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Assembly of Herpes Simplex Virus Capsids Using the Human Cytomegalovirus Scaffold Protein: Critical Role of the C Terminus

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An essential step in assembly of herpes simplex virus (HSV) type 1 capsids involves interaction of the major capsid protein (VP5) with the C terminus of the scaffolding protein (encoded by the UL26.5 gene). The final 12 residues of the HSV scaffolding protein contains an A-X-X-F-V/A-X-Q-M-M-X-X-R motif which is conserved between scaffolding proteins found in other alphaherpesviruses but not in members of the beta- or gamma-herpesviruses. Previous studies have shown that the bovine herpesvirus 1 (alphaherpesvirus) UL26.5 homolog will functionally substitute for the HSV UL26.5 gene (E. J. Haanes et al., J. Virol. 69:7375–7379, 1995). The homolog of the UL26.5 gene in the human cytomegalovirus (HCMV) genome is the UL80.5 gene. In these studies, we tested whether the HCMV UL80.5 gene would substitute for the HSV UL26.5 gene in a baculovirus capsid assembly system that we have previously described (D. R. Thomsen et al., J. Virol. 68:2442–2457, 1994). The results demonstrate that (i) no intact capsids were assembled when the full-length or a truncated (missing the C-terminal 65 amino acids) UL80.5 protein was tested; (ii) when the C-terminal 65 amino acids of the UL80.5 protein were replaced with the C-terminal 25 amino acids of the UL26.5 protein, intact capsids were made and direct interaction of the UL80.5 protein with VP5 was detected; (iii) assembly of intact capsids was demonstrated when the sequence of the last 12 amino acids of the UL80.5 protein was changed from RRIFVA ALNKLE to RRIFVAAMMKLE; (iv) self-interaction of the scaffold proteins is mediated by sequences N terminal to the maturation cleavage site; and (v) the UL26.5 and UL80.5 proteins will not coassemble into scaffold structures. The results suggest that the UL26.5 and UL80.5 proteins form a scaffold by self-interaction via sequences in the N termini of the proteins and emphasize the importance of the C terminus for interaction of scaffold with the proteins that form the capsid shell.

Herpesvirus capsid formation is the first step in viral morphogenesis. Assembly of the herpes simplex type 1 (HSV-1) capsid requires the products of six genes, UL19, UL38, UL26, UL26.5, UL18, and UL35, encoding seven proteins, VP5, VP19C, VP21 and VP24 (both encoded by UL26), VP22a, VP23, and VP26, respectively (5, 7, 9, 26, 27, 34, 35, 37, 39–41). The product of the UL26.5 gene (pre-VP22a) is a phosphorylated self-assembling protein that serves as a scaffold around which the other capsid proteins (VP5, VP19C, VP23, and VP26) condense to form the capsid shell (28, 29). The UL26.5 gene is initially translated as a 38-kDa protein which is proteolytically cleaved to a 34-kDa protein by a protease (3, 22, 38). The 80-kDa protease responsible for this cleavage is encoded by the HSV-1 UL26 gene. The catalytic site in the UL26-encoded protease is located within the amino-terminal 247 amino acids and autoproteolytically cleaves two highly conserved sequences within the full-length protein. The two sites are referred to as the release (R) site at amino acids 247 and 248 and the maturational (M) site at amino acids 610 and 611, yielding the proteolytically active 29-kDa VP24 protein and a larger 45-kDa protein, VP21 (8, 10, 23). The UL26 and UL26.5 genes are expressed as 3′-coterminal transcripts. The UL26.5 open reading frame (329 amino acids) overlaps and is in frame with the UL26 open reading frame (635 amino acids); therefore, the UL26.5-encoded protein possesses the same M site present in the full-length UL26 protein.

Homologs of the UL26 and UL26.5 genes have been identified among other members of the herpesvirus family (1, 6, 13, 16, 42, 47). As with HSV-1, these two genes are encoded as 3′-coterminal transcripts, with the larger gene encoding the autoproteolytic protease. While the degree of homology between the amino acid sequences of the scaffold proteins may differ markedly among the herpesviruses, the M site and the R site are highly conserved among the proteases or scaffold proteins (15, 47). Furthermore, the functional nature of the proteins appears to be highly conserved. For example, the scaffold protein from bovine herpesvirus (BHV), whose amino acid sequence is 41% identical to that of HSV-1, can substitute for the HSV-1 scaffold protein in a recombinant baculovirus system used to generate HSV-1 B capsids (13). The conserved nature of the proteases and scaffolding proteins among the herpesviruses suggests the prospect of a novel broadly active antiherpesvirus therapeutic agent.

As mentioned above, pre-VP22a is cleaved in *trans* at the M site by the UL26 protease, releasing a 25-amino-acid peptide (8). While cleavage of pre-VP22a is required for the production of infectious virus, it is not required for capsid assembly (11, 37, 43, 45). In fact, recent studies have shown that the C-terminal 25 amino acids of pre-VP22a are required for capsid assembly (20, 25, 44). The 25-amino-acid peptide is also required for interaction of the HSV scaffold protein with the major capsid protein, VP5 (18, 44). An essential sequence (A-X-X-F-V/A-X-Q-M-M-X-X-R) within the last 12 amino

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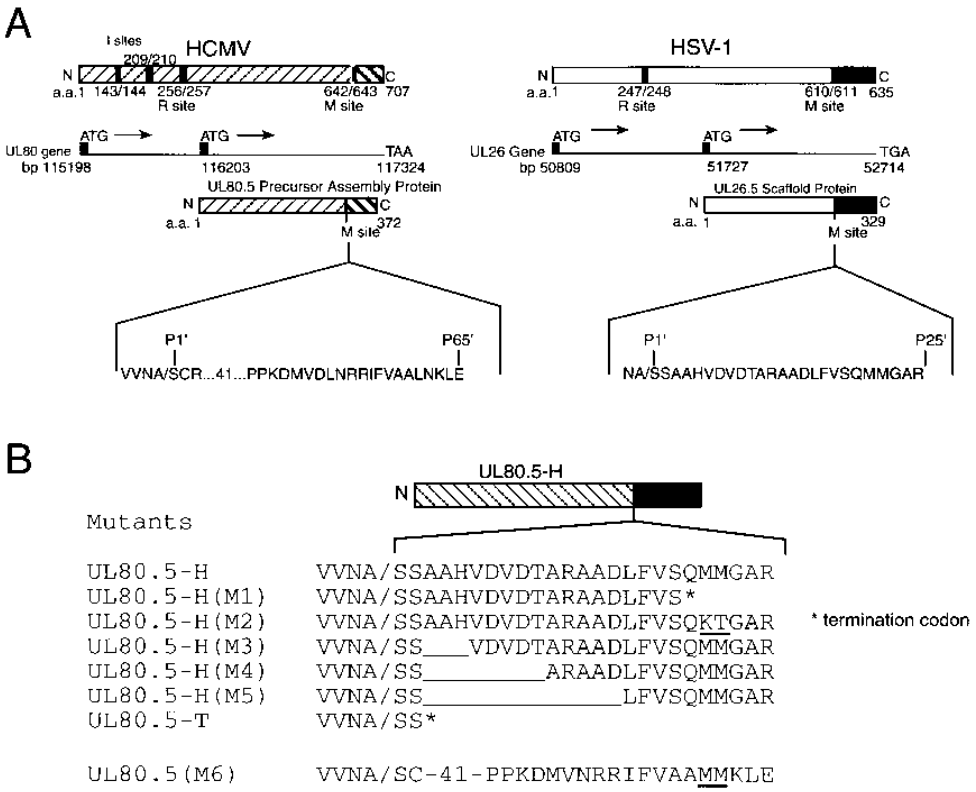

FIG. 1. (A) Top, schematic representation of the HCMV UL80 (left) and HSV-1 UL26 (right) genes encoding the protease and their respective 3′-coterminal scaffold proteins. Bottom, amino acid (a.a.) sequences of the C termini of the UL80 and UL26 proteins. The 41-amino-acid sequence not shown in the UL80.5 sequence is LATASGSEAATAGPSTAGSSSCPASVVLAAAAAQAAAASQS. (B) Amino acid sequences of UL80.5 mutants. UL80.5-T was generated by removing the C-terminal 65-amino-acid peptide from the UL80.5 gene and insertion of a stop codon. UL80.5-H was generated by replacing the C-terminal 65-amino-acid peptide of the UL80.5 protein with DNA sequences coding for the C-terminal 25-amino-acid peptide of the UL26.5 gene. UL80.5-H(M1) through UL80.5-H(M5) are UL80.5-H mutants with deletions or substitutions of amino acids within the HSV-1 C-terminal 25-amino-acid peptide. UL80.5(M6) is a UL80.5 mutant in which amino acids P61′ and P62′ were substituted with methionine residues.

acids of pre-VP22a has been identified (18). This minimal domain is conserved among the scaffold proteins within the alphaherpesvirus family, while scaffold proteins from members of the betaherpesviruses and gammaherpesviruses lack the conserved domain (18).

