of antibodies with antigens were done at room temperature for 10 min, and then the immune complexes were mixed with protein A-Sepharose CL-4B for 10 min and washed five to six times in buffers containing 2% CHAPS in HBS. Calreticulin immunoprecipitates were passed through a 2 M sucrose cushion and then washed twice with 2% CHAPS.

Carbohydrate analysis. Endoglycosidase H (endo H) and peptide-N-glycosidase F (PNGase F) were used as instructed by the manufacturer (Boehringer Mannheim). Immunoprecipitated proteins from approximately 10^6 transfected cells were eluted in 50 mM sodium phosphate buffer (pH 6.0) containing 1% SDS-2% β -mercaptoethanol and boiled for 5 min. Then, the denatured samples were divided into three aliquots for endo H, PNGase F, or mock digestion. For endo H digestion, samples were diluted to final concentrations of 0.1% SDS, 1% β -mercaptoethanol, 100 μ g of phenylmethylsulfonyl fluoride per ml, 0.5% Nonidet P-40, and 50 mM sodium phosphate (pH 5.5); for PNGase F digestion, the buffer was pH 7.2. Digestions were done at 37°C for 9 h with 5 mU of endo H or 1 U of PNGase F.

RESULTS

Site-directed mutagenesis of the hydrophobic domain of HCMV gB. The carboxyl-terminal hydrophobic sequences within the domain of the herpesvirus gB homologs are shown (Fig. 1A and B). To explore the functions of the carboxyl-terminal hydrophobic region of HCMV gB, we first constructed site-specific deletion and substitution mutations (Fig. 1C). The two hydrophobic segments, which are separated by the amino acids Lys-Asn-Pro, were deleted separately or together by introducing two *NruI* sites (TCG CGA) into the gB gene at codons 716, 747, 751, and 772. The replacement of two amino acid codons with TCG CGA, encoding Ser and Arg, created an *NruI* site. Cleavage of this site produced blunt ends, which maintained the reading frame after the fragment was deleted. Three deletion mutations were constructed: deletion of hydrophobic segment I (aa 717 to 747) (gB Δ I), deletion of hydrophobic segment II (aa 751 to 771) (gB Δ II), and deletion of both hydrophobic segments (aa 717 to 772) (gB Δ I-II). As a result of these deletions, Lys-748 was changed to Arg in the Δ I mutation and Thr-772 was changed to Ser in the Δ II mutation. These conservative substitutions were not expected to change the effects of the large deletions. Next, we constructed site-specific mutations, which changed K-748 to V, N-749 to A, and

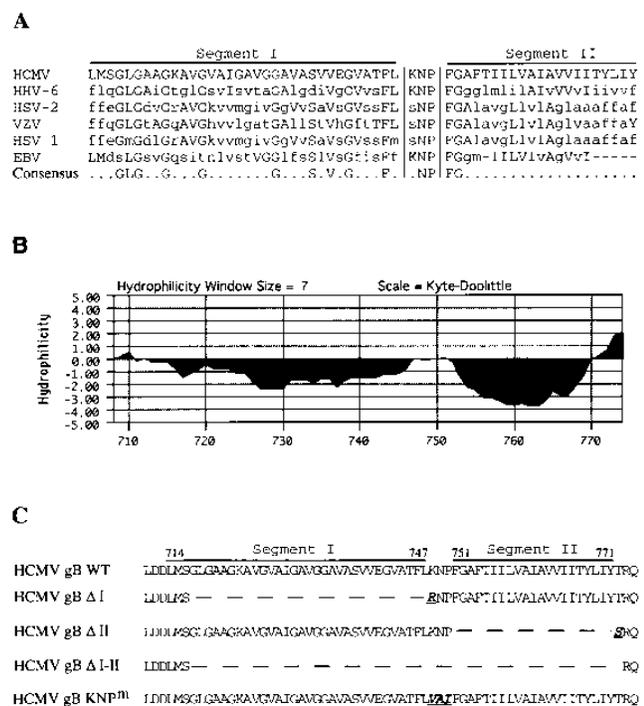


FIG. 1. Sequences in the carboxyl-terminal hydrophobic region (aa 714 to 771) of HCMV gB. (A) Alignment of the hydrophobic sequences of human herpesvirus gB homologs. HCMV gB hydrophobic segments I and II are indicated with horizontal bars separated by KNP. HHV-6, human herpesvirus 6; VZV, varicella zoster virus; EBV, Epstein-Barr virus; Consensus, most-conserved amino acid residues. (B) Kyte-Doolittle hydropathy plot of the HCMV gB hydrophobic region. (C) Mutations in the hydrophobic region of gB. Lines indicate deletions; substitutions are underlined and indicated in boldface italic type.

P-750 to I as described in the Materials and Methods section. The three residues that separate hydrophobic segments I and II, KNP, are of particular interest. K is positively charged, N is highly hydrophilic, and P tends to break the membrane-spanning α -helix structure by favoring a β -turn structure (11). The substitution of VAI for these three amino acid residues (yielding the mutant KNP^m) would result in a continuous hydrophobic sequence from aa 714 to 771.

Deletion of hydrophobic segment II alone or of I and II together causes secretion of gB. In the first set of experiments, we analyzed the synthesis of gB and determined whether the mutated forms were secreted by immunoprecipitating gB from cell extracts and culture fluids. U373 cells were transfected with plasmid DNA encoding the gB constructs, infected with vaccinia virus vTF7-3, and the glycoproteins were precipitated with a pool of MAbs to HCMV gB. Analysis of the immunoprecipitates electrophoresed in denaturing SDS-PAGE gels revealed that the expressed forms of gB were present in cell extracts and that some were secreted into the culture medium, depending on the mutation (Fig. 2). Cell extracts alone contained wild-type (WT) gB (Fig. 2, lane 2) and the mutated forms gB Δ I (Fig. 2, lane 4) and gB KNP^m (Fig. 2, lane 10), whereas gB Δ II and gB Δ I-II were present in the cell extracts (Fig. 2, lanes 6 and 8) and also secreted into the culture medium (Fig. 2, lanes 5 and 7). Deletion of segment II alone was sufficient to release gB into the medium, which indicates that hydrophobic segment II anchors gB in the membrane. In this experiment (Fig. 2, lanes 5 to 8) and others (Fig. 5, lanes 7 to 12), it appeared that less gB Δ II than gB Δ I-II was secreted

