Cleavage profiles of tobacco etch virus (TEV)-derived substrates mediated by precursor and processed forms of the TEV NIa proteinase

T. Dawn Parks, 1 Holly A. Smith 1 and William G. Dougherty 1,2,∗

1Department of Microbiology and 2 The Center for Gene Research and Biotechnology, 220 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3804, U.S.A.

Nucleotide sequences coding for proteins containing the tobacco etch virus (TEV) NIa proteinase were generated by polymerase chain reaction amplification and/or site-directed mutagenesis. These coding regions contained sequences for the proteinase alone or as part of higher Mr precursors. Following transcription and translation of these sequences in a cell-free system, the various polyproteins, all containing an active small nuclear inclusion protein (NIa) proteinase, were used to process a TEV substrate series. Most substrates were processed in a similar fashion by all proteolytic forms. However, one substrate which contained the TEV 50K/71K protein junction was differently processed by several of the polyproteins containing NIa proteinase. Substrates which previously had no identified TEV NIa proteinase cleavage sites also were tested and were not cleaved by any of the proteinase-containing polyprotein forms.

Introduction

Differential processing of particular cleavage sites by viral polyproteins containing proteinase has been proposed to be important in the replication cycle of a number of animal viruses. In the alphavirus family, there is a clear pathway for polypeptide processing by precursors containing the nsP2 proteinase. It has been hypothesized that this differential processing scheme is responsible for regulation of RNA synthesis (for review, see Strauss & Strauss, 1990). The involvement of proteinase precursors in the differential cleavage of polyproteins has also been demonstrated with picornaviruses. During poliovirus genome expression, the 3CD precursor form of the 3C proteinase is required for processing of the P1 polyprotein into the VP0, VP1 and VP3 capsid proteins. Other members of the picornavirus group may require 3C proteinase alone or as part of the 3ABC polyprotein for maturation of the structural P1 polyprotein into capsid proteins (reviewed by Harris et al., 1990; Palmenberg, 1990).

The ability of a plant viral proteinase to process different cleavage sites, with varying cleavage profiles, has been demonstrated with the proteolytic activity associated with the small nuclear inclusion protein (NIa) of tobacco etch virus (TEV) (Dougherty & Parks, 1989). TEV differential processing is determined by those particular amino acids found in the cleavage site heptapeptide consensus sequence, and does not appear to be influenced by amino acid sequences which flank the cleavage site.

This study was conducted to determine whether the TEV NIa proteinase domain or polyproteins containing this proteolytic activity differentially processed substrates derived from the TEV genome. In the TEV proteinase study cited above, the source of proteolytic activity was the 49K NIa derived from partially purified nuclear inclusion bodies isolated from TEV-infected plants. This 49K protein has recently been shown to be a polyprotein containing the domain for the VPg (viral protein, genome-linked) moiety in the N-terminal portion, in addition to a proteinase function which resides in the C-terminal portion of the polyprotein (Murphy et al., 1990; Dougherty & Parks, 1991). In the study described here, TEV NIa-associated proteolytic activity was generated in several contextual forms and tested in a cell-free system. These proteolytic activities were used to process various TEV-derived substrates. These studies indicated that, for most TEV substrates, there were no detectable differences in cleavage by various TEV polyprotein proteinases. However, one substrate did exhibit differential cleavage profiles for several proteolytic forms. Based on observations made in the cell-free studies, we would propose that, unlike the polioviral 3CD proteinase system, the TEV system would not have a prerequisite for particular proteinase precursors in the processing of its genome-derived polyprotein.

Methods

Construction of plasmids containing proteinase coding sequences. All proteinase coding sequences used in this study were generated by polymerase chain reaction (PCR) amplification (Erlich, 1989) of TEV...
sequences previously described, and are shown in Fig. 1. The proteinase coding sequence found in plasmid pTL-5473 (Carrington & Dougherty, 1987a), was used as a template for construction of plasmids pN49 and pN27, with pTL-5473CA (Carrington & Dougherty, 1987b) used in generating pN49CA. The TEV coding region contained in pTL5473-11N (Carrington et al., 1988), which had a mutation inactivating the cleavage site which delineates 6K from 49K amino acid sequence, was used in creating plasmid p6 + 49. Two other plasmids with altered cleavage site coding sequences were used as sources of TEV cDNA. Plasmids pTL5473-12C (with an altered C-terminal 49K cleavage site) and pTL5473-11N/12C (containing altered N- and C-terminal 49K cleavage sites (Carrington et al., 1988)) were cleaved with the restriction enzymes Sall (TEV nucleotide 7166) and PstI (vector sequences). A cDNA fragment corresponding to TEV nucleotides 7166 to 9176 was then inserted. The resulting proteinase transcripts were then translated in the presence of [35S]methionine in a rabbit reticulocyte lysate as described by Dougherty & Hiebert (1980), and protein production was quantified by TCA precipitation. Proteinase transcripts were then translated in the presence of unlabelled amino acids, and substrate molecules were translated in the presence of labelled methionine. Based on TCA-precipitable counts, equimolar amounts of each proteinase were incubated with various substrates in a 5:1 ratio of proteinase to substrate.

