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Human Cytomegalovirus (HCMV) Smallest Capsid Protein Identified as Product of Short Open Reading Frame Located between HCMV UL48 and UL49

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The capsid of cytomegalovirus contains an abundant, low-molecular-weight protein whose coding sequence within the viral genome had not been identified. We have used a combination of biochemical and immunological techniques to demonstrate that this protein, called the smallest capsid protein in human cytomegalovirus, is encoded by a previously unidentified 225-bp open reading frame (ORF) located between ORFs UL48 and UL49. This short ORF, called UL48/49, is the positional homolog of herpes simplex virus ORF UL35 (encoding capsid protein VP26) and shows partial amino acid sequence identity to positional homologs in human herpes viruses 6 and 7.

Capsids of cytomegalovirus (CMV), like those of herpes simplex virus (HSV), contain an abundant, low-molecular-weight protein that in CMV is called the smallest capsid protein (SCP) (15). In HSV this 12-kDa protein (6, 13) is called VP26 and is encoded by a 336-bp open reading frame (ORF), UL35 (7, 16). VP26 has a computer-predicted pI of 11.6, is phosphorylated (17), and is present in approximately equimolar amounts with the major capsid protein, VP5 (HSV type 1 UL19 product) (18). Computer-aided reconstruction experiments of the HSV capsid, imaged by cryoelectron microscopy, indicate that VP26 is located at the outer tips of the hexameres but not the pentameres (3, 26, 28, 29). Although the function of this protein has not yet been determined, it has been shown that VP26 is not essential for HSV capsid formation in the recombinant baculovirus-insect cell assembly system (23, 24) or for HSV replication in cell culture (8).

The ORF that encodes the CMV SCP had not been identified; however, by analogy with its putative HSV counterpart, the CMV ORF was predicted to be downstream and in the opposite transcriptional orientation of the longest ORF in the genome and to encode a small, electropositive protein. Human CMV (HCMV) UL49, which is downstream and in opposite transcriptional polarity of the longest CMV ORF (UL48), is nearly five times longer than needed to encode the CMV SCP. Moreover, antisera to the predicted amino and carboxyl ends of the UL49 protein were nonreactive with the SCP in Western immunoassays (WIA) (unpublished data).

When we looked more closely at the nucleotide sequence (GenBank accession number X17403) in the UL48-to-UL49 region of the genome, we found a 225-bp sequence between UL48 and UL49 that has the characteristics listed above for a CMV homolog of HSV UL35. This 225-bp ORF escaped recognition as a likely gene in the original sequence analysis because it did not satisfy the selection criterion of having recognized upstream transcriptional regulatory sequences (5). We show here that this HCMV sequence, which we have called UL48/49 because of its location between the previously designated UL48 and UL49 ORFs, encodes the HCMV SCP.

Our experimental approach was to clone and express the HCMV UL48/49 ORF in vitro, demonstrate that the resulting protein is recognized by a monoclonal antibody (MAb) specific for the SCP (MAb11.2.23: made in BALB/c mice injected with gradient-purified virions; screened by immunofluorescence on HCMV-infected human fibroblasts), and establish that the amino acid sequence of the SCP is consistent with the computer-predicted sequence of the UL48/49 protein.

Our first experiment was done to demonstrate that MAb11.2.23 recognizes the SCP. We recovered virions from the maintenance medium of human foreskin fibroblast cell cultures infected with HCMV, strain AD169. Two noninfectious aberrant particles were also collected for comparative purposes: (i) noninfectious enveloped particles (NIEPs), which closely resemble virions in structure and protein composition but contain no DNA and have an additional protein species (i.e., the assembly protein, ORF UL80.5), and (ii) dense bodies (DBs), which are essentially solid spheres composed predominantly of the lower matrix protein (ORF UL83), lacking both a capsid structure and its constituent proteins, and surrounded by a normal viral envelope (10, 14).

