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Cytomegalovirus "Missing" Capsid Protein Identified as Heat-Aggregable Product of Human Cytomegalovirus UL46

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Capsids of human and simian strains of cytomegalovirus (HCMV and SCMV, respectively) have identified counterparts for all but one of the protein components of herpes simplex virus (HSV) capsids. The open reading frames (ORFs) for the CMV and HSV counterpart proteins are positionally homologous in the two genomes. The HSV capsid protein without a recognized counterpart in CMV is VP19c, a 50-kDa element of the intercapsomeric "triplex." VP19c is encoded by HSV ORF UL38, whose positional homolog in the HCMV genome is UL46. The predicted protein product of HCMV UL46, however, has essentially no amino acid sequence similarity to HSV VP19c, is only two-thirds as long, and was not recognized as a component of CMV capsids. To identify and learn more about the protein encoded by HCMV UL46, we have expressed it in insect cells from a recombinant baculovirus and tested for its presence in CMV-infected human cells and virus particles with two UL46-specific antipeptide antisera. Results presented here show that this HCMV protein (i) has a size of ≈ 30 kDa as expressed in both recombinant baculovirus-infected insect cells and HCMV-infected human cells; (ii) has a homolog in SCMV; (iii) is a capsid component and is present in a 1:2 molar ratio with the minor capsid protein (mCP), encoded by UL85; and (iv) interacts with the mCP, which is also shown to interact with itself as demonstrated by the GAL4 two-hybrid system; and (v) aggregates when heated and does not enter the resolving gel during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a characteristic that accounts for it eluding detection until now. We call this protein the mCP-binding protein, and on the basis of the characteristics that it shares with HSV VP19c, we conclude that the HCMV mCP-binding protein is the functional as well as genetic homolog of HSV VP19c.

Cells infected with herpes group viruses characteristically contain two intranuclear capsid forms, called A- (or light) and B- (or intermediate) capsids (18, 21, 23, 43, 49). It is generally held that A-capsids represent aborted intermediates in the process of DNA packaging (34, 44, 55, 56) and that B-capsids have striking similarities to bacteriophage proheads which are precursors of the mature virus (8, 20, 34, 38, 49). In terms of protein composition, A-capsids are more closely related to the capsid within mature virions (18, 23). B-capsids and their putative precursors, pre-B-capsids (20, 21), are distinguished by a group of closely related proteins that direct capsid assembly and which include (i) the maturational proteinase and its autoproteolytic cleavage products and (ii) the precursor assembly protein (pAP) and its cleavage product, the assembly protein (AP) (13, 18, 23, 45, 63).

B-capsids of herpes simplex virus (HSV) are composed of seven proteins, and there is good evidence based on image reconstructions from cryoelectron micrographs for the location of each within the capsid (2, 40, 54, 69). The amino (VP24, HSV UL26) and carboxyl (VP21, HSV UL26) halves of the proteinase precursor (Pra), together with the genetically related VP22a (HSV UL26.5) (35, 36, 48, 64, 66), are cleavage products of Pra and the VP22a precursor (ICP35 c,d), respectively, which are required to direct proper formation of the capsid shell (15, 59, 60). The major capsid protein (MCP, VP5, HSV UL19) makes up the capsomeres, and VP26 (HSV UL35) forms a cap on the outer tips of the hexamers (62, 68). The two other capsid shell proteins, VP19c (HSV UL38) and VP23 (HSV UL18), form a triplex that is located between the capsomeres (2, 40) and is composed of two copies of VP23 per copy of VP19c (23, 39, 40).

Cytomegalovirus (CMV) B-capsids have recognized counterparts for all of the HSV B-capsid proteins except VP19c (18, 30). The amino ($NP1_n$) and carboxyl ($NP1_c$) halves of the proteinase precursor are encoded by human CMV (HCMV) UL80a (5, 66), and the genetically related AP, which is encoded by HCMV UL80.5 (64, 66), is the homolog of HSV VP22a (10, 18, 30); the MCP is encoded by HCMV UL86 (9, 10); the smallest capsid protein (SCP, HCMV UL48/49) is the homolog of HSV VP26 (3, 22); and the minor capsid protein (mCP) (19, 30) is the homolog of HSV VP23 and is encoded by HCMV UL85 (3, 27). A CMV homolog of HSV VP19c, however, was not apparent from protein analyses of CMV capsids and virions (18, 19, 30), nor was it obvious from the original sequence analysis of the HCMV genome (10). The HCMV open reading frame (ORF) UL46, which is the positional homolog of the ORF that encodes VP19c, is predicted to encode a protein that is much smaller (33 kDa) than VP19c (50 kDa) and has essentially no amino acid sequence similarity to it. Nevertheless, the fact that the ORF for each of the other HCMV capsid proteins is colinear with its HSV counterpart prompted us to reexamine this issue by directly testing the possibility that the protein encoded by HCMV UL46 is the counterpart of HSV VP19c.

In the work described here, we have used a combination of physical, immunological, and biochemical analyses to show that the HCMV UL46 protein is in fact the "missing" capsid constituent and the CMV counterpart of HSV VP19c.

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MATERIALS AND METHODS

Cells and viruses. HCMV strain AD169 and simian CMV (SCMV) strain Colburn were grown in human foreskin fibroblast (HFF) cells as previously described (18, 19, 29). Virions, noninfectious enveloped particles (NIEPs), and dense bodies were prepared by the positive density-negative viscosity gradient procedure (4) and concentrated by pelleting, all as described before (29, 58). SCMV B-capsids were recovered from the Nonidet P-40 (NP-40) nuclear fraction of infected cells ($\approx 10^8$ per gradient) by rate-velocity sedimentation (190,000 × *g*, 25 min, 4°C) in 15 to 50% sucrose gradients as described before (34).

