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Regulation of Poliovirus 3C Protease by the 2C Polypeptide

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Poliovirus-encoded nonstructural polypeptide 2C is a multifunctional protein that plays an important role in viral RNA replication. 2C interacts with both intracellular membranes and virus-specific RNAs and has ATPase and GTPase activities. Extensive computer analysis of the 2C sequence revealed that in addition to the known ATPase-, GTPase-, membrane-, and RNA-binding domains it also contains several "serpin" (serine protease inhibitor) motifs. We provide experimental evidence suggesting that 2C is indeed capable of regulating virus-encoded proteases. The purified 2C protein inhibits $3C^{pro}$-catalyzed cleavage of cellular transcription factors at Q-G sites in vitro. It also inhibits cleavage of a viral precursor by the other viral protease, $2A^{pro}$. However, at least three cellular proteases appear not to be inhibited by 2C in vitro. The 2C-associated protease inhibitory activity can be depleted by anti-2C antibody. A physical interaction between 2C and His-tagged $3C^{pro}$ can be demonstrated in vitro by coimmunoprecipitation of 2C with anti-His antibody. Deletion analysis suggests that the 2C central and C-terminal domains that include several serpin motifs are important for $3C^{pro}$-inhibitory activity. To examine the 2C protease inhibitory activity in vivo, stable HeLa cell lines were made that express 2C in an inducible fashion. Infection of 2C-expressing cells with poliovirus led to incomplete (or inefficient) processing of viral precursor polypeptides compared to control cell lines containing the vector alone. These results suggest that 2C can negatively regulate the viral protease $3C^{pro}$. The possible role of the 2C protease inhibitory activity in viral RNA replication is discussed.

Poliovirus is the prototype member of the picornavirus family with a plus-sense RNA genome of 7,440 nucleotides, which is covalently linked to a small viral protein (VP_g) at its 5′ end and polyadenylated at its 3′ end (34, 56). The positive-strand mRNA, which lacks VP_g, codes for a single large polyprotein, which is processed into mature proteins by viral proteases $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$ (reviewed in references 39 and 71). Biochemical and genetic evidence suggests that most of the poliovirus nonstructural proteins are involved in viral RNA replication in the form of precursors, mature polypeptides, or both (71). The viral RNA polymerase precursors $3CD^{pro}$, the VP_g precursor 3AB and a cellular polypeptide, poly(rC)-binding protein, have been shown to interact with the 5′-cloverleaf structure of viral RNA, leading to the formation of a functional complex important for viral RNA replication (3, 4, 31, 47, 72). The precursor proteins 3AB and $3CD^{pro}$ also interact with the 3′-untranslated region of viral RNA in the absence of other proteins (31). The initiation of poliovirus RNA synthesis appears to be primed by a protein-nucleotidyl covalent complex (VP_g-pU or VP_g-pUpU) (10, 50, 52).

Although it was initially thought to be a member of the cysteine protease family, mutational analyses, amino acid sequence comparison, and three-dimensional modeling have suggested that $3C^{pro}$ adopts a fold similar to that found in serine proteases such as chymotrypsin (12, 28, 30, 32, 33, 37, 45). The other viral protease, $2A^{pro}$, also possesses a chymotrypsin-like fold that is related to smaller serine proteases such as α-lytic proteinase (12). Although the major function of these proteases is to process viral precursor polypeptides, both proteases are also involved in the shutoff of host cell metabolism. Although $2A^{pro}$ is involved in the shutoff of host cell translation (13, 29, 41), $3C^{pro}$ has been shown to cleave and inactivate a number of host cell transcription factors leading to the inhibition of cellular transcription (19, 61, 70, 75).

The 2C protein of poliovirus is 329 amino acids long (37.5 kDa) and is highly conserved among picornaviruses (5). Genetic analyses have implicated the 2C polypeptide in a number of functions during viral replication such as uncoating (40), host cell membrane rearrangement (18), RNA replication (reviewed in reference 71), and encapsidation (69). The exact role of 2C in these processes, however, is not known. Many mutations in the 2C region have been found to be lethal. Studies with nonlethal 2C mutants suggest that 2C has at least two functions in RNA replication: a *cis*-acting guanidine-sensitive function required for initiation and a *trans*-acting function required for elongation (71). More recent results utilizing an in vitro translation/replication system (10, 43), which produces viable poliovirus, have shown that 2C is required prior to or during initiation of minus-strand RNA synthesis (11). During poliovirus infection, 2C and its precursor 2BC migrate to the rough endoplasmic reticulum, where they induce the formation of smooth membrane vesicles that bud off and become the site of viral RNA synthesis: the replication complex (2, 14–16, 18, 62, 65). Although the 2C protein lacks a defined membrane-binding domain, the N-terminal region encompassing amino

