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Assemblin Homolog of Herpes Simplex Virus Type 1 Retains Proteolytic Activity When

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The herpes simplex virus type 1 (HSV) maturational proteinase is synthesized as a precursor that undergoes two autoproteolytic cleavages; one at its (M)aturational site, which eliminates its carboxyl "tail," and a second at its (R)elease site, which separates the amino proteolytic half of the precursor from its nonproteolytic carboxyl half. In cytomegalovirus (CMV) the proteolytic half of the precursor, called assemblin, undergoes a third cleavage at an (I)nternal site that converts it from a single-chain to a two-chain enzyme that retains activity. The HSV assemblin homolog has no I site and therefore does not form a counterpart two-chain enzyme. In the work reported here we have cloned and expressed HSV sequences that encode mimics of the A_n and A_c subunits of two-chain CMV assemblin. We show that when these HSV sequences are coexpressed in eukaryotic cells, the resulting subunits associate spontaneously to form an active two-chain enzyme. We also show that the two-chain HSV enzyme, like the natural one-chain form, retains its marked preference for HSV over CMV substrates, and that intertypic recombinant two-chain assemblin (e.g., HSV A_n/CMV A_c) does not form because the cross-species subunits do not interact. We conclude from these results that (i) there are not intrinsic structural differences in the HSV assemblin homolog that preclude its functioning as a CMV-like two-chain enzyme, (ii) the substrate selectivity shown by the single-chain HSV enzyme was not noticeably relaxed in the HSV two-chain mimic, and (iii) the interactive domains, through which the A_n and A_c portions of the single-chain enzymes associate, differ between HSV and CMV.

INTRODUCTION

The maturational proteinase of herpes simplex virus type 1 (VP24, HSV UL26 product) and all other herpesgroup viruses is synthesized as an enzymatically active precursor that undergoes at least two autoproteolytic cleavages (Liu and Roizman, 1991; Preston *et al.,* 1992; Welch *et al.,* 1991). One cleavage, at the (M)aturational site, eliminates the carboxyl end of the precursor and the second cleavage, at the (R)elease site, separates the proteolytic amino half of the precursor from the nonproteolytic carboxyl half. Active site labeling (DiIanni *et al.,* 1994; Holwerda *et al.,* 1994; Stevens *et al.,* 1994) and site-directed mutagenesis studies (Cox *et al.,* 1995; Welch *et al.,* 1993) have established that the enzyme is a serine proteinase and have identified an absolutely conserved, essential His as a putative second member of the catalytic site (Cox *et al.,* 1995; Welch *et al.,* 1993).

The proteolytic amino half of the cytomegalovirus (CMV) proteinase, called assemblin, is distinguished from its homologs in the other herpesgroup viruses by having an (I)nternal cleavage site (Baum *et al.,* 1993; Burck *et al.,* 1994; Welch *et al.,* 1993). I-site cleavage occurs near the midpoint of the 28-kDa assemblin and divides it into an amino (A_n, 15.5 kDa) subunit, which contains the absolutely conserved CD3 Ser and CD2 His, and a carboxyl (A_c, 12.5 kDa) subunit that remain associated as an active, two-chain enzyme (Holwerda *et al.,* 1994).

When the coding sequences for the A_n and A_c subunits of human CMV (HCMV) and simian CMV (SCMV) assemblins are separately cloned and coexpressed in eukaryotic cells, they associate to form proteolytically active two-chain assemblin (Hall and Gibson, 1996). The ability of the A_n and A_c subunits to associate spontaneously indicates that they are able to fold without prior synthesis as a one-chain enzyme and may represent distinct domains within assemblin. Further support for this notion was provided by the finding that both A_n and A_c are able to rescue inactive mutants of assemblin by intermolecular complementation (Hall and Gibson, 1997). Two-chain HCMV assemblin has also been formed *in vitro* from bacterially synthesized GST fusion proteins but, in that case, enzyme activity was obtained only when the A_n and A_c subunits were first denatured and then combined and renatured together (O'Boyle *et al.,* 1995). Characterization of the CMV two-chain assemblins indicated that these enzymes do not differ significantly in either their catalytic properties or range of substrates when compared with

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one-chain CMV assemblin (Hall and Gibson, 1996; Holwerda *et al.,* 1994; O'Boyle *et al.,* 1995). Thus cleavage of CMV assemblin does not appear to be a mechanism to alter its substrate specificity as it is in several other two-chain serine proteinases (Ponting *et al.,* 1992; Tate *et al.,* 1987).