B-capsids were recovered from HCMV-infected cells by essentially the same procedure as used for SCMV B-capsids but with the following additional step. After NP-40 nuclei had been prepared from infected cells (18), they were resuspended in 1 ml (per 5×10^8 cells) of 150 mM NaCl–40 mM phosphate buffer, pH 7.4 (PB), containing 1 mM phenylmethylsulfonyl fluoride and subjected to three rapid cycles of freezing and thawing. Particulate material was recovered by centrifugation (2,000 × *g*, 5 min, 4°C), suspended in 1 ml (per 5×10^8 starting cells) of PB containing 1 mM phenylmethylsulfonyl fluoride, sheared by forcing four times through a 23-gauge needle, and cleared of remaining particulate material (2,000 × *g*, 5 min, 4°C). The resulting supernatant fraction was subjected to rate-velocity gradient centrifugation in 15 to 50% sucrose gradients as referenced above, and light-scattering bands of HCMV B-capsids were collected by aspiration through the wall of the tube from near the middle of the gradient. This procedure has yielded particles more consistently than has the freeze-thaw lysis method (30). Radiolabeled virus particles were prepared from infected cells grown in complete medium containing 50 μCi of [^{35}S]methionine (no. 51001H; ICN, Cleveland, Ohio) per ml that was present continuously from 3 days after infection until cell processing 3 days later. This represents a period of essentially maximal steady-state labeling of the CMV capsid proteins.

Virus-infected and noninfected HFF cells were fractionated to obtain material for analyzing intracellular proteins. This was done by scraping the cells from the bottles into the medium and collecting them by low-speed centrifugation; suspending the pellet in PB and 0.5% NP-40 (0.5 ml per $\approx 5 \times 10^7$ cells, 10 min, 4°C, with occasional vortexing); separating the lysate, by low-speed centrifugation, into supernatant (NP-40 cytoplasmic) and pellet (NP-40 nuclear) fractions; and combining the resulting material with an equal volume of 2× protein sample buffer (see below) and storing it at −80°C without prior heating, all as detailed before (18).

Rate-velocity sedimentation analysis. The nuclear pellet resulting from NP-40 fractionation of infected cells, as described above, was suspended by vortex mixing in PB (0.5 ml/5×10^7 cells) and dispersed by sonication (three 5-s pulses, setting 4, Branson Sonifier 185). The resulting lysate was cleared of large debris by low-speed centrifugation (2,000 × *g*, 10 min, 4°C), layered onto a 15 to 50% (wt/vol in PB) sucrose gradient, and subjected to centrifugation (190,000 × *g*, 20 min, 4°C, Beckman SW41 rotor). The 12-ml gradient was collected from the top, by displacement from the bottom (gradient fractionator model 185; ISCO, Lincoln, Neb.), and portions of each 0.5-ml fraction were combined, 3 volumes to 1, with 4× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and analyzed by SDS-PAGE followed by Western immunoassay (WIA) as described below.

Yeast strain and methods. *Saccharomyces cerevisiae* PCY2 (*MAT*α Δ*gal4* Δ*gal80 URA3*::*GAL1-lacZ lys2-801*^{amber} *his*-Δ200 *trp1*-Δ*63 leu2 ade2-101*^{ochre}) was used (11). Yeast cells were grown in yeast extract-peptone-dextrose or in minimal medium containing dextrose (SD) supplemented with an amino acid mixture selective for growth of transformants (1).

Yeast transformation was by the lithium acetate method performed essentially as described previously (1) except that 50 μl of competent cells was transformed with 5 μg of plasmid DNA without carrier DNA.

Yeast colonies were assayed for β-galactosidase activity by using the yeast colony filter assay as described previously (7) except that the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was used at 1 mg/ml and incubation was at 37°C (11). The liquid β-galactosidase assay of crude yeast lysates using the chromogenic substrate *o*-nitrophenyl-β-D-galactoside (ONPG) was performed essentially as described previously (50) except that yeast colonies were grown on SD-Leu-Trp plates and scraped into SD-Leu-Trp liquid medium (14), and protein concentrations were determined by the Lowry method rather than the Bradford method (52). The β-galactosidase activity given for a transformant that was positive by the filter assay represents the average of values obtained from assays of at least three dilutions of yeast lysate; only one dilution of yeast lysate was assayed if the transformant was negative by the filter assay.

Plasmids used in the GAL4 two-hybrid system. The GAL4 two-hybrid vectors used in this study were obtained from Daniel Nathans and have been described previously (11). Vector pPC86 encodes the GAL4 transactivation domain (TA). Vector pPC97 encodes the GAL4 DNA-binding domain (DB) and is a derivative of pPC62 (11). pPC97 has been modified so that its polylinker is identical to that of pPC86.

Subcloning of viral genes into the two-hybrid vectors. The plasmid TA–mC-BP encodes TA fused to the minor capsid protein (mCP)-binding protein (mC-BP) and was constructed in the following manner. The 5′ end of the UL46 gene was PCR amplified by using a forward primer that introduced a *Bgl*II site just upstream of the start ATG and a reverse primer that included the unique internal *Bsm*I site. The remainder of the UL46 gene was excised from pAT153M (obtained from Mark Chee and Bart Barrell), which contains the HCMV genomic *Hin*dIII M fragment, by digestion with *Bsm*I and *Xba*I. These two gene fragments were ligated into *Bgl*II-*Spe*I-digested pPC86. DB-mC-BP was produced by excising UL46 from TA-mC-BP by using *Bgl*II and *Not*I and ligating it into *Bgl*II-*Not*I-digested pPC97.

TA-mCP was made by PCR amplification of the gene, using a forward primer that introduced a *Sal*I site upstream of the start codon of UL85 and a reverse primer that introduced a *Bgl*II site downstream of the stop codon; the *Sal*I-*Bgl*II-digested PCR product was then ligated into *Sal*I-*Bgl*II-digested pPC86. The portion of the gene from the unique internal *Sfi*I site onward was then replaced with the same gene fragment from a clone of UL85 derived from the HCMV genomic *Eco*RI D fragment (obtained from Deborah Spector). DB-mCP was produced by excising UL85 from TA-mCP by using *Sal*I (a genomic *Sal*I site downstream of the UL85 stop codon is included in the TA-mCP insert) and ligating it into *Sal*I-digested, alkaline phosphatase-treated pPC97.

TA-pAP and DB-pAP are the HCMV UL80.5 gene cloned into pPC86 and pPC97, respectively; the construction of these plasmids is described elsewhere (67).

