Oligomerization of the Human Cytomegalovirus Major Envelope Glycoprotein Complex gB (gp55-116)

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The disulfide-linked glycoprotein B (gB; gp55-116) complex of human cytomegalovirus represents the most abundant and immunogenic component of the virion envelope. We have studied the oligomerization and transport of this molecule, using a series of murine monoclonal antibodies. Our results indicated that oligomerization of this molecule occurred shortly after its synthesis, with a half-time of maximal formation of approximately 25 min. The oligomeric form had an estimated mass of 340,000 Da and likely consisted of a homodimer of the gp55-116 complex. By using a conformation-specific monoclonal antibody, postoligomerization folding of this molecule was demonstrated. This event exhibited an unusually prolonged half-maximal time of approximately 160 min. Both oligomerization and folding occurred in the endoplasmic reticulum. Oligomerization and folding occurred in the absence of carbohydrate modifications, although likely at lower efficiency. Finally, the oligomeric and folded forms were shown to be transported to the surface of infected cells and infectious virions.

Human cytomegalovirus (HCMV) continues to represent a major cause of disease in immunocompromised hosts. It is a leading cause of morbidity and mortality in allograft recipients (16, 21, 24, 25). In addition, as the survival of human immunodeficiency virus-infected individuals has been extended by more-effective therapies for other opportunistic infections, HCMV has become an increasingly important cause of disease in this population (17). Although less widely appreciated, HCMV is the most common cause of congenital viral infection in humans. This not-uncommon infection results in approximately 5,000 to 7,000 severely infected infants each year in the United States (1).

Numerous approaches have been employed to limit HCMV-associated disease. Effective antiviral chemotherapy has recently been introduced for several of these populations, but the significant toxicity of these compounds and the development of resistance have made these therapies less than ideal. Attempts to modulate disease with passively transferred antiviral antibodies have proven successful in organ allotransplantation patients but remain controversial therapy for bone marrow transplant populations (2, 26). Although unknown, the targets of protective antiviral antibodies are thought to be components of the virion envelope because of their accessibility on the surfaces of both infectious virions and infected cells. Likewise, the induction of protective immunity by active immunization has focused on the use of envelope components of HCMV as subunit vaccines (5, 27). The major component and most immunogenic protein of the envelope of HCMV, gB, is a disulfide-linked glycoprotein complex consisting of a 116- and a 55-kDa component (4, 14, 22). The gB is a type I glycoprotein, with the gp116 representing the surface (SU) component and the gp55 being the transmembrane (TM) portion of the complex. Because of the importance of this glycoprotein complex in the structure of the envelope and its immunogenicity in vitro and in vivo, a complete understanding of its structure, including its intracellular transport, will be re-

quired to define native forms of this protein complex as well as to identify novel targets of future antiviral compounds. In addition, these studies are essential to our understanding of the morphogenesis of the complex envelope structure of this large mammalian DNA virus.

The HCMV gB has been shown to be synthesized from an approximately 105,000-Da polypeptide which is cotranslationally modified to a 150,000-Da precursor protein (gp150) containing only simple, high-mannose sugars (7, 13). This precursor form is then transported from the endoplasmic reticulum (ER) into the Golgi complex where terminal sugar modifications are made and then endoproteolytic cleavage into the gp116 and gp55 components occurs (7, 27). Initial pulse-chase experiments were somewhat difficult to interpret because of the unusually long processing time and the inability to completely chase material from the precursor into the mature forms (6). Furthermore, we noted that approximately 80 to 100 min were required prior to detection of endoglycosidase H (endo H)-resistant forms of the gp150 (7). The appearance of the endo H-resistant forms also coincided with the presence of the two cleavage products, gp55 and gp116 (7). These initial findings suggested that the transport of the gp150 from the ER was unusually slow; however, the mechanism(s) for this retarded transport was unknown. Because previous studies of other herpesviruses, including pseudorabies virus (PRV) and herpes simplex virus, have described the oligomerization of the gB envelope glycoprotein (20, 31), we investigated the oligomerization of the HCMV gB as a mechanism of delayed transport of this molecule.

