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Virus-Specific Interaction between the Human Cytomegalovirus Major Capsid Protein and the C Terminus of the Assembly Protein Precursor

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We previously identified a minimal 12-amino-acid domain in the C terminus of the herpes simplex virus type 1 (HSV-1) scaffolding protein which is required for interaction with the HSV-1 major capsid protein. An α-helical structure which maximizes the hydropathicity of the minimal domain is required for the interaction. To address whether cytomegalovirus (CMV) utilizes the same strategy for capsid assembly, several glutathione *S*-transferase fusion proteins to the C terminus of the CMV assembly protein precursor were produced and purified from bacterial cells. The study showed that the glutathione *S*-transferase fusion containing 16 amino acids near the C-terminal end was sufficient to interact with the major capsid protein. Interestingly, no cross-interaction between HSV-1 and CMV could be detected. Mutation analysis revealed that a three-amino-acid region at the N-terminal side of the central Phe residue of the CMV interaction domain played a role in determining the viral specificity of the interaction. When this region was converted so as to correspond to that of HSV-1, the CMV assembly protein domain lost its ability to interact with the CMV major capsid protein but gained full interaction with the HSV-1 major capsid protein. To address whether the minimal interaction domain of the CMV assembly protein forms an α-helical structure similar to that in HSV-1, peptide competition experiments were carried out. The results showed that a cyclic peptide derived from the interaction domain with a constrained α-helical structure competed for interaction with the major capsid protein much more efficiently than the unconstrained linear peptide. In contrast, a cyclic peptide containing an Ala substitution for the critical Phe residue did not compete for the interaction at all. The results of this study suggest that (i) CMV may have developed a strategy similar to that of HSV-1 for capsid assembly; (ii) the minimal interaction motif in the CMV assembly protein requires an α-helix for efficient interaction with the major capsid protein; and (iii) the Phe residue in the CMV minimal interaction domain is critical for interaction with the major capsid protein.

An essential step during herpesvirus replication is capsid assembly, which occurs exclusively in the nuclei of infected host cells (5, 45, 47). The herpes simplex virus type 1 (HSV-1) capsids have been characterized extensively, and the outer icosahedral shell of each capsid contains 162 capsomers, 150 of which are hexons and 12 of which are pentons (1, 31, 34, 46, 62). Three types of HSV-1 capsids, A, B, and C, have been identified by electron microscopy and can be separated by sedimentation centrifugation on a sucrose gradient (11, 16). C capsids contain the entire viral genome and can mature into infectious virions, while A capsids lack viral DNA, which is the only difference between A and C capsids. B capsids also lack DNA; however, a unique feature of B capsids is the presence of an abundant core protein, designated scaffolding protein ICP35 (VP22a) (16, 30, 31, 34). The scaffolding protein plays an essential role in capsid assembly which is believed to resemble that of the double-stranded DNA bacteriophage P22 (3, 18). Deletion or null mutation of the scaffolding protein results in aberrant shell structures and very inefficient capsid formation (8, 27, 29, 50–52). It is postulated that B capsids are involved in packaging viral DNA to form C capsids, thus suggesting that B capsids may be the important intermediates in virus assembly (38, 39, 41, 48).

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HSV-1 B capsids are composed of seven proteins, VP5, VP19c, VP21, VP22a (ICP35), VP23, VP24, and VP26 (16). The major capsid protein, VP5, makes up both hexons and pentons of the capsomers in the outer shell and accounts for approximately 70% of the mass of the shell. Most of the remainder of the shell is made up of VP19c and VP23, which form a trivalent structural unit (triplex) linking the adjacent hexons and pentons together (31, 34, 53, 54, 62). Transcapsomeric channels or openings are found in the centers of pentons and hexons, providing a potential mechanism for entry of DNA and exit of the scaffolding proteins (32, 62). VP21 and VP24 are encoded by a single gene (UL26) and are autoproteolytic products of the HSV-1 protease, Pra (6, 9, 25, 40, 56). The HSV-1 protease cleaves itself at two different sites: one separating VP24 and VP21 (R site) and one at the C-terminal end (M site) (9, 25, 56). The protease activity is essential in DNA packaging and virus assembly (8, 14, 41). The release of the protease domain, VP24, from its precursor is essential for DNA packaging (28). Recent evidence suggests that the protease begins the proteolytic process only after it enters the capsids, and it may have structural functions in addition to the proteolytic activity (43). It is believed that the protease inside a sealed capsid may undergo a conformational change which drastically augments its activity and triggers the proteolytic processing of itself as well as its substrate, the scaffolding protein. This notion is supported by recent studies showing that protease activity is significantly enhanced in the presence

of antichaeotrophic salts, with concomitant conformational changes in the protease domain (4, 61).

Similar proteolytic processing is also found in cytomegalovirus (CMV), in which the protease (also known as assemblin, product of UL80a, the counterpart of the HSV-1 protease) cleaves itself as well as its substrate, the assembly protein (product of UL80.5, the counterpart of the HSV-1 scaffolding protein) (2, 15, 58, 59). A unique autocleavage site (I site) has been identified around the center of the catalytic domain of the CMV protease (2, 58). Cleavage at the I site results in two fragments which remain physically associated. The activity of the self-cleaved CMV protease domain (two-chain enzyme) is similar to the intact protease domain (one-chain enzyme) (19, 20, 36), and so the role of this cleavage is still not clear.

