Autocatalytic activity of the tobacco etch virus NIa proteinase in viral and foreign protein sequences

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The small nuclear inclusion (NIa) protein of the tobacco etch virus (TEV) is synthesized initially as part of a genome-derived high Mr precursor. The NIa protein releases itself from this genome-derived precursor by self-cleavage, or an autocatalytic processing event. Cleavage between specific glutamine-glycine dipeptides at the N and C termini generates the 430 amino acid or 49000 Mr (49K) NIa protein. The requirements of this autocatalytic release, or cis cleavage, were examined by constructing gene cassettes encoding the TEV NIa protein which could be ligated into particular locations in cDNA of the TEV genome and also into foreign gene DNA sequences. Using cell-free transcription and translation systems, polyproteins containing TEV NIa sequences were synthesized and assayed for (i) autocatalysis and (ii) the ability of a functional NIa proteinase, purified from plant tissue, to cleave in bimolecular or trans reactions various artificial polyproteins which contained an inactive form of the NIa proteinase. The NIa self-cleavage events required an active proteinase sequence and a consensus TEV cleavage site sequence at the N and C termini. These results were consistent for NIa protein sequences placed at a foreign TEV cleavage site or in unrelated proteins. Differences were noted in the trans cleavage of these sites.

Introduction

Tobacco etch virus (TEV), a potyvirus and member of the picornavirus-like virus supergroup (Goldbach, 1987), expresses its genetic information from an ssRNA molecule possessing an uninterrupted open reading frame (ORF) of 9126 nucleotides (Allison et al., 1986). A single 346Kc polyprotein precursor is encoded, but cis- and trans-mediated proteolytic cleavage reactions result in the formation of a number of discrete lower Mr polyprotein precursors and products. Two of the three proteinases involved in the processing of the TEV polyprotein probably excise themselves from the genome-derived polyprotein initially as intermediate polyproteins (Dougherty & Carrington, 1988; Carrington et al., 1989). One of these two proteolytic activities is associated with the 430 amino acid (or 49K) small nuclear inclusion (NIa) protein encoded by an internal region of the TEV genomic ORF. The N-terminal 189 amino acids of the NIa polyprotein represent the genome-linked protein (VPg) moiety, and a proteolytic activity, similar to the picornaviral 3C activity, has been mapped to the C-terminal 240 amino acids (Carrington & Dougherty, 1987a; Murphy et al., 1990; Dougherty & Parks, 1991). The NIa protein processes itself out of a higher Mr, precursor by cleaving between specific glutamine-glycine dipeptides located at the N and C termini. These events have been shown to be obligatory self-cleavage reactions that probably occur co-translationally in cell-free translation studies (Carrington & Dougherty, 1987b). The NIa-flanking cleavage sites, and the internal NIa protein cleavage site, are not readily processed in a bimolecular or trans fashion in our standard co- or post-translational processing assays. This is in contrast to three additional TEV NIa cleavage sites which are efficiently processed by the TEV NIa proteolytic activity in reactions that are bimolecular (Carrington et al., 1988).

The requisites governing the trans cleavage events mediated by the NIa proteinase have been analysed and require a specific consensus cleavage sequence which spans the seven amino acid sequence E-X-V/I/L-Y-X-Q*S/G (X can be a variety of different amino acids) (Dougherty et al., 1988, 1989a). However, the requirements governing autocatalysis are not well understood. In this study we have used molecular genetics and cell-free expression systems to begin to examine the parameters that affect the self-cleavage reaction. The proteolytic activity associated with the TEV NIa protein was capable of releasing itself from any of seven
Fig. 1. A schematic representation of the recombinant DNA molecules used in this study. (a) The TEV genome is presented. The segments of the genome encoding particular gene products have been filled in differently. The Ms of the TEV gene products are presented. Below the genome are four regions of the RNA which have been transcribed into cDNA and used to construct the TEV recombinants. Mutations were introduced at the cleavage junctions and resulted in the generation of specific restriction endonuclease sites and codon changes as indicated. (b) The recombinant cDNA molecules described in (a) were used to generate the expression plasmids pTL-50/71, pTL-58/30 and pTL-combo. The restriction sites that were cleaved are indicated and the religated sequences and representative reading frames are shown. (c) The 49K NIa nucleotide sequence was ligated to two foreign nucleotide sequences. Plasmid pPROMag was generated by insertion of the coding sequence for a magainin peptide at the 3' end of the coding sequence of the NIa protein. For pVVPRO, the NIa coding sequence was inserted in-frame into the VV L4R ORF at an NaeI restriction site. pVVPRO + 6aa was similar, except the coding sequence for six amino acids of a TEV NIa cleavage site was introduced just before the codon for the N-terminal amino acid of the NIa protein.

polyproteins tested, with a complete seven amino acid cleavage consensus sequence at the N and C termini required for the most efficient cleavage.

