

Autocatalytic Processing of the Potyvirus Helper Component Proteinase in *Escherichia coli* and In Vitro

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The virus-encoded proteins of tobacco etch virus (TEV), a plant potyvirus, arise by proteolytic processing of a large polyprotein precursor. The TEV genome codes for two proteinases, a 49-kilodalton proteinase and helper component proteinase (HC-Pro), which cleave the polyprotein at specific sites. The only known cleavage event catalyzed by HC-Pro occurs at the HC-Pro carboxyl terminus. The proteolytic activity of HC-Pro was analyzed by expression of the enzyme in bacterial and cell-free systems. The carboxyl-terminal domain of HC-Pro exhibited proteolytic activity in *Escherichia coli* with a processing half-time of approximately 100 s. The processing kinetics of HC-Pro expressed in vitro by cell-free transcription and translation was variable, depending on the presence or absence of TEV polypeptide sequences at the amino terminus of the proteolytic domain. Cleavage of the HC-Pro carboxyl terminus appeared to proceed exclusively by an autocatalytic mechanism; the proteinase synthesized in vitro exhibited little or no proteolytic activity when reacted with the HC-Pro cleavage site in *trans* or bimolecular reactions.

Several groups of animal and plant RNA viruses employ proteolytic processing as a means to produce their mature proteins. These viruses encode large polyprotein precursors that are cleaved by proteolytic reactions at specific sites (20, 29). In general, the proteinases responsible for these reactions are virus encoded and exhibit specificities that are restricted to their respective polyprotein substrates. The proteolytic processing mechanisms of the picornaviruses, comoviruses, and potyviruses have been examined in detail (12, 20, 29). The potyviruses and (at least some) picornaviruses encode two or more proteinases (7, 10, 16, 23, 26), while the comoviruses encode only one (28). These viruses share a number of common features, including a viral protein (VPg) covalently linked to the 5' terminus of genomic RNA, a genetically derived poly(A) tail at the genome 3' terminus, and a conserved organization of nonstructural proteins within the polyprotein (15).

Among the potyviruses, tobacco etch virus (TEV) is one of the best characterized at the molecular level. The TEV genome of 9,496 nucleotides codes for at least eight mature gene products, each of which arises by proteolytic processing (1, 10). This mode of genome expression has been demonstrated also for other potyviruses, including tobacco vein mottling virus (11, 19), papaya ringspot virus (31), and soybean mosaic virus (27). Most of the TEV polyprotein cleavage events occur at Gln-Gly or Gln-Ser dipeptides and are catalyzed by a 49-kilodalton (kDa) proteinase (6, 9, 10). This enzyme autoexcises from the polyprotein precursor by a monomolecular mechanism (9) and cleaves at additional sites in bimolecular or *trans*-catalytic reactions (6, 10). Since the 49-kDa proteinase exhibits specificity toward a subset of Gln-Gly/Ser positions in the TEV polyprotein, the recognition signals within the polyprotein have been studied. A heptapeptide sequence that contains four conserved residues is found surrounding each 49-kDa proteinase-mediated cleavage site and is required for optimal substrate recognition (8, 13). Each site processed by the 49-kDa proteinase resides within the carboxyl-terminal two-thirds of the polyprotein.

A second TEV-encoded proteinase has been identified recently by using a cell-free expression system (7). This enzyme, termed helper component proteinase (HC-Pro), functions to excise an 87-kDa segment from the amino terminus of the polyprotein. Deletion and point mutational analyses indicated that the proteolytic moiety of HC-Pro resides within a 20-kDa domain at the carboxyl terminus of the 87-kDa protein; HC-Pro thus cleaves its own carboxyl terminus. Cleavage in vitro occurs between a Gly-Gly dipeptide. The 87-kDa protein is processed further in vivo to products of approximately 31 and 56 kDa, the latter being HC-Pro. HC-Pro also has been proposed to function as the aphid transmission helper component (hence the HC in HC-Pro) which is required for aphid-mediated, plant-to-plant transmission of TEV in nature (24).

Expression of active HC-Pro in *Escherichia coli*. The proteolytic activity of HC-Pro has been demonstrated by using cell-free expression strategies involving translation of defined synthetic RNA transcripts. To further study HC-Pro and its activity, we expressed the proteolytic domain in *E. coli*. DNA representing TEV nucleotides 1435 to 2681 was inserted into the nonfusion expression vector pKK233-2 (3). This construct, termed pKK1427, codes for a 47-kDa protein consisting of the carboxyl-terminal 37 kDa of HC-Pro (containing the proteolytic domain) and a 10-kDa fragment of the adjacent protein (Fig. 1). Additionally, the proteinase-inactivating mutation termed 33-20, resulting in a substitution of Pro-Gly-Arg for Cys-Tyr-Leu at amino acid positions 694 to 696 in the TEV polyprotein, was subcloned into pKK1427 to construct pKK1427/33-20 (Fig. 1).