The results of our studies suggested that the HCMV gB molecule dimerizes in the ER with a half-maximal time of formation just slightly slower than its rate of synthesis. In contrast to the intracellular transport of envelope proteins such as the G protein of vesicular stomatitis virus or the hemagglutinin of influenza virus, this oligomerization step appeared not to be rate limiting in the transport of gB from the ER. Our results suggested that postoligomerization folding of gB was necessary for transport from the ER and that the half-maximal time of formation of this form was esti-

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mated to be eight times longer than that of oligomerization. In addition, we found that oligomerization and folding were not absolutely dependent on glycosylation of the precursor polypeptide, suggesting that all necessary information for the oligomerization and folding of HCMV gB was represented within the primary sequence. Finally, our results indicated that the mature virion and cell surface form of gB was dimeric and that this form expressed antibody-binding sites unique among other forms of the gB, including monomeric forms, suggesting that natural immunity to this protein may require inclusion of this dimeric form in future subunit vaccine candidate formulations.

MATERIALS AND METHODS

Cells and virus. The HCMV AD169 laboratory strain was used for all experiments (4). Virus was propagated in, and all experiments were done with, low-passage primary human fibroblast cells (HF cells). The HF cells were maintained in medium 199 supplemented with 10% calf serum and antibiotics. The recombinant vaccinia virus containing the intact coding sequence of HCMV AD169 gB has been described and characterized in previous studies (8).

Radioactive labelling of infected-cell proteins in the presence and absence of inhibitors of glycoprotein processing. HF cells were labelled 4 days following infection with AD169 at a multiplicity of infection of 4 to 5. After a brief methionine deprivation, infected cells were labelled with 100 μ Ci of [35 S]methionine per ml in methionine-free medium. For pulse-chase experiments, the radioactive medium was removed after 10 min of labelling (unless otherwise specified), the monolayer was washed, and maintenance medium was added. Following the chase periods, the monolayers were washed and placed at -70°C to stop protein synthesis.

Infected monolayers were preincubated in methionine-free media containing 10 μ g of tunicamycin (Calbiochem, San Diego, Calif.) per ml or 1 μ M monensin (Calbiochem) for 60 min prior to labelling. The medium was removed and replaced with medium containing the same concentration of inhibitor and radioactive methionine. The labelling was carried out for 4 to 6 h, and the cells were harvested as above.

Infected cells were treated with carboxylcyanide-*m*-chlorophenylhydrazine (CCCP; Calbiochem) as previously described (28). Briefly, infected monolayers were methionine deprived in the presence of various concentrations of CCCP ranging from 0 to 20×10^{-6} M. Following a 30-min preincubation, the cells were pulse-labelled for 4 h in the presence of similar concentrations of CCCP.

SDS-PAGE immunoprecipitation and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoprecipitation and immunoblotting techniques have been described in a previous report from our laboratory (6). SDS-PAGE was carried in the presence of 0.1% SDS in the electrophoresis buffer and utilized 7.5% polyacrylamide gels. The amount of precipitated protein was quantitated on an LKB laser densitometer. All scanning of the gp150 was done by using autoradiograms exposed for identical lengths of time. The monoclonal antibodies used in this study have been described previously: antibody 27-156 (28), antibody 9-3 (4), and antibody 27-39 (30).

Velocity sedimentation analysis of gB oligomers. Infected-cell proteins from pulse-chase experiments were solubilized in 1.0% Nonidet P-40-Tris-buffered saline (0.05 M Tris, 0.15 M NaCl, 0.001 M EDTA [pH 7.5]) at 4°C . One milliliter of solubilized infected-cell proteins was placed on the top of 9

FIG. 1. Immunoblot of HCMV virions. Gradient-purified virions were disrupted in buffer containing 0.2% (lane 1), 0.4% (lane 2), and 2% (lane 3) SDS, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The disruption buffer contained 5% 2-ME in the results presented in the upper panel. The membranes were developed with monoclonal antibody (Ab) 27-156, 9-3, or 27-39 and then with rabbit anti-mouse IgG and ^{125}I -protein A. The sizes of immunoreactive proteins were estimated by comparisons with the migration of prestained molecular weight markers.

ml of 5 to 30% sucrose-Tris-buffered saline gradients containing 0.1% Nonidet P-40 and spun in a Beckman (Spinco Division, Palo Alto, Calif.) SW 41 rotor at 35,000 rpm for 16 h. Fractions (1 ml, unless otherwise specified) were collected from the bottom of the gradient immunoprecipitated with the indicated monoclonal antibody.