Construction of recombinant baculovirus. The UL46 baculovirus (BV) transfer plasmid (JM1) was prepared by digesting TA (GAL4 transactivating domain)–mC-BP (mCP-binding protein) with *Bgl*II and *Not*I, isolating a 970-bp fragment, and ligating the fragment into the pVL1392 BV transfer plasmid cut with the appropriate restriction enzymes. The UL46 sequence was introduced into the *Autographa californica* strain of nuclear polyhedrosis virus by homologous recombination, using the Baculogold system (no. 21100K; Pharmingen, San Diego, Calif.) as recommended by the manufacturer. Several recombinant BVs (rBVs) were isolated after plaquing on *Spodoptera frugiperda* (Sf9; American Type Culture Collection, Rockville, Md.) cells; each was plaque purified again (25, 41) and then used to prepare high-titer stocks, which were stored at 4°C protected from light.

Gel electrophoresis, WIA, and antisera. Proteins were separated by SDS-PAGE essentially as described by Laemmli (32); 10 to 20% polyacrylamide gradient Tricine gels (Tricine SDS-PAGE; Novex, San Diego, Calif.) were used except where noted; SDS in the electrode buffer was from Bio-Rad (Melville, N.Y.); and 2× protein sample buffer consisted of 4% SDS, 20% β-mercaptoethanol, 20% glycerol, 50 mM Tris (pH 7.0), and 0.02% bromophenol blue. Unless specified, samples were not heated above 37°C prior to SDS-PAGE because of the tendency of the CMV UL46 protein to aggregate when heated.

WIAs were done essentially as described by Towbin et al. (61). A semidry transfer unit was used, the membrane was Immobilon-P (no. IPUH 00010; Millipore, Bedford, Mass.), the buffer was 50 mM Tris–20% methanol, and the time of transfer was calculated by the following formula: gel width × height × 2.5 = milliamperes per 30 min. When electrotransfer included a tacky stacking gel, a piece of Whatman 1MM filter paper was placed between it and the Immobilon-P to prevent adherence to the membrane.

Following electrotransfer, the membrane was blocked in a solution containing 10 mM Tris, 0.9% NaCl, and 5% bovine serum albumin, pH 7.4 (TN/BSA); reacted sequentially with antiserum and then ^{125}I-protein A (no. IM144; Amersham, Arlington Heights, Ill.), both in TN/BSA; and exposed to Kodak Biomax MR single-sided X-ray film, to XAR5 X-ray film with a calcium tungstate intensifying screen (33), or to a Fuji BAS-IIIs detection screen that was scanned with a Fuji BAS 1000 phosphorimager using MacBAS 2.0 software (Stamford, Conn.). [^{35}S]methionine-labeled proteins were detected as described in Results. Quantification of radiolabel in gels and in WIA was done by phosphorimage analysis, using the system described above.

Two rabbit antipeptide antisera to the HCMV UL46 protein were prepared. One was to the amino-terminal 15 residues of the ORF (anti-UL46N), and the other was to the carboxy-terminal 15 residues of the ORF (anti-UL46C). Both antisera were made by injecting the peptide, conjugated to keyhole limpet hemocyanin (no. 77100G; Pierce, Rockford, Ill.) with γ-maleimidobutyric acid *N*-hydroxysuccinimide ester (no. M-7642; Sigma, St. Louis, Mo.), into rabbits; boosting the rabbits at scheduled intervals; collecting blood once a month, beginning 1 month after immunization; and preparing the serum fraction for storage at −80°C until needed, all as described before (53). Rabbit antipeptide antisera to the HCMV MCP and mCP were prepared similarly, to synthetic peptides representing the carboxyl 15-amino-acid sequence of each protein. Other antisera have been described before: anti-N1, to the amino terminus of pAP (53); anti-N2, to the amino terminus of assemblin or $NP1_n$ (26); and anti-C2, to the carboxy terminus of assemblin (65).

Peptide comparisons following protein cleavage by NCS. In-gel protein cleavage by *N*-chlorosuccinimide (NCS; Aldrich, Milwaukee, Wis.), followed by peptide separation in a second-dimension gel, has been described before (53). A 0.75-mm-thick, 10% mini-analytical gel was used for the first-dimension SDS-PAGE; a 1.0-mm-thick 10 to 20% Tricine preparative gel (Novex, EC6626) was used for the second-dimension SDS-PAGE. The treated proteins were then electrotransferred to Immobilon-P and analyzed by WIA as described above. Additional details are described in Results.

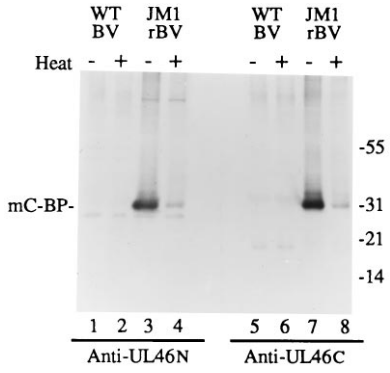

FIG. 1. HCMV ORF UL46 expressed in rBV-JM1-infected insect cells aggregates when heated and reacts with antipeptide antisera. Sf9 cells infected with rBV-JM1, containing ORF UL46, were solubilized and subjected to SDS-PAGE followed by WIA (lanes 3, 4, 7, and 8); a lysate of wild-type (WT) BV-infected cells was used as a control (lanes 1, 2, 5, and 6). Half of each sample was heated (+) in a boiling water bath for 3 min prior to Tricine SDS-PAGE (lanes 2, 4, 6, and 8); the other half was not heated (−) (lanes 1, 3, 5, and 7). Equal volumes of each sample were analyzed; the membrane was cut between lanes 4 and 5 after electrotransfer; the half containing lanes 1 to 4 was probed with anti-UL46N, and the half containing lanes 5 to 8 was probed with anti-UL46C. Shown is an autoradiogram prepared from the resulting membrane. Numbers on the right indicate the molecular weights (10^3) of protein markers (no. LC5677, Mark 12; Novex) which were separated in the same gel, transferred to the membrane, sectioned from it before the immunoassay, and stained with CBB. The position of the protein product of HCMV UL46, called mC-BP, is indicated on the left.

