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Biochemistry

Poliovirus thiol proteinase 3C can utilize a serine nucleophile within the putative catalytic triad

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ABSTRACT **The picornavirus 3C proteinases are substrate-specific thiol proteases that have been shown by secondary structure predictions and protein modeling studies to be similar to the trypsin-like serine proteases. We have examined several mutations of the 3C proteinase at putative active site and non-active site residues. The effect on 3C-mediated protein processing supports the model of serine protease similarity. In particular, we have shown that 3C can utilize a serine at position 147, which is predicted to supply the nucleophilic residue of the catalytic triad. We suggest that picornavirus 3C proteinases may represent a class of enzymes that have maintained the catalytic mechanism characteristic of a proposed enzyme ancestral to the highly divergent class of serine proteases.**

Positive-stranded RNA viruses employ the use of genomically encoded proteolytic enzymes to mediate all or part of the protein processing events necessary for the production of functional virus proteins from larger precursor polyproteins (reviewed in ref. 1). For poliovirus type 1 (PV1), the prototype member of the family Picornaviridae, the single-stranded message-sense genomic RNA encodes a large polyprotein that, upon synthesis in the infected host cell, is processed co- and posttranslationally by three proteolytic activities. Two of the proteolytic enzymes are the genomically encoded 2A and 3C proteinases, and the third proteolytic activity has yet to be ascribed to any virus or host cell enzyme (Fig. 1). Protease inhibitor studies have identified the 3C proteinase, the major viral proteinase of picornaviruses, as a cysteine protease, although serine protease inhibitors do affect catalytic activity (reviewed in ref. 2). However, activity of purified 3C proteinase has also been shown to be resistant to the highly specific thiol protease inhibitor E-64, suggesting it may have properties distinct from those of the papain-like family of cysteine proteases (3). Recent protein modeling studies have shown a clear similarity among the picornavirus 3C proteinases and the trypsin-like serine proteases (4, 5). The two independently derived models of 3C structure predict a 12-strand β-sheet secondary conformation similar to the bilobular structure of the trypsin-like serine proteases. One model (4) predicts three residues of the PV1 3C proteinase, H40, D85, and C147, to be analogous to the trypsin-like serine protease catalytic triad of H57, D102, and S147 (chymotrypsin numbering system). These residues are highly conserved among picornaviruses. An alternative model (5) suggests the use of the less conserved E71 residue of 3C to function as the serine protease D102 analog. The lack of an experimentally determined structure of a picornavirus 3C proteinase prevents direct verification of these models of 3C structure. Both models strongly suggest the divergent evolution of the trypsin-like serine proteases and several positive-strand RNA virus proteinases.

The PV1 3C proteinase is a highly specific enzyme that exhibits efficient cleavage activity when expressed as part of a normal polyprotein in an *in vitro* translation system (6). We have utilized *in vitro* expression to test the model of picornavirus 3C/serine protease homology by mutagenesis of putative catalytic triad and substrate-binding amino acid residues of PV1 3C. Our data provide genetic and biochemical evidence that support the predictions of an active site geometry for picornavirus 3C proteinases that resembles that of the trypsin-like serine proteases. We discuss the implications of our findings in the context of the proposed evolution of serine proteases from a putative ancestral cysteine-utilizing proteolytic enzyme.

METHODS

Enzymes and Lysates. Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. Rabbit reticulocyte lysate and RNasin were purchased from Promega. Bacteriophage T7 RNA polymerase and Bacteriophage T4 polynucleotide kinase were purchased from Pharmacia LKB Biotechnology.