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TABLE 1. Serpin domain alignment of 2C sequences

Protein (accession no.)	Local alignment score	No. of aligned aa[b] residues (% identity)	Alignment of poliovirus 2C (NP 740473.1) with putative serpin domains		
			gi designation(s)		Sequence[a]
Ovomucoid serpin (SW P05572)	22	54 (22.2)	gi|25121845|ref|NP_740473.1|	264	MATEMCKNCHQPANFKRCCPLVCGKAIQLMDKSSRVRYSIDQITIMIINE
			gi|124764|sp|P05572|IOVO_CYGAT	1	VATVDCSDYPKPACTMEYMP-LCGSDNKTYGN--KCHFCNAVVDS---NG
			gi|25121845|ref|NP_740473.1|		RNRRSNIGNC 323
			gi|124764|sp|P05572|IOVO_CYGAT		TLTLSHFGKC 54
Serine protease inhibitor (GB P09006)	68	133 (18.8)	gi|25121845|ref|NP_740473.1|	100	RIQKLEHTINNYIQFKSKHRIEPVCLLVHGSPGTGKSVATNLIARAIAER
			gi|25121845|ref|GB_P09006|	79	KMQQVEASLQPETLRRWKDSLRPSIDELY-LPKFSISADYNLEEDVLPEL
			gi|25121845|ref|NP_740473.1|		ENTSTYSLPPDPSHFDGYKQQGVVIMDDLNQNP-DGADMKLFCQMVSTVE
			gi|25121845|ref|GB_P09006|		GIKEAFSTQADLSGITGDKD--LMVSEVVHKAVLDVAETGTEAAAATGVK
			gi|25121845|ref|NP_740473.1|		FIPPMASLEEKGILFTSNYVLASTNSSRISPPTVAHS 235
			gi|25121845|ref|GB_P09006|		FVPVSAKLDPLIIAFDRPFLMIISDTETAIAPFLAK- 212
Pancreatic trypsin inhibitor (SW P00998)	37	18 (38.9)	gi|25121845|ref|NP_740473.1|	210	GILFTSNYVLASTNSSRI 227
			gi|124857|sp|P00998|IPK1_PIG	28	GITYSNECVLCSENKKRQ 45
Fowl pox-virus antithrombin homolog (SW P141269)	47	66 (18.2)	gi|25121845|ref|NP_840473.1|	35	KIIPQA----RDKLEFVTKLRQLEMLENQISTIHQSCPSQEHQEILFNNV
			gi|19856142|sp|P14369|V251_AFO	30	KIISKKDMTLKEIVLYLPKFELEDDVDLKDALIHMGCND------LFKSG
			gi|25121845|ref|NP_740473.1|		RWLSIQSKRFAPLYAVEAKRIQKLEH 106
			gi|19856142|sp|P14369|V251_AFO		ELVGISDTKTLRIGNIRQKSVIKVDE 99

[a] Symbols: *, identical in sequence and alignment; :, conserved substitutions observed; ., semiconserved substitutions.
[b] aa, amino acid.

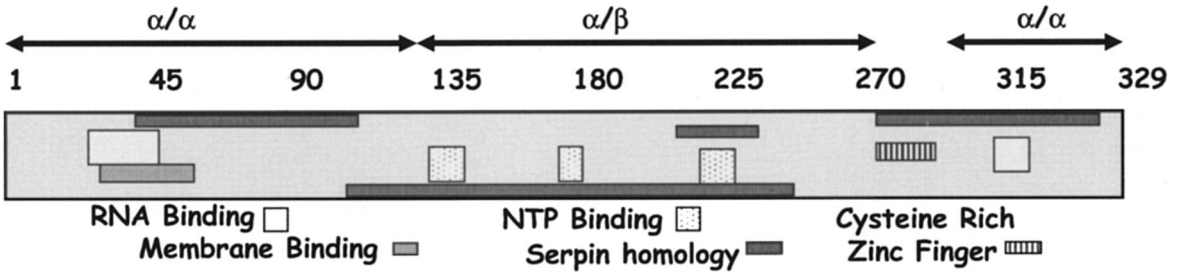

FIG. 1. Schematic diagram of domain structures of 2C. A linear diagram of 2C shows the locations of putative domains as determined by computer analyses and prior studies from various laboratories. The numbers at the top indicate the amino acids from N (1) to C (329) termini of the 2C polypeptide.

