

Cytomegalovirus Assemblin: the Amino and Carboxyl Domains of the Proteinase Form Active Enzyme when Separately Cloned and Coexpressed in Eukaryotic Cells

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The cytomegalovirus (CMV) serine proteinase assemblin is synthesized as a precursor that undergoes three principal autoproteolytic cleavages. Two of these are common to the assemblin homologs of all herpes group viruses: one at the maturational site near the carboxyl end of the precursor and another at the release site near the midpoint of the precursor. Release-site cleavage frees the proteolytic amino domain, assemblin, from the nonproteolytic carboxyl domain of the precursor. In CMV, a third autoproteolytic cleavage at an internal site divides assemblin into an amino subunit (A_n) and a carboxyl subunit (A_c) of approximately the same size that remain associated as an active “two-chain” enzyme. We have cloned the sequences encoding A_n and A_c as separate genes and expressed them by transfecting human cells with recombinant plasmids and by infecting insect cells with recombinant baculoviruses. When A_n and A_c from either simian CMV or human CMV were coexpressed in human or insect cells, active two-chain assemblin was formed. This finding demonstrates that A_n and A_c do not require synthesis as single-chain assemblin to fold and associate correctly in these eukaryotic systems, and it suggests that they may be structurally, if not functionally, distinct domains. An interaction between the independently expressed A_n and A_c subunits was demonstrated by coimmunoprecipitation experiments, and efforts to disrupt the complex indicate that the subunit interaction is hydrophobic. Cell-based cleavage assays of the two-chain assemblin formed from independently expressed A_n and A_c also indicate that (i) its specificity for both CMV and herpes simplex virus native substrates is similar to that of single-chain assemblin, (ii) R-site cleavage is not essential for the activity of two-chain recombinant assemblin, and (iii) the human CMV and simian CMV A_n and A_c recombinant subunits are functionally interchangeable.

The maturational serine proteinase of herpes group viruses (20, 28, 49) is essential for virus replication (10, 27) and functions during capsid assembly by cleaving an abundant capsid phosphoprotein called the assembly protein precursor (pAP). The proteinase is synthesized as an enzymatically active precursor (e.g., pNP1 [Fig. 1]) that undergoes several autoproteolytic cleavages. Two of these are common to all herpes group viruses: one occurs toward the carboxyl end of the precursor at the maturational (M) cleavage site, and the other occurs near the middle of the precursor at the release (R) cleavage site (2, 8, 49). R-site cleavage divides the precursor into a proteolytic amino half, called assemblin (NP1_n or A) in cytomegalovirus (CMV), and a nonproteolytic carboxyl half (pNP1_c) (Fig. 1) (8, 49). Because of the overlapping arrangement of their genes, the entire amino acid sequence of the pAP substrate is also expressed as the carboxyl half of the proteinase precursor (19, 46). The M and R sites of the 16 sequenced herpesvirus assemblin homologs are well conserved and have the core consensus sequences (V/L/I)-(D/E/N/Q)-A ↓ S and Y-(V/L/I)-(K/Q)-A ↓ (S/N/T), respectively (12, 49).

In addition to these two herpesvirus group-common cleavages, CMV proteinases undergo a third autoproteolytic cleavage at an internal (I) site that divides assemblin into approximately equal-size amino (A_n) and carboxyl (A_c) subunits (2, 3, 47). Unlike the core consensus sequences of the M and R sites,

that of the I site has an alanine in the P1' position (32) and is only modestly conserved among the human CMV (HCMV) (2, 3), simian CMV (SCMV) (47), baboon CMV (sequence submitted to GenBank), and murine CMV (22) proteinases (i.e., DVEA ↓ ATSL, DINA ↓ ADGA, DINA ↓ ADSA, and DAVA?AAAA, respectively). The cleavage order is M site before R site before I site both in transfection assays (47) and in virus-infected cells (16), but none of these cleavages is absolutely required for enzyme activity (16, 47).

Efforts to purify bacterially expressed HCMV assemblin led to the observation that A_n and A_c remain associated after I-site cleavage (3, 15, 31, 35). Further work showed that the resulting “two-chain” assemblin is still proteolytically active (15). More recently it has been found that HCMV A_n and A_c , synthesized in bacteria as glutathione S-transferase fusions, can associate to form an active two-chain proteinase but, in contrast to the results reported here, only if they are denatured and then renatured together (24).

This report describes experiments done to determine (i) whether active two-chain assemblin can form intracellularly when expressed from the individually cloned A_n - and A_c -coding sequences, (ii) whether the recombinant forms of A_n and A_c directly interact to form active two-chain assemblin, (iii) whether two-chain assemblin has a substrate specificity different from that of one-chain assemblin, and (iv) whether the A_n and A_c subunits can be interchanged between HCMV and SCMV.

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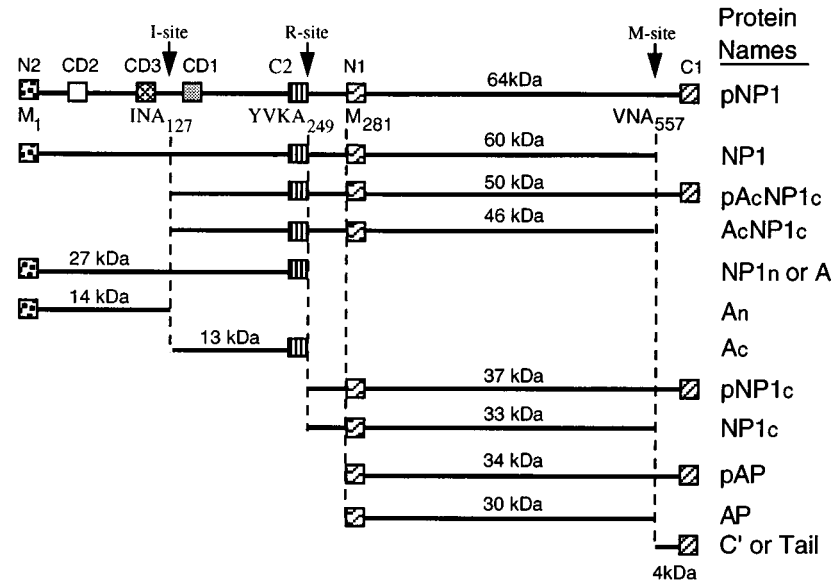


FIG. 1. Cleavage products of the SCMV assemblin precursor. Shown are the protein products that could result from cleavage of the SCMV assemblin precursor (pNP1) at any one or more of its three self-processing sites (M, R, and I sites). The abbreviations used for the cleavage products (49) are given at the right (A, assemblin); computer-predicted molecular masses are given immediately above or below the corresponding protein. The precursor assembly protein (pAP) and its M-site cleavage products, AP and C' (Tail), are also shown. Homologous products are generated from cleavage of the HCMV proteinase and assembly protein precursors (12) and from the HSV-1 assembly protein precursor.

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MATERIALS AND METHODS

Cells and viruses. The DNA sequences used in these studies were from HCMV strain AD169, SCMV strain Colburn, and herpes simplex virus type 1 (HSV-1) strain 17. Human foreskin fibroblast cells were used to propagate these viruses and to prepare the virus-infected cell lysates used as markers (11).

