Journal of Virology, Sept. 1996, p. 6097–6105 Vol. 70, No. 9
0022-538X/96/$04.00+0
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Proteins Associated with Purified Human Cytomegalovirus Particles

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Received 21 February 1996/Accepted 12 June 1996

Virion-associated proteins isolated from purified human cytomegalovirus particles (strain AD169) were used as substrates for chemical sequence analysis. Extracellular virions, noninfectious enveloped particles, and dense bodies were purified by negative viscosity-positive density gradient centrifugation, and their component proteins were separated by denaturing polyacrylamide gel electrophoresis. The deduced amino acid sequence of individual protein bands was used to identify six corresponding viral genes whose products have not previously been identified as virion constituents: UL47, UL25, UL88, UL85, UL26, and UL48.5. In addition, a 45-kDa cellular protein was identified, and the protein fragments sequenced have a high degree of amino acid identity with actin. However, antiactin monoclonal and polyclonal antibodies did not react with a specific protein in the virus preparations, suggesting that this 45-kDa protein is an immunologically distinct isoform of actin. The newly identified viral and cellular proteins were resistant to protease treatment of purified virions, suggesting that they are unlikely to be contaminants of the viral preparations.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is a structurally complex virus (for a review, see reference 38). The 230-kbp double-stranded linear DNA genome is packaged within a 100-nm-diameter icosahedral capsid which in turn is surrounded by a poorly characterized protein structure known as the tegument or matrix. A lipid envelope containing viral glycoproteins further surrounds the tegument, rendering the diameter of the mature virion 150 to 200 nm. In total, it is estimated that at least 30 proteins, ranging in size from 11 to more than 200 kDa, form the complete infectious particle (12, 13, 27).

In addition to releasing infectious virions, HCMV-infected cells release two types of aberrant particles: noninfectious enveloped particles (NIEPs) and dense bodies (DBs) (22). NIEPs are defective particles that have enveloped an immature form of the capsid, referred to as a B capsid (22, 23). B capsids lack DNA but contain an additional assembly protein encoded by the UL80a gene (45, 62). The assembly protein is absent from mature capsids (23), suggesting that this protein forms a temporary scaffolding platform for nucleocapsid assembly (15, 23, 45). In all other respects, the NIEPs appear to be structurally similar to infectious virions. DBs are enveloped particles that reportedly lack an assembled nucleocapsid and are predominately composed of one tegument phosphoprotein, ppUL83 (pp65 or lower matrix protein) (22, 33). The ratios of the three forms of HCMV particles released from infected cells vary, depending upon the viral strain and the number of infectious cycles in culture (22, 28, 49).

By using capsids purified from the nuclei of HCMV-infected cells, four proteins in addition to the assembly protein have been identified as capsid components (23). The 155-kDa major capsid protein (MCP) has been mapped to open reading frame (ORF) UL86 on the basis of homology to MCP genes of other herpesviruses (3). The gene encoding the 34-kDa minor capsid protein (MCP) has not been definitively assigned but has been proposed to be UL46 by virtue of its positional homology with the UL38 capsid protein gene of herpes simplex virus type 1 (HSV-1) (4). The corresponding genes for the 11-kDa smallest capsid protein (SCP) and the 28-kDa capsid protein have also not been mapped.

Inspection of the sequence of the AD169 strain of HCMV revealed 54 ORFs which encode potential glycoproteins (4). To date, only four virus-encoded envelope glycoproteins have been identified and mapped to specific genes: gpUL55 (gB), gpUL75 (gH), gpUL100 (gM), and gpUL4 (gp48) (for a review, see reference 53). Glycoproteins encoded by UL115 (gL), UL18, US10, and US11 have also been identified and may be incorporated into virions, but evidence localizing these proteins to the envelope does not yet exist.

The remaining 20 to 25 structural proteins are probably localized to the tegument of HCMV virions; however, the functions of nearly all of these proteins remain unknown. While some tegument proteins are almost certainly involved in assembly or egress of the progeny virions, others may have important effects on viral or cellular processes following infection of host cells. For example, the protein product of the UL82 gene (pp71 or upper matrix protein) (47) is a transcriptional activator of promoters with ATF or AP1 binding sites and consequently up-regulates the HCMV immediate-early promoter in cotransfection assays (35). Tegument protein pp65 (47), which is translocated to the nucleus immediately after infection (11, 18, 65), is thought to be a protein kinase (50, 52). However, this gene is nonessential for replication in tissue cultures (50). Along with pp71 and pp65, three other virion tegument proteins have been identified: ppUL32 (pp150), ppUL99 (pp28), and pUL56 (p130) (1, 2, 24, 37). In addition to the known tegument proteins, gene products encoded by the UL98a (pp58), UL48 (p212), and UL65 (pp67) genes have also been shown to be viral structural components (1, 2, 7, 17, 30, 31). Although these proteins likely reside within the tegument, their location within virions has yet to be clearly established. Many of these tegument and putative tegument proteins are phosphorylated by a virion-associated protein kinase (13, 25, 46).

In addition to structural proteins encoded by the virus, a small number of cellular proteins have been shown to associate with HCMV virions. Beta-2-microglobulin has been detected on the surface of HCMV particles in vivo (19, 20, 36) and found to be associated with the tegument in vitro (55, 56). The presence of virus-associated beta-2-microglobulin has been

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proposed to increase infectivity (20, 64). Another cellular protein, annexin II, has been localized to the virion envelope (63). While annexin II may play a role in cell membrane fusion events, the functional significance of its association with HCMV particles is unknown. Finally, complement control proteins CD55 and CD59 have also been detected in purified HCMV preparations (54).