Methods

Many of the plasmids used in this study have been described previously (see Fig. 1a). The TEV 49K NIa protein coding sequence and flanking 6K and Nib sequences were contained in the plasmid pTL-37/5473. Sequence coding for an inactive proteinase was contained in plasmid pTL-37/5473C339A (Carrington & Dougherty, 1987b). Terminal portions of the TEV 50K and 71K proteins and the junction between them were encoded by cDNA sequences contained in the plasmid pAS (Dougherty & Parks, 1989). The plasmid pTL-37/5473 contained a portion of the NIb coding sequence, the cleavage site between the NIb and capsid proteins, and the entire capsid protein (30K) coding sequence (Carrington & Dougherty, 1987a).

Generation of recombinant DNA molecules. To facilitate the construction of hybrid proteins containing the TEV NIa coding sequence, plasmid pTL-37/5473 was altered by site-directed mutagenesis (Taylor et al., 1985a,b) to generate a Smal restriction site at the 5' end of the 49K coding sequence and a PvuII restriction site at the 3' end of the NIa coding sequence. This altered the codons for the Q*G dipeptide (CAAGGG) where cleavage occurred to P-G (CCC-GGG, Sinai) at the 5' end of the coding sequence and to Q*L (CAG-CTG, PvuII) at the 3' terminus of the coding sequence (Fig. 1a). Digestion of this plasmid, referred to as pTL-37/5473B, with the restriction enzymes PvuII and Smal resulted in the formation of a blunt-ended cDNA fragment that contained the authentic NIa coding sequence exactly. In the plasmid pAS, a PvuII restriction site was introduced into TEV cDNA by site-directed mutagenesis, resulting in Q*S to Q*L codon changes (CAAGT to CAG-CTG, PvuII) at the 3' terminus of the coding sequence (Fig. 1a). Digestion of this plasmid, referred to as pTL-37/5473, with the restriction enzymes PvuII and Smal resulted in the formation of a blunt-ended cDNA fragment that contained the authentic NIa coding sequence exactly. In the plasmid pAS, a PvuII restriction site was introduced into TEV cDNA by site-directed mutagenesis, resulting in Q*S to Q*L codon changes (CAAGT to CAG-CTG, PvuII) at the 3' terminus of the coding sequence (Fig. 1a). Digestion of this plasmid, referred to as pTL-37/5473B, with the restriction enzymes PvuII and Smal resulted in the formation of a blunt-ended cDNA fragment that contained the authentic NIa coding sequence exactly. In the plasmid pAS, a PvuII restriction site was introduced into TEV cDNA by site-directed mutagenesis, resulting in Q*S to Q*L codon changes (CAAGT to CAG-CTG, PvuII) at the 3' terminus of the coding sequence (Fig. 1a). Digestion of this plasmid, referred to as pASB. In plasmid pTL-37/8595, the codons for the Q*S dipeptide, which delineated the junction between the Nib and capsid proteins, were altered such that a Smal site was introduced and the codons for a P-G dipeptide were generated. This resulting plasmid was referred to as pTL-37/8595B. Digestion of these three plasmids with PvuII or Smal resulted in the formation of various blunt-ended cDNA fragments which could be religated to maintain the translational reading frame. Thus, the TEV NIa protein could be positioned in different coding sequences, often with the regeneration of an authentic TEV cleavage site sequence. Schematic representations of the coding regions of recombinant
plasmids generated in this manner are presented in Fig. 1 (b) and are referred to as pTL-50/71, pTL-58/30 and pTL-combo. All three plasmids positioned the coding sequences downstream of a TEV 5' leader cassette to promote efficient translation in rabbit reticulocyte lysates (Carrington et al., 1987).