Recombinant baculoviruses (rBVs) were constructed as described below. Propagation, plaque assay, and protein expression studies of rBVs were done with *Spodoptera frugiperda* Sf9 cells (American Type Culture Collection, Rockville, Md.) grown in suspension at 28°C in 100-ml spinner flasks containing supplemented Grace's medium (no. 350-1605AJ; GIBCO, Grand Island, N.Y.) with additions to give 10% fetal calf serum (HyClone, Logan, Utah), 50 µg of gentamicin (no. 600-5750AD; GIBCO) per ml, and 125 ng of amphotericin B (Fungizone) (no. 600-5295AE; GIBCO) per ml. rBVs were plaque purified twice (4, 38); high-titer stocks were prepared for each (38) and stored at 4°C protected from light until used. Sf9 cells ($\approx 2.5 \times 10^5$ cells per well) were infected in 24-well plates (no. 3047; Becton Dickinson Labware, Oxnard, Calif.) at a multiplicity of infection of approximately 5 to 10 by adding 100 µl of virus in single infections or 50 µl of each virus in multiple-virus infections. The cells were harvested 3 days after infection by aspirating the medium, adding 70 µl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (described below) to the cell layer, and collecting the lysate. The resulting samples were heated in a boiling water bath for 3 min and stored at -80°C until analyzed by SDS-PAGE and Western immunoassay.

Plasmid constructions. Standard techniques were used to construct, clone, and propagate the plasmids (30). The new plasmids described here were derived from AW4 and AW1 (49), which are constructs of the RSV.5(neo) expression vector (21) that contain the SCMV strain Colburn genes APNG1 (full-length assembly protein nested gene; encodes precursor proteinase [pNP1]) and APNG.5 (encodes assembly protein precursor [pAP]), respectively.

BJ1 encodes the short form of the SCMV proteinase assemblin (Fig. 2A) and was derived from AW4 by digesting it with *Esp31* and *Bam*HI, isolating the large fragment by agarose gel electrophoresis, and ligating the fragment to a pair of annealed synthetic oligonucleotides. The sense oligonucleotide, 5'-CTAAGC TGCGCTATGACAAGCGACTAGTAGGGGTCACGGCTCGGGAGTCGT ACGTAAAAGCTTAAAG-3', was annealed with an antisense oligonucleotide, yielding a double-stranded fragment having 5' *Esp31* and 3' *Bam*HI overhangs. The oligonucleotide pair also contained a stop codon (TAA, double underlined) and five third-nucleotide codon changes (underlined) that conserve the amino acid sequence but introduce three new restriction endonuclease sites (i.e., *Spe*I, *Sna*BI, and *Afl*III).

MH14 was used as an intermediate in constructing MH28, a plasmid (described below) that encodes the amino subunit of SCMV assemblin, A_n (Fig.

2A). MH14 was derived from AW4 by site-directed mutagenesis and contains a conservative change in the Ser-120 codon (i.e., TCC to TCG [underlined below]) that creates a new *Xho*I site. Site-directed mutagenesis (40, 41) was done, as specified by the manufacturer (Amersham, Arlington Heights, Ill.; catalog no. RPN1523), on a single-stranded M13 DNA clone (AW12) that contains the APNG1 gene. The oligonucleotide used for mutagenesis was 5'-CTGTC GCTCTCGAGCCGCCGAGA-3'. A region of the M13 single-stranded DNA clone surrounding the mutation, and including the 5' *Eco*RV and 3' *Ssr*II cleavage sites, was converted to double-stranded DNA and PCR amplified (23) by using suitable oligonucleotide primers. The resulting mutant fragment was treated with *Eco*RV and *Ssr*II to generate the needed 5' and 3' overhangs, respectively, and then used to replace the corresponding small *Eco*RV-to-*Ssr*II fragment of wild-type AW4. This construct was then sequenced between the *Eco*RV and *Ssr*II sites to confirm that no errors were introduced during the PCR.

MH28 encodes the amino subunit of SCMV assemblin, A_n (Fig. 2A), and was made by replacing the insert-containing *Xho*I-to-*Bam*HI fragment of MH14 (described above) with an oligonucleotide pair having a stop codon (TAA, underlined below) following the Ala-127 codon and ending with the 5' *Xho*I and 3' *Bam*HI overhangs needed for ligation into MH14. The sense oligonucleotide of the pair was 5'-TCGAGCCGCCGAGATATCAACGAGTAAAG-3'.

MH29 encodes the carboxyl subunit of SCMV assemblin, A_c (Fig. 2A), and was made by removing a small *Sall*I-to-*Ssr*II fragment from BJ1 and replacing it with a pair of annealed synthetic oligonucleotides that contain a start codon (underlined below) in a newly created *Nde*I site next to the codon for Ala-128 of SCMV assemblin. The sense oligonucleotide of the pair was 5'-TCGACG GATCCATATGGCCGATGGCGCCGC-3' and when annealed with the antisense oligonucleotide gave the 5' *Sall*I and 3' *Ssr*II overhangs needed for ligation into the corresponding sites in BJ1.

MH44 encodes an amino-terminal deletion mutant of SCMV pNP1 from which the coding sequence for A_n has been eliminated (Fig. 2A). This mutant protein is called pA_cNP1_c and was made by replacing the small *Sall*I-to-*Ssr*II fragment of MH14 (described above) with the oligonucleotide pair described above for making MH29.

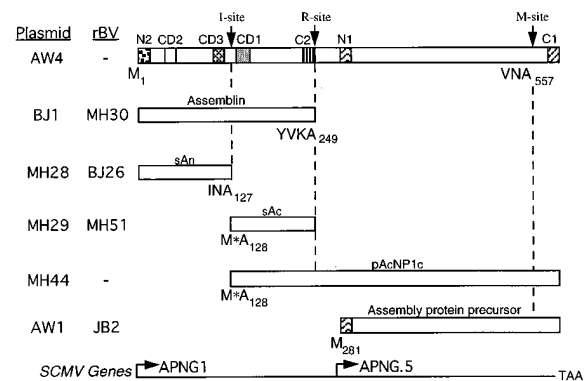
Construction of rBVs. rBVs were made by using the Baculogold system (no. 21001K; Pharmingen, La Jolla, Calif.) as instructed by the manufacturer. CMV and HSV genes were introduced into the BV genome via either the pVL1392 or pVL1393 transfer vector (25). Construction of transfer plasmids was done as follows.

JB2 encodes the SCMV pAP. It was made by removing the APNG.5 gene from a pGEM-4z construct (AW33) by *Bam*HI digestion and then ligating it into the pVL1393 *Bam*HI site.

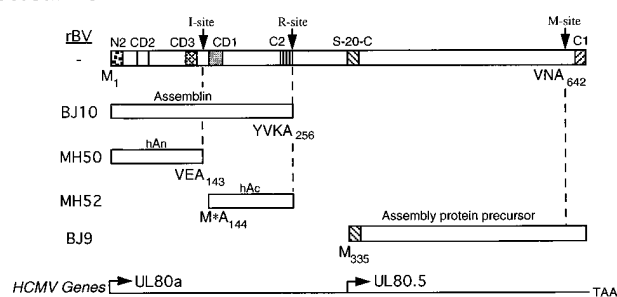
BJ9 encodes the HCMV pAP. It was made by removing the UL80.5 gene from LM11 (48) by *Xba*I digestion and then ligating it into the pVL1393 *Xba*I site.

