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Investigation of the Specificity of the Herpes Simplex Virus Type 1 Protease by Point Mutagenesis of the Autoproteolysis Sites

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The herpes simplex virus type 1 (HSV-1) protease is cleaved at two autoprocessing sites during viral maturation, one of which shares amino acid identity with its substrate, ICP35. Similar autoprocessing sites have been observed within other members of the *Herpesviridae*. Introduction of point mutations within the autoprocessing sites of the HSV-1 protease indicated that specificity resides within the P4-P1′ region of the cleavage sites.

Assembly of herpes simplex virus type 1 (HSV-1) begins late during the infectious cycle (2). Prior to packaging of genomic DNA, the interior of the immature particle (B capsid) is filled primarily with infected cell protein 35 (ICP35) (13). Similar to the gp22 protein of bacteriophage T4 (9), ICP35 is thought to function as a scaffold (14) for the assembly of capsid proteins. A temperature-sensitive mutant which demonstrated that post-translational processing of ICP35 is required for production of infectious virions has been described (15). Overlapping open reading frames, UL26 and UL26.5, were shown to encode the HSV-1 protease and its substrate, ICP35, respectively (11, 12, 16). We previously described the expression of the HSV-1 protease and substrate in *Escherichia coli* and demonstrated cleavage of two autoprocessing sites, depicted in Fig. 1 (3). These two processing sites, first alluded by Welch et al. (20), have been mapped by amino acid sequencing to positions A-247/S-248 and A-610/S-611 (5). To investigate the sequence specificity of the HSV-1 protease, we examined the effects of cleavage site point mutations on autoprocessing of the *E. coli*-expressed protein.

Point mutations were introduced into the P5-P4′ positions of the C-terminal cleavage site and the P4-P1′ positions of the N-terminal cleavage site, using degenerate oligonucleotides and PCR amplification. Purified PCR fragments containing C-terminal point mutants were digested with *Afl*III and *Pst*I, and the 364-bp band was gel purified again prior to ligating into the unique *Afl*III-*Pst*I sites of pT7635C, a full-length HSV-1 protease construct derived from pT7635A (3), and pLysS (Novagen). Mutagenic oligonucleotides (Genosys; National Biosciences) were 40 bp in length and spanned the cleavage sites. Individual point mutants were identified by DNA sequencing and transformed into *E. coli* BL21(DE3) for expression. Bacterial expression was induced with isopropyl-thiogalactopyranoside (IPTG; 0.5 mM) at $t = 0$, and HSV-1 protease products were detected by Western blot (immunoblot) analysis with monoclonal antibody MCA406 (Serotec, Inc.) and a polyclonal antibody to the C-terminal 25 amino acids (aa) (data not shown) as previously described (3). The four proteins revealed by MCA406 (Fig. 2a, lanes 13 to 16; Fig. 2c, lanes 9 to 12) correspond to full-length (Pra; aa 1 to 635), C-terminally cleaved (Prb; aa 1 to 610), N-terminally cleaved (Na; aa 248 to 635), and N- and C-terminally cleaved (Nb; aa 248 to 610) proteins. Effects of mutagenesis at the C-terminal site were noted by an increase or decrease in intensity of bands corresponding to Prb and Nb, the products of C-terminal cleavage. Cleavage at the nonmutated site served as a control for expression of active protease. Since the bacterial mutation-expression system does not require substrate assembly or interaction with other viral components, results can be interpreted only in relation to processing by the protease. A similar technique has been used successfully to study mutations of the cleavage site of a genetically engineered HIV-1 protease substrate (18).

Several phenotypes resulted from mutational analysis at the C-terminal cleavage site: no cleavage, poor cleavage, enhanced cleavage, and wild-type cleavage (Fig. 2a). A summary of the C-terminal mutagenesis data (Fig. 3) reveals that substrate specificity at the C-terminal cleavage site localized to the P4-P1′ positions. At these positions, many of the substitutions resulted in the uncleaved phenotype, whereas amino acids could be freely substituted in the P5 and P2′-P4′ positions, indicating that little specificity is found at these positions of the cleavage site.

