Subdomain Specific Functions of the RNA Polymerase Region of Poliovirus 3CD Polypeptide

Christopher T. Cornell and Bert L. Semler

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92697-4025

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The 3D polymerase domain of the poliovirus 3CD polypeptide plays a role in modulating its RNA binding and protein processing activities, even though the proteinase catalytic site and RNA binding determinants appear to reside within the 3Cpro portion of the molecule. In this study, we have generated recombinant 3CD polypeptides that contain chimeric 3D polymerase domains representing suballelic sequence exchanges between poliovirus type 1 (PV1) and coxsackievirus B3 (CVB3) to determine which portions of the 3D domain are responsible for influencing these activities. By utilizing these recombinant protein chimeras in protein processing and RNA binding studies in vitro, we have generated data suggesting the presence of separate subdomains within the polymerase domain of 3CD that may independently modulate its RNA binding and protein processing activities. In predicting where our sequence exchanges map by utilizing the previously published three-dimensional structure of the PV1 3D polymerase, we present evidence that sequences contained within the RNA recognition motif of the polymerase are critical for 3CD function in recognizing the 5’ RNA cloverleaf. Furthermore, our protein processing data indicate that at least some of the substrate recognition and processing determinants within the 3D domain of 3CD are separate and distinct from the RNA binding determinants in this domain.

Key Words: proteinase; picornavirus; RNA polymerase; protein processing; RNA binding; poliovirus; coxsackievirus; chimera; RNA recognition motif (RRM).

INTRODUCTION

Positive-strand RNA viruses possess genomes of limited coding capacity yet are able to radically rearrange the environment of the infected cell and allow rapid and efficient RNA replication and subsequent progeny virus production (Bienz et al., 1987; Cho et al., 1994). In doing so, these viruses rely not only on their own virally encoded gene products, but also on a number of host factors to perform specific functions with high fidelity. Similar to other members of the Picornaviridae family of viruses, the poliovirus genome codes for a polyprotein to generate these necessary viral protein products. Upon entry into the cell and subsequent translation of the viral RNA into a 247-kDa polyprotein, two virally encoded proteinases, 2A and 3C (and its immediate precursor 3CD), process this polyprotein at distinct dipeptide sequences in a manner that yields polypeptide products that go on to participate in events necessary for viral replication (Dougherty and Semler, 1993).

The relatively small size of the genome of poliovirus, as well as other picornaviruses, may require that the precursor and mature forms of viral proteins serve separate and distinct functions during the viral life cycle (Wimmer et al., 1993). One example of this is the activity of viral proteins 2BC and 2C. Both 2BC and 2C possess ATPase activity (Pfister and Wimmer, 1999) and are able to induce the rearrangement of membranes in the infected cell (Aldabe and Carrasco, 1995; Barco and Carrasco, 1995). 2C and 2BC also bind specific sequences in the 3' noncoding region of negative strand viral RNA (Banerjee et al., 1997, 2001), an interaction thought to be critical for RNA replication and presumably mediated by two RNA binding motifs within 2C (Rodriguez and Carrasco, 1995). However, the presence of the 2B domain within the 2BC precursor form seems to alter the RNA sequence and structure recognition properties of the 2C protein (Banerjee et al., 1997; Banerjee et al., 2001).

More relevant to this study is the striking contrast between the activities of poliovirus 3CD and 3C polypeptides. 3C, a virally encoded proteinase, has been shown to possess RNA binding determinants. Furthermore, it has been shown that sequences essential for proteolytic activity are separable from those necessary for RNA binding (Blair et al., 1998; Mosimann et al., 1997; Blair et al., 1996). Despite the fact that both the proteinase and RNA binding sequences reside within 3C alone, 3CD differs greatly from 3C with respect to these activities. In functioning as RNA binding proteins, both 3C and 3CD recognize the RNA cloverleaf element at the 5' end of the positive strand RNA genome, in concert with host protein PCBP2, to form a ternary complex (Parsley et al., 1997). However, 3CD possesses an enhanced ability to form...
the ternary complex over that of 3C, and for this reason the 3CD/PCBP2/RNA complex is thought to be the biologically relevant one (Andino et al., 1990). In functioning as a proteinase, 3CD has a dramatically enhanced ability to cleave the structural (P1) protein precursor over that of 3C (Ypma-Wong et al., 1988). Due to this appreciable difference in activity, it is thought that the 3C-containing polypeptide primarily responsible for capsid precursor cleavage during a viral infection is 3CD (Ypma-Wong et al., 1988).