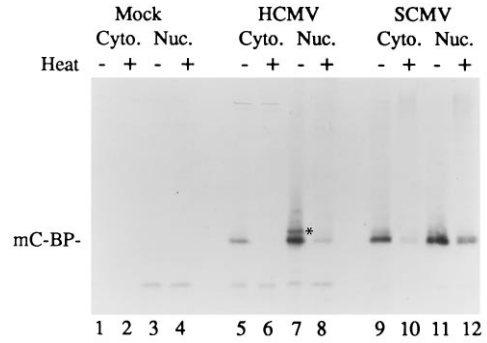

FIG. 2. CMV-infected cells contain a 30-kDa, heat-aggregatable protein reactive with anti-UL46C. Cytoplasmic (Cyto.) and nuclear (Nuc.) fractions (18) of HFF cells infected with HCMV or SCMV, or not infected (Mock), were prepared (see Materials and Methods) and analyzed by Tricine SDS-PAGE and WIA with anti-UL46C as described in the text and in the legend to Fig. 1. Half of each sample was heated (+) (lanes 2, 4, 6, 8, 10, and 12) prior to SDS-PAGE, and half was not heated (−) (lanes 1, 3, 5, 7, 9, and 11). Shown is an autoradiogram of the resulting membrane. The position of the 30-kDa protein detected in HCMV- and SCMV-infected cells, called mC-BP, is indicated on the left; the asterisk indicates the position of the band described in text.

RESULTS

The experiments described here show that the protein encoded by HCMV ORF UL46 is a component of the capsid and interacts with the protein product of HCMV ORF UL85, called the mCP. On the basis of these characteristics, which are shared with its HSV homolog VP19c and presumably with its homologs in other herpesviruses, we call this HCMV protein the mCP-binding protein (mC-BP). We have found it convenient to use a descriptive name to designate protein counterparts because the sizes and ORF numbers of homologous proteins vary between different herpesviruses and even between different strains of the same virus (19). We have used the name mC-BP from the beginning of the report, even though the supporting evidence is not presented until Fig. 4 and Table 2.

HCMV UL46 protein expressed from rBV aggregates when heated. We began our studies by making an rBV that expresses the UL46 protein in insect cells and by preparing rabbit antisera to peptide mimics of the amino-terminal 15 amino acids (anti-UL46N) and the carboxy-terminal 15 amino acids (anti-UL46C) of the protein predicted for HCMV UL46, all as described in Materials and Methods.

Initial attempts to detect mC-BP expression in rBV-JM1-infected insect cells were unsuccessful; no new Coomassie brilliant blue (CBB)-stained or immunoreactive proteins were detected following SDS-PAGE (data not shown). However, when the stacking gel was left attached to the resolving gel during electrotransfer, WIA showed that immunoreactive material had been retained in it (data not shown). Our recent experience in characterizing the HCMV UL33 G protein-coupled receptor homolog, GCR33 (37), suggested that this property of the recombinant protein may be due to intermolecular hydrophobic interactions and might be overcome by omitting the sample heating step prior to SDS-PAGE.

This possibility was tested by comparing a nonheated lysate of rBV-JM1-infected insect cells with one that had been heated in a boiling water bath prior to SDS-PAGE followed by WIA. A protein with an estimated size of 30 kDa (close to 33 kDa, the computer-predicted size) was detected in the nonheated samples (Fig. 1, lanes 3 and 7) but was reduced by 80 to 90% in the heated samples (Fig. 1, lanes 4 and 8). Antipeptide antisera Anti-UL46N (Fig. 1, lanes 1 to 4) and anti-UL46C (Fig. 1, lanes 5 to 8) both reacted with this 30-kDa protein, indicating that it contains both the amino and carboxyl ends of the predicted UL46 protein. This protein was not present in lysates of Sf9 cells infected with wild-type baculovirus (Fig. 1, lanes 1, 2, 5, and 6). On the basis of its size, its presence in only rBV-JM1-infected cells, and its reactivity with both antisera, this protein is the product of UL46 and is called mC-BP, for the reasons explained above and documented below. Anti-UL46C reacted more strongly and specifically than anti-UL46N and was used in most subsequent experiments.

CMV-infected cells and virus particles contain a 30-kDa, heat-aggregatable protein. A heat-aggregatable 30-kDa protein was also detected by WIA of HCMV-infected HFF cells. This protein comigrated with rBV-expressed mC-BP (data not shown) and was present in the cytoplasmic and more so in the nuclear fractions of HCMV-infected cells (Fig. 2, lanes 5 and 7) but not in noninfected cells (Fig. 2, lanes 1 to 4). A protein of about the same size was also present in the corresponding preparations of SCMV-infected cells (Fig. 2, lanes 9 and 11), indicating that SCMV encodes an immunologically cross-reactive homolog. The intensities of these bands were reduced by ≈65 to 90% in the samples that were heated prior to SDS-PAGE (Fig. 2, lanes 6, 8, 10, and 12). We have not investigated the somewhat larger immunoreactive proteins seen in these preparations (most noticeable in the HCMV nuclear fraction [Fig. 2, lane 7, asterisk]), but we suspect that the different bands represent different denaturation states of the protein.

mC-BP protein is present in NIEPs and virions but not dense bodies. Our next experiment was done to determine whether mC-BP is a virion protein and capsid constituent, as would be expected if it were the counterpart of HSV VP19c (23, 57). We prepared HCMV NIEPs, virions, and dense bodies and tested them for the presence of mC-BP by Tricine SDS-PAGE and WIA with anti-UL46C. As seen in Fig. 3, mC-BP was detected in NIEPs and virions but not in dense bodies (Fig. 3, lanes 1, 3, and 5, respectively) and was reduced by 80 to 90% when the samples were heated prior to SDS-

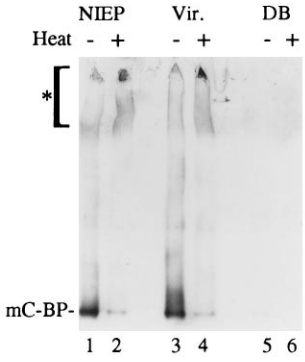

FIG. 3. HCMV virions and NIEPs, but not dense bodies, contain a 30-kDa, heat-aggregatable protein reactive with anti-UL46C. NIEPs, virions (Vir.), and dense bodies (DB) were prepared and analyzed by Tricine SDS-PAGE followed by WIA with anti-UL46C as described in the text. Half of each sample was heated (+) (lanes 2, 4, and 6) and half was not heated (−) (lanes 1, 3, and 5) prior to Tricine SDS-PAGE. Shown is an autoradiogram prepared from the resulting membrane. The bracket indicates the protein stacking portion of the gel.