The study of HCMV-encoded proteins has been greatly facilitated by the determination of the genome sequence of the AD169 strain (4). Although the genome contains 208 ORFs, for only a few viral proteins have the products been characterized and the corresponding genes been identified (for reviews, see references 4, 38, and 53). As a first step toward understanding the functions of the viral structural proteins, the genes encoding these proteins must be identified. Here, using amino acid sequence analysis of purified virus-associated proteins, we report the identification and mapping of six HCMV structural proteins that have not been previously described. In addition, a protein of cellular origin which shares some characteristics with members of the actin-related protein family was found (for reviews, see references 21 and 51). Experiments which demonstrate the specificity of the associations of these proteins with purified viral particles and argue against copurification contaminants are also presented.

MATERIALS AND METHODS

Cells and virus. Primary human foreskin fibroblasts (HFF) were passaged as monolayers in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.). A plaque-purified AD169 strain of HCMV was used. Viral titers were measured by plaque assays on HFF as follows. Cell monolayers were infected with 10-fold serial dilutions of virus stock. Following a 1-h adsorption period, the cells were overlaid with medium containing 1% low-melting-point agarose (Life Technologies). Approximately 21 days postinfection, the cells were fixed with 10% formalin–phosphate-buffered saline and stained with 0.03% methylene blue (Sigma Chemical Co.).

Extracellular HCMV particle purification. Rate-velocity centrifugation used for particle purification was performed essentially as previously described (22, 57) with some modifications. HFF (approximately 80% confluent) were infected with HCMV AD169 stocks at a multiplicity of infection of 1 to 5. Typically, 10 15-cm-diameter dishes were used for each preparation. Following a 1-h adsorption, Dulbecco's modified minimal essential medium containing 5% fetal calf serum was added and the infected cells incubated at 37°C for 8 to 10 days. The medium was then collected and cleared of cells and cell debris by centrifugation at 12,000 × *g* for 10 min at 4°C. Viral particles were concentrated with a stirred-cell concentrator (model 8200; Amicon, Inc.) with a filtration membrane with a cutoff molecular mass of 100 kDa. The concentrated medium was then layered onto 9-ml glycerol-tartrate gradients formed in TN (50 mM Tris [pH 7.4], 100 mM NaCl) and centrifuged at 40,000 rpm for 15 min at 4°C in a Beckman SW41 rotor with slow acceleration and braking. NIEPs, virions, and DBs were visualized with incandescent light and were removed from the gradients. In one experiment, virions isolated in this manner were further purified by an 18-h equilibrium banding step on the same type of glycerol-tartrate gradients. Particles extracted from the gradients were then diluted two- to threefold with TN and centrifuged at 55,000 × *g* for 1 h. The pelleted particles were resuspended in 5 μl of TN per 15-cm-diameter plate and stored at −80°C.

Protein gel electrophoresis. Proteins were solubilized by the addition of an equal volume of 2× sample buffer (4% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol, 100 mM Tris [pH 7.0], 10% glycerol, 0.005% bromophenol blue), heated to 90°C for 3 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (29). The majority of the viral proteins could be resolved on standard 10% polyacrylamide gels (acrylamide–methylene-bisacrylamide, 37.5:1). High-bis 10% polyacrylamide gels (acrylamide–methylene-bisacrylamide, 28:1) were used to separate the MCP from pp150 as described previously (22). Gradient gels of 10 to 20% polyacrylamide (Bio-Rad Laboratories) were used to resolve low-molecular-weight viral proteins. Gels were fixed, stained with Coomassie brilliant blue R, and dried between cellophane sheets. Broad-range SDS-PAGE markers (New England Biolabs) were used as standards.

Protein sequencing. Gradient-purified HCMV particles were separated on polyacrylamide gels as described above and transferred to ProBlott membranes (Applied Biosystems) in protein sequence transfer buffer (12 mM Tris [pH 8.3], 96 mM glycine, 10% methanol). The membranes were then stained with 0.1% amido black (Bio-Rad)–40% methanol–1% acetic acid for 1 min and destained with several changes of H_2O. Bands of interest were excised and submitted for analysis. Protein quantification, tryptic digestion, peptide separation, and sequencing were performed by the Harvard Microchemistry Facility (Cambridge, Mass.). The University of Wisconsin Genetics Computer Group package was used for computer-based DNA-protein library searches.

Western blot (immunoblot) analysis. Proteins were solubilized and separated by 10% SDS-PAGE as described above and transferred to Hybond-ECL nitrocellulose (Amersham Corp.) in Tris-glycine transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 20% methanol, 0.1% SDS). The membranes were blocked in 10% dry milk–TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 1 h. Two different antiactin antibodies, diluted in 1% dry milk–TBST, were employed: (i) the mouse monoclonal antibody C4 (Boehringer Mannheim Corp.) recognizes an epitope in the amino-terminal portion of actin and (ii) the rabbit polyclonal antibody A-2066 (Sigma Chemical Co.) recognizes the highly conserved carboxy-terminal 11 amino acids of actin. Both antibodies recognize the α-, β-, and γ-actin isoforms from a wide variety of species. Anti-mouse or anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham Corp.) was used as the secondary antibody. The ECL system (Amersham Corp.) followed by autoradiography was used to detect antibody-antigen complexes.