Construction of Mutant cDNA. Mutations engineered into the 3C coding region of the PV1 cDNA are diagramed in Fig. 1. A subgenomic cDNA clone of PV1, pT7-PV1-3CH, containing the entire 3C and a portion of the 3D coding regions ending at nucleotide (nt) 6516 of the chimeric PV1/coxsackievirus B3 cDNA clone pT7-PC3D (7) was constructed by ligation of the 1.3-kilobase-pair (kbp) *Sca* I–*Hin*dIII fragment into the bacteriophage T7/SP6 RNA polymerase transcription vector pGEM-1 (Promega) at the *Sma* I and *Hin*dIII sites of the multiple cloning site. Purified pT7-PV1-3CH plasmid DNA was then used as a template for site-directed mutagenesis of 3C according to the protocol of double-stranded plasmid mutagenesis (8). Twenty-base synthetic oligonucleotides containing one- or two-base changes were used to create a C → A transversion at nt 5555 (H40Y), an A → G transition at nt 5556 (H40R), an A → C transversion at nt 5691 (D85A and D85E), a C → A transversion at nt 5692 (D85E), or a T → G transversion and G → C transversion at nt 5876 and nt 5877 (C147A). Positive mutant cDNA clones were engineered into the PV1 cDNA transcription vector pT7-1 (P1:ΔNS) (9) by replacing the *Apa* I and *Nde* I fragment from nt 5340 to nt 6426 or the *Bgl* II (nt 5601) to *Nde* I fragment of pT7-1 (P1:ΔNS) with the equivalent fragment from the mutant cDNA. Replacement was confirmed by dideoxynucleotide sequencing of purified plasmid DNA. The preparation of PV1-infected or uninfected HeLa S3 cell extracts, *in vitro* transcription of wild-type and mutant PV1 cDNA clones, and translation of RNA *in vitro* have been described (7).

RESULTS AND DISCUSSION

The conserved C147 residue of picornavirus 3C proteinases is predicted in all previous amino acid sequence and struc-

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Abbreviations: PV1, poliovirus type 1; nt, nucleotide(s).

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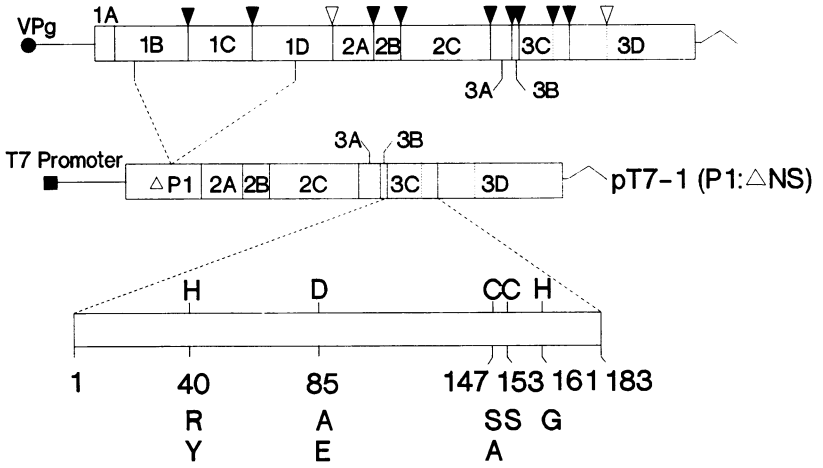

FIG. 1. Genomic structure and polyprotein processing map of PV1. The 247-kDa polyprotein coding region of the PV1 genomic RNA is shown flanked by the VPg (3B) protein-linked 743-base 5′ nontranslated region and the 71-base 3′ nontranslated region and poly(A) tract. Map positions of the encoded structural P1 and the nonstructural P2 and P3 region precursor proteins are indicated. Solid vertical lines indicate sites of cleavage in the polyprotein. Dashed vertical lines indicate infrequent cleavage. Solid triangles (▼) above the polyprotein indicate sites of 3C-mediated cleavage. Open triangles (▽) indicate sites cleaved by 2A. A third proteolytic activity not yet ascribed to any viral or host cell protein mediates the cleavage between the 1A and 1B proteins. Diagramed below the genome is the structure of the cDNA clone pT7-1 (P1:ΔNS) (9) used in these studies. The cDNA contains an in-frame deletion of the P1 sequences that increases the translational efficiency of transcribed RNA *in vitro*. An exploded view of the 3C coding region is shown below with the sites of amino acid substitution.

