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Herpes Simplex Virus Type 1 Protease Expressed in *Escherichia coli* Exhibits Autoprocessing and Specific Cleavage of the ICP35 Assembly Protein

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The UL26 gene of herpes simplex virus type 1 (HSV-1) encodes a protease which is responsible for the C-terminal cleavage of the nucleocapsid-associated proteins, ICP35 c and d, to their posttranslationally modified counterparts, ICP35 e and f. To further characterize the HSV-1 protease, the UL26 gene product was expressed in *Escherichia coli*. The expressed protease underwent autoproteolytic processing at two independent sites. The first site is shared with ICP35 and results in removal of 25 amino acids from the C terminus of the protease. The second unique site gives rise to protein species consistent with deletion of a 28-kDa fragment at the N terminus. A mutant protease, which showed no activity in a mammalian cell cotransfection assay (F. Liu and B. Roizman, Proc. Natl. Acad. Sci. USA 89:2076–2080, 1992), failed to exhibit autoproteolytic processing at either site when expressed in bacteria. The inactive mutant was able to serve as a substrate in a *trans* assay in which the substrate and protease were coexpressed in bacteria. This experiment demonstrated that the unique N-terminal processing was mediated exclusively by the HSV-1 protease. ICP35 c,d also served as a substrate in this assay and was correctly processed by HSV-1 protease in *E. coli*. This *trans*-cleavage assay will aid in the characterization of HSV-1 protease and assist in investigation of the role of proteolytic processing in the virus.

Herpesviruses are double-stranded DNA viruses which appear to share a common pathway of assembly. The DNA genome is inserted into preformed B-capsids which are assembled in the nucleus. The full capsids are enveloped as they bud through the nuclear envelope into the cytoplasm and are subsequently released from the cell (2).

The protein composition of the various capsid intermediates has been characterized (1, 8, 14), and several virally encoded proteins have been implicated in the early stages of capsid assembly. These include an assembly protein, ICP35, which is present in large amounts in B-capsids prior to packaging DNA (17, 18), but is detected as a thin coat (1) or is entirely absent (7, 17) in encapsidated, mature particles. ICP35 has been suggested to play an analogous role to the scaffolding protein of double-stranded bacteriophage, which facilitates empty prohead assembly (9). The ability of ICP35 to self-assemble into defined structures in vitro (14) makes it a likely candidate to serve as one of the instrumental nucleating components of capsid assembly.

ICP35 is proteolytically processed during the assembly process by a virally encoded protease to remove 25 amino acids from its C terminus (6, 11). C-terminal cleavage of ICP35 is thought to be essential to virus maturation since a viral mutant, *ts*1201, which is incapable of processing ICP35 at the nonpermissive temperature is also unable to package DNA (15). The protease responsible for cleavage of ICP35 has been identified and localized to the 5′ end of the UL26 gene (11). A remarkable property of this protease is that the coding region and promoter of the substrate, ICP35, are entirely contained within the C-terminal half of the protease-coding region (10). The substrate shares amino acid sequence identity with the C-terminal 329 amino acids of the protease. The protease itself therefore contains a processing site at its C terminus which is susceptible to proteolytic cleavage. Autoproteolytic cleavage at this site has been used to assess the activity of the herpes simplex virus type 1 (HSV-1) protease in vitro in a transcription-translation assay (11).

In this report, we show the results of expression of the UL26 gene in *Escherichia coli* and assess the activity of the HSV-1 protease by evaluating autoproteolysis and *trans* processing in vivo.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmids pRB4090 (11), pRB8077 (12), and pRB4140 (11) were gifts from F. Liu and B. Roizman of the University of Chicago. The T7 vector pET-11d was used for the expression of HSV-1 protease in *E. coli* (Novagen). In this construct, an in-frame fusion was made by inserting a 1,966-bp *Xba*I-*Eco*RI fragment of pRB4090 into the *Nhe*I-*Eco*RI sites of the vector. This created pT7HSV, a fusion which contained two amino acids of the vector, the in-frame, upstream seven amino acids of the UL26 gene, and then the start Met and coding region of HSV-1 protease.

