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A second proteinase encoded by a plant potyvirus genome

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The RNA genome of tobacco etch virus (TEV) encodes a large polyprotein precursor that is processed to mature proteins by virus-specific proteinases. Cleavage sites located within the carboxyl-terminal two-thirds of the polyprotein are processed by a TEV-encoded 49 kd proteinase, while the enzyme(s) responsible for cleaving the remaining sites has not been found. In this study, a second TEV-encoded proteinase has been identified based on cell-free expression of defined RNA transcripts. The boundaries of this proteinase have been delineated by deletion analysis and site-directed mutagenesis. The proteolytically active domain has been localized to the carboxyl-terminal half of the 56 kd aphid-transmission helper component. A cleavage site that is recognized by this proteinase has been identified in the polyprotein adjacent to the carboxyl-terminus of the enzyme, and the proteinase appears to cleave by an autocatalytic mechanism. Proteolysis *in vitro* occurs between a Gly-Gly dipeptide as determined by radiochemical sequencing at the amino-terminus of the proteolytic product.

Key words: potyvirus/proteinase

Introduction

A number of medically or agriculturally important RNA-containing viruses synthesize polyprotein precursors as primary products during translation. In all cases that have been examined, virus-encoded proteinases are responsible for most or all polyprotein cleavage events (Wellink and van Kammen, 1988). The plant potyviruses use this strategy of genome expression. One of the best studied potyviruses at the molecular level is tobacco etch virus (TEV), which has a genome length of 9495 nucleotides (Allison *et al.*, 1986). The TEV genome contains only one translational unit which is sufficient to direct synthesis of a polyprotein of ~346 kd. TEV particles are long, flexuous rods that consist of a single positive-sense RNA molecule, a small protein (VPg) covalently linked to the RNA, and over 2000 copies of a capsid protein monomer (Dougherty and Carrington, 1988). The closest relatives of the potyvirus group are the plant comoviruses and the animal picornaviruses, each of which possess RNA genomes that are linked to VPg molecules and that encode large polyproteins.

The TEV genome encodes possibly eight mature proteins which arise by controlled proteolytic processing (Dougherty and Carrington, 1988). At least five of the processing events, all within the carboxyl-terminal two-thirds of the polyprotein, are catalyzed by a virus-encoded proteinase of 49 kd (Carrington and Dougherty, 1987a; Carrington *et al.*, 1988). This enzyme is analogous to the picornaviral proteinase 3C (Allison *et al.*, 1986; Carrington and Dougherty, 1987b; Domier *et al.*, 1987). The 49 kd proteinase releases from the polyprotein by an autocatalytic mechanism and functions in *trans* at additional cleavage sites (Carrington and Dougherty, 1987a,b). All five sites recognized by the 49 kd proteinase are defined by a heptapeptide sequence that contains four conserved amino acid residues (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988). The entire conserved sequence has been shown to constitute the cleavage site recognition signal. Although experiments have been carried out to identify 49 kd proteinase-mediated cleavage sites within the amino-terminal 135 kd of the TEV polyprotein, none have been found to date.

It is reasonable to postulate the existence of a second TEV proteinase that might catalyze proteolysis within the amino-terminal region of the polyprotein. Several picornaviruses have been shown to encode a second proteinase in addition to 3C. The L peptide encoded by foot-and-mouth disease virus exhibits proteolytic activity to release itself from the remainder of the polyprotein (Strebel and Beck, 1986; Vakharia *et al.*, 1987). Poliovirus protein 2A functions as a proteinase to separate precursor polypeptides P1 and P2 at a Tyr-Gly dipeptide (Toyoda *et al.*, 1986). Proteinase 2A also plays a role in the cascade of events in which the cellular cap-binding protein p220 is cleaved and inactivated, thus restricting translation of capped (cellular) mRNAs within infected cells (Krausslich *et al.*, 1987). A limited degree of amino acid sequence similarity has been detected between poliovirus proteinase 2A and the TEV putative 50 kd protein (Domier *et al.*, 1987).

Cell-free translation of TEV genomic RNA, or synthetic transcripts containing the 5'-region of the genome, results in accumulation of an 87 kd proteolytic product which represents the amino-terminus of the viral polyprotein (Dougherty and Hiebert, 1980a; this paper). This study was initiated to determine if a viral-encoded proteinase was responsible for cleavages within the amino-terminal domain of the TEV polyprotein. We have applied the techniques of cell-free transcription and translation to express wild-type and site-specifically mutagenized forms of the TEV genome 5'-region. A proteolytic step was identified that functions to sever the 87 kd product from the proposed 50 kd protein mentioned above. Molecular genetic analysis has revealed that the carboxyl-terminus of the 87 kd protein serves as the proteinase to release itself from the polyprotein. This represents the first report of a plant virus expressing two proteinases.

