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

WILLIAM J. BRITT* AND LEONE G. VUGLER

Department of Pediatrics and Microbiology, the University of Alabama at Birmingham, Suite 752, Children's Hospital Tower, UAB Station, Birmingham, Alabama 35294

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The processing pathway of the major envelope glycoprotein complex, gp55-116 (gB), of human cytomegalovirus was studied using inhibitors of glycosylation and endoglycosidases. The results of these studies indicated that the mature gp55-116 is synthesized by the addition of both simple and complex N-linked sugars to a nonglycosylated precursor of estimated M_r 105,000. In a rapid processing step, the M_r 105,000 precursor is glycosylated to a protein of M_r 150,000 (gp150) which contains only endoglycosidase H-sensitive sugar linkages. The gp150 is then processed relatively slowly to a M_r 165,000 to 170,000 species (gp165-170), which is then cleaved to yield the mature gp55-116. Monensin prevented the final processing steps of the gp150, including cleavage, suggesting that transport through the Golgi apparatus is required for complete processing. Digestion of the intracellular forms of this complex as well as the virion forms confirmed the above findings and indicated that the mature virion form of gp55 contains 8,000 daltons of N-linked sugars. The virion gp116 contains some 52,000 to 57,000 daltons of N-linked carbohydrates and approximately 5,000 daltons of O-linked sugars.

Human cytomegalovirus (HCMV) is an important human pathogen accounting for severe disease in immunocompromised hosts. In addition, HCMV is also the most common cause of congenital viral infections in humans (1). Many infants suffer permanent neurologic damage as a result of this intrauterine infection (1). Little is known about protective immunologic responses which limit HCMV infection in vivo, but it is likely that both cellular and humoral immune responses against the envelope glycoproteins of HCMV play an important role in curtailing the severity of infection with HCMV. The envelope glycoproteins of HCMV have been incompletely characterized. In fact, there remains considerable controversy as to the exact number of electrophoretically unique glycoproteins within the virion envelope (2, 18, 22). More recent studies utilizing immunologic techniques have indicated that at least four electrophoretically separable glycoproteins can be identified in the envelope of HCMV (2–4, 11, 17, 18, 20). In addition, other studies have documented the presence of families of glycosylated infected-cell proteins (18), but the relationship between several of the infected-cell proteins and virion glycoproteins has not been definitively established. Of the virion glycoproteins, the most widely studied and most abundant is a disulfide-linked heterodimer consisting of two glycosylated proteins of estimated M_r 55,000 and 116,000 (6). We have termed this complex gp55-116; a similar glycoprotein complex have been referred to as gp58, gA, or gB by other investigators (8, 15, 17, 18). Although we have continued to use our original nomenclature for this disulfide complex, we have shown that monoclonal antibodies utilized in this and previous studies react with the gB gene product (7). Several properties of the gp55-116 complex make it a likely target of protective immunologic responses. These include abundant expression on the surface of infected cells and infectious virions (3, 9, 18), expression of epitope(s) recognized by neutralizing antibodies (3, 17, 18, 20), and immunogenicity in humans (3).

The structure of the gp55-116, including its synthesis from a higher-molecular-weight polyprotein, has been detailed in previous studies (6). Recent reports have provided some information on the types of carbohydrates present in the precursor polyprotein and the mature forms (2, 10, 18). These studies have suggested that both simple and complex sugars can be found in the mature forms, but the processing of the immature precursors has been incompletely elucidated. In contrast, more recent studies have indicated that only simple N-linked sugars are present on the intracellular forms of this molecule (21). Because several laboratories have begun studies utilizing heterologous expression systems to synthesize the gp55-116 (gB) gene product, it is essential to define its processing within permissive human cells. In the absence of such an understanding, the interpretation of immunological and structural studies involving recombinant-derived gp55-116 (gB) will be difficult at best. In this study we have utilized the ionophore monensin to study the processing of the gp55-116 complex. In addition, endoglycosidase H (Endo H) and glycopeptidase F were used to characterize the carbohydrate modifications present in mature and immature forms. Our results indicated that the mature gp55-116 complex is synthesized by addition of N-linked sugars to a M_r 105,000, nonglycosylated precursor. A stable intracellular intermediate of M_r 150,000 which contains only simple sugars is further processed to a penultimate M_r 165,000 to 170,0000 species containing both simple and complex sugars. The fully glycosylated form, gp165-170, is then cleaved into the mature gp55-116. Inhibition of glycosylation and processing prevented the synthesis of gp165-170 as well as its cleavage.

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* Corresponding author.

MATERIALS AND METHODS

Cells and virus. The propagation of human foreskin fibroblast (HF) cells has been described previously (3). HCMV strain AD169 was utilized for all studies.