The open reading frame (ORF) of the HSV-1 scaffolding protein, ICP35 (product of UL26.5), nests within that of the protease (UL26) and has amino acid sequence identity with the C-terminal end of the protease (23, 24). Cleavage at the C terminus of ICP35 (the same cleavage site as the M site in Pra) releases a C-terminal 25-amino-acid peptide. Genetic studies demonstrated that deletion of the coding region for the C-terminal 25 amino acids from the HSV-1 genome is lethal to the virus (29). ICP35 can self-assemble into a core-like structure which is present transiently in the inner space of B capsids (31, 50, 52). Interestingly, its disappearance coincides with the entry of DNA (14, 27, 41, 42). Thus, it is believed that ICP35 not only serves as a scaffolding protein (a functional analog of the scaffolding protein in bacteriophage P22) for capsid assembly but also plays a role in DNA packaging. Notably, the function of ICP35 can be partially compensated for by VP21, which is longer than ICP35 by 59 amino acids at the N terminus (27). The assembly protein of CMV (product of UL80.5) is similar in genomic arrangement to the HSV-1 scaffolding protein (57). It overlaps the CMV protease (product of UL80a) at the C-terminal domain and has the same maturation cleavage site (M site). Cleavage at the M site removes 65 amino acids from the C terminus of the protease or the assembly protein (2, 15, 58, 59), and this proteolytic processing is also believed to be essential for viral capsid assembly and viral growth.

In vitro cell-free or virus-free capsid assembly for HSV-1 has demonstrated that only six of the capsid proteins (VP5, VP19c, VP21, VP22a, VP23, and VP24) are required for proper capsid assembly (33, 50–52). Thus, these proteins are thought to possess all of the structural information needed for capsid formation. Additional viral gene products may participate in capsid assembly in vivo (37, 55), but they are not required in the in vitro assays. The interactions between the major capsid protein, VP5, and the scaffolding protein, ICP35, have been shown to be very important in transporting VP5 into the nucleus and in capsid assembly (22, 29, 33, 35, 51). One of the interaction domains is located in the C-terminal 25-amino-acid region of ICP35 or the protease (Pra), which is sufficient for the interaction with VP5 (7, 21, 29, 51). Removal of the C-terminal 25 amino acids by genetic engineering abolishes capsid assembly and inhibits viral growth. Interestingly, another interaction domain which is present N terminal to the M site has also been implicated, because the scaffolding protein without the C-terminal domain is still able to translocate the major capsid protein into the nucleus (22).

In a previous report, we characterized the interaction between the C-terminal end of the HSV-1 scaffolding protein and the HSV-1 major capsid protein. A minimal 12-amino-acid domain was identified. Several hydrophobic residues in the minimal domain were shown to be important for the interaction, particularly the Phe residue in the center, which is absolutely conserved among various herpesviruses (21). An α-helical structure which maximized the hydropathicity of the minimal domain was also required for efficient interaction with the major capsid protein (21).

In this report, we have characterized the interaction between the C terminus of the CMV assembly protein precursor (functional analog of the HSV-1 scaffolding protein) and the CMV major capsid protein, using the glutathione *S*-transferase (GST) fusion system previously described (21). Our results demonstrate that CMV also utilizes an α-helical domain in the C-terminal end of its assembly protein precursor for interaction with the major capsid protein, suggesting that CMV and other herpesviruses may have developed similar strategies for capsid assembly.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* (Sf9) insect cells (Life Technologies) were maintained in Sf-900 II SFM complete medium (Life Technologies) on an ISS Orbital Shaker (Integrated Separation Systems) at 27°C. The recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus), AcUL19, expressing the HSV-1 major capsid protein was a generous gift from Valerie G. Preston, MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom. The recombinant baculovirus, rbvMH18, expressing the CMV major capsid protein was created from a PCR-modified CMV UL86 gene. This modified UL86 gene was isolated as follows: (i) the entire UL86 ORF was amplified by PCR and subcloned into the RSV.5(neo) expression vector; (ii) an *Sgr*AI-digested fragment (~3,400 bp) from the CMV genomic DNA containing all but about 700 bp of the UL86 ORF was used to replace the corresponding *Srg*AI fragment in the PCR amplified insert; and (iii) the remaining 700 bp which were not replaced with the wild-type sequence were subjected to sequencing confirmation. The UL86 gene can be excised by *Xba*I digestion and subcloned into any suitable vectors. All of the recombinant baculoviruses were plaque purified, and high-titer viral stocks were obtained from Invitrogen Corp.

Construction of GST/CT fusion plasmids containing the C-terminal ends of HSV-1 and CMV. The HSV-1 GST/CT fusion plasmids were described previously (21). Several GST/CT fusion plasmids were constructed to contain different C-terminal domains of the CMV assembly protein precursor. The CMV GST/CT77 plasmid contains the coding region of the C-terminal 77 amino acids, which was isolated by PCR. The CMV GST/CT48 and GST/CT20 plasmids, containing the C-terminal 48 and 20 amino acids, respectively, were constructed by using long DNA polylinkers encoding the corresponding C termini. The codons in the polylinkers were optimized with bacterial preferred ones as described previously (17, 21). Both the PCR fragment and DNA polylinkers were then subcloned into pGEX-2T (Pharmacia Biotech) between *Bam*HI and *Eco*RI in frame with the GST ORF. For CMV GST/CT48, two pairs of complementary DNA oligomers were synthesized (GenoSys Biotechnologies, Inc.) and annealed to generate two polylinkers with compatible cohesive ends. The oligonucleotide sequences are as follows: top strand of the 5′ polylinker, 5′-GATCCCCGTCTACTGCAGGTTC TAGCTCTTGCCCGGCTAGCGTAGTTCTGGCTGCAGCTCAGGCTGCA GCT-3′; bottom strand of the 5′ polylinker, 5′-GCATCCCAGAGCCCGCCGA AAGACATGGTAGATCTGAACCGCCGCATCTTCGTTGCTGCACTGAA CAAACTGGAATAAG-3′; top strand of the 3′ polylinker, 5′-GGATGCAGC TGCAGCCTGAGCTGCAGCTGCAGCCAGAACTACGCTAGCCGGGCA AGAGCTAGAACCTGCAGTAGACGGG-3′; and bottom strand of the 3′ polylinker, 5′-AATTCTTATTCCAGTTTGTTCAGTGCAGCAACGAAGAT GCGGCGGTTCAGATCTACCATGTCTTTCGGCGGGCTCTG-3′. Similarly, GST/CT20 was constructed by using one pair of complementary DNA oligomers. The sequences are as follows: top strand, 5′-GATCCCCCAAAGACATGGTA GATCTGAACCGCCGCATCTTCGTTGCTGCACTGAACAAACTGGAAT AAG-3′; and bottom strand, 5′-AATTCTTATTCCAGTTTGTTCAGTGCAG CAACGAAGATGCGGCGGTTCAGATCTACCATGTCTTTGGGG-3′. Mutations were introduced into GST/CT20 by creating specific changes in the codons of the polylinkers, which were then cloned into the vector for expression. The sequences of all of the GST/CT fusion plasmids were confirmed by using a fluorescence-based automated sequencer (model 373A; Applied Biosystems, Inc.).