Results

Translation of TEV RNA in a cell-free system yields an 87 kd product that is encoded by 5′-proximal sequences (Dougherty and Hiebert, 1980b). Since the TEV genome possesses a single open reading frame which codes for a large polyprotein precursor, proteolytic processing is the probable mechanism whereby the 87 kd product is formed. No amino acid sequence resembling the conserved cleavage site signal motif for the previously described TEV 49 kd proteinase (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988) has been identified in this region of the polyprotein, suggesting that a second proteinase functions to release the 87 kd polyprotein. To test this hypothesis, we have expressed a series of wild-type and mutagenized cDNA segments representing various regions near the 5′-end of the TEV genome. As in previous studies, cDNA fragments were inserted into the vector pTL-8 and expressed using cell-free transcription and translation (Carrington and Dougherty, 1987a,b).

Plasmid pTL-0027 contained cDNA representing the initial 2681 nucleotides of the TEV genome (Figure 1A). Transcripts from pTL-0027 possessed the coding potential for a polypeptide of ~97 kd. If this protein were cleaved accurately in the cell-free system, products of 87 and 10 kd would be released. In fact, translation *in vitro* resulted in accumulation of 87 and 10 kd products, as well as others greater than 20 kd in size (Figure 1B, lane 1). Based on the results of numerous repetitions of this experiment, the 87 and 10 kd proteins are believed to have resulted from proteolysis of the 97 kd precursor, whereas the other species probably arose by premature termination of translation.

To test the possibility that the proteolytic activity responsible for cleavage at the 87 kd boundary resided within the 87 kd protein itself, deletion constructs lacking various lengths of the 5′-proximal TEV sequence were expressed *in vitro*. Each plasmid harbored cDNA that terminated at the same position (corresponding to nucleotide 2681), but that possessed different 5′-ends. pTL-1527 transcripts had the coding capacity for a polypeptide of ~47 kd (Figure 1A). Translation *in vitro*, however, resulted in accumulation of products at 37 and 10 kd (Figure 1B, lane 2). Translation of pTL-2027 transcripts, with a predicted coding capacity of 30 kd resulted in accumulation of products of 20 and 10 kd (Figure 1B, lane 3). On the other hand, translation of pTL-2127 transcripts with a predicted capacity to encode a 25 kd product, yielded a 25 kd polypeptide upon translation (Figure 1B, lane 4). It appeared that the primary translation products encoded by pTL-0027, pTL-1527, and pTL-2027 transcripts were undergoing proteolysis to release a 10 kd polypeptide from the carboxyl-terminus, whereas the pTL-2127-derived protein was not processed. This suggested that a carboxyl-terminal 20 kd domain of the 87 kd protein might function as a proteinase.

To further examine the potential proteolytic activity within the 87 kd protein, two clustered point mutations were introduced into the coding sequence of pTL-1527. Both mutations were within the sequence coding for the carboxyl-terminal 20 kd domain of the 87 kd protein (Figure 2A). The positions that were mutagenized were selected because they contained cysteine residues; several classes of cellular and viral proteinases possess conserved cysteine residues that reside at the proteinase active center or at structurally important positions (Argos *et al.*, 1984; Barrett, 1986).