The other cleavage counterpart of 3CD is 3D, the RNA-dependent RNA polymerase. 3D mediates the uridylylation of VPg and subsequently utilizes VPg-pUpU-primed templates to initiate RNA replication (Paul et al., 1998). Interestingly, it has been shown that unlike 3D polymerase, the 3CD polypeptide possesses no elongation activity in vitro (Flanegan and Van Dyke, 1979; Harris et al., 1992). However, it should be noted that this might not be the case for all picornalike positive-stranded RNA viruses. Recent evidence suggests that for feline calicivirus, the precursor (pro-pol) form of the polymerase is the most active and biologically relevant form of the enzyme (Wei et al., 2001).

In addition to its functions in forming the 5' ternary complex and in acting as a viral proteinase, 3CD is also thought to bind an RNA element utilized in the process of protein-primed RNA replication initiation. Recent evidence has suggested that the 3Dpol-catalyzed uridylylation of VPg is greatly stimulated by the presence of the 3CD polypeptide (Rieder et al., 2000). Although the exact role of 3D in this reaction is not clear, it is thought that 3CD might interact directly, or indirectly, with an RNA element termed cre (cis-acting replication element; see Fig. 1). Predicted to form a hairpin structure, the PV1 cre element has been shown to contain adenylate nucleotides that are absolutely required for the uridylylation of VPg and initiation of negative-strand RNA synthesis (Rieder et al., 2000). In addition to the one recently described for PV1 (Paul et al., 2000), cis-acting replication elements have been found previously in human rhinovirus type 14 (McKnight and Lemon, 1998), cardioviruses (Lobert et al., 1999), and poliovirus type 3 (Goodfellow et al., 2000). Finally, 3CD has also been shown to interact with the 3' NCR of poliovirus, which could provide a source for polymerase molecules proximal to the 3' end of the RNA to initiate negative-strand RNA synthesis (Harris et al., 1994). This 3' NCR interaction may be stabilized further by 3D domain-mediated contacts with viral polypeptide 3AB. 3D–3AB protein–protein interactions have been previously shown in a yeast two-hybrid screen (Hope et al., 1997; Xiang et al., 1998).

It is not known how the 3D polymerase domain of 3CD enhances or modulates 3C activities. The presence of polymerase sequences could serve to transmit structural alterations to the 3C proteinase domain of the 3CD molecule such that it recognizes RNA and protein sub-

![FIG. 1. Possible functions of the 3CD polypeptide in the poliovirus life cycle. Represented in the diagram are critical 3CD functions that include processing the 247-kDa viral polyprotein at specific Q-G dipeptide sequences and forming RNP complexes with 5' and 3' RNA structures that could possibly mediate circularization of the genomic RNA and initiation of negative strand synthesis (Andino et al., 1990; Xiang et al., 1995; Andino et al., 1993; Harris et al., 1994). This circularization event may involve protein–protein contacts with poly(A) binding protein (PABP) (Herold and Andino, 2001). Other putative functions for 3CD include acting as the immediate precursor form of the 3D polymerase, stimulating the VPg uridylylation reaction critical for protein-primed RNA synthesis (Paul et al., 2000; Rieder et al., 2000), and possibly regulating internal-ribosome-entry site-mediated translation (Gamarnik and Andino, 1998, 2000).]
strates with greater efficiency. Alternatively, the polymerase domain may possess secondary RNA binding determinants involving direct polymerase domain-RNA contacts, thereby allowing 3CD to form the ternary complex more efficiently than 3C. Yet another explanation is the possibility that the 3D polymerase domain could stabilize the ternary complex via protein–protein contacts with host protein PCBP2. Such 3D-mediated protein–protein contacts could also act at the level of substrate recognition, thereby explaining differences in 3CD versus 3C proteolytic activity.

We have previously undertaken studies to determine which portions within the 3D polymerase domain might be responsible for enhancing RNA recognition and protein processing by 3CD (Parsley et al., 1999). Results from these studies suggested the existence of subdomains within the polymerase portion of the 3CD molecule that could be responsible for enhanced RNA binding and protein processing. To further elucidate which subdomains within the 3D domain of the poliovirus 3CD polypeptide are responsible for these enhanced activities, we have generated recombinant 3CD polypeptides that have chimeric sequences in their polymerase domains representing suballelic exchanges between PV1 and the closely related enterovirus coxsackievirus B3 (CVB3). Our results presented here demonstrate the presence of separate and distinct subdomains within the polymerase domain required for efficient protein processing versus ternary complex formation. Specifically, sequences contained within the RNA recognition motif (RRM) of the polymerase appear to be critical for ternary complex formation while additional sequences required for polymerase–protein interactions may be necessary for protease substrate recognition.

