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Isolation of Human Cytomegalovirus Intranuclear Capsids, Characterization of Their Protein Constituents, and Demonstration that the B-Capsid Assembly Protein Is Also Abundant in Noninfectious Enveloped Particles

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Two types of intranuclear capsids have been recovered from human cytomegalovirus (HCMV, strain AD169)-infected cells. By analogy with strain Colburn (simian CMV) particles, these have been designated as A- and B-capsids. Both types of capsids are composed of proteins with molecular weights of 153,000 (major capsid protein), 34,000 (minor capsid protein), 28,000, and 11,000 (smallest capsid protein). In addition to these species, B-capsids contain a 36,000-molecular-weight (36K) protein which has been designated as the HCMV "assembly protein," based on its similarities to counterparts in strain Colburn CMV (i.e., 37K protein) and herpes simplex virus (i.e., VP22a/p40/NC-3/ICP35e). Peptide comparisons established that the assembly protein of HCMV B-capsids and the 36K protein that distinguishes HCMV noninfectious enveloped particles from virions are the same, providing direct evidence that noninfectious enveloped particles are enveloped B-capsids.

In an earlier report, we described the recovery and characterization of a noninfectious enveloped particle (NIEP) produced by human foreskin fibroblasts infected with human strains of cytomegalovirus (HCMV) (16). Although similar to virions in architecture and protein composition, NIEPs lack DNA and are consequently noninfectious. A second major difference between these two particles is that NIEPs contain an abundant protein species of molecular weight 35,000 to 36,000 (i.e., a 35K to 36K protein; molecular weight is strain dependent) that is not present in virions. This additional protein shares a comparatively unusual set of properties with previously described capsid proteins of strain Colburn CMV (i.e., a 37K protein) (8) and herpes simplex virus (HSV, i.e., the 38K to 40K protein VP22a/p40/NC-3/ICP35e [see references 12, 14, 6, and 4, respectively]). All three proteins are approximately the same molecular weight (i.e., 35,000 to 40,000), are located in the infected cell nucleus (4, 9, 11), are phosphorylated (4, 13, 16; unpublished data), fluoresce when stained with Coomassie brilliant blue (8, 13; unpublished data), and stain comparatively poorly with ammoniacal silver (procedure of Wray et al. [32]), exhibiting a "cafe au lait" color (unpublished data). Based on their suspected involvement in nucleocapsid assembly, envelopment, or both (8, 12, 13, 26), we have referred to these Colburn and HSV capsid proteins as "assembly proteins" (9, 16). Apparent counterparts of this protein have been identified in other herpesviruses, including pseudorabies virus (35K protein) (1, 19), equine herpesvirus 1 (45K protein) (24), and possibly herpesvirus saimiri (29K to 31K protein) (2, 27). This provisional name is used primarily as a convenient alternative to molecular weight, which varies, depending on both virus and strain.

A hallmark of this species is its presence in capsids of intermediate density (e.g., Colburn and HSV B-capsids) which are believed to be involved in virus assembly. The similarities between the assembly proteins of Colburn CMV and HSV B-capsids and the 36K protein of HCMV NIEPs suggested that these unusual HCMV particles arise by envelopment of B-capsids, a possibility with significant implications concerning the role of B-capsids in the virion assembly pathway (see Discussion and reference 16). To substantiate this hypothesis, it was necessary to demonstrate that the 36K protein of NIEPs is in fact the HCMV assembly protein. In this report, we describe the recovery of HCMV nuclear A- and B-capsids, identify the HCMV assembly protein counterpart, and establish that the HCMV B-capsid assembly protein and the 36K protein of NIEPs are the same.

(A preliminary report of this work was presented at the Ninth International Herpesvirus Workshop, 24 to 29 August 1984, Seattle, Wash.)

MATERIALS AND METHODS

Cells and virus. Procedures for human foreskin fibroblast culture and infection with CMV have been described previously (8). The sources of strain Colburn CMV and the human strain AD169 (HCMV) have been described elsewhere (8, 30).

