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Glycoprotein H-Related Complexes of Human Cytomegalovirus: Identification of a Third Protein in the gCIII Complex

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Previous studies have described three disulfide-bonded glycoprotein complexes within the envelope of human cytomegalovirus (HCMV). These have been designated gCI, gCII, and gCIII. Although gCI has been identified as homodimeric glycoprotein B (gB, gpUL55), the compositions of gCII and gCIII remain incompletely defined. Earlier studies suggested that gCIII was composed of glycoprotein H (gH, gpUL75) complexed with a second glycoprotein, the gL homolog of HCMV. We characterized the gCIII complex of HCMV using recombinant vaccinia virus-expressed gH and gL. Our results indicated that authentic gCIII was not reconstituted by coexpression of gH and gL. The presence of a third, structurally and antigenically unique glycoprotein with an estimated molecular mass of 125,000 Da in virion-derived gCIII complexes suggested that at least three proteins were necessary for formation of this envelope glycoprotein complex. This third glycoprotein, gp125, contained both simple and complex N-linked carbohydrates and had an estimated deglycosylated mass of 64,000 Da. Furthermore, we demonstrated that mature gH existed as both a covalently complexed and noncovalently associated component of the gCIII complex within the envelope of infectious extracellular virions. These findings provide further evidence for the structural complexity of the envelope of HCMV and emphasize the uncertainties associated with the previous assignment of specific functions to envelope proteins of HCMV.

The envelope of human cytomegalovirus (HCMV) is a complex structure of an as yet undefined composition. Studies from several laboratories have defined major protein components of the envelope (3, 10, 11, 18, 19, 23, 28, 32). These proteins include the major envelope protein of HCMV, glycoprotein B (gB, gpUL55), and the next most abundant component, gH (gpUL75) (3, 10, 23, 30, 32). Together, gB and gH represent the most readily detected protein constituents of the viral envelope and also represent the dominant targets of host-derived virus-neutralizing antibody responses (3, 7, 10, 27, 29, 33, 34). Several additional glycoproteins have been detected in the envelope of HCMV, including gM (IMP, gpUL100) and gp65 (4, 25); however, these two proteins represent minor components of the envelope and can only be detected with specific immunological reagents. Interestingly, computer-aided analysis of the genomic sequence of HCMV has suggested the presence of approximately 50 additional open reading frames (ORFs) which could encode proteins modified with N-linked carbohydrates (9).

Analysis of the glycoproteins of the HCMV envelope with gB-, gH-, and gCII-specific monoclonal antibodies (MAbs) revealed the presence of several high-molecular-weight complexes (2, 3, 10, 19, 22). These complexes were shown to consist of disulfide-linked glycoproteins and were initially designated gCI, gCII, and gCIII (19, 22). Subsequent studies from several laboratories defined gCI as the mature, homodimeric form of gB and gCIII as a gH-related complex (2, 3, 10, 11, 19). The composition of gCII is as yet undefined but is thought to include gM (21). Studies by Gretch et al. and later by Bogner have documented the complexity of the gCIII complex (2, 19). Glycoprotein H-specific MAbs defined a high molecular complex with an estimated mass of 240 kDa when immune precipitates were solubilized in the absence of reducing agents (2, 19). Disruption of disulfide bonds within this complex resulted in the appearance of at least two proteins of estimated masses of 86 kDa (gH) and higher-molecular-weight species which were thought to be differentially modified forms of gH (2, 19).

Investigations into the intracellular transport of the gH homolog of herpes simplex virus (HSV) provided evidence that intracellular transport and acquisition of native antigenicity of gH required a second protein, gL (15, 17, 20, 31). Studies in varicella-zoster virus also confirmed the presence of a gL homolog, and recently it has been shown that coexpression of gH and gL can promote fusion between adjacent cells (13, 14). Thus, it appeared that in alphaherpesviruses, gL played an important role in the folding and transport of gH. Shortly after the descriptions of gL in HSV, two groups of workers independently identified the product of the UL115 ORF as the HCMV gL homolog (23, 32). Both groups demonstrated that expression of gL could facilitate cell surface transport of gH; however, neither study quantitatively compared results from recombinant systems with gH expression in HCMV-infected cells (23, 32). Because gH and gL were shown to be disulfide bonded within infected cells, it was assumed that an oligomeric form of these two proteins constituted the gCIII complex of HCMV (23, 32). When the data in these original descriptions of the gCIII complex were examined more closely, it was of interest to note the presence of additional higher-molecular-weight proteins in this complex which appeared to be antigenically unrelated to gH. These results suggested that the gCIII complex could be composed of additional non-gH-, non-gL-related proteins.

Our initial studies were directed toward definition of the functional roles of gH and gL in the early steps of virus infec-

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tivity. Toward this end, we attempted to reconstitute the gCIII complex with recombinant derived gH and gL. Our experiments suggested that efficient intracellular transport of gH and therefore, assembly of the gCIII complex could not be achieved by coexpression of gH and gL. In addition, we noted that the gCIII complex found in mature virion- and HCMV-infected cells appeared to be larger than the high-molecular-weight complexes formed following coexpression of the gH and gL ORFs as recombinant vaccinia viruses. Additional studies determined that a third, non-gH-, non-gL-related protein, gp125, was a component of the gCIII complex. In addition, we also found that a significant amount of noncovalently linked gH was present in the envelope of HCMV, raising several questions about the processing pathway of this glycoprotein. Together, our findings have emphasized the structural complexity of the envelope of this large herpesvirus. Furthermore, these results suggested that both the composition and stoicheometry of glycoprotein components within envelope complexes must be considered when assigning specific functions, such as membrane fusion, to individual virion glycoproteins.

MATERIALS AND METHODS

Cells and virus. Human primary fibroblast (HF) and monkey kidney (BSC-1) cells were maintained in medium M199 supplemented with 10% calf serum and antibiotics. HeLa and TK-143 cells were grown in Dulbecco modified Eagle media supplemented with 10% fetal calf serum and antibiotics. The AD169 laboratory strain of HCMV was used for all experiments. Viruses were propagated in low-passage primary HF cells, and viruses from extracellular supernatant were gradient purified in sorbitol gradients as described previously (3). The infectivity of the virus was determined by a microtiter assay (6).