Expression and purification of the GST/CT proteins. The expression and purification of the GST/CT fusion proteins were described previously (21). Briefly, the GST/CT expression plasmids were electrotransformed into *Escherichia coli* DH10B cells (Gibco-BRL), using a Gene Pulser (Bio-Rad). Cell cultures (50 ml) were grown at 37°C to mid-log phase (optical density at 600 nm of 0.6) and induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Cells were harvested 2 h after the induction and lysed in 20 ml of binding buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol [DTT], 150 mM NaCl, 300 nM antipain, 200 nM leupeptin, 10% glycerol) by passage through a microfluidizer (Microfluidics Corp.). Two milliliters of equilibrated glutathione-Sepharose beads (GSH beads; Pharmacia Biotech) was added to the cell lysates, and the mixtures were incubated at 4°C for 1 h on a hematology/chemistry mixer (Fisher Scientific). The GSH beads were spun

down and washed twice with 50 ml of binding buffer and once with 50 ml of high-salt buffer (50 mM Tris-HCl [pH 8.0], 1 M NaCl, 1 mM EDTA, 1 mM DTT, 300 nM antipain, 200 nM leupeptin, 10% glycerol). The GST/CT proteins were eluted off the beads with 2 ml of elution buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 300 nM antipain, 200 nM leupeptin, 10% glycerol, 10 mM reduced glutathione). All of the GST/CT fusion proteins were purified to homogeneity, dialyzed into phosphate-buffered (PBS) saline solution supplemented with 10% glycerol, and stored at −80°C.

Labeling of cell cultures and preparation of cell lysates. Sf9 cells were seeded at 2×10^6 per well in a six-well dish and incubated for 30 min to allow attachment. Cells were infected with baculovirus recombinants at a multiplicity of infection of 10 in 1 ml of fresh Sf-900 II SFM complete medium. At 24 h postinfection, the virus inoculum was removed and 1 ml of labeling medium (50 μCi of [^{35}S]methionine per ml, 50 μCi of [^{35}S]cysteine per ml, and 10% Sf-900 II SFM complete medium in Sf-900 II SFM methionine- and cysteine-free medium) was added to each well, and cells were incubated for an additional 24 h. To prepare the cell lysates, the labeling medium was removed and 0.5 ml of lysis buffer (Dulbecco's PBS without calcium and magnesium [pH 7.3] and 50 mM EDTA) was added to each well. Cells were lysed by freezing (at −80°C)-thawing (at 4°C) at least three times, and the cell lysates were then cleared by centrifugation at 10,000 rpm (Sorvall MC 12V Microcentrifuge; Du Pont) for 2 min. The cell lysates were pooled, and glycerol was added to a final concentration of 10%. The resulting lysates were preabsorbed with 50 μl of GSH beads (50% suspension in PBS) per 500 μl of lysate at 4°C for 1 h on a hematology/chemistry mixer (used for all coprecipitations; Fisher Scientific). The GSH beads were removed by centrifugation at 700 rpm for 2 min at 4°C in an IEC Centra GP8R centrifuge (International Equipment Company). The lysates were then ready for affinity precipitation using GSH beads or storage at −80°C.

Affinity precipitation. Eppendorf tubes were first coated with 250 μl of lysis buffer containing 1% bovine serum albumin, then 250 μl of the preabsorbed lysates was added to each tube, and the tubes were incubated with 20 μg of purified GST or GST/CT fusion protein at room temperature for 2 h. The lysates were then mixed with 20 μl of GSH beads (50% suspension in PBS) and incubated at room temperature for an additional 30 min. The GSH beads were spun for 2 min at 3,000 rpm and washed once with 500 μl of lysis buffer; 50 μl of 2× sodium dodecyl sulfate (SDS) protein sample buffer (Integrated Separation Systems) was added to the beads, and the samples were boiled for 5 min. Then 20 μl of the samples was loaded on an SDS–10 to 20% polyacrylamide gradient gel (Integrated Separation Systems), and the autoradiograph was analyzed by using X-ray film (Du Pont).

Peptide synthesis. Linear peptide CT19 derived from the C terminus of the CMV assembly protein precursor was synthesized on a peptide synthesizer (model 433A; Applied Biosystems), using FastMoc chemistry as described previously (21). The dilactam-constrained peptides, CT(F) and CT(A), cyclic analogs of CT19 (see Fig. 5A), were synthesized by using the orthogonal *tert*-butoxycarbonyl–9-fluorenylmethoxycarbonyl scheme (12), with slight modifications (21). Briefly, the peptide chains were assembled on the peptide synthesizer and sequentially cyclized by using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate/1-hydroxyl-7-azabenzotriazole as the coupling reagent. The peptide was then cleaved and deprotected in hydrogen fluoride-anisole (9:1). The peptide was washed with anhydrous ethyl ether, extracted from the resin with dimethylformamide, water, and acetic acid, and finally lyophilized in water. All of the peptides were purified by reversed-phase high-pressure liquid chromatography and confirmed by mass spectroscopy.

