Human Cytomegalovirus: Glycoproteins Associated with Virions and Dense Bodies

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The glycoproteins associated with the membranes of cytomegalovirions and dense bodies were characterized by their relative mobility, percentage of glucosamine incorporation, and molecular weight. Eight glycopolypeptides were repeatedly detectable. Three glycopolypeptides of higher molecular weight with low levels of glucosamine incorporation were occasionally detectable. These latter glycopolypeptides may be precursors or aggregates of the glycopolypeptides with lower molecular weights. The glycoproteins associated with the membranes were on the surface, as determined by iodination with ^{125}I of virions and dense bodies partially purified in gradients of D-sorbitol. Velocity centrifugation in linear gradients of D-sorbitol was used to obtain concentrated and partially purified preparations of infectious cytomegalovirus. Viral infectivity and the membranes of cytomegalovirions and dense bodies were stable in gradients of sorbitol, but cellular contaminants were not completely removed. Additional centrifugation in CsCl separated both cellular contaminants and viral nucleocapsids from virions and dense bodies. Many dense bodies, which are considered to be aberrant forms of cytomegalovirus, had the same size, sedimentation properties, and density as virions. Consequently, they were not separable from virions by various centrifugation techniques. Electron microscopy demonstrated that purified virions and dense bodies were qualitatively free of extraneous material and that each dense body was bounded by a membrane, as evidenced by its double-track appearance. Antisera to a preparation of purified virions and dense bodies, or to their glycoproteins, contained antibodies that neutralized viral infectivity and reacted with antigens in cells infected with cytomegalovirus. However, these same antisera did not contain antibodies that reacted with uninfected cells. The glycoproteins associated with the membranes of cytomegalovirions and dense bodies are considered to be specified by the cytomegalovirus genome.

Cells infected with cytomegalovirus (CMV) accumulate in the cytoplasm masses of homogeneous electron-dense material as viral capsids are assembled and enveloped (4, 17, 22, 32, 38). This electron-dense material, referred to as dense bodies of CMV, becomes enveloped by budding into cytoplasmic vacuoles. Viral nucleocapsids and dense bodies are both enveloped in the Golgi region of infected cells by morphologically similar mechanisms during the same period of the infectious cycle (38). Nucleocapsids of CMV are also seen budding from the nuclear membrane, but envelopment of the dense bodies by the nuclear membrane has not been detected (4, 17, 38). Membrane-bound virions and dense bodies are released from the infected cell in the same manner (38).

Dense bodies are of variable size and lack a morphologically identifiable nucleocapsid (4, 17, 34, 39) or detectable DNA (34). They do

contain a surface antigenic determinant(s) also detectable on the virions of CMV as demonstrated by immune electron microscopy (4). In addition, the purified dense bodies have structural polypeptides with molecular weights identical to those of the purified virion (34). Since both the virions and dense bodies of CMV contain membranes, methods were designed in our laboratory to purify these two particles as a source of CMV-specific glycoproteins. This report describes the purification of virions and dense bodies, and the isolation and characterization of the glycoproteins associated with their membranes. It is proposed that the glycoproteins associated with the membranes of cytomegalovirions and dense bodies are specified by the CMV genome. The characterization of these glycoproteins and the preparation of specific antiserum to CMV glycoproteins should facilitate future studies on CMV glycoprotein syn-

thesis in productively or persistently infected cells (29) or in transformed cells (1).

MATERIALS AND METHODS

Virus. The Towne strain of human CMV was obtained from S. A. Plotkin (Wistar Institute of Anatomy and Biology). Isolation, passage, and focus purification of the virus have been described (28). Virus was propagated by infecting human fibroblast cells, either WI38 or human foreskin, with 1 to 4 PFU/cell. After adsorption of the virus for 90 min at 36°C, Eagle minimal essential medium containing 2% fetal calf serum and aureomycin (50 µg/ml) was added. When 30 to 100% of the cells demonstrated cytopathic effects, the medium was changed. The virus was harvested from the extracellular culture fluid 3 to 4 days after detecting 100% cytopathic effects in the inoculated cultures. The amount of infectious virus was determined by assays for plaques (47) or tissue culture infective doses (8). Virus and cells were found to be devoid of mycoplasma contamination by inoculating 0.5 ml of cells or virus into tubes of PPLO broth (Difco) containing 0.5% arginine, 2.5% yeast extract, 20% pretested horse serum, 0.002% phenol red, 10 units of penicillin per ml, 50 µg of fungizone per ml, and thallium acetate (one part/2,000 parts), pH 6.7. The growth of mycoplasma causes the media to turn from orange to red.

Tissue culture, viral infection, and labeling. Human WI38 fibroblast cells were obtained from L. Hayflick (Stanford University). Primary human fibroblast cells were prepared from human foreskins. The cells were cultivated in roller bottles with Eagle minimal essential medium supplemented with 15% fetal calf serum and aureomycin (50 µg/ml). To label viral proteins, the cells were infected with CMV at a multiplicity of 1 to 4 PFU/cell and ¹⁴C-labeled L-amino acids, 1 µCi/ml (New England Nuclear Corp.), or [³H]glucosamine, 5 µCi/ml (20 Ci/mmol, Amersham/Searle Corp.), were added 18 to 24 h after infection. To label the viral DNA, [³H]thymidine, 3 µCi/ml (20 Ci/mmol, New England Nuclear Corp.), was added to the cell cultures at approximately 24 h after infection with CMV.