Cell surface and intracellular immunofluorescence. These techniques have been described in detail previously (29). Briefly, infected HF cells grown on 13-mm-diameter glass coverslips were reacted with monoclonal antibody at 4°C , washed, and fixed in 0.5% buffered paraformaldehyde prior to the addition of fluorescein-anti-mouse immunoglobulin G (IgG) conjugate. Intracellular antigens were detected by first fixing the cells and then permeabilizing them with 0.5% saponin. Monoclonal antibody staining and development were then carried out as described above.

RESULTS

Identification of oligomeric forms of the gp55-116 (gB) complex. Several HCMV-specific monoclonal antibodies with neutralizing activity were shown to precipitate the gp55-116 (gB) complex from detergent-solubilized virus or virus-infected cells (data not shown). However, none of these antibodies was reactive in immunoblots when either virions or infected cells were solubilized in 2-mercaptoethanol (2-ME)-containing buffers. Because of the extensive disulfide bonding present in this glycoprotein complex (4, 14), we examined the possibility that reactivity of this group of monoclonal antibodies was dependent on disulfide-linked gp55-116 complexes. Gradient-purified virions were disrupted in buffer containing various concentrations of SDS, ranging from 0.2 to 2.0% with and without 2-ME, and subjected to analysis by immunoblotting by using three gB-specific monoclonal antibodies. In the presence of 2-ME, only monoclonal antibody 27-156, which is directed against the large linear antigenic site AD-1 located in the gp55 component, exhibited reactivity (Fig. 1) (19). This reactivity was unaltered by increasing the concentration of SDS in the

FIG. 2. Oligomerization of gB. Infected-cell proteins were pulse-labelled for 15 min with [35 S]methionine and chased in nonradioactive media for the indicated times (T). Following solubilization, the labelled infected-cell proteins were subjected to sedimentation through 10 ml of sucrose gradients, 1-ml fractions were collected from the bottom of the gradient, and the fraction was immunoprecipitated and analyzed by SDS-PAGE. The gradient fractions are represented from the bottom to the top of the gradient (lanes 1 to 10). Precipitations with monoclonal antibody 27-156 (A) and 27-39 (B) are shown.

disruption buffer (Fig. 1). When the same preparations of gradient-purified virions were disrupted in the absence of 2-ME, we could demonstrate reactivity for at least three high-molecular-mass complexes, with the fastest migrating complex having an estimated molecular mass of 170 kDa (Fig. 1). Two higher-molecular-weight complexes were also identified by both antibodies 27-156 and 9-3 (Fig. 1). These higher-molecular-weight complexes were seen only following disruption in buffer containing lower concentrations of SDS, suggesting the presence of noncovalently linked oligomeric structures in the gp55-116 (gB) complex. Antibody 27-39 reacted only with the highest-molecular-weight complex and not with the two smaller complexes (Fig. 1). Disruption of the complexes with 2-ME or increasing concentrations of SDS (>0.2%) in the disruption buffer destroyed the recognition site for antibody 27-39, suggesting that this antibody recognized only higher-order structures in this complex (Fig. 1). We could not estimate the size of these complexes because of the nonlinear rates of migration of high-molecular-weight proteins in this gel system. It can, however, be appreciated that the sizes of these complexes were considerably larger than those of the gp170 complexes (Fig. 1). Interestingly, we found no evidence that increasing temperatures influenced the abundance of these higher-molecular-weight complexes (data not shown). In addition, the same-size complexes could be demonstrated in cells infected with a recombinant vaccinia virus containing the gp55-116 gene and disrupted in the absence of 2-ME (data not shown). These data indicated that higher-order structures of the gp55-116 (gB) complex could be detected in the envelope of HCMV and by virus-neutralizing monoclonal antibodies.