CD spectropolarimetry. Lyophilized peptide samples were suspended in 10 mM potassium phosphate buffer (pH 7.4). Peptide concentrations were determined by total amino acid analysis using vapor-phase hydrolysis and OPA–9-fluorenylmethoxycarbonyl chemistry. Circular dichroic (CD) spectra were obtained at room temperature, using a Jasco model J-500A spectropolarimeter. The CD parameters included a scan range at 260 to 195 nm, a path length of 0.1 cm, a step resolution of 0.2 nm, a scan speed at 50 nm/min, a band width of 2.0 nm, and 16 scans per spectrum. A blank spectrum of potassium phosphate buffer was subtracted from each peptide spectrum. The peptide spectra were then converted from ellipticity (millidegrees) to mean residue ellipticity (Θ; °Ccm^2/dmol) and subjected to a smoothing algorithm provided by Jasco J-600 software. The CD spectra were plotted as mean residue ellipticity (Θ) versus wavelength (in nanometers). The percentage of helicity was determined at 222 nm by using a modification of the formula described by Stevenson et al. (49), with the Θ^{coil} and Θ^{helix} values at −2,878 and −30,000, respectively:

$$\text{relative helicity} = \frac{\Theta^{obs} - \Theta^{coil}}{\Theta^{helix} - \Theta^{coil}} = \frac{\Theta^{obs} + 2{,}878}{-27{,}660}$$

Θ^{coil} is the mean residue ellipticity of the unconstrained CMV peptide (CMV-CT19), Θ^{obs} is the mean residue ellipticity of the test peptides, and Θ^{helix} is the mean residue ellipticity of an ideal helical peptide.

RESULTS

The interaction between the C terminus of the HSV-1 scaffolding protein, ICP35, and the HSV-1 major capsid protein, VP5, plays an essential role during capsid assembly and viral growth (29, 51). It is of great interest to know whether other herpesviruses have similar schemes for capsid assembly. Similar genomic arrangements in CMV and Epstein-Barr virus (10, 57) for genes of the proteases and the assembly proteins suggest that such an interaction may exist in other herpesviruses. Amino acid alignment analysis revealed that the interaction domains are well conserved among various herpesviruses (21). In this study, we decided to focus on the interaction between the C terminus of the CMV assembly protein precursor and the CMV major capsid protein and attempt to address whether human CMV has developed a strategy similar to those employed by other herpesviruses.

Specific interaction between the C terminus of the CMV assembly protein precursor and the CMV major capsid protein. To investigate the interaction between the CMV major capsid protein and the CMV assembly protein, we used the surrogate GST fusion system developed for our previous studies of HSV-1 (21). The C-terminal 77, 48, or 20 amino acids of the CMV assembly protein precursor were expressed as fusion proteins with GST. The CMV assembly protein precursor, like that of HSV-1, has amino acid sequence identity with the C-terminal part of the CMV protease. It also consists of a maturation site near the C-terminal end which can be cleaved by the CMV protease. An interesting property of the CMV assembly protein precursor is that it has a longer C-terminal tail (65 amino acids) after the M site compared with that of HSV-1 (25 amino acids). The longest fusion, GST/CT77, consists of the entire 65 amino acids as well as 12 amino acids upstream of the M cleavage site. GST/CT48 and GST/CT20 consist of the last 48 and 20 amino acids, respectively, at the C-terminal end (Fig. 1A). These fusion constructs were designed to roughly map the location of the interaction domain in the C terminus of the CMV assembly protein precursor. All of the GST/CT fusion proteins as well as the vector GST protein were expressed in bacterial cells and purified to homogeneity (data not shown). Results in Fig. 1 demonstrated that all of the GST/CT fusion proteins (Fig. 1B, lanes 3 to 5; Fig. 1C, lanes 2 to 4) were able to specifically precipitate the CMV major capsid protein which was produced in Sf9 insect cells and labeled with [^{35}S]methionine and [^{35}S]cysteine, whereas the GST vector protein failed to specifically precipitate the major capsid protein (Fig. 1B, lane 2; Fig. 1C, lane 1). This result demonstrates that the C-terminal 20 amino acids alone are sufficient for interaction with the major capsid protein.

Mapping the minimum interaction domain by deletion analysis. The CMV GST/CT20 fusion protein was used as the starting point for constructing a series of deletion mutants in which amino acids were removed sequentially in pairs either from the C-terminal end or from amino acid P46′, which is located at the N-terminal border of the GST/CT20 interaction domain (Fig. 2A). The deletion mutations spanned the entire interaction domain. The results in Fig. 2B revealed that deletion of the first two amino acids from the N-terminal border of the interaction domain (P46′ and P47′; CNΔ2) did not affect the interaction with the major capsid protein at all (lane 4). Deletion of an additional two amino acids (P48′ and P49′; CNΔ4) abolished the interaction (Fig. 2B, lane 5), as did any further deletions from the N-terminal border of the interaction domain (CNΔ6, CNΔ8, and CNΔ10 [lanes 6 to 8]). On the other hand, deletion of first two amino acids from the C-terminal end (CTΔ2) significantly reduced but did not abolish the interaction with the major capsid protein (Fig. 2B, lane 9). Any further deletions (CTΔ4, CTΔ6, CTΔ8, and CTΔ10) abolished the interaction (lanes 10 to 13). These results suggest that the minimal interaction domain in the CMV assembly