Radiolabeling of virion and infected-cell proteins. Confluent monolayers of HF cells were infected at a multiplicity of infection of 1 4 days before labeling. The cultures were washed three times in Dulbecco modified phosphate-buffered saline (pH 7.4) and methionine starved by preincubation in Dulbecco modified Eagle medium without methionine for 45 min. The medium was then removed, and Dulbecco modified Eagle medium without methionine but containing 30 μCi of [^{35}S]methionine per ml and 1% dialyzed fetal calf serum was then added. The labeling period was for 3 to 6 h unless otherwise specified. Pulse-chase analysis was carried out as described (6). Extracellular virions were collected by centrifugation of clarified supernatants through sorbitol gradients as described (6). Virions were labeled with ^{125}I by the chloramine T method as described (6).

Inhibitors of glycosylation and intracellular transport. Experiments utilizing 1-deoxymannojirimycin (DMJ) and monensin (Boehringer-Mannheim, Indianapolis, Ind.) were performed by first preincubating infected cell cultures for 90 min in the presence of DMJ or monensin before radiolabeling. The radiolabeling was carried out in the presence of either DMJ or monensin.

Immune precipitation, SDS-PAGE, and immunoblotting. The details of the immune precipitation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and immunoblotting procedures have been described previously (6). All gels were of 7.5% acrylamide cross-linked with bisacrylamide. Monoclonal antibody 7-17, a mouse immunoglobulin G3 antibody, and control antibody 48 have been previously described (3, 6). Relative migration (M_r) was estimated by comparison with molecular weight standards (Sigma Chemical Co., St. Louis, Mo.).

Endo H, glycopeptidase F, and O-glycanase digestions. Endo H (endo-β-*N*-acetylglycosaminidase H; Miles Laboratories, Naperville, Ill.) digestions were performed on immune-precipitated samples under conditions specified by the manufacturer. Briefly, immune-precipitated proteins were eluted in 1.0% SDS–0.1 M sodium citrate (pH 5.5) and diluted to a final SDS concentration of 0.1%. Endo H (0.01 U) was then added, and digestion was allowed to proceed for 16 h at 37°C. The samples were then analyzed by SDS-PAGE. Glycopeptidase F (Boehringer-Mannheim) digestions were performed on identically prepared immune-precipitated samples under conditions detailed by the manufacturer. Immune-precipitated proteins were solubilized in 0.1 M sodium phosphate (pH 6.1) containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol. Approximately 1 U of glycopeptidase F was added, and the digestions were allowed to proceed for 5 h at 37°C. Samples were analyzed as described above. To inhibit proteolytic activity which may be present in preparations of Endo H, glycopeptidase F, or O-glycanase, all reaction mixtures contained 1 mM phenylmethylsulfonyl fluoride and 0.01 U of aprotinin (Sigma). Additional endoglycosidase treatments were carried out on the virion forms of the gp55-116 complex after separation of ^{125}I-labeled virion proteins by SDS-PAGE. After visualization, individual proteins were eluted from the gel slices and collected following acetone precipitation as described (5). The protein preparations were then subjected to endoglycosidase treatment as described above. Digestions with Endo H and glycopeptidase F were monitored for completeness by including ^{125}I-labeled fetuin (Sigma) as an internal control. O-glycanase (Genzyme, Boston, Mass.) digestion was carried out after neuraminidase (provided by Richard Compans, University of Alabama at Birmingham) treatment under conditions similar to those recently described (19). Radiolabeled virions were immune precipitated with antibody 7-17 and eluted from staphylococcal protein A pellets by being heated to 100°C in 0.5% SDS–0.1 M citrate buffer (pH 5.5); then they were diluted to a final SDS concentration of 0.05% before digestion. The solubilized proteins were then digested overnight with 0.01 u of neuraminidase, followed by 0.01 U of O-glycanase for an additional 12 h. The digestion products were analyzed as described above.

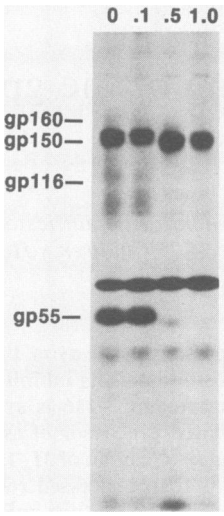

FIG. 1. Monensin treatment of HCMV-infected cells. Infected HF cells were preincubated with 0, 0.1, 0.5, or 1.0 μM monensin for 90 min and then labeled with [^{35}S]methionine in the presence of monensin for 5 h. Infected-cell proteins were solubilized, immune precipitated with antibody 7-17, and analyzed by SDS-PAGE as described in Materials and Methods.