The kinetics of proteolysis of the 47-kDa polyprotein expressed in *E. coli* carrying pKK1427 was analyzed in pulse-chase experiments. Cultures were radiolabeled with [³⁵S]methionine for 2 min, followed by a chase with nonlabeled methionine for 32 min. A comparison of proteins labeled after the 2-min incubation with [³⁵S]methionine from cells containing pKK233-2 and pKK1427 is shown in Fig. 2A. The polyprotein precursor (47 kDa) and proteolytic product (37 kDa) were the major ³⁵S-labeled proteins produced in cells containing pKK1427. In the pulse-chase experiment, a decay of the 47-kDa precursor protein was

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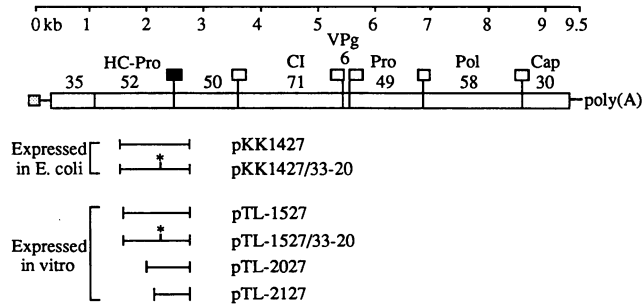


FIG. 1. Genetic map of the TEV genome and regions expressed in *E. coli* or in vitro. The linear RNA genome is represented below the scale bar. The shaded box at the 5' end of the RNA is the virus-encoded, genome-linked protein (VPg) (18). The rectangular box spanning nearly the entire length of the RNA is the open reading frame, which programs synthesis of a 346-kDa polyprotein (1). The vertical lines attached to boxes represent sequences that code for proteolytic cleavage sites within the polyprotein. The open boxes indicate cleavage sites processed by the 49-kDa proteinase, while the black box shows the position of the HC-Pro cleavage site. The approximate sizes (in kilodaltons) of the mature gene products are shown above the open reading frame. A polyadenylate region of 20 to 120 nucleotides resides at the 3' terminus of the genome (2). Several constructs containing TEV cDNA were expressed in the present study and are shown to scale underneath the genome map. pTL-1527 (containing TEV genome nucleotides 1531 to 2681), pTL-1527/33-20 (1531 to 2681), pTL-2027 (1966 to 2681), and pTL-2127 (2116 to 2681) were constructed by insertion of TEV cDNA fragments into vector pTL-8 (7, 10). This vector contains the TEV 5'-nontranslated region and start codon for translation (downstream from a bacteriophage SP6 promoter), such that inserts positioned in frame with the initiator codon may be transcribed and translated efficiently in vitro. The position of mutation 33-20 is indicated by the asterisk. kb, Kilobase; Pol, polymerase; Cap, capsid protein; CI, cylindrical inclusion protein.

seen concomitant with an accumulation of the 37-kDa product (Fig. 2B). Quantitation (by densitometer scanning) of the precursor/product ratio over time indicated that the upper limit of polyproteins that underwent proteolysis was only 67% (Fig. 2C), even with incubation times longer than those shown. Considering only the fraction of polyproteins (67%) that appeared to be processing competent, the reaction proceeded with a half-time of approximately 100 s. A pulse-chase analysis also was conducted with *E. coli* cells harboring pKK1427/33-20. Although the 47-kDa polyprotein precursor was labeled after the 2-min pulse, a proteolytic cleavage product of 37 kDa failed to accumulate (Fig. 2B).

Based on apparent sizes of the proteolytic products, HC-Pro is functional in the procaryotic system and appears to recognize the correct polyprotein processing site. This provides additional evidence for the proteolytic activity of HC-Pro beyond that which was obtained with the cell-free system (7). The fact that HC-Pro appears to function in the procaryotic system suggests that host factors (that normally would be supplied by plant cells) are not involved in the catalytic mechanism. Such a suggestion has been made by others (25) for proteolysis of a cowpea mosaic virus polyprotein. A fraction of the precursor remained stable in *E. coli*, indicating that some of the enzyme may be incorrectly folded or sequestered within insoluble granules (30) prior to cleavage.