The HSV assemblin homolog, in contrast, has no I site (DiIanni *et al.,* 1993) and shows no evidence of forming a natural counterpart of CMV two-chain assemblin. In addition, the HSV enzyme has a narrower substrate range than CMV assemblin. Whereas CMV assemblin will cleave both CMV and HSV substrates, the HSV enzyme is highly specific for HSV substrates (Welch *et al.,* 1995). These differences between the HSV and CMV enzymes raised questions of (i) whether there may be intrinsic structural differences between the two that could preclude formation of a functional two-chain HSV enzyme, and (ii) if a two-chain HSV proteinase could form, would its substrate specificity be more relaxed than the natural single-chain enzyme.

To test these questions, and with the objective of learning more about the functional significance of I-site cleavage, we attempted to make an artificial two-chain form of HSV assemblin by cloning and coexpressing the coding sequences for mimics of the CMV A_n and A_c subunits and testing for proteolytic activity toward both HSV and CMV substrates.

MATERIALS AND METHODS

Cells, viruses, and antisera

The DNA sequences used in these studies were from HCMV strain AD169, SCMV strain Colburn, and HSV-1 strain 17. Human foreskin fibroblast (HFF) cells were used to propagate these viruses and to prepare the virus-infected cell lysates used as markers (Gibson, 1981).

Propagation, plaque assay, and protein expression studies of recombinant baculoviruses (rBVs) were done in *S. frugiperda* cells (Sf9, CRL 1711, American Type Culture Collection, Rockville, MD) grown in suspension at 27° in 100-ml spinner flasks containing supplemented Grace's medium (No. 350-1605AJ, GIBCO, Grand Island, NY) with additions to give 10% fetal calf serum (HyClone, Logan, UT), 50 μg/ml gentimicin (No. 600-5750AD, GIBCO), and 125 ng/ml Fungizone (No. 600-5295AE, GIBCO). rBVs were plaque purified twice (Carrascosa, 1994; Summers and Smith, 1987); high titer stocks were prepared for each (Summers and Smith, 1987) and stored at 4° protected from light until used. Sf9 cells ($\approx 2.5 \times 10^5$ cells/well) were infected in 24-well plates (No. 3047, Becton–Dickinson Labware, Lincoln Park, NJ) at a multiplicity of infection of approximately 5–10 by adding 100 μl of virus in single infections or 50 μl of each virus in multiple virus infections. Infected cells were harvested 3 days after infection by aspirating the medium, adding 70 μl of 2× SDS–PAGE sample buffer (described below) to the cell layer, and collecting the lysate. Resulting samples were heated in a boiling water bath for 3 min and stored at −80° until analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoassay.

The polyclonal antisera anti-N1 (to the N-terminus of the SCMV pAP), anti-N2 (to the N-terminus of SCMV assemblin), anti-C2 (to the C-terminus of SCMV assemblin), and a cocktail of monoclonal antibodies (MAbs) against the HSV pAP have been described previously, (Schenk *et al.,* 1991; Welch *et al.,* 1993, 1995). Antiserum 37-93 was generated against a synthetic peptide corresponding to residues 4 to 18 of the HSV-1 strain 17 assemblin homolog and was made available by R. LaFemina (Merck Research Labs, West Point, PA). Antisera were used at a dilution of 1:40 in 10 m*M* Tris, 0.9% NaCl, and 5% bovine serum albumin (No. 126602, Calbiochem, San Diego, CA), pH 7.4 (TN/BSA), except antiserum 37-93, which was used at a dilution of 1:500.

Construction of recombinant baculoviruses

Recombinant baculoviruses were made using the Baculogold system (No. 21001K, Pharmingen, La Jolla, CA) as instructed by the manufacturer. CMV and HSV genes were introduced into the BV genome via either the pVL1392 or the pVL1393 transfer vector (O'Reilly *et al.,* 1992). Construction of the following rBVs has been described previously (Hall and Gibson, 1996): BJ26, encodes SCMV A_n; MH51, encodes SCMV A_c; JB2, encodes SCMV pAP; MH50 encodes HCMV A_n; MH52, encodes HCMV A_c; and MH60, encodes the HSV pAP. Construction of new transfer plasmids is described in the following paragraphs.

