Modulation of the RNA Binding and Protein Processing Activities of Poliovirus Polypeptide 3CD by the Viral RNA Polymerase Domain*

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To study the role of the RNA polymerase domain (3D) in the protease activity of poliovirus polyprotein 3CD, we generated recombinant 3C and 3CD polyproteins and purified them to near homogeneity. By using these purified proteins in vitro cleavage assays with structural and non-structural viral polyprotein substrates, we found that 3CD processes the poliovirus structural polyprotein precursor (P1) 100 to 1000 times more efficiently than 3C processes P1. We also found that trans-cleavage of other 3CD molecules and sites within the non-structural P3 precursor is more efficiently mediated by 3CD than 3C. However, 3C and 3CD appear to be equally efficient in the processing of a non-structural polyprotein precursor, 2C3AB. Four mutated 3CD polyproteins with site-directed lesions in the 3D domain of the proteinase were analyzed for their ability to process viral polyprotein precursors and to form a ternary complex with RNA sequences encoded in the 5′ domain of the viral genome. Analysis of mutated 3CD polyproteins revealed that specific mutations within the 3D domain affect differential effects on 3CD activity. All four mutated 3CD proteins tested were able to process the P1 structural precursor with wild type or near wild type efficiency. However, three of the mutated enzymes demonstrated an impaired ability to process some sites within the P3 non-structural precursor, relative to wild type 3CD. One of the mutant 3CD polyproteins, 3CD-3DK127A, also displayed a defect in its ability to form a ternary ribonucleoprotein complex with poliovirus 5′-RNA sequences.

The positive strand RNA genome of poliovirus contains a single open reading frame that codes for a 247-kDa viral polyprotein. Proper expression of poliovirus gene products requires specific protein processing of the viral polyprotein by the enzymatic activities of virally encoded proteinases. These enzymes cleave the polyprotein co- and post-translationally into the structural and non-structural viral gene products (Fig. 1). The majority of cleavage events within the viral polyprotein occur at Q–G bonds and is mediated by a proteinase function associated with the viral-encoded 3C protein (1–6). The rate at which 3C-mediated proteolytic processing occurs at various sites within the viral polyprotein controls the temporal expression of viral gene products (7). This regulation of gene expression is essential for viral replication since polyprotein precursors have functions in replication that are distinct from those of the mature cleavage products (8, 9). An example of a molecule exhibiting these differential functions is the viral polyprotein 3CD, which is an active proteinase containing the entire amino acid sequences of the 3C proteinase and the RNA-dependent RNA polymerase, 3D. Polypeptide 3CD is a multi-functional protein required for both proteolytic processing of the capsid precursor protein and, although it has no detectable elongation activity in vitro, viral RNA replication. The role of 3CD in viral RNA replication is dependent on its ability to form a ternary ribonucleoprotein (RNP) complex with the 5′-terminal sequences of genomic RNA (the 5′-cloveleaf structure) and a cellular RNA-binding protein termed poly(rC)-binding protein 2 (PCBP2) (10, 11).

Previous biochemical studies on poliovirus 3C and 3CD enzymes and structural studies on poliovirus 3C have suggested that, whereas the proteinase active site and RNA binding domains reside in 3C, processing of the viral capsid precursor (P1) and RNP complex formation with the 5′-terminal RNA sequences are more efficiently mediated by 3CD than 3C (10, 12–16). These findings have led to the hypothesis that structural domains within the 3D portion of the 3CD polyprotein contribute to the enhanced 3C activities. Although the exact biochemical roles of specific 3D amino acid sequences and domains within 3CD are necessary for proteinase and RNA binding activities are poorly understood, the 3D domain is necessary for the complete processing of the poliovirus capsid precursor polyprotein P1, as has been demonstrated using both in vitro synthesized 3CD enzyme (13, 14) and purified 3C expressed in Escherichia coli (17). In these previous studies, neither purified 3C nor carboxyl-terminal truncations of 3CD expressed in vitro were able to efficiently mediate processing of the P1 precursor. Studies detailing the precise function of specific residues or structural motifs in 3D necessary for mediating 3CD processing of the P1 precursor have been limited. An early report described a four-amino acid insertion in the 3D domain of the poliovirus polyprotein that eliminated cleavage of the P1 precursor while still retaining 3C-mediated processing of the non-structural polyproteins (12). Two reports describing the effects of specific mutations in poliovirus 3D on RNA polymerase activity and P1 proteolytic processing found that whereas mutations in 3D had effects on both processing and RNA replication, no single mutation in 3D sequences affected processing without also affecting RNA replication (18, 19). In this