Recovery of CMV NIEPs and A- and B-capsids. The following procedure is a modification of one previously used to recover Colburn CMV capsids (8, 12). Five days after infection with AD169 or Colburn CMV, cells were scraped from the surface of a 32-oz (0.946-liter) bottle, collected by low-speed centrifugation (1,500 × *g*, 10 min, 4°C), washed once by suspension in 0.5 ml of phosphate buffer (PB; 0.04 M phosphate [pH 7.4], 0.15 M NaCl), again collected by low-speed centrifugation, and then fractionated with 0.5 ml of 0.5% Nonidet P-40 in PB (5 min, 0°C). The resulting

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"Nonidet P-40 nuclei" were recovered by low-speed centrifugation (5 min), resuspended in 0.4 ml of PB, and lysed by three cycles of freezing (−70°C, 4 min), thawing (37°C, 2 min), and vortex mixing. After low-speed centrifugation, capsids were recovered from the clarified preparations by sedimentation through a 15 to 50% sucrose (wt/wt in PB) gradient in a Beckman SW41 rotor (40,000 rpm, 4°C, 20 min). The gradients were scanned at 280 nm and fractionated into 0.3-ml samples with an ISCO density gradient fractionator (no. 185) equipped with an absorptimeter. Radiolabeled particles were recovered in the same way from infected cells growing in medium containing [^{35}S]methionine (25 μCi/ml, Amersham SJ.204; added at 36 h postinfection, 20 ml per bottle). Gradients containing radiolabeled capsids were fractionated, and trichloroacetic acid-precipitable radioactivity was measured as described previously (16) with an LKB 1216 Rackbeta II liquid scintillation counter. Radiolabeled NIEPs were recovered by ultracentrifugation of 3 ml of clarified medium from the same cells used in preparing capsids (see above). The NIEP band was located by its light-scattering properties and removed by aspiration with a syringe.

Protein iodination. B-capsids and NIEPs were recovered from sucrose gradients and disrupted by heating (60°C, 1 min) in the presence of 0.1% Bio-Rad sodium dodecyl sulfate (SDS). These preparations were enzymatically iodinated for 30 min at 20°C with the following mixture: 37 μl of disrupted particles (approximately 0.5 to 1.0 μg of protein), 25 μl of PB, 25 μl of Enzymobeads (Bio-Rad Laboratories; hydrated according to directions), 12 μl of 2% (wt/wt in H_2O) alpha-D-glucose (allowed to mutarotate 18 h, 20°C), and 1 mCi of [^{125}I]sodium iodide (Amersham IMS.300). The reaction was quenched by adding 25 μl of tyrosine (0.4 mg/ml in H_2O), and the Enzymobeads were removed by centrifugation (1,500 × *g*, 10 min, 20°C). The resulting supernatant was combined with an equal volume of twofold-concentrated solubilizing solution (see below) and heated for 3 min at 100°C.

SDS-PAGE. Extracellular NIEPs as well as intracellular A- and B-capsids were concentrated by centrifugation in a Sorvall AH650 rotor (35,000 rpm, 4°C, 2 h) and suspended in 100 μl of solubilizing solution (2% SDS, 50 mM Tris hydrochloride [pH 7.0], 10% beta-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue). Electrophoresis of proteins in SDS-containing, polyacrylamide gels (SDS-PAGE) was done essentially as described by Laemmli (20). Gels were cross-linked with methylenebisacrylamide (bis) or *N,N'*-diallyltartardiamide in ratios to acrylamide of 0.735:28 and 1.09:28, respectively. "High-bis" gels contained an increased ratio of bis to acrylamide (1.09:28) and were used initially to resolve the HCMV basic phosphoprotein from the major capsid protein (16). Bio-Rad SDS (no. 161-0301) was used for the peptide comparison gel (see Fig. 5) to allow the protease to work. Otherwise, Pierce SDS (no. 28365) was used to better separate the Colburn matrix proteins (31). After electrophoresis, gels containing [^{35}S]methionine-labeled proteins were processed for fluorography (3) and exposed to Kodak XAR film at −70°C (21). Detection of iodinated proteins was enhanced with a calcium tungstate intensifying screen (22). Fluorograms were scanned at 540 nm with an EC910 transmission densitometer (E-C Apparatus Corp.). Further details of conditions and procedures used for electrophoresis are presented in the figure legends and elsewhere (8, 16).