Both [^{35}S]methionine-labeled (50 μCi/ml of complete medium) and nonlabeled particles were prepared by a positive-density/negative-viscosity gradient system (2), essentially as described before (14, 22). Radiolabeled particles were banded only once in the tartrate-glycerol gradients, to maximize yield, and then collected as a pellet following 1:1 dilution with phosphate buffer (0.15 M NaCl, 0.04 M sodium phosphate, pH 7.4) and sedimentation (≈150,000 × *g*, 4°C, 4 h) through a 30% sucrose (wt/vol in phosphate buffer) cushion. Nonradiolabeled particles were banded three times in tartrate-glycerol gradients, as usual, to maximize purity.

Radiolabeled and nonlabeled pairs of virions, NIEPs, and DBs were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tricine-buffered 10 to 20% polyacrylamide gradient gels (EC66255; Novex, San Diego, Calif.) followed by electrotransfer of the proteins to Immobilon-P (Millipore, Bedford, Mass.) (25, 27) and WIA (4,

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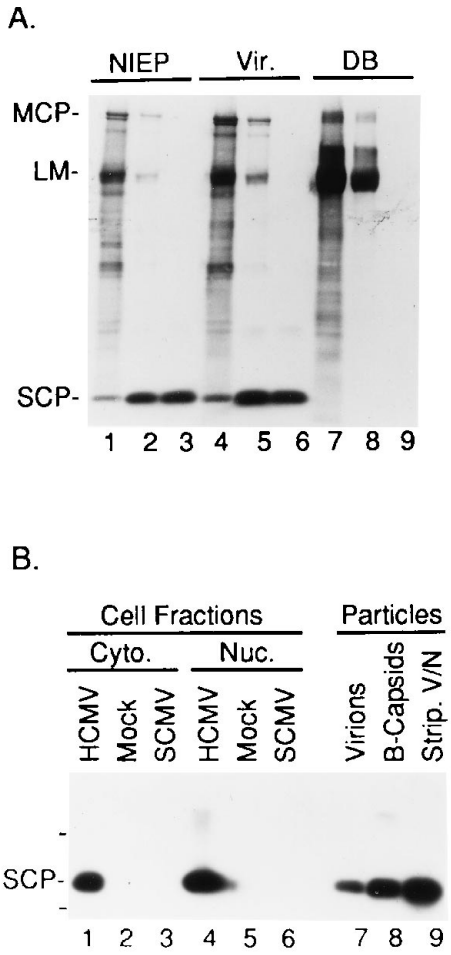

FIG. 1. (A) HCMV SCP is recognized by MAb11.2.23. [^{35}S]methionine-labeled and nonlabeled virions, NIEPs, and DBs were prepared as described in the text and subjected to Tricine-SDS-PAGE, and the proteins were electrotransferred to Immobilon-P. An autoradiogram prepared from the membrane before WIA shows the protein patterns of the radiolabeled NIEPs (lane 1), virions (Vir., lane 4), and DBs (lane 7). A fluorogram prepared after probing the membrane with MAb11.2.23. shows the lanes detected in the first autoradiogram after they had been reacted with the antibody and ^{125}I-protein A (lanes 2, 5, and 8, respectively). Proteins from the corresponding nonlabeled particles are present in lanes 3 (virions), 6 (NIEPs), and 9 (DBs). Shown here is a direct overlay composite of the autoradiogram and fluorogram. The positions of the SCP, major capsid protein (MCP), and lower matrix protein (LM) are indicated in the left margin. (B) SCP detected in HCMV-infected cells and virus particles. Cytoplasmic (Cyto.) and nuclear (Nuc.) fractions of noninfected human foreskin fibroblast cells (Mock) and human foreskin fibroblasts infected with either SCMV (strain Colburn) or HCMV (strain AD169) were subjected to Tricine-SDS-PAGE. Three types of virus particles were included in the experiment: extracellular virions, nuclear B-capsids, and a mixture of detergent-stripped virions and NIEPs (Strip. V/N). The proteins were electrotransferred to Immobilon-P and probed with MAb11.2.23, followed by ^{125}I-protein A, in a WIA. Shown here is a fluorogram prepared from the processed membrane. The position of the SCP is indicated; the molecular mass markers (indicated by dashes above and below SCP) were lysozyme (14.4 kDa) and aprotinin (6 kDa).