Aliquots were taken at 5, 10, 15, 30, 60, 90 and 120 min after the addition of proteinase for reactions using substrates derived from pAS-58/30, pAS and pTL37-8595. Additionally, substrates were incubated for 120 min with TEV Nia proteinase in the form of a partially purified nuclear inclusion body preparation. Substrates and products were separated on 12.5% polyacrylamide gels containing SDS using the buffer system of Laemmli (1970). The gels were impregnated with ENHANCE (NEN), dried and exposed to X-ray film. Autoradiographs of these gels were scanned using a Zeineh soft laser scanning densitometer, and the amount of substrate present as product was

```plaintext
plasmids pN27 and pN27 + 58 were generated using a 5' oligonucleotide primer which created an NcoI restriction site corresponding to nucleotides 6251 to 6257 of the TEV genome. Two 3' oligonucleotide primers were used. One corresponded to vector sequences and was used in generating pN49, pN49CA, p6 + 49 and pN27. A second oligonucleotide created a PstII restriction site at the coding sequence corresponding to the 58K/30K protein junction and was used in the synthesis of p27 + 58, p49 + 58 and p6 + 49 + 58. Standard recombinant DNA techniques (Sambrook et al., 1989) were used to insert the PCR-generated products described above into the transcription vector pTL27N. This plasmid vector was similar to pTL17 described previously (Carrington et al., 1987), except that an origin of replication for single-stranded DNA production was inserted and a site-directed mutation creating an NcoI restriction site at the ATG translation start codon (TEV nucleotides 43 to 145) was introduced (Taylor et al., 1985a, b).

Construction of plasmids used in substrate production. Six plasmids were used for the production of substrate molecules in this study and are shown in Fig. 3(a). Three of these plasmids (pAS, pAS-58/30 and pTL37-8595) have been described previously and contain cleavage sites recognized by the Nia proteinase (Dougherty & Parks, 1989). Briefly, pAS contained sequences corresponding to the region of the TEV genome surrounding the 50K/71K cytoplasmic cylindrical inclusion (CI) protein junction. The plasmid pAS-58/30 was similar, except that it contained a cleavage site sequence which had been mutated such that it coded for the seven amino acid cleavage site sequence found at the junction of the 58K and capsid (30K) proteins. This cleavage sequence was found in its normal polyprotein context in pTL37-8595. Two substrates derived from pTL0036 and pN71 did not contain any previously identified TEV Nia protease cleavage sites. The plasmid pTL0036 contained coding sequences from the 5' region of the TEV genome, which included an 87K polyprotein [31K and 56K helper component-proteinase (HC-Pro)] and a 50K protein, with a TGA termination codon insertion at the 3' end of the 50K coding sequence. The coding sequence of the 71K CI protein (nucleotides 3634 to 5532) found in pN71 was generated by PCR amplification using primers which created an NcoI restriction site, an AUG start codon at the 5' end and a stop codon at the 3' end. The inactive proteinase molecule described above (pN49CA) also served as a substrate source. Diagrams of these molecules are shown in Fig. 3(a).

Transcription, translation and processing. Following linearization with restriction enzymes, phenol extraction and ethanol precipitation, all plasmid DNAs were transcribed with T7 RNA polymerase as described (Carrington & Dougherty, 1987a). After ethanol precipitation, aliquots of all transcripts were translated in the presence of [35S]methionine in a rabbit reticulocyte lysate as described by Dougherty & Hiebert (1980), and protein production was quantified by TCA precipitation. Proteinase transcripts were then translated in the presence of unlabelled amino acids, and substrate molecules were translated in the presence of labelled methionine. Based on TCA-precipitable counts, equimolar amounts of each proteinase were incubated with various substrates in a 5:1 ratio of proteinase to substrate.

Aliquots were taken at 5, 10, 15, 30, 60, 90 and 120 min after the addition of proteinase for reactions using substrates derived from pAS-58/30, pAS and pTL37-8595. Additionally, substrates were incubated for 120 min with TEV Nia proteinase in the form of a partially purified nuclear inclusion body preparation. Substrates and products were separated on 12.5% polyacrylamide gels containing SDS using the buffer system of Laemmli (1970). The gels were impregnated with ENHANCE (NEN), dried and exposed to X-ray film. Autoradiographs of these gels were scanned using a Zeineh soft laser scanning densitometer, and the amount of substrate present as product was
were incubated together at 30 °C for 2 h and 24 h. For samples which terminated by the addition of RNase A. Proteinases and substrates pTL0036 and pN71 were treated slightly differently. Translations were incubated for 24 h, PMSF was added to a final concentration of 0.5 mM. Substrates and products were separated using SDS-PAGE and gels were visualized by fluorography.

