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Cytomegalovirus Protein Substrates Are Not Cleaved by the Herpes Simplex Virus Type 1 Proteinase

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The herpesvirus maturational proteinase, assemblin, is made as a precursor that undergoes at least two autoproteolytic cleavages—one in a sequence toward its carboxyl end, called the maturational (M) site, and one in a sequence toward its midpoint, called the release (R) site. The M- and R-site sequences are both well conserved among the herpesvirus proteinase homologs, suggesting that the proteinase of one herpesvirus might be able to cleave the substrates of another. To test this possibility, we cloned and expressed in human cells the long (i.e., full-length open reading frame of proteinase gene) and short (i.e., proteolytic domain, assemblin) forms of the proteinase from human and simian cytomegalovirus (HCMV and SCMV, respectively) and from herpes simplex virus type 1 (HSV-1), as well as the genes for their respective assembly protein precursor substrates. Data from cotransfections of these proteinase genes with appropriate homologous and heterologous substrates showed that although the SCMV and HCMV enzymes cleaved the M-sites of the assembly protein substrates of all three viruses and an SCMV R-site substrate, the HSV-1 proteinase cleaved only its own substrate. This finding demonstrates that the substrate specificity properties of the HSV-1 enzyme differ from those of the two CMV enzymes.

Herpesviruses encode a maturational proteinase (18, 25, 35) whose function is essential for the production of infectious virus (7, 25). The gene that encodes the proteinase is approximately 2 kb in length and contains nested, 3′-coterminal, in-frame genes (17, 33). The proteinase is translated as the full-length product of the longest open reading frame (ORF) in this nested family and then undergoes autoproteolytic cleavage at two highly conserved sites. One site is located near the carboxyl end of the full-length precursor and is called the maturational cleavage site (M-site [Fig. 1]). It has the consensus sequence V/L-X-A$^{\downarrow}$S (35). The second autoproteolytic site is located near the middle of the full-length precursor and is called the release cleavage site (R-site [Fig. 1]). It is more highly conserved than the M-site and has the consensus sequence Y-V/L-K/Q-A$^{\downarrow}$S (35). Cleavage at the R-site releases the proteolytic half of the molecule, called assemblin in cytomegalovirus (CMV), from the nonproteolytic carboxyl half (12, 20, 32, 35). CMV assemblin undergoes a third autoproteolytic cleavage at a site near its midpoint that is not conserved in the herpes simplex virus type 1 (HSV-1) proteinase (1, 2, 34). Because cleavage at this site was initially thought to inactivate the enzyme, it was called the inactivation site. However, recent studies have established that cleavage at this site, now called the internal cleavage site (I-site [Fig. 1]), yields an enzymatically active two-chain form of assemblin (10, 11). The I-site sequence is only loosely conserved between the human and simian strains of CMV (HCMV and SCMV, respectively) (i.e., VEA$^{\downarrow}$AT and INA$^{\downarrow}$AD, respectively) and, with the possible exception of ANA?AN in herpesvirus saimiri, has no obvious counterpart among the other herpesvirus assemblin homologs (see Fig. 9 of reference 34). None of these cleavages is absolutely essential to activate the proteinase; therefore, it does not appear to be made as an inactive zymogen (12, 20, 32, 34).

Site-directed mutagenesis (34) and affinity labeling (6) experiments have identified a conserved serine residue, the only one that is absolutely conserved among the herpesvirus assemblin homologs, as being the catalytic site nucleophile. An absolutely conserved and essential histidine has also been identified by site-directed mutagenesis (19, 34) and may contribute to the active site of this apparently new subclass in the serine proteinase superfamily (34).

In addition to the autocatalytic cleavage sites within its precursor, the herpesvirus proteinase also cleaves an abundant capsid phosphoprotein, called the assembly protein (AP) precursor (pAP). The gene encoding pAP is about 1 kb in length and is nested as the 3′ half of the ORF that encodes the proteinase (Fig. 1) (17, 33). Because of the nested, in-frame relationship of these ORFs, the pAP cleavage site is identical to the M-site of the full-length precursor (Fig. 1). M-site cleavage of the pAP appears to be an essential event in the assembly of infectious virus (7, 24). Given the similarity of the M-site sequences among the different homologs of this herpesvirus gene, it was of interest to determine whether the proteinase of one herpesvirus could cleave the M-site of another.

Experiments described in this report were done to test the ability of the long (i.e., precursor) and short (i.e., assemblin) forms of the SCMV, HCMV, and HSV-1 proteinases to cleave substrates of the other viruses. Substrate specificity was assayed by transient transfections, and the resulting data indicate that (i) the CMV proteinases have a less restricted substrate specificity than that of HSV-1, and (ii) additional structural information, such as amino acids surrounding the core cleavage site, or the conformation of the region containing the core sequence, or both, is required for recognition and cleavage by the HSV-1 proteinase.