RESULTS

Monensin inhibits glycosylation and processing of the gp55-116 complex. To further investigate the intracellular processing of the gp55-116 complex, we used the ionophore monensin to inhibit the normal intracellular transport of glycoproteins within HCMV-infected cells. In the absence of monensin, the four forms of the gp55-116 complex, gp160, −150, −116, and −55, were apparent, with gp160 migrating as a diffuse, heterogenous smear slightly slower than gp150, and gp116 migrating as a diffuse smear in this gel system (Fig. 1). The migration of gp116 has been arbitrarily defined by comigration of its slowest migrating boundary with the SDS standard β-galactosidase. Increasing concentrations of monensin inhibited the synthesis of gp160 as well as generation of the mature cleavage products gp116 and gp55. This finding was consistent with our previously proposed pathway for the synthesis of the mature disulfide-linked gp55-116 complex by cleavage of the penultimate gp160 protein (6). These results indicated that processing of gp150 into gp160 and subsequent cleavage into the mature gp55-116 envelope complex was dependent on normal transmit through intracellular compartments, most likely the Golgi apparatus. Furthermore, the absence of cleavage of the gp150 protein

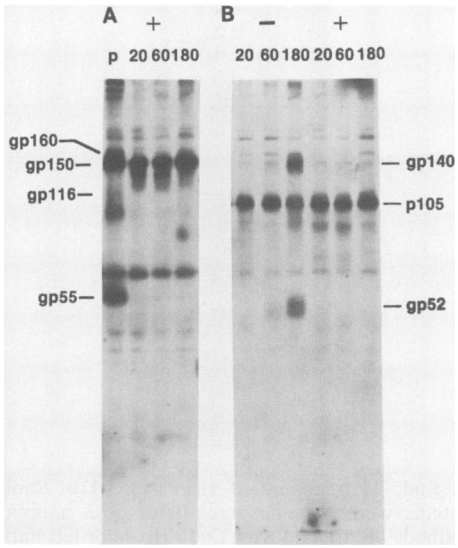

FIG. 2. Pulse-chase analysis followed by Endo H treatment of the gp55-116 complex. (A) Infected cells were preincubated with 5 μM monensin for 90 min, pulse-labeled with [^{35}S]methionine for 5 min, and then chased in medium containing monensin and nonradiolabeled methionine for 20, 60, or 180 min. The cultures were harvested, immune precipitated with antibody 7-17, and analyzed by SDS-PAGE as described in the legend of Fig. 1. For the purposes of comparison, the extreme left lane (p) contains an HCMV-infected culture pulse labeled for 180 min in the absence of monensin and analyzed in a similar fashion. (B) Duplicate cultures of HCMV-infected HF cells were preincubated either in medium (−) or in medium containing 5 μM monensin (+). The cultures were pulse-labeled with [^{35}S]methionine for 5 min and then chased in medium containing unlabeled methionine with (+) or without (−) 5 μM monensin. The cultures were harvested and immune precipitated with antibody 7-17. The immune precipitates were then digested with Endo H as described in Materials and Methods and analyzed by SDS-PAGE.

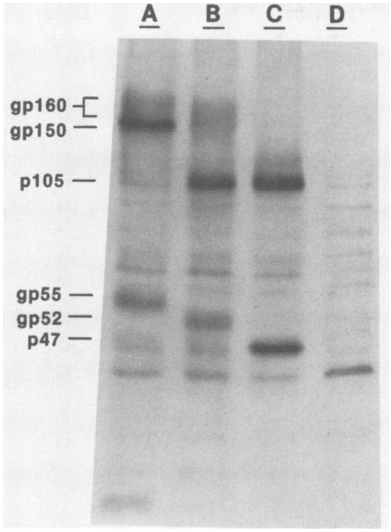

FIG. 3. Endo H and glycopeptidase F treatment of intracellular components of the gp55-116 complex. [^{35}S]methionine-labeled HCMV-infected HF cells were immune precipitated with antibody 7-17 or control antibody 48 (lane D). The immune precipitates were then left untreated (lane A), or treated with Endo H (lane B) or glycopeptidase F (C), and then separated by SDS-PAGE. Molecular weights were estimated from relative migration of molecular weight standards.

into more rapidly migrating forms suggested that cleavage of the HCMV envelope polyprotein complex was dependent on complete glycosylation or normal intracellular transport, or both, of this molecule.

The gp150 precursor protein contains Endo H-sensitive carbohydrate linkages. We next investigated the processing of the intracellular forms of the gp55-116 complex by using a combination of pulse-chase analysis in the presence of monensin followed by Endo H digestion of immune-precipitated proteins. When HCMV-infected HF cells were continuously labeled for 3 h in the absence of monensin, immune precipitation of infected-cell proteins with antibody 7-17 demonstrated the intracellular forms of the gp55-116 complex, gp160, −150, −116, and −55 (Fig. 2A). Again gp116 migrated diffusely as described in Fig. 1. A pulse-chase analysis in the presence of monensin indicated that drug treatment inhibited both the glycosylation of gp160 and the generation of mature gp55-116 over a time interval sufficient for synthesis of these mature forms (Fig. 2A).