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1 The abbreviations used are: 3Cpro, 3C proteinase; RNP, ribonucleoprotein; PCBP2, poly(rC)-binding protein 2; bp, base pair; DTT, dithiothreitol.
study, we have expressed and purified recombinant poliovirus 3C and 3CD proteins. We have used these proteins in a comparative biochemical analysis of their proteolytic processing activities on viral polypeptide substrates and their RNA binding properties in the presence of 5' noncoding region sequences of poliovirus RNA and purified recombinant PCBP2 protein. In order to determine the contribution of specific amino acid residues in 3D on 3CD functions, this analysis was extended to a number of recombinant 3CD polypeptides containing site-specific mutations in 3D previously shown to have deleterious effects on viral RNA replication (19, 20).

**Experimental Procedures**

**Design of P1 'Stop' Plasmid and 3CD Expression Plasmids—**pEnt-PV1-1(Stop) was constructed by placing sequences coding for tandem stop codons (in bold, below) at the 3' terminus of the P1 coding region in pET-PV1 using oligonucleotide mismatch mutagenesis (21, 22) with the Stratagene QuickChange site-directed mutagenesis kit and oligonucleotides as follows: P1stop + (5'-CCACATATTGATAAACGACC-3') and P1stop − (5'-GGTTGCTCCTACATAGTG G-3').

All 3CD expression plasmids coding for mutations in 3D sequences were sub-cloned into the pET15b-3CD10 expression plasmid. This plasmid, which encodes a histidine-tagged 3C protein containing a serine insertion proximal to the 3C/3D junction that essentially eliminates the expression of a histidine-tagged 3D protein containing 2 mg/ml lysozyme, and incubated on ice for 1 h. Histidine-7.4, 0.5 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% (v/v) glycerol. When necessary, gel filtration fractions were concentrated with Centricon 50 concentration of 100 nM, and incubation was continued at 30 °C in RNA binding buffer containing 1 mg/ml BSA (24). 1 μl of 10× cleavage buffer (final concentration of 20 mM HEPES, pH 7.4, 1 mM DTT, and 150 mM KCl, 0.05 mM EDTA, 1 mM DTT, 5% glycerol (25)). Peak fractions resulting from gel filtration were then used in RNA binding assays. All purified proteins were quantified using the Bio-Rad protein assay, and the percent purity of each protein was calculated by densitometric analysis of SDS-polyacrylamide gels stained with Coomassie Blue.

**Expression and Purification of Recombinant Poliovirus 3C and 3CD Proteins—**Wild type and mutated histidine-tagged 3CD10 proteins were purified as described previously (11) with the following modifications. Peak nickel affinity purified recombinant 3CD proteins were subjected to gel filtration on an Amersham Pharmacia Biotech Superdex 200 column with constant buffer flow (5 mM Tris, pH 7.4, 100 mM KCl, 0.05 mM EDTA, 1 mM DTT, 5% glycerol (26)). Peak fractions resulting from gel filtration were then used in RNA binding assays. All purified proteins were quantified using the Bio-Rad protein assay, and the percent purity of each protein was calculated by densitometric analysis of SDS-polyacrylamide gels stained with Coomassie Blue.