Repositioning of the Nla protein coding sequence into unrelated protein-coding sequences was influenced by the availability of convenient blunt-end restriction sites which allowed in-frame insertion of the TEV Nla coding sequence. Two plasmids were generated for this study: One plasmid, pPROMag, contained the coding sequence for magainin-2, a 23 residue peptide (Zasloff, 1987), positioned downstream and in-frame with the Nla coding sequence. This plasmid was constructed by ligation of a DNA fragment coding for magainin, which was generated by annealing two complementary oligonucleotides, into the Smal site of pTL37-5473/12C (Carrington et al., 1988). This resulting intermediate plasmid was then altered by site-directed mutagenesis to code for an Nla proteinase cleavage site at the Nla protein–magainin protein junction. A second recombinant molecule contained the vaccinia virus (VV) L4R gene which encoded a p25 late protein (Goebel et al., 1990). The Nla sequence, generated by digesting the plasmid pTL-37/5473B with Smal and PvuII, was inserted in-frame into the L4R nucleotide sequence at an NaeI restriction site. This resulted in the recombinant DNA molecule referred to as pVVPRO. A schematic representation of the coding region of pVVPRO is presented in Fig. 1 (c). Additionally, by site-directed mutagenesis, a TEV seven amino acid cleavage sequence (E-N-L-Y-F-Q*G) was generated at the 5’ terminus of the inserted TEV Nla coding sequence in pVVPRO. This plasmid was referred to as pVVPRO + 6aa.

For each of the hybrid Nla-containing plasmids presented in Fig. 1 (b and c), a similar recombinant plasmid was generated which would encode an inactive Nla proteinase (Zamains). Inactive proteolytic domains were generated by single amino acid changes, whereby an alanine replaced a cysteine (at TEV Nla amino acid position 339), or tyrosine substituted for histidine (at position 234) at key catalytic residues (Dougherty et al., 1989b). This is denoted in the text by "n" (In for inactive) following the plasmid designation.

Transcription, translation and processing assays. Plasmids were linearized with the restriction enzymes SalI (pVVPRO, pVVPRO n, pVVPRO + 6aa, opVVPRO + 6aa n) or NheI prior to transcription with T7 RNA polymerase. RNA transcripts were translated in a rabbit reticulocyte extract. Labelled products were detected by autoradiography. Trans processing assays were carried out as described previously (Carrington & Dougherty, 1987a). TEV nuclear inclusion bodies were used as the source of Nla proteolytic activity. The extent of processing was quantified using an Ambis Beta Scanning system (Automated Microbiological Systems) (Dougherty et al., 1988).

Results

The Nla proteolytic activity is released from the TEV genome-derived polyprotein in a rapid autocatalytic reaction as part of a 49K polyprotein. We sought to determine whether sequences adjacent to the Nla sequence were critical for this self-cleavage, or if the requirements for autocatalysis were contained within the Nla protein sequence. The strategy adopted and outlined in Fig. 1 permitted in-frame insertion of Nla-related sequences at foreign locations. These sequences were then expressed using cell-free systems. Self- or cis-processing (with a functional Nla proteinase) and trans processing (with an inactive Nla proteinase) of the artificial polypeptides were examined.

Placement of the TEV Nla proteolytic activity in the TEV polyprotein

We first asked whether the TEV Nla proteinase would be functional in a cis fashion at other TEV cleavage sites. In its normal location between the 6K and Nlb TEV proteins, the Nla protein rapidly cleaves itself from the genome-derived polyprotein precursor (Fig. 2a, lane 2). Fig. 2 presents the results of positioning the Nla protein sequence at two different naturally occurring TEV cleavage sites. Sequences coding for the proteolytically active and inactive Nla protein were inserted at the junction for the TEV 50K/71K gene products resulting in the plasmids pTL-50/71 and pTL-50/71 n (Fig. 1b). These recombinant plasmids encoded a 91K protein containing an authentic TEV cleavage site sequence at the N terminus of the inserted Nla sequence and an altered cleavage site at the C terminus, with leucine replacing the P'1 serine. (In this article we use the convention P1 position meaning -1 amino acid and P'1 position meaning +1 amino acid relative to peptide bond cleaved.) Expression of transcripts encoding the artificial polyprotein containing the functional Nla proteinase sequence in the presence of [35S]methionine resulted in radiolabelled precursors and products which could be separated by SDS–PAGE. The protein pattern suggested incomplete self-cleavage and the formation of a partially processed 59K product, the 49K Nla protein, the 32K portion of the 71K TEV gene product, and smaller products (doublet band at 27K and 28K and band at 21K) which have been shown to be Nla-related products (Fig. 2a, lanes 4 and 5). Quantifying the radioactivity associated with the different protein products suggested the C-terminal site was processed nearly to completion, whereas approximately 90% of the N-terminal sites were cleaved.