RESULTS

Experimental rationale for generating PV1/CVB3 chimeric polypeptides

To examine the role of the polymerase domain in modulating the function of 3CD in RNA binding and protein processing, we generated seven recombinant 3CD polypeptides containing a chimeric 3D polymerase domain for use in our *in vitro* electrophoretic mobility shift and substrate processing kinetic experiments. Previous studies have utilized either chimeric polypeptides or RNA constructs containing poliovirus and sequences from other closely related picornaviruses to analyze important determinants of viral macromolecular interactions (Dewalt et al., 1989). Poliovirus type 1 (PV1) and coxsackievirus B3 (CVB3) share 75% amino acid identity in their polymerase sequences (Fig. 2). In addition to displaying a high degree of amino acid identity, it is also thought that both the PV1 and CVB3 polymerases adopt a conserved three-dimensional structure that is characteristic of all polymerases, including picor-
FIG. 3. Ribbon diagram representation of the previously published crystal structure of the PV1 polymerase. This structure was adapted from (Hansen et al., 1997). (A–E) Motifs within the RNA recognition motif (RRM) required for polymerase activity. Structural domains (thumb, palm, and fingers) characteristic of all polymerases are indicated.

FIG. 4. Domain substitutions made in PV1/CVB3 chimeric polypeptides. Sequence exchanges are represented schematically and in the context of the poliovirus 3D polymerase crystal structure. All versions of 3CD generated in this study contain a 3C domain that consists entirely of poliovirus amino acid sequences as well as a serine insertion just upstream of the 3C/3D junction (indicated by the asterisk). Restriction enzyme sites in 3D used to create PV1/CVB3 sequence junctions are indicated on the left half of the panel. (CT) The carboxy terminus of 3CD. The right half of the panel predicts where these regions of substituted sequence are located in the polymerase domain of 3CD based on the poliovirus 3D polymerase crystal structure.
naviral RNA-dependent RNA polymerases (RdRps). Shown in Fig. 3 is the crystal structure for the poliovirus 3D polymerase solved by Schultz and colleagues (Hansen et al., 1997), which possesses the characteristic “palm,” “fingers,” and “thumb” subdomains, analogous to a right hand. Within the palm subdomain of the polymerase is the RNA recognition motif, or RRM, that is also a characteristic of all RdRps. Also highlighted in Fig. 3 are motifs A–E of the RRM, the functions of which have been well characterized for many viral polymerases (for review see O’Reilly and Kao, 1998).

Due to the sequence and structural similarities between the polymerases of PV1 and CVB3, we predicted that generating a 3CD polypeptide possessing a PV1/CVB3 chimeric polymerase domain would not cause structural disruptions in the 3CD molecule severe enough to globally misfold the polypeptide. Furthermore, one might predict that these sequence changes could impact certain surface-exposed residues in the polymerase domain critical for virus-specific protein–protein or protein–RNA contacts. The sequence alignment in Fig. 2 shows the junctions (indicated by Roman numerals I, II, and III) utilized in our suballelic exchanges between PV1 and CVB3 sequences. These junctions were positioned based on sequence identity in and around these regions of the polymerases, along with the presence of conve-
nient restriction sites in the PV1 cDNAs used to produce these constructs. Figure 4 summarizes the chimeras generated for this study in schematic form. Figure 4 also shows the predicted locations, based on the crystal structure of the PV1 3D RNA polymerase, of the affected sequences. In these constructs, one-third, two-thirds, or the entire 3D polymerase domain (chimera C-C-C) of poliovirus 3CD polypeptide has been substituted with the corresponding sequences from the CVB3 3D polymerase. It should be noted that data interpretations made in this study are based on the three-dimensional structure of the polymerase alone, since a crystal structure for the 3CD polypeptide has not yet been solved.

In vitro protein processing time course experiments with PV1/CVB3 chimeras

We first analyzed the effects of our chimeric substitutions at the level of protein processing. Chimeras that are deficient in their abilities to process structural or non-structural protein precursors could highlight regions of the polymerase domain of 3CD that may directly contact substrate or, alternatively, might serve to structurally alter the 3C proteinase domain so that it can effectively recognize polyprotein.