27) using MAb11.2.23. A composite of two images resulting from this experiment is shown in Fig. 1. An autoradiogram was prepared from the Immobilon sheet immediately following electrotransfer and shows only the [^{35}S]methionine-labeled proteins. It demonstrates that the SCP is present in the capsid-containing virions and NIEPs but not in the capsid-deficient DBs (Fig. 1A, lanes 1, 4, and 7, respectively). Because larger proteins are not electrotransferred efficiently, they are underrepresented in this figure. A fluorogram was prepared after probing the same sheet with MAb11.2.23 and shows that the immunoreactive protein in the nonlabeled particles (Fig. 1A, lanes 3 and 6) comigrated with the [^{35}S]methionine-labeled SCP (Fig. 1A, lanes 1 and 4). Lanes 2, 5, and 8 in Fig. 1A show the appearance of lanes 1, 4, and 7, respectively, after the sheet had been probed with MAb11.2.23 and demonstrate the coincidence of the ^{35}S-labeled and immunoreactive SCP bands in the NIEP and virion preparations. Calculations based on the mobility of the SCP in these Tricine gradient gels, relative to those of marker proteins (Mark 12; Novex) (e.g., Fig. 1B), indicate that the SCP is approximately 9 kDa.

We next used MAb11.2.23 to determine the intracellular distribution of the SCP in HCMV-infected cells, to see whether simian CMV (SCMV)-infected cells contain an immunologically cross-reactive protein, and to confirm that the protein recognized by the antibody is a capsid constituent. This was done by separating noninfected cells, and cells infected with either HCMV or SCMV, into cytoplasmic and nuclear fractions with Nonidet P-40 (9) and subjecting the resulting preparations to Tricine-SDS-PAGE (Fig. 1B, lanes 1 to 6). Three HCMV particle types were compared in the same gel (Fig. 1B, lanes 7 to 9). (i) Virions were recovered from HCMV-infected cell maintenance medium (14). (ii) HCMV B-capsids were recovered from the nuclear fraction of infected cells by freeze-thaw rupture, followed by rate-velocity sedimentation in 15 to 50% (wt/vol in phosphate buffer) sucrose gradients (9, 15). (iii) Detergent-stripped virions and NIEPs were prepared by treating a mixture of virions and NIEPs with a solution containing 0.5% Nonidet P-40, 10 mM dithiothreitol, and 0.5 M NaCl in 50 mM Tris, pH 7.4 (stripping solution), and then sedimenting the resulting particles through a 10 to 30% glycerol gradient (prepared in stripping solution) and collecting the pellet from the bottom of the tube (18a).

Following Tricine-SDS-PAGE, proteins in the cell fractions and particle preparations were electrotransferred to Immobilon-P and probed in a WIA with MAb11.2.23. A fluorogram prepared from the resulting membrane shows that the SCP was somewhat more abundant in the nuclear fraction than in the cytoplasmic fraction of HCMV-infected cells (Fig. 1B, compare lanes 1 and 4). This experiment also shows that although SCMV has a counterpart SCP (15), it was not recognized by MAb11.2.23 in SCMV-infected cells (Fig. 1B, lanes 3 and 6) or B-capsids (data not shown). Finally, this experiment demonstrates that the SCP is present in two preparations that contain predominantly capsid proteins, nuclear B-capsids (Fig. 1B, lane 8), and detergent-stripped virions and NIEPs (Fig. 1B, lane 9).