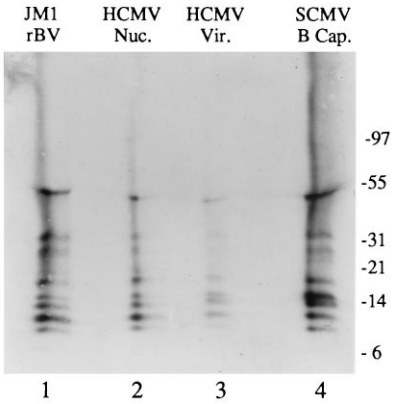

FIG. 4. Peptide comparison of the 30-kDa proteins from rBV-JM1-infected insect cells and from CMV-infected human cells and virus particles. Preparations containing the 30-kDa protein expressed in rBV-JM1-infected insect cells (lane 1), present in the nuclear fraction of HCMV-infected human cells (Nuc.) (lane 2), or contained in HCMV virions (Vir.) (lane 3) or SCMV B-capsids (B Cap.) (lane 4) were subjected to NCS cleavage and peptide analysis as described in the text. Shown is an autoradiogram prepared from the membrane following WIA with anti-UL46C. Molecular weights (10^3) indicated on the right were determined as described in the legend to Fig. 1.

PAGE (Fig. 3, lanes 2 and 4). An increased amount of high-molecular-weight material was detected in the stacking gel above the lanes containing the heated NIEP and virion preparations (Fig. 3, lanes 2 and 4, asterisk), but its intensity is not directly proportional to that of the mC-BP band in the non-heated samples, presumably because of the low efficiency of electrotransferring such high-molecular-weight material from the gel. Because virions and NIEPs both contain capsids but dense bodies contain neither a capsid nor capsid proteins, presence of mC-BP in only virions and NIEPs is consistent with it being a capsid constituent.

Peptide comparisons indicate that the ≈30-kDa protein in CMV-infected cells and virus particles is the UL46 product. To establish that the 30-kDa protein detected in CMV-infected cells and virus particles is the product of HCMV UL46, we used a protein cleavage assay to compare the 30-kDa protein expressed by rBV-JM1 with the 30-kDa proteins detected in the nuclear fraction of HCMV-infected cells, in HCMV virions, and in SCMV B-capsids. This was done by subjecting the infected-cell and virus particle preparations to SDS-PAGE, excising the 30-kDa protein-containing portion of each lane, cleaving the target proteins in their respective gel fragments with NCS, separating the resulting peptides by Tricine SDS-PAGE in a second-dimension gel, and then comparing the resulting peptide patterns following WIA with anti-UL46C, all as described in Materials and Methods.

Results of this experiment showed that although there appear to be some quantitative differences, the peptide patterns of the 30-kDa proteins in the nuclear fraction of HCMV-infected cells (Fig. 4, lane 2), in HCMV virions (Fig. 4, lane 3), and in SCMV B-capsids (Fig. 4, lane 4) are essentially indistinguishable from that of the rBV-expressed ORF UL46 protein (Fig. 4, lane 1). These data provide evidence that the 30-kDa immunoreactive protein in HCMV virions and infected cells is the product of UL46 and indicate that the 30-kDa SCMV homolog is present in B-capsids. The immunoreactive band at ≈50 kDa seen in this experiment may be a dimer of the 30-kDa protein or an aggregate of one or more of its NCS cleavage products.

mC-BP is a B-capsid protein. We next tested more directly for the presence of mC-BP in capsids. Nonlabeled and [^{35}S]methionine-labeled HCMV B-capsids were prepared as described in Materials and Methods and subjected to Tricine SDS-PAGE followed by WIA with anti-UL46C. Immediately after electrotransferring the proteins to an Immobilon-P membrane, we made an autoradiographic exposure to visualize the labeled B-capsid proteins. A second exposure was made after WIA for mC-BP, and the two images were superimposed. The results of this experiment indicate that mC-BP is present in B-capsids (Fig. 5). Comparison of the bands in the radiolabeled preparation before and after WIA with anti-UL46C (Fig. 5A, lanes 2 and 3, respectively) identified the [^{35}S]methionine-

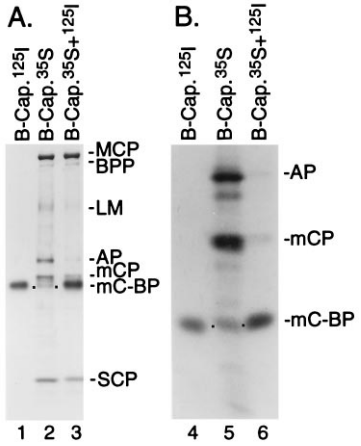

FIG. 5. HCMV B-capsids contain mC-BP. (A) Nonlabeled (lane 1) and [^{35}S]methionine-labeled (lane 2) HCMV B-capsid proteins were subjected sequentially to Tricine SDS-PAGE, electrotransfer to Immobilon-P, and autoradiography (lane 2), followed by WIA with anti-UL46C and fluorography (lanes 1 and 3). Shown is a composite of the two resulting images. (B) [^{35}S]methionine-labeled HCMV B-capsid proteins (same preparation used for panel A) were subjected sequentially to SDS-PAGE in a 10% gel, electrotransfer to Immobilon-P, and autoradiography (^{35}S radioactivity only; lane 2), followed by WIA with anti-UL46C and then fluorography with (i.e., primarily ^{125}I radioactivity; lane 4) and without (i.e., both ^{35}S and ^{125}I radioactivity; lane 6) a barrier sheet of film to attenuate the ^{35}S signal; lane 6 is shown in the mirror orientation to lanes 4 and 5. Shown is a composite of the three resulting images. Protein abbreviations to the right in each panel are as follows: MCP, AP, mCP, mC-BP, and SCP are as defined in the text; BPP is the basic phosphoprotein, which is encoded by UL32 (31); LM is the lower matrix, which is encoded by UL83 (51). Dots by lanes 2 and 5 indicate the position of mC-BP.

labeled mC-BP as a comparatively weak band (dots in Fig. 5A, lane 2) just below the mCP. This was the only protein recognized by anti-UL46C in the fluorographic exposure of the nonlabeled B-capsid proteins (Fig. 5A, lane 1). We also noted that a disproportionately large amount of the AP was lost from the membrane during the WIA procedure (Fig. 5A, compare lanes 2 and 3).