Two-dimensional electrophoresis. Solubilized B-capsid preparations were subjected to two-dimensional (charge-size) separation in denaturing polyacrylamide gels as described previously (15). Protein isoelectric points were estimated from measurements made by slicing a blank electrofocusing gel into 1.0-cm sections, submerging each piece in 0.5 ml of distilled water for 1 h at room temperature, and measuring the pH of the resulting solution. In general, the pH gradient extended from 4 to 8.

Peptide comparison. After SDS-PAGE (10% gel; bis cross-linked), the iodinated proteins were located by autoradiography, sliced out of the gel, and subjected to partial proteolysis as described by Cleveland et al. (5) with a range (5, 10, 25, or 50 μg/ml) of *Staphylococcus aureus* V-8 protease (Miles Laboratories). Molecular weights of the resulting peptides were estimated by the method of Weber and Osborn (29) with the following standards (Sigma Chemical Co.): myosin, 205,000; beta-galactosidase, 116,000; phosphorylase *b*, 97,400; bovine albumin, 66,000; egg albumin, 45,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; alpha-lactalbumin, 14,200; and insulin, 11,300.

RESULTS

Our approach was to identify HCMV B-capsids, determine whether they contain a counterpart assembly protein, and, if so, establish whether it is the same as the NIEP 36K species.

Recovery of HCMV A- and B-capsids. Freeze-thaw lysis of infected cell nuclei was used as an alternative to the detergent-DNase procedure (8, 12) for releasing HCMV capsids, since it reproducibly yielded more particles. Rate-velocity centrifugation of the clarified freeze-thaw lysate in 15 to 50% sucrose gradients yielded two light-scattering bands in preparations from AD169-infected cells. Based on the cosedimentation of the two AD169 bands with Colburn A- and B-capsids (Fig. 1; see Fig. 3) and on the similarity of their protein compositions with those of the two Colburn particles (Fig. 2), these AD169 particles have been designated as HCMV A- and B-capsids. Figure 1 also shows that the yield of capsids was lower from AD169-infected cells than from Colburn (see A_{280} profiles, insets) but that the ratio of B- and A-capsids in both preparations was approximately 4. As previously noted (8), treatment of the residual Colburn freeze-thaw pellet material with 1% deoxycholate–DNase (1 mg/ml)–0.5 M urea–1% Brij 58 yielded additional A-capsids but only trace amounts of B-capsids. Parallel treatment of the AD169 freeze-thaw pellet material, however, did not release additional capsids of either type.

To verify that these particles were composed of newly synthesized protein, A- and B-capsids were recovered from [^{35}S]methionine-labeled, infected cells. Peaks of radioactivity coincident with the A- and B-capsid absorbance maxima (Fig. 1), taken together with data in Fig. 2 showing that the radiolabel is present in CMV proteins, established that these particles arise de novo and are not degradation products of the inoculum.

Identification of HCMV assembly protein. The protein composition of the two bands recovered by freeze-thaw lysis of AD169-infected cell nuclei was determined by SDS-PAGE and compared with those of AD169 NIEPs and Colburn A- and B-capsids. Figure 2 shows the protein patterns observed for the two Colburn particles and extends our initial description (8) as follows. First, a previously undetected, low-molecular-weight protein (i.e., a 12K protein) was identified in both A- and B-capsid preparations. This protein migrates at the dye front in the *N,N'*-diallyltartardiamide-cross-linked, 14% polyacrylamide gels used in previous studies