tural modeling studies (4, 5, 10) to provide the nucleophile necessary for peptide bond cleavage and is thought to function analogously to the active site serine residue of the trypsin-like proteases. The processing of *in vitro* synthesized polyprotein by wild-type 3C activity is shown in Fig. 2*A*, lane 3. The initial polyprotein cleavage products P2 and P3 are evident, as are the intermediate and fully processed counterparts 2BC, 2C, and 2A and 3BCD (which migrates between P3 and 3CD and is not designated), 3CD, 3D, 3C, 3AB, and 3A, respectively. The 2B protein contains only one methionine residue and comigrates with the 3A protein in this gel system. The 3B protein does not contain methionine and is not visualized in this analysis. Examination of the effect on processing by mutation of 3C C147 to serine (C147S) or alanine (C147A) shows that the C147S mutation does not abrogate 3C activity on nonstructural polypeptides (Fig. 2*A*, lane 9). All of the above mentioned proteins are produced by the mutant 3C C147S proteolytic activity from *in vitro* synthesized polyprotein. However, in comparison to wild-type processing, there is an alteration in the relative amounts of authentic protein products, as illustrated by the change in the ratio of P3 precursor to 3CD and 3AB and the increased amount of 2BC protein to 2C. The presence of high molecular weight precursor proteins suggests that 3C C147S is not capable of rapid polyprotein processing *in vitro*. The proteins labeled 3ABC′, 3C′, and 3D′ are derived from 2A-mediated cleavage of P3 and 3CD within the 3D sequences (refer to Fig. 1). The increased activity of 2A on P3 proteins synthesized from RNA encoding 3C mutations has been reported (7). No processing of the P1 structural precursor is observed following incubation with *in vitro* generated 3C containing the C147S lesion (Fig. 2*B*, lane 9). Processing of the P1 precursor has been shown to be more sensitive than the processing of nonstructural P2 and P3 proteins to mutations that alter 3C activity (6, 11). Such an effect may be due to the constraints of cleavage in trans versus cleavage in cis, since the processing of nonstructural proteins occurs through inter- and intramolecular pathways, whereas P1 is processed in an intermolecular (i.e., trans) manner (reviewed in ref. 2).

The activity of 3C C147S is in striking contrast to the processing ability of the 3C C147A enzyme, which is unable to produce authentic nonstructural proteins (Fig. 2*A*, lane 8). A major aberrant cleavage product that comigrates with 3CD (72 kDa) can be seen in this translation. Altered conformation of 3C-containing polyprotein caused by mutation can be examined by incubation of uncleaved polyprotein with wild-type 3C activity supplied from cytoplasmic extracts of PV1-infected HeLa cells. All of the polyproteins produced from wild-type and mutant 3C-encoding RNA did function as substrates for wild-type 3C activity supplied from such an extract. Treatment of protein products derived from *in vitro* translation of RNA containing the C147A mutation with a PV1-infected cell extract generates authentic nonstructural proteins as well as an aberrant cleavage product (data not shown). The enzymatic activity responsible for this aberrant cleavage event has not been identified. Conversion of a nonconserved cysteine at position 153 of 3C (C153S) to serine has no detectable effect on protein processing in any assay described here (Fig. 2*A*, lane 10 and Fig. 2*B*, lane 10). In addition, transfection of a full-length poliovirus cDNA containing the C153S lesion into cultured primate cells produces a virus with growth characteristics indistinguishable from those of wild-type poliovirus (V. H. Johnson and B.L.S., unpublished results). These data suggest that the presence of a nucleophilic amino acid residue at position 147 in the 3C proteinase is required for catalytic activity, supporting the proposed role of C147 in forming part of the catalytic center.