The Nla coding sequence also was inserted at the naturally occurring TEV Nlb–capsid protein cleavage site to generate the construct pTL-58/30. The predicted artificial polyprotein produced had an authentic TEV seven amino acid cleavage sequence at the C terminus of the Nla protein (Nla–30K junction) whereas the N-terminal junction (Nlb–Nla junction) had an altered cleavage site as a proline replaced the naturally occurring P1 glutamine. Transcription and translation of pTL-58/30 n transcripts resulted in the formation of a radiolabelled polyprotein with an estimated Mr of 85K whereas the recombinant molecule containing the
Fig. 2. Cis and trans cleavage of TEV polyproteins containing the TEV Nia protein. Transcripts derived from the cDNA recombinant molecules described in Fig. 1 (b) were translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. Radiolabelled products were separated by electrophoresis on a 12-5% polyacrylamide gel and detected by autoradiography. (a) Autoradiogram of cis-processed products. Cell-free translations were primed with transcripts derived from plasmids as follows: lane 1, no exogenous RNA added; lane 2, pTL-37/5473; lane 3, pTL-37/5473c339A; lane 4, pTL-50/71; lane 5, pTL-50/711n; lane 6, pTL-combo; lane 7, pTL-combo In. Lanes 8 to 10 are added as size references and represent translation products derived from: lane 8, pAS transcripts (Parks & Dougherty, 1989); lane 9, pAS with subsequent processing of the 32K precursor by TEV Nia proteinase to generate a 22K product; lane 10, the cell-free translation precursor of pTL-37/8595 which had been processed with TEV Nia proteinase to generate the 30K product shown. Mrs of prominent products are presented on the left and right sides of the figure. (b) Autoradiogram of trans-processing reactions. Radiolabelled translation products derived from pTL-37/5473; lane 3, pTL-37/5473c339A; lane 4, pTL-50/71; lane 5, pTL-50/711n; lane 6, pTL-combo; lane 7, pTL-combo In. Lanes 8 to 10 are added as size references and represent translation products derived from: lane 8, pAS transcripts (Parks & Dougherty, 1989); lane 9, pAS with subsequent processing of the 32K precursor by TEV Nia proteinase to generate a 22K product; lane 10, the cell-free translation precursor of pTL-37/8595 which had been processed with TEV Nia proteinase to generate the 30K product shown. M,s of prominent products are presented on the left and right sides of the figure. (b) Autoradiogram of trans-processing reactions. Radiolabelled translation products derived from pTL-37/5473c339A, pTL-50/711n and pTL-combo In were incubated without (lanes 3, 5 and 7 respectively) or with (lanes 4, 6 and 8 respectively) TEV Nia proteinase derived from TEV nuclear inclusion bodies. Other lanes contain: lane 1, no exogenously added RNA; lane 2, self-processed 49K product derived from pTL-37/5473 transcripts; lane 9, the 30K processed product derived from a precursor using pTL-37/8595 transcripts; lane 10, 32K unprocessed precursor derived from pAS transcripts.

Functional Nia proteinase sequence (pTL-58/30) generated two major products of 30K and 53K (data not shown). The 30K product was the TEV capsid protein, suggesting that processing was complete at the C-terminal Nia site. The 53K product represented Nib amino acid sequences that remained attached to the N terminus of the 49K Nia protein. These results suggested that cleavage at the N-terminal site did not occur.

A plasmid construction was made such that the resulting polyprotein would have foreign TEV sequences and functional heptapeptide cleavage sites at both the N and C termini of the inserted Nia protein. This plasmid, designated pTL-combo, positioned part of the 50K gene at the 5' end of the Nia gene insert and the entire 30K capsid protein gene at the 3' end. Translational expression of this hybrid TEV sequence containing a non-functional Nia proteinase (pTL-combo In) resulted in the formation of the expected 89K polyprotein. A radio-labelled protein of this size was not observed with the translation product derived from pTL-combo, as the Nia proteinase cleaved itself from the polyprotein as a 49K protein and generated the 30K coat protein product (Fig. 2a, lanes 6 and 7). This protein pattern suggested cleavage was complete at the N and C cleavage sites. Provided the seven amino acid cleavage site sequence was present at the N or C terminus, the Nia protein was able to process itself from TEV sequences.