**RNA Mobility Shift Assays—**For this analysis, recombinant purified proteins to form a ternary complex with RNA sequences representing the 3' terminal 128 residues in the noncoding region sequences (27). The reactions were analyzed by polyacrylamide gel electrophoresis, and gels were subjected to fluorography, dried, and exposed to XAR film (Eastman Kodak Co.).

**RNA Mobility Shift Assays—**The ability of recombinant 3C and 3CD proteins to form a ternary complex with RNA sequences representing the first 108 nucleotides of the poliovirus genome (the 5' cloverleaf) was assessed using modifications (11, 29) of a previously reported RNA mobility shift assay (10). For this analysis, recombinant purified PCBP2 (200 nm) was preincubated with 0.1 nM radiolabeled RNA representing the first 108 nucleotides of the poliovirus genome for 10 min at 30 °C in RNA binding buffer containing 1 mg/ml E. coli tRNA and 0.5 mg/ml bovine serum albumin. Following preincubation, recombinant histidine-tagged 3C or 3CD protein was added, and the incubation was continued at 30 °C for 5 min at 50 °C (30). The gels were exposed to autoradiography films, and complexes were resolved on a non-denaturing 4% polyacrylamide gel at 4 °C. Following electrophoresis, gels were dried and exposed to XAR film (Kodak).
RESULTS

Protein Processing Assays with Purified Recombinant Poliovirus 3C and 3CD Proteinases—Due to limitations in the quantities of substrate produced using in vitro translation, we were unable to perform kinetic experiments under conditions of saturating substrate. Therefore, we used enzyme dilution and time course experiments to assess the relative proteolytic activities of purified recombinant 3C and 3CD proteinases to process viral polyprotein precursors. Complete processing of the P1 structural precursor by 3C-containing enzymes results in the production of the capsid proteins VP0, VP1, and VP3 (Fig. 1). For the first set of experiments, we used different enzyme dilutions and incubated the cleavage reactions for 2 h at 30 °C. The results of incubation of an in vitro translated P1 precursor with various concentrations of purified 3C (authentic or amino-terminally His-tagged) or 3CD (amino-terminally His-tagged) are shown in Fig. 2A. These data demonstrate that the two purified proteinases display differential activities in the processing of P1, a result that is consistent with P1 processing assays utilizing in vitro synthesized 3C and

![Proteolytic processing map of poliovirus](image)

**FIG. 1. Proteolytic processing map of poliovirus.** Diagrammatic representation of the ~7.5-kilobase pair poliovirus genome and the viral gene products. The dark triangles represent 3C<sup>pro</sup>- or 3CD<sup>pro</sup>-mediated processing events at Q–G peptide bonds within the viral polyprotein. The diamonds represent 2A<sup>pro</sup>-mediated processing events at two Y–G peptide bonds within the polyprotein. The star represents a virion maturation event, which occurs through an undefined mechanism.

![In vitro processing of P1 precursor by purified 3CD, His-3C, and 3C proteinases](image)

**FIG. 2. In vitro processing of P1 precursor by purified 3CD, His-3C, and 3C proteinases.** A, for cleavage analysis, ~25 fmol (in 1 μl) of P1 precursor protein (translated in vitro in the presence of [35S]methionine) and 24 μg of protein from a HeLa S10 extract were incubated for 2 h at 30 °C in the absence (lane 2) or presence of decreasing amounts of each protease (lanes 3-17, enzyme concentrations of each protease (in μM) are indicated above each lane) in a total reaction volume of 10 μl. Cleavage products were resolved on an SDS-12.5% polyacrylamide gel. Lanes 3–7, incubation of P1 with histidine-tagged 3CD; lanes 8–12, incubation of P1 with histidine-tagged 3C; lanes 13–17, incubation of P1 with 3C. Lane 1 shows poliovirus marker proteins. B, graphical representation of P1 processing results. Note that the enzyme concentrations (x axis) are shown on a log scale. Percentages of cleavage products were determined by scanning the autoradiograph of A with a Hewlett-Packard Desk Scan. Amounts of P1 and VP1 were calculated as the intensity of the scanned band multiplied by the area of the region scanned (I*A) using Sigma plot software. The amounts of P1 and VP1 were then normalized for methionine content by dividing the I*A values by the total number of methionines in each molecule. These normalized values were then used to calculate the percentage of VP1 using the formula % VP1 = VP1/(P1 + VP1) × 100%. To calculate the production of VP3 as a function of enzyme concentration normalized I/A values (calculated as above) for P1, 1ABC, and VP3 were used. Percent of VP3 was then calculated using the formula % VP3 = VP3/(P1 + 1ABC + VP3) × 100%. 3CD (□), His-3C (●), 3C (△).
3CD proteinases (12–14). As can be seen in lanes 3–7, the P1 precursor was faithfully processed into VP0, VP1, and VP3 capsid proteins by 3CD proteinase, even at the lowest concentration of enzyme (lane 7). However, both the His-3C (lanes 8–12) and 3C proteinases (lanes 13–17) were only able to process P1 at the highest concentrations of enzyme (lanes 8 and 13), and processing of P1 by His-3C and 3C was incomplete, as indicated by the presence of 1ABC (VP0-VP3, lanes 8, 9, and 13).