To determine whether the higher-molecular-weight struc-

tures detected in the HCMV envelope gB complexes represented oligomeric forms of this complex as have been described for a variety of mammalian viruses, including herpes simplex virus and PRV, we analyzed oligomer formation of gB in HCMV-infected fibroblasts. Infected-cell proteins were pulse-labelled with [35 S]methionine and chased for various times in nonradioactive media. The labelled proteins were then fractionated by sedimentation in sucrose gradients, and individual fractions were precipitated with monoclonal antibody 27-156. Following a 15-min pulse-labelling, we could detect the gB precursor, gp150, spread across the gradient in fractions 3 to 6 (Fig. 2A). After a 20-min chase period, the gp150 was most abundant in fraction 4, but considerable amounts of labelled gp150 were also present in fractions 3 to 6 (Fig. 2A). By 40 min, this distribution was even more apparent, with almost all radioactive gp150 being present in fraction 4 (Fig. 2A). Following a 180-min chase, the gp55 and gp116 cleavage products could be seen (Fig. 2A). At this last time point, more than 90% of the labelled gB products could be found in fraction 4, indicating the formation of more rapidly sedimenting, higher-molecular-weight complexes. These complexes were most likely formed as a result of oligomerization of the gp55-116 (gB) complex during its biosynthesis and transport. Although we could readily demonstrate the formation of high-molecular-weight complexes during the chase period with the coincidental loss of forms of the complex sedimenting near the top of the gradient, we could not demonstrate a unique, low-molecular-weight form which could be designated as a monomer in HCMV-infected fibroblast cells. Although there could be several explanations for our inability to clearly demonstrate a monomeric form of the gB precursor, the most likely reason was the use of an extended

FIG. 3. Pulse-chase analysis of forms of gB identified by antibodies 27-156 and 27-39. (A) HCMV-infected HF monolayers were pulse-labelled for 10 min and then chased with nonradioactive media for 0 to 320 min and 16 h. The monolayers were solubilized and immunoprecipitated with either monoclonal antibody 27-156 or 27-39 and analyzed by SDS-PAGE. (B) The amount of the gp150 precipitated was determined by laser densitometry and the time to half-maximal formation was estimated for the time interval between 0 and 320 min.

pulse-labelling period. We attempted shorter pulse-labelling times but could not attain sufficient incorporation of [^{35}S]methionine in gB in HCMV-infected cells for use in this type of analysis (data not shown). We have measured the half-maximal time of the oligomerization of the gB by scanning densitometry and have calculated it to be approximately 25 min (data not shown). This rate of oligomer formation was similar to that observed for the gB homolog of PRV (31).

The availability of a monoclonal antibody which reacted only with high-molecular-weight forms of the gp55-116 (gB) complex (Fig. 1) prompted us to examine the reactivity of this monoclonal antibody against the products of this pulse-chase experiment. Identical fractions of the gradients described above were precipitated with antibody 27-39 and analyzed by SDS-PAGE. In contrast to our previous results, antibody 27-39 precipitated complexes only from fractions 3 to 5 and only at the latter time points of the chase (Fig. 2B). This finding was in agreement with our earlier immunoblot results and indicated that antibody 27-39 recognized only an oligomeric form of the gp55-116 (gB) complex.

The size of the oligomeric form of gB was estimated by comparing the migration of radiolabelled, oligomeric gB from pulse-labelled infected cells and protein standards in sucrose density gradients. After several attempts with various standards, including β -galactosidase, we chose secretory IgA from human colostrum as a standard because of its size (380,000 Da) and relatively high carbohydrate content (10%). Identical gradients were formed, sedimented together, and then fractionated. Fractions containing radiolabelled infected-cell proteins were immunoprecipitated with antibody 27-156 and analyzed by SDS-PAGE. Gradients containing sedimented secretory IgA were fractionated, and aliquots of each fraction were analyzed by immunoblotting with radiolabelled anti-human IgA as a detector antibody. We found that secretory IgA and the oligomeric form of gB sedimented in nearly the same fractions (data not shown). Thus, as estimated by this method, the gB oligomer was approximately 380,000 Da, suggesting that the gB oligomer was likely a dimer consisting of two gp55-116 molecules with

a calculated mass of approximately 340,000 Da. This size estimate was based on previous estimates of the size of the uncleaved complex of gB (7). Identical results were obtained when cells infected with a vaccinia recombinant virus expressing gB were used as an antigen source (data not shown).