Protease assays. Gradient-purified virions (18 μg of total protein) were treated with trypsin (90 μg/ml) or alkaline protease (36 μg/ml) (Promega Corp., Madison, Wis.) in 10 μl of TN supplemented with either 1 mM (trypsin) or 5 mM (alkaline protease) $CaCl_2$. Although this resulted in the maximum ratio of trypsin to protein (1:20) or alkaline protease to protein (1:50) recommended by the manufacturer, we obtained similar results using 10-fold less protease (data not shown). Following a 1-h incubation at 37°C, trypsin digestions were terminated by adding phenylmethylsulfonyl fluoride and soybean trypsin inhibitor to 0.5 mM and 0.5 mg/ml, respectively. Alkaline protease digestions were terminated by addition of phenylmethylsulfonyl fluoride and aprotinin to 0.5 mM and 5 μg/ml, respectively. In some experiments, Triton X-100 was also added to a final concentration of 1% to remove the viral envelope and expose the tegument-nucleocapsid to the proteases (66). In some experiments, 1 μg of purified bovine muscle actin (Sigma Chemical Co.) was incubated with or without virions for 15 min at room temperature before protease digestion. The products were analyzed by SDS-PAGE followed by Coomassie brilliant blue R staining, as described above.

Glycoprotein detection. Exposed carbohydrate residues on purified virions (18 μg of total protein), purified transferrin (1 μg), or purified bovine muscle actin (1 μg) was labeled in solution with biotin with a glycoprotein detection system (Amersham Corp.). The protease sensitivity of the labeled glycoproteins on the virions was determined by trypsin digestion as described above. The products were then solubilized and separated on an SDS–10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with streptavidin conjugated to horseradish peroxidase, and the glycoproteins were visualized with the ECL system as described by the manufacturer.

RESULTS

Purification of HCMV particles released from infected cells. Following the infection of permissive cells with the AD169 strain of HCMV, three forms of viral particles are released into the medium: NIEPs, virions, and DBs. In order to analyze and compare the protein constituents of these particles, they were separated and purified via negative viscosity-positive density glycerol-tartrate gradient centrifugation (22, 57). This process facilitates both the isolation of viral particle types and the removal of any contaminating host cell proteins.

An SDS-PAGE (10% polyacrylamide) analysis of aliquots of the purified particles (Fig. 1A) revealed protein compositions similar to those described previously (10, 12, 22, 25, 26). Comparison of our data with data from these reports allowed us to tentatively identify several of the protein bands, and experiments described below led to positive identifications. NIEPs contained the 39-kDa assembly protein which was absent or whose levels were reduced in virion preparations (Fig. 1A; compare lanes 1 and 2) (23). However, all other visible bands appeared to be present in virions and NIEPs in approximately equal proportions. DB preparations (lane 3) were predominately composed of pp65 as reported previously (22, 33), although many other bands seen in NIEPs and virions were also present in DBs in various amounts. Equivalent fractions of the recovered particles were applied to each lane, indicating that NIEPs, virions, and DBs were released from HCMV-infected HFF in nearly equal proportions. Other types of gels were employed to visualize proteins not seen or resolved on standard polyacrylamide gels. By increasing the concentration of

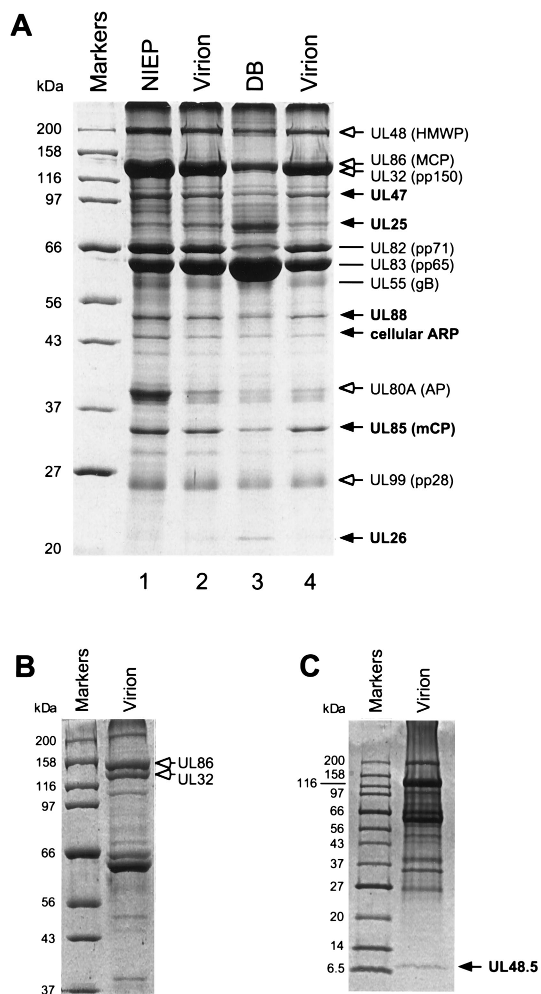

FIG. 1. Protein composition of gradient-purified HCMV particles. Extracellular particles were purified by rate-velocity glycerol-tartrate gradient centrifugation and solubilized, and the individual proteins were separated on SDS-polyacrylamide gels. The gels were fixed and stained with Coomassie brilliant blue R. (A) NIEP (lane 1), virion (lane 2), and DB (lane 3) proteins separated on a 10% polyacrylamide gel. The virion preparation used in lane 4 was subjected to an additional equilibrium centrifugation step on a second glycerol-tartrate gradient. (B) Virion proteins separated on a 10% high-bis gel. (C) Virion proteins separated on a 10-to-20% gradient polyacrylamide gel. Proteins submitted for sequence analysis are indicated by arrows at the right of the gel. Arrows with filled heads indicate newly identified viral particle-associated proteins. Arrows with open heads indicate previously identified viral particle-associated proteins. Labels lacking arrowheads indicate proteins that were not sequenced but whose identity was confirmed by immunological methods. The corresponding HCMV ORF and common designations are also shown. Sizes of the molecular mass markers are shown at the left. HMWP, high-molecular-weight protein; ARP, actin-related protein; AP, assembly protein.

bisacrylamide to cross-link the gel (high-bis gel) (22), the 155-kDa MCP and pp150 protein, which normally comigrate, could be resolved (Fig. 1B). In addition, a 10-to-20% gradient gel was used to identify low-molecular-mass proteins migrating between 20 and 6 kDa. Only one protein of approximately 8 kDa was visible (Fig. 1C).