MH30 encodes SCMV assemblin bearing an amino-terminal peptide handle (N'MHWHWH-, for chromatographic purification) (34). It was made by removing the SCMV assemblin gene, with the handle sequence, from a pET11c con-

A. SCMV Constructs



B. HCMV Constructs



C. HSV-1 Construct



FIG. 2. Genes, protein products, and landmarks of SCMV, HCMV, and HSV open reading frames. Shown are the recombinant proteins described in the text and their plasmid or BV designations. (A) SCMV, strain Colburn, constructs: M-1 (M_1) is the translational start methionine of the proteinase precursor encoded by APNG1 and its amino-terminal cleavage products, assemblin and A_n ; M^* is a translational start methionine that results from adding an ATG codon to the 5' ends of the sA_n - and $pAcNP1_c$ -coding sequences; M_{281} (M_{281}) is the start methionine for pAP encoded by APNG.5. A-127 (A_{127}), A-249, and A-557 denote the P1 (see nomenclature in reference 32) residues of the internal (I), release (R), and maturational (M) cleavage sites. N1, N2, C1, and C2 represent amino acid sequences used to prepare the antipeptide antisera anti-N1, -N2, -C1, and -C2, respectively. Conserved domains (CDs) 1, 2, and 3 (47, 49) are also indicated for reference. (B) HCMV, strain AD169, constructs: M-1 is the translational start methionine for the proteinase precursor encoded by UL80a and its amino-terminal cleavage products, assemblin and A_n ; M^* is a translational start methionine that results from adding an ATG codon to the 5' end of the hA_n -coding sequence, and M_{335} is the start methionine for the precursor assembly protein encoded by UL80.5. A-143, A-256, and A-642 denote the cleavage site P1 residues. S-20-C represents the amino acid sequence used to prepare the antipeptide antiserum, anti-S-20-C, to the amino end of the HCMV pAP. Other abbreviations are as explained for panel A. (C) HSV-1, strain 17, construct: M-307 is the translational start methionine for the precursor assembly protein encoded by UL26.5, and A-610 is the M-site P1 residue. The names of the corresponding plasmids and rBVs that encode them (Fig. 2). We have used the term two chain (15) to describe I-site-cleaved assemblin, the term subunit in reference to the A_n and A_c

structure (MH12) by *XbaI*-*Bam*HI digestion and then ligating it into those sites in pVL1392. In agreement with comparisons of HCMV assemblins with and without an amino handle (35), in these assays the proteolytic activity of the MH30 protein was found to be indistinguishable from that of the wild-type assemblin encoded by JB3 (data not shown).

BJ10 encodes HCMV assemblin. It was made by removing the HCMV assemblin-coding sequence from LM12 (48) by *XbaI* digestion and then ligating it into the pVL1393 *XbaI* site.

BJ26 encodes SCMV A_n and was made by removing the A_n -coding sequence from MH28 (see above) by *HincII*-*Bam*HI digestion and then ligating it into the compatible *SmaI*-*Bgl*III sites in pVL1393.

MH50 encodes HCMV A_n and was made by removing the A_n -coding sequence from an RSV.5(neo) construct (MH46) by *XbaI* digestion and then ligating it into the pVL1393 *XbaI* site.

MH51 encodes SCMV A_c and was made by removing the A_c -coding sequence from an RSV.5(neo) construct (MH47) by *Bam*HI digestion and then ligating it into the pVL1393 *Bam*HI site.

MH52 encodes HCMV A_c and was made by removing the A_c -coding sequence from an RSV.5(neo) construct (MH49) by *XbaI* digestion and then ligating it into the pVL1393 *XbaI* site.

MH60 encodes HSV-1 pAP and was made by removing the UL26.5-coding sequence by *XbaI* digestion of EV3 (48) and then ligating it into the pVL1393 *XbaI* site.

Transfection assay. The calcium phosphate transfection procedure (6) was done with human embryonal kidney (HEK) cells (line 293; American Type Culture Collection) essentially as described before (13). The amount of DNA used in single-plasmid transfections was 1 μ g for pAP-encoding plasmids or 2 μ g for plasmids encoding A_n , A_c , or the proteinase and mutants. In multiple-plasmid transfections the amount of each plasmid encoding A_n , A_c , or the proteinase and mutants was 0.5 μ g, and the amount of plasmid encoding pAP was 1.0 μ g. Each transfection had 0.2 μ g of a plasmid encoding the simian virus 40 (SV40) large T antigen added to increase the plasmid copy number (13).

SDS-PAGE and Western immunoassay. SDS-PAGE was done essentially as described by Laemmli (17); the ratio of *N,N'*-methylene-bisacrylamide to acrylamide was 0.735:28, SDS was from Bio-Rad (Melville, N.Y.), and 2 \times SDS-PAGE sample buffer was 4% SDS, 20% β -mercaptoethanol, 20% glycerol, 50 mM Tris (pH 7.0), and 0.02% bromophenol blue.

Western immunoassays were done essentially as described by Towbin et al. (44). A semidry transfer unit was used, the membrane was Immobilon P (Millipore, Bedford, Mass.), the buffer was 50 mM Tris-20% methanol, and the time of transfer was calculated by the formula gel width \times height \times 2.5 = milliamperes per 30 min. The membrane was blocked in a solution containing 10 mM Tris, 0.9% NaCl, and 5% bovine serum albumin (pH 7.4) (TN-BSA), reacted sequentially with antiserum and then 125 I-protein A (both in TN-BSA), and exposed to X-ray film, usually with a calcium tungstate intensifying screen (18).

Antisera were prepared by injecting rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin (no. 77100G; Pierce, Rockford, Ill.). Anti-N2 was made by immunization with a peptide corresponding to amino acids 2 to 15 of SCMV assemblin with a carboxy-terminal cysteine residue added for coupling to keyhole limpet hemocyanin. Anti-N1, an antiserum to the amino end of SCMV pAP (33), anti-S-20-C, an antiserum to the amino end of HCMV pAP (48), anti-C2, an antiserum to the carboxyl end of SCMV assemblin (47); and a cocktail of monoclonal antibodies to the HSV-1 AP (48) have all been described before. Antisera were used alone or in mixtures, as indicated in Results and figure legends, at a dilution of 1:40 in TN-BSA.

Immunoprecipitation. Immunoprecipitations from rBV-infected cells were done as follows. Approximately 2×10^6 Sf9 cells were grown at 28°C in 6-cm-diameter plastic petri dishes and then infected with one or more rBVs at a multiplicity of infection of 5 to 10. Infected cells were harvested 2 days after infection by removing the medium and adding 100 μ l of 0.5% Nonidet P-40 (NP-40) in 10 mM Tris (pH 7.4)-0.9% NaCl. The disrupted cells were scraped from the dish, transferred to a microfuge tube, and kept on ice for 5 min. The NP-40 nuclear fraction (pellet) was separated from the NP-40 cytoplasmic fraction (supernatant) by centrifugation (16,000 \times g, 4°C, 10 min). The resulting NP-40 cytoplasmic fraction was used for subsequent immunoprecipitation reactions.

Immunoprecipitations were done by combining 50 μ l of the NP-40 cytoplasmic fraction with 20 μ l of anti-C2 antiserum, rocking the solution at room temperature for 1.5 h, and then adding 70 μ l of protein A beads (Sigma, St. Louis, Mo.) (100 mg/ml in calcium- and magnesium-free phosphate-buffered saline [PBS]) and rocking the mixture for 1 h at room temperature. The beads were collected by centrifugation (30 s at 3,000 \times g), washed four times with 200 μ l of IP wash buffer (0.5% deoxycholate [DOC], 1% NP-40, and 300 mM KCl in calcium- and magnesium-free PBS), transferred to a new tube and, washed once more with IP wash buffer. A volume of 2 \times SDS-PAGE solubilizing buffer approximately equal to the bead-pellet volume (i.e., \approx 40 μ l per tube) was added to the beads, and the samples were heated in a boiling water bath for 3 min and stored at -80°C until analyzed by SDS-PAGE and Western immunoassay.

RESULTS

The protein products derived from cleavage of the SCMV proteinase and assembly protein precursors are shown in Fig. 1, and the CMV and HSV genes, proteins, and specific landmarks discussed in this report are shown in Fig. 2. These figures also show the protein names (Fig. 1) and those of the corresponding plasmids or rBVs that encode them (Fig. 2). We have used the term two chain (15) to describe I-site-cleaved assemblin, the term subunit in reference to the A_n and A_c

cleavage products of assemblin, and the abbreviation A_n/A_c to denote the recombinant two-chain enzyme.