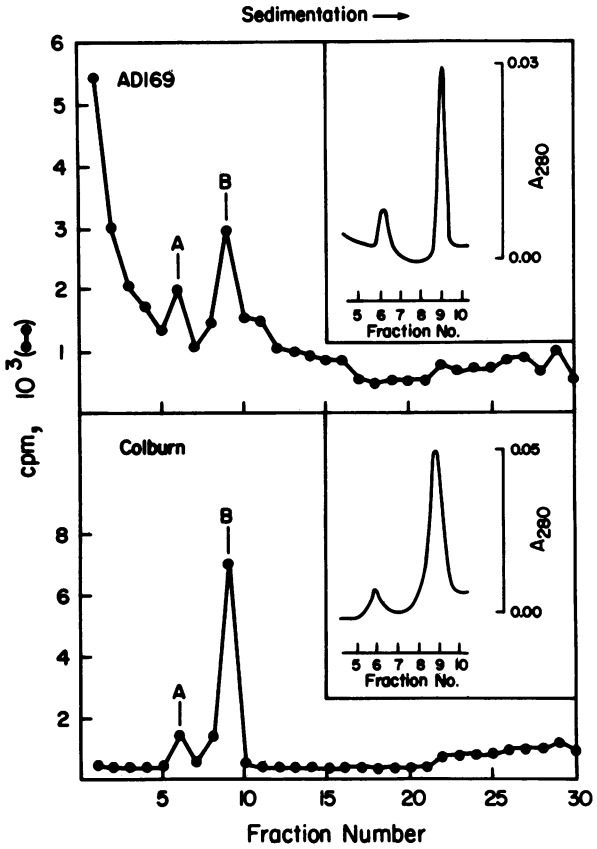

FIG. 1. Separation of CMV capsids by rate-velocity sedimentation. Nuclear lysates prepared from [^{35}S]methionine-labeled cells, infected with strain AD169 (upper panel) or Colburn (lower panel) CMV, were sedimented through 15 to 50% sucrose gradients. The gradients were fractionated and assayed for radioactivity, all as described in Materials and Methods. Shown here are the resulting optical absorbance profiles (only the capsid-containing portion of the gradient is shown in the insets) and the distribution of measured radioactivity in the gradients. Positions of A- and B-capsids are indicated.

and has been designated SCP (smallest capsid protein). Based on its radiolabeling with [^{35}S]methionine, its molar abundance in B-capsids was estimated to be 1.7 and 0.5 relative to the major and minor capsid proteins, respectively. Second, two minor protein bands migrating just slower than the assembly protein (i.e., 38K and 39K proteins, indicated by dots; also see Fig. 3, Colburn B-capsid lane), are better resolved. Third, comparison of Fig. 2 with Fig. 3 and 10 in reference 9 reveals that the relative mobility of the minor capsid protein (mCP) is strongly affected by the amount of contaminating sulfates present in the SDS used for electrophoresis (i.e., migrates slower in Pierce SDS [no. 28365] containing 34.1% tetradecyl and hexadecyl sulfates [17]).

The protein patterns for AD169 A- and B-capsids were similar to those of the Colburn particles. Based on their likeness in size, relative amount, and charge (Fig. 2 and 4), AD169 counterparts to the Colburn major capsid protein (MCP), assembly protein (AP), mCP, 28K, and SCP proteins have been designated (Fig. 2). These HCMV capsid proteins were estimated to have molecular weights of 153,000, 36,000, 34,000, 28,000, and 11,000, respectively. Further, AD169 B-capsids did not contain a recognized counterpart to the Colburn 45K protein, a minor species that is closely related to the 37K assembly protein (8). The increased amounts of higher-molecular-weight proteins in the HCMV preparation are attributed to cosedimenting noncapsid proteins which are distributed throughout the capsid-containing portion of the gradient (e.g., the band designated MP in Fig. 3). A cosedimenting lower-molecular-weight protein (i.e., the band immediately below the AD169 assembly protein in Fig. 3) was not concentrated with the particles when they were "pelleted" (cf. HCMV A-capsids in Fig. 2 [pelleted] and 3 [not pelleted]). Finally, Fig. 2 shows that the AD169 B-capsid assembly protein comigrated with the NIEP 36K protein. Densitometric measurements of these fluorograms indicate the two proteins are present in similar amounts