Purification of virions and dense bodies. Virions and dense bodies of CMV were purified from the extracellular fluid of infected human fibroblast cells. All manipulations were conducted at 18 to 25°C because low temperatures inactivate the virus. Particulate cellular material was removed from the extracellular fluid by centrifugation at 7,000 rpm for 20 min in a Sorvall GSA rotor. After pooling the supernatant fluids, virions and dense bodies were centrifuged in a Spinco T-21 rotor at 21,000 rpm for 1 h through 20% (wt/vol) D-sorbitol in 0.05 M Tris-hydrochloride, pH 7.2, containing 0.001 M MgCl₂ and 100 µg of bacitracin per ml. Bacitracin was added to suppress aggregation of virions and dense bodies. The pellets were suspended in 2 to 4 ml of Tris-buffer saline (TBS, 0.05 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl) by mixing with a Vortex mixer until no particulate matter remained visible. To dissociate aggregates, urea (final concentration 0.5 M) was added and the mixture was sonicated for 10

to 15 s. The suspension was layered onto a 20 to 70% (wt/vol) linear D-sorbitol gradient containing 0.05 M Tris-hydrochloride, pH 7.2, 0.001 M MgCl₂, and 100 µg of bacitracin per ml. Velocity centrifugation was at 22,500 rpm for 1 h in either a SW25 or a SW27.1 rotor. Virions and dense bodies in the region of 55% D-sorbitol were collected and then diluted by the addition of 2 volumes of TBS, pH 7.4. This suspension was layered onto a preformed, discontinuous gradient consisting of 1.4 and 1.2 g of CsCl per ml. After velocity centrifugation as described above, one wide band was detectable. The band was collected and examined by electron microscopy. Contaminating nucleocapsids were detectable. Nucleocapsids were separated from the virions and dense bodies by adding CsCl to a density of 1.5 g/ml and then adding layers 1.4 g/ml and 1.2 g/ml of CsCl. Centrifugation was at 22,500 rpm for 18 h in either the SW25 or SW27.1 rotor.

Before purification, human fibroblast cells labeled with [³H]arginine, [³H]uridine, and [³H]thymidine or with [¹⁴C]acetate were suspended into their extracellular fluid and homogenized to lyse the cells. Approximately 2×10^6 counts per min (cpm)/ml of the uninfected cell lysate was added to the extracellular fluid of CMV-infected cells. Removal of the radioactively labeled cell contaminants at the various stages in the purification was evaluated by determining the cpm per milligram of protein in the virion-dense body preparations. Protein concentrations were determined according to the procedure of Lowry et al. (20) using crystalline bovine serum albumin as the standard protein.

Electron microscopy. To remove sorbitol or CsCl from preparations of virions and dense bodies, the sample was placed onto a 50-nm membrane filter (Millipore Corp.) floating on water. After 20 to 30 min to allow diffusion of the sorbitol or CsCl through the filter, most of the sample was in water. A drop of this suspension was then placed on a 400-mesh copper grid for 20 min. The grid contained a thin film of Formvar that was coated with a light layer of carbon. This material was finally stained with 2% sodium phosphotungstate containing 100 µg of bacitracin per ml. Other material was pelleted by ultracentrifugation and then fixed with 2% glutaraldehyde and 1% osmium tetroxide in sodium cacodylate buffer, pH 7.3, according to a modification of the method of Trump and Bulger (44). The fixed pellet was dehydrated in ethanol, embedded in Epon (21), thin-sectioned, and stained with saturated uranyl acetate and lead citrate by the method of Reynolds (30).

Antisera, indirect immunofluorescence, and viral neutralization. Antibody to CMV antigens was produced by inoculating New Zealand white rabbits either subcutaneously or intramuscularly at four sites with 0.5 to 1 mg of purified antigen mixed with equal volumes of Freund complete adjuvant (Difco). The rabbits were inoculated six to eight times at weekly intervals and were bled 10 days after the final inoculation.

To prepare glycoprotein antigens of CMV, purified virions and dense bodies were first treated with 4% Triton X-100 (TX-100) for 1 h at room tempera-

ture. This mixture was placed onto a 15 to 60% linear sucrose gradient in 0.1 M Tris-hydrochloride, pH 7.8, containing 0.001 M EDTA, and centrifuged at 25,000 rpm for 1 h in the SW56 rotor. Rabbits were inoculated with the nonsedimentable glycoproteins as described above. All of the antisera were heated at 56°C for 30 min and then stored at -20°C.

The indirect fluorescent antibody method of Weller and Coons (46) was used with the following modifications. Monolayers of human fibroblast cells on cover slips were inoculated with approximately 2×10^3 PFU of cytomegalovirus and incubated at 36°C. After 72 h of incubation, the cultures were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4, and fixed with ice-cold acetone. Antisera to CMV antigens, preimmune serum, and fluorescein-conjugated goat anti-rabbit gamma globulin serum contained 0.2 M α -methyl-D-mannoside (Pfaltz and Bauer). This treatment was found to inhibit a nonspecific reaction between rabbit or goat immunoglobulin G (IgG) and IgG receptors induced by CMV. After treating the cultures for 30 min at room temperature with the various antisera, they were washed with PBS and examined by fluorescent microscopy according to the following criteria: (-), no detectable fluorescence; ($\pm$), questionable fluorescence; (+), up to 10%; (++) , 10 to 25%; (+++), 25 to 50%; (++++) , 50 to 100% fluorescence detectable in infected cells. Cover slips were number coded and the degree of fluorescence was evaluated by two individuals; one individual knew the code and the other did not.

The plaque-reduction method was used to test for neutralizing antibody in the antisera prepared against cytomegalovirions and dense bodies, or CMV glycoproteins. Approximately 1,400 PFU/ml of virus was mixed with an equal volume of either anti-cytomegalovirion-dense body serum (1:2), anti-CMV glycoprotein serum (1:2), preimmune serum (1:2), or medium containing 2% fetal calf serum, and incubated at 37°C for 30 min. Monolayers of human fibroblast cells were inoculated with 0.1 ml of the above mixtures for plaque assay as previously described (47).

Iodination with ^{125}I . Cytomegalovirions and dense bodies were iodinated with ^{125}I in the presence of lactoperoxidase and H_2O_2 after density gradient centrifugation in sorbitol. Since the viral preparation was highly infectious at this stage of purification and few of the membranes were penetrable by phosphotungstic acid, as determined by electron microscopy, it was concluded that a majority of the membranes of the virions and dense bodies were intact. The method of iodination used was that of Phillips and Morrison (27) with modifications as described previously (42). The concentration of sorbitol in the virion-dense body preparation was reduced by brief dialysis against 0.005 M Tris-hydrochloride, pH 7.8, containing 0.001 M EDTA. Unconjugated ^{125}I was removed from the cytomegalovirions and dense bodies by velocity and equilibrium centrifugation in CsCl as described above. The virions and dense bodies were also dialyzed extensively against several changes of 0.05 M Tris-hydrochloride, pH 7.4, containing 0.001 M EDTA.