We next asked whether a proteolytically inactive form of the Nia protein could be cleaved from higher Mr precursors in a bimolecular or trans reaction. One feature of cleavage sites flanking the Nia protein in its normal location is that they are refractory to cleavage in a bimolecular reaction (Fig. 2b, lanes 3 and 4) (Carrington & Dougherty, 1987b). Radiolabelled substrates derived from pTL-50/711n, pTL-58/301n and pTL-combo1n were made and incubated with TEV nuclear inclusion bodies. The samples were then analysed by SDS-PAGE for processing. The pTL-50/711n-derived 91K precursor polyprotein, containing the inactive Nia proteinase, was processed poorly in the trans reaction and only at the N-terminal site. This is presented in Fig. 2(b), lanes 5 and 6. The polyprotein product derived from the plasmid pTL-58/301n was also used as a substrate. In this case, the C-terminal Nia cleavage site was partially processed by the proteinase provided in trans, whereas the N-terminal Nia cleavage site containing a P1 proline was not processed in trans (data not shown). Finally, trans processing of the pTL-combo1n-derived polyprotein was also examined. Cleavage at the C-terminal site was evident (approx. 60% of the sites were processed) whereas processing at the N-terminal site was limited (less than 10%). Smaller Mr products represent cleavage and further processing of the internal Nia protein site.
Table 1. *Summary of NIa proteinase processing experiments: placement in the TEV polyprotein*

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<tr>
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<tr>
<td>pTL-58/30</td>
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<tr>
<td>pTL-50/71</td>
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<td>Complete</td>
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<td>pTL-combo</td>
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* Schematic representation of the hybrid constructs and their designation used in this study. Each TEV gene is labelled using the format described in Fig. 1. The construct which expresses a polyprotein containing a non-functional NIa proteinase is presented below each recombinant molecule shown. Proteinase gene sequences which encode an inactive 49K proteolytic activity are indicated (C/A). The resulting dipeptides which mark the gene boundaries are indicated below the line. All coding sequences were placed downstream of a cDNA copy of the TEV 5' untranslated leader and initiating codon.

† Results from processing studies are presented for autocatalysis (cis) and for bimolecular cleavage (trans) of the polyprotein displayed. Cleavage at the N and C termini was monitored and the degree of processing is described. The percentage of processing was determined by quantifying the radioactivity associated with products, precursors and intermediate products. ND, Not detected; ‘-‘, unable to test.

These cis and trans processing results are summarized in Table 1.

Placement of the TEV 49K NIa polyprotein in foreign protein sequences

The experiments above located the NIa protein at previously described TEV cleavage sites. These cleavage sites, although distinct from those which flank the NIa protein, could contain structural motifs associated with all TEV cleavage sites. Therefore, gene sequences distinct from the TEV coding sequence were selected for fusion to the NIa coding sequence in order to assay for autocatalysis (Fig. 1c).

A small DNA fragment containing the coding sequence for a magainin-2 peptide (23 amino acids) (Zasloff, 1987) was ligated to the 3' terminus of two 49K coding sequences, coding for either a functional or non-functional NIa proteinase. pPROMag and pPROMag<sup>ln</sup> reconstituted the heptapeptide TEV cleavage sequence at the gene fusion site. Expression of the hybrid protein derived from pPROMag<sup>ln</sup> transcripts, containing an...
domain were translated in a rabbit reticulocyte cell-free system in the polymerase-generated transcripts from the following plasmids were used to programme the rabbit reticulocyte cell-free translation system. Lane 1, no exogenous RNA transcripts added; lane 2, pTL-37/5473; lane 3, pTL-37/5473c339A; lane 4, pPROMag; lane 5, pPROMagln; lane 6, pVVPRO; lane 7, pVVPROln; lane 8, pVVPRO + 6aa; lane 9, pVVPROln + 6aa; lane 10, no exogenous RNA added. Mrs of major products formed are presented on the left and right sides of the figure. (b) Autoradiogram of trans-processing reactions. Polyproteins containing a non-functional form of the NIa proteinase were incubated for 2 h with TEV nuclear inclusion bodies as a source of TEV NIa proteolytic activity. The precursors and processed products were separated by SDS-PAGE and detected by autoradiography. Cell-free translation products were derived as follows: lanes 1, 7 and 12, no exogenous RNA added; lane 2, pTL-37/5473 transcripts; lanes 3 and 4, translation products of pTL-37/5473c339A transcripts incubated without or with TEV NIa proteinase added in trans, respectively; lanes 5 and 6, translation products of pPROMag transcripts without and with added TEV NIa; lanes 8 and 9, translation products of pVVPROln incubated without and with TEV NIa proteinase; lanes 10 and 11, translation products of pVVPROln + 6aa incubated without and with TEV NIa proteinase. The Mrs of the major products are presented on the left and right sides of the figure.