Our analysis of processing the structural polyprotein precursor (P1) revealed a differential effect on 3CD activity. Figures 5B, 5C, and 6A show the results of an 8-h kinetic experiment in which wild-type or chimeric 3CD polypeptide was incubated in the presence of in vitro translated radiolabeled P1 substrate. Processing of P1 by wild-type 3CD reveals that even at the earliest time of sample analysis (15 min), appreciable levels of the mature cleavage products VP0, VP1, and VP3 could be detected (Fig. 5B, lanes 9–14; Figure 6A, lanes 3–8). In contrast to processing by wild-type 3CD, several of the chimeras were deficient in their P1 cleavage activity. P-C-C showed a slight deficiency in the kinetics of processing P1 when compared to processing by wild-type 3CD.
3CD (Fig. 5B, compare lanes 9–14 to lanes 3–8). The activity of C-C-P was also deficient to an extent similar to that of P-C-C (compare Fig. 5C lanes 29–34 to Fig. 5B lanes 9–14). What is striking, however, are the processing profiles for four of the chimeras, C-P-P, C-P-C, P-P-C, and C-C-C, which were dramatically different compared to wild-type 3CD (Fig. 5B, lanes 15–20; Fig. 5C lanes 23–28 and 42–47; and Fig. 6A, lanes 9–14, respectively). Interestingly, these chimeras, as well as polypeptides P-C-C and C-C-P, displayed a processing deficiency that seemed to center around cleavage of the VP0–VP3 junction. This is apparent when looking at the level of VP0–VP3 (refer to Fig. 5A or Fig. 6A) intermediate present in each time course experiment involving these chimeric polypeptides. To further substantiate this claim, Figs. 5D and 6B show a quantitative analysis of these data. Each graph shows the relative amounts of VP0 or VP1 produced during each incubation reaction. Indeed, a quantitation of our data revealed that the major difference between each of the chimeras and wild-type 3CD occurs at the level of %VP0 produced. In contrast, the percentage of VP1 produced during the time-course experiment indicated only minor differences in cleavage of the VP3–VP1 junction by our 3CD chimeras. We also quantitated the total percentage of P1 products, which takes into account the sum total of all precursor and mature cleavage products generated during this experiment. These results supported the notion that the overall activity of each chimeric polypeptide was quite comparable to that of wild-type 3CD (data not shown). Only the C-P-C chimera seemed to be unusually deficient in processing the structural precursor (see Fig. 5C, lanes 22–28), which might have suggested a misfolding of this polypeptide. However, such a conclusion is not substantiated by subsequent data presented in this report. Furthermore, the processing kinetics of chimera C-C-C support previous data (Bell et al., 1999) which showed that a chimeric PV1 cDNA harboring a 3D polymerase gene entirely from CVB3 is severely deficient in P1 processing.

Combined with our previously published results (Parsley et al., 1999), the results shown in Figs. 5 and 6 provide consistent evidence that one of the primary functions of the polymerase domain of 3CD is to enhance the recognition and/or cleavage at the VP0–VP3 junction by the 3C proteinase. Furthermore, these results suggest that the first amino-terminal one-third and the carboxy-terminal one-third of the 3D domain may contain poliovirus sequences or structural elements responsible for enhancing cleavage of the P1 structural precursor.

We next compared the activities of our PV1/CVB3 chimeric polypeptides at the level of P3 (nonstructural) protein processing. The results demonstrated that differences between wild-type 3CD and the PV1/CVB3 chimeras were not as apparent as those seen with P1 as a substrate (data not shown). All of the chimeras tested displayed efficiency in processing the P3 precursor at or
Ternary complex formation by chimeric 3CD polypeptides

We analyzed the abilities of the chimeric 3CD polypeptides to form the ternary complex, consisting of the 5′ RNA cloverleaf element, host protein PCBP2, and 3CD. Figures 7A and 7B show the results of an electrophoretic mobility shift analysis utilizing recombinant PCBP2 protein, radiolabeled RNA representing the first 108 nucleotides of the poliovirus plus-strand RNA, and either wild-type or chimeric 3CD protein. It should be noted that, compared to previously published work by our laboratory (Parsley et al., 1999), an increased amount of RNA probe (1.0 nM) was utilized in this study. As expected, PCBP2 protein was capable of forming a ribonucleoprotein complex when incubated with cloverleaf in the absence of 3CD (Figs. 7A and 7B, compare lanes 1 and 2). The addition of wild-type 3CD protein to this reaction resulted in the formation of two ternary complexes, with bona fide complexes appearing at the lowest 3CD concentration, 50 nM (Figs. 7A and 7B, lanes 3–8). The appearance of two isoforms of the ternary complex is consistent with previously published results from our laboratory (Parsley et al., 1997, 1999). Analogous to our protein processing results, mobility shifts carried out with different 3CD chimeras resulted in marked differences in the abilities of these proteins to form the ternary complex. Even at a concentration of 50 nM, chimeric 3CD construct C-P-P was able to form this complex with an efficiency equal to that of wild-type 3CD (Fig. 7A, lanes 11–16). The P-C-P 3CD chimeric protein was able to form the ternary complex at a slightly higher concentration of 3CD polypeptide, forming a distinct ternary complex at approximately 150 nM (Fig. 7A, lanes 17–22).