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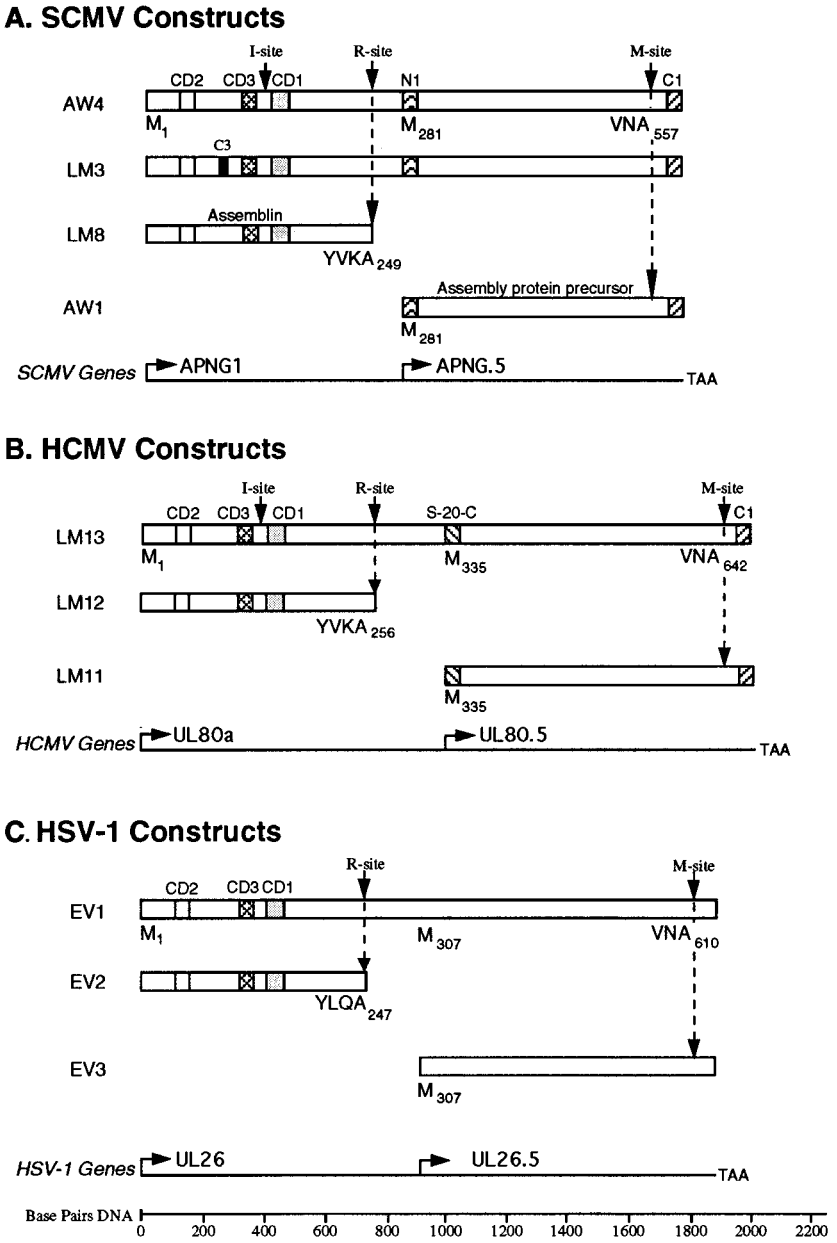