Processing of HC-Pro in vitro. The availability of cell-free systems to express viral proteinases and their substrates has enabled significant progress to be made toward understanding the mechanisms and requirements involved in TEV

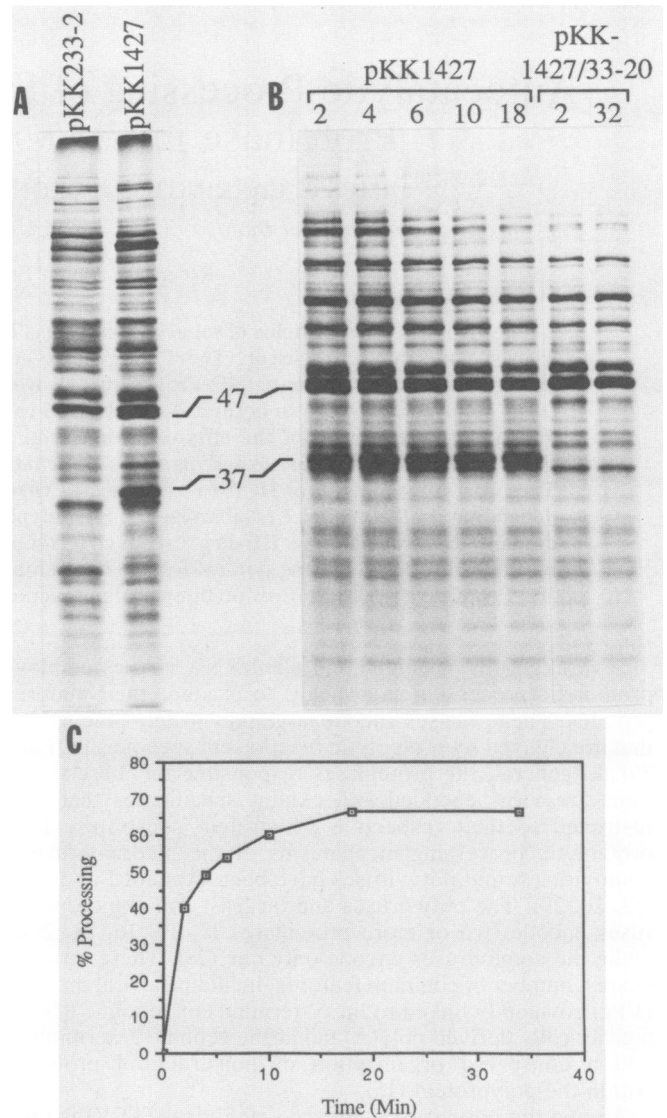


FIG. 2. Pulse-chase analysis of pKK1427-derived proteins synthesized in *E. coli*. (A) Comparison of [35 S]methionine-labeled proteins produced in bacteria containing pKK233-2 (vector without insert) and pKK1427. Cells were labeled for 2 min and collected by centrifugation. Sodium dodecyl sulfate-soluble proteins were extracted by boiling in protein dissociation buffer. (B) Bacteria containing either pKK1427 or pKK1427/33-20 were labeled with [35 S] methionine for 2 min, after which nonlabeled methionine was added to a final concentration of 20 mM. Samples were withdrawn to ice-cold stop buffer (40 mM KPO₄ [pH 7.0], 2.5% formaldehyde) immediately (2 min) and after those times indicated (in minutes) above the autoradiograph. The positions of the 47-kDa precursor and 37-kDa proteolytic product are shown between panels A and B. (C) Quantitation of proteolytic processing of the 47-kDa polyprotein over time. The intensities of the 47- and 37-kDa protein bands were determined by densitometry of the autoradiograph. Percent processing was calculated by dividing the amount of 37-kDa protein product by the amount in both the 47-kDa precursor and the 37-kDa product. The plot was extrapolated to 0% processing at 0 min.

polyprotein processing. We used SP6 transcription and cell-free translation in the present study to examine further the processing kinetics of HC-Pro and to determine whether this proteinase functions autocatalytically. The processing

rates of polyproteins encoded by transcripts of pTL-1527, pTL-1527/33-20, pTL-2027, and pTL-2127 (Fig. 1) were determined *in vitro* by pulse-chase experiments. These plasmids carry the coding sequences for amino-terminal-deletion variants of HC-Pro, the HC-Pro cleavage site (Gly-Gly dipeptide), and a 10-kDa segment of the protein neighboring the carboxyl terminus of HC-Pro. It has been shown previously that pTL-1527 and pTL-2027 encode processing-competent polyproteins, whereas pTL-2127 and pTL-1527/33-20 encode inactive polyproteins owing to infringement of a deletion into the proteolytic domain and a clustered point mutation, respectively (7). A processing time course for each polyprotein is shown in Fig. 3.