In order to determine if any potential contaminating host cell proteins could be removed by further purification, virions isolated from an initial rate-velocity centrifugation step were applied to a second glycerol-tartrate gradient. This centrifugation step was extended to 18 h to allow the virions to band at their isodense position in the gradient. An aliquot of this material (Fig. 1A, lane 4) appeared to have a protein pattern identical to that of material that had undergone only the single centrifugation step. We concluded that the additional equilibrium banding step resulted in no further purification and therefore was not included in the production of material used in subsequent protein sequence analyses. In addition, electron microscopy of negatively stained virion preparations (data not shown) showed a fairly homogeneous mixture of particles of the size and shape expected for HCMV virions (10, 22, 57). Some of the capsids were joined by what appeared to be a continuous membrane; however, no gross contamination by cell debris was evident.

Sequence analysis of proteins associated with purified HCMV particles. Less than half of the proteins suspected of constituting HCMV virions have been mapped to specific ORFs on the HCMV genome (53), with the majority of the uncharacterized proteins probably residing within the tegument. Polyacrylamide gels of the types shown in Fig. 1 indicated that many of these protein bands were well resolved and in sufficient abundance to be identified by protein sequence analysis. Three of the most-studied structural proteins are the tegument proteins pp71 and pp65 and the envelope glycoprotein gB. Western blot analysis and immunoprecipitation using monoclonal antibodies against these proteins (data not shown) allowed us to confirm the identity of these bands in our NIEP, virion, and DB preparations, and these bands were therefore not sequenced (Fig. 1A). Of the remaining bands on the polyacrylamide gels, 13 were candidates for sequence analysis. Because the protein profiles of the NIEPs and virions were visually identical (except for the presence of the assembly protein in the NIEP preparations), these two gradient fractions were pooled for the purpose of obtaining sufficient quantities of protein for sequencing. In two cases, DB preparations were utilized to obtain sequence information on proteins that seemed to be more abundant in this type of particle.

Purified HCMV particles were solubilized and run on preparative gels similar to those shown in Fig. 1, with the type of gel being determined by the protein of interest. The proteins were then transferred to membranes and stained with amido black. Sections of the membranes which contained the bands of interest were excised and submitted for tryptic digestion. Selected tryptic fragments were purified and subjected to N-terminal sequencing. Several HCMV structural proteins described by others can be recognized by their characteristic sizes in denaturing polyacrylamide gels (22, 23, 46). Nevertheless, we attempted to sequence each protein (except those identified by immunological methods described above) for which sequencing was feasible. In those cases in which the protein's identity was suspected, only one proteolytic fragment was sequenced for confirmation. In other cases, however, at least two tryptic fragments were sequenced to positively identify the corresponding viral gene. The results (Table 1) allowed us to assign these proteins to the HCMV ORFs as indicated to the right of the gels in Fig. 1. In one case (Table 2), the protein did not correspond to any ORF in the HCMV AD169 genome.

On the basis of previous studies, the proteins migrating at 212, 155, 150, 39, and 26 kDa were suspected to be encoded by the UL48, UL86, UL32, UL80a, and UL99 ORFs, respectively (2, 3, 22, 24, 25, 37, 45, 46). Sequence analysis (Table 1) confirmed their identities. Of the remaining protein bands sequenced, six were found to be encoded by HCMV genes that had not previously been shown to produce a protein product: UL47, UL25, UL88, UL85, UL26, and UL48.5. The newly identified protein product of UL47 (predicted molecular mass, 109,962 Da) appeared to be present in all three types of HCMV particles in approximately equal quantities (Fig. 1A).

TABLE 1. Comparison of N-terminal sequences of HCMV-associated protein tryptic fragments and predicted HCMV protein sequences

Fragment source[a]	Predicted ORF[b]	Sequence obtained[c]	Corresponding ORF	Corresponding sequence[d]	Residues
V+N	UL48 (1, 2)	MQ-VFDPYGR	UL48	MQVVFDPYGR	482–491
V+N	UL48 (1, 2)	LGVGPQVTELYER	UL48	LGVGPQVTELYER	927–939
V+N	UL48 (1, 2)	ITPP-ADFQQPVFK	UL48	ITPPPADFQQPVFK	1224–1237
V+N	UL86 (3, 22)	LRPPPDYEET	UL86	LRPPPDYEET	584–593
V+N	UL32 (22, 24)	VALVNFLR	UL32	VALVNFLR	14–21
V+N	?[e]	LLQTLREI	UL47	LLQTLREI	127–134
V+N	?	LLSEEVSPRA	UL47	LLSEEVSPRr	337–346
V+N	?	SATEDLDRMEAGL(L)P	UL25	SATEDLDRMEAGLsP	46–60
V+N	?	RQPQIAAGAPRGSPAT	UL25	RQPQIAAGAPRGSPAT	96–111
DB	?	GVTDG	UL25	GVTDG	323–327
V+N	?	ALVNTFR	UL88	ALVNTFR	189–195
V+N	?	QYFFLLER	UL88	QYFFLLER	196–203
V+N	UL80a (45, 62)	RYEPAPSLHPSYPVPP	UL80A	RYEPAPSLHPSYPVPP	112–127
V+N	UL46 (4)	LSIADV	UL85	LSIADV	16–21
V+N	UL46 (4)	APRQHVSPDDE-AR	UL85	APRQHVSPDDEIAR	254–267
V+N	UL99 (37)	KPAASLPS	UL99	KPAASLPf	183–190
DB	?	GRHLDLPYPR	UL26	GRHLDLPYPR	22–31
DB	?	LLVVTQGQLR	UL26	LLVVTQGQLR	55–64
DB	?	GLLHSYFEDVER	UL26	GLLHSYFEDVER	115–126
V+N	?	MSSLFNDK	UL48.5	MSSLFNDK	50–57
V+N	?	LDLLR	UL48.5	LDLLR	62–66

[a] V+N, virions plus NIEPs.
[b] Based on the reference(s) indicated in parentheses.
[c] -, not determined; (L), tentative assignment.
[d] Protein sequences predicted from HCMV AD169 DNA sequence data (GenEMBL accession number, X17403). Lowercase type indicates deviation from the sequence obtained.
[e] ?, no HCMV ORF encoding a structural protein of the observed molecular mass had been previously described.