FIG. 1. Genes, proteins, and landmarks of herpesvirus proteinases and substrates. All of the genes shown were expressed using the plasmid pRSV.neo (see Materials and Methods). The primary translation products from each of the cloned genes are diagrammed, and specific landmarks are indicated. CD1, CD2, and CD3 are the three most highly conserved domains in the long (i.e., full-length translation product of the longest ORF) and short (i.e., assemblin and its homologs) forms of the proteinase; CD3 contains the essential serine nucleophile (6, 34), and CD2 contains the putative catalytic site histidine (34). The maturational (M), release (R), and internal (I) cleavage sites are indicated, and the carboxy-terminal residues of the R-and M-sites are denoted. The nomenclature of the overlapping genes is shown at the bottom of each panel; their relative sizes (in base pairs) are indicated at the bottom of the figure. (A) Constructs of SCMV strain Colburn. M_1 and M_{281} are the start methionines of the long (APNG1 ORF product) and short (assemblin) forms of the proteinase and of the assembly protein precursor (APNG.5 ORF product), respectively; A_{249} and A_{557} are the P1 (numbering scheme in reference 28) alanine residues at the release and maturational cleavage sites, respectively. N1 denotes the amino-terminal 13 amino acids of SCMV pAP that were used to generate the anti-N1 peptide serum. Plasmids named at the left encode the long (AW4) and short (LM8) forms of the proteinase and the pAP (AW1). LM3 encodes a minimally active long form of the proteinase (35), mutated by insertion of a 15-amino-acid sequence (C3) between CD2 and CD3. (B) Constructs of HCMV strain AD169. M_1 and M_{335} are the start methionines of the long (UL80a ORF product) and short (assemblin homolog) forms of the proteinase and of the assembly protein precursor (UL80.5 ORF product), respectively; A_{256} and A_{642} are the P1 (numbering scheme in reference 28) alanine residues at the release and maturational cleavage sites, respectively. S-20-C indicates the position of amino acids 2 through 20 of the HCMV pAP that were used to generate the anti-S-20-C peptide serum. Plasmids named at the left encode the long (LM13) and short (LM12) forms of the proteinase and the pAP (LM11). (C) Constructs of HSV-1 strain 17. M_1 and M_{307} are the start methionines of the long (UL26 ORF product) and short (assemblin homolog) forms of the proteinase and of the assembly protein precursor (UL26.5 ORF product), respectively; A_{247} and A_{610} are the P1 (numbering scheme in reference 28) alanine residues at the release and maturational cleavage sites, respectively. Plasmids named at the left encode the long (EV1) and short (EV2) forms of the proteinase and the pAP (EV3).

(An initial report of these data was presented at the Fourth International Cytomegalovirus Workshop in Paris, France, 19 to 21 April, 1993.)

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts were prepared, grown, and infected with the Colburn (simian) or AD169 (human) strain of CMV or with the F strain of HSV-1, as previously described (8). The human embryonal kidney (HEK) cell line 293 (American Type Culture Collection, Rockville, Md.) was grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Nonidet P-40 nuclear and cytoplasmic lysates of infected cells were prepared as previously described (8).

Plasmid construction. Standard techniques were used to construct, clone, and propagate the plasmids (26).

(i) SCMV strain Colburn constructs (Fig. 1A). The protein products of the APNG1, APNG.5, and assemblin ORFs were expressed from the plasmids AW4, AW1, and LM8, respectively, whose construction was described before (35). LM3 expresses an inactive form of the SCMV proteinase as a result of the insertion of a 15-amino-acid coding sequence into the *Eco*RV site of AW4 (Fig. 1A) and is used as a substrate for R-site cleavage (35).

(ii) HCMV strain AD169 constructs (Fig. 1B). The protein products of UL80a and UL80.5 ORFs (33) were amplified by the PCR (23) from viral DNA of HCMV strain AD169 and cloned into the *Xba*I site of the expression vector pRSV.5 (*neo*) (21) to generate LM13 and LM11, respectively. The portion of the UL80a ORF encoding the amino-terminal 256 amino acids (i.e., the HCMV assemblin homolog) was similarly amplified with primers that introduced a stop codon immediately following the Ala-256 codon of UL80a. This sequence was then cloned into the *Xba*I site of pRSV.5 (*neo*) to create LM12.

(iii) HSV-1 strain 17 constructs (Fig. 1C). The UL26.5 gene (17) of HSV-1 was cloned by a three-way ligation as follows. (i) The 5′ half of the UL26.5 gene was PCR amplified with a 5′ primer containing an *Xba*I site and a 3′ primer overlapping a unique *Pst*I site. (ii) The 3′ half of the UL26.5 gene was amplified with a 5′ primer overlapping the unique *Pst*I site and a 3′ primer containing an *Xba*I site. (iii) These two pieces were then ligated into the *Xba*I site of pUC18 to generate a 1,020-bp *Xba*I-*Xba*I fragment, which was then subcloned into pRSV.5 (*neo*) to create EV3.

The portion of the HSV-1 UL26 ORF encoding the N-terminal 247 amino acids (i.e., HSV-1 assemblin homolog) was cloned by a three-way ligation as follows. (i) The 5′ half of the sequence encoding the HSV-1 assemblin homolog was PCR amplified with a 5′ primer containing an *Xba*I site and a 3′ primer overlapping a unique *Xho*I site. (ii) The 3′ half was amplified with a 5′ primer overlapping the unique *Xho*I site and a 3′ primer containing an *Xba*I site. (iii) These two pieces were then ligated into the *Xba*I site of pUC18 to form a 783-bp *Xba*I-*Xba*I fragment, which was subcloned into pRSV.5 (*neo*) to create EV2.