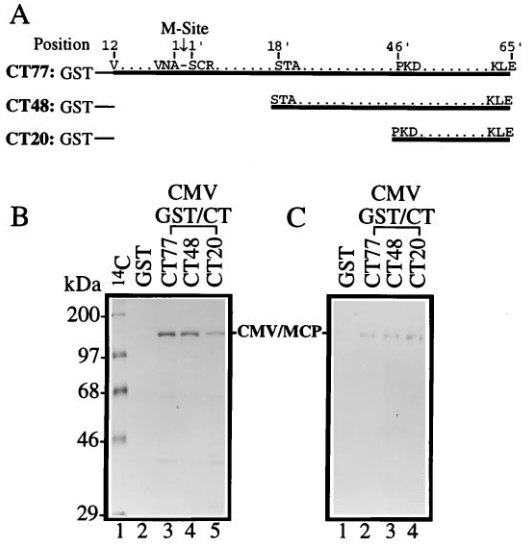

FIG. 1. Specific interaction between the CMV GST/CT fusion proteins and the CMV major capsid protein. (A) Illustration of CMV GST/CT fusion constructs. CT77, CT48, and CT20 represent GST/CT fusion proteins containing 77, 48, and 20 amino acids, respectively, from the C-terminal end of the CMV assembly protein precursor. The amino acid positions are numbered relative to the position of the M-site scissile bond as indicated. Partial amino acid sequences (in single letters) are shown to mark the M cleavage site and the border of each fusion construct. (B) Affinity precipitation of ^{35}S-labeled CMV major capsid protein. Lane 1, ^{14}C-labeled molecular weight marker (Amersham); lane 2, GST control; lane 3, GST/CT fusion protein, CT77; lane 4, GST/CT fusion protein, CT48; lane 5, GST/CT fusion protein, CT20. (C) Western blotting (immunoblotting) analysis of the affinity precipitates by CMV GST/CT fusions. Western blotting was carried out as previously described (21), using a polyclonal rabbit antiserum against the C-terminal 15 amino acids of the CMV major capsid protein. Lane 1, GST control; lanes 2 to 4, CMV GST/CT fusion proteins CT77, CT48, and CT20, respectively.

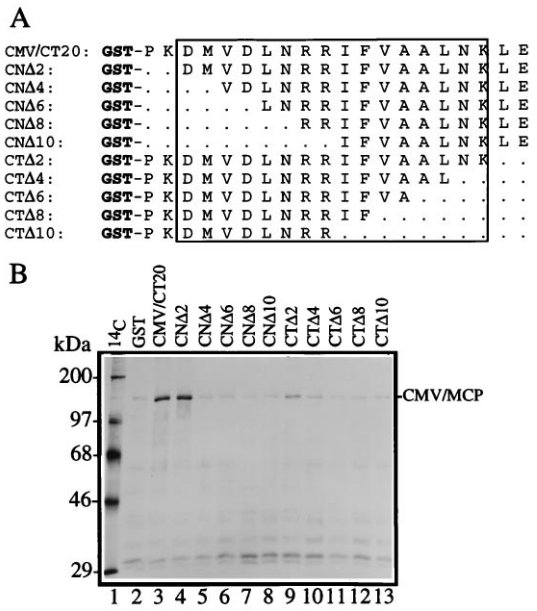

FIG. 2. Mapping the minimal interaction domain in the C terminus of the CMV assembly protein precursor. (A) Construction of deletion mutants. The sequence of the C-terminal 20 amino acids of the CMV assembly protein precursor is shown to be fused in frame with GST. Dots represent deleted amino acids, and the minimal domain is boxed. (B) Affinity precipitation of ^{35}S-labeled CMV major capsid protein with deletion mutants. Lane 1, ^{14}C-labeled molecular weight marker; lane 2, GST control; lane 3, CMV GST/CT20 fusion protein; lanes 4 to 13, GST/CT20 deletion mutants as shown in panel A.

protein precursor consists of 16 amino acids between P48′ and P63′ near the C-terminal end.

Lack of cross-interaction between the C terminus of the CMV assembly protein precursor and the HSV-1 major capsid protein. HSV-1 and CMV are related viruses in the family *Herpesviridae*. However, because of their differences in host ranges, replication cycle and cytopathicity, they belong to different subfamilies; HSV-1 is a member of the *Alphaherpesvirinae*, while CMV is a member of the *Betaherpesvirinae* (44). It is of great interest to evaluate whether HSV-1 and CMV have identical conserved domains for interaction with their major capsid proteins. It is important to address this issue, since it will provide insights for designing a broad-spectrum antiviral target. We decided to test whether the proteins from the two viruses were interchangeable. The results in Fig. 3 revealed that the C terminus of the HSV-1 scaffolding protein failed to interact with the CMV major capsid protein (Fig. 3A, lane 3), and the C terminus of the CMV assembly protein precursor also failed to interact with the HSV-1 major capsid protein (Fig. 3B, lane 3). This finding suggests that there is no cross-interaction between the HSV-1 and CMV counterpart capsid proteins. The same conclusion was reached from experiments using the yeast two-hybrid system (60). The background band which comigrates with the HSV-1 major capsid protein in Fig. 3A (lanes 1 and 3) was due to insolubility or aggregation of the protein during the incubation period, which was also observed in our previous report (21).