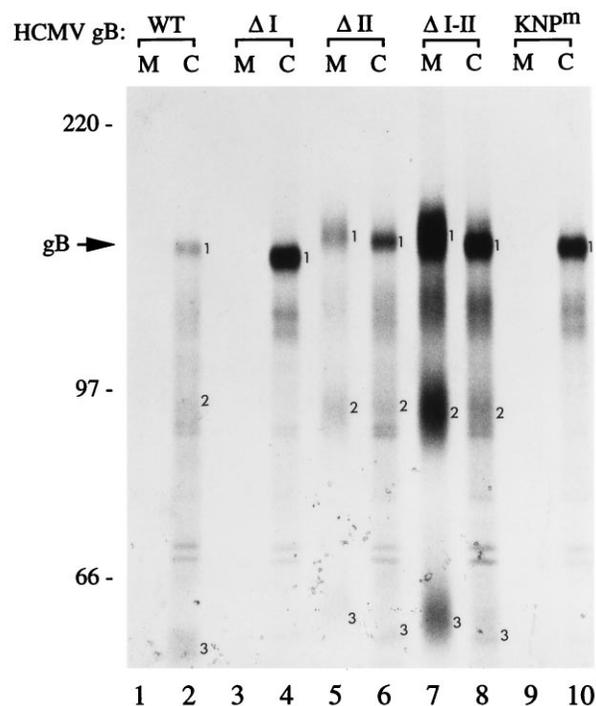


FIG. 2. Immunoprecipitation of WT gB and mutated forms gB Δ I, gB Δ II, gB Δ I-II, and gB KNP^m from the medium (M) and cell extracts (C) of transfected U373 glioblastoma cells. Radiolabeled proteins (³⁵S]Trans-label) were boiled in sample buffer with 2% SDS and β -mercaptoethanol and separated in denaturing SDS-9% polyacrylamide gels. Uncleaved forms of gB are indicated with an arrow (band 1); N-terminal (band 2) and C-terminal (band 3) endoproteolytic products are also indicated. Molecular mass markers are indicated at the left in kilodaltons.

into the medium. In addition to uncleaved gB molecules (band 1), WT gB and the Δ II and Δ I-II gB forms were cleaved into N-terminal (band 2) and C-terminal (band 3) halves. The secreted gB forms Δ II and Δ I-II gB, with deletions of 21 and 56 aa, respectively, had higher apparent molecular masses (bands 1 and 3) than WT gB. Since WT gB has 21 potential N-glycosylation sites, 20 in the ectodomain and 1 in the cytoplasmic tail (10), the decrease in electrophoretic mobility may result from use of the N-glycosylation site at Asn-863 after translocation of the mutated forms into the ER lumen.

Deletion of hydrophobic segment I and the site-specific mutation KNP^m preclude gB transport. To study the intracellular transport of the mutated forms of HCMV gB, we analyzed transfected U373 cells by immunofluorescence confocal microscopy. The transfected cells were stained with the pool of MAbs to gB and then with reagents to identify either ER or Golgi. The ER was identified with a MAb to calnexin, a resident ER protein (12), and the Golgi complex was identified with LcH agglutinin (43). We found that WT gB (Fig. 3A and D) and the mutated forms gB Δ II (Fig. 3E and H) and gB Δ I-II (Fig. 3I and L) stained predominantly with a strong perinuclear pattern comparable to that of LcH agglutinin (Fig. 3C, G, and K). Analysis of the immunofluorescence pattern of mutated gB Δ I (Fig. 3M and P) and gB KNP^m (Fig. 3Q and T) showed that they stained in a predominantly reticular pattern, which was comparable to that of calnexin in the ER (Fig. 3N and R). Comparison of the staining pattern of these mutated forms with that of LcH agglutinin (Fig. 3O and Q) showed a distinct dissimilarity, which suggested that gB Δ I and gB KNP^m were not transported to the Golgi. Results of these experiments

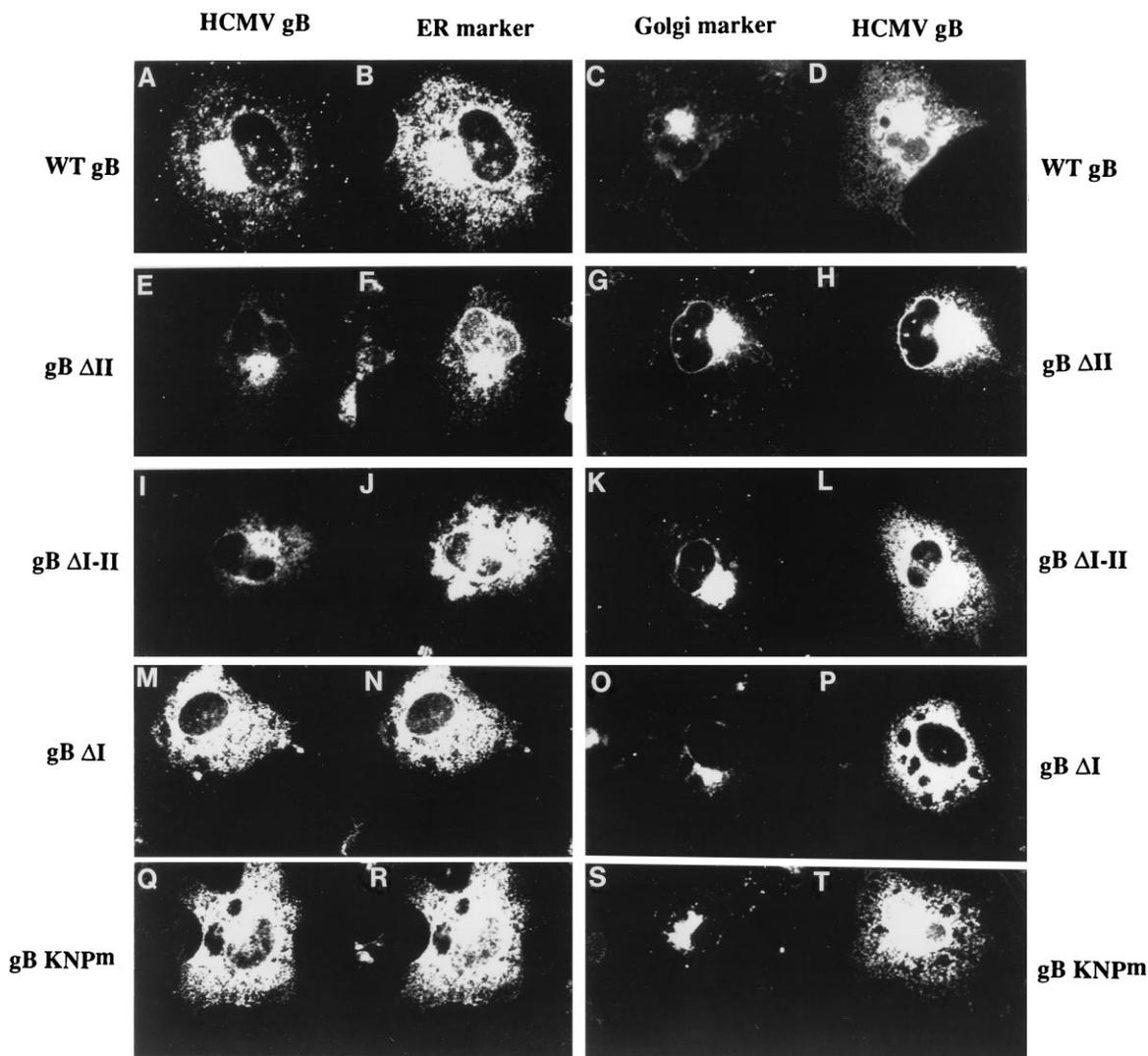


FIG. 3. Immunofluorescence confocal microscopy analysis of the pattern of staining of HCMV gB (A to D) and mutated forms gB Δ I (M to P), gB Δ II (E to H), gB Δ I- Δ II (I to L), and gB KNP^m (Q to T) in transfected U373 cells reacted with a pool of MAbs to gB (HCMV gB), antisera to calnexin, a marker for ER, and LcH agglutinin, a marker for Golgi. WT gB and the mutated forms are indicated on the left and right.

indicated that the mutated forms gB Δ II and gB Δ I-II, like WT gB, were transported to the Golgi and suggested that gB Δ I and gB KNP^m were predominantly retained in the ER and could fail to reach the Golgi.