Three of the chimeras assayed in this experiment displayed an extremely low affinity for the RNA/PCBP2 complex. Protein concentrations of C-C-P, P-C-C, and C-C-C were required at levels much higher than 50 nM or even 150 nM to successfully form the ternary complex (Fig. 7A, lanes 39–44 and 45–50; Fig. 7B, lanes 11–16, respectively). Our RNA binding results with the C-C-C construct supported previous data that demonstrated a 3CD polypeptide containing a CVB3 3D domain is deficient in ternary complex formation (Bell et al., 1999). Interestingly, two of the chimeras were enhanced in their abilities to form the ternary complex. Both P-P-C (Fig. 7A, lanes 25–30) and C-P-C (Fig. 7A, lanes 31–36) were able to effectively bind and almost quantitatively shift the RNA/PCBP2 binary complex into ternary complex between 250 nM and 350 nM concentrations of 3CD. Higher concentrations of wild-type 3CD were required to accomplish this (approximately 450 nM to 600 nM). Figure 7C shows a quantitation of the data from the electrophoretic mobility shift assay given as the percentage of ternary complex formed at each concentration of 3CD polypeptide assayed. A comparison of the RNA binding properties between wild-type 3CD and each of the chimeras is shown below the graphs. The fact that two of the chimeras displayed an enhanced ability to form the ternary complex might indicate that, for these proteins, we have altered sequence and/or structure of the 3CD polypeptide, allowing for a higher binding affinity. More interesting, however, is the fact that four of our chimeras were deficient in forming the ternary complex. We predict, based on the three-dimensional structure of the poliovirus 3D polymerase, that each of the chimeras deficient in RNA binding has an altered RNA recognition motif (refer to Fig. 3). Our results suggest that the central one-third portion, containing RRM motifs A–C of the 3D polymerase domain, may play a critical role in RNA recognition and subsequent ternary complex formation, since each deficient chimera (P-C-P, C-C-P, P-C-C, and C-C-C) has altered sequences in this domain. Near-wild-type levels of ternary complex formation were seen in the chimeras that contained poliovirus sequence in this region (C-P-P, P-P-C, and C-P-C), suggesting that the central domain contains determinants essential for protein–RNA interactions.

DISCUSSION

The polymerase subdomain: Sequence and structure requirements for protein processing