Antibodies. Mouse anti-gH MAbs 14-4b and AP86 and anti-gB MAb 7-17 were produced in our laboratory (6, 26, 33). Rabbit polyvalent sera against gL were produced as previously described (32).

Generation of the recombinant vaccinia virus expressing gH and gL. Recombinant vaccinia viruses containing the intact UL75 (gH) or UL115 (gL) ORF were constructed as described in previous studies (6, 7). Recombinant viruses were selected in 5-bromodeoxyuridine-containing media and plaque purified three times prior to their use.

Flow cytometry analysis. Cells infected with either HCMV or recombinant vaccinia viruses were collected by scraping the monolayer into 15-ml conical centrifuge tubes containing phosphate-buffered saline (PBS) (pH 7.4). Following two washes with PBS, the cells were incubated with mouse anti-gH MAb 14-4b or rabbit anti-gL polyclonal sera for 15 min at 4°C. The antibody was aspirated and the cells were washed twice with PBS and then incubated for 30 min on ice with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) antibodies. Samples were washed and then fixed in 1% paraformaldehyde in PBS. Flow cytometry was performed on a FACSCAN instrument (Becton Dickinson, Palo Alto, Calif.) as described previously (12). Data were analyzed by the software WinMDI. Plots for negative-control samples were overlaid with those of test samples on a logarithmic frequency histogram.

Radiolabeling of infected-cell and virion surface proteins. Confluent monolayers of HF cells in 35-mm-diameter tissue culture dishes were infected with HCMV AD169 at a multiplicity of infection of approximately 1. When >90% of the cells showed cytopathic effect, pulse-chase radiolabeling was carried out as described in a previous report from our laboratory (5, 6). A single culture of HCMV-infected cells was continuously labeled throughout the duration of the experiment and was designated as pulse-labeled. Labeled infected cell proteins from this culture should therefore represent the universe of labeled HCMV proteins in this experiment. Gradient-purified extracellular virions were radiolabeled with ^{125}I as described previously (6, 26).

Velocity sedimentation analysis of gCIII complex. Proteins from ^{125}I-labeled gradient-purified virions were solubilized in 0.1% Nonidet P-40–Tris-buffered saline (TBS) (0.005 M Tris, 0.15 M NaCl, 0.001 M EDTA, pH 7.5) at 4°C. One milliliter of solubilized viral proteins was placed on the top of a preformed 5 to 30% sucrose in 0.1% NP40 TBS gradients and centrifuged in an SW 41 rotor (Beckman, Palo Alto, Calif.) at 32,000 rpm for 16 h. One-half-milliter fractions were collected from the bottom of the gradient by pumping mineral oil onto the top of the gradient.

Immunoprecipitation and immunoblotting. Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (6–8). Molecular weights were estimated by comparison of migration with molecular weight standards (Sigma Chemical Co., St. Louis, Mo.).

Proteolytic peptide analysis. Labeled gH and gp125 were immunoprecipitated from ^{125}I-labeled extracellular virions by MAb 14-4b and isolated by SDS-PAGE. Gel slices containing the labeled proteins were subjected to in situ digestion with staphylococcal V8 protease (USB, Cleveland, Ohio) as described previously (5). Peptide fragments were resolved by SDS-PAGE in 15% acrylamide gels.

RESULTS

Coexpression of gH and gL does not reconstitute the gCIII complex. Previous studies have suggested that the intracellular transport and incorporation of gH into extracellular virions required a second glycoprotein, gL (UL115) (23, 32). The structure of the gH/gL complex in extracellular HCMV virions is unknown, and it remains to be determined if the gH/gL complex is required for the proposed fusion activity associated with gH (16, 24). In order to investigate the role of gH in the early events of host cell infection, we initially attempted to reconstitute the native gCIII complex by coexpression of both gH and gL in infected cells. Recombinant vaccinia virus encoding either the UL75 (gH) or the UL115 (gL) ORF was constructed and used to infect HeLa cells. We analyzed cells which had been infected simultaneously with both vaccinia gH and gL by flow cytometry using an anti-gH monoclonal antibody and a polyvalent rabbit antisera directed against gL (26, 32). As a measure of cell surface expression of gH and gL following wild-type HCMV infection, we also analyzed the expression of both molecules on the surface of primary HF cells infected with HCMV strain AD169. Both gH and gL were detected on the surface of HCMV-infected HF cells (Fig. 1a and b). In contrast, coexpression of gH and gL in cells infected with recombinant vaccinia viruses resulted in only minimal cell surface expression of gH and no detectable expression of gL (Fig. 1c and d). Expression of gH and gL in recombinant vaccinia virus-infected cells was readily detected in fixed and permeabilized cells, suggesting that the low level of gH cell surface expression was not secondary to limited expression of the protein (data not shown). These findings confirmed previous studies which have shown that coexpression of gH and gL in a heterologous system facilitated cell surface transport of gH (23, 32); however, the level of surface expression in cells coinfected with gH and gL expressing recombinant vaccinia viruses was reduced considerably compared to wild-type HCMV-infected HF cells.

The biochemical forms of the gH and gL complexes in cells infected with the recombinant vaccinia viruses were then examined. Infected cell proteins from vaccinia gH- and gL-infected cells were subjected to SDS-PAGE, transferred to membranes, and probed with a MAb (AP86) which is directed against a 10-amino-acid epitope located near the amino terminus of gH (33). When virions prepared from HCMV-infected cells were electrophoresed in the absence of reducing agents, MAb AP86 detected gH-related proteins with a high molecular weight, estimated to be approximately 240 kDa and a second 89-kDa protein (Fig. 2a). The high-molecular-weight band most likely represented the previously described gCIII complex and the smaller 89-kDa species, gH (2, 19). The anti-gL antisera which were used for the previous flow cytometry experiments also detected an identically migrating 240-kDa protein in extracellular virions which were solubilized and electrophoresed in the absence of 2-mercaptoethanol (2-ME) (data not shown). Essentially identical results were obtained when infected cell proteins from HCMV-infected cells were analyzed by immunoblotting, with the exception of inconsistent reactivity of the anti-gH antibody with a protein of estimated mass of 115 kDa (data not shown). In contrast to these results, analysis of the gH-containing complexes formed by coexpression of gH and gL in recombinant vaccinia virus-infected HeLa cells solubilized under nonreducing conditions revealed three proteins of estimated masses of 210, 115, and 86 kDa (Fig. 2a).