Homologs of the HSV-1 UL26 and UL26.5 genes in the human cytomegalovirus (HCMV) genome are encoded by the UL80 and UL80.5 genes (4, 46, 47). The 85-kDa protease encoded by the UL80 gene autoproteolytically cleaves at amino acid 256 and 257, yielding a 30-kDa protein, and at amino acids 642 and 643, releasing a 57-kDa protein (Fig. 1) and a 65-amino-acid peptide (2, 12, 48). The catalytic site resides within the 30-kDa amino-terminal peptide. Internal cleavage sites have also been identified within the protease domain at amino acid residues 143 and 144 and at amino acid residues 209 and 210 (2, 19, 47). The 143/144 cleavage site was initially referred to as an inactivation domain, although it has recently been shown that cleavage at this site does not inactivate the protease (14, 17, 32). The UL80.5 gene encodes the 44-kDa scaffold protein, which is processed by the UL80 protease to a 37-kDa protein with the release of the 65-amino-acid C-terminal peptide. In addition to the increased length of the 65-amino-acid C terminal peptide, the amino acid sequence is markedly different from the HSV-1 C-terminal 25-amino-acid peptide. To further evaluate the specificity of the herpesvirus scaffold proteins in capsid assembly, we have constructed baculovirus recombinants expressing the HCMV UL80.5 protein and tested whether the HCMV scaffold protein would support capsid assembly and if it formed a stable complex with the HSV major capsid protein.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Baculovirus *Autographa californica* nuclear polyhedrosis virus was grown in *Spodoptera frugiperda* (Sf9) cells (ATCC CRL 1711) as previously described (45). Recombinant baculoviruses expressing the HSV UL18, UL19, UL26.5, UL26.5/304, UL35, and UL38 genes have been described elsewhere (44, 45). Monoclonal antibody MCA406 (Serotec Inc.) was used to analyze the UL26.5 proteins (45). The UL19, UL38, and UL18 proteins were analyzed by using rabbit polyclonal antisera NC1 (UL19), NC2 (UL38), and NC5 (UL18), provided by Roselyn Eisenberg and Gary Cohen of the University of Pennsylvania, Philadelphia (5).

Isolation of HCMV DNA. Subconfluent monolayers of human foreskin fibroblasts cultured in Corning 150-cm^2 tissue culture flasks were inoculated with HCMV strain Ad169 (American Type Culture Collection) at a multiplicity of infection of 0.005 in medium containing 10% fetal calf serum. Medium changes were performed at 3- to 4-day intervals. Cells were harvested 10 days postinfection, and HCMV viral DNA was isolated as described by Oram et al. (33) and LaFemina and Hayward (21), with the following modifications. Supernatant from infected cells was transferred to 250-ml polypropylene bottles and centrifuged at 3500 × *g* for 15 min. Cleared supernatant was transferred to a second set of bottles and centrifuged at 12,000 × *g* for 90 min at 4°C. The supernatant was decanted, and the pellet was resuspended in 0.8 ml of 10 mM Tris-HCl–10 mM $MgCl_2$. DNase was added to a final concentration of 0.2 mg/ml, and the sample was incubated 1 h at 37°C. An equal volume of 2× lysis buffer (0.8 M NaCl, 10 mM Tris-HCl [pH 8.0], 200 mM EDTA, 1% sodium dodecyl sulfate [SDS], 4% Sarkosyl, 2 mg of pronase per ml) was then added, and the sample was incubated overnight at 37°C. The sample was extracted once with phenol and twice with $CHCl_3$-isoamyl alcohol (24:1), then dialyzed against 10 mM Tris-HCl (pH 8.4)–1 mM EDTA–300 mM NaCl, and finally dialyzed against 10 mM Tris–10 mM EDTA (pH 8.4).

Construction of recombinant baculoviruses expressing wild-type and mutant forms of the HCMV UL80.5 gene. Sequence information used for cloning follows that of Chee et al. (4) The 11.4-kb *Hin*dIII L fragment from the HCMV genome, containing the entire UL80.5 gene, was cloned into pUC18. PCR was used to introduce an *Eco*RI restriction site immediately upstream from the initiation codon and downstream from the termination codon of the UL80.5 open reading frame. Oligonucleotide primers for PCR, 5′-GGCGAATTCAATGTCGCACC CTCTGAGT-3′ and 5′-GCCGAATTCAAGCTTGAGGGAAGCCCTAAATA TAGCGTCTCTC-3′, corresponded to nucleotides 116203 to 116220 and 117325 to 117351, respectively. The 1.2-kb fragment generated by PCR was digested with *Eco*RI and inserted into the *Eco*RI site of pGEM-3Zf(+) (Promega), and the entire PCR clone was sequenced.

The UL80.5 gene was cloned for expression in a recombinant baculovirus by using the baculovirus transfer vector, pVL941. The *Eco*RI fragment from pGEM-3Zf(+) containing the UL80.5 gene was made blunt by using Klenow polymerase and inserted in the *Bam*HI site of pVL941, also made blunt by using Klenow polymerase. The resulting isolate was then screened for proper orientation of the UL80.5 gene with respect to the baculovirus polyhedron promoter.

A C-terminal truncation of the UL80.5 protein (UL80.5-T; Fig. 1B) was constructed by introducing an *Xho*I site at position 117127 of the UL80.5 gene by using PCR. This region occurs at the C-terminal cleavage site of the UL80.5 protein. Reverse and forward oligonucleotide primers were 5′-GGGCCAAGC TTGAATTCAGATCTCAACTCGAGGCGTTCACCACGCCGGCCTGAGC G-3′ and 5′-GGCGGACACAACAAACGCCGTAAGG-3′, respectively (the *Hin*dIII and *Xho*I restriction sites in the reverse primer are underlined). The forward primer corresponds to a region 30 bp upstream (nucleotides 116713 to 116737) from a unique *Sac*II site in the UL80.5 gene. The coding sequence at the 3′ end of the truncated gene generated by PCR was 5′-GC**CTCGAGT**TGA-3′. The underlined sequence is the *Xho*I site. The letters in boldface indicate the reading frame demonstrating codons for two serines followed by a stop codon. Therefore, the amino acid sequence in the UL80.5-T gene was altered from VNA/SC to VNA/SSstop, which is in identity with the UL26.5 sequence at the C-terminal cleavage site (Fig. 1B). In addition to an *Xho*I site, the reverse primer also contained a *Hin*dIII site downstream of the new stop codon. The approximately 400-bp PCR product was digested with *Sac*II and *Hin*dIII and ligated into the *Sac*II/*Hin*dIII site of UL80.5 cloned in pGEM-3Zf(+) to yield pGEM-U L80.5-T gene. The PCR amplified region of the gene was confirmed by DNA sequencing.

In the UL80.5-H mutant (Fig. 1B) the C-terminal 65 amino acids of the UL80.5 protein were replaced with the C-terminal 25 amino acids of the HSV-1 UL26.5 protein. PCR of the template plasmid pAcUL26.5 (45) was used to generate the HSV-1 UL26.5 C-terminal 25-amino-acid coding sequence, using the forward and reverse primers 5′-GGCGGCCTCGAGTGCAGCACACGTG GACGTTGACACGG-3′ and 5′-CCGGGAAGCTTAGATCTGGTACCAAA GACCGGG-3′, respectively. The forward primer contains an *Xho*I site (underlined), while the reverse primer, which corresponds to the region downstream from the stop codon of UL26.5 contains both *Hin*dIII and *Kpn*I sites (underlined). The resulting 100-bp PCR fragment was digested with *Hin*dIII and *Xho*I and ligated into the *Hin*dIII/*Xho*I site of pGEM-UL80.5-T to generate pGEM-UL80.5-H. The pGEM-UL80.5-T and pGEM-UL80.5-H constructs genes were digested with *Eco*RI and *Hin*dIII and ligated into the pVL941 baculovirus transfer vector by blunt-end ligation as described above for cloning of the wild-type UL80.5 gene in this vector.