We conclude from these first two experiments that (i) MAb11.2.23 reacts with the protein designated the HCMV SCP (15); (ii) this protein is a capsid constituent on the basis of its presence in all capsid-containing particles tested (i.e., virions, NIEPs, B-capsids, and detergent-stripped virions and NIEPs) and its absence from DBs, which contain neither a capsid nor capsid proteins (15); and (iii) the SCMV counterpart SCP is not immunologically cross-reactive with MAb11.2.23.

In order to establish a relationship between the HCMV SCP and UL48/49, we tested the ability of MAb11.2.23 to immunoprecipitate the protein product of UL48/49. To do this, we first cloned the UL48/49 ORF into a transcription vector. *Pfu* DNA polymerase (no. 600153; Stratagene, La Jolla, Calif.) was used in PCR to copy and amplify the UL48/49 nucleotide sequence from the *Hin*dIII-F fragment of HCMV strain AD169 (from Mark Chee and Bart Barrell). Appropriate primers were designed to place a *Sal*I site at the 5′ end of the resulting PCR product and a *Bam*HI site at the 3′ end. The resulting PCR fragment was digested with *Sal*I and *Bam*HI to form the spe-

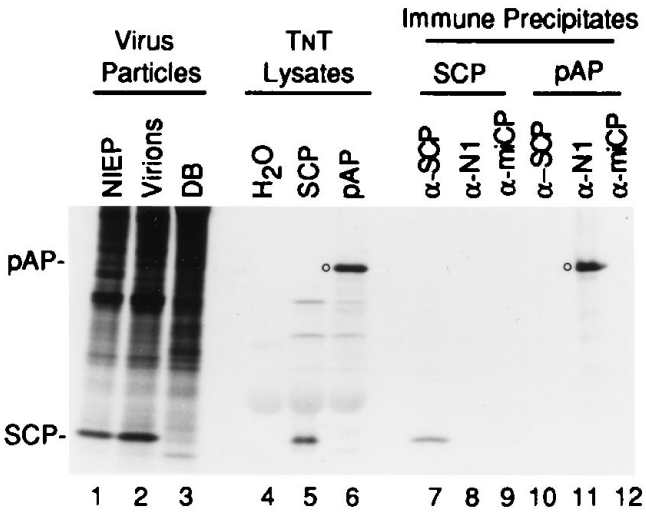

FIG. 2. Protein encoded by HCMV UL48/49 is immunoprecipitated by MAb11.2.23. [^{35}S]methionine-labeled NIEPs, virions, and DBs were subjected to Tricine-SDS-PAGE, together with samples from the immunoprecipitation experiment described in the text. The [^{35}S]methionine-labeled in vitro translation products used in the immunoprecipitations are shown in lanes 4 (no template added), 5 (UL48/49 encoding SCP), and 6 (APNG.5 encoding SCMV pAP). The SCP and pAP translation products were immunoprecipitated, separately, with protein A beads precharged with MAb11.2.23 (lanes 7 and 10), Anti-N1 (lanes 8 and 11), or Anti-miCP (lanes 9 and 12). Shown here is an autoradiogram prepared from the fixed, Coomassie brilliant blue-stained, and dried gel. The positions of SCP and pAP are indicated in the left margin; circles adjacent to lanes 6 and 11 indicate pAP.

cific 5′ and 3′ overhangs needed and ligated into the pGEM-4Z vector (Promega, Madison, Wis.), which had also been cleaved with *Sal*I and *Bam*HI and gel purified. The resulting mixture was transformed into competent *E. coli* XL1-Blue (Stratagene); colonies were picked and screened for the recombinant plasmid; and DNA from one such recombinant, pMB100, was prepared by chromatography (columns from Qiagen, Chatsworth, Calif.) as described by the manufacturer. The predicted sequence of the insert was confirmed by dideoxynucleotide sequence analysis (20) of both strands. Standard techniques (1, 19) were used in these manipulations.