acids 21 to 54 appears to be important for membrane binding (24). The N-terminal fragment of 2C containing an amphipathic helix (49) is sufficient to target a soluble protein, chloramphenicol acetyltransferase (CAT), to cellular membrane (23). A second amphipathic α-helix located at the C terminus has also been implicated in 2C membrane interaction (66). Apart from its membrane-binding ability, 2C also binds to viral RNA in vitro (57) and appears to be attached to viral RNA in the replication complex that forms on virus-induced smooth vesicles (14). Purified 2C interacts specifically with the 3′-terminal cloverleaf of the minus-strand RNA through the sequence UGUUUU when present in the context of a double-stranded structure (7). This interaction requires an intact stem-loop B within the minus-strand cloverleaf (8). In addition to 2C, two host cell proteins bind to the 3′-terminal cloverleaf of the minus-strand RNA (59, 60). The 2C protein also has ATPase and GTPase activities (42, 58). Point mutations targeted to the conserved amino acids of the nucleoside triphosphate (NTP)-binding motif result in nonviable viruses that do not replicate their RNA, although such point mutants display normal proteolytic processing patterns (42, 67). The 2C protein also contains a zinc-binding cysteine-rich motif (53). The 2C ATPase activity is inhibited by low concentrations of guanidine hydrochloride (54), a potent inhibitor of picornavirus RNA replication in vivo.

In order to gain further understanding into the functions of this multifunctional viral protein (2C), its protein sequence was analyzed extensively. The main objectives were to find distantly related proteins of known functions and align related sequences to the 2C protein to identify possible motifs or domains of interest. As expected, the first 76 matches corresponded to related single-stranded, plus-strand RNA virus sequences. A second group of homology included several known ATPases, ATP- and GTP-binding proteins, and viral helicases. A more intriguing finding was a third group of proteins that showed homology to 2C. This group included protease inhibitors, particularly serine protease inhibitors or "serpins" (26, 76). Serpins are believed to inhibit protease activity by binding to the catalytic pocket of proteases and blocking their ability to associate with their substrates. The inhibitors may be cleaved by the protease, but at much slower rates than normal substrates. Many of these proteins are characterized by having several repeats of short inhibitor domains of ca. 50 to 60 amino acids. Pairwise local alignments to the protease inhibitors showed that there were several possible serpin motifs scattered throughout the 2C sequence (Table 1 and Fig. 1). In addition, 2C was found to be homologous to the proteosome regulatory subunits from various organisms (data not shown). These observations suggest the interesting possibility that 2C may regulate viral or cellular protease activity.

Here we demonstrate that the purified 2C protein is indeed capable of inhibiting the activity of both the viral proteases $3C^{pro}$ and $2A^{pro}$ in vitro. The regions in the center of the protein and at the C terminus that include three predicted serpin motifs appear to be important for its protease inhibitory activity. A physical interaction between 3C and 2C can be demonstrated by coimmunoprecipitation of 2C by an antibody that recognizes $3C^{pro}$. Infection by poliovirus of HeLa cell lines that express 2C in an inducible fashion results in a processing pattern consistent with slower processing of a number of poliovirus precursor polypeptides. These results suggest that the poliovirus 2C protein is capable of regulating $3C^{pro}$ activity both in vitro and in poliovirus-infected cells.