The HSV-1 UL26 ORF was cloned as follows. (i) The 165-bp *Sac*I-*Xba*I fragment of EV2 was replaced with a PCR-generated 338-bp fragment to create the plasmid HSVL (this plasmid encodes the amino-terminal 307 amino acids specified by the UL26 ORF). (ii) The 952-bp *Xba*I fragment was subcloned from HSVL into EV3, which had been partially digested at the upstream *Xba*I site to create EV3L. (iii) To generate the wild-type UL26 gene (22), the sequence between the newly created *Sca*I site and the *BstE*II site of EV3L was replaced with a synthetic 49-bp fragment. The resulting plasmid is called EV1.

Transfection assays. Transfections were carried out in the human embryonal kidney (HEK) cell line 293 (American Type Culture Collection) by the modified calcium phosphate method (3), essentially as described before (9, 34).

Gel electrophoresis and Western immunoassay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done essentially as described by Laemmli (15) with 10% acrylamide gels. Nonidet P-40 nuclear and cytoplasmic fractions of mock-infected and virus-infected human foreskin fibroblast cells were prepared, as previously described (8), late in the infection cycle from cultures showing strong viral cytopathic effect.

Electrotransfer was done essentially as described by Towbin et al. (31), using a semidry transfer unit with an Immobilon P membrane and an electrotransfer buffer containing 50 mM Tris and 20% methanol (9). The membrane was blocked in 5% bovine serum albumin, probed with the indicated antiserum followed by ^{125}I-labeled protein A, and exposed to X-ray film with a calcium tungstate intensifying screen (16). Rabbit anti-N1 and anti-C1 sera directed against the amino-terminal and carboxy-terminal amino acids of SCMV pAP, respectively (Fig. 1A and B), have been described previously (29). Rabbit anti-S-20-C serum is directed against amino acids 2 through 20 of the HCMV strain AD169 pAP and has also been described before (2). HSV-1 proteins were detected with a cocktail of five monoclonal antibodies (MAbs) produced by immunizing mice with HSV-1-infected cells and were reactive with the HSV-1 assembly protein (purchased from L. Pereira, University of California, San Francisco).

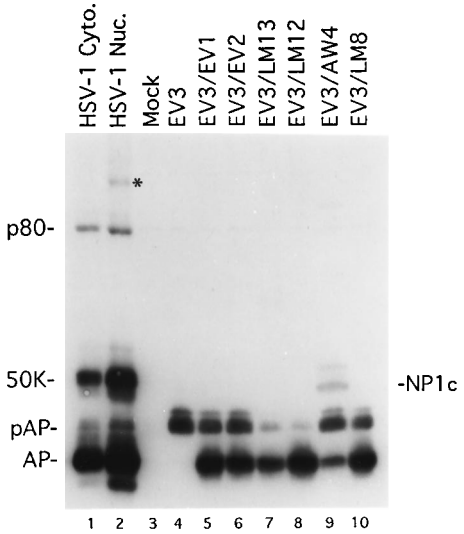

FIG. 2. Proteinases from both SCMV and HCMV cleave the HSV-1 pAP. HEK 293 cells were transfected with 2 μg of the EV3 plasmid alone or cotransfected with 2 μg of EV3 and 0.5 μg of one of the following proteinase constructs: EV1 (HSV-1 long proteinase), EV2 (HSV-1 assemblin homolog), LM13 (HCMV long proteinase), LM12 (HCMV assemblin homolog), AW4 (SCMV long proteinase), or LM8 (SCMV assemblin) as described in Materials and Methods. Western immunoassays were done with a cocktail of MAbs reactive with the HSV-1 assembly protein. Nuclear (Nuc.) and cytoplasmic (Cyto.) fractions of HSV-1-infected cells were used as a source of markers for the HSV-1 pAP and its cleavage product (AP). p80 (38) corresponds in size to Pra and Prb (18), long forms of the proteinase; the 50-kDa (50K) band corresponds in size to Na and Nb (4a), the carboxyl halves of Pra and Prb, respectively. The carboxyl half of the SCMV long proteinase (i.e., $NP1_c$ [34, 35]), which contains the entire amino acid sequence of the assembly protein, was visualized in lane 9, because the HSV-1 MAb cocktail used as a probe cross-reacts with the SCMV assembly protein (32a). The asterisk denotes a band reproducibly detected in the nuclear fraction of HSV-1-infected cells with this MAb cocktail (e.g., Fig. 4); its origin is not known, but it is not observed with the same MAb preparation in the nuclear fraction of mock-infected human fibroblasts.

RESULTS

The abilities of the SCMV maturational proteinase assemblin and its homologs from HCMV and HSV-1 to cleave the pAP substrate of a heterologous virus were tested in the experiments described below. The approach was to express each enzyme with different substrates in transient transfections and then use Western immunoassays to determine the extent of substrate cleavage to product. The full-length precursor form of SCMV assemblin (i.e., pNP1) and its HCMV and HSV-1 homologs (Fig. 1) were also tested.