Truncation and substitution mutants within the C-terminal 25 (P1′ to P25′) amino acids of the UL80.5-H gene were constructed by PCR (Fig. 1B). In the UL80.5(M1) mutant, DNA sequences coding for the last six (P20′ to P25′) amino acids were deleted and replaced with a stop codon. While in the UL80.5-H(M3), UL80.5-H(M4), and UL80.5-H(M5) mutants, DNA sequences coding for amino acids P3′ to P5′, P3′ to P10′, and P3′ to P15′, respectively, were deleted. In the mutant UL80.5-H(M2), sequences encoding amino acids P21′ and P22′ were changed, resulting in the substitution of the two methionines found at these positions with lysine and threonine residues, respectively.

Reverse primers for UL80.5-H(M1) (5′-AGAGGTACCTCAAGAGACGAA CAAATCGGC-3′) and UL80.5(M2) (5′-AGAGGTACCTCAGCGGGCCCC AGTCTTCTGAGAGACGAAC-3′) were used with the forward primer that was used to generate the UL80.5-H construct, using pAcUL26.5 as a template. The PCR amplified fragments were digested with *Xho*I and *Kpn*I (underlined) and used to replace the *Xho*I/*Kpn*I fragment of the UL80.5 gene cloned in pVL941.

Forward primers for UL80.5-H(M3) (5′-GGCGGCCTCGAGTGTGGACGT TGACACGGCCGC-3′) and for UL80.5-H(M4) (5′-GGCGGCCTCGAGTGC CCGCGCCGCCGATTTGTTCG-3′) were used with reverse primer 5′-CAAC AACGCACAGAATCT-3′. Template plasmid for PCR was pAcUL26.5. The reverse primer corresponded to sequences downstream from a unique *Kpn*I site in pAcUL26.5. The PCR amplified fragments were digested with *Xho*I (und erlined) and *Kpn*I and used to replace the *Xho*I/*Kpn*I fragment of the UL80.5-H gene that was cloned in pVL941.

UL80.5-H(M5) was generated by annealing the 54-base oligonucleotide 5′-C CGCCTCGAGTTTGTTCGTCTCTCAGATGATGGGGGCCCGCTGAGGT ACCGGGC-3′ with its complement to generate a double-stranded molecule. The DNA was digested with *Xho*I and *Kpn*I (underlined), and the resulting fragment was used to replace the *Xho*I/*Kpn*I fragment of the UL80.5-H gene cloned in pVL941. The UL80.5(M6) mutant was generated by annealing the 66-base oligonucleotide 5′-GCCTGGTAGATCTGAATCGGCGGATTTTTG TGGCTGCGATGATGAAGCTCGAGTAAGGTACCGCCG-3′ with its complement to generate a double-stranded molecule. The DNA was digested with *Bgl*II and *Kpn*I (underlined), and the resulting fragment was used to replace the *Bgl*II/*Kpn*I fragment of the UL80.5 gene that was cloned in pVL941. The resulting clone contains a mutant form of the UL80.5 gene in which amino acids P61′ leucine and P62′ asparagine of the C-terminal 65-amino-acid peptide were changed to P61′ methionine and P62′ methionine.

Capsid purification. Suspension cultures (100 ml) of Sf9 cells were infected with baculovirus recombinants at a multiplicity of infection of 5 (each virus), and at 64 h postinfection, the cells were harvested and capsid or capsid structures were purified by double banding on 20 to 65% sucrose gradients as previously described (44).

Western immunoblots and transmission EM. Sf9 cells infected with recombinant baculoviruses were prepared for Western blot analysis or for transmission electron microscopy (EM) (thin sections) as described previously (44).

Coimmunoprecipitation of VP5 with either the HSV or HCMV scaffold proteins. Cell extracts were prepared from Sf9 cells infected with baculoviruses expressing the HSV-1 UL19 gene (VP5) or from cells infected with either wild-type or mutant forms of the HSV-1 UL26.5 or HCMV UL80.5 gene. Coimmunoprecipitation of VP5 with either the HSV or HCMV scaffold proteins was performed as previously described (44). Briefly, cell extracts were prepared from cells infected with viruses expressing wild-type or mutant UL26.5 or UL80.5 genes by resuspending the cells in 4 volumes of phosphate-buffered saline (PBS), and the samples were freeze-thawed four times and then cleared by centrifugation at 16,000 × *g* for 1 min. The resulting supernatants (20 μl) were then examined by SDS-polyacrylamide gel electrophoresis (PAGE) (Coomassie-stained gel) to determine if the expressed proteins were soluble. Extracts (15 μl) containing soluble proteins alone or after mixing with UL19 extracts (15 μl) were incubated at 27°C for 1 h. Five microliters of VP5-specific monoclonal antibody 6F (30) was added to each sample, and the mixture was incubated for an additional 45 min at 27°C. The sample was then centrifuged at 16,000 × *g* for 0.5 min, and the resulting pellet was washed and resuspended in dissociation buffer prior to analysis by SDS-PAGE. Since no secondary antibodies or protein A beads are added to the assay, the 6F antibody precipitates only VP5 complexed with the scaffold protein. VP5 is most likely a monomer in solution and therefore is not large enough to precipitate when bound to 6F alone, while VP5 bound to scaffold protein forms a large complex due to self-interaction of scaffold subunits.

Production of rabbit antibody against HCMV scaffold protein. The entire coding region of the UL80.5 gene was cloned into the glutathione *S*-transferase fusion vector pGEX-3X (Pharmacia Biotech) by digesting pGEM-UL80.5 with *Eco*RI, isolating the 1.1-kb fragment generated by this digest, and inserting it into the *Eco*RI site of pGEX-3X. *Escherichia coli* (JM109) was induced with isopropylthiogalactopyranoside (IPTG), and the glutathione*S*-transferase–UL80.5 fusion protein was extracted from a 250-ml culture by sonication in PBS containing 1 mg of lysozyme per ml. The insoluble protein was recovered by centrifugation at 8,000 × *g* in a Beckman GSA rotor and resuspended in 2 ml of dissociation buffer (5 mM Tris-HCl [pH 7.5], 5% 2-mercaptoethanol, 2% SDS, 5% glycerol, 0.1 mg of bromphenol blue per ml). Seventy-five micoliters of the sample containing approximately 200 μg of fusion protein was electrophoresed on a preparative SDS–10% polyacrylamide gel. Following separation, the fusion protein was cut from the gel, crushed, and resuspended in PBS. One-third of the volume was mixed with 1 to 2 mg of alum and injected intramuscularly into a New Zealand White rabbit. A booster was repeated 2 weeks following primary injection. One month following the second injection, serum was collected. Western analysis demonstrated that the resultant antiserum (T61) reacted specifically with the UL80.5 protein expressed from a recombinant baculovirus.

RESULTS

Expression of HSV and HCMV scaffold proteins in Sf9 cells. To determine if the HCMV scaffold protein would substitute for the HSV-1 scaffold protein in an insect cell capsid assembly assay, recombinant baculoviruses that expressed the HCMV UL80.5 gene were constructed. The region of the HCMV genome that was inserted into the baculovirus transfer vector is shown in Fig. 1A. In addition to the wild-type UL80.5 gene, we constructed a truncated version of the scaffold protein (UL80.5-T) which was missing sequences coding for the last 65 amino acids of the HCMV scaffold protein (Fig. 1B). We also constructed a hybrid HCMV scaffold protein (UL80.5-H) in which sequences coding for the C-terminal 65 amino acids of the HCMV scaffold protein were replaced with sequences coding for the C-terminal 25 amino acids of the HSV scaffold protein (Fig. 1B). The HCMV proteins expressed from these recombinant baculoviruses were then examined by separately infecting Sf9 cells with each virus. At 64 h postinfection, solu-