Expression and detection of the amino and carboxyl subunits of SCMV assemblin. I-site cleavage of SCMV assemblin yields an amino 127-amino-acid subunit (A_n , residues 1 to 127) and a carboxyl 122-amino-acid subunit (A_c , residues 128 to 249). The coding sequences for these two proteins were cloned into the expression vector RSV.5(neo) as described in Materials and Methods, yielding the plasmids MH28 (encodes A_n) and MH29 (encodes A_c). Expression of A_n and A_c from these plasmids was demonstrated by an initial experiment in which HEK cells were transfected with either MH28 or MH29. As controls for expression of the substrate, pAP (encoded by AW1), and assemblin (encoded by BJ1) and for the cleavage reaction, transfections were also done with the plasmids AW1, BJ1, and AW1 plus BJ1, respectively. Lysates prepared from the transfected cells were subjected to SDS-PAGE, all in the same gel, followed by Western immunoassay with antisera that recognize the amino (anti-N2) or carboxy (anti-C2) terminus of SCMV assemblin or the amino terminus of the SCMV AP (anti-N1), all as described in Materials and Methods.

The results of the experiment showed that anti-N2 recognized both assemblin and its I-site cleavage product A_n in the BJ1 lysate (Fig. 3A, lane 7), anti-C2 recognized both assemblin and its I-site cleavage product A_c in the BJ1 lysate (Fig. 3B, lane 7), and anti-N1 recognized substrate pAP in the AW1 lysate (Fig. 3C, lane 6) and both pAP and its cleavage product AP in the AW1+BJ1 lysate (Fig. 3C, lane 8), all as expected. A mixture of the three antisera was used to detect all of the proteins simultaneously (Fig. 3D, lane 8). Although the antisera cross-reacted to some extent with host cell proteins, most notably with two species just above A_c that are especially conspicuous with anti-C2 (h in Fig. 3B), none cross-reacted significantly with targets of the other antisera.

Expression of A_n from MH28 was indicated by the presence of a protein that reacted with anti-N2 (Fig. 3A, lane 4) and comigrated with assemblin-derived A_n (Fig. 3A, lane 7). Expression of A_c from MH29 was similarly indicated by the presence of a protein that reacted with anti-C2 (Fig. 3B, lane 5 [indicated by dot]) and comigrated with assemblin-derived A_c (Fig. 3B, lane 7). The intensity of A_n in both transfected and SCMV-infected cells was reproducibly stronger than that of A_c , which was often too weak to be detected in these assays. Synthesis of A_c was better demonstrated by immunoprecipitation, but again its intensity was weak (data not shown). When expressed in rBV-infected insect cells, however, synthesis of A_c was readily detected (see Fig. 7A, lane 9). Neither SCMV A_n (9.7 kDa observed; 13.7 kDa predicted) nor A_c (15.2 kDa observed; 13.2 kDa predicted) migrated as expected during SDS-PAGE.

A_n and A_c coexpressed from separate plasmids catalyze both M- and R-site cleavage. When SCMV pAP and assemblin are coexpressed, a substantial portion of pAP is cleaved at its M site to yield AP (Fig. 4, lane 6) (13, 49). Neither A_n nor A_c alone catalyzed this M-site cleavage (Fig. 4, lanes 7 and 8), but when the two subunits were expressed together, pAP→AP cleavage was readily detected (Fig. 4, lane 9).

To determine whether coexpression of A_n and A_c could also produce R-site cleavage, we used the S-118→A (S118A) mutant (47) of the SCMV precursor proteinase as a substrate. This mutant is proteolytically inactive because it lacks the essential Ser-118 nucleophile (7, 9, 15, 36, 47), and it therefore can be used as a substrate to monitor cleavage at its M, R, and I sites (13, 48). Cells expressing S118A alone (Fig. 5, lane 4) showed only the noncleaved precursor, pNP1 (Fig. 1), a modified form of pNP1 (Fig. 5, asterisk), and a ladder of smaller

presumptive degradation products, none of which comigrated with the specific cleavage products, NP1, NP1_c, and assemblin (Fig. 1). However, when S118A was coexpressed with assemblin (Fig. 5, lane 6) both M-site (evidenced by pNP1→NP1) and R-site (evidenced by NP1→NP1_c plus assemblin) cleavages occurred. S118A was similarly cleaved when expressed with A_n + A_c (i.e., presence of NP1, NP1_c, and assemblin) (Fig. 5, lane 5), demonstrating that these subunits can catalyze both R- and M-site cleavage when coexpressed. It should be noted that the assemblin band seen in lane 6 of Fig. 5 can come both from cleaved S118A and from expression of the BJ1 plasmid, but that seen in lane 5 can come only from cleaved S118A.

R-site cleavage not required for two-chain proteinase formation. Cleavage of the precursor proteinase, pNP1, only at its I site would generate A_n and a large carboxyl product composed of A_c , still linked to pNP1_c (pA_cNP1_c [Fig. 1]). In order to determine whether A_n could interact with this carboxyl-extended form of A_c to yield an active two-chain proteinase, we cloned and expressed pA_cNP1_c . Alone, this amino-deletion mutant of the proteinase was unable to cleave SCMV pAP→AP (Fig. 6, lane 7). However, when pA_cNP1_c was coexpressed with A_n , proteolytic activity was constituted, as indicated by the processing of pAP→AP (Fig. 6, lane 8; AP is indicated by the open circle). Coexpression of pA_cNP1_c with A_c yielded no cleavage of pAP (Fig. 6, lane 9). A notably greater amount of pAP→AP cleavage was apparent when A_n was coexpressed with A_c (Fig. 6, lane 6) rather than with the extended form of A_c (i.e., pA_cNP1_c) (Fig. 6, lane 8). Given that the respective amounts of pAP plasmid and A_n plasmid were the same in the two transfections and that the amount of extended A_c (pA_cNP1_c) produced alone or with A_n (Fig. 6, lanes 7 and 8) was dramatically larger than the amounts detected for A_c (Fig. 3B, lane 5; see Fig. 7A, lane 8), these data suggest that R-site cleavage either (i) enables A_n to interact with the A_c domain more easily and thereby form more two-chain enzyme or (ii) yields a more active two-chain enzyme (i.e., $A_n/A_c > A_n/pA_cNP1_c$). Neither pNP1_c nor NP1_c, which could arise by R-site cleavage of pA_cNP1_c with or without M-site cleavage (Fig. 1), was detected in cells coexpressing both A_n and pA_cNP1_c (Fig. 6, lane 8), indicating either that R-site cleavage of pA_cNP1_c did not occur or that its frequency was below the sensitivity of detection in this system.

SCMV and HCMV A_n and A_c expressed from rBVs also show pAP→AP cleavage. To establish that the proteolytic activity of coexpressed SCMV A_n and A_c observed in transfected cells is not restricted to one experimental system, the A_n - and A_c -coding sequences of both SCMV and HCMV assemblins were cloned into rBVs and tested for activity in insect cells. Lysates prepared from Sf9 cells 3 days after infection were subjected to SDS-PAGE followed by Western immunoassay. The results showed that SCMV A_n and A_c were expressed from their rBVs (Fig. 7A, lanes 7 and 9) and that neither one alone cleaved rBV-expressed SCMV pAP→AP (Fig. 7B, lanes 7 and 9). The trace AP seen in lane 9 of Fig. 7B is spillover from adjacent wells and is not seen in other comparable samples (e.g., Fig. 7C, lane 9). However, when cells were coinfecting with both the SCMV A_n - and A_c -expressing rBVs, pAP→AP cleavage was readily demonstrated (Fig. 7B, lane 8), and the proportion of pAP cleaved to AP was consistently much higher than in transfection experiments (e.g., compare Fig. 7B, lanes 8 and 10, with Fig. 4, lane 9). We attribute the increase in efficiency of pAP→AP cleavage in the rBV system to the larger amount of subunit protein produced and the greater chance of forming two-chain enzyme.