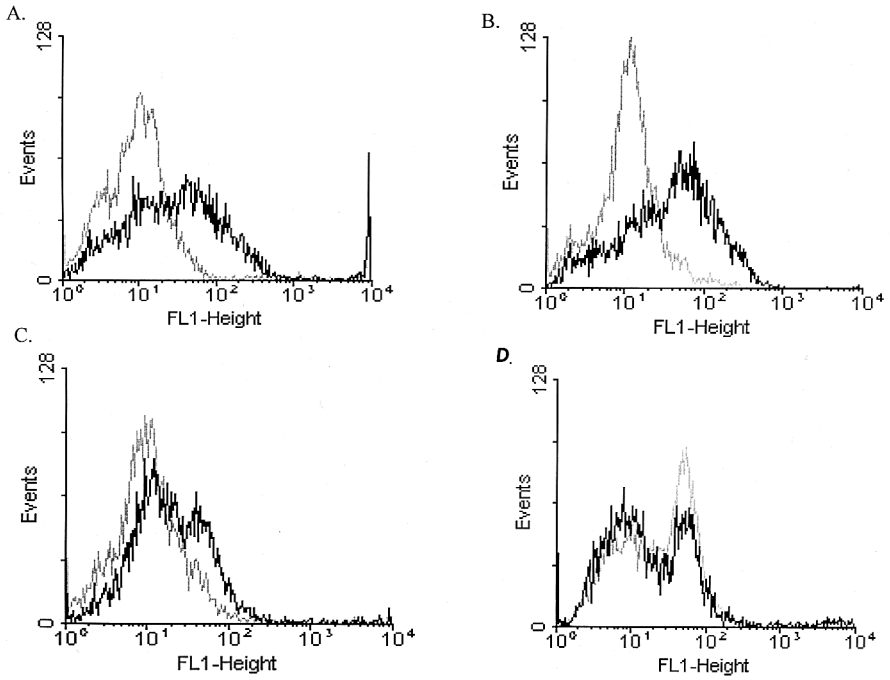

FIG. 1. Cell surface expression of gH and gL. Cell surface expression of gH and gL was examined by flow cytometry as described in Materials and Methods. (A) HF cells mock infected (gray) or infected with HCMV AD169 (black) and stained with gH-specific MAb 14-4b. (B) HF cells infected in the same manner as in panel A but stained with gL-specific polyvalent sera. (C) HeLa cells infected with recombinant gH- and gB-expressing vaccinia viruses (gray curve) or with recombinant vaccinia gH and gL viruses (black curve) and stained with MAb 14-4b. (D) HeLa cells infected as described for panel C and stained with gL-specific polyvalent sera. Fluorescence intensity is shown on the *x* axis, and the relative number of cells is indicated on the *y* axis.

The largest protein complex found in vaccinia gH- and gL-infected cells was significantly smaller than the estimated 240-kDa gCIII complex detected in HCMV-infected HF cells, whereas the estimated mass of the 115-kDa species was consistent with a disulfide-linked complex consisting of gH and gL (Fig. 2a). A prominent band identified as gH was seen in nonreduced samples from HCMV virions, vaccinia gH- and gL-coinfected cells and vaccinia gH-infected cells (Fig. 2a). A single band corresponding to gH was seen when each of the samples was treated with 2-ME prior to SDS-PAGE (Fig. 2b). It was of interest that the virion form of gH migrated slightly more slowly than the gH expressed in cells infected with the gH-containing recombinant vaccinia virus (Fig. 2b). This increase in the apparent mass of the virion gH was also noted when we compared the migration of virion gH to that of gH from HCMV-infected cells and was most consistent with additional carbohydrate modifications of the virion form of gH (data not shown). When viewed together with our flow cytometery analysis of cell surface expression of gH, these results suggested that coexpression of gH and gL in a heterologous system failed to reconstitute the gCIII complex found in extracellular virions and HCMV-infected HF cells. This observation raised the possibility that an additional virus-encoded protein(s) found in HCMV-infected cells was required for the formation of the gCIII complex.

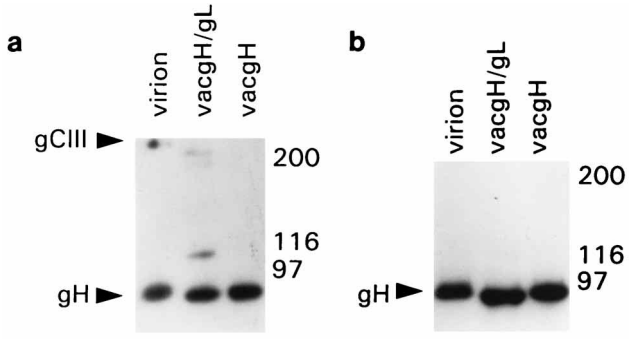

FIG. 2. Immunoblotting of gH- and gL-associated complex. Gradient-purified HCMV virions and HF cells infected simultaneously with both gH and gL vaccinia viruses or with the gH vaccinia virus alone were lysed in sample buffer and subjected to SDS-PAGE under nonreducing (a) or reducing (b) conditions. The gH-related proteins were detected by gH-specific MAb AP86 followed by anti-mouse IgG and ^{125}I-labeled protein A.