A frameshift mutant which changed all of the amino acid residues beyond the P2′ position demonstrated cleavage similar to the wild-type sequence (data not shown). To confirm that processing had occurred, this frameshift mutant was subcloned into pT7ICP35K (3) by replacing the *Pst*I-*Eco*RI fragment of the vector with the *Pst*I-*Eco*RI fragment of the frameshift and coexpressed with HSV N_0, the 247-aa catalytic domain of the protease. Processing of the frameshift mutant in which amino acids C terminal to the P2′ position were changed to THTWTLTRPAPPICSSLR was indisputable in this context (Fig. 2b). Cleavage of the frameshift mutant clearly demonstrated that amino acids C terminal to the P2′ position could be altered with no effect on processing. This result does contrast with in vitro peptide cleavage data, which showed that sequences beyond the P2′ position were required to obtain cleavage (6). However, peptide data may reflect a length dependence to induce the proper conformation for cleavage rather than a sequence requirement.

The mutagenesis data agree favorably with the cleavage site specificity predicted from the sequence homologies first identified by Gibson et al. (8). Alignment of C-terminal cleavage sites from five putative herpesvirus proteases (Fig. 4) shows

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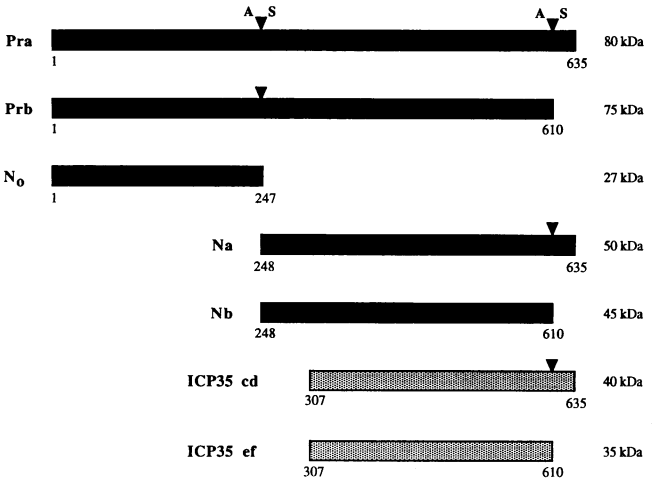

FIG. 1. Diagram of cleavage products of HSV-1 protease. Autocatalytic processing products of the protease (Pra, Prb, N_0, Na, and Nb) are shown in black. ICP35 cd and its cleavage product, ICP35 ef, are shaded. Numbering of amino acids is in relation to full-length protease, Pra.

conservative amino acids in the P4 and P2 positions, while identity is maintained in the P3, P1, and P1′ positions. Several mutations to nonconserved amino acids (Glu and Tyr at the P4 position; Ala and Phe at the P2 position) maintain wild-type processing, indicating that the cleavage site can accommodate a broader range of amino acids than would be predicted from sequence alignments. Nevertheless, the strict amino acid specificity at the P3, P1, and P1′ positions is maintained by allowing only very conservative changes at these positions.

After analyzing the data from the C-terminal mutagenesis, we introduced point mutations into the N-terminal cleavage site at P4-P1′. The purified PCR fragments containing N-terminal point mutations were digested with *Dra*I and *Nco*I, and the resulting 750-bp fragment was further gel purified prior to ligation into the unique *Nco*I and *Dra*I (created by *dut ung* mutagenesis) (17) sites of pT7635K2, a derivative of pT7635A (3), and pET-9d (Novagen). Results of mutagenesis at the N-terminal site were indicated by an increase or decrease in intensity of bands corresponding to Na and Nb, the products of N-terminal cleavage. Representative phenotypes resulting from these mutants are shown in Fig. 2c and summarized in Fig. 3. No enhanced mutations were apparent at the N-terminal cleavage site.