SCMV and HCMV proteinases can cleave the HSV-1 pAP at its M-site. Plasmids encoding both the short (i.e., LM8, LM12, and EV2) and long (i.e., AW4, LM13, and EV1) forms of the SCMV, HCMV, and HSV-1 proteinases, respectively, were individually cotransfected with plasmid EV3, which encodes the HSV-1 pAP. Three days later, lysates were prepared from the transfected cells and subsequently analyzed by Western immunoassays. Data from the assay show the following. (i) Both forms of the SCMV and HCMV proteinases cleaved the HSV-1 pAP (Fig. 2, lanes 7 to 10). (ii) Cleavage of HSV pAP (Fig. 2, lane 4) to AP was approximately equivalent for the long (Fig. 2, lane 5) and short (Fig. 2, lane 6) forms of the HSV proteinase. Parenthetically, we expected to detect either the full-length protein product of UL26 (i.e., p80) or the 50-kDa carboxyl half of p80 (i.e., 50K) or both in lane 5. Their absence here suggests that they were present at very low levels. (iii) Comparison of the relative amounts of pAP and AP in each

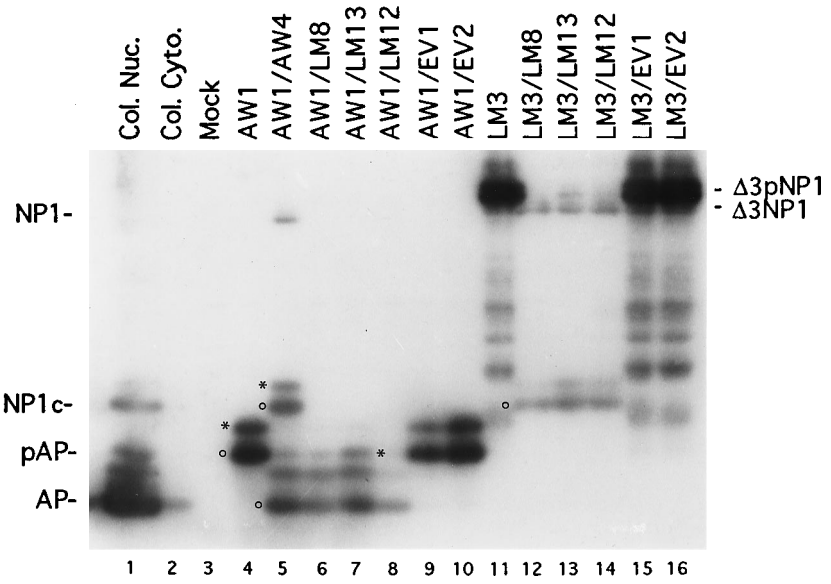

FIG. 3. HCMV proteinase cleaves SCMV M- and R-sites, but HSV-1 proteinase does not. HEK 293 cells were transfected with 2 μg of the AW1 plasmid alone (lane 4) or 2 μg of the LM3 plasmid alone (lane 11) or cotransfected with 2 μg of AW1 or LM3 and 0.5 μg of one of the following proteinase-encoding plasmids: AW4, LM8, LM13, LM12, EV1, or EV2 (lanes 5 to 10, 12 to 16, respectively), as described in Materials and Methods. Western immunoassays were done with anti-N1 (Fig. 1A). Lanes 1 and 2, nuclear (Nuc.) and cytoplasmic (Cyto.) fractions of SCMV strain Colburn (Col.)-infected cells; lane 3, lysate of mock-transfected HEK 293 cells. Bands indicated by an asterisk above $NP1_c$, pAP, and AP are modified forms of the corresponding proteins (34, 35) and increase in abundance at late times after transfection (9). Open circles indicate the positions of proteins identified in the left margin. Δ3pNP1 is the full-length protein product of the mutant proteinase gene in plasmid LM3 and is slightly larger than wild-type pNP1 because of the 15-amino-acid C3 insertion (Fig. 1A). The comparatively greater intensity of the Δ3pNP1 band in lanes 11, 15, and 16 is due to accumulation of this inactive form of the enzyme (i.e., the amounts of Δ3NP1 and $NP1_c$ in lanes 12 to 14, where active proteinase was present, are comparable to those in lane 5, which contains the counterpart products NP1 and $NP1_c$ of the wild-type full-length proteinase). The bands below Δ3pNP1 in lanes 11, 15, and 16 are attributed to residual activity of LM3, probably in combination with cellular proteinases.

transfection indicated that the HCMV proteinases (Fig. 2, lanes 7 and 8) were the most efficient of the six in cleaving at the HSV-1 M-site and that the product of the SCMV APNG1 ORF, encoded by AW4 (Fig. 2, lane 9), was the least efficient.