MH59 encodes the HSV assemblin homolog and was made by removing the coding sequence for the HSV assemblin homolog from the plasmid, EV2 (Welch, Villarreal, and Gibson, 1995) by *Xba*I digestion and ligating it into the *Xba*I site of pVL1393.

MH61 encodes HSV A_n and was made by isolating the 441-base pair (bp) *Xba*I, *Bgl*I fragment from EV2 (Welch *et al.,* 1995) and using it in a three-way ligation with pVL1392 cut by *Xba*I and *Bam*HI, and a set of annealed synthetic oligonucleotides. The sense oligonucleotide was 5′-GGGCGAGGCGTAATCTAGAG-3′ which when annealed with its complementary antisense partner gave the *Bgl*I and *Bam*HI overhangs needed for ligation. A stop codon (underlined) was added to terminate HSV A_n at Ala139.

MH62 encodes HSV A_c. PCR (Mullis *et al.,* 1986) was done using native PFU polymerase (No. 600135, Stratagene, La Jolla, CA) with the manufacturer's standard protocol. The sense primer was 5′-CGGTTGGATCCCCA-ATGCACCCCGATCGCACGC-3′; it contains a new start

methionine codon (underlined) adjacent to the codon for His140. A *Bam*HI site was added for cloning purposes (double underline). The antisense primer was made to a sequence just 3′ to the multiple cloning site of the EV2 (Welch *et al.,* 1995) plasmid. The PCR product was digested with *Bam*HI and *Xba*I and ligated into the same sites in pVL1392. The plasmid was then sequenced between the *Bam*HI and *Xba*I sites to confirm that no mutations were introduced during the PCR.

JB3 encodes SCMV assemblin and was made by digesting LM8 (Welch *et al.,* 1991) with *Xba*I and ligating the fragment containing the assemblin coding sequence into the *Xba*I site of pVL1393.

SDS–PAGE and Western immunoassay

SDS–PAGE was done essentially as described by Laemmli (Laemmli, 1970); the ratio of *N,N′*-methylene-bis-acrylamide to acrylamide was 0.735:28. SDS was from Bio-Rad (Melville, NY) and 2× SDS–PAGE sample buffer was 4% SDS, 20% β-mercaptoethanol, 20% glycerol, 50 m*M* Tris (pH 7.0), and 0.02% bromophenol blue.

Western immunoassays were done essentially as described by Towbin *et al.* (1979). A semidry transfer unit was used; the membrane was Immobilon-P (Millipore, Bedford, MA); the buffer was 50 m*M* Tris, 20% methanol; and the time of transfer was calculated by the formula: gel width × height × 2.5 = milliamperes per 30 min. The membrane was blocked in 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 (Laskey and Mills, 1977).

Immunoprecipitation

Immunoprecipitations from rBV-infected cells were done as follows. Approximately 2 × 10^6 Sf9 cells were grown at 27° in 6-cm plastic petri dishes and then infected with one or more recombinant baculoviruses at a multiplicity of infection of 5 to 10. Infected cells were harvested 2 days after infection by removing the medium and adding 100 μl of 0.5% Nonidet P-40 (NP-40) in 10 m*M* 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 *g,* 4°, 10 min). The resulting NP-40 cytoplasmic fraction was used for subsequent immunoprecipitation reactions.