A third glycoprotein is covalently linked to the gH/gL complex in HCMV-infected fibroblasts. The formation of the gCIII complex in HCMV-infected HF cells was studied by pulse-chase analysis. Virus-infected HF cells were pulse-labeled with ^{35}S-methionine for 15 min and then chased in media containing unlabeled methionine and cycloheximide. An additional culture of HCMV-infected cells was continuously labeled during the duration of this experiment to provide a complete representation of labeled infected cell proteins. This preparation was designated pulse-labeled. Infected cell proteins were precipitated with a gH-specific MAb, 14-4b, and analyzed by SDS-PAGE. Following elution of the precipitated proteins in the presence of 2-ME, gH could be detected throughout the chase intervals with an apparent increase in its rate of migration during the later chase intervals (Fig. 3A). This change in migration was most likely secondary to modifications of the carbohydrates on gH during its intracellular processing (2). Furthermore, the diffuse appearance of the gH band following prolonged pulse-labelling suggested there was a heterogeneous population of molecules, possibly secondary to differences in carbohydrate modifications (Fig. 3A). During the chase peri-

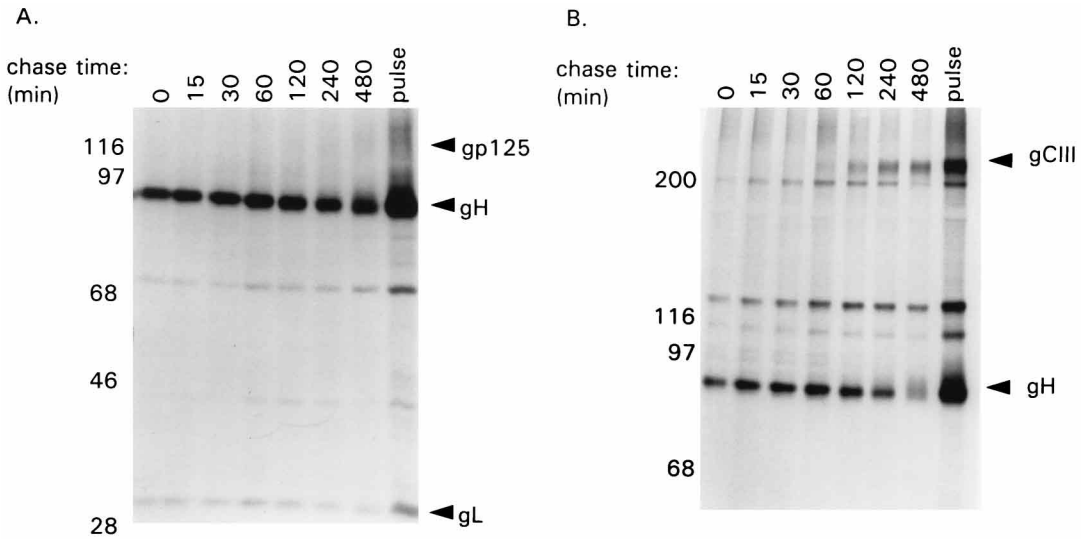

FIG. 3. Pulse-chase analysis of HCMV-infected cells. Pulse-chase experiment was performed as described in Materials and Methods. A single culture of HCMV-infected cells was continuously labeled throughout the duration of the experiment (pulse). Infected cell proteins were precipitated with gH-specific MAb 14-4b. The chase times are indicated above the respective lanes, and estimated molecular weights (in thousands) are designated in the left margin. The immune precipitates were analyzed by SDS-PAGE under reducing conditions (A) or nonreducing conditions (B).

ods we could also detect a protein migrating with the same M_r as has been reported for gL (Fig. 3A). In the later chase periods and in the lane containing pulse-labeled infected cell proteins, we also detected a protein with an estimated mass of 125 kDa (Fig. 3A). When the same precipitates were eluted in the absence of 2-ME, the kinetics of formation of the gCIII complex was apparent. The gH signal increased over the first four chase intervals and then exhibited a slow decline in signal intensity with a marked decrease during the 240- and 480-min chase intervals (Fig. 3B). Concomitant with the decrease in gH signal at a chase interval of 120 min was the appearance of the high-molecular-weight complex which we have designated gCIII based on its previously reported mass (Fig. 3B) (19). The 125-kDa protein seen under reducing conditions was no longer detected when the precipitates were eluted in the absence of 2-ME (Fig. 3B). Additional proteins with approximate masses of 140 and 200 kDa were also present. These proteins were not reactive with an anti-gH MAb or the anti-gL antiserum in immunoblot assays of infected cell proteins, suggesting that they were not antigenically related to gH or gL (data not shown). Together these data were consistent with the possibility that at least three proteins, gH, gL, and gp125, were incorporated into the gCIII complex.

We next characterized the composition of the gCIII complex in extracellular virions in order to define the relationship between gH, gL, and the third protein, gp125. Extracellular virus was purified by centrifugation through sorbitol gradients, radiolabeled with ^{125}I, and precipitated with the gH-specific MAb 14-4b. In some experiments the precipitated proteins were then treated with either endoglycosidase H (endo H) or *N*-glycopeptidase F prior to analysis by SDS-PAGE. We included immune precipitates of gB as a control for the specificity of the glycosidase activity (6). Elution of the precipitated proteins in the absence of 2-ME revealed two predominant species in the labeled virions, a high-molecular-weight protein which was designated the gCIII complex and gH (Fig. 4A) (19). Treatment of this material with endo H caused a slight increase in the migration of both the gCIII and the gH, whereas treatment with *N*-glycopeptidase F caused a readily apparent increase in the migration of both the gCIII and gH (Fig. 4A). When identically prepared samples were run in the presence of 2-ME, we could resolve three protein species of estimated masses of 125, 89, and 31 kDa (Fig. 4B). These proteins were of similar sizes as the gp125, gH, and gL in Fig. 3A. Treatment with endo H caused a minimal increase in the migration of the gp125 and gH but little, if any, change in the migration of gL (Fig. 4B). Treatment with *N*-glycopeptidase F caused a marked increase in the migration of the gp125, reducing its mass to approximately 64 kDa (Fig. 4B). Likewise, the mass of the gH was reduced to approximately 78 80 kDa, roughly the predicted core size of the product of the UL75 (gH) ORF (Fig. 4B). Finally, the size of gL was also reduced by *N*-glycopeptidase F treatment to an estimated mass of 28 kDa (Fig. 4B). From these findings we concluded that gH and gp125 contained both simple and complex sugars and gL contained only complex linkages. We did not determine if any of these glycoproteins contained O-linked sugars. These results provided additional evidence for the presence of a third protein, gp125, within the gCIII complex, as well as demonstrating the presence of both the gp125 and gL in extracellular virions.