The HSV-1 proteinase is unable to cleave at the SCMV M- or R-site. We next tested whether the proteinases of HCMV and HSV-1 could cleave SCMV substrates. This was done by cotransfecting plasmids encoding the short and long forms of the HCMV (i.e., LM12 and LM13, respectively) and HSV-1 (i.e., EV2 and EV1, respectively) proteinases individually with plasmid AW1, which encodes the SCMV pAP (i.e., M-site substrate). Lysates prepared from the cells 3 days after transfection were analyzed by Western immunoassay. The data show that proteins expressed from both HCMV plasmids were able to cleave the SCMV pAP to its product AP with efficiencies similar to those of their SCMV counterparts (Fig. 3, lanes 5 to 8) and that neither form of the HSV-1 proteinase cleaved the SCMV pAP (Fig. 3, lanes 9 and 10).

The HCMV and HSV-1 enzymes were also tested for the ability to cleave at the SCMV R-site. The substrate used for this experiment was a minimally active, mutant long form of the SCMV proteinase that contains a 15-amino-acid insertion (C3 [Fig. 1A]) between CD2 and CD3. This mutant proteinase is encoded by the LM3 construct and has been shown to serve as both an M-site substrate and an R-site substrate for SCMV assemblin (35). Cotransfections of plasmids encoding the respective HCMV and HSV-1 enzymes with the LM3 plasmid were done, and the resulting lysates were assayed as part of the experiment shown in Fig. 3. The data demonstrate that both forms of the HCMV proteinase cleaved the substrate at its R-site, as indicated by the disappearance of the mutant proteinase precursor, Δ3pNP1, and the appearance of the R-site cleavage fragment $NP1_c$ (denoted by an open circle in Fig. 3, lanes 12 to 14). However, neither form of the HSV-1 proteinase produced R-site cleavage of this substrate, as indicated by the unchanged intensity of the Δ3pNP1 band and by the absence of $NP1_c$ (Fig. 3, lanes 15 and 16).

To verify that the HSV-1 proteinases were present in an active form in cotransfections with SCMV substrates, a set of triple transfections was done. The experiments described above were repeated but with the HSV-1 construct EV3 (encodes HSV-1 pAP) added to each transfection to provide a homologous substrate indicator for HSV-1 proteinase activity. Lysates prepared from the cells 3 days after transfection were analyzed by Western immunoassay. Data from this experiment show that even though HSV-1 proteinase activity could be demonstrated in all transfections, as revealed by cleavage of the indicator HSV pAP to AP (Fig. 4A, lanes 5 to 8), neither of the SCMV substrates was cleaved by the HSV enzymes (Fig. 4B, lanes 5 to 8). Because the SCMV substrates (e.g., SCMV Δ3pNP1 band in lanes 7 and 8) cross-reacted with the MAb cocktail used to identify the HSV proteins, the blot shown in Fig. 4A was stripped (13), subjected to fluorographic exposure to verify removal of the first set of immunologic reagents, and reprobed with the anti-N1 serum (Fig. 1A), which is specific for the SCMV proteins. The absence of SCMV cleavage products is clearly seen in the resulting immunoimage (Fig. 4B) and demonstrates the inability of both forms of the HSV-1 proteinase to cleave SCMV R-site and M-site substrates.

HSV-1 proteinases are unable to cleave the HCMV pAP at its M-site. The ability of the SCMV and HSV-1 proteinases to cleave the HCMV pAP substrate was similarly tested. Plasmid LM11, encoding the HCMV pAP was cotransfected with a plasmid encoding one of the two SCMV or HSV-1 proteinases (i.e., SCMV AW4 and LM8 or HSV-1 EV1 and EV2). Lysates were prepared from the cells 3 days after transfection, and