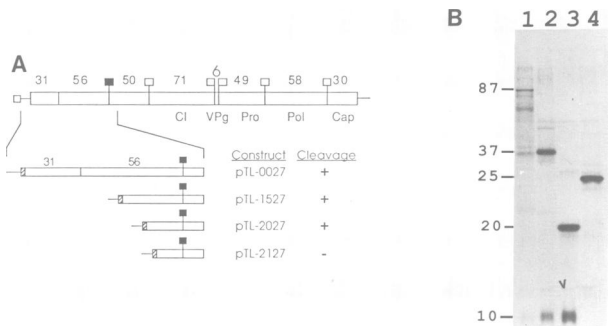

Fig. 1. Cell-free expression of TEV cDNA derived from sequences near the 5′-terminus of genomic RNA. (**A**) A genetic map of the TEV genome is shown at the top. The 5′- and 3′-untranslated regions are indicated by straight lines, and the long open reading frame covering most of the RNA is shown by the rectangular box. The VPg (shaded box) is attached to the 5′-terminus of the viral RNA. The square boxes above the open reading frame indicate the positions that encode known cleavage sites in the polyprotein. The open squares show 49 kd proteinase-mediated sites, while the black square indicates the site cleaved by the second TEV-encoded proteinase (reported in this study). DNA fragments (enlarged diagram) representing various lengths of the TEV genome were inserted into vector pTL-8, which carries cDNA representing the 5′-untranslated region and initial coding sequence (striped box) of the TEV genome. The details of each plasmid are given in Materials and methods. (**B**) ^{35}S-labeled translation products encoded by SP6 transcripts from plasmids pTL-0027 (**lane 1**), pTL-1527 (**lane 2**), pTL-2027 (**lane 3**), and pTL-2127 (**lane 4**). Translation reactions were incubated for 1 h, disrupted with protein gel loading buffer (Laemmli, 1970), and the products were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The apparent mol. wts ($\times 10^{-3}$) of the major protein products are given next to the autoradiograph. The primary translation products of transcripts from pTL-0027, pTL-1527, and pTL-2027 were processed at the cleavage site indicated in (A), whereas the pTL-2127-derived product was stable. CI, cylindrical inclusion protein; VPg, genome-linked protein; Pro, proteinase; Pol, polymerase; Cap, capsid protein.

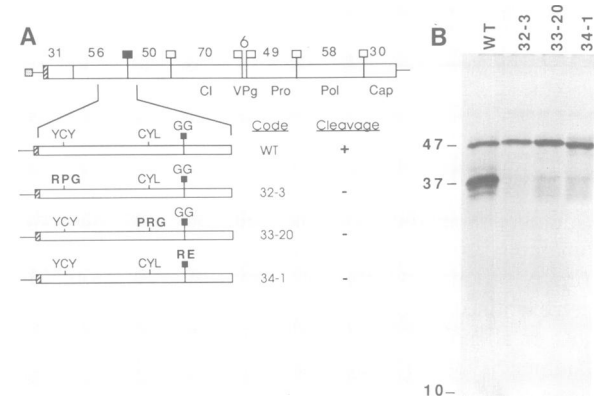

Fig. 2. Expression of site-directed mutants carrying defects in the putative proteolytic domain encoded by pTL-1527 transcripts. (**A**) Positions of wild-type (WT) and mutagenized (32-3, 33-20, and 34-1) amino acid sequences (single letter code) encoded by pTL-1527. The mutant amino acid sequences are indicated in bold. The exact position of each mutation is given in Materials and methods. (**B**) ^{35}S-labeled translation products encoded by wild-type and mutagenized forms of pTL-1527 transcripts. The wild-type pTL-1527-derived translation product (47 kd) underwent proteolysis to yield proteins of 37 and 10 kd, whereas the three mutant primary products remained stable. Abbreviations are the same as in Figure 1.