A similar experiment was then carried out in the presence or absence of monensin, followed by Endo H digestion of the immune-precipitated proteins. In the absence of monensin, the intracellular precursor gp150 was found to be sensitive to Endo H digestion as shown by the absence of gp150 and the appearance of a protein of estimated M_r 105,000 (p105; Fig. 2B). In contrast to the results observed in the initial time periods, a protein of M_r 140,000 to 150,000 (which migrated slightly faster than gp150), two proteins of M_r 90,000 to 100,000 and 52,000, and the previously detected protein of M_r 105,000 were seen during the final 180-min chase interval (Fig. 2B). Because the M_r 140,000-150,000, 90,000-100,000, and 52,000 species were seen during a chase interval in which both the mature gp55-116 complex and the gp160 were present (Fig. 2A, lane P), it was likely these more rapidly migrating proteins represented forms of gp160, −116, and −55 which contained Endo H-resistant sugar linkages. When a similar pulse-chase experiment was carried out in the presence of monensin, no Endo H-resistant forms were detected (Fig. 2B), indicating that monensin prevented the processing of the gp150 to a form containing Endo H-resistant sugars. After Endo H digestion of pulse-chase products in the presence of monensin, a discretely migrating species of M_r 90,000 was also seen (Fig. 2B). Although the origin of this protein(s) is unknown, because it was a minor species seen only after Endo H digestion we assumed it likely represented a degradation product of the higher-molecular-weight form. From these results, the intracellular processing of the gp55-116 complex appears to involve N-linked glycosylation of the M_r 105,000 nonglycosylated precursor to an abundant glycosylated intermediate, gp150, which contains only simple, high-mannose sugars. The gp150 is then processed to a form containing Endo H-resistant sugars, gp160, which is in turn cleaved into the mature gp55-116 complex.

The gp160, gp150, and mature gp55-116 complex contain complex sugars. To further characterize the carbohydrate modifications present on the intracellular forms of the gp55-116 complex, we analyzed infected-cell proteins with Endo H and glycopeptidase F, an endoglycosidase which cleaves both simple and complex sugar linkages (24). Monoclonal antibody 7-17 precipitated four proteins of previously designated M_r of 160,000, 150,000, 116,000, and 55,000 from infected cells (Fig. 3, lane A), although in this experiment

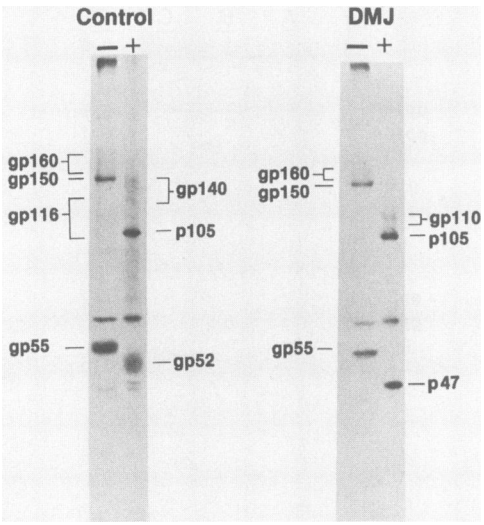

FIG. 4. Inhibition of processing of the gp55-116 complex by DMJ. Infected HF cells were radiolabeled with [^{35}S]methionine in medium (control) or medium containing 4 mM DMJ. The infected-cell proteins were then immune precipitated with antibody 7-17, treated with Endo H (+) or left untreated (−), and then analyzed by SDS-PAGE.

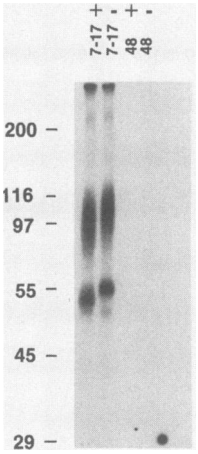

FIG. 5. Endo H treatment of virion gp55-116. Radioiodinated virion proteins were immune precipitated with antibody 7-17 or control antibody 48, treated with Endo H (+) or left untreated (−), and subjected to SDS-PAGE. Molecular weight standards are indicated on left.