The 47-kDa polyprotein precursor derived from pTL-1527 accumulated transiently after a 7-min translation period (5-min radiolabel pulse plus 2-min chase) and was processed to products of 37 and 10 kDa with a half-life of approximately 6 min after its appearance. Proteolysis of the 30-kDa precursor derived from pTL-2027 occurred much more rapidly than proteolysis of the precursor derived from the pTL-1527 polyprotein. Precursor (30 kDa) and product (20 kDa) were detected in ratios that indicated 80% processing immediately after the 5-min pulse period. The ratio of precursor to product remained constant throughout the chase period; identification of a processing half-time thus was not possible. This suggests that proteolysis of this particular polyprotein was extremely efficient, occurring immediately after ribosomes had traversed the cleavage site coding sequence. As expected from previous studies, the polyproteins encoded by pTL-1527/33-20 and pTL-2127 failed to undergo proteolysis. Cleavage of these polyproteins would have yielded products of 37 and 15 kDa, respectively. There appeared to be a slight decrease in the amount of total radioactively labeled protein during the time courses for each construct. This is attributed to the activities of endogenous nonspecific proteinases that reside in the reticulocyte lysate. Quantitation of processing, therefore, was based on the ratio of polyprotein to proteolytic product at each time point.

The reason for the differential rates of cleavage for pTL-1527- and pTL-2027-derived polyproteins was not investigated further, although we speculate that it may be due to differences in protein folding. The pTL-1527 polyprotein contains a 17-kDa protein segment at the amino terminus that is absent from the pTL-2027 polyprotein. This additional protein may interfere with or slow proper folding of the proteolytic domain of HC-Pro or possibly interfere with presentation of the cleavage site to the active center of the proteinase.

To determine whether HC-Pro functions in a *trans*-catalytic or bimolecular reaction, we conducted experiments to test the proteolytic activity synthesized from pTL-1527 and pTL-2027 transcripts using the polyprotein encoded by pTL-1527/33-20 as the substrate. Nonlabeled translation mixtures programmed with H₂O (as a negative control) or transcripts from pTL-1527, pTL-2027, or pTL-2127 were added to reaction mixtures containing the 47-kDa polyprotein (³⁵S labeled) encoded by pTL-1527/33-20 transcripts. Proteolytic cleavage of the radiolabeled substrate was predicted to yield products of 37 and 10 kDa. However, little or no processing of the precursor was detected after reaction with either pTL-1527- or pTL-2027-derived proteinase (Fig. 4, lanes 1 and 2). No differences were observed between proteinase-containing reaction mixtures and those that contained the pTL-2127-derived translation product (lane 3) or H₂O (lane 4). The lack of *trans* proteolytic activity also was seen with