Antibody 27-39 recognizes a conformational site on gB which is formed following oligomerization. The availability of a monoclonal antibody specific for oligomeric forms of gB allowed us to more conveniently and accurately examine the kinetics of oligomer formation. Pulse-chase analysis of HCMV-infected cells was carried out, and the infected-cell proteins were precipitated with either monoclonal antibody 27-156 or 27-39 and the precipitated proteins were resolved by SDS-PAGE. The gp150 precursor was detected by antibody 27-156 in the immediate pulse interval and increased through the first three chase intervals (Fig. 3A). In this experiment, a doublet was detected in the early chase intervals, suggesting the presence of differentially modified forms of the precursor (Fig. 3A). As has been previously described, the precursor was processed into the mature products between 80 and 160 min, as indicated by the initial appearance of the cleavage products gp55 and gp116 (antibody 27-156, Fig. 3A). The kinetics of synthesis of gp150 reactive with antibody 27-39 was different in that the quantity of the gp150 increased through the first five chase periods (antibody 27-39, Fig. 3A). Quantitation of the kinetics of the appearance of the gp150 by densitometry revealed a half-maximal synthesis time of approximately 20 min for the forms detected by antibody 27-156 and approximately 160 min for the forms detected by antibody 27-39 (Fig. 3B). The half-maximal time of gp150 formation was only slightly faster than the estimated half-maximal time of oligomerization (20 versus 25 min), suggesting that oligomerization occurred nearly coincidentally with synthesis of gp150. In contrast, the much slower half-maximal time of formation of antibody 27-39-reactive gp150 indicated that gB underwent additional conformational changes and/or modifications following oligomerization. This latter event was relatively slow

FIG. 4. HCMV gB oligomerization occurs in the ER. Infected-cell monolayers were radiolabelled in 1-ml aliquots in the presence of CCCP (0 , 5×10^{-6} , 10×10^{-6} , or 20×10^{-6} M), as described in Materials and Methods. Following solubilization, the infected-cell proteins were immunoprecipitated with either monoclonal antibody 27-156 or 27-39. Replicate precipitates obtained with antibody 27-156 were treated with endo H (+) as previously described (7). The precipitated proteins were resolved by SDS-PAGE. The migration of the forms of gB is shown on the right, including the previously described nonglycosylated p105 (7).

compared with the synthesis and oligomerization of the gB and was consistent with the previously reported prolonged processing time required for transport, terminal glycosylation, and cleavage of the precursor gp150 to the final components of the gp55-116 complex (7).

gB oligomerization and acquisition of additional conformation occurred in the ER and was independent of posttranslational modifications. We investigated the site of oligomerization of gB, using a combination of transport inhibitors and the two described monoclonal antibodies. Infected cells were pulse-labelled in the presence of the various concen-

trations of the ionophore CCCP and solubilized with detergent-containing buffer. CCCP is an inhibitor of oxidative phosphorylation and therefore inhibited energy-dependent transport from the ER (9, 28). Infected-cell proteins were precipitated with either monoclonal antibody 27-156 or 27-39 and analyzed by SDS-PAGE. Both antibodies precipitated the gp150 from the infected cells, including gp150 from cultures treated with the highest concentration of CCCP which prevented processing of the precursor into the cleaved, mature forms of the protein (Fig. 4, lanes 20). To ensure that this form represented the unprocessed and therefore ER form of gB, we subjected a replicate sample of the immunoprecipitated infected-cell proteins to digestion with endoglycosidase H (endo H). Endo H-sensitive forms have been previously shown to correspond to forms of gB localized to the ER (7). Digestion with endo H resulted in increased rate of migration of the gp150 to approximately 105 kDa, a size corresponding to the mass of the nonglycosylated precursor, indicating its complete sensitivity to endo H (7). Identical results were obtained when brefeldin A was used to inhibit transport from the ER (data not shown). These results indicated that the gp150 recognized by monoclonal antibody 27-39 was localized to the ER and that oligomerization and additional folding of gB as detected by reactivity with antibody 27-39 occurred within the ER.