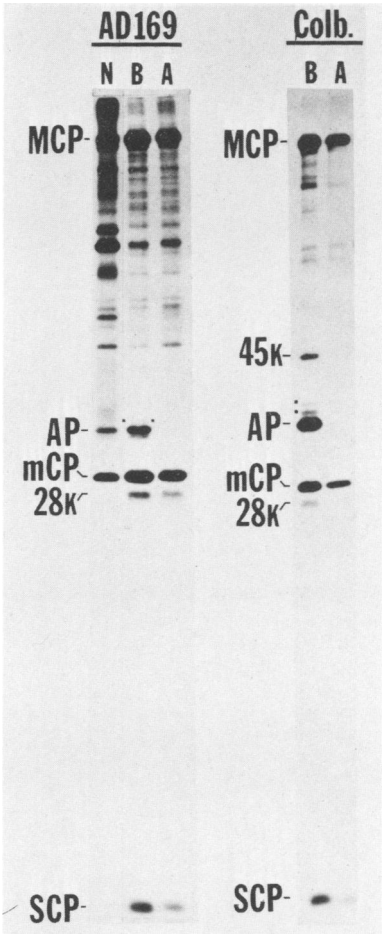

FIG. 2. Protein composition of CMV capsids. [^{35}S]methionine-labeled A-capsids, B-capsids, and NIEPs were recovered from sucrose gradients and solubilized, and the proteins were separated in an 11% high-bis polyacrylamide gel. Shown here are fluorographic exposures of the resulting gel. Lane designations indicate NIEPs (N), B-capsids (B), and A-capsids (A). Protein designations are as follows: major capsid protein (MCP), 45K protein (45K), assembly protein (AP), minor capsid protein (mCP), 28K protein (28K), and smallest capsid protein (SCP). The number of particles in the NIEP (N) lane was approximately one-half that in AD169 capsid lanes (A and B), as reflected by the intensity of mCP bands. The NIEP MCP band appears relatively too intense due to an approximately equal amount of closely migrating basic phosphoprotein (9, 16). Dots to left of panels indicate positions of the AD169 37K and Colburn (Colb.) 38K and 39K proteins.

(relative to the major and minor capsid proteins) in their respective particles.

Two-dimensional separation of B-capsid proteins. HCMV and Colburn B-capsid proteins were next subjected to two-dimensional separations to determine whether the assembly proteins as well as the other capsid counterparts are similar in charge as well as size. Figure 4 shows such comparisons of B-capsids labeled with either [^{35}S]methionine (panels A and B) or ^{125}I (panels C and D). A high degree of similarity is evident in the relative distribution of these counterpart AD169 and Colburn proteins.

This experiment provided the following additional information. First, the assembly protein of each CMV strain was approximately neutral in net charge. Second, and in agreement with the data shown in Fig. 2, among the 45K, 39K, and 38K minor proteins of Colburn B-capsids, only the 38K species has a recognized AD169 counterpart (i.e., the 37K protein; see proteins indicated by dots [Fig. 2] and arrows [Fig. 4, panel C]). Third, compared with the major capsid and assembly proteins, the minor capsid protein was only weakly iodinated (cf. upper and lower panels of Fig. 4). The following two capsid proteins do not appear in this figure: (i) the 28K species, which did not resolve with the electrofocusing conditions used here; and (ii) the smallest capsid protein (11K and 12K proteins), which migrated at the dye front. Other experiments, in which higher concentrations of acrylamide were used in the second-dimension gels, showed that the 12K protein of Colburn is slightly more acidic than the major capsid protein. Thus, as estimated by this technique, the relative net charges of the AD169 and Colburn B-capsid proteins are SCP < MCP < 39K ≤ 45K < 38K < AP < mCP and range from approximately 6.0 to 7.2, respectively. Additional results have demonstrated that the