A similar relationship between conserved amino acids in the sequence alignments and allowed amino acid substitutions in the mutagenesis data was inherent at this site (Fig. 4). The principal distinction between the N- and C-terminal sites was the Tyr at the P4 position. Although mutagenesis was not exhaustive, the result of substituting the P4 Tyr with several amino acids that were active at this position of the C-terminal site revealed a difference in specificity between the two sites. The sequence alignment of other herpesvirus proteases argues a critical role for the P4 Tyr, since identity of this amino acid is maintained in all known herpesvirus proteases. The distinct specificities of the two sites could be explained by different secondary structures in the regions of processing which may serve to regulate the timing of their cleavage.

A notable result of the mutagenesis was the finding that other amino acids can substitute at the P1 and P1′ positions to give wild-type cleavage. The protease appears to possess a

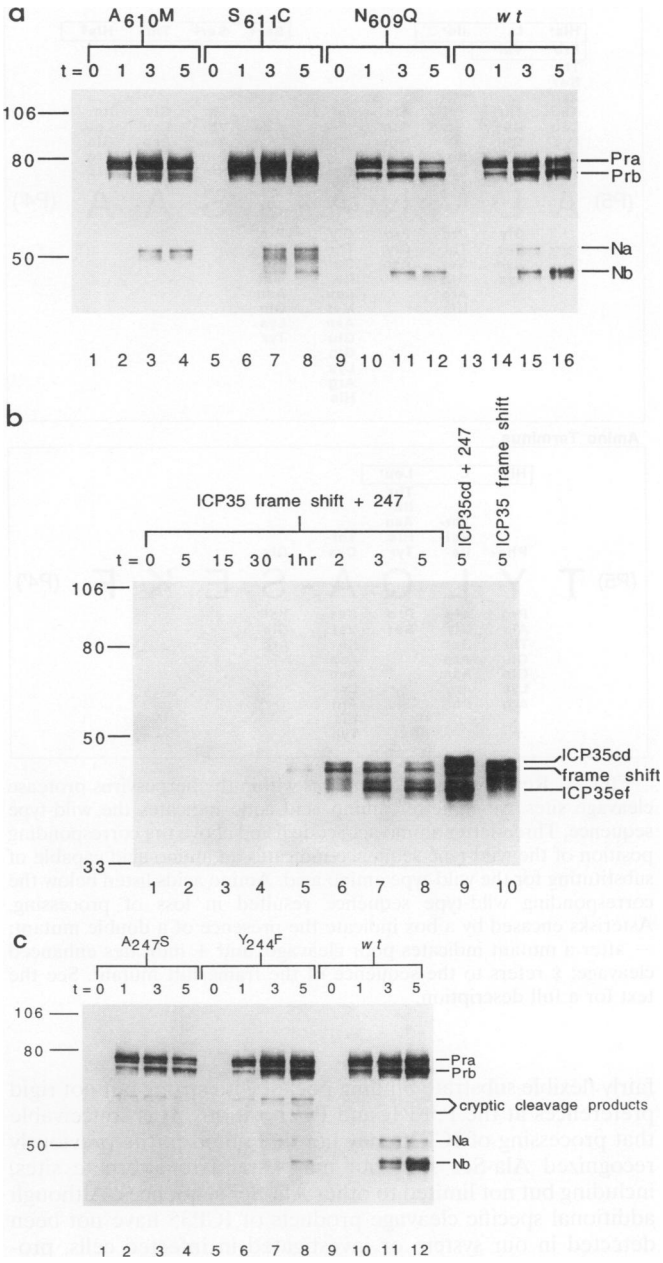

FIG. 2. The positions of full-length (Pra; aa 1 to 635), C-terminally cleaved (Prb; aa 1 to 610), N-terminally cleaved (Na; aa 248 to 635), and N- and C-terminally cleaved (Nb; aa 248 to 610) proteins are labeled at the right. Apparent molecular masses in kilodaltons are shown at the left. (a) C-terminal cleavage site mutants. Results show the time course of induction of uncleaved mutant A610M (lanes 1 to 4), poorly cleaved mutant S611C (lanes 5 to 8), enhanced-cleavage mutant N609Q (lanes 9 to 12), and wild-type (*wt*) full-length protease (lanes 13 to 16). (b) Frameshift mutant in ICP35. Results show analysis of a frameshift mutant coexpressed with HSV N_0, the active domain of HSV protease, at t = 0, 5, 15, and 30 min and 1, 2, 3, and 5 h (lanes 1 to 8), ICP35 cd coexpressed with HSV N_0 at 5 h (lane 9), and the frameshift