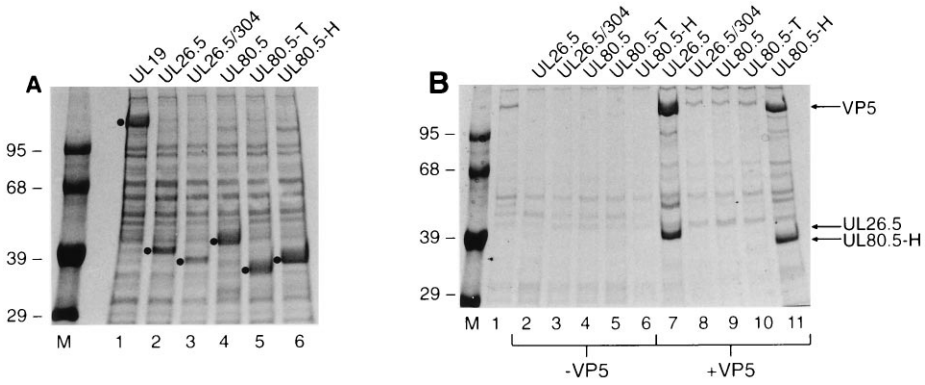

FIG. 2. (A) Polypeptides synthesized by recombinant baculovirus. Sf9 cells infected with the indicated recombinant baculovirus were lysed by repeated freeze-thawing, and the sample was cleared by centrifugation. Proteins that remained in the supernatant were separated on an SDS–12.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. The HSV or HCMV proteins expressed by each recombinant baculovirus are indicated by dots. (B) Coimmunoprecipitation of HSV or HCMV scaffold proteins with VP5. The cell extracts shown in panel A containing the indicated scaffold protein were preincubated alone (lanes 2 to 6) or following addition of an extract containing VP5 (lanes 7 to 11). Protein complexes were immunoprecipitated by addition of a VP5-specific monoclonal antibody (6F) as described in Materials and Methods. Precipitated proteins were separated on an SDS–12.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. Lane 1, VP5 extract alone showing the residual amount of VP5 that is precipitated by the 6F antibody in the absence of scaffold protein; lane M, marker proteins with molecular masses (in kilodaltons) shown at the sides. The mobilities of the capsid proteins are marked.

ble cell proteins were prepared and virus-infected cell polypeptides were separated by SDS-PAGE; after electrophoresis, the gel was stained with Coomassie blue (Fig. 2A). The UL80.5-infected cell extract contained one major band with a molecular mass of 45 kDa (Fig. 2A, lane 4). As would be predicted from DNA sequence analysis, the UL80.5 protein is slightly larger then the 38-kDa full-length HSV-1 UL26.5 protein (Fig. 2A, lane 2) or the 34-kDa truncated UL26.5/304 protein, which is missing the last 25 amino acids of the full-length protein (Fig. 2A, lane 3). Although the UL80.5-H protein (332 amino acids in length) is slightly larger than the UL26.5 protein (329 amino acids in length), the UL80.5-H protein migrated faster than the UL26.5 scaffold protein (Fig. 2A; compare lanes 2 and 6). Correspondingly, the 307-amino-acid UL80.5-T protein migrated faster than the 304-amino-acid UL26.5/304 protein (Fig. 2A; compare lanes 3 and 5).

Substitution of the HCMV UL80.5 gene for the HSV-1 UL26.5 gene in an insect cell capsid assembly model. A model for HSV-1 capsid assembly has previously been described for insect cells coinfected with recombinant baculovirus expressing the six essential HSV-1 capsid proteins (43, 45). This system was used to examine if the HCMV scaffold proteins would support assembly of HSV capsids. Sf9 cells were infected with a mixture of recombinant baculoviruses expressing the HSV-1 UL18, UL19, UL35, and UL38 genes along with either the UL26.5 or UL80.5 gene, and the cells were harvested at 64 h postinfection. The cells were either fixed and then thin sectioned for EM or layered onto 20 to 65% sucrose gradients. Following sedimentation, the B-capsid band was harvested and rebanded on a second 20 to 65% sucrose gradient. The protein composition of the double-banded sample was then determined by Western blot analysis. Capsid proteins VP5, VP19C, and VP23 were detected by using a pool of rabbit polyclonal antisera against the three proteins (Fig. 3A). Separate blots were probed with rabbit polyclonal antibody T61, for detection of the UL80.5 protein (Fig. 3B), or monoclonal antibody MCA406, for detection of the UL26.5 protein (Fig. 3C). As has been previously reported (44, 45), when Sf9 cells were coinfected with baculoviruses expressing the UL19, UL18, UL38, and UL26.5 genes, large-cored B capsids formed (Fig. 4A). Analysis of the protein composition of the capsid structures showed that they were composed of the proteins (VP5, VP19C, and VP23) that make up the capsid shell (Fig. 3A, lane 1) along with the UL26.5 scaffold protein (Fig. 3C, lane 1). Only incomplete, or aberrant, capsids were observed when the UL26.5/304 gene was used in place of the UL26.5 gene, as indicated by the presence of VP5, VP19C, and VP23 (Fig. 3A, lane 2) but lack of UL26.5/304 scaffold protein (Fig. 3C, lane 2). When the HCMV UL80.5 scaffold protein was used in place of the HSV-1 UL26.5 protein, again only aberrant capsid structures formed, as noted by the lack of scaffold protein detectable by Western blot analysis (Fig. 2B, lane 3) and by EM (Fig. 4B).

Since the C-terminal amino acid sequence in UL80.5 shares little homology with UL26.5, we wanted to determine if substitution of the C-terminal 65-amino-acid peptide of the UL80.5 gene with the C-terminal 25-amino-acid peptide of the UL26.5 gene would support formation of complete capsids. When UL80.5-H gene was substituted for UL80.5 gene, thin-section preparations of Sf9 cells demonstrated large numbers of complete, large-cored capsids (Fig. 4C). Interestingly, the inner cores of the capsids made with the UL80.5-H protein stain darker but are of the same size as the core made with the HSV scaffold protein (Fig. 4A). As expected, the capsids isolated from these cells contained the UL80.5-H protein (Fig. 3B, lane 5). In contrast only aberrant capsid structures were found when the 65-amino-acid peptide was deleted from the C terminus of the UL80.5 gene, as evidenced by the absence of the UL80.5 protein from capsid structures isolated from Sf9 cells coinfected with UL80.5-T-expressing baculovirus (Fig. 3B, lane 4). The results of these experiments demonstrated that the HCMV scaffold protein could functionally substitute for the HSV-1 scaffold protein in formation of complete capsids, although this was dependent on replacing the C-terminal 65 amino acids of the HCMV scaffold protein with the C-terminal 25-amino-acid peptide of the HSV-1 scaffold protein.

Immunoprecipitation of a UL80.5-H/VP5 complex. The HSV-1 major capsid protein, VP5, has been shown to interact with UL26.5 during capsid assembly, and this interaction is dependent on the C-terminal 25-amino-acid peptide of UL26.5 protein (20, 25, 31, 44). We have used a previously described coimmunoprecipitation assay (44) to determine if the UL80.5 scaffold protein directly interacts with the HSV major capsid protein (Fig. 2B). As previously reported (44), when the

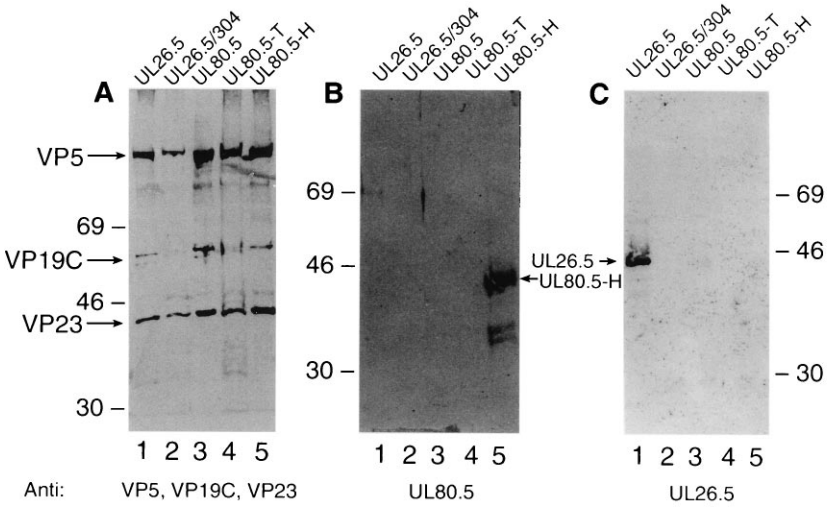

FIG. 3. Protein composition of particles harvested from sucrose gradients. Sf9 cells were coinfected with recombinant baculoviruses expressing UL18, UL19, UL35, and UL38 along with viruses expressing the indicated HSV or HCMV scaffold protein. Cells were harvested 64 h postinfection, and particles were purified by double banding on 20 to 65% sucrose gradients. Capsid proteins were electrophoresed on SDS–12.5% polyacrylamide gels and electroblotted onto nitrocellulose. (A) Western blot analysis with NC1, NC2, and NC5 as the primary antibodies identifying VP5, VP19C, and VP23. (B) Western blot analysis with T61 as the primary antibody identifying the HCMV scaffold protein UL80.5. (C) Western blot analysis with MCA406 as the primary antibody identifying the HSV scaffold protein UL26.5. Numbers at the side show molecular masses (in kilodaltons). The mobilities of the capsid proteins are marked.