The resulting plasmid DNA was used as the template in an in vitro coupled transcription and translation system (TNT; Promega) supplemented with [^{35}S]methionine (ICN, Cleveland, Ohio). As a control, a plasmid (SP1) encoding an SCMV assembly protein precursor (pAP) was similarly transcribed and translated. Equal portions of the resulting preparations were subjected to both specific and nonspecific immunoprecipitations with MAb11.2.23 (specific for the SCP), rabbit antipeptide antiserum Anti-N1 (specific for the pAP [21]), and rabbit antipeptide antiserum Anti-mCP (specific for the minor capsid protein, HCMV UL85 [12]), each prebound to protein A beads (Sigma, St. Louis, Mo.). Samples of both the starting lysates and the resulting immunoprecipitates were subjected to Tricine-SDS-PAGE, and an autoradiogram was prepared from the Coomassie brilliant blue-stained and dried gel (Fig. 2). Portions of the [^{35}S]methionine-labeled virion, NIEP, and DB preparations described above were included as markers to identify the position of the SCP.

Results of this experiment showed the following. First, a protein that comigrated with the virion and NIEP SCP (Fig. 2, lanes 1 and 2, respectively) was present in the transcription-translation lysate of UL48/49 (Fig. 2, lane 5). Second, this protein was selectively immunoprecipitated by MAb11.2.23 (Fig. 2, lane 7) and was nonreactive with the control antisera, Anti-N1 and Anti-miCP (Fig. 2, lanes 8 and 9). And third, the control pAP protein present in the starting lysate (Fig. 2, lane 6) was selectively immunoprecipitated by its specific antiserum, Anti-N1 (Fig. 2, lane 11), and was nonreactive with MAb11.2.23 and Anti-mCP (Fig. 2, lanes 10 and 12). These results establish that MAb11.2.23, which recognizes the HCMV SCP in Western assays (Fig. 1), reacts specifically with the protein product of UL48/49 synthesized in vitro, thereby providing evidence that UL48/49 encodes the SCP.

Finally, we submitted a sample of the HCMV virion SCP for amino acid sequence analysis. The sample was prepared by subjecting glycerol-tartrate gradient-purified virions to Tricine-SDS-PAGE; electrotransferring the separated proteins to Immobilon-P; locating the SCP by staining with Coomassie brilliant blue; cutting the SCP protein bands from the Immobilon sheet as rectangles (1.5 by 7 mm); and destaining them, soaking them in water, and storing them at −20°C until analyzed. These two SCP-bearing fragments were subjected to three cycles of gas-phase amino acid sequence analysis (492 Protein Sequencer; Applied Biosystems, Inc., Palo Alto, Calif.) and yielded no specific signal, suggesting that the amino terminus of the protein was blocked.

As a way around this problem, we treated the Immobilon-bound SCP with CNBr, which could cleave the UL48/49 protein into four fragments (see Fig. 3B). This was done by saturating the air-dried fragments with a solution containing 70 μg of CNBr per ml of 70% formic acid (about 10 μl per fragment) and allowing the reaction to proceed in the dark at room temperature overnight (11). Two additional virion SCP-bearing fragments of Immobilon-P^{SQ} (Millipore) were prepared and similarly treated with CNBr (omitting the first sequencing step) to increase the total amount of SCP available for analysis. After eliminating the CNBr and formic acid by evaporation (SpeedVac, room temperature, overnight), the four fragments were subjected to sequence analysis. Signals in the low picomole range were obtained, and the analysis was continued for 11 cycles. When the amino acids detected at each cycle (Fig. 3A) were matched with the computer-predicted sequences for the four UL48/49 peptides, as described before (Fig. 11 in reference 11), 17 residues could be assigned unambiguously to three of the four peptides (boldface letters, Fig. 3A; enclosed residues, Fig. 3B). Trace amounts of four nonpredicted amino acids were detected, two in cycle 1 (i.e., Glu and Ala), one in cycle 7 (i.e., Gly), and one in cycle 11 (i.e., Ala) (lightface letters, Fig. 3A).