The persistence of high molecular weight precursor proteins during mutant 3C C147S-mediated processing suggests a defect in the rate of polyprotein cleavage (refer to Fig. 2*A*, lane 9). To investigate such a possibility, a kinetic analysis of protein synthesis and processing was undertaken. Processing of the nonstructural proteins by normal 3C proteinase is extremely rapid (Fig. 3*A*). In contrast, the processing mediated by the 3C C147S mutant is slowed considerably (Fig. 3*B*). Particularly noticeable are the high molecular weight proteins visible throughout the ongoing translation and processing, which are seen only at very early times during normal 3C-mediated processing. All of the high molecular weight proteins can be immunoprecipitated with antiserum directed against the 2C and 3D proteins, and the largest may represent a primary translation product or a complete nonstructural P2–P3 precursor. Processing by the C147S mutant 3C activity is delayed, although approximately equal levels of protein synthesis occur in each reaction, as judged by the synthesis of ΔP1, which is not processed by 2A or 3C activity

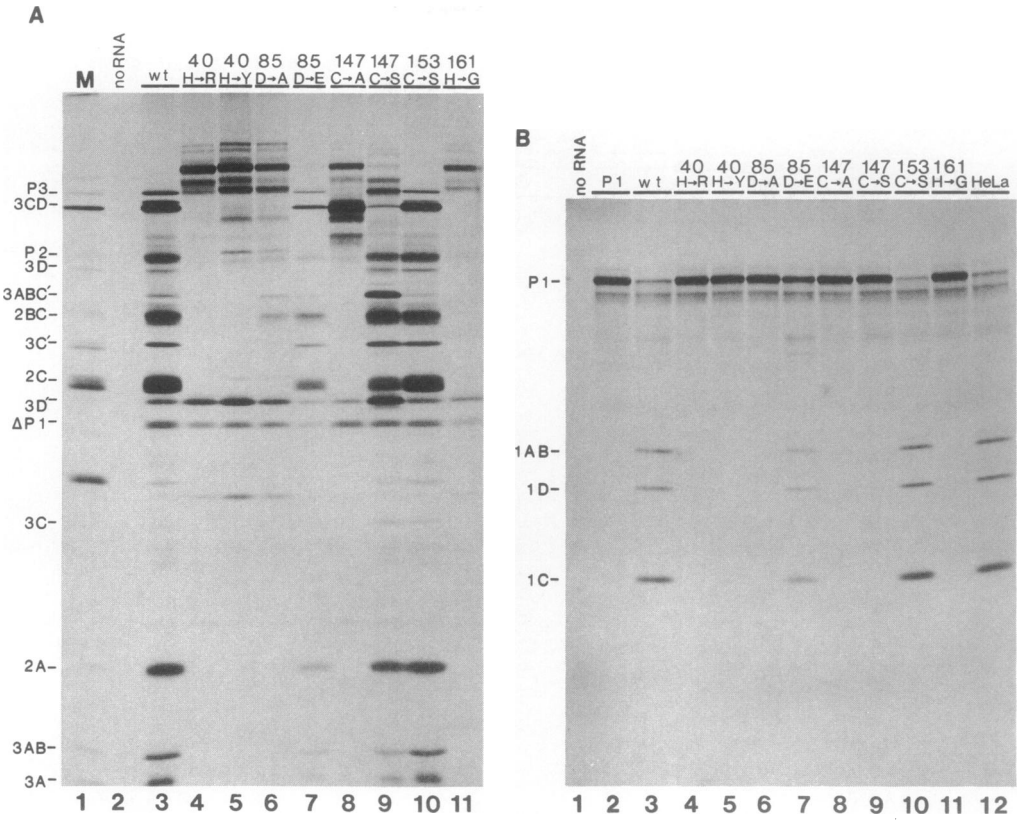

FIG. 2. Processing profiles of ^{35}S-labeled protein synthesized from *in vitro* translated PV1 RNA transcribed from pT7-1 (P1:ΔNS) containing wild-type (wt) and mutant 3C coding regions. Transcription *in vitro*, purification of RNA, and translation in the rabbit reticulocyte lysate/HeLa cell translation system was carried out as described (7). Reaction mixtures were incubated for 2 hr, stopped by the addition of RNase and cycloheximide at 200 μg/ml and 5 μg/ml, respectively, and allowed to process further for 1 hr. Reaction mixtures were then diluted 1:10 in Laemmli sample buffer, run on 12.5% SDS/polyacrylamide gels, and fluorographed with 2,5-diphenyloxazole; the resultant fluorograms are shown. (*A*) Lane 1, marker lane of proteins from PV1-infected HeLa cells ^{35}S-labeled 6 hr after infection. Only polypeptides identical to those synthesized in translation of pT7-1 (P1:ΔNS) are identified. The truncated P1 product (ΔP1) that results from translation of pT7-1 (P1:ΔNS) transcribed RNA and migrates as a doublet is also indicated. Lane 2, a no-RNA control translation reaction. Lanes 3–11, protein products after translation and processing of RNA transcribed from pT7-1 (P1:ΔNS) cDNA clones containing wild-type or mutant 3C sequences. (*B*) P1 processing ability of wild-type and mutant 3C activities. The ^{35}S-labeled P1 precursor was generated *in vitro* by transcription of the subgenomic cDNA clone PT7-P1 (9) and translation of the transcribed RNA in the rabbit reticulocyte/HeLa cell system for 2 hr. Parallel translation reactions programed with wild-type and mutant pT7-1 (P1:ΔNS) transcribed RNA were performed without [^{35}S]methionine, terminated as above, mixed 1:1 with the P1 translation reaction, and incubated for 1 hr at 30°C. Lane 1, a no-RNA control translation. Lane 2, P1 protein generated *in vitro*. Lanes 3–11, P1 protein mixed with unlabeled translation reaction mixtures identical to those in lanes 3–11 in *A*. Lane 12, P1 protein generated *in vitro* and incubated with an extract of PV1-infected HeLa cells.