mutant expressed without protease at 5 h (lane 10). The cleavage product is ICP35 ef for all species. (c) N-terminal cleavage site mutants. Results show analysis of mutant A247S showing no N-terminal cleavage (lanes 1 to 4), mutant Y244F showing poor cleavage (lanes 5 to 8), and wild-type (*wt*) full-length protease (lanes 9 to 12). The position of the cryptic cleavage site is labeled.

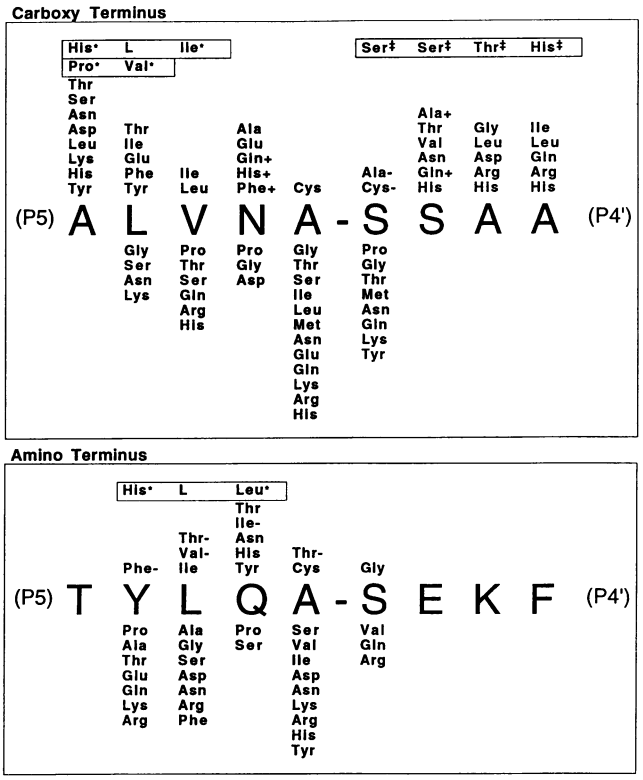

FIG. 3. Results of point mutations within the herpesvirus protease cleavage sites. Single-letter amino acid code indicates the wild-type sequence. Three-letter amino acid code listed above the corresponding position of the wild-type sequence indicates an amino acid capable of substituting for the wild-type amino acid. Amino acids listed below the corresponding wild-type sequence resulted in loss of processing. Asterisks encased by a box indicate the presence of a double mutant; − after a mutant indicates poor cleavage; and + indicates enhanced cleavage; ‡ refers to the sequence of the frameshift mutant. See the text for a full description.

fairly flexible substrate binding pocket with strong but not rigid preferences at the P3, P1, and P1′ positions. It is conceivable that processing of ICP35 may not be limited to the previously recognized Ala-Ser sites but may extend to alternate sites, including but not limited to other Ala-Ser sequences. Although additional specific cleavage products of ICP35 have not been detected in our system or investigated in infected cells, processing of ICP35 to smaller products prior to DNA packaging is an attractive hypothesis. Extensive cleavage of the T4 scaffold protein by the T4 phage protease has been reported to coincide with DNA packaging (10). In a similar fashion, further cleavage of the ICP35 at alternate cleavage sites is a conceivable means by which ICP35 is removed from the B capsids during maturation to virions.