HSV-1 scaffold proteins UL26.5 and UL26.5/304 were preincubated with VP5, UL26.5 coprecipitated with VP5 (Fig. 2B, lane 7) whereas UL26.5/304 did not (Fig. 2B, lane 8). Little specific protein was immunoprecipitated with extracts preincubated in the absence of VP5 (Fig. 2B, lanes 2 to 6). The UL80.5 protein did not coimmunoprecipitate with VP5, nor did the UL80.5-T protein (Fig. 2B, lanes 9 and 10). However, when the 65-amino-acid C-terminal peptide of the UL80.5 scaffold protein was replaced with the C-terminal 25 amino acids of the UL26.5 protein, the chimeric scaffold protein coprecipitated with VP5 (Fig. 2B, lane 11). These results demonstrated that specific sequences within the C-terminal end of the scaffold protein are essential for interaction of the HSV or HCMV scaffold proteins with the HSV major capsid protein.

Coassembly of the HSV and HCMV scaffold proteins. Since the HCMV UL80.5-H protein functionally substituted for the UL26.5 protein in capsid assembly, we wanted to determine if there was enough identity between the UL26.5 and UL80.5 scaffold proteins to enable interaction (coassembly) with one another. To test this, the UL26.5 protein was coexpressed with the truncated UL80.5 (UL80.5-T) protein in Sf9 cells, along with the UL18, UL19, and UL38 genes. Inversely, the UL80.5-H protein was coexpressed with the truncated UL26.5 (UL26.5/304) protein. Capsids were purified from sucrose gradients, and the protein composition of these capsids was analyzed. When UL26.5 was coexpressed with UL80.5-T, only the UL26.5 protein was found to be present in the capsid band (Fig. 5B, lane 4; Fig. 5D, lane 2). Alternatively, when UL80.5-H was coinfected with UL26.5/304, only the UL80.5-H protein was detected in purified capsids (Fig. 5B, lane 5; Fig. 5D, lane 7). Since removal of the C-terminal 25-amino-acid peptide may have altered the ability of the truncated scaffold protein to self-assemble with the full-length scaffold, capsid assembly assays were performed to determine if the truncated scaffold could coassemble with its homologous full-length scaffold. Capsid assembly was assayed in Sf9 cells coinfected with viruses expressing the UL26.5 and UL26.5/304 genes or with viruses expressing UL80.5-H and UL80.5-T genes. In this case, it was found that purified capsids contained the UL26.5 and UL26.5/304 proteins (Fig. 5B, lane 3) or the UL80.5-H and UL80.5-T proteins (Fig. 5D, lane 3) in addition to the three capsid shell proteins, VP5, VP19C, and VP23 (Fig. 5A, lane 3; Fig. 5C, lane 3). To determine if UL80.5 could interact with UL80.5-H to form capsids, these two recombinants were used to coinfect Sf9 cells along with viruses expressing the UL18, UL19, and UL38 genes. Both forms of the protein were detected in the capsids purified from cells infected with these two viruses (Fig. 5D, lane 5), indicating that the UL80.5 protein fails to support capsid assembly on its own because of its inability to interact (through its C terminus) with the HSV major capsid protein, VP5 (Fig. 2B, lane 9).

Coassembly of the HSV and HCMV scaffold proteins was also examined by using the VP5/scaffold protein coimmunoprecipitation assay (Fig. 6). When each of the truncated scaffold proteins was preincubated with its homologous full-length protein (UL26.5/304 plus UL26.5 or UL80.5-T plus UL80.5-H), both forms of scaffold protein coimmunoprecipitated with VP5 (Fig. 6B, lanes 5 and 6). However, when the truncated HSV or HCMV scaffold protein was incubated with its heterologous full-length scaffold protein (UL26.5/304 plus UL80.5-H or UL80.5-T plus UL26.5), only one protein which migrated with UL80.5-H (Fig. 6B, lane 7) or UL26.5 (Fig. 6B, lane 8) was observed. Taken together, the capsid assembly (Fig. 5) and immunoprecipitation (Fig. 6) data clearly demonstrated that the UL26.5 or UL80.5-H scaffold protein could coassemble with its homologous but not the heterologous truncated scaffold protein, indicating that specific amino acids within the N-terminal end of the scaffold protein (N terminal to the M cleavage site) determine self-interaction of the scaffold proteins.

Identification of essential sequences of the UL26.5 C terminus for capsid assembly. To identify critical regions in the C-terminal 25-amino-acid domain required for capsid assembly, mutations in the HSV-1 C-terminal 25-amino-acid domain were made (Fig. 1B). These mutations were maintained in the UL80.5 backbone to minimize any sequence-specific interactions with other capsid proteins that may stabilize the capsid structure. In the UL80.5(M1) mutant, the six most C-terminal

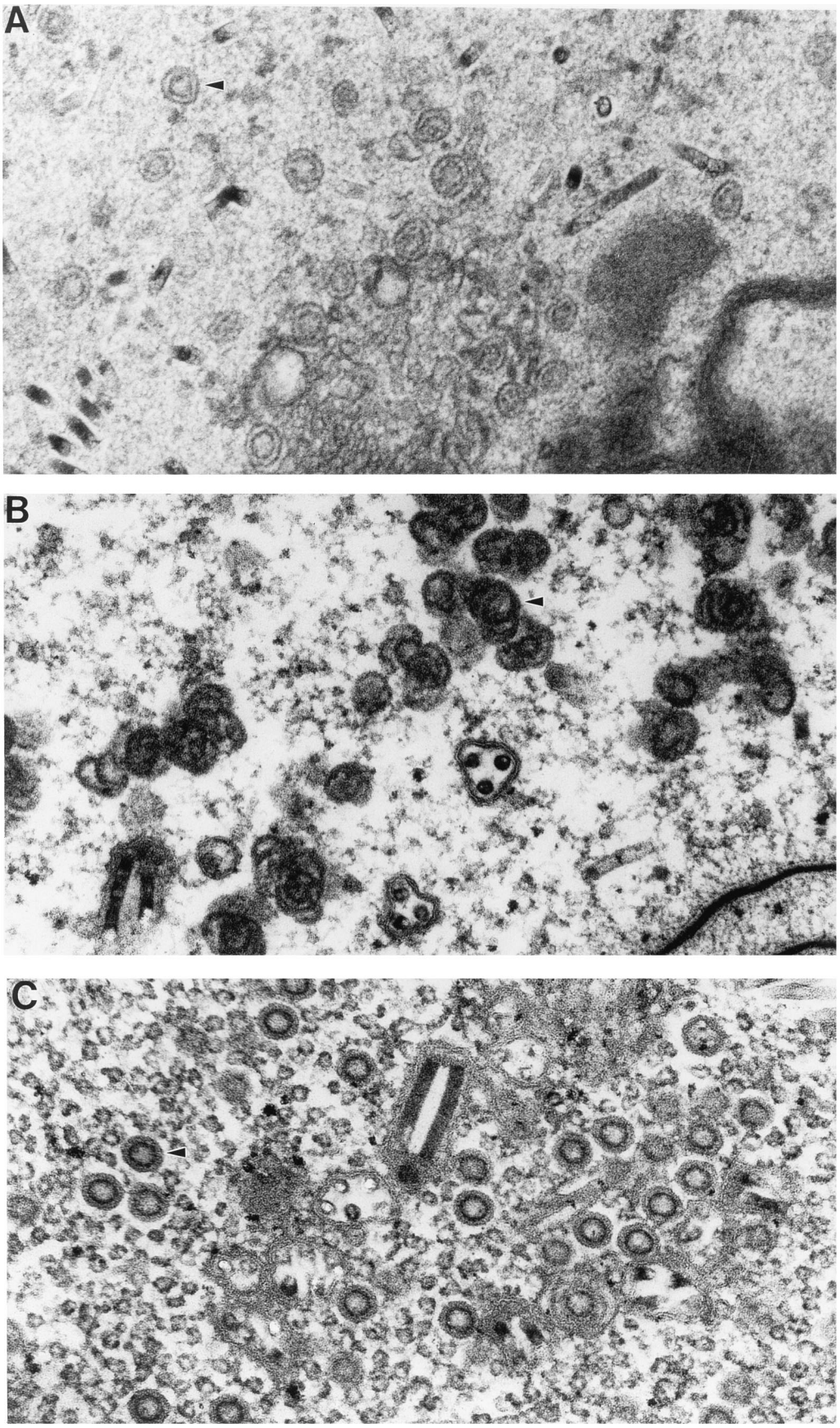

FIG. 4. Electron micrographs of thin sections of Sf9 cells infected with recombinant baculoviruses. A portion of the cells harvested for isolation of virus particles (see Fig. 3) was pelleted and fixed, and thin sections were prepared for EM. Cells were infected with recombinant baculoviruses expressing UL18, UL19, UL35, and UL38 plus UL26.5 (A), UL80.5 (B), UL80.5-H (C), UL80.5-H(M5) (D), or UL80.5(M6) (E). Magnifications were ×84,500 for panels A to D and ×107,000 for panel E. Arrowheads in panels A and C to E point to intact capsids containing a scaffold core, and the arrowhead in panel B points to aberrant capsid structures.