We next investigated the influence of inhibitors of glycosylation on the oligomerization and folding of the gB. Infected cells were radiolabelled and chased in the presence of either tunicamycin, an inhibitor of N-linked glycosylation, or monensin, an inhibitor of intracellular transport. Both antibodies reacted with the gp150 precursor synthesized in the presence of monensin (Fig. 5). Unexpectedly, antibody 27-39 also detected the nonglycosylated form of the gB precursor, p105, although the quality of precipitated protein was markedly reduced (Fig. 5). Because the amounts of nonspecifically precipitated protein migrating slightly slower than gp55 were comparable in all lanes of the experiment, it was unlikely that the decreased amount of p105 was a direct consequence of inhibition of protein synthesis by tunicamycin (Fig. 5). Alternatively, the reduced incorporation of radiolabel into the p105 forms precipitated by both antibodies 27-156 and 27-39 suggested that tunicamycin treatment resulted in additional, unmeasured effects of tunicamycin on

FIG. 5. Effects of inhibitors of glycoprotein processing on gB oligomerization. Infected monolayers of HF cells were pulse-radiolabelled in the presence or absence (control) of $1 \mu\text{M}$ monensin (mon) or $10 \mu\text{g}$ of tunicamycin per ml for 10 min. After being washed, the cultures were chased in media containing the inhibitors but without radioactive methionine. Infected-cell proteins were precipitated with either antibody 27-156 or 27-39 and analyzed by SDS-PAGE. The migration of the forms of gB is shown on the right.

FIG. 6. Cell surface expression of gB oligomeric forms. Infected cells were subjected to immunofluorescence with antibody 27-39, 7-17, or 58-15, which is directed at the cytoplasmic tail of the molecule. Cell surface analysis was done with viable cells, while intracellular analysis was done with fixed, permeabilized cells.

gB synthesis, such as inhibition of transport or aggregate formation. Regardless of these as yet undefined effects, it appears that a nonglycosylated form of gp150 can be recognized by antibody 27-39, suggesting the possibility that gB underwent oligomerization and additional folding to achieve a conformation resembling a native state. Furthermore, the reactivity of antibody 27-39 for gp150 from monensin-treated infected cells also supported the likelihood that the conformational changes observed during gp150 processing were independent of additional, previously undescribed carbohydrate modifications.

Detection of gB on the cell surface and on infectious virus. We have previously shown that antibody 27-39 can neutralize infectious virus, indicating the antigenic determinant detected by this antibody was a native structure and not an *in vitro* artifact created by detergent disruption or by alteration of intracellular transport and aggregate formation resulting from the use of pharmacologic agents. Further evidence of this finding was obtained by using antibody 27-39 to detect cell surface expression of gB on live infected cells in an immunofluorescence assay. Antibody 27-39 and control anti-gB antibody 7-17 (30) clearly reacted with the surface of infected cells and stained similar perinuclear collections of antigen in permeabilized cells (Fig. 6). As a control we also utilized a monoclonal antibody (antibody 58-15) generated against the putative cytoplasmic tail of gB (19a). Monoclonal antibody 58-15 is nonneutralizing and does not react with the surface of HCMV-infected cells (Fig. 6). Thus, it appeared that the oligomeric structures detected by antibody 27-39 were indeed native structures of the gB complex and not merely *in vitro* artifacts. Furthermore, the reactivity pattern of antibody 27-39 indicated that these gB oligomeric forms were transported to the surface of infected cells and incorporated into infectious virions.

DISCUSSION

Oligomerization of mammalian viral envelope glycoproteins is a well-described posttranslational modification of these membrane-bound proteins. Although it is better characterized in less structurally complex viruses such as influ-