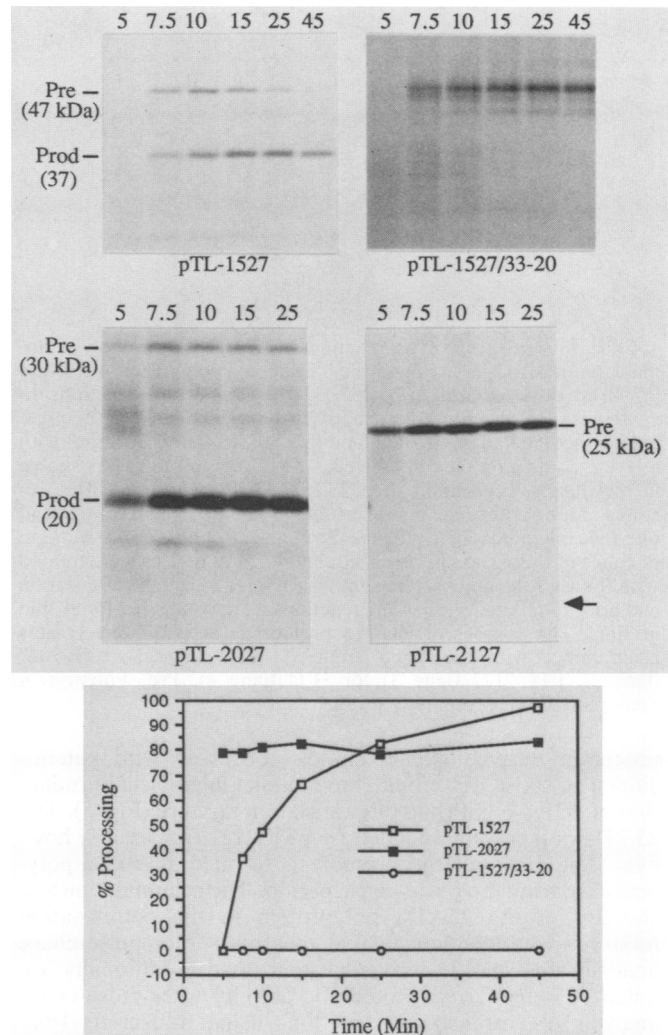


FIG. 3. Pulse-chase analysis of polyproteins synthesized *in vitro* from transcripts of pTL-1527, pTL-1527/33-20, pTL-2027, and pTL-2127. The genome segments represented by these transcripts are shown in Fig. 1. Transcripts were translated in a rabbit reticulocyte lysate system containing [³⁵S]methionine for 5 min, after which nonlabeled methionine was added to a final concentration of 20 mM. Samples were withdrawn immediately (5 min) or after the times (in minutes) indicated above the autoradiographs. The positions of the polyprotein precursors (Pre) and proteolytic products (Prod) are shown next to each autoradiograph (the markers are the same for pTL-1527 and pTL-1527/33-20). The arrow next to the pTL-2127 autoradiograph indicates the position expected for a protein that would result from processing at the HC-Pro-mediated cleavage site. Quantitation of pTL-1527, pTL-1527/33-20, and pTL-2027 polyprotein processing by using the calculation described in the legend to Fig. 2 is shown in the graph. The plot for pTL-1527 was extrapolated to 0% processing at 5 min.

the 25-kDa polyprotein substrate encoded by pTL-2127 transcripts (data not shown).

The inability of HC-Pro to function in *trans* may have been due to instability of the enzyme or to a feature inherent to the proteinase or cleavage site or both (i.e., the reaction requires an autocatalytic mechanism). To test these hypotheses, we cosynthesized proteinase encoded by pTL-1527 and substrate from pTL-2127 *in vitro* and examined the products by pulse-chase analysis. Synthesis and processing of the 47-kDa

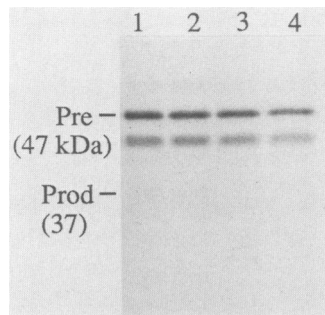


FIG. 4. *trans* proteolytic reactions with HC-Pro proteolytic domain synthesized *in vitro* and the ^{35}S -labeled polyprotein substrate (47 kDa) derived from pTL-1527/33-20. Translation reactions to synthesize HC-Pro proteolytic activity were conducted in the presence of nonlabeled methionine and then mixed (in a 5:1 ration) with a lysate containing the ^{35}S -labeled, 47-kDa polyprotein. (As shown in this figure, translation of pTL-1527/33-20 transcripts often resulted in the appearance of two proteins—one of the correct size and one that migrated slightly faster during electrophoresis. The origin or cause of this extra species is not known.) All lysates were treated with RNase A to stop protein synthesis immediately after translation and prior to mixing. Processing reactions were conducted for 90 min at 30°C. The sources of HC-Pro proteolytic activity were lysates programmed with transcripts from pTL-1527 (lane 1), pTL-2027 (lane 2), pTL-2127 (lane 3), or H₂O (lane 4). Pre, Polyprotein precursor; Prod, proteolytic product.

precursor derived from pTL-1527 occurred with kinetics similar to those described above, indicating normal production of active HC-Pro in the translation mixture (Fig. 5). The 25-kDa polyprotein encoded by pTL-2127 transcripts, however, failed to undergo proteolysis to yield a 15-kDa polypeptide during the pulse-chase period. The accumulation and stability of the 25-kDa polyprotein in the cotranslation mixtures was identical to that measured after pulse-chase analysis of pTL-2127 derived protein alone. Additionally, we failed to detect *trans*-proteolytic activity after pulse-chase analysis of cotranslation reactions using transcripts from pTL-2027 (for HC-Pro activity) and pTL-1527/33-20 (for substrate; data not shown). These findings suggest that HC-Pro strongly prefers or requires an autocatalytic mechanism for peptide bond cleavage at its carboxyl terminus.