Results of this experiment are illustrated graphically in Fig. 2B, which shows the production of either VP1 or VP3 as a function of enzyme concentration. The percentages of VP1 and VP3 were determined by scanning the autoradiograph of Fig. 2A and using calculations described in the legend to Fig. 2. As indicated by the graph, both 3C enzymes are impaired, relative to 3CD, in their abilities to process the P1 precursor. Comparable cleavage at either the VP0-VP3 or VP3-VP1 junction requires greater than 1000-fold more 3Cpro than 3CDpro, and overall processing of the P1 precursor by 3Cpro is inefficient as indicated by the presence of the 1ABC polyprotein product (Fig. 2A, lanes 8 and 13). These results illustrate the necessity of the 3D domain in 3CD for complete and efficient in vitro processing of the P1 precursor.

The diminished proteolytic cleavage properties of 3Cpro relative to 3CDpro seen with the P1 structural precursor are not observed when the non-structural polyprotein precursor 2C-3AB is used as a substrate (Fig. 3). For this analysis, reaction conditions using enzyme dilutions were identical to those detailed above for the P1 precursor. In the case of the 2C-3AB substrate, all three proteinases appear to have similar activities in processing the polyprotein into the 2C and 3AB products (Fig. 3A, lanes 3–17). Results of this experiment are illustrated graphically in Fig. 3B where production of 2C was calculated as described in the legend to Fig. 3. We have carried out a kinetic analysis of cleavage of the 2C-3AB substrate at fixed concentrations of His-3C or His-3CD and have confirmed that this substrate is cleaved with equal efficiencies by 3C and 3CD (data not shown). Note also that since the proteolytic activities of the 3C enzymes (amino-terminally histidine-tagged 3C and authentic 3C) appear to be nearly equivalent, the rest of the experiments in this study utilize the histidine-tagged 3C protein as a source of 3Cpro.