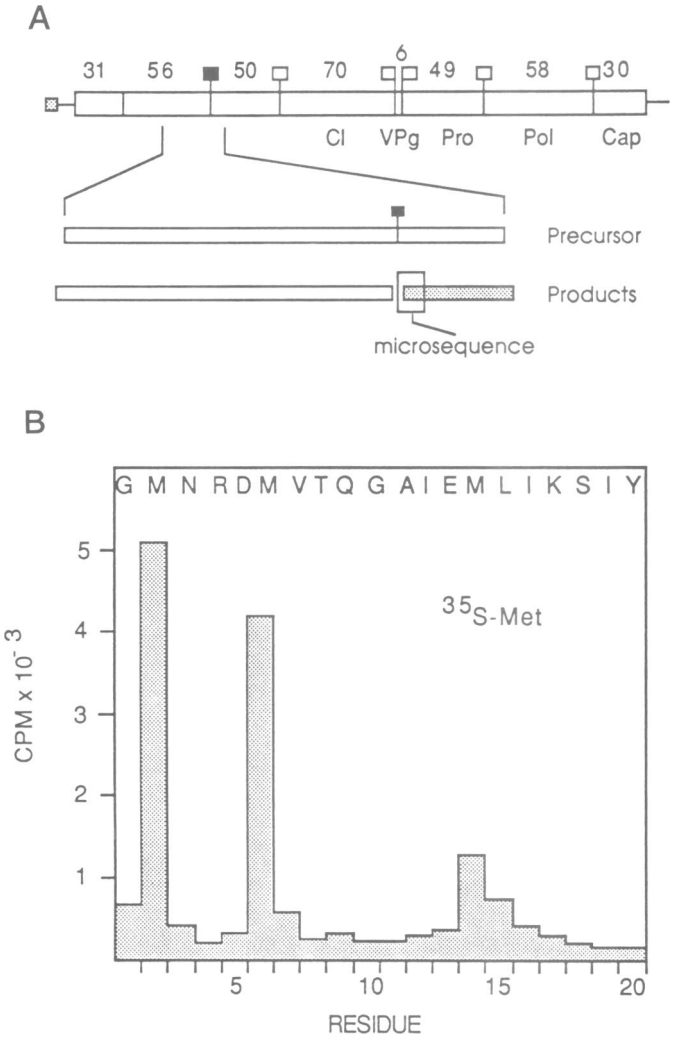

Fig. 3. Radiochemical microsequence analysis of the amino-terminus of the 10 kd polypeptide released by proteolysis of the pTL-1527-derived synthetic polyprotein. **(A)** Diagrammatic representation detailing the relationship between the precursor (47 kd) and proteolytic products (37 and 10 kd) encoded by pTL-1527 transcripts. The ^{35}S-labeled 10 kd product (shaded) was isolated using preparative polyacrylamide gel electrophoresis and subjected to automated Edman degradation. **(B)** Radioactivity released at each cycle during the sequencing run. [^{35}S]methionine was identified at positions 2, 6 and 14. The sequence of amino acids between positions 764 and 783 within the TEV polyprotein is shown above the plot.

Mutation 32-3 resulted in conversion of Tyr−Cys−Tyr to Arg−Pro−Gly at position 648−650 in the TEV polyprotein, while mutation 33−20 resulted in conversion of Cys−Tyr−Leu to Pro−Arg−Gly at position 694−696. Expression of these two mutagenized sequences yielded stable 47 kd precursor polyprotein rather than the 37 and 10 kd products seen with the wild-type construct (Figure 2B). Additionally, the 33-20 mutation was introduced into pTL-0027; expression of the construct resulted in accumulation of a 97 kd protein that was not processed (data not shown).

The cleavage site position within the pTL-1527 polyprotein was investigated by amino-terminal radiochemical sequence analysis of the 10 kd proteolytic product. The [^{35}S]methionine-labeled polypeptide was eluted from a preparative gel and subjected to automated Edman degradation. The quantity of [^{35}S]methionine released at each cycle is shown in Figure 3. Peaks of radioactivity were released during cycles, 2, 6, and 14. This combination of methionine residues occurs at only one position within the pTL-1527-encoded polyprotein, corresponding to residues 765, 769, and 777 in the TEV polyprotein. Assuming that the amino-terminus of the 10 kd product represents the actual site of proteolysis (i.e. no exoproteolytic processing), cleavage occurred between the Gly−Gly dipeptide at position 763−764 in the TEV polyprotein.

The necessity of the Gly−Gly dipeptide at the cleavage site was tested by expressing transcripts that were derived from mutagenized plasmids. The amino acid sequence Gly−Gly was substituted with Arg−Glu by site-directed mutagenesis of pTL-1527, and given the mutant code 34-1 (Figure 2A). Expression of this mutant yielded a stable 47 kd product that was not cleaved (Figure 2B). This result confirms, although does not prove, the localization of the cleavage site suggested by radiochemical sequence analysis.

Discussion

A cell-free system to express various wild-type and mutagenized segments of the TEV genome near the 5′-terminus has been employed to study proteolytic processing of the TEV polyprotein. This approach previously has permitted extensive analysis of the TEV 49 kd proteinase, and has led to identification of five cleavage sites that are recognized by the 49 kd proteinase within the TEV polyprotein (Carrington and Dougherty, 1987a,b; 1988; Carrington *et al.*, 1988; Dougherty *et al.*, 1988). However, no 49 kd proteinase-mediated substrate sites have been identified previously near the amino-terminus of the TEV polyprotein, leading to speculation that a second proteinase might be encoded by the virus genome (Dougherty and Carrington, 1988).