To resolve mC-BP from mCP and to remove any ambiguity from the identification of the mC-BP band in the B-capsid preparation, we subjected the same radiolabeled B-capsid preparation to SDS-PAGE in a 10% gel followed by WIA with anti-UL46C. An autoradiogram was prepared from the membrane before (Fig. 5B, lane 5) and a fluorogram was prepared after (Fig. 5B, lane 6) probing for mC-BP. In addition, a fluorographic exposure showing only the immunoimage of mC-BP (Fig. 5B, lane 4) was made by placing autoradiographic film between the membrane and the fluorographic film to selectively diminish ^{35}S detection. The mC-BP and mCP did separate better in the 10% gel, and a composite of the autoradiographic and fluorographic images of the membrane prepared before (Fig. 5B, lane 5) and after (Fig. 5B, lanes 4 and 6) probing with anti-UL46C shows mC-BP as a distinct single band (dots in Fig. 5B, lane 5).

To establish that mC-BP is in fact associated with B-capsids, rather than just a nonspecific contaminant aspirated from the tube with the B-capsid band, we determined its distribution in the gradient following rate-velocity sedimentation. This was done by preparing B-capsids from SCMV-infected cells as usual (33), fractionating the gradient after centrifugation, subjecting samples of each resulting fraction to SDS-PAGE in a 10% gel, electrotransferring the proteins to Immobilon-P, and using a mixture of anti-UL46C and anti-UL46N in a WIA to identify mC-BP. SCMV B-capsids were used in this experiment because we have antisera to all of the SCMV capsid proteins, which were needed to establish the location of B-capsids in the gradient (described next). This seemed justified given that the SCMV and HCMV mC-BP homologs were essentially indistinguishable on the basis of their sizes, their reactivities with anti-UL46C (Fig. 2), and their peptide patterns (Fig. 4).

A phosphorimage prepared from the resulting immunoblot showed that mC-BP was concentrated in the region of the gradient corresponding to the position of B-capsids (Fig. 6, inset, lane 11), as estimated from the location of the light-scattering capsid band prior to fractionation. To demonstrate the coincidence of mC-BP with B-capsids more directly, the membrane was wetted and probed a second time with a mixture of antisera that detect all but one (i.e., SCP not retained in 10% gel) of the other CMV capsid proteins: MCP (anti-MCP), AP and $NP1_c$ (anti-N1), mCP (anti-mCP), and assemblin or $NP1_n$ (anti-N2 or anti-C2). A phosphorimage prepared from the resulting immunoblot showed that the distribution of mC-BP in the gradient coincided with that of the B-capsid proteins (i.e., MCP, mCP, AP, $NP1_c$, and $NP1_n$), each maximal in fraction 11 (Fig. 6). The codistribution of mC-BP with the B-capsid proteins supports the conclusion that it is a capsid constituent.

The molar ratio of mC-BP to minor capsid protein is approximately 1:2. The ORFs encoding the HCMV mC-BP and mCP are positionally homologous to the HSV ORFs encoding VP19c and VP23, respectively. In HSV capsids, VP19c and VP23 are present in a molar ratio of approximately 1:2 (VP19c:VP23) (23, 39, 40) and make up the triplex (2), which is situated between and at the base of the capsomeres (69). To determine whether the HCMV mC-BP and mCP are present in a comparable ratio, we used the [^{35}S]methionine-radiolabeled HCMV B-capsid preparation described above, separated the proteins by SDS-PAGE in a Tricine 10 to 20% gradient gel and in a 10% gel, stained the proteins with CBB, dried the gels, and then subjected them to phosphorimaging as described in Materials and Methods and in Table 1, footnote *a*. The general pattern of the radiolabeled proteins was essentially the same as seen in Fig. 5A and B, lanes 2 and 5, although the less efficiently electrotransferred proteins (e.g., MCP) are underrepresented in Fig. 5A. The results of quantifying relative protein amounts in the two gels, presented in Table 1, show that (i) the ratio of HCMV mC-BP to mCP was approximately 1:2, close to the ratio of the counterpart triplex proteins in HSV (i.e., VP19c:VP23) and equine herpesvirus (EHV) (i.e., 19:23) (23, 39, 40); and (ii) other HCMV B-capsid proteins were also present in molar ratios that approximated those in HSV and EHV, e.g., mCP:MCP (≈2:3) and AP:MCP (≈1:1). The standard deviations given for the HCMV B-capsid proteins are high because our averaging method intentionally maximized parameter differences to be as inclusive as possible (see Table 1, footnote *a*). The consistently high standard deviation for measurements of the AP and its homologs VP22a (HSV) and 22 (EHV) may result from one or both of the following: (i) their characteristically unusual staining and radiolabeling properties (18, 24, 30) or (ii) variability in relative amount from preparation to preparation, because they are transient rather than integral components of the capsid.