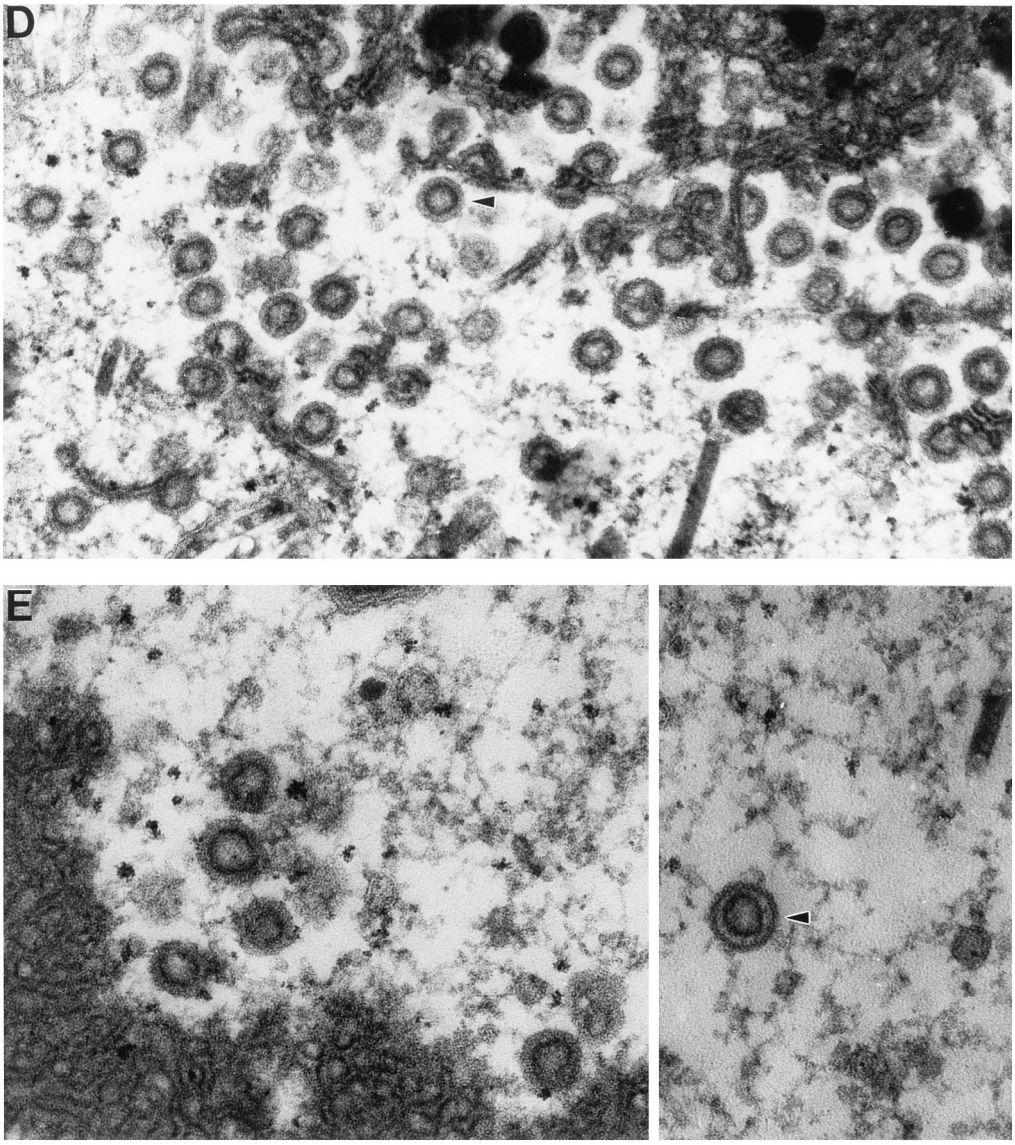

FIG. 4—*Continued.*

amino acids, P20′ to P25′ were deleted from the HSV-1 C-terminal peptide. Also, we made sequential deletions in UL80.5-H in which amino acids P3′ to P5′, P3′ to P10′, and P3′ to P15′ were removed to generate mutants UL80.5-H(M3), UL80.5-H(M4), and UL80.5-H(M5), respectively. The amino acids are numbered sequentially from P1′ to P25′ for the HSV-1 C-terminal peptide or P1′ to P65′ for the HCMV C-terminal peptide. Amino acid position P1′ is the N-terminal amino acid of the C-terminal peptide. Capsid formation in Sf9 cells was analyzed as previously discussed. Note that VP5, VP19C, and VP23 were present in all sucrose-banded material from baculovirus-infected cells, confirming their expression in baculovirus (Fig. 7A and C). Incomplete capsids formed with the UL80.5-H(M1) mutant, as indicated by the lack of detection of scaffold protein (Fig. 7B, lane 1). The UL80.5-H(M3), UL80.5-H(M4), and UL80.5-H(M5) mutants all supported capsid assembly, as indicated by the presence of the HCMV scaffold protein in capsid structures isolated from Sf9 cells (Fig. 7D, lanes 5 to 7). Intact 125-nm capsids containing a visible core structure were demonstrated by EM of thin sections for the three mutants with the P3′–P15′ deletion mutant UL80.5-H(M5) shown in Fig. 4D. These data demonstrated that the minimal domain in the C-terminal 25 amino acids of the HSV-1 peptide required for capsid assembly resides between amino acids P16′ and P25′.

The five UL80.5-H deletion mutants were also analyzed for interaction with VP5 by coimmunoprecipitation (Fig. 8). UL80.5-H(M3), UL80.5-H(M4), and UL80(M5) coimmunoprecipitated with VP5 (Fig. 8B, lanes 5 to 7), while interaction with VP5 was abolished when amino acids P20′ to P25′ were deleted (data not shown). These results correlated with the capsid assembly data indicating that the critical domain for interaction with VP5 resided within amino acids P16′ to P25′.

Substitution of two methionine residues in the C-terminal 65-amino-acid domain of UL80.5. Two methionine residues present at P21′ and P22′ within the critical binding domain of the HSV-1 C-terminal 25-amino-acid peptide appear in a motif which is conserved in scaffold protein homologs from several

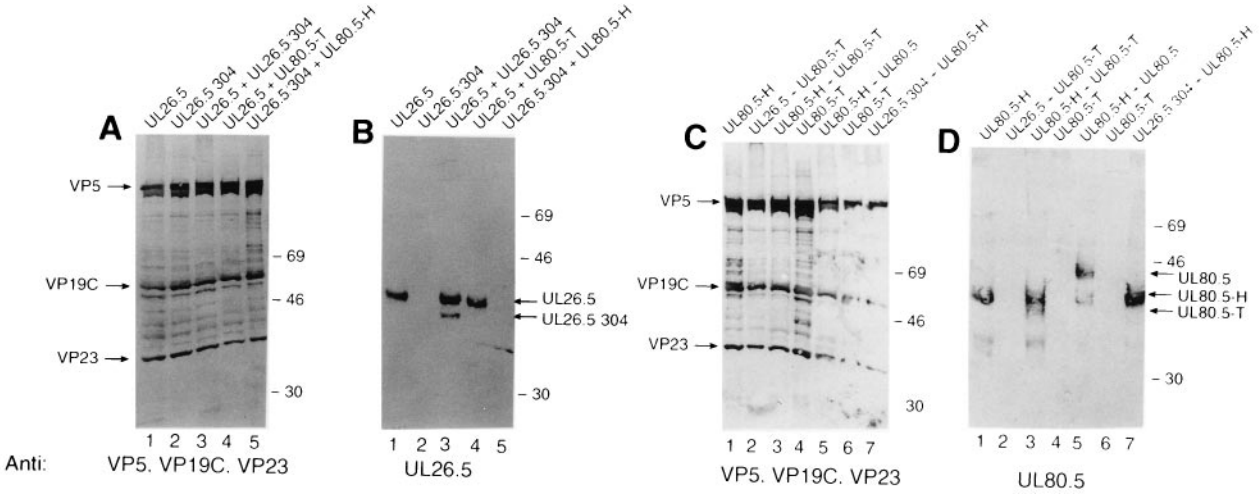

FIG. 5. Protein composition of particles harvested from sucrose gradients. Sf9 cells were coinfected with recombinant baculovirus expressing UL18, UL19, UL35, and UL38 along with the indicated scaffold proteins, and particles were purified as described in the legend to Fig. 3. Capsid proteins were electrophoresed on SDS–12.5% polyacrylamide gels and electroblotted onto nitrocellulose. (A and C) Western blot analysis with rabbit polyclonal antibodies NC1, NC2, and NC5. (B) Western blot analysis with monoclonal antibody MCA406. (D) Western blot analysis with rabbit polyclonal antibody T61. Numbers at the side show molecular masses (in kilodaltons). The mobilities of the capsid proteins are marked. The proteins that the antisera detect are listed below the blots.