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

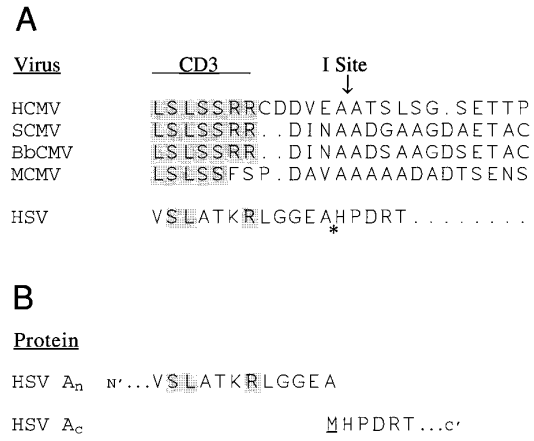

FIG. 1. Alignment of CMV and HSV-1 assemblin I-site regions and termini of recombinant HSV A_n and A_c. (A) An amino acid sequence alignment of the I-site region of CMV and HSV-1 assemblins. The arrow denotes the known (HCMV; SCMV) or probable (BbCMV; MCMV) I site of the four sequenced CMV assemblins. The asterisk (*) indicates the position in the HSV assemblin sequence that was selected as a break point to create recombinant mimics of the CMV amino (A_n) and carboxyl (A_c) subunits comprising the CMV two-chain enzyme. A gap was introduced into the computer alignment of the MCMV sequence, between Pro134 and Asp135, to bring the putative P4 Asp into alignment with those in the other three sequences and to shift the putative I cleavage site to the first Ala–Ala pair, because the P1′ Ala of all other known M, R, and I sites is the first one. (B) The carboxyl end of HSV A_n and the amino terminus of HSV A_c. The underlined "M" is the nonencoded Met added to translate HSV A_c.

RESULTS

An amino acid sequence alignment of the I-site region of the CMV assemblins and their HSV homolog is shown in Fig. 1A. The I site (Fig. 1A, arrow) has been identified by direct sequence analysis in HCMV (DVEA ↓ ATSL) (Baum *et al.,* 1993; Burck *et al.,* 1994), and by mutational analysis in SCMV (DINA ↓ ADGA) (Welch *et al.,* 1993); probable counterparts also occur in the corresponding regions of baboon CMV (DINA ↓ ADSA) (Gibson *et al.,* 1995) and murine CMV (DAVA?AAAA) (Loutsch *et al.,* 1994). There is little sequence similarity between HSV and CMV assemblin in this small region between the highly conserved domains CD1 and CD3, so for purposes of constructing recombinant genes encoding HSV mimics of the CMV A_n and A_c subunits, we defined Ala139

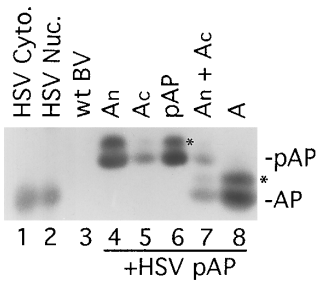

FIG. 2. Recombinant two-chain HSV assemblin analog is an active proteinase. Sf9 cells were infected with rBVs encoding HSV pAP, assemblin, A_n, or A_c, either alone or in combination, as described under Materials and Methods. Lysates of the infected cells were prepared and subjected to SDS–PAGE in a 10% polyacrylamide gel, followed by Western immunoassay using a cocktail of MAbs reactive with the HSV pAP and AP, all as described under Materials and Methods. Shown here is a fluorographic image of the resulting immunoblot. The sample order is NP-40 cytoplasmic (lane 1) and NP-40 nuclear (lane 2) fractions of HSV-infected HFFs, wild-type baculovirus-infected cells (lane 3), pAP + A_n (lane 4), pAP + A_c (lane 5), pAP alone (lane 6), pAP + A_n + A_c (lane 7), and pAP + HSV assemblin homolog (A). The positions of the precursor (pAP or ICP35c,d) and mature (AP or ICP35e,f) forms of the HSV assembly protein are indicated to the right. Band indicated by asterisk above pAP is believed to be the counterpart of a modified form of the CMV pAP that increases in amount as expression progresses (Gibson *et al.,* 1994). Band indicated by lower asterisk is cleavage product of band above pAP.

as the carboxy terminus of our HSV A_n construct and His140 (following an added translational start methionine) as the amino terminus of our HSV A_c construct (Fig. 1B). Ala139 was selected because it gave the same carboxy-terminus for HSV A_n as is found in all CMV A_n subunits and because it is approximately the same number of residues from CD3 as the P1 (Schechter and Berger, 1967) Ala of the CMV I site. The experiments described below were done to determine whether these two HSV recombinant subunit proteins can associate to form a substrate-specific two-chain proteinase when coexpressed in insect cells.