Poliovirus polypeptide 3CD is a polypeptide that could potentially cleave itself through an intramolecular processing event to generate the mature 3C and 3D protein products (refer to Fig. 1). The relatively high abundance of the 3CD polypeptide compared with the 3C and 3D protein products in a poliovirus-infected cell suggests that if cleavage of the 3C/3D junction does occur in cis, it is a very inefficient processing event (7). Structural data on the 3C proteinases of both rhabdovirus 14 (HRV14) (28) and poliovirus type 1 (16) suggest that the 3C/3D junction is positioned such that it could not fold into the active site cleft and be accessible to intramolecular cleavage. These previous data suggest that the regulation of processing of the 3C/3D junction is mediated through a trans-cleavage event. To assess the relative efficiencies of 3C and 3CD activities on trans-processing of the 3CD precursor, enzyme dilution analysis was performed on a purified, radiolabeled 3CD substrate containing an active site cysteine 147 to alanine mutation (24). This lesion renders the 3C enzyme proteolytically inactive. Fig. 4A shows the results of incubating radiolabeled 3CD(C147A) substrate in the absence (lanes 2) or presence of decreasing amounts of purified His-3CD (lanes 3–7) or His-3C (lanes 8–12). As seen in lanes 3–7, 3CD is a more effective enzyme at processing the 3C/3D junction than is 3C. Results of this experiment are illustrated graphically in Fig. 4B, where percentage of 3D was calculated as described in the legend to Fig. 4. Results of this analysis indicate that 3CD is a more effective enzyme in trans-cleavage of 3CD than is 3C with...
were removed at the indicated times. Our data demonstrate that 3CD is able to process the P1 precursor much more rapidly and efficiently than is 3C, confirming the differences detected in our enzyme dilution assays shown in Fig. 2. P1 cleavage products appeared as early as 15 min when substrate was incubated with 3CD, whereas comparable levels of processing were not achieved by 3C until 8 h. Processing of P1 by 3C was also less efficient than processing of P1 by 3CD as indicated by the accumulation of the 1ABC intermediate cleavage product in the reactions incubated with 3C (compare lanes 10, 11, and 16).

Fig. 5B shows the results of a kinetic analysis of P1 processing (identical to that described above) in the presence of 100 nM 3CD-3DK127A (lanes 3–8), 3CD-AL10 (lanes 9–14), 3CD-AL14 (lanes 15–20), or 3CD-3DN424H (lanes 21–26). Three of these mutated 3CD proteinases, K127A, AL10, and N424H, are able to process the P1 precursor at rates similar to that of wild type 3CD (compare lanes 3–8, 9–14, and 21–26 in Fig. 5B to lanes 11–16 in Fig. 5A). One mutant, AL14, is impaired in its ability to process the P1 precursor (relative to wild type 3CD and the other three mutated proteinases) as indicated by the lag in the production of VP1 and VP3 (see lanes 15–17 in Fig. 5B) and the increased relative amounts of 1ABC. These results are represented graphically in Fig. 5C.

Based on our observation that 3CD was able to perform trans-cleavage of a purified 3CD substrate more effectively than was 3C on the same substrate, we examined whether this differential cleavage activity extended to other sites in the P3 non-structural precursor. As seen in Fig. 1, the 3P polypeptide is thought to be a direct precursor to 3CD as well as a precursor to other non-structural polypeptides (e.g. 3AB and 3D) involved in poliovirus RNA replication. We performed a kinetic analysis of proteolytic cleavage using an in vitro synthesized P3 substrate (containing a proteolytically inactive 3C domain (27)) and 100 nM concentrations of different 3C-containing enzymes. Results of this analysis show that 3C and 3CD proteinases display differential proteolytic activity toward multiple cleavage sites in the P3 non-structural precursor (Fig. 6A). For the primary cleavage of the P3 precursor, 3C-mediated processing may occur at three sites in the polyprotein: (i) the 3A/3B junction producing 3A and 3BCD, (ii) the 3B/3C junction producing 3B and 3CD, and (iii) the 3C/3D junction producing 3ABC and 3D (refer to Fig. 1). Processing of the primary cleavage products or multiple processing events within the P3 precursor could also occur, producing any of the mature cleavage products 3A, 3B, 3C, or 3D. The appearance of any of these products would depend on the efficiency of cleavage at the respective sites within the polyprotein and on the stability of the cleavage product. Results of the time course processing assay show that incubation of P3 with either exogenous His-3C or His-3CD results in the production of 3CD, 3D, 3ABC, 3B, and 3C, and 3D (Fig. 6A, lanes 7–10 and 11–16, respectively). The cleavage products 3A (lane 16), 3C (lanes 14–16), and 3ABC (lanes 13–16) are also seen at later times when P3 is incubated with 3CD. As documented above for the data shown in Fig. 4, 3CD appears to be more effective than 3C at processing the 3C/3D junction with comparable levels of 3D appearing at 8 h for 3C (lane 10) and between 1 and 2 h for 3CD (lanes 13 and 14). It also appears that 3CD is more effective at processing the 3B/3C junction than is 3C (compare the levels of 3C and 3AB in lanes 7–9 with those in lanes 12–16), although this difference is not as pronounced as that for the 3C/3D junction. One potential P3 product that was not observed in this analysis is protein 3BCD (which would have an electrophoretic mobility between that of 3AB and 3CD). Previous studies using in vitro translation of a 3C precursor containing wild type 3C sequences indicate that 3BCD is rapidly produced during the course of translation and could not be detected by our gel system.