In contrast, pUL25 (predicted molecular mass, 73,541 Da) appeared to be much more abundant in DB preparations than in NIEPs or virions. The comigrating band in DB preparations was also sequenced and was confirmed to be pUL25. Another protein identified in this study, pUL88 (predicted molecular mass, 47,691 Da) was found to be present in each particle type in similar quantities. The sequence of the 35-kDa band revealed that it was produced from the UL85 gene (predicted molecular mass, 34,596 Da). This protein was also present in all three forms of particles, but in lesser amounts in the DB preparations. We suspected that UL85 encodes the 34-kDa capsid protein described by Irmiere and Gibson (23). Indeed, anti-mCP antiserum (a gift from W. Gibson) reacted with a protein of the same size on immunoblots of virion preparations (data not shown). Therefore, pUL85, rather than pUL46 as has previously been assumed (4), is probably the true mCP. One protein of approximately 21 kDa was found in amounts sufficient for sequencing only in the DBs. The corresponding gene for this protein was UL26 (predicted molecular mass, 21,156 Da). Although not readily visible in electrophoretic profiles of

TABLE 2. Comparison of N-terminal sequences of HCMV-associated actin-related protein tryptic fragments and human β- and γ-actin sequences

Fragment sequence obtained[a]	Sequence of:		Residues[e]
	Human β-actin[b,c]	Human γ-actin[c,d]	
AGFAGDDAPR	AGFAGDDAPR	AGFAGDDAPR	19–28
VMV-MTQL	VMVGMgQk	VMVGMgQk	42–49
VAPEEHPVLLTEAPLNPK	VAPEEHPVLLTEAPLNPK	VAPEEHPVLLTEAPLNPK	96–113
TGGIVMDSGDGVT--VPIY---AL	TtGIVMDSGDGGVTHTVPIYEGYAL	TtGIVMDSGDGGVTHTVPIYEGYAL	148–171
DEYDESGPGIV	qEYDESGPsIV	qEYDESGPsIV	360–370

[a] -, not determined.
[b] Human cytoplasmic β-actin (GenEMBL accession number, M10277).
[c] Lowercase type indicates deviation from sequence obtained.
[d] Human cytoplasmic γ-actin (GenEMBL accession number, M19283).
[e] Residue numbers correspond to γ-actin sequence.

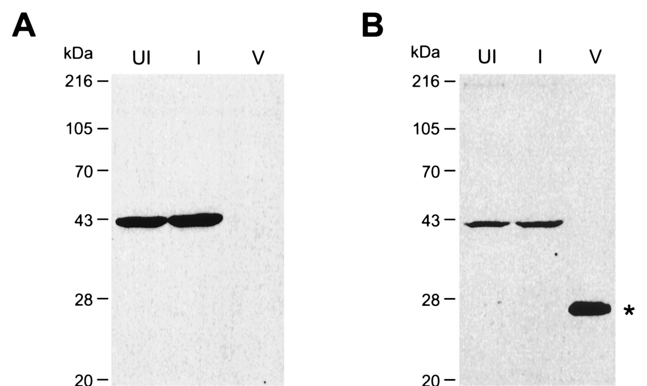

FIG. 2. Immunoblot analysis of proteins from HFF and gradient-purified HCMV virions. Total proteins from uninfected cells (UI), HCMV-infected cells (I), or purified virions (V) were separated on an SDS–10% polyacrylamide gel, transferred to nitrocellulose membranes, and incubated with an antiactin monoclonal (A) or a polyclonal (B) antibody. The positions of the prestained molecular mass markers are indicated at the left of each gel. The asterisk (B) marks a cross-reacting protein recognized by the polyclonal antibody.

proteins from NIEPs or virions, its presence has not been ruled out. The only visible protein smaller than 20 kDa migrated with an apparent molecular mass of 8 kDa (Fig. 1C). This protein, which likely represents the SCP (23), was mapped to a previously unrecognized 225-bp ORF (predicted molecular mass, 8,463 Da) located between UL48 and UL49. This ORF begins with an ATG initiation codon at position 70,407 and reads from right to left on the bottom strand of the prototype orientation of the AD169 genome (4). Because of its position on the HCMV genomic map, we have designated the ORF UL48.5. The assignment of the SCP to UL48.5 was recently established in a concomitant study by Gibson et al. (14), who have labeled the ORF UL48/49.

The protein band which was not derived from an HCMV gene migrated at approximately 45 kDa in preparations of NIEPs, virions, and DBs (Fig. 1A) and was consistently present in all preparations of HCMV particles. A total of five tryptic peptides derived from this protein band were sequenced (Table 2). Database searches revealed that the fragments showed almost complete amino acid identity to conventional α-, β-, and γ-actins from various species. In addition, the peptides sequenced corresponded to regions of the human γ-actin sequence from residue 19 to 370 of the 375-amino-acid protein. However, there were five nonconservative amino acid substitutions when the corresponding regions of the two nonmuscle cytoplasmic β- and γ-actins were compared with the sequences obtained from the 45-kDa protein. This was surprising, as the amino acid sequence among different actin isoforms is very highly conserved. Some of these differences were at amino acid residues of actin in which the sequence does not vary between the different isoforms or between species. A tryptic map of the 45-kDa band isolated from another virion preparation was identical to that of the first, and sequence analysis of a duplicate fragment gave the same sequencing results (data not shown). The presence of this band is therefore not likely to be a consequence of variable virus purity or a sequencing artifact.