and is stable in the translation reaction. Densitometric analysis of the processing of P2 after cycloheximide inhibition of translation (refer to the last four lanes in Fig. 3*A* and Fig. 3*B*) shows that the precursor–product relationships of P2 → 2BC → 2C are not disturbed (data not shown), indicating that a normal cascade of proteolytic events is carried out by the mutant 3C enzyme. The half-life of P2 protein after cycloheximide treatment is ≈65 min in the presence of wild-type 3C activity and ≈57 min in the presence of C147S proteinase activity. The significance of this observation is not clear. As the majority of P2 protein has been processed by wild-type 3C activity before the inhibition of translation, this observation may suggest a secondary method of P2 processing. Interestingly, the half-life of P3 protein containing wild-type 3C after cycloheximide treatment is ≈52 min, whereas the P3 protein containing the 3C C147S mutation is not significantly processed after the inhibition of translation. The production of 3CD in the uninhibited 3C C147S translation may indicate that processing of the mutant P3 may be concentration-dependent and may occur in trans.

The use of protease inhibitors in the classification of the proteolytic enzymes of picornaviruses has often given equivocal results due to their reactivity with substrates as well as with the proteinases (reviewed in ref. 2). The low activity and the high enzyme-to-substrate ratios needed to analyze trans-acting 3C proteolytic activity on peptides or on nonstructural proteins restrict the use of protease inhibitors in a posttranslational cleavage assay (3, 12). It can be concluded from Fig. 3 that the majority of nonstructural protein processing normally occurs immediately upon synthesis of the polyprotein. To test the effect of protease inhibitors on 3C activity, we have chosen an assay in which inhibitor is present at the time of 3C synthesis (Table 1). All inhibitors described in Table 1 did show some effect on 3C-mediated processing, and *N*-ethylmaleimide, iodoacetamide, and Zn^{2+} exhibited some inhibitory effect on translation. A differential sensitivity of C147S 3C-mediated processing compared to wild-type 3C activity is seen in translation reactions treated with Zn^{2+} and phenylmethanesulfonyl fluoride. Although the decreased sensitivity of 3C C147S activity to the presence of thiol-reactive Zn^{2+} (13) and the increased sensitivity of mutant 3C activity to the presence of phenylmethanesulfonyl fluoride provide only suggestive evidence for the role of C147 in catalysis, it is consistent with such an interpretation.