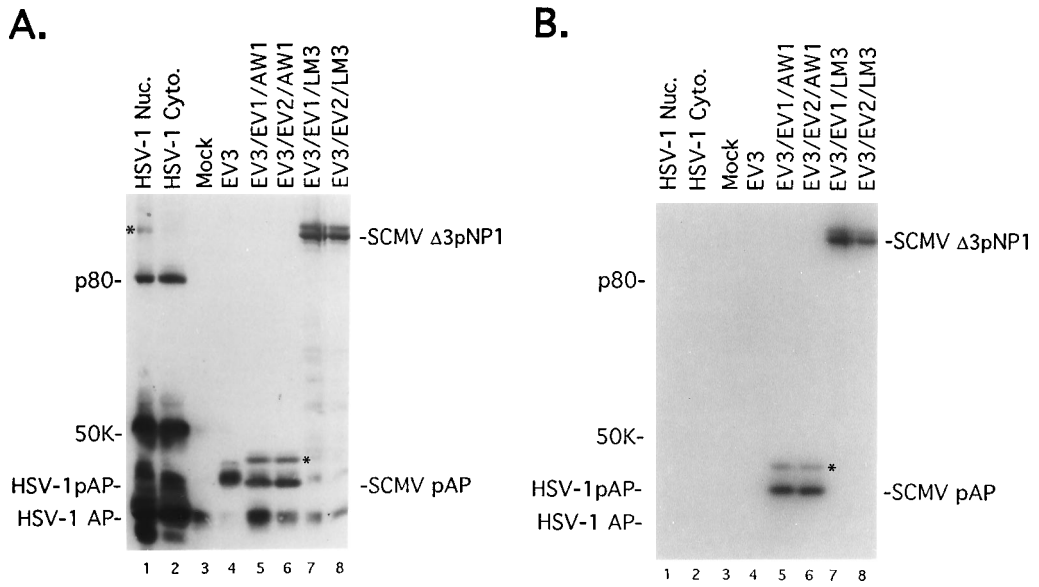

FIG. 4. HSV-1 proteinase cleaves HSV-1 pAP in the presence of SCMV substrates. HEK 293 cells were transfected with 2 μg of the EV3 plasmid alone (lane 4) or cotransfected with 0.5 μg of the EV1 or EV2 plasmid together with 2.0 μg of the AW1 or LM3 plasmid (lanes 5 to 8), as described in Materials and Methods. Western immunoassays were done initially with a MAb cocktail against the HSV-1 AP (A). The blot was then stripped and probed again with anti-N1, specific for the SCMV AP (B). Lanes 1 and 2, nuclear (Nuc.) and cytoplasmic (Cyto.) fractions of HSV-1-infected cells; lane 3, lysate of mock-transfected HEK 293 cells. p80 (38) and the 50-kDa (50K) band are explained in the legend to Fig. 2. Other protein designations in the left and right margins are explained in the legend to Fig. 3. The asterisk to the right of lane 6 indicates the band similarly labeled to the left of pAP in Fig. 3, lane 4; the asterisk to the left of lane 1 indicates the band similarly labeled to the right of lane 2 in Fig. 2.

Western immunoassays were done to visualize the results. The data show that the HCMV pAP (Fig. 5, lane 4) was efficiently cleaved to AP by all four CMV proteinases (Fig. 5, lanes 5 to 8) and that there was no cleavage of this HCMV substrate by either form of the HSV-1 proteinase (Fig. 5, lanes 9 and 10). As was done in the experiment shown in Fig. 4, expression of active HSV-1 proteinase in these cotransfections was verified by a set of triple transfections in which both HSV enzymes were tested for the ability to cleave LM11 in the presence of indicator HSV pAP. The results of this experiment showed that even though the indicator HSV pAP was cleaved, the HCMV pAP substrate was not cleaved by either form of the HSV proteinase (data not shown).

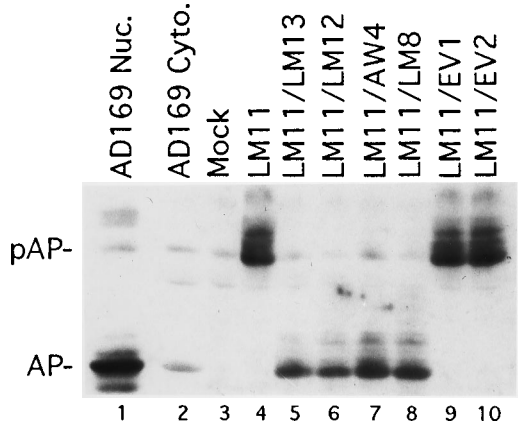

FIG. 5. SCMV proteinase cleaves HCMV pAP at its M-site, but HSV-1 proteinase does not. HEK 293 cells were transfected with 2 μg of the LM11 plasmid alone (lane 4) or cotransfected, respectively, with 0.5 μg of the plasmid LM13, LM12, AW4, LM8, EV1, or EV2 (lanes 5 to 10), as described in Materials and Methods. Western immunoassays were done with the S-20-C antiserum. Lanes 1 and 2, nuclear (AD169 Nuc.) and cytoplasmic (AD169 Cyto.) fractions of HCMV-infected cells; lane 3, lysate of mock-transfected HEK 293 cells.

DISCUSSION

Many reports have appeared now describing the cleavage of CMV and HSV substrates by their respective maturational proteinase in both cellular (e.g., 18, 20, 25, 34, 35) and in vitro (e.g., 2, 4, 5, 27) assay systems. Only one report, however, has tested the ability of the proteinase from one herpesvirus to cleave the substrate of another (2). The results of that study showed that purified HCMV assemblin will cleave peptide mimics of both the HSV-1 M- and R-sites but with reduced efficiencies compared with those of its own M- and R-site peptide mimics (2). In the work described here we used a transfection-immunoassay system to test substrate cross-recognition and cleavage by three different herpesviruses, HCMV, SCMV, and HSV-1. Our results show that HCMV and SCMV assemblin and their precursors cleave the substrate pAPs of all three viruses; however, the counterpart HSV-1 proteinases were able to cleave only the HSV-1 substrate and not those of HCMV or SCMV.