We interpret the data from this experiment as follows. First, the amino terminus of the SCP prepared in this way is blocked, as indicated by the absence of sequence from the intact protein and from CNBr-peptide 1 (Fig. 3B). Second, the absence of predicted Cys (peptide 3, cycle 8), Arg (peptide 2, cycle 6; peptide 4, cycles 7 and 8), Ser (peptides 2 and 3, cycle 2; peptide 4, cycle 4), and Thr (peptide 4, cycle 6) is attributed to the facts that Cys is destroyed during sequencing and Arg, Ser, and Thr are comparatively difficult to detect. Third, cleavage under the conditions used appears to have been complete, since the sequence that would have resulted from incomplete cleavage at Met-3 (i.e., M-S-S-L-F, cycles 7 to 11) was not detected. And finally, the unambiguous correspondence of the amino acids detected at each cycle with those predicted for three of the four UL48/49 CNBr-peptides is compelling evidence that the HCMV SCP is the product of that ORF.

In conclusion, we have presented evidence, based on immunological reactivity and amino acid sequence correspondence, showing that the HCMV SCP is encoded by the 225-bp ORF, designated UL48/49, that lies between UL48 and UL49. Hu-

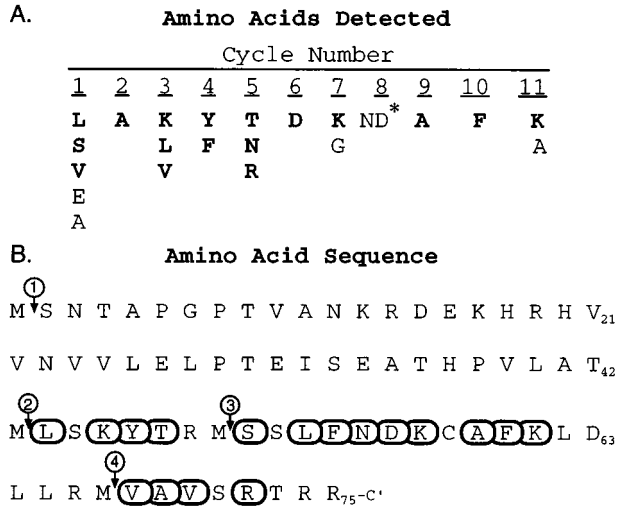

FIG. 3. Amino acid sequence data confirm that HCMV UL48/49 encodes SCP. Fragments of Immobilon membrane bearing electrotransferred HCMV SCP were prepared and treated with CNBr as described in the text. (A) Amino acids that match (boldface) those predicted at each cycle, as well as the four nonpredicted amino acids (lightface). (B) Amino acid sequence of UL48/49. Predicted residues that were detected (A) at each cycle (enclosed letters), the four methionine bonds potentially cleaved by CNBr (encircled numbers), the carboxyl end of the protein (C′), and amino acid sequence numbers (right side) are indicated.

man herpesviruses 6 and 7 both have positionally homologous ORFs (i.e., human herpesvirus 6 U32, GenBank number X83413; human herpesvirus 7 bp 46628 to 46356, GenBank number U43400) whose predicted proteins show 37 and 31% amino acid sequence identity, respectively, with the HCMV SCP. Furthermore, like their positional homolog UL35 in HSV type 1 that encodes VP26, all share the characteristics of being immediately adjacent to the largest ORF in their respective genome and in the opposite transcriptional polarity and coding for a small protein (≈10 kDa) with a basic pI. Identification of this HCMV homolog of HSV VP26 is expected to aid further studies of the structure and assembly of CMV capsids and add to our understanding of the functional similarities and differences between herpes group viruses.

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