Determination of viral specificity of the interaction between the C terminus of the assembly protein precursor and the major capsid protein. The results in Fig. 3 demonstrate that the interaction domains in HSV-1 and CMV are not exchangeable, indicating that each virus must have specific amino acids in the interaction domain which determine the specificity of the interaction. We next sought to explore the differences between the interaction domains of the HSV-1 scaffolding protein and the CMV assembly protein precursor to determine what features conferred viral specificity to the interaction. Amino acid alignments (performed with GCG PileUp, version 8) of the interaction domains from various herpesviruses re-

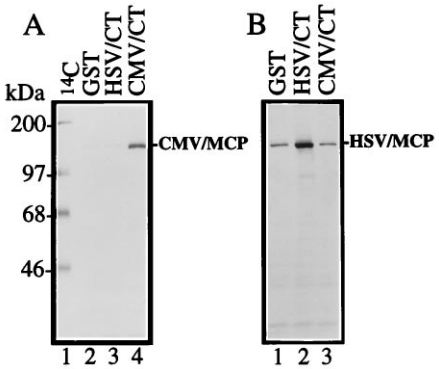

FIG. 3. Viral specific interaction between the C terminus of the assembly protein precursor and the major capsid protein. (A) Affinity precipitation of ^{35}S-labeled CMV major capsid protein (CMV/MCP). Lane 1, ^{14}C-labeled molecular weight markers; lane 2, GST control; lane 3, HSV-1 GST/CT fusion protein containing 30 amino acids from the C terminus of the scaffolding protein (HSV/CT); lane 4, CMV GST/CT fusion protein containing 20 amino acids from the C terminus of the assembly protein precursor (CMV/CT). (B) Affinity precipitation of ^{35}S-labeled HSV-1 major capsid protein (HSV/MCP). Lane 1, GST control; lane 2, HSV-1 GST/CT fusion protein; lane 3, CMV GST/CT fusion protein.

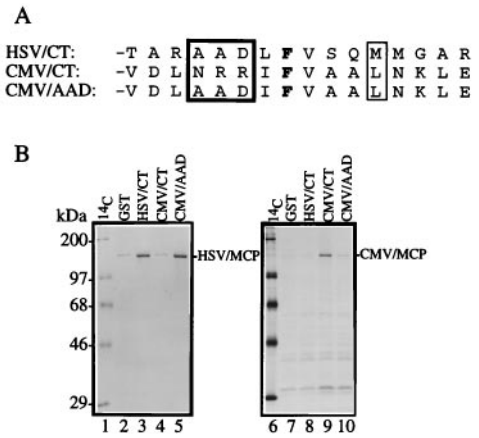

FIG. 4. Determination of viral specificity of the interaction. (A) GST/CT fusion constructs. HSV/CT consists of the C-terminal 30 amino acids of the HSV-1 scaffolding protein; CMV/CT consists of the C-terminal 20 amino acids of the CMV assembly protein precursor; CMV/AAD derives from CMV/CT by conversion of the Asn-Arg-Arg (NRR) region (boxed with thick lines) to the corresponding one, Ala-Ala-Asp (AAD), in HSV-1. The Phe residue in boldface is absolutely conserved. The conserved contact point (Met in HSV-1 or Leu in CMV) at the C-terminal side of Phe is boxed with thin lines. (B) Affinity precipitation of ^{35}S-labeled HSV-1 (lanes 2 to 5) and CMV (lanes 7 to 10) major capsid proteins (MCP). Lanes 1 and 6, ^{14}C-labeled molecular weight markers; lanes 2 and 7, GST controls; lanes 3 and 8, HSV-1 GST/CT fusion protein (HSV/CT); lanes 4 and 9, CMV GST/CT fusion protein (CMV/CT); lanes 5 and 10, converted CMV GST/CT fusion protein (CMV/AAD).

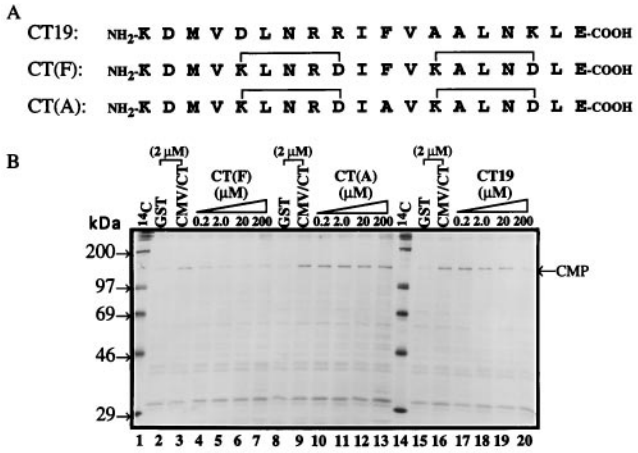

FIG. 5. Peptide competition with the CMV GST/CT fusion protein for interaction with the CMV major capsid protein (CMP). (A) Amino acid sequences of the peptides. CT19 consists of the last 19 amino acids of the CMV assembly protein precursor; CT(F) is a cyclic analog of CT19 containing two intrahelix linkages; CT(A) is a mutant peptide of CT(F) with a Phe-to-Ala substitution. The brackets represent the lactam bonds between the side chains of Lys (K) and Asp (D). (B) Peptide inhibition of the interaction between the CMV GST/CT fusion proteins and the CMV major capsid protein. The final concentration for GST or GST/CT fusion proteins was 2 μM. The peptide concentration was 0.2, 2.0, 20, or 200 as indicated. All peptides were dissolved in PBS.