Because the M-site core consensus sequence (i.e., VNA$^{\downarrow}$S) is identical for these three herpesviruses, the finding that the HSV-1 proteinase does not cleave the CMV substrates indicates that substrate recognition by the enzyme involves more than just this core sequence. Data from in vitro cleavage assays are consistent with this interpretation and suggest that the difference may be due to a comparatively extended substrate-binding pocket for the HSV-1 enzyme. It has been shown that the smallest peptide that will serve as a substrate in vitro for either HCMV or HSV-1 assemblin is larger than the 4-amino-acid core sequence (2, 27). Burck et al. (2) first demonstrated that P5-P5′ peptide mimics of the HCMV and HSV-1 M- and R-sites were cleaved by purified HCMV assemblin and that the

HSV peptides were poorer substrates than those of HCMV, indicating that amino acids flanking the core sequence play a role in recognition and cleavage. Sardana et al. (27) extended this finding by demonstrating that the smallest peptide mimic of the HCMV M-site that is cleaved by HCMV assemblin is P4-P4′, only 4 amino acids longer than the core sequence and 3 amino acids longer than the HSV peptide mimics cleaved by HCMV assemblin (2). In an earlier similar study, DiIanni et al. (5) showed that the smallest peptide mimic of the HSV-1 M-site cleaved by HSV-1 assemblin is P5-P8′ or 9 amino acids longer than the core sequence. Considered together, these results indicate that HSV-1 assemblin requires a longer recognition or cleavage sequence than the HCMV enzyme and may explain the observed inability of HSV assemblin to cleave CMV substrates reported here. An alternate explanation is that the structural context of the cleavage site affects substrate recognition by the enzyme, as has been proposed for the recognition of cleavage sites by the picornavirus (36, 37) and bacteriophage T4 (14) proteinases. This possibility can be tested through transfection assays, since they are expected to faithfully reproduce native features of the proteins that may affect presentation of the cleavage site (e.g., conformation and modification by prosthetic groups) which may not be present in assays done in vitro with synthetic peptide substrates and reconstituted, bacterially synthesized enzyme. By replacing HCMV cleavage site sequences with the corresponding sequences from HSV, for example, it could be determined whether HSV-1 peptides that are recognized and cleaved in vitro render the HCMV chimeras susceptible to cleavage by the HSV-1 proteinase or whether other factors are involved.

There are at least two differences between the CMV and HSV proteinases that may contribute to their substrate recognition differences. The first is the presence of an additional major cleavage site (i.e., I-site) in a potential loop of CMV assemblin that appears to be reduced or absent in the HSV enzyme (1, 2, 32). The I-site sequence is situated between two highly conserved domains, one that contains the active site nucleophile (i.e., CD3 Ser [6 and 34]) and the other whose function is presently unknown but that may be involved in substrate recognition and binding (i.e., CD1). This putative insertion sequence could give the CMV proteinases more flexibility in accomodating and cleaving substrates that differ somewhat from their natural recognition and cleavage sequences.

A second difference between the CMV and HSV proteinases is in the amino acid sequences of their R-sites and, consequently, the carboxyl ends of their assemblin homologs. It has been shown that substitutions in the carboxy-terminal four amino acids of assemblin or in the corresponding R-site residues of its precursor (32b, 33) or deletions or short additions at the carboxyl end of assemblin (1, 30) affect the proteolytic activity of the enzyme. A similar involvement of the carboxyl end of the HSV-1 assembin homolog is indicated by results obtained with HSV-1 constructs (20, 34). These observations suggest a functional involvement of the carboxyl end of assemblin (i.e., R-site) in its proteolytic activity. Interestingly, there is a subtype-specific difference between alpha- and betaherpesviruses in the carboxy termini of their assemblin homologs. The proteinases of the betaherpesviruses SCMV and HCMV have a valine at P3 and a lysine at P2 of their R-site, whereas the corresponding residues of the proteinases from the alphaherpesvirus are leucine and glutamine. The proteinases from the gammaherpesviruses have a sequence that is intermediate between the alpha- and betaherpesvirus sequences, a P3 leucine and a P2 lysine. These subtype-specific differences near the carboxyl end of the respective assemblin homologs and the sensitivity of this portion of the molecule to change are suggestive of a function in enzyme activity beyond R-site recognition and also would be compatible with an involvement in the substrate specificity reported here.

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