In this study, we have shown that 3CD polypeptides containing chimeric polymerase domains representing suballelic exchanges of CVB3 sequences for PV1 sequences have altered abilities to process the P1 (structural) protein precursor. In comparing our results for P1 versus P3 processing (data not shown), our sequence substitutions had a significantly greater influence overall on P1 processing by our chimeric polypeptides. This can be correlated with previous studies examining 3C versus 3CD activity in P1 and P3 processing. Results from these studies suggested the 3D domain of 3CD may be more critical in enhancing processing of the P1 precursor than in processing of the P3 precursor (Parsley et al., 1999). More importantly, these previous results also indicated that the difference between 3C versus 3CD activity in processing P1 was primarily in recognition of the VP0–VP3 junction by the proteinase. Our data from the present study suggest that changes we have made in the polymerase domain of 3CD have had an influence primarily on recognition of this very same junction. Our P1 processing results utilizing chimeras C-P-P, C-P-C, P-P-C, and C-C-C suggest that amino acid sequences within the 3D domain may interact directly with substrate determi-
FIG. 7. Electrophoretic mobility shift analysis with wild-type and PV1/CVB3 chimeric 3CD polypeptides. (A and B) Radiolabeled probe (1 nmol in a total reaction volume of 10 μl) representing the first 108 nucleotides of the poliovirus genome (RNA cloverleaf) was incubated in the absence (A: lanes 1, 9, 23, and 37; B: lanes 1 and 9) or presence of 200 nM PCBP2 protein (A: lanes 2, 10, 24, and 38; B: lanes 2 and 10). Additionally, increasing amounts (50 to 600 nM) of recombinant wild-type 3CD or chimeric 3CD polypeptide were incubated with probe and PCBP2 protein. The mobilities of free probe, binary complex (PCBP2 – RNA), and ternary complex isoforms (3CD – PCBP2 – RNA) are indicated. (C) The relative ability of each 3CD polypeptide to form the ternary complex was determined. The percentage of ternary complex formed at each concentration of 3CD protein was calculated by quantitating the amount of ternary complex as a fraction of all radiolabeled species in a given lane (ternary complex, binary complex, and free probe) using ImageQuaNT software (Molecular Dynamics). Shown below the graphs, “plus” ratings were assigned to each 3CD polypeptide by estimating the approximate concentration of 3CD protein required to shift one-half of the PCBP2-RNA complex into ternary complex. Wild-type 3CD binding is given by ++++. 
nants at or near the VP0–VP3 junction or with other protein factors that allow recognition and subsequent cleavage at this site on the polyprotein. Based upon our P1 processing results, we postulate that the fingers and thumb subdomains of the polymerase domain play a role in modulating substrate recognition and proteolytic processing by 3CD. In each instance where we have made sequence substitutions predicted to alter the fingers, thumb, or both subdomains (C-P-P, P-P-C, C-C-P, P-C-C, and C-C-C) we have, to varying degrees, affected P1 processing. Although we have not directly tested the cause(s) for these processing deficiencies, we can turn to the polymerase structure/function literature for possible answers. It is the thumb and fingers domains of the poliovirus polymerase that have been implicated as being necessary for oligomerization of the enzyme by forming polymerase–polymerase contacts (Hansen et al., 1997). More importantly, oligomerization of the polymerase has been shown to be functional in binding nucleotide and in elongation activity (Pata et al., 1995; Hobson et al., 2001). Though functional oligomerization of the 3CD polyprotein mediated by the polymerase domain has not yet been shown, it is possible that 3CD forms oligomeric structures necessary to recognize and process substrates and that substituting CVB3 polymerase sequence for PV1 sequence has changed structural or sequence motifs required for this oligomerization. Hydrophobicity plots (Kyte and Doolittle, 1982) of the amino-terminal 100 residues of CVB3 and PV1 polymerases indicate that a substitution changing these sequences of the poliovirus polymerase domain would noticeably impact its overall hydrophobicity (data not shown). This change maps to the base of the fingers domain and might influence protein–protein contacts between this region and the top of the thumb subdomain of another 3CD molecule in the context of our C-P-P, C-C-P, and C-P-C polypeptides in this study. Termed “Interface II,” these contacts have been shown to occur within the crystal packing of the 3D polymerase (Hansen et al., 1997) and are important for polymerase function (Hobson et al., 2001).

In addition to altering the base of the fingers subdomain, chimeric substitutions involving amino-terminal sequences of the polymerase are predicted to also impact the top of the thumb subdomain (see Fig. 3). Thus, we would predict that the thumb subdomain is also important for proteinase substrate recognition. Chimeras that have carboxy-terminal substitutions, P-P-C, C-P-C, P-C-C, and C-C-C (see Fig. 4), all have altered thumb subdomains, and each has been affected, albeit to a different degree, in their protein processing activities. Studies by Bresanelli and co-workers (Bressanelli et al., 1999) have indicated that there are armadillo-like repeat motifs in the largely α-helical thumb subdomain of the hepatitis C virus RNA polymerase and that these motifs may be critical for polymerase–protein contacts necessary for viral RNA replication. The thumb subdomain of the poliovirus polymerase is also highly α-helical in nature, and one might suggest that this domain could participate in host/viral protein–protein contacts that are required for RNA replication, but additionally required for protein processing. We have previously shown that P1 processing is
dependent on a cellular cofactor (Blair et al., 1993), and 3CD may participate in protein–protein contacts with this factor via 3D polymerase domain sequences. As indicated in Fig. 2, changing the identity of the thumb subdomain is not predicted to change a substantial number of amino acids in the molecule, but examination of the sequence alignment between the CVB3 and PV1 polymerase reveals that of the 42 amino acids substituted within this domain, 19 are predicted to alter charge. These charged residues are likely to be surface exposed on some of the α-helices and actively participate in protein–protein interactions required for substrate processing by 3CD. Finally, the thumb subdomain also participates in the formation of Interfaces I and II, which have been shown to be essential for polymerase oligomerization (Hobson et al., 2001). By altering the thumb subdomain, we may have affected oligomerization of 3CD, modulated by the 3D polymerase domain, required for its function.