Because of the strong sequence conservation between the

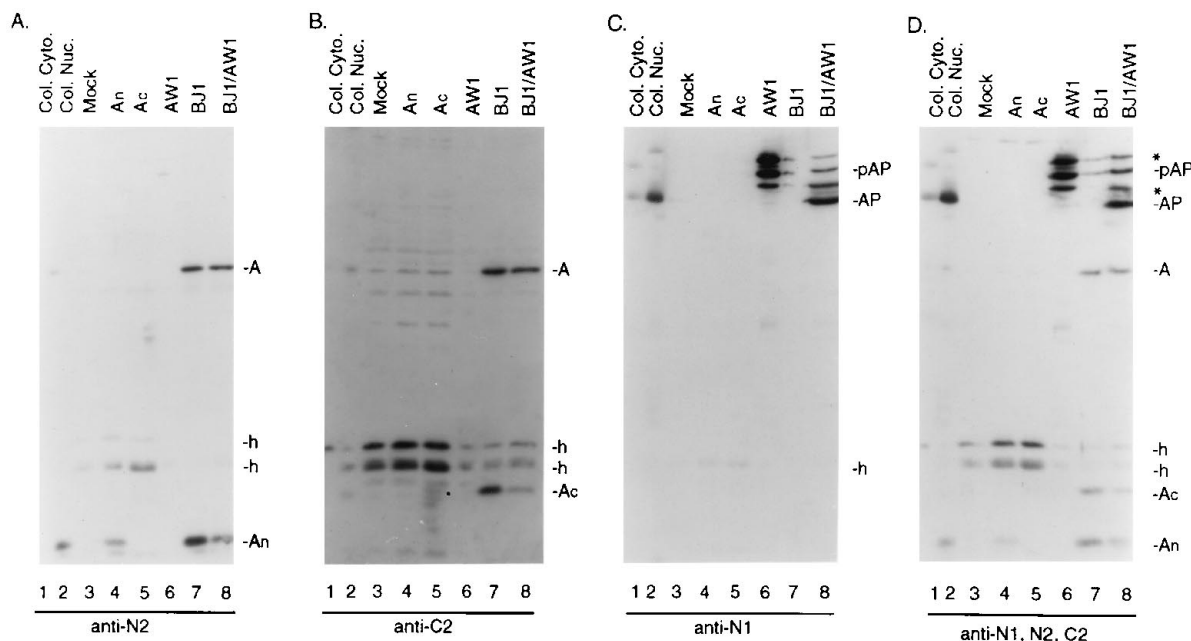


FIG. 3. Western immunoassays of SCMV constructs. HEK cells were transfected with plasmids encoding A_n (lanes 4), A_c (lanes 5), pAP (lanes 6), assemblin (lanes 7), or a mixture of assemblin and pAP (lanes 8); lysates were prepared 3 days later and subjected to SDS-PAGE in the same 14% acrylamide gel, all as described in the text and in Materials and Methods. NP-40 cytoplasmic (Col. Cyto.) and nuclear (Col. Nuc.) fractions of SCMV-infected HFF cells and a lysate of HEK cells transfected with a plasmid encoding SV40 T antigen alone (lanes 3) were analyzed in parallel as controls. Four repeats of the samples were separated in the same gel. Following SDS-PAGE, the proteins were electrotransferred onto Immobilon and subjected to Western immunoassays as described in Materials and Methods. Shown are immunoblots resulting from probing the replicas with antisera anti-N2 (A), anti-C2 (B), anti-N1 (C), and a mixture of anti-N1, -N2, and -C2 (D). The positions of assemblin (A), A_n , A_c , pAP, and AP are indicated to the right of each panel. Protein abbreviations are as shown in Fig. 1; h denotes host proteins (i.e., present in lysates of mock-transfected cells [lanes 3]) that cross-reacted in these assays. The origins of the bands immediately above and below pAP (asterisks) are unknown, although the upper one appears to arise as a modified form of pAP at late times after transfection (13), and the appearance of the lower one is more variable and seems to relate to the conditions of SDS-PAGE.

SCMV and HCMV assemblins (12, 49), anti-N2 and anti-C2 (antisera prepared by using synthetic peptides of SCMV assemblin) were also reactive with HCMV A_n and A_c and could be used to demonstrate their synthesis in rBV-infected cells (Fig. 7A, lane 10). The finding that HCMV A_n (see Fig. 9A, lane 1) migrates more slowly in SDS-PAGE than HCMV A_c (see Fig. 9A, lane 2) is consistent with their computer-predicted molecular masses of 15.5 and 12.5 kDa, respectively, and contrasts with the aberrant relative mobilities of SCMV A_n (Fig. 7A, lane 7) and A_c (Fig. 7A, lane 9). HCMV pAP was not cleaved by either HCMV A_n or HCMV A_c alone (Fig. 7C, lanes 7 and 9). However, when HCMV A_n , A_c , and pAP were expressed together by coinfection with all three rBVs, pAP→AP cleavage was readily detected (Fig. 7C, lane 8).

Cross-species cleavage properties of CMV A_n/A_c are similar to those of assemblin. We have shown previously that HCMV assemblin can cleave SCMV pAP and, conversely, that SCMV assemblin can cleave HCMV pAP (48). To determine whether the SCMV and HCMV two-chain assemblins (i.e., A_n/A_c) can also carry out this cross-species cleavage, insect cells were infected with individual or combinations of rBVs encoding HCMV and SCMV A_n , A_c , and pAP. The results showed that SCMV A_n/A_c cleaved HCMV pAP→AP (Fig. 7C, lane 10) and that HCMV A_n/A_c , likewise, cleaved SCMV pAP→AP (Fig. 7B, lane 10).

Previous work (48) had also shown that both the HCMV and SCMV assemblins can cleave HSV-1 pAP. To determine whether the SCMV and HCMV A_n/A_c s are also able to cleave HSV pAP, insect cells were infected with rBVs expressing HSV-1 pAP together with the A_n and A_c subunits of either

HCMV or SCMV assemblin. Cells coexpressing the HSV pAP together with both SCMV A_n and A_c showed pAP→AP cleavage (Fig. 8, lane 11); no cleavage of HSV pAP was seen with either subunit alone (Fig. 8, lanes 9 and 10). A parallel experiment showed that coexpression of HCMV A_n and A_c together with HSV pAP also yielded pAP→AP cleavage (Fig. 8, lane 14); again, no cleavage of HSV pAP was seen with either subunit alone (Fig. 8, lanes 12 and 13). These experiments demonstrate that the SCMV and HCMV recombinant two-chain enzymes, A_n/A_c , retain the capacity of their SCMV and HCMV assemblin counterparts to cleave the SCMV, HCMV, and HSV pAP to AP (48).

Direct association between A_n and A_c indicated by coimmunoprecipitation. Several mechanisms could explain the ability of the coexpressed A_n and A_c subunits to cleave the substrates in these experiments. The most straightforward of these is that the two subunits directly associate within the cell to form an active enzyme. If there is a direct interaction, an antibody specific for one of the subunits might be expected to coimmunoprecipitate both. This was tested by doing immunoprecipitations with lysates prepared, as described in Materials and Methods, from rBV-infected insect cells expressing the following HCMV proteins: A_n alone, A_c alone, $A_n + A_c$, or HCMV assemblin. An antiserum to the carboxyl end of A_c (anti-C2) was used. In order to identify both A_n and A_c in the resulting immunoprecipitates, they were subjected to SDS-PAGE followed by Western immunoassay with a mixture of anti-C2 and anti-N2 (an antiserum to the amino end of A_n).