Analysis of Recombinant 3CD Polyproteins Containing Site-specific Lesions in the 3D Domain. The experiments presented above demonstrated that 3D amino acid sequences contribute to the overall specificity and proteolytic activity of the 3CD proteinase. To define specific amino acid residues in 3D that contribute to 3CD function, we sub-cloned site-specific lesions coding for amino acid changes 3D-R136A/D137A (AL10), 3D-E226A/E227A/Y264C (AL14), 3D-K127A, and 3D-N424H into the pET15b-3CDp10 expression vector. Recombinant 3CD proteins containing these mutations were expressed in E. coli, purified, and used (along with wild type 3C and 3CD proteinases) in protein processing assays. For these experiments (shown in Fig. 5 and Fig. 6), we used a kinetic analysis at fixed enzyme concentrations (100 nM) since we anticipated that this more sensitive analysis might be required to detect less dramatic differences in cleavage activity between wild type and mutant 3CD polypeptides compared with differences previously observed between 3C and 3CD. Shown in Fig. 5A are the results of time course cleavage assays of the 3C polypeptide precursor.

For this experiment, in vitro synthesized P1 substrate was incubated in the absence (lanes 3 and 4) or presence of 100 nM 3C (lanes 5–10) or 3CD (lanes 11–16), and aliquots of the cleavage reaction products were resolved on a SDS-12.5% polyacrylamide gel.

- **Fig. 4. trans-Cleavage of 3CD by purified 3CD and 3C proteinases.** A. For trans-cleavage analysis, 10 pmol of radiola beled 3CD(C147A) substrate was incubated for 2 h at 30°C in the absence (lane 2) or presence of decreasing amounts of purified 3CD (3500 to 35 nM, lanes 3–7) or His-3CD (3500 to 35 nM, lanes 8–12) in a total reaction volume of 10 μl. Following incubation, Laemml sample buffer was added to each reaction mixture; the mixtures were boiled, and the cleavage products were resolved on a SDS-12.5% polyacrylamide gel. Lane 1 shows poliovirus marker proteins. Lane 2 shows 3CD(C147A) incubated in buffer alone. B, graphical representation of the production of 3D as a function of enzyme concentration. Note that the enzyme concentrations (x axis) are shown on a log scale. Percentage of 3D was calculated from normalized l*A values of 3CD and 3D using the formula 3D(3CD + 3D) × 100%. 3CD ( ), His-3C ( ).
that cleavage at the 3A/3B junction may occur in cis (28). These findings, combined with our results, indicate that cleavage of the P3 precursor at the 3A/3B junction may only occur in cis. Lack of the 3BCD product also indicates that the mature 3A protein (Fig. 6A, lane 16) results from proteolysis of a precursor other than P3 (i.e. 3AB).