Solubilization of membranous proteins. Membrane proteins of cytomegalovirions and dense bodies were solubilized with TX-100 according to the method of Simons et al. (37). Purified virions and dense bodies in 0.05 M Tris-hydrochloride, pH 7.8, containing 0.001 M EDTA were incubated in 4% TX-100 at room temperature for 1 h. With high concentrations of virions and dense bodies, 2 mg of protein/ml, 4% TX-100 solubilized the majority of the ^3H -labeled glycoproteins. Glycoproteins were separated from viral nucleocapsids, lipids, and nonsolubilized membranous material by centrifugation at $60,000 \times g$ for 1 h into 15 to 60% (wt/vol) linear sucrose gradients in 0.01 M Tris-hydrochloride, pH 7.8, containing 0.001 M EDTA. The ^3H -labeled glycoproteins were not very sedimentable under these conditions and, consequently, remained near the top of the gradient. Because TX-100 caused diffuse leading and trailing edges of glycopolypeptides in sodium dodecyl sulfate (SDS)-gels, excess TX-100 was removed from the glycoproteins with Bio-Beads SM-2 (Bio-Rad) according to the method of Holloway (13). Significant amounts of glycoprotein were lost because of hydrophobic interactions between TX-100 and the glycoproteins and subsequent adsorption of the complexes by the Bio-Beads. Excess TX-100 was not removed from material used to inoculate rabbits.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was as described previously (42) according to a modification of the method of Laemmli (18). Acrylamide was recrystallized as described by Loening (19). The gels were cross-linked with N,N' -diallyltartardiamide, which facilitates separation of glycosylated polypeptides from nonglycosylated polypeptides (11). The ratio of N,N' -diallyltartardiamide to acrylamide was 1:30. Myosin (220,000 daltons, a gift from C. Swenson, University of Iowa), β -galactosidase (130,000), phosphorylase A (94,000), bovine serum albumin (68,000), ovalbumin (43,000) and α -chymotrypsinogen (25,700) were used as standard markers for molecular weight. All protein markers with the exception of myosin were obtained from Sigma. After electrophoresis, the gels were removed from the tubes and immersed in 50% trichloroacetic acid overnight. Proteins in the gels were stained for 3 h with Coomassie brilliant blue (0.25%) in 25% methanol and 50% acetic acid. Molecular weights of the viral proteins were estimated according to the procedure of Weber and Osborn (45).

Gel filtration. Agarose (Bio Rad), 4 and 6%, was prepared in 6 M guanidine-hydrochloride (GuHCl; Schwarz/Mann Chemicals). Molecular weights of polypeptide chains were determined by gel permeation according to the method of Mann et al. (23) in columns (0.9 by 90 cm). The columns were run at room temperature at a flow rate of approximately 1 ml/h with 1-ml fractions being collected. The void volume and the total volume were marked with blue dextran and phenol red, respectively. Viral glycoproteins and standard proteins for molecular weight markers in 0.1 M Tris-acetic acid buffer, pH 8.6, containing 0.01 M EDTA and 6 M GuHCl, were reduced and alkylated in an atmosphere of N_2 with

0.1 mM dithiothreitol (Calbiochem) for 2 h at room temperature and then alkylated with 0.12 mM iodoacetamide (Sigma) at pH 8.6 for 3 h at 37°C. Thyroglobulin (160,000 daltons), phosphorylase A (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) were used as markers for molecular weights (16, 45). Molecular weights were calculated by plotting the log of the molecular weight against the distribution constant of the standards (7).

Determination of radioactivity. Gels containing ^3H were cut into 1-mm slices and solubilized with Protosol (New England Nuclear), and then Omni-fluortoluene mixture was added as previously described (42). The radioactivity in gels containing ^3H was counted in a Packard liquid scintillation spectrometer; gels containing ^{125}I were sliced as described above and the radioactivity was counted in a Packard gamma counter.

RESULTS

Recovery of infectious CMV during purification of virions and dense bodies. Infectious human CMVs are characteristically difficult to purify or store because of the marked instability of the virion. However, mixing the virus with 35% D-sorbitol suppresses the degree of inactivation of viral infectivity during long periods of storage at room temperature, 4°C or

–50 to –70°C (Stinski, unpublished data). Therefore, sorbitol was tested as a medium to provide stability during velocity centrifugation of infectious CMV. It was observed empirically that bacitracin (100 $\mu\text{g}/\text{ml}$) suppressed aggregation and also stabilized viral infectivity. Similar results were obtained with sucrose gradients containing bacitracin. After pelleting cytomegalovirions and dense bodies through 20% sorbitol in 0.05 M Tris-hydrochloride, pH 7.2, containing 0.001 M MgCl_2 and 100 μg of bacitracin per ml, titers as high as 10^{10} PFU/ml were obtained. Data in Table 1 illustrate the virion-dense body purification procedure, the recovery of infectious virus, and the removal of host-cell proteins, nucleic acids, and lipids. After velocity centrifugation in 20 to 70% linear sorbitol gradients, 30% of the viral infectivity was recovered from a diffuse light-scattering band in the region of 55% sorbitol (Table 1). Figure 1A illustrates the distribution of ^{14}C -labeled protein and ^3H]DNA in the 20 to 70% linear sorbitol gradients. Results identical to Fig. 1A were also obtained with 12 to 52% linear sucrose gradients in the above buffer containing bacitracin and with similar conditions of centrifugation. The ^{14}C -labeled protein peak

TABLE 1. Recovery of infectious cytomegalovirus during purification of virions and dense bodies and removal of radioactively labeled host-cell contaminants