The results of the experiment showed that A_n was coimmunoprecipitated with A_c from lysates of cells expressing both

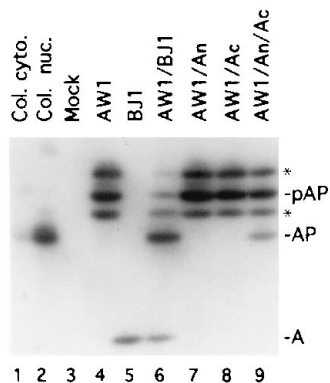


FIG. 4. Cleavage of SCM V pAP \rightarrow AP when coexpressed in human cells with SCM V A_n and A_c . HEK cells were transfected with the indicated plasmids, lysed, and subjected to Western immunoassay following separation of the proteins by SDS-PAGE in a 10% acrylamide gel. The Immobilon replica was probed with a mixture of antisera anti-N1, -N2, and -C2 as described in Materials and Methods. Shown is an autoradiographic image of the probed blot. NP-40 cytoplasmic (Col. cyto.) (lane 1) and nuclear (Col. nuc.) (lane 2) fractions of SCM V-infected HFF cells and HEK cells transfected with the SV40 T antigen-encoding plasmid alone (lane 3) were analyzed as controls. The plasmids used encoded pAP alone (lane 4), assemblin (A) alone (lane 5), pAP + assemblin (lane 6), pAP + A_n (lane 7), pAP + A_c (lane 8), and pAP + A_n + A_c (lane 9). Protein abbreviations are as shown in Fig. 1; asterisks are explained in the legend to Fig. 3.

subunits (Fig. 9B, lane 3), providing evidence that the two subunits are directly associated in a complex that can withstand the cell lysis conditions of 0.3 M KCl, 1.0% NP-40, and 0.5% DOC. Additionally, when immunoprecipitated from cells lysed with 0.5% NP-40 alone, the complex was not dissociated by any of the following (each dissolved in calcium- and magnesium-free PBS): 0.5% NP-40, 0.5% DOC, or a mixture of 1% NP-40 and 0.5% DOC containing either 0.1, 0.3, 0.5, or 2 M KCl. It was not possible to determine the stoichiometric relationship of A_n and A_c (i) because free A_c was immunoprecipitated along with that in the A_n/A_c complex and (ii) because the Western immunoassay did not provide a direct measure of protein amount. However, a comparison of the relative amounts of A_n and A_c coprecipitated from cells coexpressing the two recombinant subunits (Fig. 9B, lane 3) with the relative amounts coprecipitated from cells containing two-chain enzyme formed by I-site cleavage of assemblin (Fig. 9B, lane 5) suggests that the ratios are similar.

Controls demonstrated that (i) the starting cell lysates contained the expected proteins (A_n alone [Fig. 9A, lane 1], A_c alone [Fig. 9A, lane 2], A_n and A_c [Fig. 9A, lane 3], and assemblin, A_n , and A_c [Fig. 9A, lane 4]); (ii) A_n was not precipitated by anti-C2 from lysates of cells expressing only A_n (Fig. 9B, lane 1); (iii) only A_c was precipitated from cells expressing A_c alone (Fig. 9B, lane 2); (iv) A_n was present and coprecipitated with A_c from lysates of cells expressing assemblin (Fig. 9B, lane 5), because of the autoproteolytic conversion of single-chain assemblin to the two-chain form; (v) assemblin was precipitated by anti-C2 (Fig. 9B, lane 5), because it contains the C2 antigen at its carboxy terminus (Fig. 2); and (vi) no cross-reactive proteins were detected in wild-type-BV-infected cells (Fig. 9B, lane 4).

Formation of interspecies hybrid two-chain enzymes. On the basis of the high degree of sequence homology between the SCM V and HCM V assemblins, we tested the ability of A_n and A_c to yield proteolytic activity when coexpressed with their cross-species partner. Coinfections were done in which two-chain enzymes were generated by using either same-species subunits (e.g., SCM V A_n and A_c) or cross-species subunits

(e.g., HCM V A_n and SCM V A_c). The combinations were assayed for their abilities to cleave both the SCM V and HCM V pAPs. Coexpression of SCM V pAP either with SCM V A_n + HCM V A_c or with HCM V A_n + SCM V A_c resulted in pAP \rightarrow AP cleavage (Fig. 10, lanes 8 and 9). Similarly, coexpression of HCM V pAP either with SCM V A_n + HCM V A_c or with HCM V A_n + SCM V A_c resulted in pAP \rightarrow AP cleavage (data not shown). Cleavage of HCM V or SCM V pAP \rightarrow AP was not detected in cells expressing only the cross-species homologous pairs (i.e., HCM V A_n + SCM V A_n or HCM V A_c + SCM V A_c) (Fig. 10, lanes 11 and 12; data not shown). As expected, the same-species positive controls (i.e., HCM V A_n/A_c and SCM V A_n/A_c) cleaved both HCM V and SCM V pAP \rightarrow AP (Fig. 10, lanes 7 and 10; data not shown).

DISCUSSION

This work was begun to confirm the finding that the HCM V proteinase assemblin remains enzymatically active following cleavage at its I site and consequent conversion to a two-chain structure (15). Our approach was to separately clone the DNA sequences encoding the amino (A_n) and carboxyl (A_c) domains of assemblin that normally result from I-site cleavage, coexpress them in mammalian or insect cells, and determine whether they cleave their biological substrate. The results of our experiments show that when A_n and A_c are expressed together in eukaryotic cells, they associate to form active pro-

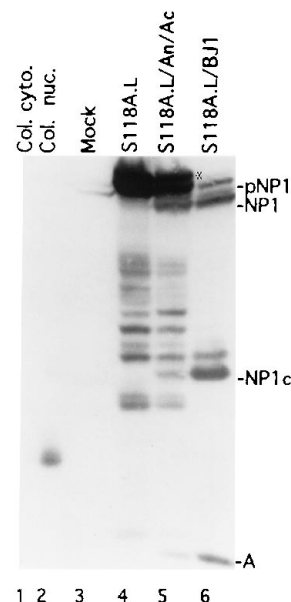


FIG. 5. M- and R-site cleavage of assemblin precursor (pNP1) mutant coexpressed in human cells with SCM V A_n and A_c . Cells were cotransfected with plasmids encoding the S118A inactive mutant precursor proteinase (S118A.L), A_n and A_c . Three days later lysates were prepared, subjected to SDS-PAGE in a 10% acrylamide gel, and analyzed by Western immunoassay with a mixture of anti-N1, -N2, and -C2, all as described in the text. NP-40 cytoplasmic (Col. cyto.) (lane 1) and nuclear (Col. nuc.) (lane 2) fractions of SCM V-infected HFF cells and HEK cells transfected with only the SV40 T antigen-encoding plasmid (lane 3) were included as controls. Lanes 4 to 6, respectively, contained lysates of HEK cells transfected with plasmids encoding the mutant precursor proteinase alone, the mutant precursor proteinase plus A_n plus A_c , and the mutant precursor proteinase plus assemblin. Full-length precursor proteinase (pNP1), its M-site cleavage product (NP1), and the R-site cleavage products of NP1 (NP1_c and assemblin [A]) are shown on the right. The asterisk denotes the band mentioned in the text and thought to be the pNP1 modification counterpart of the band above pAP (see asterisk above pAP in Fig. 4).