Plasmid pT7H148A was derived from pT7HSV by replacement of the *Bcl*I-*Bsu*36I fragment of the vector with a 1,181-bp *Bcl*I-*Bsu*36I fragment of pRB8077. This transferred the mutation of His-148 to Ala into the expression vector and resulted in an inactive protease.

Plasmid pT7HSVtag was derived from pT7HSV by replacement of the 386-bp *Sac*I-*Bsu*36I fragment of vector with the 1,052-bp *Sac*I-*Bsu*36I fragment of pRB4140. This allowed expression of an active form of HSV protease tagged with a cytomegalovirus (CMV) epitope near the C terminus. This protein was converted into a substrate for HSV-1 protease by replacing the 1,568-bp *Nco*I-*Bsu*36I fragment

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with the *Nco*I-*Bsu*36I fragment of pT7H148A to create pT7HSVSUB.

The substrate plasmid for coexpression experiments was derived from pACYC184 by ligating the 2,384-bp *Eco*RV-*Bcl*I site of pLysS (19) to the 2,924-bp *Bgl*II-*Ssp*I fragment of pT7HSVSUB. The resulting construct, pT7HSVSUBC, contained the p15A origin of replication, the Cam^r gene, and the substrate under control of the T7 promoter.

Plasmid pT7ICP35 was cloned by polymerase chain reaction mutagenesis with an oligonucleotide that created a new *Nco*I site at the start Met of ICP35, amino acid 307 of HSV-1 protease. The downstream primer hybridized to the C-terminal region of HSV-1 protease near the *Bsu*36I site. The resulting polymerase chain reaction fragment was cloned into the *Nco*I-*Bsu*36I sites of pT7HSV and resulted in the expression of ICP35. The ICP35 gene was transferred to the pACYC plasmid for coexpression as above for pT7HSVSUBC.

The *E. coli* strain used for expression was BL21(DE3) (19). Other *E. coli* strains used for expression were prepared by R. Breyer (Department of Medicine, Vanderbilt University Medical Center) and I. Deckman by lambda lysogeny of bacteriophage DE3. They include HB101(DE3), JM101 (DE3), JM101degP(DE3), OMPT5600(DE3), P453LPS(DE3) and X90(DE3). All strain manipulations were performed as described previously (13).

Protein analysis. *E. coli* strains carrying the expression plasmids were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) according to standard techniques (19), and total cell extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western (immunoblot) analysis (3). Monoclonal antibodies (MAbs) MCA406 (Serotec Inc.) and CH28.2 (purchased from Lenore Pereira) were used to analyze HSV-1 protease products and protein sequences containing the short CMV epitope, respectively. A rabbit polyclonal antibody (PAb) raised against a polypeptide containing the carboxy-terminal 13 amino acids was used to detect cleavage of the protease at the C terminus. Western analysis was performed with alkaline phosphatase-conjugated antibodies (DAKO) and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Kirkegaard & Perry Laboratories, Inc.).

Pulse-chase analysis. Cells containing the expression plasmids were grown in LB medium to an optical density at 600 nm of 0.5. Bacteria were pelleted, washed once, and resuspended in M9 minimal medium plus amino acids (no methionine) to an optical density at 600 nm of 0.15. After 1.5 h, cultures were IPTG induced (0.5 mM) for 30 min and treated with rifampin (200 μg/ml) for 10 min. Bacteria were then pulse-labelled with [^{35}S]methionine (Amersham) for 5 min and chased with cold methionine by conventional methods (20). Bacterial cells were pelleted at 15-min intervals post-chase, resuspended in sample buffer containing 65 mM Tris (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 1% SDS, and boiled for 2 min to inhibit further processing. Samples were immunoprecipitated (3) with MCA406 or C-terminal PAb and analyzed by SDS-12.5% PAGE. The gel was enhanced with Enlightening (DuPont) and subjected to fluorography.