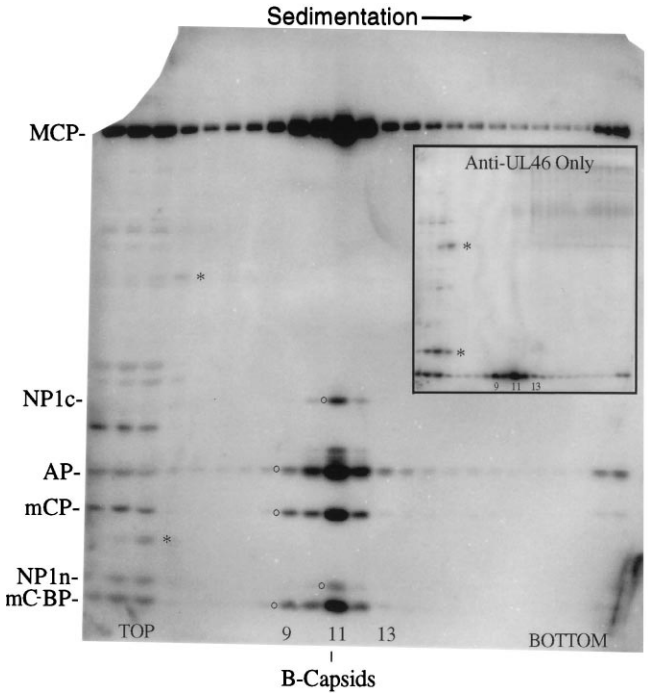

FIG. 6. SCMV mC-BP homolog cosediments with B-capsid band. SCMV B-capsids were prepared by rate-velocity sedimentation as described in the text; a light-scattering band of B-capsids (and A-capsids slightly above) near the middle of the tube was visualized following centrifugation. The gradient was collected, and samples of each fraction were analyzed by SDS-PAGE in a 10% gel, followed by WIA with a mixture of anti-UL46N and anti-UL46C and phosphorimaging (inset). The membrane was probed again to reveal the B-capsid proteins indicated by abbreviations at the left, as described in the text. Also indicated is the position of B-capsids (i.e., fraction 11), the positions in the gradient of $NP1_c$, AP, mCP, $NP1_n$, and mC-BP (circles), and reference bands (asterisks) that appear in both the figure and the inset. Shown is a print (440 Phaser; Tektronix, Beaverton, Oreg.) of a composite prepared from the computer-enhanced and superimposed first (inset) and second phosphorimages.

TABLE 1. Calculated molar ratios of four B-capsid proteins from three herpesviruses

HCMV		HSV			EHV	
Protein	Ratio[a]	Protein	Ratio[b]	Ratio[c]	Protein	Ratio[d]
MCP	2.9 ± 0.6	VP5	3.2 ± 0.6	2.6 ± 0.2	9	2.4 ± 0.2
AP	3.7 ± 0.9	VP22a	4.2 ± 0.9	3.1 ± 0.5	22	1.6 ± 0.4
mCP	2.1 ± 0.4	VP23	2.0 ± 0.4	1.5 ± 0.2	23	1.7 ± 0.3
mC-BP	1	VP19c	1	1	19	1

[a] Measurements were made from [^{35}S]methionine-labeled proteins from HCMV B-capsids, prepared as described in Materials and Methods and separated by SDS-PAGE in either a 10% gel or a 10 to 20% gradient gel. The proteins were CBB stained; the gels were dried and analyzed for radioactivity by using a Fuji BAS 1000 phosphorimager. Three measurements were made for each band in each gel: one by outlining individual bands in Quant (MacBAS 2.0) and two by integrating either the minimum or maximum peak areas obtained from a plot of the distribution of radioactivity in the sample lane made with Profile (MacBAS 2.0). To obtain a relative molar amount for each protein, these values were each then divided by the number of methionines predicted to be in that protein (either with or without the translational start methionine); i.e., MCP = 36 or 35, AP = 3 or 2, mCP = 13 or 12, and mC-BP = 5 or 4, respectively. The relative molar ratios of these four proteins were then calculated for all possible combinations (e.g., minimum radioactivity and maximum methionine number for one band, versus maximum radioactivity and minimum methionine number for another) and normalized by setting the lowest value (i.e., that for mC-BP) equal to 1. The resulting ratios were averaged, and standard deviations were calculated.

[b] Calculated from data in reference 29, Table 2.

[c] Calculated from data in reference 40, Table 2.

[d] Calculated from data in reference 39, Table 2.

Yeast GAL4 two-hybrid assays indicate mC-BP/mCP and mCP/mCP interactions. The GAL4 two-hybrid system (16) was used to determine whether the HCMV mC-BP and mCP interact, as do their HSV triplex counterparts VP19c and VP23 (14, 40). Fusion proteins were made between HCMV mC-BP and the TA and DB domains, respectively, of the GAL4 protein (i.e., TA–mC-BP and DB–mC-BP). Similar fusions were constructed for the HCMV mCP (i.e., TA-mCP and DB-mCP). These fusion proteins were tested alone, in combinations with each other, and in combination with GAL4 constructs of the MCP, pAP, and SCP that have been described elsewhere (67). The DNA cloning and assay procedures that were used are described in Materials and Methods.

Results of these experiments are presented in Table 2 and can be summarized as follows. First, there was an interaction between DB–mC-BP and TA-mCP, as indicated qualitatively by blue colony color in the filter assay and quantitatively by the liquid assay (Table 2, sample 3). Controls showed that neither of these plasmids gave false-positive reactions when cotransformed with the complementary vector alone (Table 2, samples 1 and 2) and that the intensity of the colorimetric liquid reaction for the DB–mC-BP-plus-TA-mCP cotransformation (Table 2, sample 3) was at least 1,000-fold above that of transformants that were negative for interaction in the filter assay (Table 2, samples 1, 2, 4, and 5) and even 17-fold above that of the positive control for interaction (Table 2, sample 8). We take these results as direct evidence that the HCMV mC-BP and mCP interact. Second, this interaction between mC-BP and mCP was not detected when the reciprocal vectors (i.e., TA–mC-BP plus DB-mCP) were used (Table 2, sample 4). Vector-dependent results such as this are not uncommon in the GAL4 two-hybrid assay (17, 67) and are generally attributed to differential expression, stability, or physical/chemical (e.g., conformation/charge) properties of the respective TA- and DB-domain fusion proteins. Third, DB–mC-BP did not interact with several other GAL4 TA-domain fusion proteins that were tested (data not shown); these included the HCMV MCP (TA-MCPhcmv), the HCMV pAP (TA-pAPhcmv), and the HCMV SCP (TA-SCPhcmv). Fourth, DB-mCP and TA-mCP interact as indicated by both filter and liquid assays (Table 2, sample 6). This self-interaction has not been reported before for either CMV mCP or HSV VP23.

TABLE 2. Interaction of CMV mC-BP with mCP and of mCP with itself in the yeast GAL4 two-hybrid system

Sample	Plasmid[a]		Assays[b]	
	DB domain fusion	TA domain fusion	Blue colony color[c]	Mean β-Gal activity (U)[d] ± SD
1	mC-BP	—[e]	−	0.02
2	—	mCP	−	0.03
3	mC-BP	mCP	+	46.1 ± 4.8
4	mCP	mC-BP	−	0.05
5	mCP	—	−	0.03
6	mCP	mCP	+	1.04 ± 0.04
7	mC-BP	mC-BP	−	ND[f]
8	pAP	pAP	+	2.71 ± 0.19

[a] Construction described in Materials and Methods.