The H40 residue of PV1 3C, which is predicted to lie outside any secondary structural elements, is proposed to function as a catalytic base analogous to the invariant histidine residue present in the catalytic triad of serine and

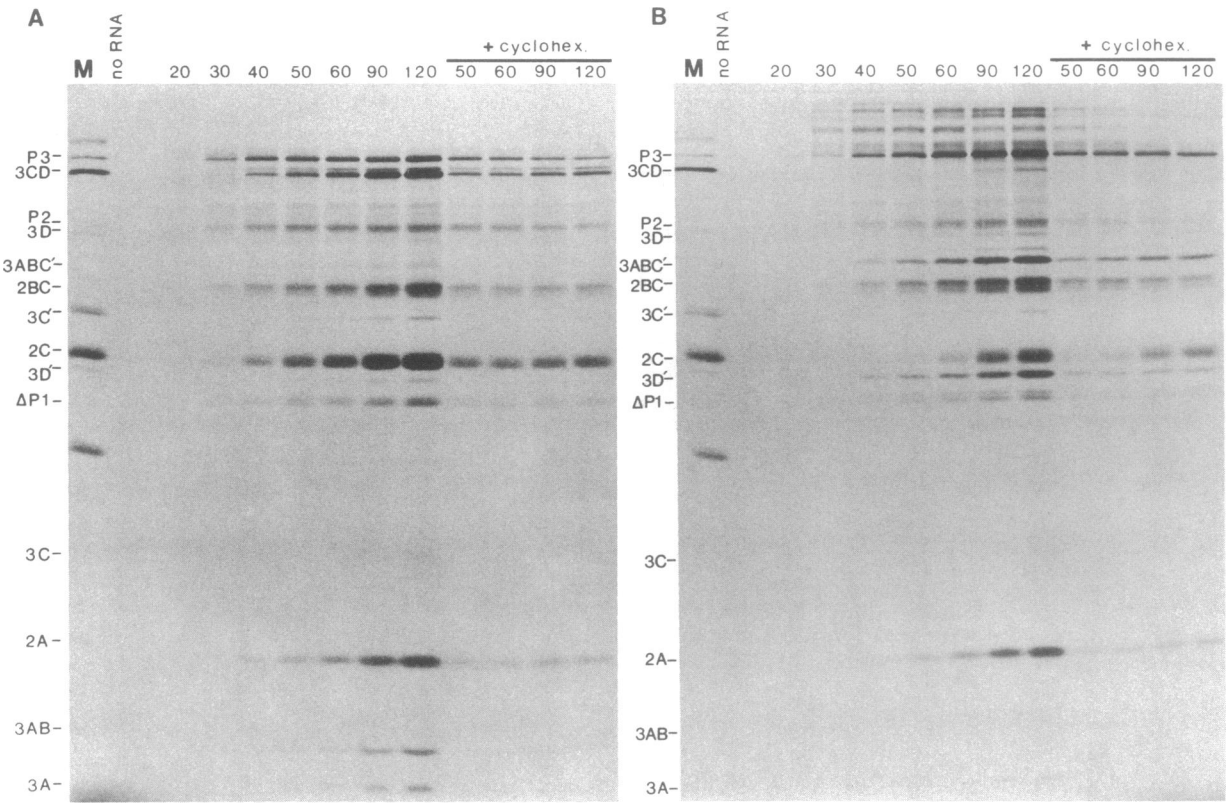

FIG. 3. Kinetic analysis of the synthesis and processing of poliovirus polyprotein by wild-type and C147S mutant 3C activity *in vitro*. Translation reactions performed as in Fig. 2 were programed with RNA transcribed from pT7-1 (P1:ΔNS) encoding wild-type or C147S mutant 3C. Aliquots were removed from the reaction mixture at the times indicated, treated with RNase A at a final concentration of 1 mg/ml for 1 min, and analyzed as in Fig. 2. Alternatively, a large aliquot was removed from the ongoing translation reaction mixture at 40 min and cycloheximide (cyclohex.) was added to 5 μg/ml. Aliquots were removed from this inhibited reaction mixture at the times indicated and treated as above. (*A*) Ongoing protein synthesis and processing profile of a translation reaction programed with pT7-1 (P1:ΔNS) transcribed RNA encoding wild-type 3C protein. (*B*) Identical translation reaction programed with RNA transcribed from pT7-1 (P1:ΔNS) encoding the C147S mutant 3C protein. The first two lanes in each panel contain ^{35}S-labeled proteins from PV1-infected HeLa cells as described in the legend to Fig. 2 and no-RNA control translation reactions, respectively.