Since malformed forms of viral glycoproteins are retained in the ER by protein chaperones that facilitate folding and monitor conformation (26, 31), we examined the integrity of epitopes on the surface of gB Δ I and gB KNP^m with a panel of MAbs to neutralizing epitopes on HCMV gB. We found that WT gB and the mutated forms reacted with immunofluorescence with a panel of 24 MAbs to conformational epitopes (data not shown), indicating that most of the surface epitopes on gB recognized by antibodies to important antigenic domains were not altered by these mutations in the hydrophobic sequences.

It was recently reported that HCMV gB with a deletion mutation in hydrophobic segment I was transported to the plasma membrane when expressed constitutively in U373 cells (47). In the present study, results of immunofluorescence confocal microscopy of the mutant forms gB Δ I and gB KNP^m suggested that their transport was arrested in the ER and that

they did not reach the Golgi and hence failed to reach the cell surface. To determine whether these mutated forms were transported to the plasma membrane, we selected U373 cells that constitutively express WT gB, gB Δ I, and gB KNP^m, using previously published procedures (56), and subjected these intact cells to flow cytometry with the pool of MAbs to gB (Fig. 4). For these experiments, 40,000 to 60,000 cells (excluding dead cells) were counted. These data showed that WT gB was detected on the cell surface, as indicated by a mean fluorescence value of 159.4 (Fig. 4A). In contrast, the mutated forms gB Δ I and gB KNP^m had mean fluorescence values of 10.1 and 8.5, respectively (Fig. 4C and D), which were comparable to the negative control value 4.4 obtained with cells that did not express gB (Fig. 4B). These results indicated that WT gB was on the cell surface whereas the mutated forms gB Δ I and gB KNP^m were not.

Together, results of immunofluorescence experiments and flow cytometry on cells expressing gB Δ I and gB KNP^m showed that deletion of hydrophobic segment I alone and mutations in KNP abolished transport of gB to the cell surface and caused

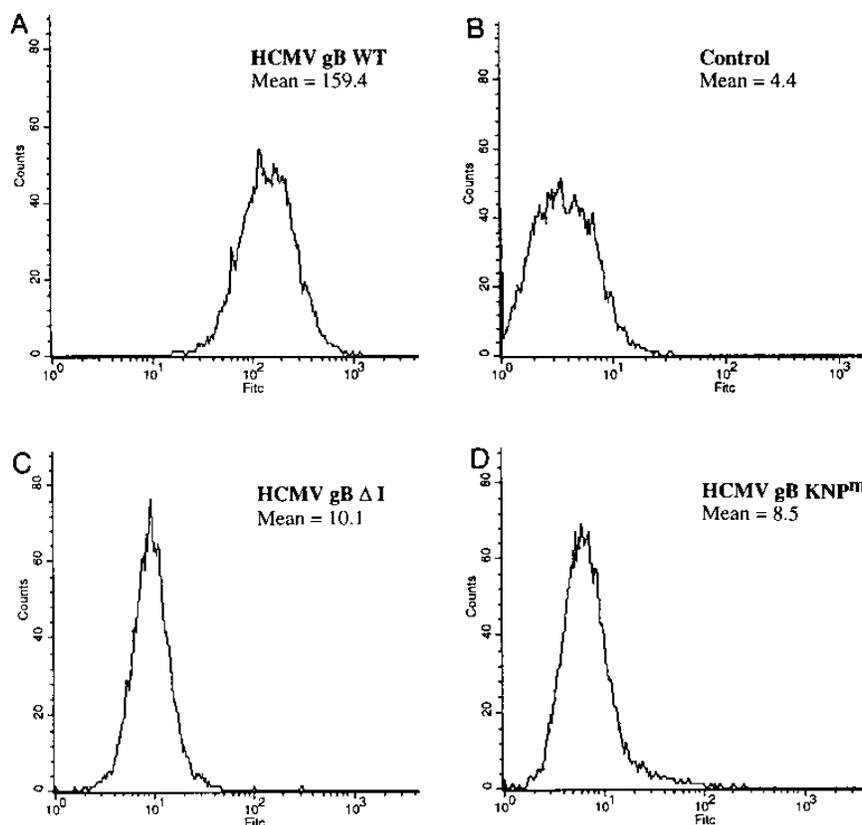


FIG. 4. Flow cytometry analysis of U373 cells constitutively expressing HCMV WT gB (A) and mutated forms gB Δ I (C) and gB KNP^m (D), labeled with a pool of MAbs to gB. (B) Negative control. Mean fluorescence intensities are indicated. Counts, cell number counted; FITC, fluorescence intensity.

retention of the mutated forms in the ER. Analysis of the conformation of gB Δ I and gB KNP^m with a panel of MAbs to epitopes on the surface of gB showed that no changes were detected, which suggests that the mutations introduced subtle alterations in the molecule. These findings indicate that hydrophobic segment I and KNP are critical elements which maintain the structural integrity of gB that is required for exit of the glycoprotein from the ER.

The deletion of hydrophobic segment I and the KNP mutation arrest gB in the ER and block cleavage and oligosaccharide processing. In HCMV-infected cells, gB undergoes cleavage in a post-ER compartment (50, 51); both the intact and cleaved forms are partially sensitive to endo H (38, 46). In the next set of experiments, we studied the cleavage and processing of the oligosaccharides on the mutated forms by digesting the immunoprecipitated proteins with endo H, which cleaves high-mannose-type N-glycans, and PNGase F, which cleaves all N-glycans including complex structures formed in post-ER compartments (53). For these experiments, cells were transfected with the DNA constructs, and gB forms were immunoprecipitated from the cell extracts and medium after 18 h, treated with endoglycosidases, and then electrophoresed in denaturing SDS-polyacrylamide gels. The results (Fig. 5) were as follows.

(i) In cells expressing WT gB (Fig. 5, lane 1), the uncleaved precursor (band 1) as well as N-terminal (diffuse band 2) and C-terminal (band 3) cleavage products were formed. These gB bands were partially sensitive to endo H (Fig. 5, lane 2). Only bands 2 and 3 were deglycosylated further by PNGase F digestion (Fig. 5, lane 3). After complete removal of the N-glycans,

both N- and C-terminal deglycosylated proteins appeared to comigrate. This finding agreed with the calculated molecular weights of deglycosylated N-terminal (aa 25 to 459) and C-terminal (aa 460 to 906) halves of gB, which are 50,000 and 49,500, respectively.