A third, cryptic cleavage described by Weinheimer et al. (19) which gave rise to proteins of 60 and 65 kDa was observed in the N-terminal mutants that had decreased or no cleavage (Fig. 2c). The appearance of two cleavage products with similar apparent molecular weight is indicative of C-terminal cleavage which results in loss of 25 aa. In addition, the faster-migrating cleavage product is not immunoreactive with the C-terminal polyclonal antibody (data not shown), but both cleavage products are immunoreactive with MCA406. Therefore, this processing site is believed to lie within the N-terminal

C-terminal Cleavage Site

	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′
HSV	Gly	Ala	Leu	**Val**	Asn	**Ala**	**Ser**	Ser	Ala	Ala	His	Val
VZV	Val	Asn	Ala	**Val**	Glu	**Ala**	**Ser**	Ser	Lys	Ala	Pro	Leu
CMV	Ala	Gly	Val	**Val**	Asn	**Ala**	**Ser**	Cys	Arg	Leu	Ala	Thr
EBV	Lys	Lys	Leu	**Val**	Gln	**Ala**	**Ser**	Ala	Ser	Gly	Val	Ala
ILTV	Arg	Glu	Thr	**Val**	Asp	**Ala**	**Ser**	Met	Pro	Lys	Arg	Leu

N-terminal Cleavage Site

HSV	His	Thr	**Tyr**	Leu	Gln	**Ala**	**Ser**	Glu	Lys	Phe	Lys	Met
VZV	His	Val	**Tyr**	Leu	Gln	**Ala**	**Ser**	Thr	Gly	Tyr	Gly	Leu
CMV	Glu	Ser	**Tyr**	Val	Lys	**Ala**	**Ser**	Val	Ser	Pro	Glu	Ala
EBV	Glu	Ser	**Tyr**	Leu	Lys	**Ala**	**Ser**	Asp	Ala	Pro	Asp	Leu
ILTV	Pro	Lys	**Tyr**	Leu	Gln	**Ala**	**Asn**	Glu	Val	Ile	Thr	Ile

FIG. 4. Sequence alignment of autoprocessing sites of herpesvirus proteases. Identity is maintained for the amino acids in boldface type. Cleavage is between the P1 and P1′ positions. VZV, varicella-zoster virus; CMV, cytomegalovirus strain AD169; EBV, Epstein-Barr virus; ILTV, infectious laryngotracheitis virus.

domain of the protease. In light of the mutagenesis results indicating the importance of the P3, P1, and P1′ positions, we predict the location of the cryptic cleavage site to be Leu-85–Ile-86–Ala-87/Cys-88. Cleavage at this sequence would result in products of the appropriate size and immunogenicity. A similar autoprocessing site has been identified within the N-terminal domain of the cytomegalovirus protease at the sequence Val-141–Glu-142–Ala-143/Ala-144 and was postulated to have a role in regulation of protease activity (1).

Several of the substitutions to the P2 or P2′ position imparted enhanced cleavage to the C-terminal site in *E. coli*. This phenotype was unanticipated and could not have been predicted by sequence alignment. If the mutagenesis data are an indication of viral enzyme specificity, then the optimal sequence for cleavage at this site has not been selected for by the virus. This suboptimal cleavage sequence may be important for the timing of cleavage during assembly or for interactions of the scaffolding protein with other viral proteins similar to those observed between the scaffold and capsid of phage P22 (7). Perhaps substrate cleavage is a well-orchestrated event in which cleavage by the protease is processive, and then selection of an optimal cleavage site may not be critical to assembly. The substrate could assemble in such a way that the cleavage sites are lined up side by side, eliminating the dissociation of the enzyme from the substrate similarly to enzymes which act processively at the surface of membranes (4). The rate of this type of burst proteolysis would be very fast, and the cleavage site would not need to be optimal. Only replacement of the C-terminal cleavage site of the viral protease with the altered sequences can offer insight into the selection of the substrate sequence.

Studies currently under way to elucidate the mechanism of action and investigate the timing of cleavage, along with the cleavage site mutagenesis presented here, will aid in the understanding of the HSV-1 protease and its role in the assembly process.

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