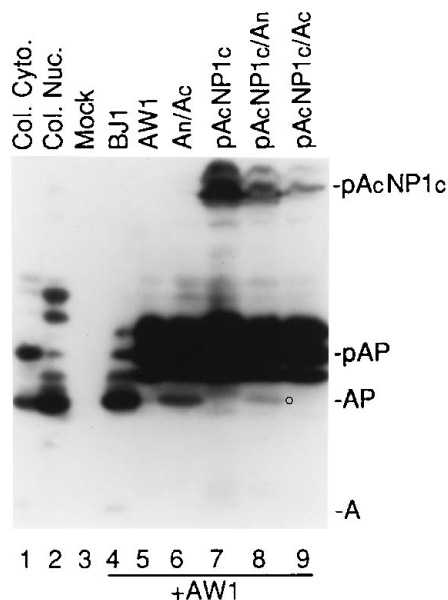


FIG. 6. R-site cleavage not required to form two-chain assemblin (A). HEK cells were transfected with the pAP-encoding AW1 plasmid alone (lane 5) or together with plasmids encoding various forms of the proteinase (lanes 4 and 6 to 9), as described in the text. Three days later, lysates were prepared and subjected to SDS-PAGE in a 10% acrylamide gel, and the proteins were electrotransferred to Immobilon and analyzed by Western immunoassay with a mixture of the antisera anti-N1, -N2, and -C2, all as described in the text. NP-40 cytoplasmic (Col. Cyto.) (lane 1) and nuclear (Col. Nuc.) (lane 2) fractions of SCMV-infected HFF cells and HEK cells transfected with the SV40 T antigen-encoding plasmid alone (lane 3) were analyzed in parallel as controls. Proteolytic activity was indicated by conversion of pAP to AP. Protein abbreviations are as shown in Fig. 1.

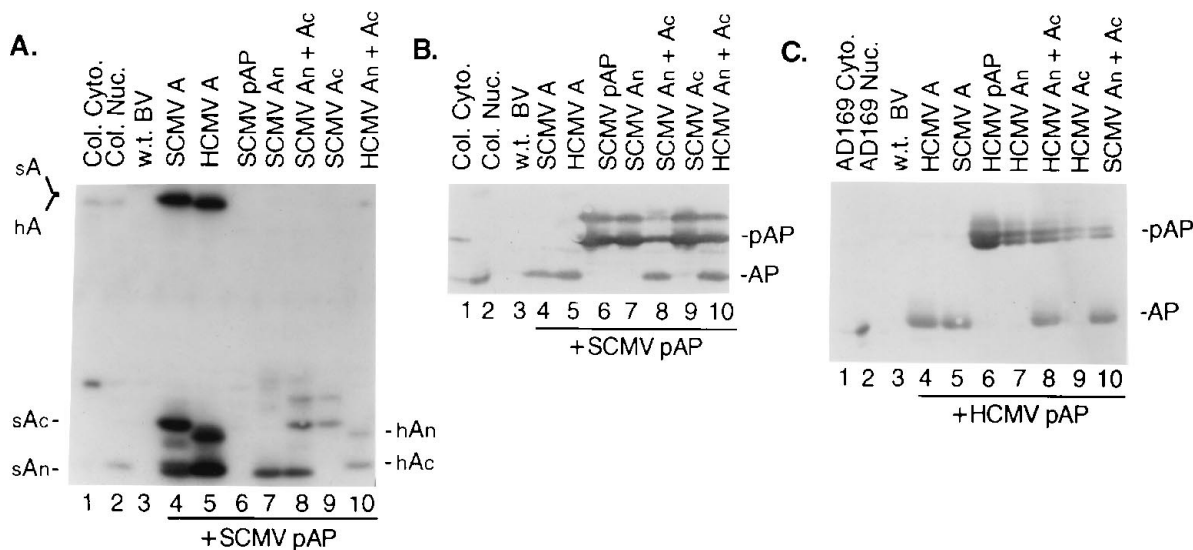


FIG. 7. Expression of one- and two-chain SCMV and HCMV assemblins in insect cells infected with rBVs. (A) Sf9 cells were coinfecting with rBVs encoding SCMV or HCMV assemblins (A) (lanes 4 and 5) or their subunits (lanes 7 to 10), together with an rBV encoding the SCMV pAP (expressed alone in lane 6). Three days after infection the cells were solubilized and subjected to SDS-PAGE in a 14% acrylamide gel, and the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with the antisera anti-N2 and anti-C2. NP-40 cytoplasmic (Col. Cyto.) (lane 1) and nuclear (Col. Nuc.) (lane 2) fractions of SCMV-infected HFF cells and wild-type (w.t.) BV-infected Sf9 cells (lane 3) were analyzed in parallel as controls. Shown is a fluorographic image of the resulting blot. Protein abbreviations: sA and hA, SCMV and HCMV assemblins, respectively; sAn and hAn, SCMV and HCMV An, respectively; sAc and hAc, SCMV and HCMV Ac, respectively. (B) The same samples used in panel A, in the same order, were subjected to SDS-PAGE in a 10% acrylamide gel to better resolve pAP and AP; the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with the antiserum anti-N1. Shown is an autoradiogram of the resulting blot. (C) Sf9 cells were coinfecting with rBVs encoding HCMV or SCMV assemblins (lanes 4 and 5) or their subunits (lanes 7 to 10), together with an rBV encoding the HCMV pAP (expressed alone in lane 6). Three days after infection the cells were solubilized and subjected to SDS-PAGE in a 10% acrylamide gel, and the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with the antiserum anti-S-20-C, which reacts with the HCMV pAP. NP-40 cytoplasmic (AD169 Cyto.) (lane 1) and nuclear (AD169 Nuc.) (lane 2) fractions of HCMV-infected HFF cells and wild-type (w.t.) BV-infected Sf9 cells (lane 3) were analyzed in parallel as controls. Shown is a fluorographic image of the resulting blot.

teinase. We have found this for both SCMV and HCMV, and we have shown that the substrate cleavage specificity of the recombinant two-chain proteinase is similar to that reported previously for single-chain assemblin (48, 49). We have also investigated the process of two-chain assemblin formation and found that the two subunits interact to form an immunoprecipitable complex, that R-site cleavage is not required for this interaction, and that the subunits of HCMV and SCMV can be interchanged and still yield active proteinase.

Our main conclusion is that enzymatically active two-chain assemblin is able to form spontaneously, in eukaryotic cells, from its independently cloned subunits. This was demonstrated to occur in both transfected human cells and rBV-infected insect cells. The spontaneous association of An and Ac to form active proteinase in these expression systems suggests that they may be structurally distinct domains and demonstrates that formation of the two-chain enzyme does not require its initial synthesis as single-chain assemblin to achieve correct folding and interaction. In contrast, when HCMV An and Ac were individually expressed as glutathione S-transferase fusion proteins in bacteria, proteolytic activity was detected only when the subunits were denatured and refolded together and not when they were refolded separately and then combined (24).

We tested the possibility that conversion of single-chain assemblin to the two-chain form could be a mechanism to alter its specificity. Our results showed, however, that the substrate recognition properties of intracellular two-chain assemblin are comparable to those of the single-chain enzyme. Like the single-chain form, two-chain assemblin cleaved the M sites of both the assembly protein and the proteinase precursors (i.e., pAP→AP and pNP1→NP1). This finding is consistent with results of studies which showed that bacterially synthesized

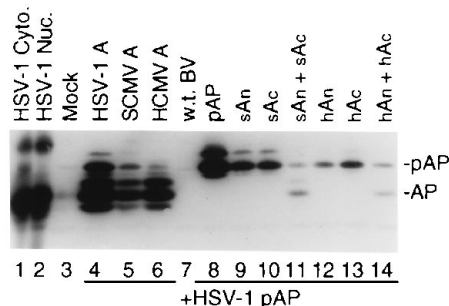


FIG. 8. SCMV and HCMV two-chain assemblins retain ability to cleave HSV-1 pAP. Sf9 cells were infected with rBVs encoding HSV-1, SCMV, or HCMV assemblins (lanes 4 to 6) or the SCMV or HCMV assemblin subunits (lanes 9 to 14), together with an rBV encoding the HSV-1 pAP (expressed alone in lane 8). Three days after infection the cells were solubilized and subjected to SDS-PAGE in a 10% acrylamide gel, and the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with a cocktail of monoclonal antibodies that react with the HSV-1 pAP and AP (48). NP-40 cytoplasmic (HSV-1 Cyto.) (lane 1) and nuclear (HSV-1 Nuc.) (lane 2) fractions of HSV-1-infected HFF cells and uninfected (Mock) (lane 3) and wild-type (w.t.) BV-infected Sf9 cells (lane 7) were analyzed in parallel as controls. Shown is a fluorographic image of the resulting blot. Other abbreviations are as in Fig. 8.

two-chain assemblin cleaves M-site peptide mimics in vitro with kinetics similar to those of the single-chain form (15, 24). Also like the single-chain form, recombinant two-chain assemblin cleaved the R site of an inactive mutant proteinase precursor used as a substrate.