RESULTS

Bacterial expression of HSV-1 protease. The UL26 gene was cloned into a bacterial expression vector driven by the strongly repressed and highly inducible T7 promoter. After 30 min of induction, a major species with an apparent molecular mass of 80 kDa, corresponding to the full-length protein, Pra, was observed by Western analysis with an ICP35 MAb, MCA406. At later time points, a specific pattern of degradation or proteolytic processing emerged (Fig. 1a, lanes 1 to 4). A smaller species migrating just below Pra was attributed to C-terminal cleavage and was observed in transcription-translation assays of the UL26 gene (11). This conclusion was supported by Western analysis with the PAb reactive against the C-terminal 13 amino acids of HSV-1 protease. The PAb displayed no reactivity to the lower band of the protein doublet, suggesting that the C terminus had been removed (Fig. 1, lanes 9 to 12). In addition to the expected C-terminal cleavage product, a second doublet of lower molecular mass (50 and 45 kDa) accumulated with time. Both proteins were immunoreactive with the ICP35 MAb but were 25 to 30 kDa smaller than full-length protein. This suggested that processing had occurred at a second N-terminal processing site or that translation had been initiated from a Met further downstream in the coding region. The 45-kDa protein was not reactive with the C-terminal PAb and appeared to be the C-terminally cleaved product of the 50-kDa protein. These proteins were referred to as Na and Nb, because of their N-terminal processing. To examine the possibility that the unexplained N-terminal processing was due to bacterial proteases, we expressed the HSV-1 protease in several strains of bacteria. Some of these strains were deficient in known *E. coli* proteases. Identical patterns of HSV-1 protease cleavage products were observed in all strains (data not shown). If N- and C-terminal

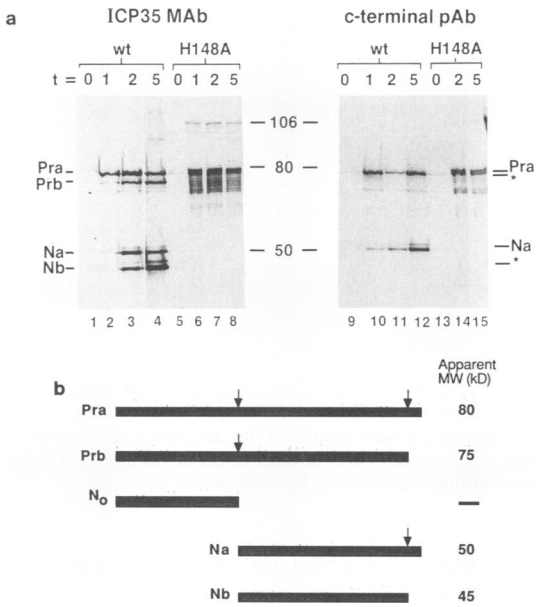

FIG. 1. Western analysis of bacterially expressed HSV-1 protease (wt) and inactive mutant H148A. (a) Time course (t = hours) of bacterially produced HSV-1 protease (lanes 1 to 4 and 9 to 12) and inactive mutant H148A (lanes 5 to 8 and 13 to 15) probed with MCA406 (lanes 1 to 8) or C-terminal PAb (lanes 9 to 15). *, position of absent C-terminally cleaved proteins. Numbers in middle show molecular mass in kilodaltons. (b) Diagram of HSV-1 protease cleavage sites. Autoproteolysis of Pra (full-length protease) at the designated arrows produces Prb (C-terminally cleaved Pra), Na (N-terminally cleaved Pra), and Nb (C-terminally cleaved Na). N_o is the N-terminal product of cleavage and is not immunoreactive with the antibodies used. kD, kilodaltons.