cysteine proteases (4, 5). The catalytic H57 of trypsin has been shown to function efficiently when stabilized in the tautomeric form, which allows the transfer of the S195 hydroxyl proton (14, 15). A similar role for the H40 of PV1 3C can be deduced from the cleavage profile of H40R or H40Y mutant enzymes shown in Fig. 2*A*, lanes 4 and 5. Both mutations at position 40 lead to severely debilitated cleavage of nonstructural proteins and elimination of P1 structural precursor processing (Fig. 2*B*, lanes 4 and 5). The extremely low proteolytic activities of 3C H40R and 3C H40Y suggest that neither the polarity nor the aromatic structure of the substituted amino acid side chains can functionally replace the histidine residue at that position. The conserved H161 residue of 3C has been proposed to be involved in formation of the substrate binding pocket. Although the exact placement of this residue varies between the two models (4, 5) of 3C structure, both predict H161 to lie within a β-sheet region. Previous analysis of 3C-mediated processing using precursor proteins expressed in *E. coli* has indicated that mutation of H161 eliminates the activity of 3C (16). The H161G mutant 3C exhibits only very low activity on nonstructural precursors (Fig. 2*A*, lane 11) and no activity on the P1 structural precursor polypeptide (Fig. 2*B*, lane 11) in the polyprotein cleavage assay. The effects of mutation at H40 and H161 support the proposed roles of these residues in formation of structures important for efficient catalytic activity of 3C.

Table 1. Effect of protease inhibitors on polyprotein processing by wild-type and C147S 3C proteinases

Inhibitor	Conc., mM	Relative inhibition	
		Wild type	C147S
Iodoacetamide	1	1.3	1.5
N-Ethylmaleimide	5	1.9	2.5
Zn^{2+}	5	8.2	3.9
PMSF	5	1.3	2.7

Translation reactions were performed as in Fig. 2. After 20 min, aliquots were removed and adjusted to the final concentrations of protease inhibitors indicated or received inhibitor-free solvent. Prior to treatment with Zn^{2+} (as $ZnSO_4$), reactions were stabilized with Hepes at 5 mM (pH 7.4). Reactions were continued for 40 additional min, then terminated by the addition of RNase A to 200 μg/ml, and allowed to incubate a further 20 min. The resultant fluorograms were scanned by laser densitometry and the peak ratios of 2BC:2C proteins were determined. The relative changes in 2BC:2C between the untreated and treated reaction mixtures at the given concentrations of inhibitor are shown as a measure of relative inhibition. PMSF, phenylmethanesulfonyl fluoride.

The highest degree of variability tolerated at any position in the catalytic triad of serine proteases is found at the position occupied by aspartic acid in trypsin and subtilisin. The proposed role of the carboxylic acid moiety is to maintain the proper orientation of the histidine residue, facilitating its participation in catalysis (14, 15). The effect of mutation of the D85 residue of PV1 3C, the amino acid suggested in one study (4) to function analogously to D102 in trypsin, is consistent with a role of D85 in catalysis (Fig. 2*A*, lanes 6 and 7). As shown in Fig. 2*A*, lane 7, the D85E mutation in 3C does not prevent the processing of nonstructural precursor polypeptides. In addition, this mutant enzyme also maintains near wild-type cleavage function on P1 structural precursors pro-