The cross-species substrate recognition characteristics of the CMV recombinant two-chain enzyme likewise did not differ noticeably in these assays from those of the one-chain form. Like their single-chain counterparts (48), the HCMV recombinant two-chain enzyme cleaved SCMV pAP and the SCMV recombinant enzyme cleaved HCMV pAP, and both CMV two-chain assemblins cleaved HSV-1 pAP. These results indicate that the range of substrates recognized and cleaved by assemblin is not substantially reduced by its conversion to a two-chain enzyme. However, they do not rule out the possibility that this structural change may render the enzyme less specific and increase its tendency to further degrade substrates, although we detected no additional fragments that might be expected if this were the case (e.g., compare the assemblin and $A_n + A_c$ lanes in Fig. 4, 5, 7, and 8).

Several mechanisms could explain the ability of the coexpressed subunits of assemblin to cleave substrate, including the possibility that a substrate may be required as a nucleation center around which an active complex could form. The immunoprecipitation data (Fig. 9) demonstrate that a viral substrate (e.g., pAP) is not required for A_n/A_c formation, and they are consistent with a direct interaction between the recombinant subunits to give active A_n/A_c . However, they do not rule out the possibility that a host cell molecule may be included in the complex. The association between A_n and A_c appears to be predominantly hydrophobic, considering that (i) assemblin does not contain disulfide bonds (3, 35), (ii) the complex is not dissociated by 7 M urea (3, 31, 35), and (iii) the immunoprecipitated A_n/A_c complex was not dissociated by KCl concentrations of 300 mM to 2 M (Fig. 9B, lane 3). The importance of hydrophobic interactions in the structure and function of assemblin is also indicated by two recent reports that antichaeotropic salts significantly enhanced the activity of the HSV-1 assemblin homolog (14, 51). We also determined that A_n can associate with A_c still attached to pNP1_c through a noncleaved R site (i.e., pA_cNP1_c [Fig. 2]), indicating that R-site cleavage is

not required for interaction of the A_n and A_c domains. Our study did not, however, enable us to determine whether the apparent reduced activity of the A_n/pA_c NP1_c form compared with the A_n/A_c form of the recombinant enzyme was due to a lower rate of subunit association, to a comparatively less efficient enzyme, or to something else. Even though this form of the enzyme can be produced in transfections (Fig. 6, lane 8) and evidence of its formation in bacteria has been presented (2), it has not been detected in HCMV-infected cells, and it seems likely that its occurrence would be infrequent given that the order of precursor cleavage is M site before R site before I site in both transfected (47) and virus-infected (16) cells.

Several two-chain serine proteinases have been described. Of these, only assemblin and tissue plasminogen activator (39) have significant proteolytic activity in both their single- and two-chain forms; others, like plasmin (26) and factor IXa (37), are derived from one-chain inactive zymogens. Assemblin differs, however, from both tissue plasminogen activator and the other two-chain serine proteinases in at least two ways. First, assemblin is converted to its two-chain form by an autoproteolytic cleavage and not by the action of an additional proteinase, and second, the subunits of assemblin are not held together by disulfide bonds (3, 15, 24, 35).

Although it is different from other two-chain serine proteinases, assemblin shows some similarities to the cysteine proteinase interleukin-converting enzyme (ICE) and its related family members (43) in terms of subunit formation and interaction. ICE is a tetramer composed of two heterodimers (45, 50), and, as with assemblin, the heterodimer is derived from an enzymatically active precursor by autoproteolysis (5, 42). Also, as demonstrated with assemblin (24), the subunits of ICE can be independently synthesized in bacteria and combined to form active enzyme when they are refolded together after denaturation (29, 45). Unlike with assemblin, however, the conversion of single-chain ICE involves autoproteolytic cleavages that remove the amino-terminal portion, as well as an internal peptide, from the precursor. More significantly, and also unlike with assemblin, the substrate specificity of ICE is dramatically altered by its conversion to a two-chain form: the precursor carries out only autoproteolytic cleavages, and the mature form cleaves only its biological substrate, interleukin-1 (5, 42).

It should be possible to identify the respective interactive domains of the assemblin A_n and A_c subunits by taking advan-

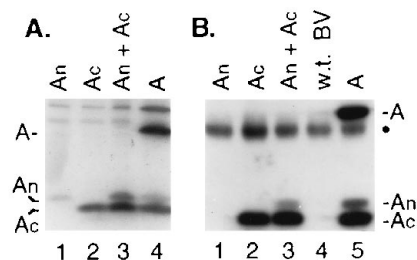


FIG. 9. Coimmunoprecipitation indicates direct interaction between A_n and A_c in recombinant two-chain assemblin of HCMV. Sf9 cells were infected with wild-type (w.t.) BV or rBVs encoding HCMV assemblin, its A_n or A_c subunit alone, or its A_n and A_c subunits together. Two days after infection, lysates were prepared from the infected cells and subjected to immunoprecipitation with the A_c -specific antiserum anti-C2, all as described in Materials and Methods. Portions of the starting lysates (A) and of the resulting immunoprecipitates (B) were solubilized and subjected to SDS-PAGE in separate 14% acrylamide minigels, and the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with the antisera anti-N2 and anti-C2, specific for A_n and A_c , respectively. Shown are fluorographic images of the resulting blots. Protein abbreviations are as shown in Fig. 1; the dot to the right of panel B indicates position of rabbit immunoglobulin G light chain from immunoprecipitates.

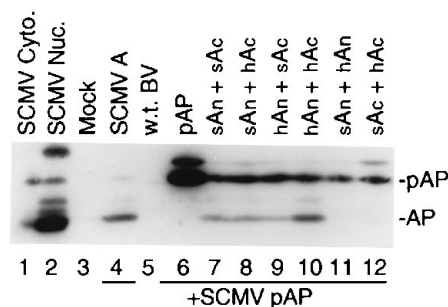


FIG. 10. SCMV-HCMV hybrid two-chain assemblins are active. Sf9 cells were infected with rBVs encoding SCMV assemblin (A) (lane 4) or with homologous (lanes 7 and 10) or heterologous (lanes 8, 9, 11, and 12) subunit pairs, together with an rBV encoding the SCMV pAP (expressed alone in lane 6). Three days after infection the cells were solubilized and subjected to SDS-PAGE in a 10% acrylamide gel, and the proteins were electrotransferred to Immobilon and subjected to Western immunoassay with the antiserum anti-N1, which reacts with SCMV pAP and AP. NP-40 cytoplasmic (SCMV Cyto.) (lane 1) and nuclear (SCMV Nuc.) (lane 2) fractions of SCMV-infected HFF cells and noninfected (lane 3) and wild-type (w.t.) BV-infected (lane 5) Sf9 cells were analyzed in parallel as controls. Shown is a fluorographic image of the resulting blot. Protein abbreviations are as in Fig. 1.

tage of their ability to spontaneously associate. Compounds able to block this interaction could have potential as assemblin-specific antiviral agents. A similar strategy is being pursued with the human immunodeficiency virus proteinase, for which attempts are being made to use small molecules to dissociate or inhibit formation of the homodimer and thereby inhibit its activity (1).

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