vealed that the phenylalanine residue in the center of the interaction domains was absolutely conserved, which was consistent with the observation that the Phe residue was critical for the interaction (21). The region C terminal to the Phe residue is well conserved at the site of interaction; HSV-1 has a Met residue, while CMV has a Leu residue at the corresponding site. A significant difference between the two viruses resides in a region N-terminal to the Phe residue, where HSV-1 consists of the sequence Ala-Ala-Asp whereas CMV consists of Asn-Arg-Arg (21). This region in CMV is positively charged with the basic amino acid Arg, while the region in HSV-1 is not. A double Lys mutant in HSV-1, in which the two Ala-Ala residues were substituted with two Lys-Lys residues, completely abolished the interaction between the C terminus of the HSV-1 scaffolding protein and the HSV-1 major capsid protein (21), suggesting that positive charge in this region is detrimental to the interaction between the HSV-1 capsid proteins. Mutants were constructed in CMV GST/CT20 to convert the Asn-Arg-Arg in the region N-terminal to the Phe residue to the corresponding HSV-1 sequence Ala-Ala-Asp (Fig. 4A). The results in Fig. 4B demonstrated that the converted CMV GST/CT20 mutant protein lost its ability to interact with the CMV major capsid protein (lane 10) but gained full interaction with the HSV-1 major capsid protein (lane 5). Sequential mutations were also made in the region to determine at which point the CMV GST/CT20 mutant protein would lose its ability to interact with its own major capsid protein and gain the ability to interact with the HSV-1 major capsid protein. It was found that a single conversion from Arg to Asp abolished the interaction with the CMV major capsid protein but failed to confer any interaction with the HSV-1 major capsid protein. A double conversion from Arg-Arg to Ala-Asp had a phenotype similar to that of the single conversion (data not shown). The CMV GST/CT20 mutant protein gained interaction with the HSV-1 major capsid protein when all of the three amino acids were converted to those of HSV-1, suggesting that these three amino acids are important in defining viral specificity. Changing the amino acid at the C-terminal side of the central Phe residue from Leu to Met resulted in slight reduction in the interaction with the CMV major capsid protein but only a slight increase in the interaction with VP5 (data not shown). Therefore, this finding suggests that the region C terminal to the Phe residue plays a less critical role in establishing the viral specificity of the interaction.

The minimal domain in the C terminus of the CMV assembly protein precursor also forms an α-helix for interaction with the major capsid protein. In our previous report, we provided evidence to establish the requirement for an α-helical structure in the interaction domain in HSV-1. We demonstrated that a helicity-enhancing peptide derived from the minimal interaction domain competes more efficiently with HSV-1 GST/CT than the linear peptide for interaction with the HSV-1 major capsid protein (21). Similar experiments were designed to address whether CMV also required such a helical structure for interaction between the C terminus of the assembly protein precursor and the major capsid protein. Three peptides, all derived from the last 19 amino acids of the C-terminal end containing the minimal interaction domain, were synthesized and purified. CT19 is a linear peptide, whereas CT(F) and CT(A) are cyclic analogs of CT19 which were synthesized and modified by introducing two intrahelix lactam bonds formed by the cyclization of the Lys and Asp side chains (12) (Fig. 5A). Such modifications are compatible with α-helix conformation (13) and have been shown to stabilize α-helical structures and increase helicity in aqueous solution (12, 13, 26). All three peptides were tested for the ability to compete with CMV GST/CT20 for interaction with the major capsid protein. The results in Fig. 5B demonstrate that the dilactam-constrained cyclic peptide CT(F) competed much more efficiently for the interaction than the linear peptide CT19. At 0.2 μM, CT(F) significantly inhibited the interaction between GST/CT20 and the CMV major capsid protein (Fig. 5B, lane 4), whereas 200 μM CT19 was required to compete significantly for the interaction between GST/CT and the major capsid protein (lane

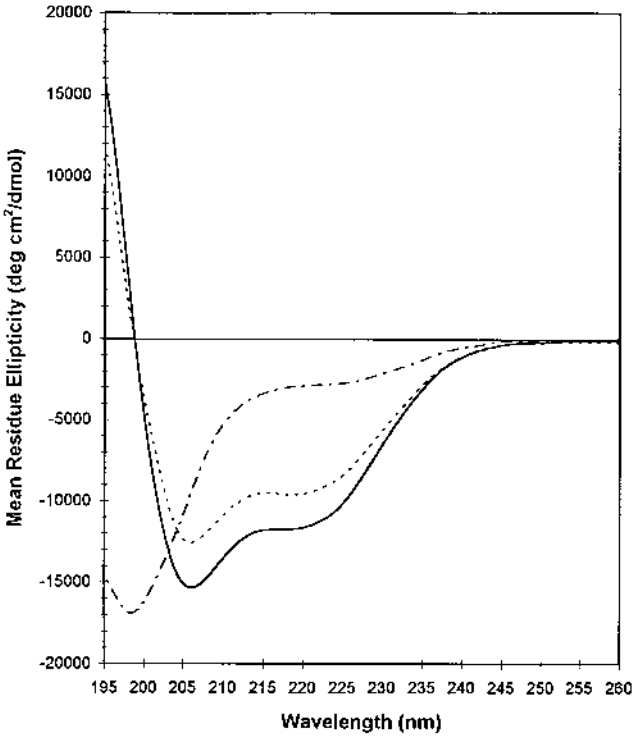

FIG. 6. Determination of the relative helicity of each peptide by CD spectropolarimetry. The CD parameters are discussed in Materials and Methods. Dash-dotted line, CT19; dotted line, CT(F); solid line, CT(A).

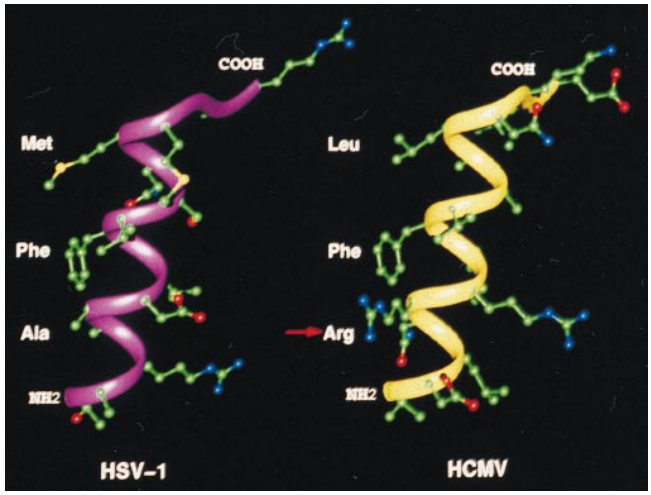

FIG. 7. Three-dimensional structural model for the minimal interaction domains of HSV-1 (left) and CMV (right). The α-helix models were constructed by using the Insight II software (version 95.0; Biosym/MSI).