Fraction	Expt 1				Expt 2			
	Volume (ml)	Infectivity (PFU/ml)	Infectivity ^a recovered (%)	^3H -labeled cellular protein and nucleic acid (cpm/mg of protein)	Volume (ml)	Infectivity (PFU/ml)	Infectivity ^a recovered (%)	^{14}C acetate-labeled cells (cpm/mg of protein) ^c
Extracellular fluid of infected cells	240.0	2.1×10^7	100	1,973.9	100.0	5.5×10^5	100	3,360.0
Supernatant after low-speed centrifugation	240.0	2.3×10^7	109	1,549.1	100.0	5.5×10^5	100	3,299.5
Supernatant after high-speed centrifugation through 20% D-sorbitol	265.0	1.6×10^6	8.4	2,991.8	120.0	2.3×10^4	5.1	2,665.0
Pellet after high-speed centrifugation through 20% D-sorbitol	2.5	5.0×10^8	24.8	1,877.4	2.0	1.5×10^7	54.5	1,381.8
Bottom band in linear 20–70% D-sorbitol gradient	1.5	9.9×10^8	29.6	139.5	1.5	1.1×10^7	30.0	165.0
Top band in preformed CsCl	1.0	2.4×10^7	0.5	None		ND ^d		None
Band after flotation to density of virions and dense bodies in CsCl	1.0	3.0×10^3	0.00006	None		ND		None

^a Percentage of total infectivity in extracellular fluid of infected cells.

^b 1×10^6 to 3×10^6 cpm of lysed human fibroblast cells labeled with ^3H]arginine, ^3H]uridine, and ^3H]thymidine was added to the extracellular fluids from CMV-infected cells.

^c 1×10^6 to 3×10^6 cpm of lysed human fibroblast cells labeled with ^{14}C]acetate was added to the extracellular fluids from CMV-infected cells.

^d ND, Not done.

FIG. 1. Centrifugation of cytomegalovirions and dense bodies in gradients of D-sorbitol and CsCl. (A) Virions and dense bodies from the extracellular fluid of infected cells containing ^{14}C -labeled amino acids ($\circ$ — $\circ$) and [^3H]thymidine ($\bullet$ — $\bullet$) were concentrated 10-fold by centrifugation at $70,000 \times g$ for 1 h at 18°C through 20% D-sorbitol in 0.05 M Tris-hydrochloride, pH 7.2, containing 0.001 M MgCl_2 and 100 μg of bacitracin per ml. The virion-dense body pellet was suspended in TBS, pH 7.4, and 0.2 ml containing 5.5×10^6 PFU/ml was centrifuged into a 4-ml linear 20 to 70% D-sorbitol gradient in a Spinco SW56 rotor at $61,400 \times g$ for 1 h at 18°C . Fractions (0.15 ml) were collected from the bottom of the tube. (B) Fractions 4 to 8 from A were pooled, diluted to 2.4 ml in TBS, and layered onto a preformed, discontinuous gradient consisting of 1.4 and 1.2 g/ml of CsCl. Centrifugation and collection of fractions were as described above. (C) Fractions 12 to 16 from B were pooled, CsCl was added to a density of 1.5 g/ml, the sample was placed at the bottom of a centrifuge tube, and equal volumes of CsCl containing 1.4 g/ml and 1.2 g/ml were layered onto the sample. Virions and dense bodies were floated to their density by centrifugation for 16 to 18 h as described above.

at 40% sorbitol contained unidentified globular material and particles of 20 and 48 nm. These particles were similar to particles isolated by others from cells infected with herpes simplex virus (HSV) (31). Virions and dense bodies of CMV were in the region of 55% sorbitol. In the region of 63% sorbitol, the ratio of protein to DNA was high (Fig. 1A). An electron microscopic analysis of this region indicated that the ratio of dense bodies to virions was approximately 2:1. However, at 53% sorbitol, the ratio of dense bodies to virions was 1.3:1. In this later region of the gradient, many of the dense bodies had a size similar to that of the virions. In an experiment not shown, maximal viral infectivity corresponded with the peak of maximal incorporation of [^3H]thymidine.

To evaluate the removal of host cell contaminants during the purification procedure, an artificial mixture was prepared containing the extracellular fluids from infected cultures and a cellular-extracellular lysate of uninfected host cells labeled with [^3H]arginine, [^3H]uridine, [^3H]thymidine, or [^{14}C]acetate. Cells grown in the presence of radioactive precursors before infection were not used because misleading results might be obtained from (i) the possible reutilization of host cell-radioactive components (14) during the slow replication of CMV and (ii) the possible acquisition of radioactively labeled host cell histones by the viral DNA (6). After velocity centrifugation, virions and dense bodies in the region of 55% sorbitol contained low levels of radioactively labeled host-cell contaminants (Table 1). The cytomegalovirions and dense bodies were diluted threefold with TBS, pH 7.4, and this suspension was layered on a preformed cushion consisting of equal volumes of 1.4 g/ml and 1.2 g/ml of CsCl. After velocity centrifugation, radioactive host-cell contaminants were removed from the virions and dense bodies but there was a considerable loss of viral infectivity (Table 1). This loss in infectivity after centrifugation in CsCl paralleled a loss of integrity of the viral envelope seen by electron microscopy in negatively stained preparations. Although not evident in Fig. 1B, a shoulder was occasionally detected toward the heavier density of CsCl. This area of the gradient contained a considerable number of viral nucleocapsids and some partially enveloped virions and dense bodies. The relative proportion of these naked and partially enveloped virions and dense bodies to virions and dense bodies with membranes depended on handling procedures; if there was a delay in purification, or if low temperatures were used, the proportion increased. Similar observations