(ii) Comparison of precipitated gB Δ II and gB Δ I-II from culture medium (Fig. 5, lanes 7 and 13) and cell extracts (Fig. 5, lanes 10 and 16) showed some differences in oligosaccharides. Like WT gB, these intracellular mutated forms were cleaved, were partially sensitive to endo H (Fig. 5, lanes 11 and 17), and were further deglycosylated by PNGase F digestion (Fig. 5, lanes 12 and 18). However, some heterogeneity in the sensitivity of the intracellular forms of gB Δ II and gB Δ I-II to endo H was observed. Following treatment, uncleaved gB (Fig. 5, lanes 10 and 16, bands 1) was resolved into two bands, one that was highly endo H resistant (Fig. 5, lanes 11 and 17, band 1a) and another that was endo H sensitive (Fig. 5, lanes 11 and 17, band 1b). Analysis of the secreted forms of gB Δ II and gB Δ I-II showed that they too were cleaved (Fig. 5, lanes 7 and 13) but were only minimally sensitive to endo H (Fig. 5, lanes 8 and 14). In contrast, bands 1, 2, and 3 were fully sensitive to PNGase F, as indicated by a dramatic shift in electrophoretic mobility following digestion (Fig. 5, lanes 9 and 15). It should be mentioned that PNGase F-treated forms of gB Δ II and gB Δ I-II migrated more rapidly than PNGase F-treated forms of WT gB, as was expected for the size of the deletion. This finding indicated that the heterogeneous appearance of the processed bands of these mutated forms resulted from differences in glycosylation patterns.

(iii) Analysis of precipitates from cells expressing gB Δ I (Fig.

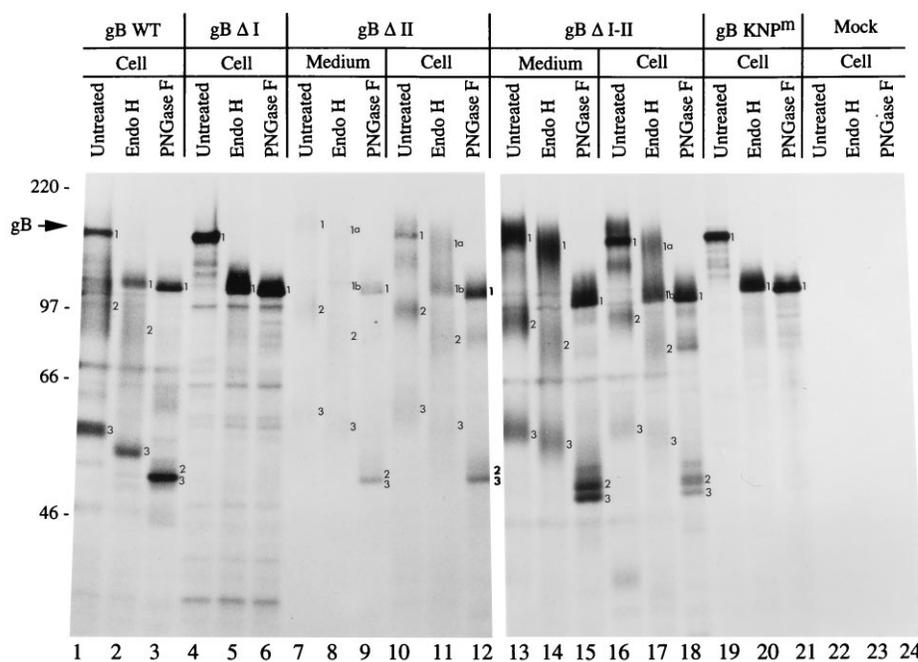


FIG. 5. Posttranslational processing of WT and mutated forms gB Δ I, gB Δ II, gB Δ I-Δ II, and gB KNP^m in transfected U373 cells. Radiolabeled proteins (³⁵S]Trans-label) were immunoprecipitated from the medium or cell extracts with MAb CH177. Immunoprecipitates were boiled in the appropriate buffers, digested with endo H or PNGase F or left untreated, and separated in denaturing SDS-9% polyacrylamide gels. Uncleaved gB molecules (band 1) and endoproteolytic products of the N terminus (band 2) and C terminus (band 3) are indicated. Bands 1a and 1b indicate endo H digestion products. Mock, no DNA. Molecular mass markers are indicated at the left in kilodaltons.

5, lane 4) and gB KNP^m (Fig. 5, lane 19) showed that only band 1 was present, which indicated that these mutated forms were not cleaved. Notably, both gB Δ I and gB KNP^m were fully sensitive to endo H (Fig. 5, lanes 5 and 20) but were not digested further by PNGase F (Fig. 5, lanes 6 and 21). Inasmuch as HCMV gB contains 21 N-glycosylation sites, the very slight differences in the migration of these mutated forms could result from the removal of all residual N-linked saccharides by PNGase F, whereas endo H does not remove the *N*-acetylglucosamine linker from Asn residues. A slight difference in the migration of endo H- and PNGase F-treated uncleaved precursor of WT gB (band 1) was also observed (Fig. 5, lanes 2 and 3). The results of carbohydrate analysis agreed with those of the flow cytometry and immunofluorescence analyses, which showed that both the mutated forms, gB Δ I and gB KNP^m, failed to exit the ER and did not transit further along the exocytic pathway. The finding that these mutated forms of gB failed to undergo endoproteolytic cleavage and remained endo H sensitive indicates that the deletion of hydrophobic segment I and the mutation in KNP between segments I and II alter the gB molecule, precluding exit of these forms from the ER.

WT gB and mutated forms of HCMV gB bind to protein chaperones in the ER. We previously reported that a mutated form of HSV-1 gB which failed to react with MAbs to conformational epitopes was arrested in the ER, where it formed stable complexes with GRP78 (63) and GRP94 (44). The present studies on the mutated forms of HCMV gB indicated that the deletion of hydrophobic segment I and the site-specific mutation in KNP result in sequestration of these mutated forms in the ER. In addition to GRP78 and GRP94, calnexin and calreticulin, which are chaperones with lectin-like properties in the ER, bind glycosylated proteins, promote their fold-

ing (33, 40), and prevent the exit of unassembled monomers and conformationally defective forms from the ER (43).

To determine whether mutations in the hydrophobic domain alter the association of HCMV gB molecules with ER chaperones, we next investigated the binding of WT gB and the mutated forms gB Δ I and gB KNP^m to protein chaperones by coimmunoprecipitation experiments. U373 cells were transfected with DNA, labeled for 12 h, and lysed with 2% CHAPS to maintain the association of proteins in the complexes. Immunoprecipitates from equal numbers of cells were loaded into each lane of SDS-polyacrylamide gels. The results (Fig. 6) were as follows.