The polymerase subdomain: Sequence/structure requirements for ternary complex formation

Data presented in this report argue that there are specific regions of the polymerase domain of 3CD that are responsible for enhancing the ability of the 3C proteinase to recognize the RNA cloverleaf and participate in ternary complex formation. Although the primary RNA binding determinants appear to reside in the 3C portion of the molecule as previously reported (Mosimann et al., 1997; Andino et al., 1993; Harris et al., 1994), protein–RNA contacts may occur that directly involve the 3D domain of the 3CD polypeptide. We have recently reported that complete replacement of the polymerase domain of poliovirus 3CD with that of CVB3 produces a replication defective 3CD/3D complex (Bell et al., 1999). In contrast, we had previously shown that the first 627 nucleotides of the coxsackievirus genome could functionally substitute for poliovirus sequences in the 5′ noncoding region of genomic RNA (Semler et al., 1986; Johnson and Semler, 1988). This finding demonstrated that structural elements in the 5′ NCR can be substituted with other structurally similar elements and still allow binding of host and viral factors necessary for replication. Additionally, other studies have also indicated that the poliovirus cloverleaf can be substituted into a full-length cDNA clone of coxsackievirus (Zell et al., 1995). Successful replication of this chimera further supports the notion of conserved protein/nucleic acid interactions between these two viruses.

Recently we have shown that recombinant CVB3 3CD polypeptide is able to bind PV1 cloverleaf with an affinity approximately equal to that of PV1 3CD, but a chimera containing a PV1 3C domain and a CVB3 3D domain does not bind efficiently (Bell et al., 1999). Thus, we would expect our PV1/CVB3 chimeras to exhibit some deficiency in RNA binding. Indeed, chimeras P-C-P, P-C-C, C-C-P, and C-C-C were noticeably reduced in their abilities to form the ternary complex with cloverleaf RNA and PCBP2. Mechanistically, the effects of these substitutions could be exerted in one or more of the following contexts: (a) structural or sequence alterations of 3D subdomains that participate in direct interactions between this domain and the RNA cloverleaf; (b) structural alterations conferred upon the 3C domain by the polymerase domain, either at a global level or by structural transmission; (c) direct physical interference of the 3C domain by the 3D polymerase domain; or (d) essential protein–protein contacts between 3CD and PCBP2 that either allow 3CD to recognize the cloverleaf RNA or are critical for stability of the ternary complex itself. Interestingly, all three of the chimeric 3CD polypeptides that are severely deficient in ternary complex formation are predicted to have an altered palm subdomain, based on the crystal structure for poliovirus 3D (Hansen et al., 1997). Specifically, these substitutions are predicted to primarily impact conserved motifs A–C that lie within the RRM.

Two of our 3CD chimeras, P-P-C and C-P-C, were able to form the ternary complex with an affinity greater than that of wild-type 3CD polypeptide. For both of these recombinant proteins, we would predict that motifs A–C of the RRM are left intact and are active in recognizing RNA cloverleaf. Given that both chimeras contain CVB3 sequences in their C-terminal domains, the increased RNA binding activities could be attributed to higher affinity protein–protein interactions mediated by these CVB3 sequences. Such interactions would be distinct from those that are necessary for efficient P1 processing, as demonstrated by P1 cleavage defects observed for these same chimeras.

RNA-dependent RNA polymerases like 3Dpol are required to bind nucleotides in a reversible manner. It is possible that picornavirus 3CD proteins have taken advantage of this nucleotide binding property in their polymerase domains to augment binding affinity or specificity to their interactions with cloverleaf RNA. It is significant that results from primed template elongation assays utilizing poliovirus 3CD have shown that this protein possesses no measurable RNA chain elongation activity in vitro (Flanegan and Van Dyke, 1979; Harris et al., 1992). Since these studies examined elongation activity rather than nucleotide or RNA binding, the possibility that 3CD itself recognizes primed template cannot be ruled out. Interestingly, work by Cameron and co-workers (Wei et al., 2001) has indicated the active form of the feline calicivirus RNA polymerase is the precursor polypeptide
(pro-pol) form, equivalent to poliovirus 3CD. These authors demonstrated that the precursor pro-pol form of the enzyme was able to recognize homopolymeric primer–template duplexes and synthesize nascent chains in vitro. It is likely that the polymerase domain portion of the calicivirus pro-pol molecule recognizes this primer–template duplex. For poliovirus, utilization of this primed template for RNA elongation must involve additional steps or reaction components not required for chain elongation by the calicivirus RNA polymerase.