have been reported by Huang et al. (15). Virions and dense bodies were separated from viral nucleocapsids by suspending the material in 1.5 g of CsCl per ml, layering 1.4 g and 1.2 g of CsCl per ml, and then floating the virions and dense bodies to their density of 1.276 g/ml by equilibrium centrifugation (Fig. 1C). Virions were not separated from dense bodies by these procedures. The centrifugation was designed to select for purified membrane-bounded virions and dense bodies which would serve as a purified source of CMV-specific glycoproteins. The final purified material contained approximately twice as many dense bodies as virions. Purified cytomegalovirions and dense bodies consisted of 21 species of polypeptides as determined by SDS-PAGE and Coomassie brilliant blue staining of the polypeptides (see Fig. 5C) and 11 additional polypeptides were detected when high concentrations of virions and dense bodies were analyzed (see Fig. 5D). A polypeptide composition of at least 23 polypeptides was reported for purified AD169 cytomegalovirions and 17 to 22 polypeptides for purified dense bodies (34). Centrifugation techniques (12 to 52% sucrose, SW27.1 rotor for 43 min at 23,000 rpm) identical to those used by others to separate AD169 cytomegalovirions and dense bodies isolated from infected WI38 human fibroblast cells (34) did not separate the Towne strain of cytomegalovirions and dense bodies isolated from infected WI38 or human foreskin fibroblast cells. Many dense bodies of the Towne strain had the same sedimentation properties and size as the virions. However, it was possible to obtain large dense bodies (300 to 350 nm) of the Towne strain relatively free from virions and nucleocapsids by velocity centrifugation in sucrose gradients as described by Sarov and Abady (34). Since dense bodies appear to be an aberrant form of viral morphogenesis, the size of these dense bodies may differ with different strains of CMV, with different kinds of human fibroblast cells, and with different multiplicities of infection.

Electron microscopy of purified virions and dense bodies. Electron microscopy was used to characterize cytomegalovirions and dense bodies and to determine qualitatively if virions and dense bodies were free of extraneous debris. Figure 2A is an electron micrograph of purified virions and dense bodies stained with sodium phosphotungstic acid. Exposure to CsCl caused a loss of integrity of many membranes. Consequently, the stain penetrated the particles (Fig. 2A). This permitted a differentiation of virions from dense bodies. Virions contained a nucleocapsid, whereas dense bodies contained no nu-

cleocapsid and stained darker than virions. Virions and dense bodies were free of extraneous globular material and small particles (20 to 50 nm). Figure 2A illustrates that the dense bodies had a spherical configuration, varied in size, stained dark if partially disrupted, and corresponded morphologically to the dense bodies found in the cytoplasm of thin sections of CMV-infected human fibroblast cells (4, 17, 22, 32, 38). The average diameter of the virions was approximately 200 nm, which is similar to that reported by Smith and deHarven (38). The dense bodies had sizes ranging from 133 to 354 nm with an average diameter of 236 nm. These results were obtained by measuring 61 dense bodies with membranes from seven individual electron micrographs of four purified samples prepared at different times and stained with sodium phosphotungstic acid.

Figures 2B and 2C are electron micrographs of the purified material fixed with glutaraldehyde and osmium tetroxide, embedded in Epon, thin-sectioned, and stained with uranyl acetate and lead citrate. Virions possessed a membrane with a nucleocapsid. Dense bodies possessed a membrane with electron-dense material and were devoid of a morphologically identifiable nucleocapsid (Fig. 2B). More of the virions and dense bodies in samples fixed and embedded in Epon had disrupted membranes. This was probably due to ultracentrifugation in the absence of a medium to insure stability. Such centrifugation was necessary to obtain a concentrated sample for embedding in Epon and subsequent electron microscopy. Figure 2C is a high-magnification electron micrograph ($\times 100,000$) of a dense body. The associated membrane had a width of approximately 11 nm, and on the surface are what appear to be projections that are approximately 15 nm apart. Surface subunits were also observed in negatively stained preparations of virions. These surface projections are probably composed of viral glycoproteins. The membrane to which these surface subunits are attached is probably a lipid bilayer, as evidenced by its double-track appearance in thin sections (Fig. 2C).

Antisera to antigens associated with cytomegalovirions and dense bodies. To determine if significant amounts of host-cell antigens were incorporated into virions and dense bodies

of CMV during their morphogenesis and to obtain a specific antiserum for future studies on CMV-glycoprotein synthesis, antisera were prepared against purified virions and dense bodies and against their glycoproteins. When CMV-infected cells were treated with preimmune rabbit sera and then with fluorescein-conjugated goat anti-rabbit gamma globulin serum, fluorescence occurred because of immunoglobulin receptors induced by the virus. IgG molecules from various species react nonspecifically with receptors in CMV-infected cells (9). This nonspecific reaction was suppressed in the present study by the addition of 0.2 M α -methyl-D-mannoside to all the sera (Stinski, unpublished data). The mannoside may inhibit a reaction between the Fc portion of immunoglobulin and CMV glycoproteins.

Monolayers of human fibroblast cells were infected with 2×10^8 PFU of CMV and incubated at 36°C for 72 h, after which 20 to 30% of the cells exhibited cytopathic changes. Uninfected cells were used as a control. Cells infected with CMV and treated with antiserum prepared against a preparation of purified cytomegalovirions and dense bodies had intense fluorescence (+++++) in their nuclei and some fluorescence in their cytoplasm (++) (Fig. 3A). Fluorescence was judged as described in Materials and Methods. Uninfected cells treated with antiserum prepared against a preparation of purified virions and dense bodies had no detectable fluorescence (-).

When CMV-infected cells were treated with antiserum prepared against the glycoproteins extracted from cytomegalovirions and dense bodies, intense fluorescence (+++++) was detected only in their cytoplasm (Fig. 3B). In addition, viable CMV-infected cells treated with anti-CMV glycoprotein serum had intense cytoplasmic membrane fluorescence (+++++; data not shown). When uninfected cells were treated with this serum, no fluorescence (-) was detected. Infected cells treated with preimmune serum had little or no cytoplasmic ($\pm$) and no nuclear fluorescence (-) (Fig. 3C). Since antisera to CMV antigens contained no detectable anti-host cell antibodies, it is proposed that purified cytomegalovirions and dense bodies contain little or no significant amounts of cellular antigens.

FIG. 2. Electron microscopy of purified CMV (large arrow) and dense bodies (small arrow). (A) Stained with 2% sodium phosphotungstic acid containing 100 μ g of bacitracin per ml. (B) Fixed with 1% osmium tetroxide and 2% glutaraldehyde in 0.2 M sodium cacodylate-hydrochloride buffer, pH 7.0. Fixed samples were dehydrated in ethanol, embedded in Epon, and thin-sectioned. Sections were stained with uranyl acetate and lead citrate as described in Materials and Methods. (C) Dense body prepared as described in B. All marker bars represent 100 nm.