Coexpression of HSV A_n and A_c yields proteolytic activity

The recombinant genes encoding HSV A_n and A_c were cloned into baculoviruses, as described under Materials and Methods. Insect cells were infected with an rBV encoding the HSV pAP (Fig. 2, lane 6) or were coinfected to express the HSV pAP together with (i) the HSV assemblin homolog (Fig. 2, lane 8), (ii) HSV A_n (Fig. 2, lane 4), (iii) HSV A_c (Fig. 2, lane 5), or (iv) both HSV A_n and A_c (Fig. 2, lane 7). Three days after infection the cells were harvested and analyzed by SDS–PAGE and Western immunoassay. Results of the experiment showed that substrate-specific cleavage (i.e., HSV pAP → AP) occurred when HSV pAP was coexpressed with both HSV A_n and A_c (Fig. 2, lane 7; also see Fig. 4, lane 6), but not when coexpressed with just A_n or A_c alone (Fig. 2, lanes 4 and 5, respectively).

Although we could detect only A_n directly (Fig. 5B, lanes 1 and 2; no antibodies to HSV A_c), the finding that HSV A_n is proteolytically active only when coexpressed with HSV A_c (Fig. 2, lane 7) is taken as reasonable evidence that the HSV A_c subunit is also expressed. Variation in the amount of pAP in the different samples was not consistent from experiment to experiment and is attributed to differences in cell numbers among the starting samples. It is likely that the reduced amount of pAP → AP cleavage seen with the two-chain enzyme, compared to the single enzyme, is due to the comparatively lower efficiency of forming the two-chain enzyme from two independently expressed subunits. These findings are similar to those obtained from coexpression experiments done with the A_n and A_c subunits of HCMV and SCMV assemblin (Hall and Gibson, 1996) and indicate that the HSV assemblin homolog is proteolytically active as an artificial recombinant two-chain enzyme.

Artificial two-chain HSV proteinase has substrate specificity comparable to that of the natural single-chain enzyme

We have shown before that the substrate specificity of the HSV assemblin homolog is more restricted than those of HCMV or SCMV (Welch *et al.,* 1995). Whereas HCMV and SCMV assemblins can cleave SCMV, HCMV, and HSV pAP → AP, the HSV enzyme shows a much stronger preference for its own substrate, to the extent that its ability to cleave CMV pAP → AP was undetected in transfection assays (Welch *et al.,* 1995), and only became apparent at the higher expression levels achieved in the baculovirus system (e.g., Fig. 3, lane 10). We have shown that conversion of one- to two-chain assemblin does not restrict the substrate range of the CMV enzyme; it still cleaves both CMV and HSV substrates (Hall and Gibson, 1996). However, because we had no pAP substrates that the single-chain enzyme was unable to cleave, we were unable to determine whether conversion to the two-chain form might have given the CMV enzyme a broader substrate range. The restricted range of the HSV assemblin homolog provided an opportunity to test this possibility.

To evaluate the substrate recognition range of the two-chain HSV enzyme, we tested its ability to cleave SCMV and HCMV pAPs. This was done by infecting insect cells with appropriate combinations of rBVs and monitoring the extent of substrate cleavage by Western immunoassay of cell lysates prepared 3 days after infection. Results showed that although coexpression of HSV [A_n + A_c] yielded cleavage of HSV pAP → AP (e.g., Fig. 2, lane 7), coexpression of the HSV subunits together with either SCMV pAP (Fig. 3, lane 7) or HCMV pAP (data not shown)

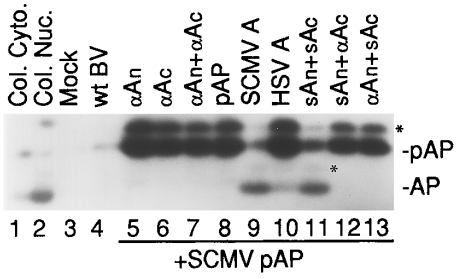

FIG. 3. Recombinant two-chain HSV assemblin analog shows strong species specificity similar to the single-chain enzyme. Sf9 cells were infected with rBVs, processed, subjected to SDS–PAGE in a 10% polyacrylamide gel, and analyzed by Western immunoassay using anti-N1 (reactive with the SCMV pAP and AP), all as described under Materials and Methods. Shown here is a fluorographic image of the resulting blot. One-chain assemblin is abbreviated "A" (e.g., HSV A). "α" indicates an HSV protein (e.g., αA_n is HSV A_n) and "s" denotes SCMV proteins (e.g., sA_n is SCMV A_n). Mock and wtBV are lysates of noninfected and wild-type baculovirus-infected cells, respectively. Col. Cyto. and Col. Nuc. are NP-40 cytoplasmic and nuclear fractions of SCMV-infected HFF cells, shown for reference. The positions of the SCMV pAP and AP bands are indicated in the right margin. The asterisk is as explained in the legend to Fig. 2.