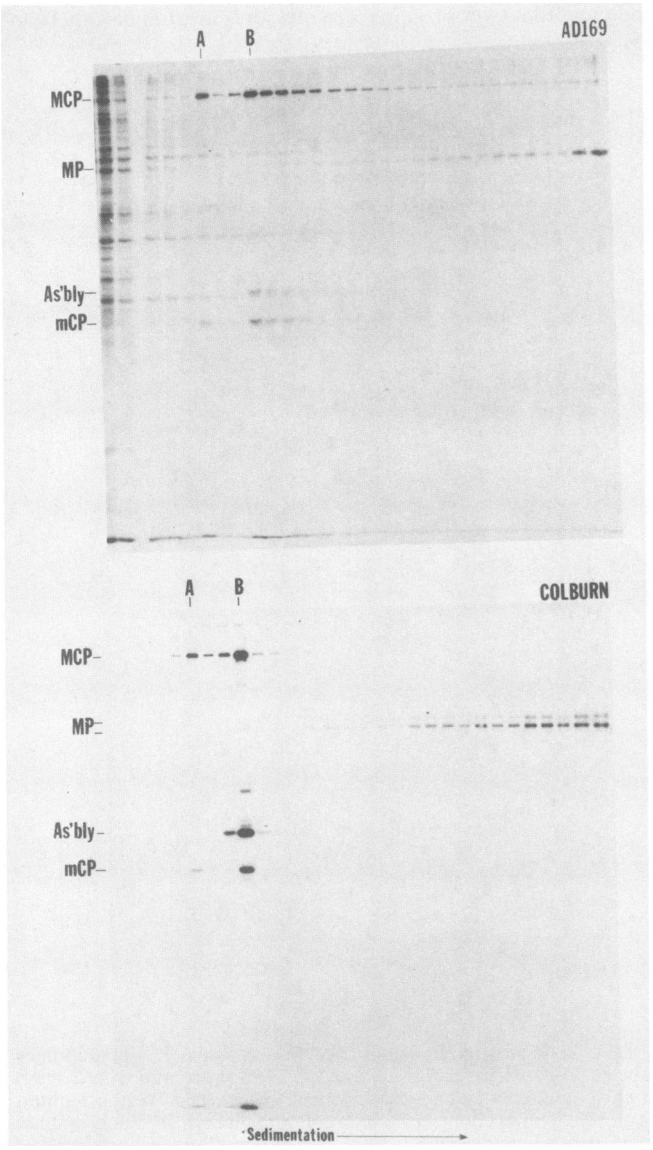

FIG. 3. Proteins in sucrose gradient fractions after separation of CMV capsids by rate-velocity sedimentation. Fractions from the two gradients shown in Fig. 1 were solubilized and subjected to SDS-PAGE through a 10% high-bis polyacrylamide gel. Shown here are fluorograms of the resulting gels. The positions of AD169 (upper panel) and Colburn (lower panel) A- and B-capsids are indicated. Protein designations are as follows: major capsid protein (MCP), matrix protein (MP [69K protein in AD169; 66K and 69K proteins in Colburn]); assembly protein (As'bly); and minor capsid protein (mCP).