Results

The six proteinase sources used in this study presented the NIa proteolytic activity in different contextual forms (Fig. 1). These forms included the 27K NIa proteinase domain expressed alone and five additional sources which presented the proteolytic activity in TEV polyprotein precursor forms. These five polyproteins were generated from plasmids containing site-directed mutations (single amino acid changes) which eliminated cleavage sites and consisted of the following: (i) a 49K NIa polyprotein [(21K VPg and 27K NIa proteinase activity), p49], (ii) 49K NIa proteinase with the additional amino acids of the 6K protein product at the N terminus (p6 + 49), (iii) 49K polyprotein with the 58K putative replicase sequences attached at the C terminus (p49 + 58), (iv) a 6K–49K–58K polyprotein (p6 + 49 + 58) and (v) the 27K NIa proteolytic domain with the 58K protein at the C terminus (p27 + 58). After in vitro transcription and translation in a rabbit reticulocyte lysate containing [35S]methionine, aliquots of each translation were analysed by SDS-PAGE. Each proteinase form containing the TEV NIa proteolytic activity were precipitated with TCA and radioactivity was counted. Based on the level of incorporation and the number of methionine residues, containing polyprotein were used in processing reactions.

We investigated whether TEV cleavage sites, demonstrated to be processed in trans by the proteolytic activity associated with the TEV NIa polyprotein (49K), would be processed differently by the various artificial polyprotein sources described above. Processing reactions using substrates derived from pAS, pAS-58/30 and pTL37-8595 were conducted. Substrate precursor and processed products were separated by PAGE and visualized by fluorography, with examples shown in Fig. 2(a). For pAS and pAS-58/30, the 32K substrate precursor was converted over time into 20K and 12K (not detected in our gel system) products. These autoradiographs were subjected to densitometric analysis. The percentage of substrate converted to product was determined for each time point sample and is presented for each proteinase in Fig. 2(b). These graphs represent the mean percentage values of processed product at each time point, based on four or five independent processing reactions. For processing reactions in which pAS-58/30 was used as a substrate source, each proteinase appeared to cleave the substrate to generate a similar cleavage profile. This also was the case with processing of pTL37-8595 (data not shown).

In contrast, there was differential processing of the cleavage site contained in the pAS substrate source when the various proteinase forms were analysed. In these processing reactions, the p49 + 58-derived proteinase appeared to process the pAS substrate at an enhanced rate, while the pN27-derived proteinase processed somewhat slower. The other four proteinase forms were similar and intermediate in processing efficiency. These results are presented in Fig. 2. The cleavage profiles generated by proteinases derived from p49 + 58 and pN27 were statistically faster and slower, respectively, when compared to the other proteinases (P < 0.001).

Another substrate molecule, encoded by sequences contained in the plasmid pN49C/A, consisted of amino acid sequences corresponding to the 6K protein linked to a proteolytically inactive NIa 49K polyprotein and 62 amino acids of the amino terminus of the large nuclear inclusion protein (NIb) (58K). This molecule contained two cleavage sites (6K/49K and 49K/NIb junctions) normally processed in an autocatalytic or cis reaction, as well as an inefficiently processed internal cleavage site which delineates the VPg and NIa proteinase domains. Processing of this precursor molecule by nuclear inclusion bodies proceeded slowly, with limited processing evident after 2 h. Some processing was evident after 24 h, with approximately half of the sample remaining in the 62K unprocessed form, as shown in Fig. 3(b). Polyacrylamide gel electrophoretic analysis of the cleavage products of this reaction indicated that limited cleavage occurred at both sites normally processed in cis, as well as at the internal site in the 49K proteinase form. The predicted Ms of the bands shown in Fig. 3(b) correspond to 62K for the uncleaved substrate, 56K representing the product formed by cleavage at the N terminus of the 49K polyprotein, 49K representing cleavage at both the N and C termini of the 49K polyprotein, 34K representing cleavage at the internal site only (C-terminal fragment), 27K for a doublet representing the product formed by cleavage at only the internal site (N-terminal fragment) and the 27K proteinase fragment, and 21K representing the product formed by cleavage at the N terminus and internal site. Cleavage profiles similar to that observed for nuclear inclusion bodies were exhibited when the pN49C/A substrate was incubated with the six proteinase forms (data not shown).