We next attempted to detect the presence of cellular actin in our virus preparations by immunoblot analysis using antiactin monoclonal or polyclonal antibodies. The antibodies chosen recognize conserved epitopes on either the amino- or carboxy-terminal portions of all actin isoforms. Each antibody recognized a 42-kDa actin band in both uninfected or HCMV-infected HFF (Fig. 2); however, neither antibody reacted with a band of this size in a purified mixture of virions and NIEPs. Together, these results suggest that the protein associated with HCMV particles is an unconventional form of actin which deviates in amino acid sequence from prototype actins and is not recognized by two different antiactin antibodies. We suspect that the protein could be a member of the actin-related protein family (21, 51).

Protease sensitivity of HCMV virion-associated proteins. To further demonstrate that the newly identified proteins were unlikely to be copurification contaminants of the HCMV particle preparations, we monitored the total protein composition of the virions following protease treatments. Other studies have shown that exposed herpesvirus envelope glycoproteins are sensitive to proteases, while internal capsid and tegument proteins are protected in the intact virions (66). It would be assumed that any contaminating proteins that nonspecifically copurify with the HCMV preparations, as well as any viral envelope glycoproteins, would also be exposed and thus degraded by protease treatment.

Purified virions were mixed with either trypsin or alkaline protease in order to degrade any proteins exposed on the surfaces of the virions. The sensitivity of all virion-associated proteins was examined by treating a duplicate sample with the detergent Triton X-100 in order to remove the envelope and expose the capsid and tegument proteins. Following a 1-h incubation at 37°C, the products were analyzed by SDS-PAGE (Fig. 3A). While protease treatment of virions with intact envelopes produced some novel proteolytic fragments (presumably products of viral envelope glycoproteins), the virus-associated proteins pUL47, pUL25, pUL88, and pUL85 and the actin-related protein identified in this study did not appear to be sensitive to the proteases. To further demonstrate the protease sensitivity of exposed virus-associated proteins in our assay, carbohydrate residues of intact virions were labeled with biotin (Fig. 3B). Following SDS-PAGE, glycoproteins with molecular masses of approximately 50 to 60 kDa and 100 kDa were detected. The size of these bands correlates with the sizes of known HCMV envelope glycoproteins (53). However, following digestion with trypsin these glycoproteins were no longer visible, indicating that viral envelope glycoproteins of intact particles are protease sensitive. The carbohydrate specificity of biotinylation was confirmed in separate reactions by labeling of the 81-kDa glycoprotein transferrin, while the nonglycosylated bovine muscle actin was not labeled. Although treatment with the detergent (Fig. 3A) rendered all or nearly all of the viral proteins sensitive to the proteases, the interpretation with respect to some specific protein bands is complicated by the position of the proteolytic fragments generated. For example, pUL47, pUL88, and the actin-related protein appear to be protease sensitive in the presence of detergent. In contrast, potential proteolytic fragments comigrating with pUL25, pUL85, and pUL48.5 make their sensitivities unclear. However, the UL85 and UL48.5 gene products are likely to be capsid proteins; as such, they are bona fide virion constituents. Because pUL26 was only visible in DB preparations and not in infectious virions, the localization of this protein was not examined further.

Although it is reasonable to assume that any virion-associated proteins of viral origin are likely to be specifically packaged in the viral particles, it is possible that host cell proteins found as components of purified virions could be nonspecifically associated. Consequently, we considered the possibility that the 45-kDa actin-related protein could have become nonspecifically associated with the extracellular HCMV particles during the host cell destruction. The actin-related protein is not degraded by proteases in intact virions (Fig. 3A). However,

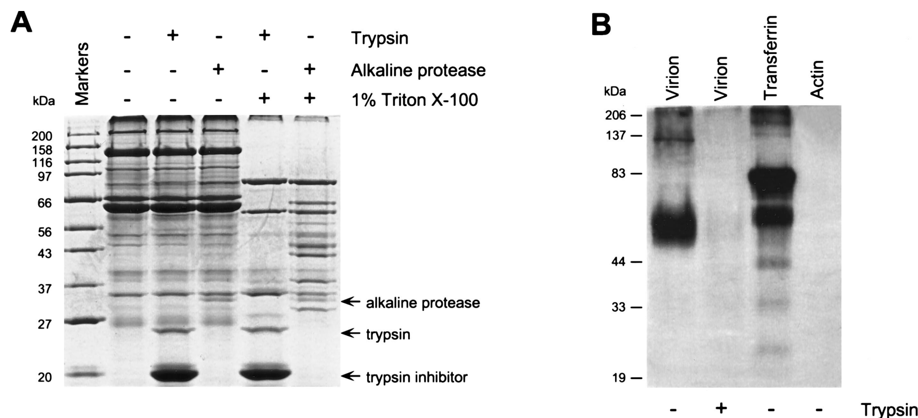

FIG. 3. Protease sensitivity of gradient-purified HCMV virions. (A) Purified virions were treated as indicated with trypsin or alkaline protease, either in the presence (+) or absence (−) of Triton X-100. Following a 1-h incubation, the reactions were stopped and the products were separated on an SDS–10% polyacrylamide gel. The positions of the added alkaline protease, trypsin, and soybean trypsin inhibitor are indicated at the right. (B) Purified virions, transferrin, or actin was incubated with a carbohydrate-specific labeling reagent and subsequently treated as indicated with trypsin. Following a 1-h incubation, the reactions were stopped and the products were separated on an SDS–10% polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane, and the glycoproteins were visualized by chemiluminescence and autoradiography. For both panels, sizes of the prestained molecular mass markers are indicated at the left.