We have identified in this study a proteinase that is responsible for at least one additional processing event. This enzyme catalyzes release of an 87 kd protein from the amino-terminus of the TEV polyprotein. A carboxyl-terminal 20 kd domain within the 87 kd protein is required for proteolysis as shown by deletion analysis. Site-directed mutations that introduce clustered substitutions within this 20 kd domain greatly reduce or eliminate proteolytic activity. These results suggest that the proteinase resides at the carboxyl-terminus of the 87 kd protein and adjacent to the cleavage site. The fact that cleavage occurs at the proteinase carboxyl-terminus suggests that processing proceeds by an autocatalytic or intramolecular mechanism. Preliminary attempts to show activity in *trans* (i.e. in a bimolecular reaction) with the proteinase synthesized from pTL-1527 transcripts, and using the substrate polyprotein from pTL-2127 transcripts, have been unsuccessful. This could be due to restrictively low quantities of proteinase synthesized *in vitro*, instability of the proteinase, or a requirement for autocatalytic or *cis* processing. A requirement *in vitro* for *cis* processing apparently exists at the termini of the 49 kd proteinase, since proteolysis in *trans* occurs with extreme inefficiency (Carrington and Dougherty, 1987a,b).

Identification of a second TEV proteinase at the carboxyl-terminus of the 87 kd protein was unexpected, considering that another function for this region has been proposed. The 87 kd protein *in vivo* apparently is processed to products of 31 and 56 kd, corresponding to the amino- and carboxyl-

terminus of the precursor, respectively (Dougherty and Carrington, 1988). The homologue of the 56 kd protein encoded by two other potyviruses [tobacco vein mottling (TVMV) and potato virus Y] has been proposed to function as an aphid-transmission helper component (Thornbury *et al.*, 1985). This protein is required for successful potyvirus transmission from plant-to-plant by aphids, probably by a mechanism that involves interaction of the virus and helper component with the aphid stylet (Berger and Pirone, 1986). The proteinase identified in this study would reside at the carboxyl-terminus of the helper component protein, raising some intriguing possibilities. In addition to releasing the 87 kd precursor from the TEV polyprotein, this proteinase may function during the aphid-transmission process in an as yet unidentified manner. The affinity of this proteinase for alternative substrates that might be cleaved during aphid transmission has yet to be investigated. An alternative hypothesis suggests that the helper component and proteinase reside in separate domains within the 56 kd protein. Consistent with this is the localization of the proteinase to a 20 kd segment at the carboxyl-terminus, and identification of limited amino acid sequence similarities between amino-terminal sequences of the TVMV helper component and the helper component of cauliflower mosaic virus (CaMV), a plant caulimovirus (Domier *et al.*, 1987). Since the helper component and proteinase both reside within the 56 kd protein, we will refer henceforth to this protein as HC-Pro.

The cleavage site processed by HC-Pro was mapped by amino-terminal sequence analysis of the 10 kd proteolytic product released from the pTL-1527-derived polyprotein. Assuming that the protein termini remain unaltered after proteolysis (i.e. no trimming), the proteinase cleaves at a Gly−Gly dipeptide located at position 763−764 in the TEV polyprotein. This dipeptide differs from those recognized by the 49 kd proteinase (Gln−Gly or Ser). The amino acid sequence context within which the HC-Pro cleavage site resides also differs from the conserved sequence around each of the 49 kd proteinase sites (Figure 4). HC-Pro thus possesses a substrate specificity quite different from the 49 kd proteinase.

Although poliovirus and TEV both encode second proteinases that release precursor polyproteins (P1 for poliovirus, 87 kd protein for TEV) from the amino-terminus of the large viral polyprotein, they function in substantially different capacities. Poliovirus proteinase 2A cleaves at its amino-terminus, whereas HC-Pro cleaves at its carboxyl-end. Poliovirus 2A functions to sever the capsid proteins from non-structural proteins, while HC-Pro separates precursors for two sets of non-structural proteins. The most significant difference biochemically is found by comparison of their amino acid sequences. Bazan and Fletterick (1988) have proposed that the 2A proteinases from rhino- and enteroviruses are structurally similar to the 'small' subclass of trypsin-like serine proteinases. Comparing five picornavirus 2A proteinases with three small serine proteinases, they identified ten residues that were conserved in all eight sequences. Importantly, the viral 2A proteinases have Cys as their active center nucleophile while the cellular serine proteinases carry Ser. The most highly conserved sequence

Pro−Gly−Asp−Cys/Ser−Gly−Gly

is positioned around the active centers of the picornaviral 2A and small serine proteinases. Alignment of all Cys and