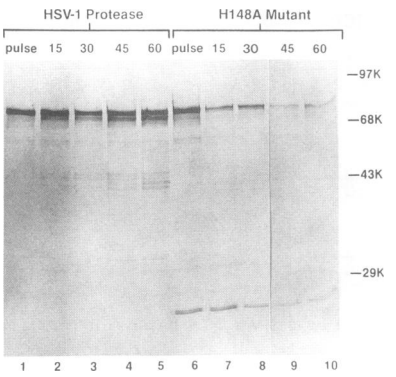

FIG. 2. Pulse-chase of HSV-1 protease and inactive mutant H148A. Bacteria expressing HSV-1 protease (lanes 1 to 5) or inactive mutant H148A (lanes 6 to 10) were pulse-labelled as described in the text. Time is expressed in minutes. Numbers on right show molecular mass in kilodaltons.

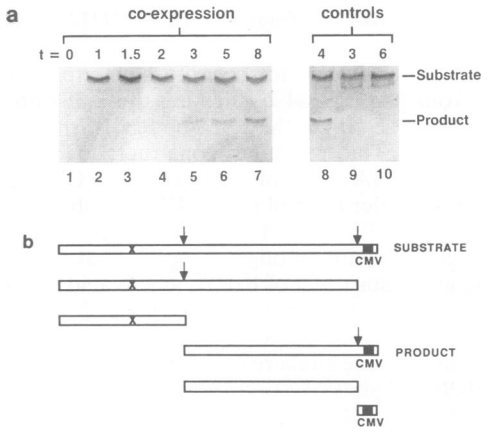

FIG. 3. *trans*-cleavage assay in *E. coli*. (a) Time course (t = hours) of HSV-1 protease coexpressed with the inactive, tagged mutant HSVSUB (lanes 1 to 7). Controls include a 4-h time point of HSVtag (lane 8) and HSVSUB at 3 h (lane 9) and 6 h (lane 10) after induction. Western blots were probed with CH28.2. Positions of full-length substrate and N-terminally processed product are marked accordingly. (b) Diagram of HSVSUB. Positions of cleavage sites are marked by arrows. The location of the point mutation which renders the protease inactive is depicted by an X. The dark box represents the CMV epitope used as a tag to detect N-terminal cleavage.

processing was due to a bacterial protease, it was present in all strains tested.

Pulse-chase analysis. Pulse-chase experiments were performed to establish the identity of the immunoreactive proteins that appeared at later time points after induction of HSV-1 protease expression. A 5-min pulse-label resulted in a single protein species corresponding to full-length protease. Immunoprecipitation with the ICP35 MAb (Fig. 2, lanes 1 to 5) and C-terminal PAb (data not shown) at 15-min intervals postchase showed that the C- and N-terminal cleavage products appeared with time. These data established that the smaller protein species, Prb, Na, and Nb (Fig. 1b), were processed products of the full-length HSV-1 protease. The N-terminally cleaved proteins appeared to migrate as doublets on an SDS-PAGE gel, although sequencing data suggested a single amino terminus (6).

Expression of inactive point mutant. Mutational analysis of the HSV-1 protease has previously established that a mutant with a point mutation at His-148 to Ala was unable to process ICP35 in a mammalian cotransfection assay (12). This inactive mutant (a gift from F. Liu and B. Roizman), H148A, was expressed in bacteria and resulted in a single full-length protein (Fig. 1a, lanes 5 to 8 and 13 to 15). It was significant that none of the cleavage products which accumulated during expression of the wild-type protease appeared with the inactive mutant. Pulse-chase analysis of the mutant protease revealed no posttranslational processing. However, in contrast to wild type, the species labeled in the pulse rapidly degraded, with no species accumulating during the chase (Fig. 2, lanes 6 to 10). These data implied that both the C- and N-terminal cleavage products of the wild-type protein were processed specifically by the HSV-1 protease. A role for a bacterial protease could not be entirely ruled out since the greater lability of the mutant Pra suggested a folding difference between it and wild-type protease.