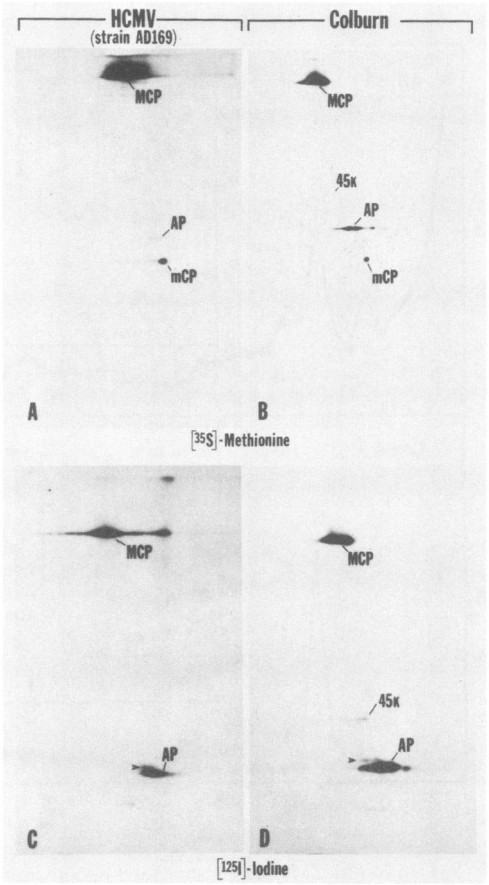

FIG. 4. Two-dimensional separation of CMV B-capsid proteins. AD169 (left panels) and Colburn (right panels) capsids, labeled with [^{35}S]methionine (upper panels) or ^{125}I (lower panels), were subjected to isoelectric focusing from right (basic proteins) to left (acidic proteins). [^{35}S]methionine-labeled proteins were then separated by size in a 10% high-bis gel, and ^{125}I-labeled proteins were separated in a normal 10% polyacrylamide gel. Note the relatively poor iodination of both AD169 and Colburn mCP. Protein designations are as in the legend to Fig. 2. Arrowheads indicate the positions of the AD169 37K and Colburn 38K proteins.

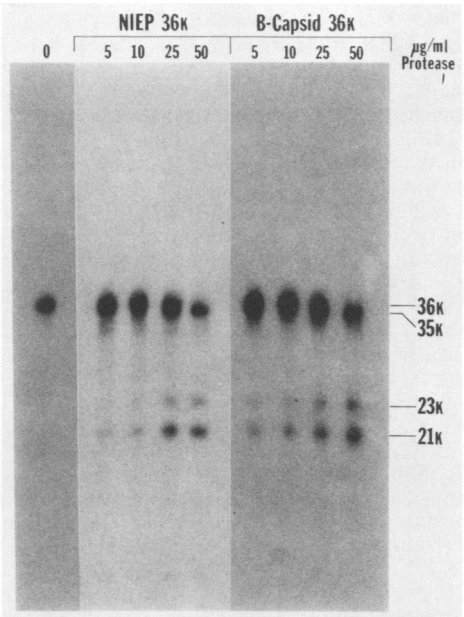

FIG. 5. Comparison of the AD169 NIEP and B-capsid 36K proteins by partial proteolysis. ^{125}I-labeled NIEPs and B-capsids were subjected to SDS-PAGE. The 36K bands were located by fluorography, excised, and subjected to partial proteolysis with increasing amounts of *S. aureus* V-8 protease. A 14% polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide was used to separate the proteolytic fragments, and a fluorogram prepared from the resulting gel is shown here. Molecular weights of the resulting peptides are indicated in the right margin.

AD169 NIEP 36K protein as well as the other NIEP and virion capsid proteins (i.e., MCP and mCP) have the same relative distribution as their B-capsid equivalents after two-dimensional separation in denaturing polyacrylamide gels(9; data not shown).

HCMV assembly protein and NIEP 36K protein are the same. Direct evidence for the relatedness of the assembly protein of HCMV B-capsids and the 36K protein of NIEPs was obtained by comparing the two by partial proteolysis. AD169 NIEPs and B-capsids were disrupted with SDS, iodinated, and subjected to SDS-PAGE. The proteins of interest were located by autoradiography, cut from the gel, rehydrated and subjected to partial proteolysis with *S. aureus* V-8 protease essentially as described by Cleveland et al. (5). Results of this experiment (Fig. 5) demonstrate that the patterns of ^{125}I-radiolabeled peptides were essentially indistinguishable for the NIEP 36K and B-capsid assembly proteins. Three iodinated fragments with molecular weights of 35,000, 23,000, and 21,000 were released in increasing amounts from both 36K proteins as the concentration of protease was increased from 5 to 50 μg/ml.

DISCUSSION

We have recovered nuclear B-capsid assembly intermediates from HCMV-infected cells, identified their assembly protein constituent, and demonstrated that it is the same as the 36K phosphoprotein that distinguishes NIEPs from virions. At the outset, we found that HCMV B-capsids were recovered in greater yield from nuclei ruptured by freezing and thawing rather than by detergent treatment. Even with this modification, however, fewer capsids were obtained from cells infected with HCMV as compared with Colburn (Fig. 1). While we do not know the reason for the lower yield, the broad distribution of HCMV capsid proteins in rate-velocity gradients (Fig. 3) suggests that these particles are more labile or perhaps more tightly associated with the nucleus than those of Colburn.