gp116 could not be distinguished from comigrating proteins nonspecifically precipitated by the immunosorbent. When identically prepared precipitates were digested with Endo H, abundant gp150 was no longer present; however, a new species migrating at M_r 105,000 (p105) could be seen (Fig. 3, lane B). In addition, the migration of gp160 and gp55 were increased, resulting in the presence of proteins of estimated M_r 140,000 to 160,000 and 52,000 (gp52), respectively (Fig. 3, lane B). Digestion of similarly prepared precipitates with glycopeptidase F resulted in no change in the migration of p105, but an increase in the M_r 140,000-160,000 protein to a diffusely migrating band of approximate M_r 110,000 (Fig. 3, lane C). The migration of the gp52 protein also increased further, with its size estimated at M_r 47,000 (Fig. 3, lane C). A control antibody failed to precipitate any of these proteins (Fig. 3, lane D). From these results and those presented above, it appears that the intracellular forms, gp160 and gp55, contain significant quantities of the N-linked sugars, as has been reported previously (2, 10, 21). If the digestions by glycopeptidase F were complete, then the gp160 contains some 50,000 daltons of carbohydrates and the gp55 some 8,000 daltons, representing approximately 30 and 15%, respectively, of their mass. The carbohydrate content of the gp116 could not be determined because of the poor resolution of this protein in these experiments.

Additional evidence for the presence of complex sugars on the intracellular forms of the gp55-116 protein was obtained by use of DMJ, an inhibitor of α-mannosidase I. Because this enzymatic activity is required for complex sugar processing, inhibition should prevent full processing of the gp55-116 complex. Immune precipitation of infected-cell proteins radiolabeled in the presence of DMJ revealed little alteration in the migration of the previously identified forms gp160, gp150, gp116, and gp55 as compared with control cultures (Fig. 4). Treatment with Endo H of identically prepared immune precipitates from control untreated cultures resulted in the appearance of the previously described proteins of M_r 140,000-150,000, 105,000, and 52,000 (Fig. 4). When immune precipitates from DMJ-treated cultures were digested with Endo H, species of M_r 110,000, 105,000, and 47,000 were present (Fig. 4). Because these species were also found in glycopeptidase F-treated infected-cell proteins from untreated cultures (Fig. 3), these results provide confirmatory evidence for the presence of complex sugars on gp160 and gp55. Furthermore, because the migration of the gp150 protein in DMJ-treated cultures after Endo H digestion was not increased, these findings indicate that the N-linked carbohydrates present on the gp150 are of the simple and not complex form.

The virion gp55-116 complex contains both N-linked and O-linked carbohydrates. Because we could not adequately resolve the gp116 precipitated from infected cells, we attempted to more fully characterize its carbohydrate modifications in extracellular virions. Extrinsically radiolabeled gradient purified virions were disrupted, immunoprecipitated with antibody 7-17 or control antibody 48, and analyzed by SDS-PAGE. The two components of the complex, the gp116 and gp55 proteins, were precipitated by antibody 7-17 (Fig. 5). When identically prepared immune precipitates were digested with Endo H, the migration of both proteins increased, indicating that both proteins contain simple N-linked sugar modifications (Fig. 5). A control antibody failed to precipitate either of these forms (Fig. 5). Further definition of the carbohydrate modifications of these proteins was obtained by immune precipitation of radiolabeled virions, isolation of the individual proteins by SDS-PAGE, and digestion with either Endo H or glycopeptidase F. Endo H digestion again resulted in a minimal increase in the migration of the gp116 and gp55 to species of estimated M_r 100,000 and 52,000, respectively (Fig. 6). When these proteins were digested with glycopeptidase F, the migration of gp55 was increased, yielding a protein of M_r 47,000, whereas the migration of gp116 increased dramatically and resulted in the appearance of a new species of estimated M_r 52,000-60,000 (Fig. 6). These findings were in agreement with our earlier results and indicated that gp55 contains some 8,000 daltons of N-linked simple and complex sugars. However, the finding that the gp116 contained nearly 56,000 daltons (calculated by assigning the molecular size of gp116 as 116,000 daltons and that of the glycopeptidase F digestion product as 60,000) was somewhat surprising in that previous calcula-

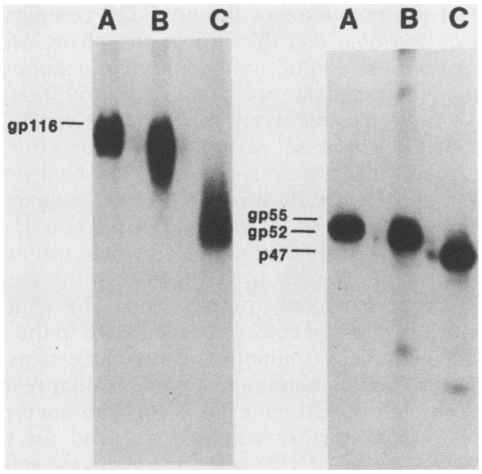

FIG. 6. Endo H (left lanes) and glycopeptidase F (right lanes) digestion of virion gp55-116. Radioiodinated virion proteins were immune precipitated with antibody 7-17 and separated by SDS-PAGE, and the individual proteins were eluted from the gel as described in Materials and Methods. After digestion with Endo H (lanes B), glycopeptidase F (lanes C), or buffer alone (lanes A), the proteins were analyzed by SDS-PAGE.