The content of the gCIII complex was further analyzed by excising the band corresponding to the gCIII complex and subjecting this material to a second SDS-PAGE after boiling in the presence of a reducing agent. As a control, the band corresponding to gH from the same gel was excised and analyzed in an identical manner. Reduction of the gCIII complex resulted in the appearance of three proteins, gp125, gH, and gL, whereas we could detect only gH within the reduced gH band (Fig. 4C). These results confirmed our previous findings and provided additional evidence that the gCIII complex consists of at least three disulfide-linked glycoproteins.

The gp125 and gH components of the gCIII complex differ in primary sequence. Although the ORF encoding the gp125 has not been identified, we attempted to define its structural relationship to gH by proteolytic peptide mapping. Radiolabeled virions were immune precipitated with a gH-specific MAb, and the gp125 and gH were separated by SDS-PAGE. The labeled gp125 and gH were excised from the gel, subjected to digestion

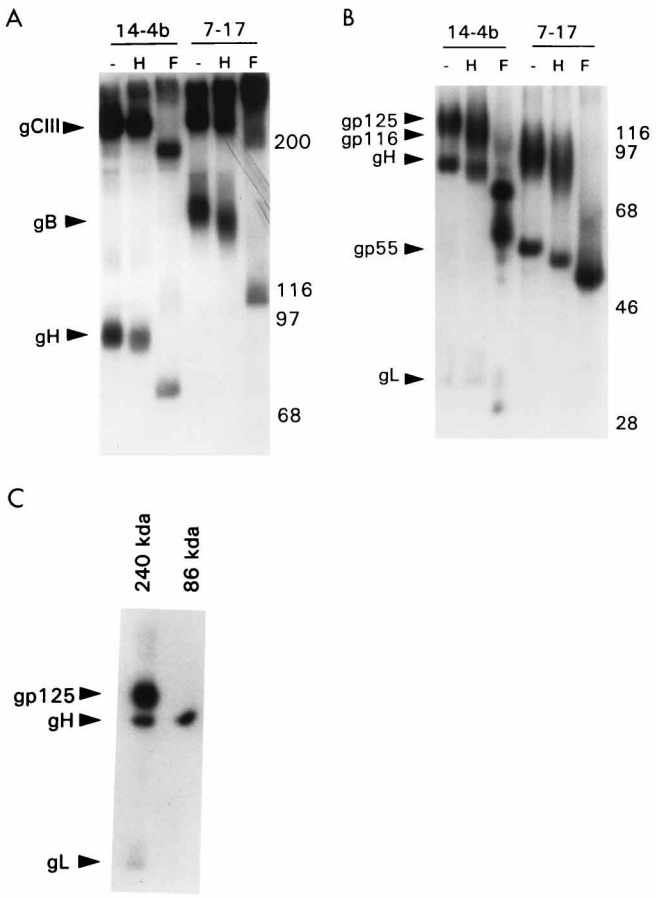

FIG. 4. Analysis of carbohydrate modifications on components of the gCIII complex. Gradient-purified extracellular HCMV virions were labeled with ^{125}I as described in Materials and Methods. Viral proteins were precipitated by gH-specific MAb 14-4b or gB-specific MAb 7-17. Aliquots of precipitates were then treated with endo H (H) or glycopeptidase F (F) or left untreated (−). The proteins were then resolved on SDS-PAGE under nonreducing conditions (A) or reducing conditions (B). The estimated molecular weights (in thousands) are indicated in the right margin. The migration of the gCIII-specific proteins gp125, gH, and gL is indicated in the left margin along with the components of gB, gp116, and gp55 (C). The bands corresponding to gCIII complex and gH were excised and electrophoresed following boiling in sample buffer containing 5% 2-MER.

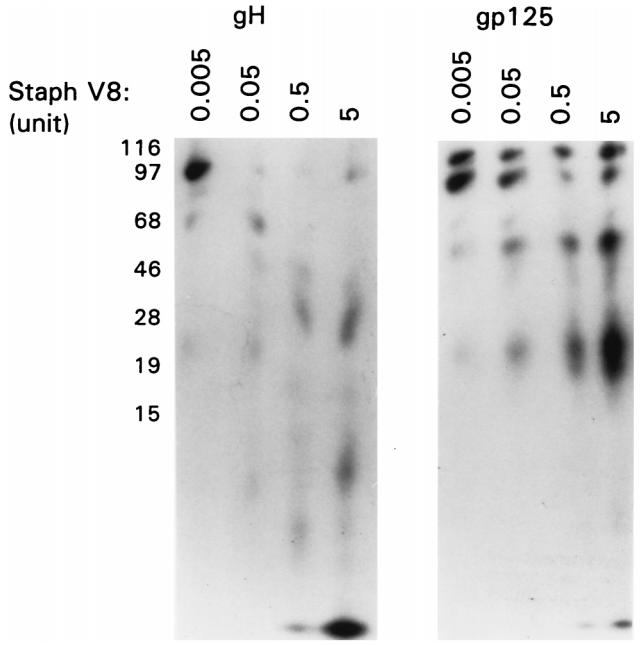

FIG. 5. Proteolytic peptide analysis of gH and gp125. Following identification of ^{125}I-labeled gH and gp125 by autoradiography, the gel slices containing gH or gp125 were excised and digested with increasing amounts of staphylococcal V8 protease. The resulting fragments were then separated by SDS-PAGE in 15% acrylamide gels. Estimated molecular weights (in thousands) are shown in the left margin.

with varying amounts of staphylococcal V8 protease, and the resulting fragments were resolved by SDS-PAGE. Comparison of the proteolytic peptide map of gp125 with that of gH suggested that these two proteins differed in primary sequence (Fig. 5). Together with the differences in the masses of these two proteins following *N*-glycopeptidase F treatment (Fig. 3B), our results indicated that gp125 and gH were unrelated antigenically or structurally. Thus, the previously defined gCIII complex contained at least three unrelated proteins.