[b] Procedures described in Materials and Methods.

[c] Blue colony color (+) indicates an interaction; − indicates no interaction.

[d] β-Galactosidase (β-Gal) activity units = nanomoles of ONPG cleaved per minute per milligram of protein in the yeast lysate (see Materials and Methods).

[e] —, GAL4 domain not fused to another protein.

[f] ND, not done.

DISCUSSION

This report describes experiments that were done to identify and characterize the protein product of the HCMV UL46 open reading frame. Our interest in this ORF stemmed from its positional homology with HSV UL38, which encodes the essential HSV capsid protein, VP19c—a species once thought to be missing a CMV counterpart (18, 19, 30). Our initial results showed that the protein product of HCMV UL46, called mC-BP, forms high-molecular-weight aggregates when heated in preparation for SDS-PAGE and provided an explanation for why it had not been detected before. When the sample heating step was omitted, we identified a 30-kDa protein in both rBV-JM1-infected insect cells (Fig. 1) and HCMV-infected human fibroblasts (Fig. 2).

Our evidence that this 30-kDa protein is encoded by UL46 is threefold: (i) its size is close to the 33-kDa computer-predicted size of the UL46 protein (Fig. 1), (ii) it was recognized in WIA by antipeptide antisera specific for the amino and carboxyl ends of the predicted UL46 protein (Fig. 1), and (iii) the peptide patterns obtained by NCS cleavage of the 30-kDa proteins from infected cells and virus particles were essentially the same as that from the protein product of cloned UL46 expressed by rBV-JM1 in insect cells (Fig. 4). SCMV was shown to encode a closely related homolog that is the same size (Fig. 2), is immunologically cross-reactive (Fig. 2), and has a similar NCS peptide pattern (Fig. 4).

In HSV, VP19c is an integral capsid protein (12, 23, 38) that interacts with VP23 (14) to form a complex, called the triplex, in which the two proteins are in a molar ratio of 1:2 (VP19c: VP23) (40). As discussed below, HCMV mC-BP shares these characteristics, indicating that it is the functional, as well as genetic, homolog of HSV VP19c.

Our first series of experiments demonstrated that mC-BP is a capsid protein. We showed that it is present in two enveloped particles that contain a capsid (i.e., virions and NIEPs) but is not found in dense bodies, a third type of enveloped particle

that contains neither a capsid structure nor capsid proteins. More definitively, it was shown that mC-BP is a constituent of both HCMV and SCMV B-capsids (Fig. 5 and 6); this was demonstrated best by its codistribution with the other B-capsid proteins following rate-velocity sedimentation (Fig. 6).

A second set of experiments provided estimates of the molar ratios of the mC-BP with the other B-capsid proteins and indicated that, as with HSV VP19c and VP23, there is one copy of mC-BP per approximately two copies of the mCP. The experiment was done by first establishing the identity of the B-capsid mC-BP band by a combination of [^{35}S]methionine biosynthetic labeling and WIA (Fig. 5) and then measuring the ^{35}S radioactivity of the mC-BP and other B-capsid proteins by phosphorimaging (Table 1).

Although the phosphorimager provides a high degree of sensitivity and accuracy in data quantification, uncertainty was introduced into our calculations by the fact that we do not know whether the translational start methionine is present or absent on each protein. Because of this, four sets of calculations were averaged to obtain values compatible with either situation (Table 1, footnote *a*). Additional calculations showed that the molar ratio for at least four of the HCMV B-capsid proteins (i.e., MCP:AP:mCP:mC-BP) approximates those for the counterpart proteins of HSV and EHV (i.e., ≈3:3:2:1, respectively) (Table 1).

Finally, we used the GAL4 two-hybrid system to demonstrate that the HCMV mC-BP and mCP can interact with each other, as can their respective HSV counterparts, VP19c and VP23 (14). Considering that these proteins are in a 1:2 molar ratio and that the triplex that they form is thought to interact with the MCP (14, 42), it seemed plausible that additional intermolecular interactions (e.g., mCP/mCP, MCP/mCP, or MCP/mC-BP) might be detected by this method. Although no interactions were identified between either the mC-BP or mCP and the MCP, AP, or SCP, we did observe an mCP self-interaction.

These findings are consistent with the calculated molar ratio of 1:2 for mC-BP:mCP and indicate that the mCP has two sites for intermolecular interaction: one for an mCP/mCP self-interaction and one for an mCP/mC-BP interaction. This is suggestive of the pAP, which self-interacts through a conserved domain near its amino end (67) and interacts with the MCP through a conserved domain at its carboxy terminus (14, 28, 67). In the case of the pAP/MCP interaction, one apparent functional consequence is that the MCP (which lacks a nuclear localization signal [NLS]) can be cotranslocated into the nucleus with its pAP escort, which contains two NLSs (47). Unlike the MCP, which is too large to enter the nucleus without an NLS, both mCP and mC-BP could enter by diffusion. However, a triplex composed of two mCP and one mC-BP (i.e., ≈100 kDa) would be larger than the 60-kDa nuclear pore cutoff for entry by diffusion and would require an NLS. It may be relevant, in this connection, that the CMV mCP lacks an obvious NLS, but the mC-BP contains two potential NLSs. Whether either mC-BP NLS is functional and whether an mCP/mC-BP triplex forms in the cytoplasm are questions of interest.

In summary, the results presented in this report show that HCMV mC-BP has at least three characteristics in common with HSV VP19c and provide evidence that the two proteins are functional as well as genetic homologs. We have not tested whether mC-BP has DNA-binding properties, as does VP19c (6), nor have we determined whether it is essential for capsid formation, as has been shown for VP19c (46, 59, 60). It will be important to determine whether these two characteristics are also general features of VP19c homologs. The two most conspicuous differences between the CMV mC-BP and HSV VP19c homologs are their sizes and sensitivities to heating. The significance of these differences, and an understanding of whether the two properties are related, is likely to come from further studies of their function and their interactions with other components of the capsid or virion.

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