inactive TEV NIa proteinase fused to the magainin sequence, resulted in the formation of the expected 65K polyprotein (Fig. 3a, lane 5). A similar plasmid construct (pPROMag), coding for a functional NIa proteinase, resulted in the formation of the 65K precursor which underwent rapid processing to generate a 49K protein. (Fig. 3a, lane 4).

The nucleotide sequences encoding an active or inactive form of the TEV NIa proteinase also were inserted into the VV L4R gene which encodes a 25K late protein (Goebel et al., 1990). These recombinant plasmid constructs, pVVPRO and pVVPROln (Fig. 1c), when transcribed and translated resulted in the formation of hybrid polyproteins which possessed an authentic TEV seven amino acid cleavage site only at the C terminus of the inserted NIa protein (Fig. 1c). The polyprotein containing the inactive NIa proteinase resulted in the formation of the expected 66K precursor (Fig. 3a, lane 7). Expression of the sequences containing a functional form of the NIa proteinase resulted in the formation of the 66K precursor and partial cleavage at the C-terminal cleavage site to generate a 52K product (Fig. 3a, lane 6). These two recombinant DNA molecules were modified further such that the nucleotides coding for a six amino acid sequence were added to the coding sequence just upstream of the region coding for the N terminus of the NIa protein. This insertional mutagenesis resulted in the codons for an authentic TEV cleavage site being added to the N terminus of the NIa protein gene. Translational expression of these sequences (pVVPROln + 6aa) resulted in the formation of a 66K precursor, whereas translation of pVVPRO + 6aa transcripts formed a 49K protein and smaller Mr products (Fig. 3a, lanes 8 and 9). This suggested cleavage had occurred at both the N and C termini.

Trans-processing reactions using polyproteins containing the inactive form of the NIa proteinase and authentic cleavage sites at the C, or N and C termini, were conducted. The 65K pPROMagln-derived polyprotein was incubated with NIa proteinase provided in the form of nuclear inclusion bodies. Processing at the N- and C-terminal sites was detected as the 49K protein accumulated after the 2 h incubation period. Approximately 20% of the precursor was processed in trans (Fig. 3b, lanes 5 and 6). Limited cleavage of the pVVPROln-derived polyprotein was detected with approximately 5% of the substrate cleaved (Fig. 3b, lanes 8 and 9). In contrast, the pVVPROln + 6aa-derived polyprotein was processed at the N-terminal site (about 50% of the sites were cleaved) and to a limited degree (about 10%) at the C-terminal site (Fig. 3b, lanes 10 and 11).

Therefore, in a foreign protein setting, the NIa proteolytic activity was able to excise itself from the polyprotein provided a TEV seven amino acid cleavage sequence was present at the N and/or C termini. Trans processing of these sites proceeded to a limited degree and was influenced by the flanking amino acid sequence.
Table 2. Summary of NIa proteinase processing experiments: placement in foreign proteins*

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<td>pVVPRO+6aa</td>
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<td>cleavage site (6aa) + 49</td>
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* The recombinant molecules used in this study are described in Fig. 1. See Table 1 for abbreviations. The pPROMag construct has a single codon change in which the histidine (H) at 49K amino acid position 234 was changed to a tyrosine (Y).

These cis- and trans-processing results are summarized in Table 2.

Discussion

This study demonstrated that the autocatalytic or self-cleavage reaction of the TEV 49K polyprotein required the 49K NIa proteolytic activity and an authentic TEV heptapeptide cleavage sequence at the N and C termini. For hybrid molecules in which the 49K protein and N- and C-terminal cleavage sites were placed in different TEV sequences or in foreign proteins, amino acid sequences found outside the cleavage sites did not appear to have an effect on the autocatalytic release of the NIa protein from a higher Mr precursor. This was illustrated by the results with pTL-37/5473, pTL-combo and pVVPRO+6aa. Providing only a single functional cleavage sequence at the N or C terminus usually resulted in complete cleavage at this site. This implied that flanking sequences could be present at one terminus and autocatalytic cleavage would occur at the opposite site. Proper folding for proteolytic activity and autocatalysis would appear to be a function dictated by the NIa protein in these studies.