Results of time course processing assays (identical to those described above) with P3 precursor polypeptides as substrates and mutated 3CD proteinases are shown in Fig. 6, B and C. One of the mutated 3CD proteins studied, 3CD-N424H (Fig. 6C, lanes 3–8), displays P3 processing activities that are similar to those of the wild type 3CD proteinase (compare lanes...
Another mutant, 3CD-AL10 (Fig. 6B, lanes 9–14), appears to process the 3B/3C junction within the P3 precursor with an efficiency similar to that of wild type 3CD, as indicated by the appearance of 3CD and 3AB cleavage products at 0.5 and 1 h, respectively. These times of appearance are similar to the appearance of 3CD and 3AB seen in wild type 3CD processing of P3 (compare Fig. 6B, lanes 9–14, with Fig. 6A, lanes 12–16). However, the AL10-mutated proteinase appears to be impaired in cleavage activity at the 3C/3D junction since both the 3D and 3C cleavage products are delayed in appearance in the reaction incubated with 3CD-AL10 relative to the reaction incubated with wild type 3CD proteinase (compare Fig. 6B, lanes 9–14, with Fig. 6C, lanes 9–14). Both of the other two mutated 3CD proteinases, 3CD-3DK127A (Fig. 6B, lanes 3–8) and 3CD-AL14 (Fig. 6C, lanes 9–14), showed a decreased cleavage efficiency of the P3 substrate relative to cleavage of this substrate by wild type 3CD proteinase. The 3CD-AL14 proteinase processes the P3 precursor with an efficiency nearly identical to that of the 3C proteinase (compare lanes 9–14 in Fig. 6C with lanes 5–10 in Fig. 6A). The 3CD-3DK127A proteinase is severely impaired in its ability to process the P3 precursor relative to the wild type 3CD proteinase activity on P3 substrates, with only the 3CD and 3AB cleavage products being produced at late times during the incubation (Fig. 6B, lanes 3–8). The data from Fig. 6, A—C, are summarized graphically in Fig. 6D.

RNA Mobility Shift Assays Using 3C and 3CD Proteinases—The ribonucleoprotein (RNP) complex formed by poliovirus 3CD proteinase and the 5'9 terminal RNA sequences of the viral genome (the 5' cloverleaf) was first reported by Andino et al. (10, 30). These authors concluded that poliovirus 3CD bound to the first 100 nucleotides of viral RNA in conjunction with an
Modulation of Poliovirus Polypeptide 3CD Functions

In this study, we have used purified recombinant poliovirus 3C and 3CD proteinases in enzyme dilution and time course protein processing experiments to assess the relative contribution of the 3D domain to the proteolytic activities of 3CD. Our results with wild type 3C and 3CD proteinases show that 3CD is able to process the structural polyprotein precursor more rapidly and efficiently than is 3C. 3CD is also able to perform trans-cleavage of other 3CD molecules more efficiently than is 3C, and it processes sites within the P3 precursor more rapidly. However, 3C and 3CD appear to be equally efficient in the processing of a non-structural polyprotein precursor, 2C3AB. Moreover, cleavage of the non-structural precursors 2C3AB and P3 by 3C and 3CD appears to be less efficient than the processing of the structural precursor by 3CD.

We examined four mutated 3CD polypeptides containing site-directed lesions in 3D amino acid sequences in time course processing and RNA binding assays. Our rationale for examining these particular mutations for possible defects in specific 3CD functions is based on the following observations. (i) Analysis of virus and recombinant poliovirus 3D RNA polymerase containing the N424H mutation revealed that this mutation causes a defect in the synthesis of positive strand RNA unrelated to the RNA elongation activity of 3D polymerase and viral protein processing (18). This suggested a defect in the initiation of viral RNA synthesis. We therefore hypothesized that the N424H mutation may affect the ability of 3CD to interact with 5’ poliovirus RNA sequences. (ii) Mutant virus containing the AL14 mutation in 3D has been shown to display a severe defect in viral yield and RNA accumulation; however, this mutation also confers a defect in polyprotein processing, suggesting that these residues might function in 3CD proteinase activity (19). (iii) The individual 3D-K127A mutation had not been previously characterized; however, a basic residue (Lys or Arg) at 3D amino acid position 127 is conserved throughout the picornavirus family. This residue is centered in a highly conserved basic domain (126-KKKRD-130) found within the flexible fingers domain of all enterovirus and rhinovirus 3D polymerases.
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(33), indicating that it may be exposed on the surface of the molecule and could play a role in RNA-protein or protein-protein interactions. Transfections of HeLa cell monolayers with full-length poliovirus cDNA constructs coding for 3D-K127A or 3D-K126A, or 3D-K127A in combination with mutations encoding 3D-R128A and 3D-D129A did not yield virus, suggesting that in the presence of these other mutations, the K127A mutation is lethal for viral replication (19). (iv) Mutant viruses containing the AL10 mutation in 3D has been shown to display a severe temperature-sensitive defect in both viral yield and RNA accumulation while displaying wild type protein processing and translation levels at the non-permissive temperature (19).