The molecular basis for an autocatalytic requirement is a matter for speculation. Presumably, the carboxyl-terminal cleavage site adopts an orientation that effectively encounters a *cis*-linked proteinase domain. This could be rationalized by envisioning the cleavage site region of the polypeptide chain extending across the active site, perhaps as the last step during protein folding. The amino acid residues at or surrounding the cleavage site would interact selectively with a binding-site pocket near the active center of the enzyme. The cleavage site would assume a sufficiently ordered local structure dictating that nonlinked or exogenous proteinase molecules are excluded from interaction with the substrate. This mechanism may have evolved to ensure efficient and complete processing at the HC-Pro carboxyl terminus. A similar self-cleavage requirement has been documented for both the amino and carboxyl ends of the TEV 49-kDa proteinase (9). Additionally, cleavage between NS1 and NS2a proteins of dengue virus may require an autocatalytic pathway involving a *cis*-dominant proteolytic activity of NS2a (14). Although the picornavirus proteinase 3C has been shown to function autocatalytically (17, 21), a strict requirement for this mode apparently does not exist (22).

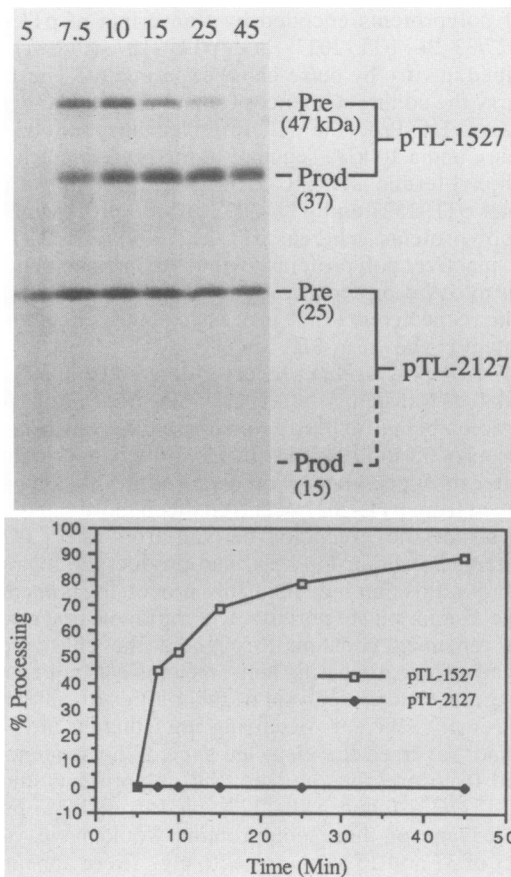


FIG. 5. Pulse-chase analysis of polyproteins synthesized by cotranslation of transcripts from pTL-1527 and pTL-2127 *in vitro*. ^{35}S labeling was conducted for 5 min, after which nonlabeled methionine was added to a final concentration of 20 mM. Samples were withdrawn immediately after the addition of methionine (5 min) or after the times indicated (in minutes) above the autoradiograph. The positions of the polyprotein precursors (Pre) and proteolytic products (Prod) are indicated at the right. Quantitation of processing (as described in the legend to Fig. 4) is shown in the graph. The plots were extrapolated to 0% processing at 5 min.

The class of proteinases in which HC-Pro belongs has not yet been resolved. Based on protein modeling, Bazan and Fletterick (5) have postulated that the proteinases encoded by the picornaviruses and cowpea mosaic virus, as well as the 49-kDa proteinase of the potyviruses, resemble serine proteinases that contain cysteine rather than serine at their active sites. The signature amino acid sequence motifs that characterize these other proteinases are not evident in the HC-Pro proteolytic domain, suggesting that HC-Pro belongs within a separate classification of viral proteinases. HC-Pro does contain the sequence Gly-Asn-Ser-Gly (positions 623 to 626 in the TEV polyprotein), which resembles the sequence Gly-Asp-Ser-Gly surrounding and including the active-site serine residue found in serine-type proteinases; however, the spatial relationship of this sequence motif relative to the rest of the HC-Pro proteolytic domain is entirely different from that found in the cellular serine proteinases (4). The nature of HC-Pro currently is being investigated by systematic mutagenesis of this and other domains within the molecule.

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