TABLE 2. *Effect of antisera to cytomegalovirus antigens on infectivity of cytomegalovirus*

Inoculum ^a	Virus titer (PFU/ml)	Viral survivors (%)
Virus + anti-cytomegalovirion-dense body serum	1.7×10^2	25.0
Virus + anti-cytomegalovirus glycoprotein serum	$<1.0 \times 10^1$	<14.7
Virus + preimmune serum	5.7×10^2	83.8
Virus + medium containing 2% fetal calf serum	6.8×10^2	100.0

^a Approximately 1,400 PFU of CMV per ml plus equal volumes of anti-cytomegalovirion-dense body serum (1:2), anti-cytomegalovirus glycoprotein serum (1:2), preimmune serum (1:2), or medium containing 2% fetal calf serum were incubated at 37°C for 30 min and then 0.1 ml of each mixture was inoculated into each of three human fibroblast cell cultures for plaque assay.

Antisera against a purified preparation of cytomegalovirions and dense bodies and against CMV glycoproteins neutralized viral infectivity as determined by plaque reduction. Preimmune sera did not significantly neutralize viral infectivity (Table 2). Thus, these antisera were specific for CMV antigens.

Glycoproteins associated with virions and dense bodies of cytomegalovirus. To characterize the glycoproteins associated with virions and dense bodies, the glycoproteins were fractionated according to electrophoretic mobility in SDS-polyacrylamide gels. Purified virions and dense bodies labeled with [³H]glucosamine were dissociated with SDS in the presence of mercaptoethanol at 100°C for 2 to 3 min and then were analyzed by SDS-PAGE in 9% gels. The relative mobilities of the glycopolypeptides are illustrated in Fig. 4. Glycopolypeptides between relative mobilities of 0.2 and 0.3 acquired little label even during long incubations with [³H]glucosamine and were not always detectable or well resolved. Glycopolypeptides between relative mobilities of 0.3 and 0.5 had greater amounts of incorporated glucosamine and migrated through the gel as distinct species. A considerable amount of material labeled with [³H]glucosamine migrated just behind the dye

marker. This material was considered a low-molecular-weight glycopolypeptide for the following reasons: (i) it was not removed by delipidation with TX-100 (see Fig. 6A), suggesting it was not a glycolipid; (ii) a molecular weight of approximately 12,000 daltons, which is higher than that of glycolipids, was estimated by gel filtration in 4 and 6% agarose containing 6 M GuHCl (Table 3); (iii) it contained protein since iodination with ¹²⁵I (Fig. 5) and Coomassie brilliant blue staining (Fig. 6B) was possible. Furthermore, it contained a considerable amount of incorporated [³H]glucosamine (Table 3), and it was stainable by periodic acid-Schiff reagent (data not shown).

Surface polypeptides of virions and dense bodies of CMV. To characterize the polypeptides exposed to the surface of cytomegalovirions and dense bodies, partially purified material was used from the region of 55% sorbitol after velocity centrifugation. The virus in this material was highly infectious (10^8 PFU/ml), suggesting that the membranes were intact. In addition, electron microscopic analysis of negatively stained preparations indicated that the majority of the membranes were intact. Virions and dense bodies were iodinated with ¹²⁵I in the presence of lactoperoxidase and H₂O₂ and then were further purified by two centrifugations in preformed discontinuous gradients of CsCl as described previously. The purified virions and dense bodies were dissociated to polypeptide chains and analyzed by SDS-PAGE. Figure 5 illustrates that the majority of the iodinated polypeptides had relative mobilities similar to the glycopolypeptides associated with cytomegalovirions and dense bodies (Fig. 4). These data suggested that (i) the CMV glycopolypeptides are on the surface of the membranes of virions and dense bodies and (ii) nonglycosylated proteins with relative mobilities significantly different from that of the glycopolypeptides are not detectable on these surface membranes.

Membranous glycoproteins extracted from virions and dense bodies of CMV. To prepare antiserum to CMV-specific glycoproteins, virions and dense bodies were treated with 4% TX-100 as described previously to isolate glycopro-

FIG. 3. Immunofluorescence photomicrographs of human CMV-infected human fibroblast cells treated with rabbit anti-cytomegalovirion-dense body serum, anti-cytomegalovirus glycoprotein serum, or preimmune serum. All sera contained 0.2 M alpha-methyl-D-mannoside to inhibit a nonspecific reaction between rabbit or goat IgG and IgG receptors induced by CMV. Cells were treated with antiserum 72 h after infection and stained with fluorescein-conjugated goat anti-rabbit gamma globulin mixed with rhodamine-labeled bovine albumin. (A) Virus infected cells reacted with anti-cytomegalovirion-dense body serum. $\times 250$. (B) Virus-infected cells reacted with anti-cytomegalovirus glycoprotein serum. $\times 250$. (C) Virus-infected cells treated with preimmune serum. $\times 100$. The arrow designates CMV-infected cells.

FIG. 4. Discontinuous SDS-PAGE of [^3H]glycopolypeptides associated with cytomegalovirions and dense bodies. Purified virions and dense bodies were dissociated with SDS in the presence of mercaptoethanol and electrophoresed at 3 mA in cylindrical 9% polyacrylamide gels cross-linked with N,N' -diallyltartardiamide as described in Materials and Methods. Gels were sliced into 1-mm fractions and analyzed as described in Materials and Methods. The mobility of the glycopolypeptides was relative to a bromophenol blue dye marker. Molecular weight standards possessed the following relative mobilities: myosin (220,000 daltons), 0.12; β -galactosidase (130,000), 0.21; phosphorylase A (94,000), 0.33; bovine serum albumin (68,000), 0.40; ovalbumin (43,000), 0.62; α -chymotrypsinogen (25,700).