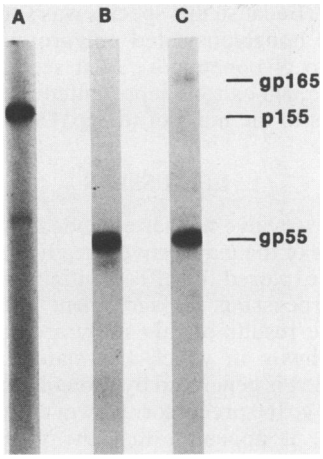

FIG. 7. Immunoblot analysis of extracellular virions. Gradient-purified virions (lanes A and B) or a partially purified high-speed pellet of culture supernatant (lane C) were heated to 100°C for 4 min in buffer containing 2% SDS and 5% 2-mercaptoethanol and subjected to SDS-PAGE. After transfer to nitrocellulose, the strips were incubated with monoclonal antibody 28-4 (lane A), which is reactive with the major capsid protein of HCMV, or antibody 7-17 (lanes B and C). Antibody binding was detected by ^{125}I-protein A, followed by autoradiography.

tions had suggested that the gp160-penultimate precursor of both components of the complex could contain only 55,000 daltons of total carbohydrate. A simple explanation of this discrepancy was that the assignment of the mass of the gp160 was an underestimate and the gp160 was actually a larger molecule with a greater carbohydrate content. This possibility was not unlikely because small differences in migration in this area of the acrylamide gel can result in major differences in the estimation of the mass of a protein. We examined this possibility by analyzing extracellular virions: both partially purified preparations, which have been shown to contain the uncleaved gp160 without the closely migrating gp150 (3, 6), and gradient-purified virions. Immunoblot characterization of these two preparations revealed the gp55 and a higher molecular-weight form of estimated M_r 165,000 to 170,000 (gp165) in partially purified extracellular virions, which were not found in the gradient-purified virions (Fig. 7). As a marker for this high-molecular-weight form we also developed one of the nitrocellulose membranes with monoclonal antibody 28-4, which is reactive with an M_r 155,000, nonphosphorylated capsid protein (Fig. 7). Thus it appears that the previously designated gp160 was likely of M_r 165,000 to 170,000. When the carbohydrate content of the penultimate precursor was calculated based on this mass, the precursor contained some 60,000 to 65,000 daltons of carbohydrates, of which 8,000 daltons was present on the gp55 and approximately 52,000 to 57,000 daltons on the gp116, values approximately those calculated from the results of glycopeptidase F digestions (Fig. 6).

We next examined the possibility that the gp55-116 complex contained a small amount of O-linked oligosaccharides, because it has been reported that other human herpesviruses contain O-linked sugars within their envelope glycoproteins (13). Extrinsically labeled virions were disrupted and then immune precipitated with antibody 7-17, and the precipitated proteins were treated with either neuraminidase or neuraminidase plus O-glycanase, according to previously published methods for removal of O-linked sugars (19). Analysis of the treated proteins revealed a small but perceptible increase in the migration of gp116, suggesting the presence of O-linked sugars (Fig. 8). Digestion with neuraminidase alone did not alter the migration of either gp55 or gp116 as compared with that of untreated precipitates (data not shown). From these results it appears that gp116 contains a small amount of O-linked carbohydrates, although it was impossible to accurately estimate the quantities of O-linked sugars present in the gp116. However, several findings indicated this type of carbohydrate modification represents at most 5,000 daltons of the mass of the gp116. First, removal of both simple and complex N-linked sugars from the gp160 (gp165-170)-penultimate form of the gp55-116 complex generated a species of M_r 110,000 (Fig. 3). In addition, inhibition of complex sugar addition by DMJ, followed by Endo H digestion, generated an M_r 110,000

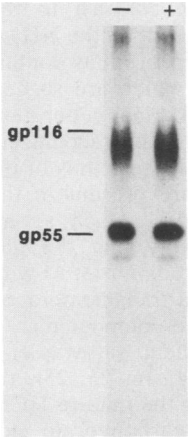

FIG. 8. Neuraminidase and O-glycanase digestion of virion gp55-116. Radioiodinated virion proteins were precipitated with antibody 7-17 and treated with neuraminidase (−) or neuraminidase followed by O-glycanase (+). The treated proteins were subjected to SDS-PAGE.

species (Fig. 4). Because this species was some 5,000 daltons larger than the nonglycosylated polyprotein precursor and was resistant to glycopeptidase F, it seemed possible that this additional 5,000 daltons represented the contribution of O-linked sugars to the mass of the gp116.