(i) Immunoprecipitates of WT, gB Δ I, and gB KNP^m formed by MAb CH177-3 from transfected-cell extracts showed that the glycoproteins were expressed equally and contained comparable amounts of the WT and mutated forms (Fig. 6A, lanes 1 to 3). Faster-migrating cleavage products of WT gB were observed (Fig. 6A, lane 1), whereas gB Δ I and gB KNP^m were not cleaved (Fig. 6A, lanes 2 and 3). Three slightly faster-migrating bands were detected in precipitates of the mutated forms; their closely spaced, ladder-like appearance (Fig. 6A, lanes 2 and 3, bands 1a to c) suggests they may be partially glycosylated forms.

(ii) Analysis of immunoprecipitates of calnexin showed that the synthesis of this chaperone was induced in tunicamycin-treated, mock-transfected U373 cells (Fig. 6A, lanes 9 and 10). WT gB and the mutated forms gB Δ I and gB KNP^m coprecipitated with calnexin in approximately equal amounts (Fig. 6A, lanes 6 to 8). gB Δ I, having the large deletion of 30 aa (Fig. 6A, lane 7), migrated slightly faster than WT gB and gB KNP^m (Fig. 6A, lanes 6 and 8). We next determined whether calreticulin bound to gB as calnexin did. In this separate experiment, extracts of transfected cells were immunoprecipitated

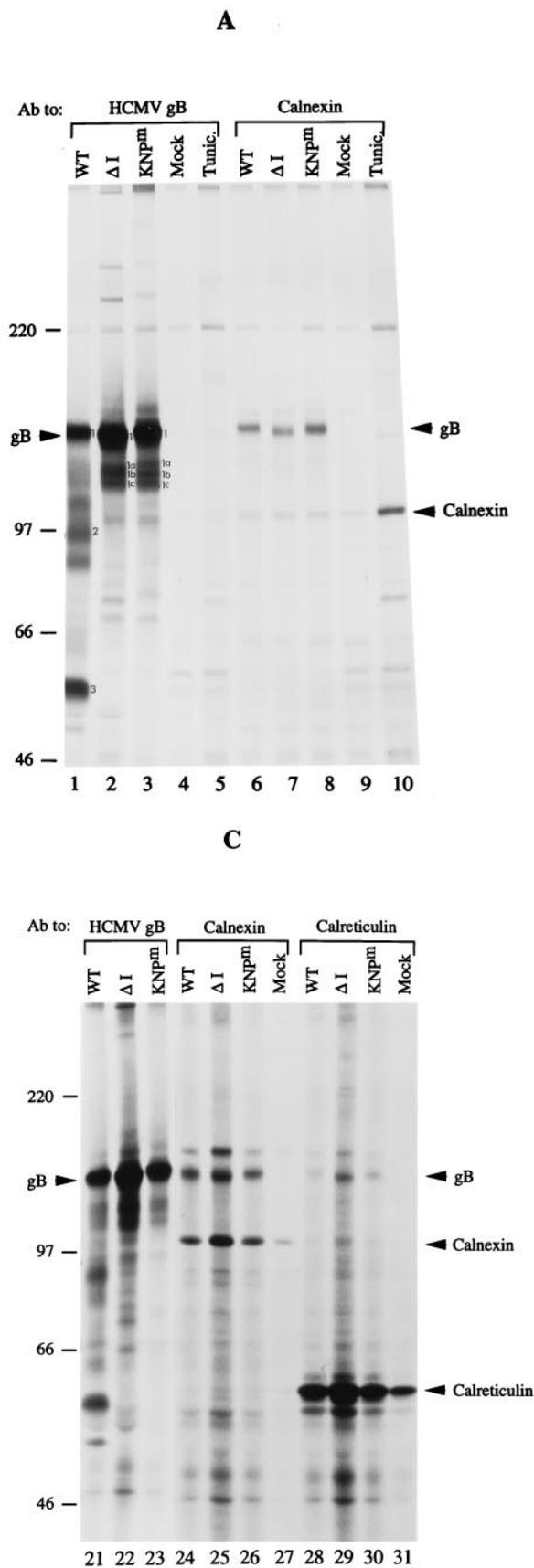


FIG. 6. Coimmunoprecipitation of molecular chaperones in the endoplasmic reticulum with WT gB and mutated forms gB Δ I and gB KNP^m. Transfected U373 cells were radiolabeled with [³⁵S]Trans-label for 12 h and lysed in 2% CHAPS. A negative control sample was mock transfected alone (Mock). Another mock-transfected sample contained tunicamycin (Tunic.) (1.5 μ g/ml) for 12 h prior to radiolabeling and was maintained at the same concentration during radiolabeling. Lysates were divided into aliquots and immunoprecipitated with antibodies (Ab) to gB (A and C), calnexin (A and C), calreticulin (C), GRP78 (B), and GRP94 (B). Precipitated proteins were boiled in SDS sample buffer containing 2% SDS and β -mercaptoethanol and separated in SDS-9% polyacrylamide gels. HCMV gB is indicated with an arrow. Uncleaved gB molecules (band 1) and endoproteolytic products of the N terminus (band 2) and the C terminus (band 3), as well as faster-migrating bands of mutated forms gB Δ I and gB KNP^m (bands 1a to c) are indicated. Molecular mass markers are indicated at the left in kilodaltons.

with antibodies to calnexin and calreticulin; however, the lanes containing gB Δ I (Fig. 6C, lanes 22, 25, and 29) were slightly overloaded. With this taken into account, WT gB, gB Δ I, and gB KNP^m coprecipitated in approximately equal amounts with calreticulin (Fig. 6C, lanes 28 to 30). Notably, only the intact form of WT gB and the mutated glycoproteins coprecipitated with calnexin and calreticulin. The intensity of the gB band in the calnexin precipitates (Fig. 6C, lanes 24 to 26) compared with those of the calreticulin precipitates (Fig. 6C, lanes 28 to 30) suggested that more gB is bound to calnexin than to calreticulin. Interestingly, trace amounts of calnexin were contained in the calreticulin precipitates (Fig. 6C, lanes 28 to 30), but no calreticulin was detected in the calnexin precipitates (Fig. 6C, lanes 24 to 26), suggesting that the calreticulin-bound gB is also bound to calnexin. Results of these experiments indicate that calnexin and calreticulin bind to nascent, intact gB molecules in the ER and confirm that cleavage occurs in a