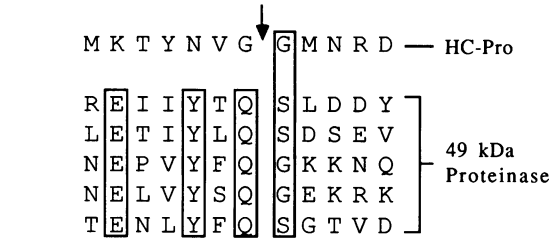

Fig. 4. Comparison of cleavage sites recognized by the TEV 49 kd proteinase and HC-pro. The scissile bond is indicated by the arrow. Conserved amino acid residues are indicated by the boxes.

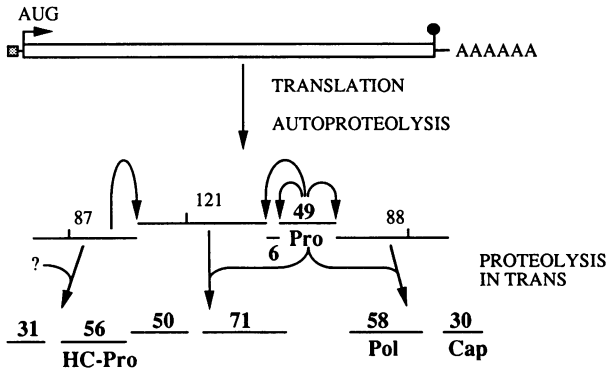

Fig. 5. Diagrammatic representation of TEV polyprotein processing. The genomic RNA, with the positions of the start (AUG) and stop (black circle) codons, the VPg (shaded box), and polyadenylate region (AAA...), is shown at the top. Based on the behavior *in vitro* of the 49 kd proteinase and HC-Pro, as well as the cleavage sites recognized by each enzyme, the primary translation product is cleaved by autoproteolysis at four positions, yielding three polyprotein precursors (light type) of 87, 121, and 88 kd, and two mature products (bold type) of 6 and 49 kd (proteinase). Cleavage of the remaining sites occurs by intermolecular or *trans*-proteolytic mechanisms. The 121 and 88 kd precursors are processed by the 49 kd proteinase, while the 87 kd precursor is cleaved in an undefined manner. Except for the function of HC-Pro, most of these events are reviewed by Dougherty and Carrington (1988). Abbreviations are the same as in Figure 1.

Ser residues in the proteolytic domain of the HC-Pro with the active center Cys or Ser of the 2A/small serine proteinase conserved sequence clearly shows the absence of this motif. Furthermore, the additional residues in common between the 2A and small serine proteinases are absent in HC-Pro within the context of each Cys and Ser residue.

Although HC-Pro catalyzes cleavage at its own carboxyl-terminus, cleavage at the proteinase amino-terminus (between the 31 and 56 kd proteins) apparently is carried out by another enzyme or complex. The 87 kd polyprotein precursor remains stable in the cell-free translation mixture for several hours, and products of 31 and 56 kd fail to accumulate. The enzyme required for this reaction also appears not be the 49 kd proteinase since incubation of the 87 kd precursor with this proteinase, in quantities sufficient to cleave rapidly a substrate containing the 58−30 kd processing site, has no detectable effect (unpublished data). It is conceivable that specific conditions *in vivo* are required to promote HC-Pro or 49 kd proteolytic activity at the 31−56 kd precursor cleavage site.

Alternatively, auxiliary factors may be necessary to complement one of the proteinases to stimulate cleavage of the 87 kd precursor. Two viral proteinases, poliovirus 3C and cowpea mosaic virus (CpMV) 24 K proteinase, require additional proteins to affect cleavage at certain processing sites. Proteinase 3C of poliovirus will cleave P1 precursor sites only when linked within a polyprotein with protein 3D (Ypma-Wong and Semler, 1987; Ypma-Wong *et al.*, 1988), while the CpMV 24 K proteinase requires the viral 32 K protein for processing at a Gln−Met cleavage site (Vos *et al.*, 1988). The possibility that a third proteinase is encoded by the TEV genome has not been excluded. Neither have we excluded the notion that a novel proteolytic activity, such as the RNA-assisted mechanism proposed for VPO cleavage in picornavirus capsids (Arnold *et al.*, 1987), functions at the 31−56 kd polyprotein processing site. The identity of the enzyme responsible for this cleavage will be a subject for further study.