20), confirming the requirement for an α-helical structure in CMV GST/CT20 for efficient interaction with the major capsid protein. In contrast, the dilactam-constrained control peptide CT(A) containing a substitution of the critical Phe with Ala failed to compete for the interaction (lanes 10 to 13), suggesting that, as in HSV-1, the Phe residue in the interaction domain of the CMV assembly protein precursor is also critical for the interaction. The peptides were further analyzed by CD for the presence of secondary structures. As predicted, the results in Fig. 6 show that the linear peptide, CT19, exhibits a typical spectrum for random coil, whereas CT(F) and CT(A) both have characteristic spectra for an α-helical structure, confirming that both cyclic peptides are locked in a predominantly α-helical structure. The relative helicity for each peptide was calculated by using the formula of Stevenson et al. (49). The linear peptide, CT19, lacks secondary structure, and thus its helicity is normalized to 0%. The helicities for CT(F) and CT(A) relative to that of the linear peptide are calculated to be approximately 24 and 31%, respectively. These results suggest that the minimal domain requires an α-helix for efficient interaction with the major capsid protein.

DISCUSSION

The primary findings of this study are as follows: (i) the C-terminal 20 amino acids of the CMV assembly protein precursor (or those of the maturation protease) are sufficient for interaction with the CMV major capsid protein, and the minimal interaction domain has been mapped to a 16-amino-acid region near the C-terminal end; (ii) the Phe residue in the center of the interaction domain is critical for the interaction; (iii) an α-helix is required for efficient interaction with the CMV major capsid protein; (iv) there are no cross-interactions between HSV-1 and CMV counterpart capsid proteins; (v) a region at the N-terminal side of the central Phe residue which plays a role in determining the viral specificity of the interaction has been identified. Results consistent with these have been obtained in a study using the yeast two-hybrid system, which localized the assembly protein-major capsid protein interaction domain to the carboxy-terminal 21 amino acids of the human CMV assembly protein precursor and detected no cross-interactions between the HSV-1 and CMV counterpart proteins (60).

Thus far, we have demonstrated that the HSV-1 scaffolding protein and the CMV assembly protein have acquired similar schemes for interaction with the major capsid protein. They both require a Phe residue and the formation of an α-helical structure in the minimal domain for efficient interaction with the major capsid proteins. In HSV-1, it has been shown that the interaction with the major capsid protein occurs on one side of the helix and requires the hydrophobic amino acids centered around the central Phe residue which forms a hydrophobic interface (21). In CMV, the central Phe residue is also critical (Fig. 5B), and the Leu residue at the C-terminal side of the Phe seems to play a role similar to that of the Met residue at the corresponding position in HSV-1 (Fig. 4A). At the N-terminal side of the Phe residue, however, CMV has an Arg residue rather than the Ala at the corresponding position in HSV-1. We propose a structural model (Fig. 7) for the interaction domains in both viruses whereby (i) they both form an α-helix in their minimal domains, and the interfaces with the major capsid proteins are located on one side of the α-helix, and (ii) the interfaces mainly consist of three-amino-acid side chains which form the contact points for interaction with the major capsid protein. The middle contact point is made up of the Phe side chain, the C-terminal one is made up of either the Met side chain in HSV-1 or the Leu side chain in CMV, and the N-terminal one is either the Ala in HSV-1 or the Arg in CMV. Our data suggest that the N-terminal site plays a critical role in determining the viral specificity of the interaction in different viruses.

A capsid assembly assay for HSV-1 which provides very useful information on both the structure and the assembly pathway of the capsids has been established (50, 52). It has been shown that interactions between different capsid proteins

play critical roles in dictating the proper assembly of the capsids (8, 21, 22, 29, 35, 51, 62). An in vitro capsid assembly for CMV has yet to be established. To move ahead without extensive characterization of CMV capsid assembly, we chose to focus on one critical aspect of the assembly pathway: the interaction between the C terminus of the assembly protein precursor and the major capsid protein. We hope that this will allow us to probe any intrinsic difference or similarity between the two viruses in capsid assembly. The results presented here demonstrate that although the two viruses have developed similar schemes for the interaction between the assembly proteins and the major capsid proteins, the specificities of the interactions are different (Fig. 3). We hypothesize from this finding that while CMV and HSV-1 may have similar strategies leading to capsid assembly, the information dictating the execution of each step may not be exchangeable between the two viruses. It will be interesting to test this hypothesis in an in vitro capsid assembly assay using a mixture of heterologous capsid proteins from both viruses.

Pileup alignment of the interaction domain from various herpesviruses (21) has revealed some interesting features: (i) the Phe residue is absolutely conserved; (ii) the interaction site or contact point at the C-terminal side of Phe is well conserved, being occupied by Met in alphaherpesviruses and by Leu in beta- and gammaherpesviruses; and (iii) the interaction site at the N-terminal side of Phe is not conserved, consisting of Ala in alphaherpesviruses and of Arg, Lys, or Gln in beta- and gammaherpesviruses. Our data demonstrate that the N-terminal interaction site plays a role in determining the viral specificity of the interaction between viruses from two different subfamilies, such as HSV-1 and CMV. However, within the same subfamily, the N-terminal site is conserved. For example, HSV-1, HSV-2, and varicella-zoster virus have the same Ala at the N-terminal interaction site. It is of great interest to determine whether viruses from the same subfamily interact with each other for the interaction between the assembly protein and the major capsid protein. Alternatively, this hypothesis on viral specificity could be tested by examining whether Epstein-Barr virus (a member of the gammaherpesviruses) also lacks cross-interaction with HSV-1 capsid proteins.

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