DISCUSSION

In this study we have further extended our understanding of the synthesis of the major envelope glycoprotein complex of HCMV and explored the effects of inhibitors of glycosylation on its processing. In agreement with our previous findings (6), the results of this study are consistent with a processing pathway in which the mature disulfide-linked gp55-116 complex is generated by proteolytic cleavage of the fully processed gp160 precursor. However, in contrast to our earlier reports, it appeared that the gp160 precursor is actually of higher molecular weight, probably in the range of M_r 165,000 to 170,000. This fully processed form contained both N-linked complex and simple sugars and possibly also O-linked carbohydrates. The sizes of the polypeptide components of the mature gp55 and gp116, as determined by digestion with glycopeptidase F, were M_r 47,000 and 52,000 to 60,000, respectively. In addition, the gp116 appeared to contain some 5,000 daltons of O-linked sugars, suggesting that the nonglycosylated protein would be of M_r 47,000 to 55,000. Because of the divergent size of these two components of the complex and their vastly different levels of glycosylation, it is likely the mature gp160 is cleaved asymmetrically to yield the gp55 and gp116. Thus, the mature complex is likely a heterodimer and not a homodimer as proposed by other investigators (17). This interpretation is also consistent with our previous results which indicated that gp55 and gp116 share little structural or antigenic relatedness (6). Additional support for these previous findings was obtained from analysis of the nucleotide sequence encoding the gp55-116 complex (gB), which revealed divergent sequences of the proteins produced by cleavage at the putative cleavage site (M. Mach, personal communication). Furthermore, potential N-linked glycosylation sites of the gp55-116 precursor predicted from the nucleotide sequence are more frequent on the N-terminal two-thirds of the molecule (8). Of the 17 potential N-linked sites, all but 4 are located on the NH_2-terminal portion of the cleaved precursor molecule (8). In contrast to the abundant N-linked glycosylation site present on the NH_2-terminal portion of the precursor, the COOH terminus contains a stretch of hydrophobic amino acids which are suggestive of a membrane-spanning region (7, 8). Finally, our initial results have clearly indicated that the epitope recognized by antibody 7-17 is located within the NH_2 terminus of the gp55, and no cross-reacting epitope(s) are present in the gp116 (U. Utz, L. Vugler, W. Britt, and M. Mach, submitted for publication). Thus, it appears likely that the gp55-116 complex is a disulfide-linked heterodimer (3, 6) with a heavily glycosylated ectodomain and a transmembrane region located within the smaller of the two components.

Similar to the synthetic pathway of glycoproteins of other herpesviruses (12, 14, 16, 23, 25), the initial steps in the synthetic pathway of the mature HCMV gp55-116 complex involve addition of carbohydrate side chains through N-linkages to a nonglycosylated M_r 105,000 precursor. This size estimate agrees closely with the M_r 105,000 species detected by Endo H digestion of the gp150 protein. This result is also consistent with previous estimates for the mass of HCMV gA (18) and gB (8), as well as with our findings of the size of the precursor of the gp55-116 complex synthesized in *Escherichia coli* (7). Processing of the M_r 105,000 precursor involves addition of simple, high-mannose sugars until a stable intracellular precursor of M_r 150,000 (gp150) is formed. This step is relatively rapid, as the gp150 could be detected within a chase period of 10 min after a short pulse-labeling with [^{35}S]methionine (6). The final steps in the processing of the gp150 involve terminal sugar addition leading to the synthesis of the partially Endo H-resistant gp165-170. We also detected an intermediate form migrating slightly faster (M_r 160,000 to 165,000) than the penultimate gp165-170, containing only simple, Endo H-sensitive sugar linkages, when infected cells were incubated in the presence of castanospermine, an inhibitor of the early steps of complex sugar processing (data not shown). Similar results were previously noted by Gretch et al. (10), who have proposed that this form is further trimmed to yield an Endo H-sensitive species of M_r 138,000. These investigators have also suggested that the M_r 138,000 species might represent the penultimate form of the virion gp55-116 complex, a processing pathway initially proposed by Pereira et al. (18). However, our results indicated that the gp165-170 form contains both simple and complex sugars, suggesting that cleavage of the penultimate precursor into the mature gp55 and gp116 occurs after its complete processing. From the experiments presented in this manuscript we cannot definitively exclude either proposed processing pathway; however, the hypothesis we have proposed provides an unidirectional pathway involving increasingly complex processing steps instead of one with a branch point occurring early in the pathway (18). Previous pulse-chase analysis (6) and the results presented in Fig. 2 suggest that the mature cleavage products gp55-116 are generated either simultaneously or shortly after synthesis of the Endo H-resistant gp160 (165-170). Because it seems inefficient for a large mammalian virus such as HCMV to coordinate two processing pathways such that all products are synthesized simultaneously, we favor the simpler pathway, involving synthesis of a processed polyprotein followed by cleavage into the mature forms. Our results also indicate that some 5,000 daltons of O-linked sugars is added to the gp116. Although we have not determined whether this addition occurs before or after cleavage of the precursor, the results obtained with DMJ were consistent with the addition of O-linked carbohydrates prior to cleavage of the gp165-170 precursor (Fig. 4). These processing steps after the synthesis of the gp150 are the slowest in the synthetic pathway and are most likely the rate-limiting step in synthesis of the gp55-116 complex. Consistent with the kinetics of this pathway is the finding that gp150 could not be efficiently chased into gp165-170 even after prolonged chase intervals (6). This result indicates that gp150 is relatively stable within infected cells or is present as a large pool, or both. The failure to deplete this pool during prolonged chase periods suggests that gp150 is transported through the Golgi apparatus by some type of saturable transport mechanism. Whether this transport mechanism is specific for gp150 or includes other envelope HCMV glycoproteins remains to be determined.