***trans* cleavage assay in *E. coli*.** To prove that the HSV-1 protease was responsible for the cleavage products observed during expression in *E. coli*, we designed a *trans* assay with the inactive mutant protease as a substrate. In this assay, the inactive mutant was tagged by insertion of the unique CMV epitope (11) to create a substrate that could be easily monitored in the presence of wild-type protease. The tag allowed cleavage of the substrate to be observed by Western analysis with the CMV MAb, CH28.2. Wild-type protease tagged in the same manner retained activity in the *E. coli* system (Fig. 3, lane 8). The protease and substrate were expressed from different vectors in the same cell by using plasmids containing different origins of replication and antibiotic resistance genes. In this way, processing of the inactive mutant by the wild-type HSV-1 protease could be monitored.

As early as 1 h postinduction, a band corresponding to the N-terminal cleavage product was detected when the substrate was coexpressed with the authentic protease. This band was not detected in the substrate alone, even after 6 h of expression. This proved that the HSV-1 protease possesses a second self-processing site at the N terminus. C-terminal cleavage was difficult to assess in this system since the C-terminal cleavage products are small and N- and C-terminally cleaved products could not be distinguished immunologically from those of wild type.

Expression and coexpression of ICP35. A second coexpression system was established to evaluate *trans* processing of the C-terminal cleavage site. The authentic substrate for HSV-1 protease, ICP35, was cloned into the T7 expression vector by polymerase chain reaction mutagenesis. Bacterially expressed ICP35 migrated with some heterogeneity (Fig. 4a, lanes 1 to 5), similar to uncleaved ICP35 c,d of HSV-1-infected cells. Although differences in phosphorylation may account for the differences in migration of members of the ICP35 family of proteins in infected cells (1), this cannot explain the protein pattern observed in *E. coli* since such posttranslational modifications should not occur in prokaryotes. There are no other methionines to serve as a second site of initiation, and use of different stop codons could not explain the protein pattern since ICP35 e,f also appears as several bands after C-terminal cleavage. Therefore, it appears that something inherent in the amino acid sequence is responsible for the observed banding pattern.

When coexpressed with HSV-1 protease, ICP35 c,d was cleaved to a faster-migrating form which was not observed when ICP35 was expressed alone (Fig. 4a, lanes 6 to 9).

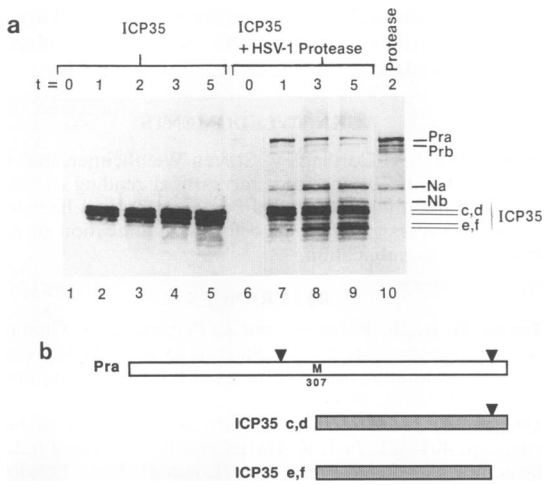

FIG. 4. *trans*-cleavage assay with ICP35. (a) Time course of induction (t = hours) of ICP35 (lanes 1 to 5), ICP35 coexpressed with HSV-1 protease (lanes 6 to 9), and HSV-1 protease (lane 10). Western blots were probed with MCA406. HSV-1 protease proteins are labeled according to the diagram in Fig. 1b. ICP35 proteins are labeled according to the diagram below. (b) Diagram of ICP35 cleavage by HSV-1 protease. The location of the start Met within HSV-1 protease for the ICP35 proteins is indicated by M.