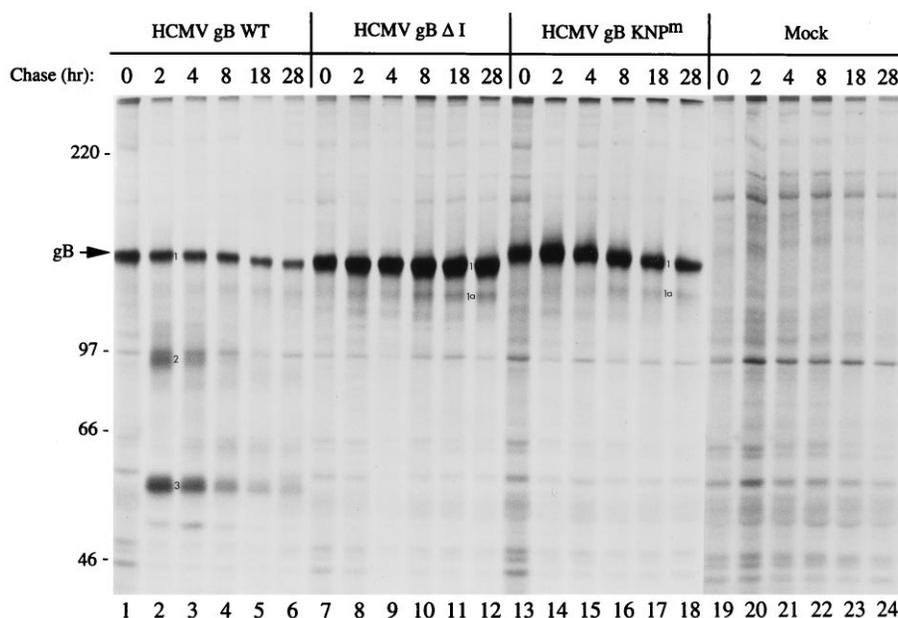


FIG. 7. Pulse-chase experiments of WT gB and mutated forms gB Δ I and gB KNP^m synthesized in transfected U373 cells. Cells were radiolabeled with [³⁵S]Trans-label for 30 min and chased for 0, 2, 4, 8, 18, and 28 h. HCMV gB was immunoprecipitated with MAb CH177. Precipitated proteins were boiled in SDS sample buffer containing 2% SDS and β -mercaptoethanol and separated in SDS–9% polyacrylamide gels. Uncleaved gB molecules (arrow, band 1) and endoproteolytic products of the N terminus (band 2) and C terminus (band 3) of WT gB as well as slightly faster-migrating bands of mutated forms (band 1a) are indicated. Mock, no DNA. Molecular mass markers are indicated at the left in kilodaltons.

post-ER compartment. That WT gB and the mutated forms coprecipitated with calnexin and calreticulin in approximately equal amounts suggests that these chaperones bind transiently to folding intermediates of these molecules and that the binding sites recognized on gB are unaltered by the Δ I and KNP^m mutations.

(iii) We found that tunicamycin treatment of mock-transfected U373 cells induced the synthesis of GRP78 (Fig. 6B, lane 15) as previously published (63). Analysis of precipitates formed by GRP78 and GRP94 showed that different amounts of WT gB, gB Δ I, and gB KNP^m coprecipitated with GRP78 (Fig. 6B, lanes 11 to 13) and GRP94 (Fig. 6B, lanes 16 to 18). Only uncleaved molecules were associated with GRP78 and GRP94, as would be expected for gB complexes formed with resident ER proteins. The proportions of the mutated gB forms sequestered in precipitates with GRP78 (Fig. 6B, lanes 12 and 13) and GRP94 (Fig. 6B, lanes 17 and 18) were greater than those of WT gB (Fig. 6B, lanes 11 and 16). Enhanced production of GRP78 was found in tunicamycin-treated, mock-transfected cells. These results suggest that transient complexes were formed between WT gB and GRP78 and GRP94, whereas the mutated forms showed prolonged or stable association with these chaperones. The finding that the Δ I and KNP^m mutations increase the association of mutated gBs with GRP78 and GRP94 suggests that these mutations subtly modify the structure of gB. Inasmuch as the chaperones GRP78 and GRP94 monitor the quality of proteins in the ER and block the transport of unassembled subunits and improperly folded forms, it is likely that the accumulation of gB Δ I and gB KNP^m in the ER stems from their sequestration into stable complexes with these chaperones.

Mutated forms of gB sequestered in the ER were not degraded. We reported that the mutated form of HSV-1 gB with altered conformation, mentioned above, coprecipitated with GRP78 and GRP94, was blocked from exiting the ER and was

rapidly degraded (31, 41). To determine whether the mutated forms of HCMV gB that were associated with GRP78 and GRP94 were degraded in the ER, we performed pulse-chase experiments. Transfected U373 cells expressing gB were pulse-labeled for 30 min as described in Materials and Methods and chased for intervals up to 28 h. The results (Fig. 7) were as follows.

(i) WT gB made during the pulse was cleaved after 2 h of chase (Fig. 7, lanes 1 to 3, bands 1 to 3). The intact precursor (band 1), the 97-kDa N terminus (band 2), and the 55-kDa C terminus (band 3) appeared to be slightly reduced after 8 h. Band 1, the only form present in the ER, was not degraded, i.e., lost, but migrated slightly faster by 18 h. Presumably, this resulted from trimming of the mannose-rich carbohydrates, which occurs in proteins residing in the ER for a long period (25). By 28 h, the intensity of the N- and C-terminal cleavage products in post-ER compartments was diminished. Results of these experiments show that the fraction of WT gB that is in the ER for over 28 h is not degraded.

(ii) By contrast, the mutated forms gB Δ I and gB KNP^m were not cleaved and they accumulated stably in the ER (Fig. 7, lanes 7 to 18). The quantity of the uncleaved gB Δ I band was relatively constant during the chase intervals (Fig. 7, lanes 8 to 12), but the KNP^m band was diminished somewhat by 18 h although still present (Fig. 7, lanes 14 to 18). Like WT gB, both mutated forms migrated slightly faster after 8 h. The results show that these mutated forms of HCMV gB are not rapidly degraded and appear to be quite stable in the ER. Compared with our previous finding that a defective form of HSV-1 gB lacking an important conformational domain on the surface of the molecule is rapidly degraded (within 60 min) in the ER (31), results of the present study suggest that some sequestered proteins are degraded whereas others are not, and whether degradation occurs could depend on the extent of the defect. This result supports the idea that the ER contains subcompartment-

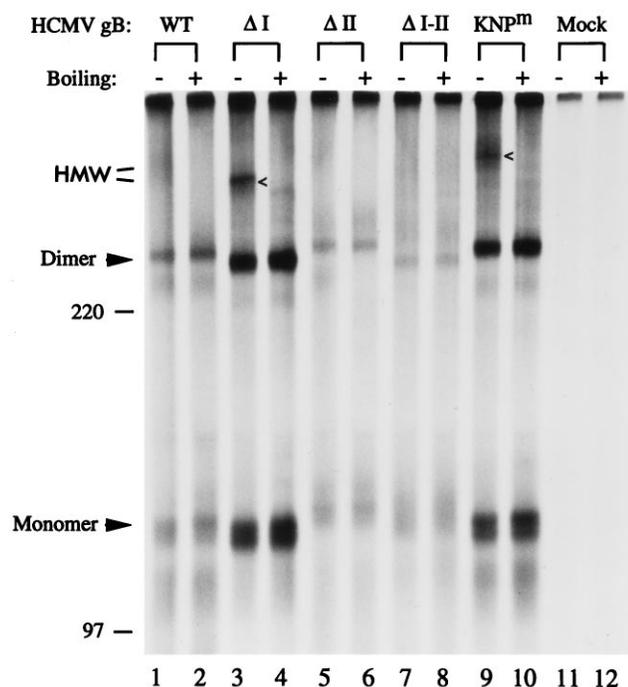


FIG. 8. Immunoprecipitation of oligomers formed by WT and mutated forms of gB. Transfected U373 cells were radiolabeled with [³⁵S]Trans-label, and gB was immunoprecipitated with MAb CH177. Immunoprecipitates were eluted in 0.2% SDS without β -mercaptoethanol at room temperature (10 min). Eluates were divided into equal samples and boiled for 3 min (+) or not boiled (-) before separation in denaturing SDS-7% polyacrylamide gels. Monomeric and dimeric forms of WT and mutated gB forms are indicated by filled arrowheads to the left. <, HMW bands (lanes 3 and 9); Mock, no DNA. Molecular mass markers are indicated at the left in kilodaltons.

ments, some in which proteins that are blocked from exit accumulate and others in which they undergo rapid degradation (3, 24, 60).