Our findings are for the most part in agreement with earlier reports detailing the synthesis of an abundant HCMV glycoprotein which was likely identical to the gp55-116 (10, 11, 18, 20). In contrast to our findings and these earlier results, a more recent report has indicated that the gp55-116 complex (gB) contains only N-linked, high-mannose sugars (21). Furthermore, this report also noted little if any effect on the processing of the gp55-116 complex (gB) by monensin,

whereas we observed a dramatic effect. It is difficult to reconcile our results with these findings, although our experiments did utilize higher concentrations of monensin. We could not detect a measurable difference in protein synthesis in treated versus untreated cells (data not shown), and nonspecifically precipitated proteins were unaffected by increasing concentrations of monensin (Fig. 1). Thus, it seems unlikely that we were observing a toxic effect secondary to higher drug concentrations. Additional findings, including susceptibility to digestion with glycopeptidase F and inhibition of complex sugar by addition of DMJ, also indicated that the mature forms of the gp55-116 complex as well as the penultimate precursor contain complex N-linked sugars. From these results, we have concluded that the majority of carbohydrate linkages present on the mature gp55-116 complex are N-linked complex sugars.

Several lines of evidence initially suggested that the cleavage of gp165-170 into the mature gp55-116 complex required complete processing of gp150 to gp165-170. First, tunicamycin inhibited the glycosylation of the M_r 105,000 precursor and likewise prevented cleavage of the nonglycosylated precursor into more rapidly migrating forms (data not shown). Second, monensin inhibited the processing of gp150 to gp165-170 and also prevented the generation of more rapidly migrating cleavage products. Finally, the cleavage-produced gp55 and gp116 were detected only following the appearance of Endo H-resistant sugar linkages on gp165-170, indicating that complete processing was associated with cleavage. Although several mechanisms could account for the dependence of the cleavage step on complete processing, a possible explanation would be the acquisition, during the final processing steps, of conformational determinants recognized by specific proteases. A more likely mechanism would be that cleavage and the final processing of the gp165-170 are independent events which are both dependent on transport to a specific site within the Golgi apparatus. Because treatment with either tunicamycin or monensin could be expected to inhibit transport through the Golgi apparatus, either agent could inhibit both the final processing steps and cleavage. Studies of glycoprotein processing of other herpesviruses have also provided conflicting results. A recent investigation of the processing pathway of bovine herpesvirus GVP6 in the presence of monensin indicated that complete glycosylation of the GVP6 was not necessary for proteolytic cleavage of this polyprotein into GVP11a and GVP16 (25). In contrast to this report, monensin clearly inhibited both the processing and cleavage of pseudorabies virus gB (14). In later experiments we attempted to separate the events of cleavage and glycosylation by treating infected cells with DMJ. This compound inhibited complete glycoprotein processing but allowed transport through the Golgi. Our results indicated that cleavage occurred even in the absence of complete glycosylation. Thus, it appears that the two processing events, glycosylation and cleavage, are independent and associated only with transport through the Golgi apparatus. Furthermore, the finding of a fully glycosylated gp165-170 in a subpopulation extracellular virions provides additional evidence for the independence of carbohydrate modification and proteolytic cleavage in the synthesis of the mature gp55-116 complex. Whether inhibition of complete glycosylation or proteolytic cleavage of the gp55-116 precursor adversely affects functions of the gp55-116 such as virion attachment, adsorption, and penetration remains to be determined.

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