Western blot analysis of ICP35 cleavage products with the C-terminal PAb confirmed that they resulted from C-terminal processing of the ICP35 primary translation product and therefore correspond to ICP35 e,f (data not shown). The increased mobility of the ICP35 cleavage products resulting from C-terminal cleavage therefore appears to mimic the processing of ICP35 observed in vivo. Coelectrophoresis with ICP35 from infected cells confirmed that the shift in molecular weight between the ICP35 c,d and e,f forms was identical to the bacterial coexpressed species (data not shown). It was also noted that HSV-1 protease was autoprocessed to a greater extent during coexpression with ICP35. This enhanced cleavage was more pronounced at the N-terminal site (compare lanes 9 and 10, Fig. 4a), while the C-terminal cleavage product of HSV-1 protease, Prb, was barely detectable during time courses of coexpression.

DISCUSSION

Proteolytic C-terminal processing of the putative scaffolding protein, ICP35, of HSV-1 is believed to be an essential step in viral assembly (15). The maturational protease responsible for cleavage of ICP35 c,d to ICP35 e,f is virally encoded and contains the entire amino acid sequence of ICP35 within its own C terminus (10). The identity shared between these proteins mandates that the HSV-1 protease is also susceptible to a proteolytic cleavage at its C terminus. *E. coli*-expressed HSV-1 protease mimicked the processing at this C-terminal cleavage site. In addition to the expected C-terminal autoproteolysis of HSV-1 protease, a second cleavage was observed in the bacterially expressed protein. This cleavage generated proteins with apparent molecular masses of 50 and 45 kDa, referred to as Na and Nb, respectively. These species were also shown to have a precursor-product relationship with the primary translation product, Pra, by pulse-chase analysis.

Only a portion of the full-length HSV-1 protease was processed to product within 1 h, suggesting that the efficiency of cleavage is poor. This is also apparent in vitro from transcription-translation assays in which complete C-terminal cleavage is only achieved after an 18-h incubation (5a). The slow kinetics of cleavage observed in bacteria may stem from the lack of protein modification to the enzyme or substrate in this system. Phosphorylation of ICP35 has been demonstrated in HSV-1-infected cells and may be important in maintaining the active conformation of these proteins. The environment of *E. coli* may also exclude or impede an important activation step, such as assembly of the protease into a more ordered structure, which may be important for cleavage. Alternatively, the protease may be an intrinsically slow enzyme in the virus, as well as in *E. coli*, and this delayed activity may serve a role in viral assembly.

An *E. coli trans* assay verified that HSV-1 protease was indeed responsible for both the C- and N-terminal cleavages observed in the expression of the UL26 gene product. A similar *E. coli trans* assay has been employed to study processing of the human immunodeficiency virus type 1 (HIV-1) *gag* polyproteins by HIV-1 or simian immunodeficiency virus proteases (4, 5). Coexpression of the *gag* substrate with HIV-1 or simian immunodeficiency virus protease lead to the complete processing of the polyprotein to form the structural entities, MA and CA. The bacterial assay was able to mimic the protease processing that occurs during assembly of HIV-1. To study and confirm *trans* processing at the two identified cleavage sites of HSV-1 protease, we required two substrates for coexpression with the protease. Cleavage of the authentic substrate, ICP35, could only provide the C-terminal cleavage site and would not confirm the existence of the N-terminal cleavage site. For this purpose, the substrate used in the HSV-1 *trans* assay was a tagged inactive form of the protease, incapable of autocleavage when expressed alone. When this substrate was coexpressed with HSV-1 protease, a species corresponding to the N-terminally cleaved product accumulated with time.

The identification of a second cleavage site in HSV-1 protease led to a homology search of the related proteases of HSV-1, varicella-zoster virus, CMV, and Epstein-Barr virus to locate the amino acid sequence involved. Proteolytic processing of the assembly protein has been shown to occur between the Ala and Ser of a conserved sequence, XVXAS, of CMV (21) and, more recently, HSV-1 (6). A similar sequence, YXXAS, is located within the N-terminal domain of these proteins and has been postulated as a second protease recognition site for the CMV protease (22). Cleavage at this homologous sequence in HSV-1 would result in removal of 247 amino acids from the N terminus and could account for the presence of Na and Nb in HSV-1 protease expressed in bacteria. In fact, an N-terminal cleavage product of HSV-1 protease expressed in *E. coli* has recently been sequenced and corresponds exactly to the postulated second cleavage site (6).