Deletion of hydrophobic segment I and mutation of KNP promote aggregation. HCMV gB forms a homodimer that is assembled cotranslationally and undergoes relatively slow folding before exiting the ER (2, 7). To determine whether the mutated forms of gB in transfected cells formed dimers, the immunoprecipitated glycoproteins were analyzed in nonreducing 7% polyacrylamide gels (Fig. 8). We found that WT gB formed dimers that remained intact with or without heat (Fig. 8, lanes 1 and 2). All the mutated forms of gB also assembled into dimers, including the secreted forms, gB Δ II (lanes 5 and 6) and gB Δ I-II (lanes 7 and 8), and the mutated forms arrested in the ER, gB Δ I (lanes 3 and 4) and gB KNP^m (lanes 9 and 10). These dimers (too were resistant to heat, indicating that the monomers were associated by covalent disulfide bonds. It is notable that unheated samples of the mutated forms gB Δ I (lane 3) and gB KNP^m (lane 9) contained an additional high-molecular-weight band, designated HMW, which migrated more slowly than the dimers. Band HMW was absent from precipitates of WT gB and from heat-treated samples of gB Δ I and gB KNP^m. The protein profile in the corresponding heat-treated samples showed that the intensity of the monomeric gB band increased, which indicated that band HMW comprises primarily gB aggregates. Our results are consistent with the idea that gB oligomers assemble in the ER and show that mutations in the hydrophobic domain do not detectably alter oligomer formation but cause aggregation of a fraction of the gB molecules.

DISCUSSION

Role of the carboxyl-terminal hydrophobic domain of HCMV gB. In this study, we analyzed the effect of site-specific mutations in the carboxyl-terminal hydrophobic sequence (aa 714 to 771) of HCMV gB. This domain is separated into two hydrophobic segments, I and II, by Lys-748-Asp-749-Pro-750 (KNP) (Fig. 1A). The deletion of hydrophobic segment II alone (aa 751 to 771) resulted in secretion of the mutated glycoprotein, which confirmed a recent report (47) that this sequence functions as a membrane anchor. However, the deletion of hydrophobic segment I (aa 717 to 747) or the mutation of KNP blocked the exit of gB from the ER and abrogated its cell surface expression. Interestingly, gB Δ II and gB Δ I-II both lack the membrane anchor sequence and were secreted from cells, but the finding that less gB Δ II was released suggests that the remaining segment I may hinder secretion or that the spatial relationship between segments I and II may be important for anchoring gB in the membrane. Immunoprecipitation studies revealed that the molecular chaperones calnexin, calreticulin, GRP78, and GRP94 formed complexes with gB molecules in the ER. Whereas WT gB appeared to bind transiently to these chaperones, complexes of gB Δ I and gB KNP^m with GRP78 and GRP94 were detected in the ER, which suggested that these associations were stable. Consequently, a fraction of WT gB was present in the ER but disproportionate amounts of gB Δ I and gB KNP^m accumulated and were retained there. These arrested molecules were not endoproteolytically cleaved, a step in gB processing, which agrees with the observation that the endoprotease is located in a post-ER compartment (50, 58). Our results indicate that aa 717 to 747 maintain a critical feature that is required for the quality of gB structure, permitting its release from GRP78 and GRP94 and transit from the ER to the Golgi and cell surface.

Our results on the role of the hydrophobic domain at the carboxyl terminus of HCMV gB agree with the analysis of a comparable region of HSV-1 gB (Fig. 1A) (45). Segment 3 of the 69-aa hydrophobic region of HSV-1 gB, which is equivalent to HCMV gB segment II, is sufficient for stable membrane anchoring. Mutated forms with deletions in segments 1 or 2, which are equivalent to HCMV gB segment I, were not expressed on the cell surface; in addition, they failed to complement an HSV-1 gB-null mutant, suggesting that they had functional defects as well. Our results differ in important aspects from a report by Reschke et al. on mutated forms of HCMV gB with deletions in hydrophobic regions I and II (47). As in our study, the deletion construct lacking hydrophobic region II, aa 751 to 771, was secreted into the medium, but their finding that a deletion construct comparable to gB Δ I, lacking aa 714 to 747, was transported to the plasma membrane differs from ours. In our hands, immunofluorescence confocal microscopy and flow cytometry showed that gB Δ I was arrested in the ER. This result was supported by endo H sensitivity and the failure of this mutated form to undergo cleavage, which is a post-ER step in gB processing (5, 58). Moreover, the observation that increased amounts of GRP78 and GRP94 formed a complex with gB Δ I strongly implies that, unlike WT gB, this mutated form was sequestered in the ER. Reschke et al. (47) also found that the deletion construct lacking hydrophobic region I of gB was not cleaved, which suggested that it was not transported from the ER to the Golgi. A possible explanation for the discrepant finding in cell surface expression is that the surface biotinylation reaction they used was not completely quenched prior to Nonidet P-40 extraction or that the procedure itself or the expression of these mutated forms damaged the cells. Under these conditions, some intracellular gB would be labeled

and result in precipitation of the intracellular forms of gB during the subsequent streptavidin-mediated affinity precipitation reactions. This seems a plausible explanation inasmuch as the HCMV-infected cell controls showed that intact gB was precipitated from the biotinylated sample by streptavidin even though only the cleaved form, which is processed in a post-ER compartment, should be available for biotinylation on the surface of infected cells.