vided in trans (refer to Fig. 2B, lane 7). The ability of the 3C D85E enzyme to function would suggest that, if D85 were indeed a participant in catalysis, the active site of 3C is sufficiently flexible to allow the γ-carboxylate group of E85 to perform the identical function as the β-carboxylate of the wild-type D85. The limited activity of the D85A enzyme indicates that the carboxylate moiety is necessary for efficient catalytic activity. Precedence for the observed limited activity of the D85A mutant can be found in mutational analyses of the serine proteases trypsin (14, 15) and subtilisin (17), which maintain some activity when this residue is altered, and in the primary structure of the papain-like thiol proteases, which do not strictly conserve the N175 residue, analogous to the D102 of trypsin, in the catalytic triad (18). Others have proposed that the PV1 D85 residue participates in substrate recognition rather than in catalysis (5). Site-directed mutagenesis of D85 and E71 provides some support for the proposed use of E71 rather than D85 in the 3C catalytic triad (12, 19). However, a recent report, in which a polyprotein cleavage assay similar to that presented here was employed, showed that mutation of neither E71 nor D85 could eliminate nonstructural protein cleavage activity (19). Final identification of the third member of the catalytic triad as D85 or E71 will require structural analysis and a more extensive genetic analysis.

On the basis of our data presented here, we conclude that both models of 3C proteinase secondary structure are generally correct in their identification of H40 and C147 as potential catalytic residues. Previous mutational analyses of picornavirus 3C proteinases expressed in *E. coli* have not detected activity of a proteinase bearing a putative active site C → S mutation (12, 16, 20). The readily detectable activity of such a 3C mutant in the polyprotein cleavage assay described in this study underscores the importance of temporal factors, higher order structural interactions, and intra- versus intermolecular processing in determining the specificity and the activity of this unique class of enzymes (7, 9, 21, 22). The failure to detect activity in previous studies can be attributed to the elimination of one or more of these factors in the assay systems used. The kinetic analysis of protein processing presented in Fig. 3 shows that the majority of nonstructural protein processing occurs rapidly upon synthesis of the polyprotein. The relatively low rate of wild-type 3C-mediated processing that occurs after cycloheximide-mediated inhibition of translation suggests that nonstructural protein processing is carried out in a predominantly cis-like fashion, possibly in a complex formed by the nascent polyprotein. Alternatively, efficient processing of nonstructural proteins may be linked to active translation. The inefficient activity of exogenously added 3C on nonstructural proteins supports this interpretation (6, 7). These observations may explain the inability to detect nonstructural cleavage activity of mutant 3C C147S and 3C H40Y proteins synthesized in or purified from *E. coli* (12, 16, 20), whereas activity was observed when these same proteinase mutants were expressed in the context of a nascent polyprotein.

A reciprocal mutation, in which the active site serine of rat trypsin (23) and the putative active site serine of the sindbis virus autoprotease (24) were converted to cysteine, did not abolish activity of these enzymes. The tobacco etch virus 49-kDa proteinase, a potyvirus enzyme that also shows significant similarity to the serine proteases (4), exhibits a very low level of activity upon conversion of its putative active site cysteine to serine (25). Conversion of the active site serine residue of subtilisin to alanine did not completely eliminate activity (17). Tolerance of more drastic amino acid substitutions would suggest that an active site cysteine sulfhydryl and a serine hydroxyl reactive group might be interchangeable within the 3C proteinase.

The serine proteases are a diverse class of proteolytic enzymes that maintain a consistent tertiary structure and active site geometry throughout nature. It has been suggested that the variant codon usage for serine (UCN, AGY) within the conserved active site amino acids of serine proteases indicates divergent lines of descent of modern serine proteases from an ancestral enzyme bearing a cysteine nucleophile utilizing the codon set UGY, which links the serine codon sets by a single base change (26). Although alternate phylogenetic classifications of serine enzymes differ in some instances, the discovery that viral thiol proteases are related to the trypsin-like serine proteases (4, 5) and the demonstration in this study and others (24, 25) of the functional exchange of cysteine and serine in related viral proteinases provide strong evidence that such an intermediate enzyme could have existed and that the proteinases of positive-strand RNA viruses and the trypsin-like serine proteases may represent a divergent group of homologous proteolytic enzymes.

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