The envelope of HCMV contains both covalently and noncovalently complexed gH. During our studies of the kinetics of formation of the gCIII complex, we noted that a substantial amount of gH was not converted into the higher-molecular-weight gCIII complex. To determine if noncomplexed gH was also present in infectious virions, ^{35}S-methionine-labeled extracellular virions were gradient purified as previously described and individual gradient fractions were assayed for infectivity and gH. When the fractions containing the peak infectivity were assayed by SDS-PAGE following immune precipitation with a gH-specific mab, we could detect both gCIII and noncomplexed gH (Fig. 6). A third protein with an estimated mass of 125 to 140 kDa could also be detected (Fig. 6). This protein was antigenically unrelated to either gH or gL (Fig. 2). Although the ratio of the noncomplexed gH to gCIII was not quantitated, the relative signal intensities suggested that a significant amount of gH in the envelope of HCMV was noncovalently linked to the gCIII complex. We further investigated the distribution of noncomplexed gH in the envelope of HCMV by centrifugation of radioiodinated envelope glycoproteins through 5 to 30% sucrose density gradients. The gradients were then fractionated and individual fractions were precipitated with a gH-specific MAb. Analysis of the precipitated proteins by SDS-PAGE under reducing conditions revealed the three previously described components of gCIII: gp125, gH, and gL (Fig. 7a). When the same precipitates were eluted in the absence of 2-ME, we could again detect gCIII and noncovalently complexed gH (Fig. 7b). Of note was the cosedimentation of gH and gCIII in fraction 9, indicating that these molecules were physically associated in the envelope of the virus (Fig. 7). This finding indicated that the gCIII complex contained a covalently linked complex of gp125, gL, and gH as well as noncovalently linked gH. Finally, it was also of interest that noncomplexed gH sedimented in the higher fractions which contained little gCIII (Fig. 7b). This observation raised the possibility that gH may also exist in the envelope of HCMV in a free, uncomplexed form.

DISCUSSION

Several previously reported studies have suggested that within infected cells and extracellular virus, gH was complexed to additional but antigenically unrelated proteins (2, 19). The identification of a gH-containing high-molecular-weight complex designated gCIII by Gretch and coworkers was the first study to demonstrate that gH was complexed with at least one

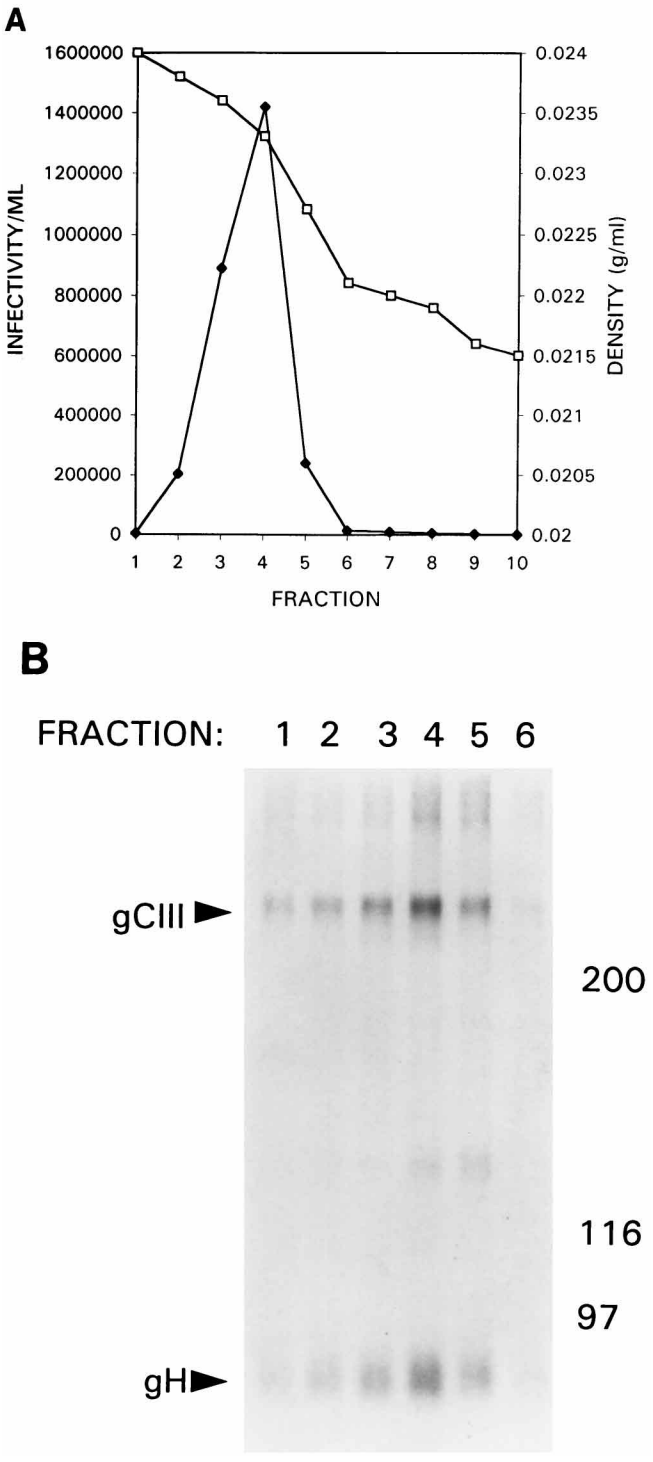

FIG. 6. Expression of nonconvalently complexed gH in gradient-purified infectious HCMV virions. HF cells infected with HCMV AD169 were metabolically labeled with ^{35}S-methionine. The extracellular virus was loaded onto the top of a preformed 25 to 70% sorbitol gradient and centrifuged in an SW 41 rotor at 22,000 rpm for 1 h. Ten fractions were collected from the bottom of the gradient. (A) Infectivity (◆) and density (◇) of each fraction measured as described in Materials and Methods. (B) gCIII and gH content in each fraction determined by precipitation with gH-specific MAb 14-4b, followed by SDS-PAGE in the absence of reducing agents. The migrations of the gCIII complex and gH are shown in the left margin, and migrations of molecular weight standards (in thousands) are shown in the right margin.