yielded no detectable pAP → AP cleavage. Controls were done to verify that the SCMV and HCMV two-chain assemblins had cleaved both the SCMV (Fig. 3, lane 11; data not shown) and the HCMV pAPs (data not shown), as shown before (Hall and Gibson, 1996). Thus, like the natural HSV single-chain assemblin homolog (Fig. 3, lane 9), the artificial two-chain enzyme showed a strong preference for its own substrate over those of CMV.

HSV A_n and A_c are not interchangeable with their CMV counterparts

Previously, we showed that the A_n and A_c subunits of HCMV and SCMV can be interchanged and still give active two-chain proteinases (e.g., HCMV A_n + SCMV A_c = active hybrid two-chain proteinase) (Hall and Gibson, 1996). To determine whether an intertypic two-chain enzyme could be formed, consisting of one HSV subunit and one CMV subunit, we coexpressed either [SCMV A_n + HSV A_c] or [HSV A_n + SCMV A_c] together with SCMV pAP. Neither intertypic combination resulted in SCMV pAP → AP cleavage (Fig. 3, lanes 12 and 13).

Considering that the inability of the intertypic two-chain enzymes to cleave SCMV pAP may be due to the strong substrate specificity of the HSV enzyme for HSV substrates, we also tested their ability to cleave HSV pAP. Of the four possible combinations of HSV and CMV subunits, none yielded HSV pAP → AP cleavage (Fig. 4, lanes 7–9, 12). In contrast, both of the SCMV/HCMV hybrid enzymes cleaved HSV pAP → AP (Fig. 4, lanes 11, 13). Controls showed that all three homotypic two-chain enzymes cleaved the HSV pAP → AP (Fig. 4, lanes 6, 10, 14).

HSV A_n and CMV A_c do not form a complex when coexpressed

To examine the possibility that the intertypic two-chain enzymes were inactive because the cross-species subunits did not interact, we coexpressed the HSV A_n and HCMV A_c and tested for their interaction by immunoprecipitation with anti-C2, specific for CMV A_c (Hall and Gibson, 1996), as described under Materials and Methods. The resulting immunoprecipitates were subjected to SDS–PAGE followed by Western immunoassays using anti-C2 to visualize the HCMV A_c subunit, and antiserum 37-93 to visualize the HSV A_n subunit.

The HCMV A_c subunit was immunoprecipitated by anti-C2 when expressed alone (Fig. 5A, lane 2) or when coexpressed with HSV A_n (Fig. 5A, lane 3) or when produced by I-site cleavage of assemblin (Fig. 5A, lane 5). HCMV single-chain assemblin was also immunoprecipitated by anti-C2 (Fig. 5A, lane 5), as expected, because it contains the A_c subunit domain, including the 15 amino acid sequence used to produce the antiserum. No proteins were specifically immunoprecipitated by anti-C2 from wild-type baculovirus-infected cells (Fig. 5A, lane 4). The HSV A_n subunit was not coimmunoprecipitated with the HCMV A_c subunit (Fig. 5B, lane 7). To confirm that HSV A_n was present in the starting lysate, it was tested by Western immunoassay using the antiserum 37-93. This immunoassay showed that HSV A_n was present in cells where it was expressed alone (Fig. 5B, lane 1) and in cells where it was coexpressed with HCMV A_c (Fig. 5B, lane 2). Only single-chain assemblin was detected when the HSV assemblin homolog was expressed alone (Fig. 5B, lane 4), because the natural enzyme does not undergo