MATERIALS AND METHODS

Cloning of PV1/CVB3 3CD expression constructs

Chimeric 3CD expression constructs were based on pET15b-3CD (μ10), a previously described plasmid (Parsley et al., 1997, 1999; Ypma-Wong et al., 1988) encoding a histidine-tagged poliovirus 3CD protein containing a serine insertion just upstream of the 3C/3D junction, thereby eliminating autoprocessing at this junction without affecting proteinase and RNA binding activity. CVB3 polymerase sequences were specifically polymerase chain reaction (PCR) amplified using primers that produced nucleotide mismatches resulting in a product containing restriction enzyme recognition sequences compatible with sites found within the PV1 3D polymerase sequence of pET15b-3CD (μ10). Table 1 summarizes the fragments (and their origins) that were incubated in the presence of T4 DNA ligase to generate the seven chimeric constructs used in this study. In each case except for pET15b-3CD (μ10-CCC), CVB3 sequences were PCR amplified from plasmid pT5T-3CD[CV] (Bell et al., 1999), which was derived from an infectious cDNA clone of CVB3 (Chapman et al., 1994). The PCR products were gel purified, treated with the appropriate restriction enzymes, phenol/chloroform extracted, and ethanol precipitated. pT5T-3CD[CV]KO contained a C7205A mutation in the presence of this site internal to the PCR fragment upon treatment with PshAI enzyme. This mutation was introduced by oligonucleotide site-directed mutagenesis. To generate substrate for polymerase domain chimeras, the precursor pro-pol form, equivalent to poliovirus 3CD. These authors demonstrated that the precursor pro-pol form of the enzyme was able to recognize homopolymeric primer–template duplexes and synthesize nascent chains in vitro. It is likely that the polymerase domain portion of the calicivirus pro-pol molecule recognizes this primer–template duplex. For poliovirus, utilization of this primed template for RNA elongation must involve additional steps or reaction components not required for chain elongation by the calicivirus RNA polymerase.

Protein processing assays

To generate substrate for in vitro protein processing kinetic experiments, pT7-PV1-P1(stop) (Parsley et al., 1999) was linearized and used as template for an in vitro transcription reaction with T7 RNA polymerase. The RNA from these reactions was used to program HeLa S10 translation extracts in the presence of [35S]methionine (Todd et al., 1997).

Processing assays were carried out in a total volume of 60 μl. Briefly, P1 substrate (~25 fmol) was diluted into 1X cleavage buffer [20 mM HEPES (pH 7.4) 0.15 M KOAc, 1 mM EDTA, 1 mM DTT] and preincubated at 30°C for 10 min. Recombinant 3CD polypeptide was then added to the reaction to a final concentration of 0.5 mM and the culture was incubated overnight at room temperature. Wild-type and chimeric histidine-tagged 3CD proteins were purified from this culture as previously described (Parsley et al., 1999).

Purification of recombinant polypeptides expressed in bacteria

Each His-3CD expression plasmid was transformed into BL21(DE3) bacteria, colony purified, and used to produce a 2-liter culture grown in Luria–Bertani medium containing ampicillin. At an A600 of approximately 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM and the culture was incubated overnight at room temperature. Wild-type and chimeric histidine-tagged 3CD proteins were purified from this culture as previously described (Parsley et al., 1999).
sample buffer [20% glycerol, 125 mM Tris–HCl (pH 6.8) 4% SDS, 10%  \( \beta \)-mercaptoethanol, 0.01% bromophenol blue] (Laemmli, 1970). Additionally, substrate was incubated for the duration of the reaction (8 h) in the absence of 3CD proteinase. All samples were boiled and resolved on a sodium dodecyl sulfate–12.5% polyacrylamide gel. Gels were subjected to fluorography in dimethylsulfoxide, dried, and exposed to XMR film (Eastman Kodak Co.).

**Electrophoretic mobility shift analyses**

Recombinant 3CD polypeptides were analyzed for ternary complex formation with RNA sequences representing the first 108 nucleotides of the poliovirus genome (RNA cloverleaf) and recombinant host protein PCBP2 as previously described (Parsley et al., 1999). Briefly, 200 nM PCBP2 protein was incubated in the presence of 1 nM radiolabeled cloverleaf in binding buffer [5 mM HEPES–KOH (pH 7.4), 3.8% glycerol, 2.5 mM MgCl2, 125 mM KCl, and 20 mM dithiothreitol] containing 1 mg/ml *Escherichia coli* tRNA and 0.5 mg/ml bovine serum albumin. Following a 10-min preincubation, increasing amounts of recombinant 3CD polypeptide were added to bring the final reaction volume to 10 \( \mu \)l, and the mixtures were incubated for an additional 10 min, at which time 2.5 \( \mu \)l of 50% glycerol was added. Samples were resolved on a non-denaturing 4% polyacrylamide gel at 4°C, dried, and exposed to XMR film (Eastman Kodak Co.).

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