additional protein in mature virions (19). Bogner et al. also described the presence of a 125-kDa protein within the gCIII complex which was thought to be a differentially modified form of gH (2). More recently, two studies have demonstrated formation of a complex between the product of the UL115 ORF (gL) and gH within infected cells (23, 32). It should be emphasized that neither of these later two studies demonstrated reconstitution of the gCIII complex when gH and gL were coexpressed in cells (23, 32). Thus, the precise composition of the gCIII complex has remained controversial. Our findings have demonstrated complexity of the forms of the gCIII complex within infected cells and virions in that we have defined at least three proteins within this complex. All three proteins appear antigenically and structurally unrelated. In addition, all three proteins were found within extracellular virions. Because the three protein complex cosedimented with virus infectivity, it was likely that this complex represented a native structure within the HCMV envelope and not an aggregate of proteins formed during the lysis and analysis of infected cells.

Several findings in this study indicated that the three protein components of the gCIII complex were structurally distinct proteins. First, we demonstrated by immunoblotting that the gH, gL, and gp125 were antigenically unrelated. Secondly, endoglycosidase digestions indicated that the estimated core size of gH was approximately 80 kDa and that of gL was 28 kDa, sizes that were in agreement with previous reports and predicted sizes based on sequence analysis (23, 32). In contrast, the core size of the gp125 was estimated to be 64 kDa. Because this estimate did not take into account the possibility of O-linked sugars in this molecule, the core polypeptide of gp125 could have been even smaller. Therefore, it is difficult to image how this molecule could be a differentially modified form of gH, as was previously suggested (2). Finally, comparison of peptides from limited proteolysis of the gp125 and gH indicated that these two proteins differed in primary sequence. When viewed together, these experimental findings strongly suggested that the gCIII complex consisted of at least three different glycoproteins. Furthermore, all three proteins were presumably virus encoded because of their abundance in the envelope of infectious virus; however, we cannot designate gp125 a virus-encoded protein at this time prior to identification of its respective ORF within the HCMV genome.

The formation of the gCIII complex within infected cells appeared to require an extended period as suggested by an estimated half-life ($t_{1/2}$) of formation of 120 to 240 min. Interestingly, this $t_{1/2}$ approached that described for the formation of the mature gB complex within infected cells (8). We have not defined the cellular compartment associated with complex formation between gp125, gH, and gL; however, inspection of the kinetics of formation of the gCIII complex suggested that the gCIII complex was first detected in chase intervals in which the gH contained incompletely processed carbohydrates. Because these forms of gH have been shown to localize in the endoplasmic reticulum (ER), it was likely that complex formation also occurred in the ER. This model would also be consistent with the current understanding of the formation of intermolecular disulfide bonds between glycoproteins which ultimately enter the secretory pathway. A similar interpretation of the pulse-chase analysis of the formation of the gCIII complex has been suggested in an earlier report (2). Finally, we could not demonstrate convincingly the processing pathway of the gp125 because of its limited incorporation of radiolabeled methionine; however, we could estimate its rate of synthesis by the appearance of the gCIII complex. Two possibilities could explain the poor incorporation of ^{35}S-methionine into gp125. The first was that the protein had a limited number of methi-

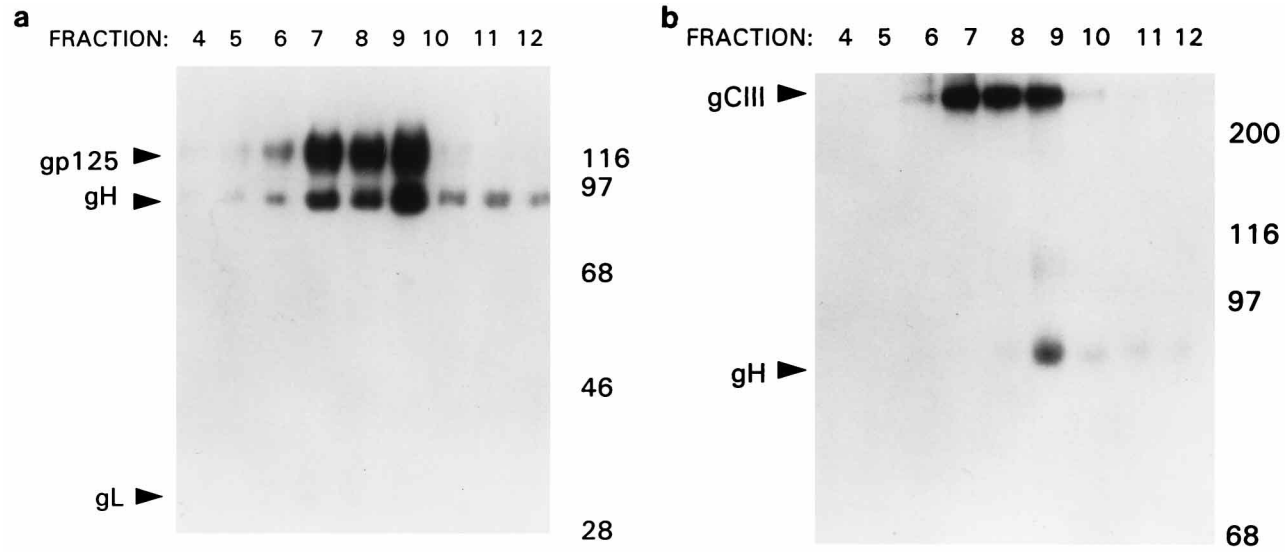

FIG. 7. Velocity sedimentation analysis of gH and gCIII complex. Proteins from ^{125}I-labeled virions were solubilized in 0.1% NP40 containing TBS and subjected to centrifugation in sucrose density gradients as described in Materials and Methods. Eighteen fractions were collected from the bottom of the gradient and precipitated with the gH-specific MAb 14-4b. The precipitates were then analyzed by SDS-PAGE under reducing conditions (a) or nonreducing conditions (b). Estimated molecular weights (in thousands) are shown in the right margin, and the migration of protein components of the gCIII complex is indicated in the left margin. The gL signal was poorly reproduced in this photograph but readily detectable on the original fluorogram.