this could hypothetically be caused by inhibitors in the virion preparations or a conformational phenomenon that causes actin to fold into a form in which trypsin cleavage sites are not exposed. To test these possibilities, bovine muscle actin was added to purified virions and the mixture was incubated with trypsin as described above. The exogenous actin, whether alone or in the presence of virions, was completely degraded by the trypsin, while the virion-associated actin-related protein remained intact (Fig. 4). Therefore, if the actin-related protein was exposed on the envelope surface, it likely would have been degraded by tryptic digestion. Glycosidase treatment of the virions, to remove carbohydrate residues from exposed glycoproteins, did not increase the sensitivity of the actin-related protein to proteases (data not shown). We conclude that the actin-related protein is specifically associated with the virion particles, probably residing internal to the envelope.

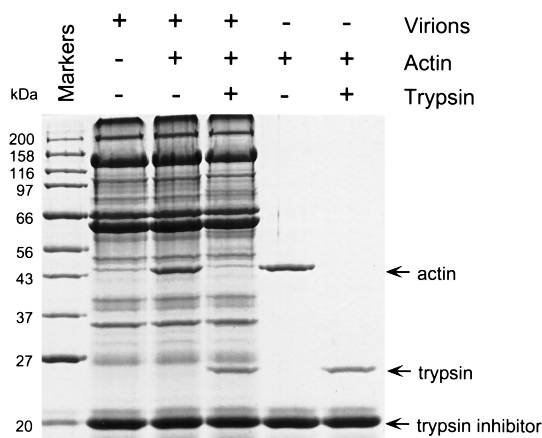

FIG. 4. Trypsin sensitivity of the virion-associated actin-related protein and exogenous purified actin. Gradient-purified virions and purified bovine muscle actin were treated with trypsin as indicated. In lanes in which actin and virions were mixed, the samples were incubated for 15 min at room temperature before trypsin addition. The positions of the added actin, trypsin, and soybean trypsin inhibitor are indicated to the right. Sizes of the molecular mass markers are shown at the left.

DISCUSSION

In this study, we have used protein sequencing to identify six new HCMV-encoded proteins that are associated with purified viral particles: pUL47, pUL25, pUL88, pUL85, pUL26, and pUL48.5. In addition, a 45-kDa cellular protein with a high degree of amino acid sequence homology to actin was found. For two reasons, we believe that these proteins are specifically and internally associated with HCMV. (i) The HCMV particles were rigorously purified. Extracellular NIEPs, virions, and DBs recovered from infected HFF were purified by glycerol-tartrate centrifugation, a separation process based on both size and density differences between particles (57) that would be expected to substantially separate viral particles from host cell debris. Virions recovered from these single rate-velocity gradients appeared identical in protein composition, as determined by SDS-PAGE, to virions subjected to an additional equilibrium centrifugation step (Fig. 1). Any contaminating proteins which could adhere to the particles, via nonspecific ionic interactions, prior to their application to the glycerol-tartrate gradient would be unlikely to remain attached in the 0.66 to 1.55 M potassium tartrate encountered during the centrifugation. (ii) The newly identified proteins appear to be insensitive to protease treatment of intact virions and therefore are not exposed (Fig. 3 and 4). Although pUL85 and pUL48.5 are likely capsid components, the exact localization of the remaining proteins within the virion must await the production of specific antibodies. However, the lack of putative glycosylation sites and transmembrane anchor sequences in these viral proteins suggests that they do not reside in the envelope (4).

SWISS-PROT protein database searches performed with the six newly identified HCMV-encoded virion proteins revealed statistically significant similarities with only proteins encoded by other members of the herpesvirus family (Table 3). The greatest degree of amino acid similarity was found between HCMV and another human betaherpesvirus, human herpesvirus 6 (HHV-6). HCMV pUL47 has a 23.8% amino acid identity to the predicted protein product of the HHV-6 U30 ORF. Furthermore, these ORFs are positionally conserved between the two viruses. The predicted protein product of the HHV-6 U14 gene is the homolog of HCMV pUL25, with a 24.1% amino acid identity. However, the location of

TABLE 3. Comparisons between newly identified HCMV proteins and proteins or predicted proteins of HHV-6 and HSV-1[a]

HCMV ORF[b]	HHV-6[c]			HSV-1[d]			Positional conservation[e]
	ORF[b]	Residues	% Identity	ORF[b]	Residues	% Identity	
UL47 (982)	U30 (1,082)	148–1001	23.8	—	—	—	+
UL25 (656)	U14 (609)	19–297	24.1	UL9 (851)	95–204	21.4	HHV-6, −; HSV-1, −
UL88 (429)	U59 (350)	9–99	36.4	—	—	—	+
UL85 (306)	U56 (296)	4–296	43.0	UL18 (318)	16–285	22.5	HHV-6, +; HSV-1, +
UL48.5 (75)	U32 (88)	9–80	36.5	UL35 (112)	—	—	HHV-6, +; HSV-1, +

[a] For ORF UL26 (188 amino acids [based on the size of the predicted translation product]), no HHV-6 or HSV-1 ORFs were identified.
[b] Values in parentheses are numbers of amino acids (based on the sizes of the predicted translation products).
[c] GenEMBL accession number for HHV-6, X83413.
[d] GenEMBL accession number for HSV-1, X141112. —, no homolog identified.
[e] Positional conservation was (+) or was not (−) demonstrated.