enza virus and vesicular stomatitis virus, recent studies of PRV and early reports of herpes simplex virus have clearly demonstrated the oligomerization of the gB analogs of these herpesviruses (20, 31). In both cases, the gB glycoprotein has been reported to oligomerize into a homodimer (20, 31). We have only crudely attempted to determine the size of the oligomeric form of HCMV gB; these size estimates were most consistent with the oligomeric form also being a dimer. The composition of the oligomeric complexes has not been determined because the stoichiometry of the two structurally unrelated components, the TM component, gp55, and the SU component, gp116, in the mature gB remain undefined. Because these proteins have been shown to be synthesized as a polyprotein precursor which is cleaved in the Golgi complex following transport from the ER and because the oligomerization occurred in the ER, it seemed most likely that the oligomeric form of HCMV gB consisted of equimolar amounts of the two components. Sedimentation through hypertonic sucrose could have conceivably disrupted additional higher-molecular-weight, noncovalently linked complexes; thus, we could not exclude the possibility that more-complex forms of gB existed. In fact, we and others have described very-high-molecular-weight complexes in different viral envelope preparations (4, 14, 18). The consistent reactivity of the neutralizing monoclonal antibody 27-39 for all of the forms characterized in this study, however, suggested that the mature envelope component was most likely the homodimer and that some of the previously described high-molecular-weight forms were possibly aggregated protein produced during the sample preparation. This interpretation was further supported by the results of immunoblot analysis of virion preparations disrupted in limiting amounts of detergents (Fig. 1). These experiments defined a number of high-molecular-weight complexes, but only the highest-molecular-weight complex was reactive with antibody 27-39. In contrast to this finding, antibody 27-156, which is directed against a linear determinant, detected many smaller complexes but none larger than those detected by antibody 27-39. Finally, we have analyzed recombinant HCMV gB expressed in a recombinant vaccinia virus and have found identical kinetics and sizes of the oligomeric

protein in a variety of different cell lines, suggesting that the oligomer formation was not dependent on additional viral proteins or viral functions. At this time we cannot rule out the possibility that the oligomeric complexes of gB contained host-derived proteins.

Several lines of evidence suggested that gB oligomerization and folding occurred in the ER. First, the kinetics of synthesis and oligomer formation both were relatively rapid and much faster than cleavage of the molecule, a processing step which has been shown to take place in the Golgi complex (7, 13). Secondly, the use of the metabolic inhibitor CCCP prevented transport of the gB precursor into the Golgi complex, as evidenced by its sensitivity to endo H. Furthermore, the ER-retained form of the molecule was readily recognized by antibody 27-39, which was specific for folding intermediates formed following oligomerization. These findings were consistent with much of the information from the studies of intracellular transport of other viral glycoproteins, which have definitively shown that oligomerization was an ER event and was required for exit from the ER (12, 15, 23). Somewhat surprising in our studies was the finding of a relatively slow oligomerization step followed by further folding, which was remarkably slow in comparison to previously reported folding pathways (11, 15). Our evidence from utilizing the conformation-specific antibody 27-39 also suggested that this folding took place in the ER and might be a prerequisite for transport from the ER.

Although the kinetics of the HCMV gB oligomerization was noticeably slow compared with those of similar processes observed in vesicular stomatitis virus and influenza virus, its half-maximal time of formation was quite comparable to that of PRV and, interestingly, to that of the gp95 of Rous sarcoma virus (10, 31). However, of more interest was the prolonged postoligomerization folding pathway followed by HCMV gB prior to its exit from the ER. This folding pathway could be easily monitored from its initiation to its conclusion by the use of monoclonal antibodies which identified these two forms. A third monoclonal antibody, antibody 9-3, apparently identified additional folding intermediates in this pathway and is currently being more thoroughly characterized (data not shown). The prolonged folding event of HCMV gB prior to its transport from the ER was quite consistent with our previous studies of the synthesis of this glycoprotein. We noted the extremely prolonged processing pathway of this molecule and speculated that the rate-limiting step of intracellular transport may be the exit from the ER (6). In addition, we could not explain the observation that significant amounts of the uncleaved gB precursor remained even after extended chase periods (6). Results from the current study clarified many of these early observations and suggested that postoligomerization folding of gB was a potential explanation for its prolonged intracellular transport and processing.