FIG. 5. Discontinuous SDS-PAGE of the surface polypeptides associated with cytomegalovirions and dense bodies. After partial purification in a sorbitol gradient, virions and dense bodies were iodinated with ^{125}I in the presence of lactoperoxidase and H_2O_2 as described in Materials and Methods and then they were further purified by velocity and equilibrium centrifugation in CsCl as described in Fig. 1. Purified ^{125}I -labeled virions and dense bodies were dissociated, electrophoresed, and analyzed with molecular weight standards as described in Fig. 3.

teins. TX-100 was selected for isolating lipid-free glycoproteins because it has notably mild effects on proteins and conformational changes in water-soluble-proteins are not induced (10).

Figure 6A and B illustrate the distribution of the extracted glycopolypeptides in 9% gels after SDS-PAGE. The peaks of [^3H]glycopolypeptides were not sharply defined and the relative mobilities increased slightly since the Bio-Beads SM-2 did not adsorb out all of the TX-100, and it is assumed that some TX-100 was bound to the hydrophobic segments of these proteins. The presence of TX-100 in the sample

and not in the gel may have effected the migration of the glycoproteins and consequently altered slightly the electrophoretic profile. Alternatively, the manipulations used in extraction of the glycoproteins may have caused some degradation. The percentage of [^3H]glucosamine incorporated into the various glycopolypeptides corresponded to that detected in the SDS-PAGE analysis of virions and dense bodies labeled with [^3H]glucosamine (Fig. 4, Table 3). Glycoproteins extracted from the membranes of virions and dense bodies were also stained with Coomassie brilliant blue after

SDS-PAGE (Fig. 6B). Proteins were detectable with molecular weights (Table 4) corresponding to the glycopolypeptides detected in Fig. 6A. Figure 6C and D illustrate the polypeptide composition of untreated purified cytomegalovirions and dense bodies as analyzed by SDS-PAGE and subsequent staining of polypeptides with Coomassie brilliant blue. High and low concentrations of cytomegalovirions and dense bodies were analyzed to evaluate their polypeptide profile in SDS-gels. The glycoproteins extracted from the membranes of virions and dense bodies were free of detectable nonglycosy-

lated proteins. These methods for the isolation of CMV glycoproteins were also used to prepare CMV-glycoprotein antigens and subsequently anti-CMV glycoprotein serum as described previously.

Properties of the glycoproteins associated with cytomegalovirions and dense bodies. Unlike proteins, glycoproteins behave anomalously in SDS-PAGE due to a decreased binding efficiency of SDS to the glycoproteins. If the amount of bound SDS is insufficient, the glycoproteins do not migrate in the same manner as the standard proteins. At higher concentrations

FIG. 6. Discontinuous SDS-PAGE of glycopolypeptides extracted from cytomegalovirions and dense bodies and of polypeptides associated with cytomegalovirions and dense bodies. Purified virions and dense bodies labeled with [^3H]glucosamine (A) or unlabeled (B) were treated with 4% TX-100 for 1 h at room temperature and then centrifuged into 15 to 60% linear sucrose gradients at $60,000 \times g$ for 1 h at 18 C. Glycoproteins were near the top of the gradient. Excess TX-100 was removed from the glycoproteins with Bio-Beads SM-2. (C) Low concentration, 0.75 mg of protein, of cytomegalovirion-dense body polypeptides. (D) High concentration, 1.5 mg of protein, of cytomegalovirion-dense body polypeptides. The glycoproteins and proteins were dissociated, electrophoresed, and compared with molecular weight standards as described in Fig. 3. Gels containing unlabeled proteins were stained with Coomassie brilliant blue. The positions of the Coomassie brilliant blue-stained glycoproteins are represented diagrammatically. The arrow designates the direction of electrophoresis.

of acrylamide, the charge of the SDS-protein complex becomes less important relative to the sieving effect of the gel (24, 25, 36). Therefore, only high concentrations of gel were used to characterize the molecular weight properties of the CMV glycopolypeptides. The efficiency of fractionation of these glycopolypeptides was better in gels with 9% acrylamide than in gels with lower or higher concentrations of acrylamide.

Gel filtration of reduced and alkylated glycopolypeptides in 6 M GuHCl was used as an alternative technique for fractionating glycopolypeptides, but the quality of fractionation was inferior to that of 9% acrylamide gels. A column of 4% agarose with an exclusion limit of approximately 220,000 daltons in the presence of 6 M GuHCl was used to fractionate nine of the CMV glycopolypeptides. Table 3 lists the determined molecular weights of the CMV-glycopolypeptides in 9, 10, and 12% polyacrylamide gels and in 4 and 6% agarose containing 6 M GuHCl. Table 3 also contains the frequency of detection of each glycopolypeptide and their percentage of incorporated [^3H]glucosamine. Eight glycopolypeptides with unique molecular

weights containing significant amounts of [^3H]glucosamine were detected. Three additional glycopolypeptides with molecular weights of 140,000 to 190,000 were occasionally detectable but these contained low amounts of [^3H]glucosamine (Table 3). These glycopolypeptides may represent precursors or aggregates of the glycopolypeptides with lower molecular weights. Experiments are in progress to determine if precursor glycoproteins of CMV are synthesized.

DISCUSSION

The glycoproteins associated with the membranes of cytomegalovirions and dense bodies are considered to be specified by the CMV genome. Observations indicating that the membranes of these virions and dense bodies have a similar glycoprotein composition are (i) the membranes of both share a common antigen(s) as determined by immune electron microscopy (42), (ii) the membranes of both attach to and fuse with the membranes of host cells as determined by electron microscopy (39), and (iii) purified dense bodies have structural polypeptides

TABLE 3. *Molecular weight determinations of glycoproteins associated with cytomegalovirions and dense bodies*

Frequency of detection ^a (%)	Glucosamine ^b (%)	Mol wt $\times 10^{-3c}$					
		SDS-PAGE				Gu-HCl agarose	
		9% ^d	9% TX-100 ^e	10% ^f	12% ^g	4% ^h	6% ⁱ
	(Top of gel) 13.5						
36.8	1.0	190.0	NS $\leftrightarrow$ 155.0	NS	177.5	190.0	>100
52.6	1.0	165.0	141.5	153.0	152.5	157.5	>100
42.1	3.1	141.0	132.0	140.0	NS	NS	>100
84.2	3.4	128.3	110.0	131.0	132.5	128.3	>100
84.2	3.1	111.6	99.0	115.0	116.7	113.0	>100
89.5	22.0	98.8	75.5	107.5	103.7	95.0	98.0
94.7	7.0	83.3	67.0	81.0	83.0	78.0	78.0
89.5	6.1	63.1	60.8	65.5	65.5	64.0	69.0
68.4	6.8	57.3	54.8	62.0	60.8	NS	NS
78.9	10.4	53.3	NC	NS	53.0	54.7	56.0
100.0	22.6	NC ^k		NC	NC	11.8	11.5

^a (Total number of detections/total number of analyses) $\times 100$ in 9% gels.