The N-terminally cleaved products Na and Nb are similar in size and composition to the ICP35 proteins, but are rarely revealed immunologically in HSV-1-infected cells. Since the protease and ICP35 are expressed from different promoters, the protease and its products may not be observed because they are inherently less abundant in the cell. Support for the presence of an N-terminal cleavage site comes from a recombinant mutant, R1247, in which the UL26 gene was linked to an immediate-early promoter and the protease was overproduced at the nonpermissive temperature as a result of a temperature-sensitive mutation (16). Under these conditions, a pattern of four proteins identical to bacterially

expressed HSV-1 protease autoprocessing was detected upon immunoprecipitation of whole-cell lysates with an ICP35 MAb. A 5-h chase of these proteins showed complete processing to the Nb-like species of the recombinant virus. Similar results were observed with HSV-1 protease expressed in *E. coli*. Processing also proceeds to completion, with a single species corresponding to Nb remaining after 24 h (data not shown).

What purpose does the N-terminal cleavage serve in the protease function and in the assembly of the virus? Sequence alignment of the proteases of CMV, HSV, Epstein-Barr virus, and varicella-zoster virus reveals that most of the homology is concentrated in the N-terminal 250 amino acids. Mutational analysis by truncation of the C terminus revealed that the activity of HSV-1 protease is confined to the N-terminal 307 amino acids (12) and suggests that the active site may be N terminal to the first cleavage site. Processing at the N terminus may therefore result in release of an active protease and could serve to make the protease more mobile. In fact, a protein corresponding to the N-terminal cleavage product, N_o (Fig. 1b), has been expressed in *E. coli* and retains wild-type activity in the bacterial *trans* assay (unpublished data). Similar results have been observed in C-terminal truncation mutants of the CMV protease (22). N-terminal cleavage has not been observed in the transcription-translation assays, suggesting that the protein produced in vitro may be folded or constrained in a way that hides the cleavage site from autoproteolysis. If this type of folding occurs in the virus, then the two processing sites may provide two separate functions during viral assembly.

The C-terminal 329 amino acids of the HSV-1 protease are identical to ICP35 and may serve several important functions for the protease. Immunoelectron microscopy of *ts*1201 shows nuclear localization of unprocessed ICP35 (17). Therefore, it can be inferred that the C-terminal cleavage is not necessary for nuclear localization and that ICP35 contains the sequences necessary for transport to the nucleus. Several runs of positively charged amino acids similar to the nuclear localization signal for simian virus 40 are found in the N-terminal region of ICP35 and may serve as signals for transport. If the nuclear localization signal for the protease is found within this domain, then the small N-terminal cleavage product, N_o, would be excluded from the nucleus. Second, ICP35 isolated from B-capsids has been observed to self-associate into defined structures in vitro (14). It is possible that the sequence at the C terminus of the protease is also likely to mediate an association between ICP35 and the protease. The protease may be assembled as part of the scaffolding of the B-capsids to form a complex containing both substrate and enzyme. Such an association between substrate and enzyme would ensure that the protease was present when conditions for cleavage were favorable. Assembly may be a prerequisite for cleavage, or it could put the protease into a conformation or environment that facilitates cleavage. This might explain the enhanced N-terminal cleavage of the protease we observed when it is coexpressed with ICP35. Additional experiments are necessary to address these issues rigorously.

It is still not understood why ICP35 is cleaved at its C terminus to remove 25 amino acids or how it is eliminated from the capsids during assembly. Cleavage of ICP35 occurs prior to DNA encapsidation and is necessary, but not sufficient, for DNA encapsidation (18). Perhaps, as in bacteriophage, the scaffold is associated with the capsid proteins and it is in this association that cleavage serves a function. Whatever the purpose, it is clear that cleavage of ICP35 can be mimicked in a bacterial system. This should help in the characterization of the enzyme and substrate as well as in elucidating their role in viral assembly.

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