We subsequently investigated whether there were existing cryptic cleavage sites in the TEV polyprotein which could be processed by only one of the different NIA proteinase sources. Several additional substrates were tested for the ability to be processed by the six proteolytic forms. Two of these, derived from plasmids pTL0036 and pN71, contained sequences corresponding to the 31K–HC-Pro–50K TEV polyprotein and the 71K TEV cytoplasmic inclusion protein, respectively. The cDNA contained in these two particular plasmids did not code for a previously experimentally identified NIA proteinase cleavage site. Plasmid pTL0036 did contain sequences which coded for an active HC-Pro. Translation products derived from this plasmid were autocatalytically processed to generate 87K and 50K products, and the pN71 translation product migrated at a position corresponding to 71K. The substrates generated from these two plasmids were incubated for 2 h with the six
proteinase forms, and with the proteolytic activity associated with TEV nuclear inclusion bodies. There was no evidence that processing by any of the NIa proteolytic forms had occurred. Even after 24 h incubation with the six proteinase forms or with TEV nuclear inclusion bodies, there was no apparent cleavage of either substrate. Incubation of these substrates for 24 h with five times the normal amount of proteinase also did not result in any detectable processing (Fig. 3b).

Discussion

We have used a cell-free processing system to examine the possible role of processing by TEV NIa proteinase precursors. This experimental system has been used previously to determine that specific amino acids within TEV NIa cleavage sites affect the rate at which cleavage occurs (Dougherty & Parks, 1989). It has been proposed that these varying rates of cleavage may be important in the production of various viral proteins, hence providing a mechanism for regulation of gene expression. This study was undertaken to examine the possibility that an additional level of control over protein production might exist, whereby differential processing by proteinase precursor forms could enhance or inhibit the production of specific viral proteins.

The results of this study with various TEV NIa proteinase forms indicated that, for most substrates, all tested proteinase forms cleaved with essentially equivalent profiles. The substrates derived from pAS-58/30 and pTL37-8595 were cleaved by all six proteinase activities to generate similar processing profiles. Both of these substrates contain the seven amino acid cleavage sequence E-N-L-Y-F-Q/S, which has been identified previously as a rapidly processed TEV cleavage site sequence (Dougherty & Parks, 1989; Dougherty et al., 1989). However, different reaction profiles were consistently observed when substrate derived from pAS was tested. The substrates derived from pAS and p2AS-30 are identical except for three amino acid differences localized within the heptapeptide cleavage sequence. The cleavage sequence (E-I-I-Y-T-Q/S) encoded in pAS has been demonstrated to be a slowly processed site. In cleavage assays utilizing pAS-derived substrate, two of the proteolytic forms showed somewhat different efficiencies. Proteinase derived from p49 + 58 cleaved the pAS substrate more rapidly and to a greater extent than had been observed when either TEV nuclear inclusion bodies or other polyprotein forms were used as a proteinase source. Essentially, p49 + 58 proteinase cleaved the slowly processed site of pAS with the same profile as the rapidly processed site sequence (with averages of 81% and 84% substrate converted to product after 2 h, respectively). The proteinase derived from pN27 cleaved the pAS substrate more slowly and to a lesser extent than any other proteinase tested over the 2 h assay period. This was considerably slower than the processing exhibited when this same proteinase was used in cleavage assays with pAS-58/30-derived substrate (average cleavages of substrate to product of 26% and 82%, respectively).

It can only be speculated as to why the different proteinase forms vary in their ability to cleave the pAS substrate and yet cleave the pAS-58/30 translation product with similar profiles. This suggests that the three amino acid differences which are proximal to the cleavage site are important in the processing phenotypes observed. One possibility is that the pAS-58/30 cleavage site is so efficiently recognized and processed that differences in proteinase form are not important and cleavage proceeds regardless of the specific proteinase.
We would suggest that the pAS-58/30 cleavage site takes on a conformation which maximizes proteinase:substrate interaction at the heptapeptide cleavage sequence, relegating the rest of the substrate molecule to an indifferent state, unimportant in defining cleavage.