these ORFs within their respective genomes is not conserved between the two viruses. HCMV pUL25 also has a short region (residues 289 to 399) which shares a 21.4% amino acid sequence identity with HSV-1 UL9 gene product. However, the lack of more extensive amino acid homology and ORF positional conservation between HCMV UL25 and HSV-1 UL9 makes additional comparisons questionable. In the HCMV genome, the UL25 and UL35 genes have a high degree of DNA sequence homology, and together they constitute the UL25 family (4). The similarity also extends to the amino acid level, with a 19.7% identity over the length of the proteins (data not shown). The murine cytomegalovirus homolog of UL25 has been shown to encode a viral structural protein which probably resides within the tegument (5). HCMV pUL88 is homologous to the predicted protein product of the positionally conserved HHV-6 ORF U59, with a 36.4% amino acid identity. The protein encoded by the HCMV UL85 gene has significant homologies to both the HHV-6 U56 and the HSV-1 UL18 gene products (43.0 and 22.5% amino acid identity, respectively, over the length of the proteins). In HSV-1, the UL18 gene product (VP23) has been shown to be a capsid protein (43) which is essential for capsid formation (9, 58). The HCMV pUL85 is also likely to be a capsid protein and is probably the mCP (23) previously thought to be encoded by UL46, the positional homolog of the HSV-1 UL38 capsid protein gene (4). In addition, HCMV UL85 is positionally conserved in HHV-6 and HSV-1 with respect to the MCP genes in each virus. HCMV pUL26 has no identifiable homologs in HHV-6 or HSV-1. Finally, the smallest HCMV-encoded structural protein identified in this study, pUL48.5, has a 36.5% amino acid identity to the predicted product of the HHV-6 U32 ORF. In HSV-1, UL35 (VP26) is the positional homolog of HCMV UL48.5, although similarity at the amino acid level is limited. The 12-kDa HSV-1 protein has been shown to be the smallest constituent of mature capsids (8); however, it is not required for the formation of basic capsid structures (58). Gibson et al. (14) have also identified HCMV UL48.5 as the SCP gene.

The protein composition of our DB preparations (Fig. 1) indicates a greater degree of polypeptide complexity than has been described by others (22, 32, 33). Of particular surprise is the presence of the MCP and mCP (pUL85), since DBs reportedly lack a nucleocapsid (22, 49). It is possible that the DB fraction taken from the glycerol-tartrate gradients was contaminated with aggregated virions, although electron micrographs of purified virions did not reveal gross contamination with DBs (data not shown). On the basis of the relative ratios of many of the other virion proteins visible in Fig. 1, however, the degree of contamination would have to be significant. Alternatively, a fraction of the DB particles could contain a capsid or capsid proteins. The UL25-encoded protein was more abundant in DBs than in either NIEPs or virions. In this respect, it is similar to pp65 and could indicate an interaction between these two proteins within the tegument. Although the significance of increased levels of pp65 and pUL25 in DBs is unknown, overexpression of pp65 has been attributed to continuous serial passage in tissue culture (28). The DBs also contained pUL26, which is not visible in the NIEP or virion preparations. This protein, if truly unique to DBs, may have a role in the assembly of more mature virus particles (e.g., NIEPs or virions).

The only nonviral protein identified during the sequence analysis migrates at approximately 45 kDa (Fig. 1A). Five tryptic fragments generated from this protein were sequenced and showed a variable but high degree of identity to the cellular α-, β-, and γ-actin isoforms (Table 2). In vertebrates, these conventional isoforms of actin differ in amino acid sequence in only a few positions, with >90% amino acid identity (21). In addition, conventional actin sequence variation lies predominately at the amino terminus, although there are some substitutions throughout the length of the actin molecules (60). The amino acid deviations found when we compared sequences of nonmuscle cytoplasmic β- and γ-actin with those of the fragments derived from the 45-kDa band were inconsistent with the conserved nature of actin. The lack of reactivity of the 45-kDa band with monoclonal and polyclonal antiactin antibodies (raised against different epitopes) led us to suspect that the virus could have acquired an unconventional form of actin. Actin-like isoforms with these characteristics, called actin-related proteins, have been found in widely divergent species, including protozoan, yeast, and vertebrate species (21, 51). The best characterized vertebrate actin-related protein, actin-RPV or Arp-1, is a component of the dynein-driven microtubule transport system (34). In lower eukaryotes, actin-related proteins are involved in nuclear transport or migration (39, 42, 44). Although speculative, it is conceivable that the 45-kDa unconventional actin found associated with HCMV is involved in the transport of viral particles from the nucleus to the cytoplasmic membrane.

There are several examples of negative-stranded RNA viruses and retroviruses which incorporate actin into their virions (6, 40, 41, 59, 61), with this incorporation in some cases being mediated by viral matrix proteins (16) or envelope proteins (48). At present, the role of actin in these viral particles and the mechanism by which it is incorporated are unknown. The substantial amount of the 45-kDa actin-related protein relative to virus-encoded proteins in our preparations suggests that the protein is specifically acquired. Determining the role of this cellular protein, as well as those of the virus-encoded proteins newly described here, should provide new insights into the biology of the HCMV replication cycle.

ACKNOWLEDGMENTS

We thank C. Patterson for HFF; W. Gibson for anti-mCP antiserum and sharing of SCP data prior to publication; L. Enquist for helpful discussions; and L. Bullions, R. Hyde-DeRuyscher, A. Marchini, and C. Patterson for commenting on the manuscript.

C.J.B. is a recipient of an NIH postdoctoral fellowship (AI09156) from the National Institute of Allergy and Infectious Diseases, and T.S. is an American Cancer Society Professor and an Investigator of the Howard Hughes Medical Institute.

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