One of the mutated proteinases studied, 3CD-3DN424H, displayed activities similar to those of wild type 3CD in protein processing and RNA binding. These results indicate that the defect in the initiation of RNA synthesis previously observed for virus containing this mutation is not the result of a defect in the RNA binding or protein processing activities of 3CD. The location of residue Asn-424 in the reported structure of poliovirus 3D RNA polymerase (33) is at the base of the L α-helix in the thumb domain of the molecule, proximal to two other residues, Met-394 and Val-391, which have been shown to give rise to conditional polymerase mutants in 3D proteins containing single site substitutions of M394T and V391L (34, 35). Characterization of the M394T mutant using an in vitro replication assay revealed that this mutation confers a temperature-sensitive defect in the initiation of negative strand RNA synthesis (34). The conditional RNA synthesis mutant 3D-V391L was initially identified by Hope et al. (35) based on its inability to interact with poliovirus 3AB protein in a two-hybrid screen. Poliovirus 3AB contains the amino acid sequences of a small basic protein, termed VPg (also called 3B), which is covalently linked to the 5′ terminus of all virion RNAs and newly synthesized viral RNAs in infected cells. It has been hypothesized that 3AB serves as a donor of 3B to the RNA replication complex of poliovirus and that the 3B portion of the molecule is utilized as a primer for the initiation of RNA synthesis (9, 32). Based on the location of residues Val-391, Met-394, and Asn-424 in the structure of 3D and the conditional phenotypes exhibited by polymerase molecules harboring single site mutations at these positions, it is possible that residue Asn-424 functions in 3D-3AB interactions necessary for initiation of RNA synthesis. This function appears to be independent of the ability of 3CD to process P1 or P3 structural precursors or to form a ternary RNP complex with the 5′-terminal poliovirus RNA sequences and PCBP2.

Another mutated proteinase examined in this study, 3CD-AL14, was deficient in all aspects of 3CD activity, although not as deficient as 3C(Pro) in processing the P1 capsid precursor. These results make it difficult to assign a specific function for 3D residues, since efficient complex formation was not observed even at the highest concentration of protein tested. These results suggest that the conserved 3D-KKRDI (amino acid residues 126–130) may comprise a structurally important RNA binding domain. Studies on the effects of the K127A mutation on 3D RNA polymerase function are ongoing.

The data presented in this study demonstrate that amino acid residues in the 3D portion of poliovirus 3CD form functionally distinguishable domains that contribute to the differential activities of the 3CD polypeptide. Wild type 3CD proteinase and two of the mutated 3CD proteinases examined, 3CD-AL10 and 3CD-3DK127A, displayed altered abilities to process sites within the non-structural P3 precursor and to form a ternary RNP complex with viral RNA sequences relative to those of wild type 3CD. These findings show that 3D amino acid sequences contribute to the proteolytic processing of both the structural P1 precursor and the non-structural P3 precursor. The observed ability of the 3CD-AL10 and 3CD-3DK127A proteinases to process the P1 precursor as efficiently as wild type 3CD also suggests that substrate recognition determinants in 3D required for processing of P1 may be different from those required for the processing of P3 precursors and for RNP complex formation. Collectively, our results underscore the multi-functional nature of picornavirus non-structural proteins involved in proteolytic cleavage and viral RNA replication. This functional overlap, necessitated by the limited coding capacity of picornavirus genomes, contributes to the exquisite specificity of proteins like 3CD for viral polypeptide and RNA substrates.

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