The amino acid sequences which constituted the cleavage site at the N or C terminus had a major influence on autocatalysis. As we have observed in trans-processing studies (Dougherty et al., 1989a), the consen- sus TEV cleavage sequence, E-X-V/I/L-Y-X-Q*G/S, was crucial for autocatalysis. An analysis of the seven polyproteins generated in this study revealed that whenever the 'optimal' cleavage site consensus sequence was maintained, cleavage was rapid and complete, or nearly complete, as over 95% of the substrate cleavage sites were processed. In the complete absence of this site, no cleavage was detected. The importance of the consensus cleavage site sequence in autocatalysis was best illustrated with the polyprotein derived from pVVPRO, which completely lacked this sequence at the N terminus, resulting in no cleavage at this position. However, cleavage at this junction was efficient when a seven amino acid cleavage sequence was introduced at the N terminus (pVVPRO+6aa). This result was similar
to those obtained when an artificial TEV cleavage site was generated in the analysis of TEV trans-processing (Carrington & Dougherty, 1988). Alteration of the 'optimal' consensus cleavage sequence resulted in a reduced amount or elimination of cleavage. In the case of the polyprotein derived from pTL-50/71, the C-terminal cleavage sequence was altered at the P1 position with glycine replaced by leucine. Cleavage proceeded efficiently with this substitution. Previous studies examining trans cleavage have shown that a variety of amino acids can be tolerated at the P1 position (Dougherty et al., 1988). Synthesis of the polyprotein derived from pTL-58/30 replaced the P1 glutamine with a proline residue, resulting in the elimination of cleavage at the N-terminal cleavage site. This observation was consistent with previous results in which trans and cis cleavages were eliminated by most single amino acid replacements at the P1 position (Dougherty et al., 1988). Collectively, these results suggested cleavage site requirements for cis cleavage will be similar to those described for the trans cleavage reaction.

Analysis of the TEV NIa autocatalytic activity reveals a number of similarities to and differences from RNA viruses that use a similar protease during genome expression. Animal picornaviruses and plant comoviruses, all members of the picorna-like virus supergroup, have co-evolved such that their genomes are organized and expressed in a similar fashion (Goldbach, 1987; Strauss & Strauss, 1988). The proteolytic activity responsible for most of the cleavage events required to process the genome-derived polyprotein is encoded by an internal portion of each of the RNA genomes and is initially expressed as part of a higher M, polyprotein (for reviews see Harris et al., 1990; Goldbach, 1990; Palmenberg, 1990). All three viral proteases share extensive amino acid similarities and are predicted to have a structure which mimics that of members of the serine proteinase family (Bazan & Fletterick, 1988, 1990). In the case of picornaviruses, the proteolytic activity is associated with a 3C protein, but the P3 precursor (3ABCD) and the 3CD polyprotein have been implicated to be crucial in a number of cleavage events (Ypmawong et al., 1989; Parks et al., 1989). In the study of the comovirus cowpea mosaic virus, a 24K proteinase has been identified (Verrier et al., 1987). However, higher M, polyproteins which contain this proteolytic activity can be readily detected. Additionally, a 32K scaffolding protein has been implicated as necessary for particular cleavage events associated with the polyprotein derived from M-RNA (Vos et al., 1988). In the case of potyviruses, it appears that most of the NIa proteinase is released as part of a stable 49K protein in plant and cell-free studies. The NIa protein analogue in the comovirus and picornavirus system would be the VPg–proteinase precursor (i.e. the picornaviral 3B–3C polyprotein or the comoviral 3K–24K polyprotein). The release of this polyprotein in TEV cell-free studies was much more rapid than that observed for similar polyproteins in these other RNA virus systems. Previous studies with the TEV NIa proteinase have revealed a similar proteolytic activity regardless of the polyprotein the proteinase was 'locked into' (Parks et al., 1992). It may be that in evolving a highly ordered cleavage site sequence, potyviruses are not dependent on accessory proteins or various polyprotein forms required to mediate specific processing events in other plant and animal virus systems.

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References


TEV NIa proteinase autocatalysis


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