The prolonged folding pathway of the HCMV gB remains undefined. Initially, we considered the possibility that gB transport was dependent on other virus-encoded proteins which influenced the kinetics of gB folding; however, expression of HCMV gB in a recombinant vaccinia virus revealed identical processing and transport, with similar, if not identical, kinetics. Additional possibilities include extensive remodeling of the gB within the ER. A recent study by Braakman et al. provided findings which are consistent with this possibility (3). In this study, the investigators provided evidence for energy-dependent molecular remodeling within the ER (3). This process potentially included the remodelling of aberrant intramolecular and intermolecular disulfide

bonds formed within the oxidizing environment of the ER. If such disulfide bond remodeling is a universal phenomenon which occurs during the synthesis and processing of membrane glycoproteins, then the prolonged folding pathway of gB could be consistent with rearrangement of the extensive disulfide bonding present in this molecule. Preliminary findings with a series of deletion mutants of gB suggested that formation of the post-ER forms of gB as well as some of the folding intermediates was exquisitely dependent on the intactness of disulfide bonding. Thus, one could envision a pathway in which disulfide bonds formed cotranslationally would be broken and reformed prior to the molecule assuming a near-native conformation. This would undoubtedly be an inefficient process and likely require an extended period of time as the molecule could assume many intermediate conformations as a result of aberrant disulfide bonding. Eventually, extensive remodeling of these bonds would result in a conformationally stable molecule which could then be transported from the ER. Alternatively, the prolonged folding pathway of HCMV gB could have resulted from the inefficiency of its interaction with ER resident chaperone proteins, thus requiring a prolonged ER stay to achieve a transportable form. At this time, we have no evidence that these folding events were chaperone protein mediated; however, this aspect of HCMV gB is currently under investigation, as its importance in the intracellular transport of other proteins has been well established (12).

It was of considerable interest that the conformational-dependent antibody-binding site of antibody 27-39 was formed even in the absence of carbohydrate modification of the gB. We have also observed the oligomerization of the gB during a pulse-chase analysis in the presence of tunicamycin (data not shown). These results indicated that the gB molecule oligomerized and folded even in the absence of extensive glycosylation, which accounts for some 30% of the mass of the gp150 (7). This finding was consistent with previously proposed requirements for oligomerization in that it appeared that the primary structure of the HCMV gB molecule was the determining feature of this process (12). Although our data were consistent with this hypothesis, the possibility that glycosylation was necessary for efficient folding and transport was also consistent with our results. In fact, our results suggested much-less-efficient folding of gB in the ER in the presence of TM (Fig. 6). Thus, glycosylation may not be an absolute requirement for the oligomerization and folding of gB in the ER but may greatly increase the efficiency of the process and prevent the generation of large amounts of misfolded intermediates, perhaps by limiting aberrant disulfide bond formation.

Although the determinant expressed on the ER form of gB which was detected by antibody 27-39 was also present on the mature virion form of gB, it was likely that the molecule underwent additional folding once it left the ER. Cleavage of the precursor and carbohydrate modifications, including O-linked sugar addition, occurred in the Golgi complex and made it probable that new conformations were assumed (7). To date we have not identified a monoclonal antibody which was reactive with post-ER forms of gB and nonreactive with uncleaved gB precursors trapped in the ER. However, many of our assays have been done in the presence of various detergents; thus, it was conceivable that conformations dependent on integrity of membrane structures were lost. It was clear, however, that antibody 27-39 defined a conformationally dependent antibody-binding site which was expressed on the surface of both infected cells and infectious virions. Previous studies have shown that humans produced

antibodies reactive with this or a nearby site following natural infection (19). Thus, this conformation-dependent antibody-binding site on gB was immunogenic in humans and represented a significant antibody-binding site on gB. Preparations of HCMV gB which will be considered for use as vaccine candidates must be examined for a structure(s) defined by antibodies with reactivity similar to that displayed by antibody 27-39 if the goal of such immunization is to mimic natural immunity by providing a native antigen.

Our results indicated a complex folding pathway for HCMV gB within the ER. These folding events were essential for transport of the gB out of the ER and to other intracellular structures, including the cell surface. The unique features of this pathway for HCMV gB, especially its prolonged oligomerization and folding within the ER, may allow a more complete analysis of the intermediates and mechanisms of protein folding in the eukaryotic cell. Furthermore, the availability of defined reagents will greatly simplify the study of this folding pathway and aid in the antigenic analysis of this important viral envelope protein of a clinically important human herpesvirus.

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