members of the alphaherpesvirus family. The two methionine residues have been shown to be critical in the interaction with VP5 (18). To assess their role in capsid assembly, the two methionines were substituted in mutant UL80.5-H(M2) with lysine at P21′ and threonine at P22′. Only incomplete capsids formed in the baculovirus capsid assembly model when cells were coinfected with the UL80.5-H(M2) gene, as indicated by the lack of detection of scaffold protein (Fig. 7B, lane 2). The dual substitution also abolished interaction with VP5 in the coimmunoprecipitation assay (Fig. 8B, lane 4).

The highly conserved phenylalanine and valine residues of the UL26.5 protein located at P17′ and P18′ are found in the same position of the HCMV UL80.5 protein, relative to the very C-terminal end of the two proteins. In contrast, the two conserved methionines found in the HSV protein are not found within the C terminus of HCMV protein (Fig. 1A). We wanted to determine if capsid assembly would occur when the P61′ and P62′ amino acids, corresponding to P21′ and P22′ of the HSV-1 C-terminal peptide, were changed to methionines. UL80.5(M6) was constructed such that the amino acid sequence at the C terminus of the UL80.5 protein was altered from . . .FVAALNKLE to . . .FVAAMMKLE (Fig. 1B). Complete capsids were observed with UL80.5(M6) by EM of thin sections (Fig. 4E), although the number of capsids found was low. In fact, we had to make a thorough search of the sections to find intact capsids (Fig. 4E). Both the major capsid protein (Fig. 9A, lane 1) and the UL80.5(M6) protein (Fig. 9B, lane 1) were readily found in the double-banded capsid particles. In contrast, only a small amount of VP19C and no VP23 were detected in these samples (Fig. 9A, lane 1), although when the amount of protein run on the gel was increased, VP23 appeared. These results indicate that the UL80.5(M6) protein

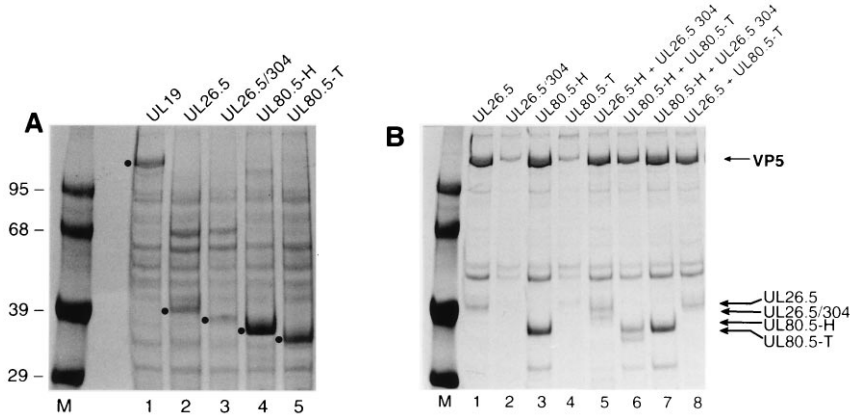

FIG. 6. (A) Polypeptides synthesized by recombinant baculovirus. Insect cell extracts were prepared from cells infected with recombinant baculoviruses expressing the indicated proteins as described in the legend to Fig. 2A. Proteins were separated on a SDS–12.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. The HSV or HCMV proteins expressed by each recombinant baculovirus are indicated by dots. (B) Coimmunoprecipitation of HSV or HCMV scaffold proteins with VP5. The cell extracts shown in panel A containing the indicated HSV or HCMV scaffold proteins were preincubated alone (lanes 1 to 4) or with extracts containing homologous or heterologous scaffold proteins (lanes 5 to 8) along with an extract containing the HSV major capsid protein, VP5. Proteins were immunoprecipitated with monoclonal antibody 6F and separated on a SDS–12.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. Lane M, marker proteins with molecular masses (in kilodaltons) shown at the sides. The mobilities of the capsid proteins are marked.

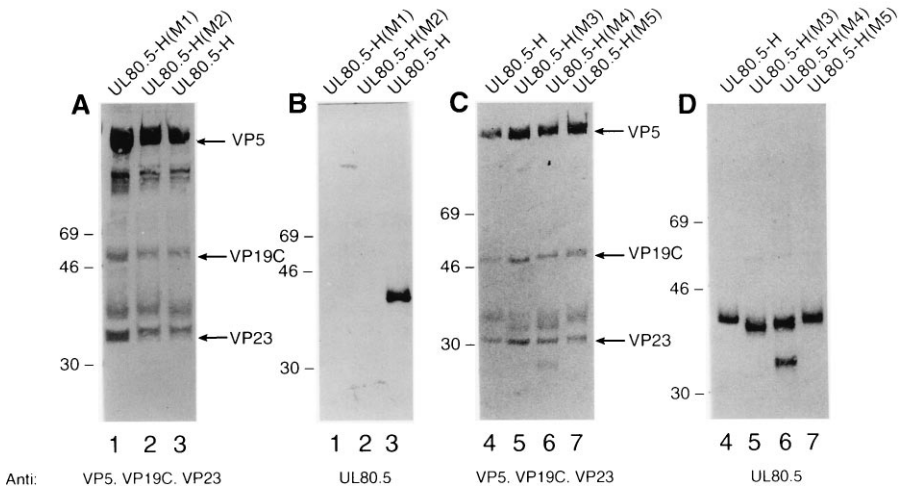

FIG. 7. Effects of UL80.5-H C-terminal mutants on HSV-1 capsid formation. Shown is the protein composition of particles harvested from sucrose gradients. Sf9 cells were coinfected with recombinant baculovirus expressing UL18, UL19, UL35, and UL38 along with the indicated scaffold proteins, and particles were purified as described in the legend to Fig. 3. Capsid proteins were electrophoresed on SDS–12.5% polyacrylamide gels and electroblotted onto nitrocellulose. (A and C) Western blot analysis with rabbit polyclonal antibodies NC1, NC2, and NC5. (B and D) Western blot analysis with rabbit polyclonal antibody T61. Numbers at the side show molecular masses (in kilodaltons). The mobilities of the capsid proteins are marked. The proteins that the antisera detect are listed below the blots.

supports assembly of intact capsids but with reduced efficiency compared to UL26.5 (Fig. 4A) or UL80.5-H (Fig. 4C). Moreover, it appears that the majority of the particles isolated from the sucrose gradient are structures composed of VP5 and UL80.5(M6).

The UL80.5(M6)-expressed protein proved to be insoluble, precluding testing of this protein in the coimmunoprecipitation assay. The UL80.5(M6) protein was expressed at high levels, but all of the protein remained in the pellet following centrifugation of the freeze-thawed extracts (data not shown). To ensure that the UL80.5(M6) band detected in the gradient-purified capsid sample was not due to an aggregate of the insoluble protein, extracts of the Sf9 cells infected with just the UL80.5(M6) gene were run on a 20 to 65% sucrose gradient and the region of the gradient that corresponded to where B capsids should band was isolated. The sample was concentrated by centrifugation and rebanded on a second gradient. In the absence of the UL18, UL19, and UL38 genes, no UL80.5(M6) protein was detected in the sample taken from the second gradient (Fig. 9A and B, lanes 2), indicating that the UL80.5(M6) protein was associated with capsid structures. These results emphasize the critical role of the C-terminal methionine residues in HSV-1 capsid formation.