Molecular chaperones participate in folding of HCMV gB. It is widely accepted that molecular chaperones, which include the soluble proteins GRP78, GRP94, and calreticulin and the membrane-bound protein calnexin, among others, are components of the quality control system in the ER that assists protein folding and precludes the exit of molecules that fail to attain the proper conformation (reviewed in reference 20). It has been well established that GRP78 and GRP94 bind transiently to cellular and viral glycoproteins during the process of folding and oligomerization (16) and bind stably to misfolded proteins that fail to exit the ER (26). It is thought that these chaperones prevent intramolecular and intermolecular interactions caused by exposed hydrophobic sequences. Calnexin is a lectin-like chaperone that binds to nascent glycoproteins in the ER (1, 9, 12, 19, 33, 43). Calnexin assists in the folding of monomeric influenza hemagglutinin subunits by binding partially trimmed, N-linked oligosaccharides (9). Treatment of cells with inhibitors of α -glucosidases blocks binding of calnexin to hemagglutinin and to vesicular stomatitis virus G, indicating a requirement for glucose trimming of newly synthesized carbohydrates (17). Calnexin binds $\text{Glc}_1\text{Man}_6\text{GlcNAc}_2$ oligosaccharides as an initial step in recognizing unfolded glycoproteins in the ER (61). Both the reduced and the oxidized forms of major histocompatibility complex (MHC) class I heavy chain are recognized by calnexin, but it is not involved in the assembly of class I molecules with β -2 microglobulin (55) or the assembly of hemagglutinin homotrimers (54). It was recently reported that calnexin assists in the folding of HCMV gB in infected cells (64). Calnexin associated transiently with nascent gB molecules in the ER, binding the reduced form and dissociating from the completely oxidized form. It is interesting that MHC class I heavy chains and invariant chains of MHC class II heterodimers that were associated with calnexin were protected from rapid degradation; this revealed a central role for calnexin in protecting unassembled molecules prior to formation of protein complexes (22, 48). Calreticulin is a soluble calcium-binding protein in the ER which is similar to calnexin in sequence (12) and function (59). Like calnexin, calreticulin plays an important role in the maturation and quality control of newly synthesized cellular and viral glycoproteins (29, 32, 40).

Calnexin and calreticulin are components of a unique quality control system, which assists in folding proteins and retains intermediates in the ER (9, 17, 40). It was recently reported that most of the human immunodeficiency virus gp160 molecules bound to calreticulin are also bound to calnexin, but only a portion of the gp160 associated with calnexin is bound to calreticulin (32). Our observation that calnexin and calreticulin both form complexes with uncleaved forms of HCMV gB suggests that a fraction of the newly synthesized glycoprotein is bound by both chaperones. Considerably more gB was present in complexes with calnexin than with calreticulin, which suggests that the chaperones have somewhat different substrate specificities. Several reasons for the substrate selectivity of the lectin-like chaperones have been suggested (40). Calnexin may facilitate the initial folding steps, resulting in a more prolonged association with calnexin than with calreticulin. Alternatively, the soluble chaperone calreticulin may prefer soluble sub-

strates, whereas calnexin may be better suited to interact with membrane-bound glycoproteins. Another variable is the location of the N-linked glycans, which could determine whether they are more accessible to binding by calnexin (close to the membrane) than calreticulin (far from the membrane). Whether HCMV gB forms complexes with several chaperones simultaneously or participates in a series of sequential interactions with one or more chaperones remains to be elucidated in detailed pulse-chase experiments.

HCMV gB forms disulfide-linked dimers cotranslationally in the ER, which is followed by slow postdimerization folding prior to exit from this subcellular compartment (2, 7). Results of our experiments showed that both calnexin and calreticulin form complexes with newly synthesized HCMV gB and also with the mutated forms gB Δ I and gB KNP^m in the ER. Although the rate of dissociation of the chaperones from gB was not determined, this finding indicates that both these protein chaperones participate in gB folding and that the mutations did not alter their recognition sites. Our results suggest that gB molecules bound to calreticulin form complexes with calnexin as well. The membrane-anchored calnexin could tether regions of newly translated gB that are proximal to the ER membrane, whereas calreticulin could bind later and influence folding of the luminal portion of glycoprotein, comparable to the role postulated for these lectin-like chaperones in the folding of vesicular stomatitis virus G (40) and human immunodeficiency virus gp160 (32). Although HCMV gB Δ I and gB KNP^m were retained in the ER, they did not undergo degradation. It is possible that the association of calnexin with glycans on the WT and mutated forms of gB protects them from degradation, as has been shown for MHC class I molecules and invariant chains of the MHC class II (22, 48).

Significantly more of the mutated forms of gB that were retained in the ER formed complexes with the chaperones GRP78 and GRP94 than did WT gB. In a study of HSV-1 gB, we showed that GRP78 and GRP94 play an important role in gB folding (31) and that a mutated form, gB-(LK479), induced the synthesis of GRP78 and GRP94, which formed more abundant complexes with gB-(LK479), retaining the mutated form in the ER (44, 63). Unlike the mutated forms of HCMV gB, which had no detectable misfolding, HSV-1 gB-(LK479) showed a defect in a major conformational region in the ectodomain and was rapidly degraded in the ER. In the present study, we demonstrated that GRP78 and GRP94 also play an important role in the folding of HCMV gB and that they monitor the exit of selected mutated forms of gB from the ER. One sequence of events could be that GRP78 and GRP94 associate with nascent gB monomers, followed by calnexin and calreticulin to assist conformation maturation, and that the chaperones either dissociate from correctly folded forms or bind stably to misfolded ones, sequestering them in the ER. Sequential binding of GRP78 and calnexin to thyroglobulin (23) and to vesicular stomatitis virus G (18) was important for efficient folding of G protein and retention of partially folded forms in the ER. Interestingly, HCMV gB Δ I and gB KNP^m showed no evident conformational defects, as indicated by reactions with MAbs to HCMV gB. We conclude that the subtle conformational changes resulting from mutations in hydrophobic domain I and KNP led to their sequestration in the ER but not to rapid degradation.

The gB homolog is the most conserved gene in the herpesvirus family, which is consistent with its essential function in virus infectivity by promoting fusion of the virion envelope with the plasma membrane. Hydrophobic segment I of the known gB homologs is highly conserved (Fig. 1A). It was suggested that hydrophobic segment I (aa 714 to 747) of HCMV gB has

the potential to function in syncytium formation, on the basis of the sequence similarity with viral fusion peptides (47). Part of HCMV gB segment I (L-718–GAAGKAVGV–A-728), in particular the glycines at positions 719, 722, and 726, is similar to the fusion peptide (L-523–GAAGSTMGA–A-533) of HIV gp160. However, when we replaced this sequence with aa 718 to 728 of HCMV gB and analyzed syncytium formation by the gp160 chimera using published procedures (62), this sequence failed to induce fusion of cocultivated CD4⁺ cells and produced results comparable to those obtained when a random sequence (AGVDEDGLVRE) was substituted (65). Although negative results are not conclusive, these experiments suggest that segment I alone lacks syncytium-inducing activity. Also of interest is the sequence Asp-749–Pro-750 separating hydrophobic segments I and II that is conserved among all herpesvirus gB homologs (Fig. 1A). Asp is highly hydrophilic and Pro tends to break the membrane-spanning α -helix structure by favoring a β -turn structure (11). Substitution of the hydrophobic residues Val-Ala-Ile for aa 748 to 750 should result in a continuous hydrophobic sequence from aa 714 to 771. This substitution in gB KNP^m altered the relationship between hydrophobic segment I and segment II, the membrane anchor, causing gB to be sequestered in the ER. Our findings indicate that the structural organization of this region of gB is central to the maturation and release of the folding intermediates from the molecular chaperones GRP78 and GRP94.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grants EY10138 and EY11223 from the National Institutes of Health (L.P.) and also by grant R94-SF-051 (L.P.) and a postdoctoral fellowship (F94-SF-013) (Z.Z.) from the Universitywide AIDS Research Program.

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