Our understanding of TEV polyprotein processing has advanced to the point where we have identified six of the seven known or postulated cleavage sites, as well as the proteinases responsible for their processing. A polyprotein cleavage map is proposed in Figure 5. Five cleavage sites are recognized by the 49 kd proteinase while one position is cleaved by the HC-Pro. We have proposed that four sites are processed by autocatalysis, while the others are cleaved in bimolecular reactions. A number of questions concerning TEV proteolytic processing are currently being addressed, including identification of the cleavage pathway *in vivo* and mapping of functional domains that regulate cleavage site specificity within the proteinases.

Materials and methods

Construction of plasmids

Complementary DNAs respresenting various lengths of the TEV genome were inserted into the cell-free expression vector pTL-8. This plasmid contains the 5′-proximal sequence of the viral RNA, including the untranslated leader, the initiator codon, and the first 60 nucleotides of the coding region, inserted downstream from an SP6 promoter and upstream from a polylinker (Carrington *et al.*, 1987). DNA sequences containing portions of open reading frames are inserted into pTL-8, and SP6 transcripts are translated *in vitro* using the TEV initiator codon.

Four plasmids were constructed for this study. A *Sac*I−*Hpa*I restriction fragment was inserted into pTL-8 to assemble pTL-0027. This plasmid harbored cDNA representing the initial 2681 nucleotides of the TEV genome. The numbering system for these plasmids derives from the 5′- and 3′-ends of the insert; the first two digits (e.g. pTL-0027) indicate the coordinate of the 5′-terminus of the insert, to the nearest 100, while the last two digits (e.g. pTL-0027) refer to the coordinate of the 3′-end relative to the genomic RNA. pTL-1527 contains an insertion of a *Nde*I−*Hpa*I cDNA fragment (nucleotides 1531−2681). pTL-2027 contains an insertion of a *Ssp*I−*Hpa*I cDNA fragment (nucleotides 1966−2681), while pTL-2127 harbors an insertion of a *Spe*I−*Hpa*I cDNA fragment (nucleotides 2116−2681). Each insert terminated at the same *Hpa*I site. Restriction digests, ligations, transformation of *Escherichia coli* HB 101 cells, and analysis of recombinant plasmids were carried out using previously described procedures (Carrington and Dougherty, 1987a; Maniatis *et al.*, 1982).

Site-directed mutagenesis

Three clustered point mutations were introduced into the pTL-1527 insert. The cDNA first was excised from pTL-1527 with *Sac*I and *Pst*I (both cut in flanking vector sequences) and subcloned in pTL-37 (Carrington *et al.*, 1987). This vector resembles pTL-8 except that the M13 IG region has been added (thus allowing single-stranded DNA production with the aid of helper phage M13K07) and the TEV leader has been positioned adjacent to a T7 phage promoter. Oligonucleotide-directed mutagenesis was conducted using the procedure of Taylor *et al.* (1985). Mutation 32-3 resulted in conversion of the sequence Tyr−Cys−Tyr at position 648−650 in the TEV polyprotein to Arg−Pro−Gly. Mutation 33-20 resulted in conversion of Cys−Tyr−Leu (position 694−696) to Pro−Gly−Arg. Mutation 34-1 yielded a conversion of Gly−Gly (position 763−764) to Arg−Glu.

Cell-free transcription and translation

Specific details of transcription with SP6 and T7 phage polymerases and translation in rabbit reticulocyte lysate or wheat germ systems have been described (Carrington and Dougherty, 1987a; Dougherty and Hiebert, 1980a; Melton *et al.*, 1984). Since transcripts containing the TEV leader sequence translate *in vitro* efficiently without a 5′-cap structure, cap precursor was not added to the transcription reaction. All translation reactions were conducted in the presence of [^{35}S]methionine (>600 Ci/mM, New England Nuclear). Protein products were analyzed by discontinuous SDS−PAGE (Laemmli, 1970) and autoradiography. ^{14}C-labeled size standards, consisting of myosin (200 kd), phosphorylase B (97 kd), gamma globulins (heavy chain, 53 kd), carbonic anhydrase (29 kd), and lactoglobin A (18 kd), were included with each gel.

Protein microsequence analysis

The positions of methionine residues relative to the amino-terminus of the 10 kd proteolytic product encoded by pTL-1527 transcripts was determined by radiochemical microsequence analysis as described (Carrington and Dougherty, 1987b; Carrington *et al.*, 1988).

Acknowledgements

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