^b Determined as [^3H]glucosamine in 9% gels by calculating the percentage of radioactivity under each peak.

^c The molecular weight of each polypeptide was relative to standard proteins as described in Materials and Methods.

^d Average of six separate determinations.

^e TX-100-extracted glycopolypeptides; average of four separate determinations.

^f Average of two separate determinations.

^g Average of three separate determinations.

^h Average of three separate determinations with 6 M GuHCl in 4% agarose.

ⁱ One determination with 6 M GuHCl in 6% agarose.

^j NS, Not separated or not containing sufficient radioactivity to be identified as a unique species of glycopolypeptide.

^k NC, Not calculated because the peak of [^3H]glucosamine was one or two fractions before the dye mark.

with relative mobilities and molecular weights similar to the structural polypeptides of purified virions as determined by SDS-PAGE (34). Therefore, methods were designed to purify virions and dense bodies of CMV and characterize their glycoproteins. Purity was defined with respect to specific host cell contaminants such as protein, RNA, DNA, and lipid, and therefore, by definition, the pure preparations were free of detectable cellular and extracellular contaminants. However, the final product was not homogeneous since two morphologically different particles with similar sedimentation coefficients and densities were purified. Virions and dense bodies of the Ad 169 strain of CMV have been separated by velocity centrifugation in linear sucrose gradients because this strain of CMV induces dense bodies that are larger and have a higher sedimentation coefficient than the virions (34). This type of separation was not possible with the Towne strain of CMV used in our investigation. The infected cells yielded dense bodies having a size varying from 133 to 354 nm and many of the dense bodies had the same size and sedimentation coefficient as the virions of CMV. Therefore, both particles were purified and analyzed together.

Electron microscopic analysis of purified virions and dense bodies demonstrated that most extraneous globular material and small particles were removed during the purification. Antisera prepared against a preparation of purified virions and dense bodies or against the glycoproteins extracted from their membranes reacted specifically with CMV-infected cells as determined by indirect immunofluorescence. The immunofluorescent studies support the contention that dense bodies of CMV do not contain host cellular antigens since the above antisera did not react with uninfected cells. These results favor the conclusion of Sarov and Abady (34) that dense bodies consist solely of CMV proteins, which is based on the observation that purified dense bodies have approximately the same number of polypeptides as purified virions and both particles have polypeptides with similar molecular weights. If residual host antigens are present in dense bodies of CMV, they are relatively minor structural constituents. In addition, antiserum against CMV glycoproteins precipitated ^{14}C -labeled glycoproteins from CMV-infected cells, but not from uninfected cells. Precipitation of ^{14}C -labeled glycoproteins from CMV-infected cells was significantly higher with anti-CMV glycoprotein serum than with preimmune serum. However, precipitation of ^{14}C -labeled glycoproteins from infected cells was detectable with preimmune serum. This precipitation may be

due to an interaction between an Fc receptor synthesized in CMV-infected cells (9) and rabbit or goat gamma globulin (Stinski, to be published). Presently, it is not known whether the Fc receptor is a viral or host cellular protein(s).

Cellular proteins are not found in the viral membranes of Semliki forest virus (10), a virus that derives its envelope at cytoplasmic membranes, and the virions of HSV, a virus that derives its envelope at nuclear membranes, contain also little or no host-derived protein (41). Morphological evidence (5, 26, 35) suggests that the virion of HSV acquires its glycoprotein-containing envelope only at the nuclear membrane, whereas CMV obtains an envelope from either the nuclear membranes or the cytoplasmic membranes (38). In the HSV-infected cells, HSV glycoproteins and cellular proteins are contiguous on the plasma membrane (12) and smooth internal membranes (40). Thus, in CMV-infected cells, viral proteins and cellular proteins may be in juxtaposition on the membranes and, consequently, the viral proteins may occupy a topologically unique space from which all host cell proteins are excluded. The viral glycoprotein patch on the membrane may simply exclude the cellular proteins for steric reasons. The acquisition of these viral membranes may depend upon the presence of viral nucleocapsid proteins. These proteins may be necessary as a nucleation site for viral glycoproteins. A similar mechanism has been proposed for Semliki forest virus (10). Although dense bodies of CMV have no morphologically identifiable nucleocapsid, they may contain nucleocapsid structural polypeptides for the acquisition of a viral membrane.

The membranes of cytomegalovirions and dense bodies were found to be composed of eight glycoproteins repeatedly detectable by either SDS-PAGE or gel filtration. In addition, three glycoproteins of high molecular weights containing low amounts of incorporated [^3H]glucosamine were occasionally detectable. These occasionally detectable glycoproteins of high molecular weights may be precursors or aggregates of glycoproteins of lower molecular weights. Glycopolypeptides that can be differentiated electrophoretically may be related antigenically. The number of antigenically distinct viral-specified glycopolypeptides remains to be determined.

How dense bodies of CMV are generated during CMV infection is presently unknown. Since the dense bodies accumulate only in the cytoplasm (4, 17, 38), it is possible that structural proteins of CMV are produced in excess and not all of these viral proteins are transported to the nucleus for viral morphogenesis. The absence of

DNA in dense bodies (34) suggests that without DNA the viral structural proteins are assembled incorrectly. The appearance of cytoplasmic dense bodies in human fibroblast cells infected with CMV but not HSV (38) implies an intrinsic difference in the nature of these two viruses. Other herpesviruses that also induce the synthesis of dense bodies are murine CMV (33), varicella-zoster (2, 3), and herpes-type virus of frogs (43).

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