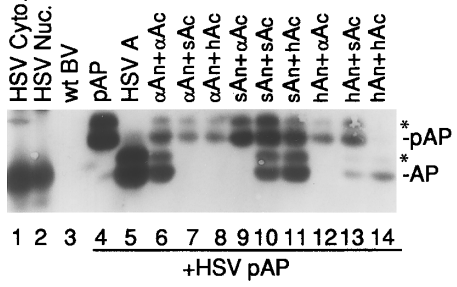

FIG. 4. Coexpression of HSV and CMV subunits gives no evidence of intertypic HSV/CMV two-chain assemblin. Sf9 cells were infected with rBVs, harvested, subjected to SDS–PAGE in a 10% polyacrylamide gel, and analyzed by Western immunoassay using a cocktail of MAbs reactive with the HSV pAP and AP, all as described under Materials and Methods. Shown here is a fluorographic image of the resulting blot. HSV A is the single-chain HSV assemblin homolog. "α" indicates HSV proteins (e.g., αA_n is HSV A_n), "h" and "s" denote HCMV and SCMV proteins, respectively. Mock and wt BV are lysates of noninfected and wild-type baculovirus-infected cells, respectively. HSV Cyto. and HSV Nuc. are NP-40 cytoplasmic and nuclear fractions of HSV-infected HFF cells, shown for reference. The positions of the HSV pAP and AP homologs (i.e., ICP35c,d and ICP35e,f) are indicated in the right margin. The asterisks are as explained in the legend to Fig. 2.

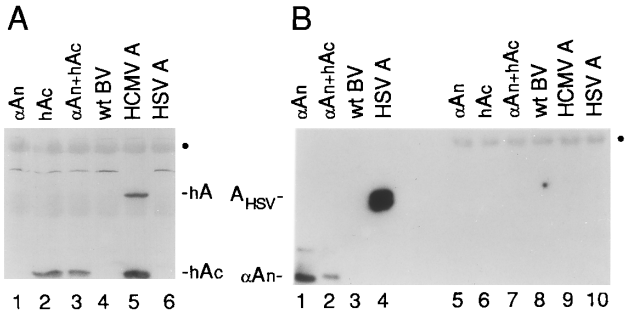

FIG. 5. HCMV A_c does not coimmunoprecipitate HSV A_n. Sf9 cells were infected with rBVs encoding wild-type BV, HCMV assemblin (HCMV A), HSV assemblin (HSV A), HSV A_n, or HCMV A_c, either alone or in the indicated combinations. The infected cells were processed, subjected to SDS–PAGE in 14% polyacrylamide mini-gels, electrotransferred to Immobilon, and probed in a Western immunoassay with either anti-C2 (A) or antiserum 37-93 (B), all as described under Material and Methods. (A) The anti-C2 immunoprecipitates after Western immunoassay with the anti-C2 antiserum. (B) An immunoimage prepared from the blot showing both the starting lysates (lanes 1–4), and the anti-C2 immunoprecipitates after Western immunoassay with the antiserum 37-93 (lanes 5–10). Shown here is a fluorographic image of the resulting blot. "α" indicates an HSV protein (i.e., αA_n is HSV A_n), and "h" denotes HCMV proteins (i.e., hA_c is HCMV A_c). The positions of the HSV A_n (αA_n) and assemblin (A_{HSV}) and HCMV A_c (hA_c) and assemblin (hA) are indicated between the panels. Dots indicate the position of the IgG light chain.

I-site cleavage and there are no A_n and A_c counterparts produced. The absence of detected coimmunoprecipitation of HSV A_n by HCMV A_c indicates that these cross-species subunits do not interact to form a two-chain enzyme and explains why no pAP → AP cleavage was detected when these HSV and CMV subunits were coexpressed.

DISCUSSION

CMV assemblins appear to be distinguished as a group by autoproteolytic cleavage at an (I)nternal site (Baum *et al.,* 1993; Burck *et al.,* 1994; Welch *et al.,* 1993) that converts the enzyme from a one-chain to a two-chain form that retains activity (Holwerda *et al.,* 1994). None of the α- or γ-herpesviruses for which the proteinase sequence is available (Gibson *et al.,* 1995), or even β-herpesviruses other than the CMVs (e.g., HHV-6, and HHV-7), has an obvious I-site equivalent. Correspondingly, no evidence of a counterpart two-chain enzyme has been found among the α- (i.e., HSV-1; Fig. 5B, lane 4) and γ-(i.e., EBV) herpesvirus assemblin homologs that have been cloned and expressed (DiIanni *et al.,* 1993; Donaghy and Jupp, 1995).