In the case of the pAS substrate, a suboptimal configuration may be assumed by the heptapeptide sequence, and secondary interactions, not important in pAS-58/30 cleavage, come into play. Additional sequences contained in the proteinase polyprotein might interact with the substrate, or with other regions of the polyprotein, to enhance or inhibit proteinase-substrate contact. Thus, various proteinase polyproteins might have different affinities for a given substrate. The final cleavage profile obtained would reflect the summation of these various interactions.

Two substrates, derived from pTL0036 and pN71, which had no known NIa proteinase cleavage sites, were incubated with the polyprotein proteinase forms to determine whether cryptic cleavage sites existed which could be cleaved by only one of these precursor forms. A possible cleavage site in the carboxyl-terminal portion of the TEV 50K protein (TEV amino acids 1110 to 1111) has been proposed by Lain et al. (1989). We found no cleavage by any proteinase form, even when NIa proteinase was added in a large excess to the substrate synthesized from pTL0036, as shown in Fig. 3(b). This putative cleavage sequence is present in substrates derived from pAS-58/30 and pAS, but we were also unable to detect a second cleavage event in either of these substrates (data not shown). It is unlikely that a functional NIa cleavage site is located in this region of the TEV genome-derived polyprotein. However, we cannot rule out the possibility that substrate molecules larger than those used in this study are necessary to direct cleavage, as has been shown in processing studies of the poliovirus P1 polyprotein (Ypma-Wong & Semler, 1987). Additionally, this region of the 50K protein may be located on the interior of the protein and be inaccessible to the proteinase in the absence of an accessory factor. This factor, not present in our cell-free processing system, may be required to facilitate cleavage, as has been demonstrated for cowpea mosaic virus (Vos et al., 1988).

In the poliovirus system, proteolytic processing by proteinase precursor forms has been shown to be important in cleavage of viral polyproteins in the generation of specific viral proteins. In some picornaviruses (poliovirus), the 3CD polyprotein is required for efficient cleavage during the generation of capsid protein subunits VP0, VP3 and VP1 (Jore et al., 1988; Ypma-Wong et al., 1988), while other members of this virus family (genera aphtho- and cardiovirus) appear to produce capsid proteins quite efficiently via cleavage by 3C proteinase alone, or as a component of a larger polyprotein such as 3ABC (reviewed by Harris et al., 1990; Palmenberg, 1990).

In comparing the results presented here with the picornaviral studies, several similarities and differences are apparent. Both virus groups make extensive use of a proteinase in a polyprotein form. In the case of potyviruses, the 49K NIa protein is a VPg-proteinase or 3BC polyprotein; in picornaviruses it is often a proteinase-replicase (3CD) polyprotein. In the poliovirus studies, precursor proteinase forms are necessary to mediate the cleavage required to generate capsid protein subunits, while the single cleavage necessary to generate the TEV capsid appears to proceed efficiently with a variety of proteinase forms, although the potyvirus 49K polyprotein is probably involved in vivo. The cleavage of the pAS substrate by the various proteinase forms reflects differences in the rate and extent of processing, not an absolute requirement for a particular proteinase form. This has also been exhibited in in vitro studies of poliovirus, where the 3C proteinase, when added in large excess, was able to cleave the P1 precursor (Nicklin et al., 1988). The differential processing of the 50K/71K cleavage site sequence found in pAS-derived substrate would be analogous to a faster cleavage by a 3BCD precursor (p49 + 58), with slower cleavage by the 3C proteinase (pN27).

It must be emphasized that the cell-free studies reported here are based on artificial proteinase forms, which may or may not reflect proteins actually present in significant amounts in infected cells. With this caveat in mind, we would suggest that during TEV genome expression and replication, autocatalysis of the 49K NIa proteinase from the genome-derived polyprotein is essentially complete and likely to be cotranslational, with larger precursor molecules usually undetectable. The cleavage of the 49K protein to the N-terminal VPg and C-terminal 27K proteinase proceeds much more slowly. Therefore, it is probable that in TEV-infected cells, cleavage of the genome-derived polyprotein is directed by the abundant 49K polyprotein or 27K NIa proteinase and that larger polyprotein precursors play a minimal role in gene expression.

We gratefully acknowledge Stephen S. Whitehead for his invaluable assistance with the statistical analysis. This work was supported by a grant from the Department of Energy to W.G.D. This is Oregon Agricultural Experiment Station Paper number 9637.

References


(Received 8 July 1991; Accepted 23 September 1991)