onine residues, an explanation consistent with its limited incorporation of label even under prolonged pulse-labelling conditions. A second explanation would be a slow rate of synthesis. If this latter possibility contributed to its limited incorporation of ^{35}S-methionine, then the slow synthetic rate of the gp125 could also provide an explanation for the prolonged kinetics of formation of the gCIII complex.

Comparison of the migration of gH obtained from extracellular virions with forms of gH from HCMV-infected cells or recombinant derived gH indicated that virion gH was approximately 2 to 3 kDa larger in size. This difference in size was believed to be secondary to the presence of additional carbohydrate modifications on virion gH. The processing of infected cell gH has previously been thought to include both simple and complex carbohydrate modifications of an estimated four acceptor sites for N-linked sugar addition (2). Our findings were consistent with these proposed posttranslational modifications, yet the increased size of the virion gH compared to the infected cell form of gH indicated that the virion molecule was further modified. Careful examination of the pulse-chase analysis of gH provided some support for this interpretation in that the migration of gH became more diffuse in the later chase intervals (Fig. 3B). In addition, the diffuse migration of gH was even more apparent in immune precipitates from cells pulse labelled with ^{35}S-methionine, and a distinct band migrating slightly more slowly than the majority of gH could be appreciated (Fig. 3B). The carbohydrate modification on virion gH which accounted for its increased mass has not been identified, but the delay in its addition and its presence only on extracellular virions suggested that this modification occurred late in the processing of this glycoprotein. A potential candidate for such a modification was O-linked sugars.

We consistently noted a significant quantity of gH remaining in a noncomplexed form following extended chase periods. Similarly, we found that a significant amount of gH in extracellular virions was not complexed to gCIII. Moreover, infectivity in gradient-purified extracellular virus cosedimented with both the gCIII complex and noncovalently complexed gH. A second experiment using extrinsically radiolabeled virions as a source of envelope proteins confirmed this finding. From these results it would appear that within the envelope of infectious virus, gH was present both as a covalently linked and a noncovalently complexed component of the gCIII complex. The cosedimentation of the noncovalently linked gH with the gCIII complex suggested that the majority of gH was complexed with gCIII. However, we did note that gH was also detected in gradient fractions containing only minimal amounts of gCIII, raising the possibility that a noncomplexed, free form of gH was present in the envelope of HCMV. These findings illustrated the structural complexity of the envelope of this large herpesvirus and stressed the difficulty in assignment of a function for gH in the initial stages of HCMV infectivity. Furthermore, it also calls into question the biological relevance of studies which have attempted to define domains of gH which interact with gL (1). The finding of a readily detectable gH-related protein with an approximate mass of 115 kDa in cells infected with gH and gL recombinant vaccinia viruses was in contrast to results obtained with HCMV-infected fibroblasts. The 115-kDa protein represented only a minor species compared to gH and gCIII when HCMV-infected cell proteins were analyzed by immunoblotting with gH- and gL-specific antibodies (data not shown). Thus, the abundant formation of complexes between gH and gL in recombinant systems could be associated with the overexpression of gH and gL in the absence of gp125.

The definition of a noncovalently linked form of gH within the gCIII complex raised several questions about the processing pathway and intracellular transport of this molecule. Based on studies in recombinant systems, it has been assumed that gH must interact with gL for efficient transport from the ER (23, 32). Our findings were consistent with these earlier studies; however, we also noted that gH isolated from cells coinfected with gH and gL recombinant vaccinia viruses migrated more rapidly than gH isolated from extracellular HCMV virions. This result suggested that the processing and therefore intracellular transport of the recombinant derived gH differed from gH produced by HCMV-infected cells. Moreover, the efficiency of cell transport of gH in cells infected with the

recombinant gH and gL vaccinia viruses was markedly decreased compared to that in wild-type HCMV-infected cells. Because the level of expression of these two proteins in recombinant vaccinia virus-infected cells was comparable to the expression seen in HCMV-infected cells, we believe that the failure to efficiently transport gH within the infected cell was secondary to the absence of gp125 and possibly other viral proteins. To account for the presence of both complexed and noncomplexed fully processed gH in the envelope of HCMV, either gp125 or gp125 complexed with gL must have facilitated transport of gH in HCMV-infected cells. Transport of gH covalently linked to the gp125/gL complex would be consistent with previous models of gH processing, and from our findings, noncovalently linked gH would likely follow a similar route of intracellular transport. Several models could be envisioned, including noncovalent dimerization of gH with one molecule of the dimer becoming disulfide linked to the gCIII complex. The presence of three molecules within this complex has presented additional complexity which thus far precluded any definitive description of the stoicheometric relationship between components of the complex.

In summary, we have characterized the composition of the gH-related gCIII complex in HCMV virions. In contrast to earlier studies, we found that the gCIII complex was composed of three structurally and antigenically unrelated proteins. In addition, we have shown that gH was present in two forms within the complex, a covalently linked form and a noncovalently linked form. A lesser although detectable amount of gH might also be present as a free noncomplexed molecule. The presence of this trimolecular complex in extracellular HCMV virions further illustrated the structural and antigenic complexity of this viral envelope. Previous studies which have suggested that gL and gH formed a functional complex in HCMV-infected cells must be re-examined in light of the finding of an abundant but unrelated protein in this complex.

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