The objectives of the work described in this report were to determine (i) whether there might be intrinsic differences in the non-CMV assemblin homologs, specifically HSV, that preclude them from functioning as an analogous two-chain enzyme; (ii) whether the inability of the HSV assemblin homolog to form a two-chain enzyme accounts for its narrower substrate range, as compared with CMV assemblins; and (iii) whether intertypic two-chain assemblins (e.g., CMV A_n + HSV A_c) can be formed. This was accomplished by cloning HSV sequences that approximate those encoding CMV A_n and A_c and expressing them with one another to test for active two-chain HSV proteinase, or with CMV subunits to test for active HSV/CMV intertypic two-chain proteinase. Our results (i) establish that a two-chain HSV assemblin analog can be formed in insect cells by coexpressing recombinant HSV counterparts of the CMV A_n and A_c subunits, (ii) show that the two-chain HSV enzyme retains the comparatively restricted substrate range of the single-chain enzyme, and (iii) show that HSV and CMV subunits do not associate to form an active intertypic two-chain assemblin.

The ability of the HSV assemblin homolog to function as a two-chain enzyme indicates that the absence of a natural two-chain form is due to the lack of an I site, or to the inability of the enzyme to recognize and cleave an I-site sequence, or to both. The spontaneous association of the HSV recombinant subunits within the cell indicates that they can assume a suitably interactive conformation without prior synthesis and folding as a single-chain enzyme and suggests that they represent structurally and perhaps functionally distinct domains, as noted before with CMV two-chain assemblin (Hall and Gibson, 1996). General structural similarities of this sort are expected among the proteinase homologs of different members of the same virus group (Bazan and Fletterick, 1989).

The inability of the HSV and CMV subunits to interact and form intertypic two-chain enzymes indicates that the interfaces through which A_n and A_c interact differ between the HSV and CMV enzymes, or that the cross-species subunits are conformationally incompatible, or both. It may be possible to exploit this incompatability to map the interactive domains by using chimeras of one subunit (e.g., HSV A_n having a specific sequence replaced with the corresponding sequence from CMV A_n) to identify residues that enable it to interact with a normally incompatable cross-species subunit (e.g., HSV A_n chimera + CMV A_c) to form active enzyme. Identifying these interactive domains will have importance in understanding assemblin function and possibly providing additional targets for inhibiting the enzyme.

Three lines of evidence now indicate that I-site cleavage is not likely to be a mechanism for altering enzyme specificity. The first is that expression of CMV assemblin as just the two-chain form (i.e., coexpression of A_n and A_c) did not narrow its substrate range (i.e., still cleaves CMV M, R, and I sites, and HSV M site) (Hall and Gibson, 1996) or appreciably alter its kinetics *in vitro* (Holwerda *et al.,* 1994; O'Boyle *et al.,* 1995). The second is the finding reported here that expression of the HSV assemblin ho-

molog as a two-chain enzyme did not broaden its substrate range (i.e., strong selectivity for HSV substrates retained). Third, a set of collary experiments to these HSV studies showed that when expressed as just the single-chain form (e.g., I-site mutant A127Q unable to be cleaved to two-chain enzyme), the substrate range of SCMV assemblin was not decreased (i.e., still cleaved CMV M, R, and I sites and HSV M site; M. R. T. Hall and W. Gibson, unpublished data; Welch *et al.,* 1993). An alternative explanation for its evolutionary retention or selection is that I-site cleavage is a mechanism to eliminate or reduce the amount of assemblin in CMV capsids in order to accomodate its comparatively large genome (i.e., ≈25% larger than that of Epstein–Barr virus, the largest of the characterized non-CMV herpesviruses). Preliminary findings are consistent with this possibility and indicate that, in contrast to virions of HSV that retain their assemblin homolog VP24 (Davison *et al.,* 1992; Person *et al.,* 1993), virions of HCMV and SCMV either lack or contain substantially reduced amounts of assemblin (J. Borchelt, K. Clopper, and W. Gibson, unpublished data).

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