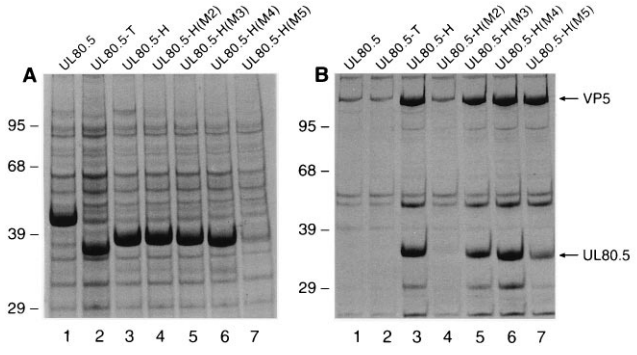

FIG. 8. (A) Polypeptides synthesized by recombinant baculovirus. Insect cell extracts were prepared from cells infected with recombinant baculoviruses expressing the indicated proteins as described in the legend to Fig. 2A. Proteins were separated on an SDS–12.5% polyacrylamide gel, and proteins were visualized with Coomassie blue stain. (B) Coimmunoprecipitation of HCMV scaffold proteins with VP5. The cell extracts shown in panel A containing the indicated HCMV scaffold proteins were preincubated with a cell extract containing the HSV major capsid protein, VP5. Proteins were immunoprecipitated with monoclonal antibody 6F and separated on an SDS–12.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. Numbers at the side show molecular masses (in kilodaltons). The mobilities of the capsid proteins are marked.

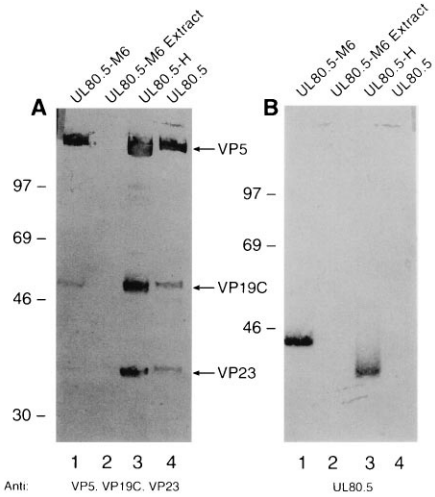

FIG. 9. Capsid assembly with the UL80.5(M6) mutant. Shown is the protein composition of particles harvested from sucrose gradients. Sf9 cells were coinfected with recombinant baculovirus expressing UL18, UL19, UL35, and UL38 along with the indicated scaffold proteins, and particles were purified as described in the legend to Fig. 3. Lane 2, Sf9 cells infected with just the UL80.5(M6)-expressing baculovirus (see text). Capsid proteins were electrophoresed on SDS–12.5% polyacrylamide gels and electroblotted onto nitrocellulose. (A) Western blot analysis with rabbit polyclonal antibodies NC1, NC2, and NC5. (B) Western blot analysis with rabbit polyclonal antibody T61. Numbers at the side show molecular masses (in kilodaltons). The mobilities of the capsid proteins are marked. The proteins that the antisera detect are listed below the blots.

DISCUSSION

The primary findings of this report are that (i) the UL80.5 scaffold protein from HCMV will not substitute for the HSV-1 UL26.5 protein in a baculovirus capsid assembly model; (ii) the UL80.5 protein can serve as a scaffold for HSV-1 capsid assembly if its C-terminal 65-amino-acid peptide is replaced with the C-terminal 25-amino-acid peptide of the HSV-1 scaffold protein; (iii) self-assembly requires amino acid specificity within the portion of the scaffold protein amino terminal to the maturational cleavage site; (iv) the critical domain for VP5 interaction with the scaffold protein is located within the last 10 amino acids of the HSV-1 C-terminal 25-amino-acid peptide, and (v) two methionine residues within this conserved domain are required for HSV-1 capsid assembly and VP5 interaction.

Previous reports have demonstrated that the C-terminal 25-amino-acid peptide of the HSV-1 UL26.5 protein is required for interaction with VP5 (18, 44) and for capsid assembly (20, 25, 44). Studies by our group have addressed the virus specificity of this interaction in the formation of capsids, demonstrating that the UL26.5 homolog of the BHV scaffold protein can substitute for the HSV-1 scaffold in a baculovirus capsid assembly model (13). The BHV scaffold protein has 41% amino acid sequence identity with HSV-1 scaffold protein, with extensive homology noted within the C-terminal 25-amino-acid peptide (13). In fact, the scaffold proteins from members of the alphaherpesvirus family contain a conserved Phe-Val or Phe-Ala motif 8 to 10 amino acids from the C terminus along with conserved Met-Met residues 4 to 6 amino acids from the C terminus. In contrast, the HCMV (a member of the gammaherpesvirus class) scaffold contains the conserved Phe-Val amino acids within its C terminus but is missing the other two conserved amino acids noted among members of the alphaherpesviruses. We have shown that the HCMV UL80.5 scaffold will not substitute for the HSV-1 scaffold. We subsequently demonstrated that when the C-terminal 65-amino-acid domain of the HCMV scaffold was replaced with the HSV-1 C-terminal 25-amino-acid peptide or when the C terminus of the HCMV scaffold protein was modified to contain the two conserved Met residues, the HCMV scaffold was able to substitute for the HSV-1 scaffold in the formation of complete capsids. These results demonstrate that virus-specific interactions occur between the C terminus of the scaffold protein with the proteins comprising the capsid shell. The coimmunoprecipitation experiments (Fig. 2) demonstrated that the scaffold protein interacts directly (through its C terminus) with the major capsid protein, VP5. The inability of the HSV-1 scaffold protein to coassemble with the homologous HCMV scaffold suggests that oligomerization of the scaffold protein is also linked to virus-specific amino acid sequence(s). This specificity appears to reside within the amino acids N terminal to the maturational cleavage site since the HSV-1 C-terminal peptide is not required for self-assembly (36). Determination of the amino acid domains required for this self-assembly may be useful for targeted antiviral research.

Both the cleaved and uncleaved forms of the scaffold protein were present in capsids purified from insect cells dually infected with recombinant baculovirus expressing both the full-length and truncated UL26.5. These two forms of the assembly protein were also shown to coassemble in a coimmunoprecipitation assay using VP5. Previous reports suggest that the cleaved scaffold protein, VP22a, cannot function in capsid assembly, although it can form scaffold-like structures (36). Our results suggest that VP22a may function in capsid assembly through coassembly with full-length scaffold protein. The interaction of the cleaved scaffold protein with the uncleaved form may be a mechanism of circumventing the loss of the C-terminal end through proteolytic cleavage by the protease prior to capsid assembly.

We have demonstrated a direct correlation of VP5-UL26.5 interaction and the formation of complete capsids and have used this correlation to define the minimal domain within the C terminus of the UL26.5 protein. In these studies, we observed that amino acids P3′ to P15′ are not required for formation of complete capsids since removal of these amino acids did not alter capsid assembly or the ability of scaffold protein to interact with VP5 (Fig. 6 and 7). Hong et al. (18) have shown that P12′ to P23′ are essential for UL26.5 interaction with VP5, and they noted that when the two hydrophobic methionines at positions P21′ and P22′ were substituted with alanine or lysine residues, the interaction was abolished. We also observed similar results in that alteration of the two methionines abolished both interaction with VP5 and the formation of complete capsids. Our substitutions, lysine and threonine, were more severe than the alanine substitutions and likely interrupted the critical hydrophobic region defined by Hong et al. (18) in a manner similar to substitution with lysine residues. The critical methionines are not present within the C-terminal 65-amino-acid domain of the HCMV scaffold (Fig. 1). Instead, the amino acids in the same position relative to the absolutely conserved phenylalanine residue are leucine and asparagine. Based on the findings of Hong et al. (18), one would predict that the polar, charged asparagine would disrupt interaction with the VP5. In accordance with this prediction, when we substituted the leucine and the asparagine with two methionines, we observed the formation of complete capsids in the baculovirus capsid assembly model.

As noted above, the HCMV and HSV scaffolds have little amino acid sequence identity. Therefore, the ability of the chimeric HCMV scaffold protein to substitute for the HSV protein in assembly of intact capsids strongly suggests that the capsid shell interacts with the scaffold protein solely via the C terminus of the scaffold protein. Identification of the region of VP5 that the scaffold protein interacts will be an important next step. This information would be useful in design of antiviral agents targeted against capsid assembly.

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