Next, the HCMV B-capsid assembly protein was identified based on its characteristics shared with the Colburn and HSV counterpart proteins, namely (i) presence in B-capsids but not A-capsids or virions (Fig. 2; unpublished data), (ii) efficiently iodinated in vitro (Fig. 4C and D), and (iii) neutral to slightly acidic in net charge (Fig. 4). Comparisons of HCMV and Colburn B-capsid proteins by one- and two-dimensional separations led to the designation of HCMV counterparts to the Colburn B-capsid proteins (summarized in Table 1), including an abundant, low-molecular-weight species (11K protein) that had not been previously detected. This smallest capsid protein (SCP) is present in all virus particles except dense bodies (Fig. 2; data not shown), which do not contain a capsid, and thus appears to be an integral capsid constituent. A capsid protein of similar size has been reported in HSV-1 and HSV-2 (6, 14). Like the other proteins (i.e., MCP, mCP, and 28K) that form the simplest capsid structure (i.e., the A-capsid), this low-molecular-weight species is not phosphorylated (unpublished data).

Finally, our demonstration that the NIEP 36K protein is the same as the B-capsid assembly protein substantiates our hypothesis that NIEPs are enveloped B-capsids (16) and is of particular interest because of the insight that it provides into the role of this protein. Evidence from previous studies indicates that B-capsids are precursors of virions (7, 18, 23, 25, 26, 28) and that their maturation involves the modification or elimination of the assembly protein from the particle (4, 8, 13, 19, 26, 33, 34). Based on these and other results, it has been suggested that the assembly protein may play a role in capsid assembly, DNA packaging, or envelopment (4, 12, 19), but the stage at which it participates in these processes has not been established. Data presented here bear on this question as follows. First, the presence of the assembly protein in NIEPs, which do not contain DNA, suggests that it becomes a B-capsid component before and independently of DNA packaging. Consistent with this suggestion are our

TABLE 1. Characteristics of primate CMV capsid proteins

Protein counterparts (mol wt [10^3])		Protein designation	Characteristics
AD169	Colburn		
153	145	MCP	Principal capsid constituent; abundant
37	45,39,38		Minor bands related to assembly protein
36	37	AP	Phosphorylated; in B-capsids and HCMV NIEPs; abundant; has precursor (i.e., 40K, Colburn[b])
34	34	mCP	Poorly iodinated; electrophoretic mobility strongly affected by nature of SDS; abundant
28	28	28K	Poorly iodinated
11	12[a]	SCP	Most acidic capsid protein; abundant

[a] The molecular weight estimate for SCP was based on its mobility in 12% high bis and 16% N,N'-diallyltartardiamide cross-linked polyacrylamide gels, in which it migrated slightly faster than cytochrome *c* (12,300 molecular weight).

[b] Results from manuscript in preparation.

observations that (i) B-capsids containing little or no DNA are recovered from CMV-infected cells and (ii) inhibitors of DNA synthesis that eliminate production of DNA-containing virions and C-capsids do not block the synthesis of NIEPs, which lack DNA, or B-capsids (10; manuscript in preparation). Second, the presence of the assembly protein in approximately the same amounts in NIEPs (enveloped) and B-capsids (nonenveloped) demonstrates that its removal or modification is not required for, nor does it necessarily follow, acquisition of the tegument and envelope layers. However, since this alteration of the assembly protein occurs during the maturation of particles which contain DNA (virions) but not of structurally similar particles which lack DNA (NIEPs), it may be a consequence of DNA packaging. Further, since NIEPs lack DNA, it is also apparent that the presence of viral DNA in the capsid, like processing of the assembly protein, is not a requirement for envelopment. In this connection, the reduced amounts of 74K (10 to 20% less) and 69K (30 to 60% less) matrix proteins (tegument components) in NIEPs, compared with virions, may be due to an interaction between those proteins and viral DNA (absent from NIEPs) or to a volumetric exclusion by the assembly protein. Taken together, these observations suggest that the assembly protein (i) becomes a capsid component before DNA packaging and (ii) is modified or removed in conjunction with DNA packaging.

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