MATERIALS AND METHODS

Plasmids. pGEM-CREB was a gift from R. Gaynor (University of Texas). Poliovirus 2A was cloned into pET-21b between BamHI and XhoI enzyme sites after PCR amplification by using the following forward and reverse primers: 5′-GTG TAA GGA TCC GGG ATT CGG ACA CCA AAA CAA AGCG-3′ and 5′-AAT TTA CTC GAG TTG TTC CAT GGC TTC TTC TTC GTAG-3′ incorporating appropriate restriction sites (underlined). 3CD was cloned into pET-15b as described earlier (7), and wild-type (wt) 2C and the 2C deletion mutants were cloned into pGEM, pET-15b, and pET-21b as previously described (7, 24). In addition, 2C and the deletion mutants were subcloned into the in vitro expression vector pET21b (Novagen) with a carboxy-terminal V5 epitope tag. The 2C coding sequence was PCR amplified by using a forward 5′-terminal primer, which annealed to the first 13 to 15 nucleotides of the coding sequence. Immediately upstream of this, an NdeI endonuclease restriction site was inserted flanked by nucleotides complementary to the parental vector. Adjacent to the 3′-terminal end of the coding sequence, a six-nucleotide spacer is located, followed by the EcoRV, BstXI, NotI, XhoI, XbaI, ApaI, and SacII restriction sites. These are immediately followed by the V5 epitope. The reverse 3′-terminal primer was complementary to the last 15 nucleotides of the V5 epitope, followed by a HindIII restriction site and 9 nucleotides of the parental vector. The 5′-NdeI and 3′-HindIII restriction sites were used to subclone the sequence into the pET21b plasmid. A similar scheme was used for each of the 2C deletion mutants.

Cell lines. 2C expressing cell lines and controls were made by using the original ClonTech Tet On/Off system vectors, pTet-On, pTRE2, and pTK-Hyg. HeLa cells were transfected with the pTet-On plasmid and selected with G418 (900 μg/ml). Selection was continued for 2 months to ensure stable integration of the plasmid. HeLa-pTet-On cell lines were clonally selected. These cell lines were then used to construct the 2C-expressing cell line and the vector-alone control cell line. The 2C gene was PCR amplified with the primers 5′-GGTCGGCCG CATATGGGTGACAGTTGGTTG and 3′-CTGATATCAGAATAGCAGTA GCAGCATGTCTAGTGGTCCTTGAAACAAAGCCTCC. The resulting PCR

product was gel purified and digested with EagI and EcoRV. The pTRE2 vector was digested with EagI and EcoRV prior to ligation. In each case, the vector pTRE2 (with or without 2C) was transfected into the HeLa-pTet-On cells along with the pTk-Hyg plasmid, which carries the hygromycin resistance gene according to the ClonTech protocol. Stable cell lines were selected by using G418 (900 μg/ml) and hygromycin B (400 μg/ml). 2C is expressed in these cell lines in the presence of doxycyclin (1-3 μg/ml). The presence of the 2C gene inside the cells was confirmed by PCR (Amersham Ready-To-Go PCR beads) of the genomic DNA purified with Qiagen's genomic DNA kit. Expression of 2C RNA in the cell line was confirmed by reverse transcription-PCR (RT-PCR) (Amersham First-Strand-Ready-To-Go beads) of the total cellular RNA purified by using the Qiagen RNA purification kit. The cell lines containing 2C or vector alone were grown in medium lacking tetracycline but containing fetal bovine serum (ClonTech). The presence of the 2C sequence in the cell line was checked by PCR before each experiment with the cell line. This was necessary because the selection marker and the 2C gene were present on different plasmids in these cells, and the possibility existed that the cell line could lose the gene upon repeated passages.

Poliovirus infection and plaque assay. The 2C cell line (E2) and vector control cell line (9E) were grown overnight (10 h) in the presence of doxycycline (2 μg/ml). The following morning the cells were infected with poliovirus at a multiplicity of infection of 10 in methionine-deficient medium. The infection was allowed to continue for 3 to 4 h. At the end of this time, 5 μg of actinomycin D/ml was added to the cells. The cells were incubated for 10 to 15 min and then pulsed with 10 μCi of [^{35}S]methionine (specific activity, 1,000 Ci/mmol; ICN Biochemicals) for 10 to 15 min, followed by a chase with 100 mM cold methionine. Samples were taken every 20 min after the chase. Extracts were made by repeated freeze-thawing of the cells, and viral proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. Labeled viral precursor polypeptides were quantified by using the Image J program provided by the NIH. For plaque assay, HeLa cells (~3.2 × 10^6) were grown in 60-mm culture plates to near confluency in Dulbecco modified Eagle medium with 10% fetal bovine serum and newborn calf serum. 2C and vector HeLa-pTet-On cell lines were incubated with 2 μg of doxycycline/ml for 10 h before the assay. Incubation of doxycycline with the cell lines for longer periods (24 h) resulted in fewer observed plaques. For the assay, cells were aspirated and washed, incubated with 200 μl of various dilutions of wt poliovirus (Mahoney) for 30 min at 37°C in a CO_2 incubator. The plates were rocked every 5 min during this time. The virus was then aspirated off, and an agarose overlay consisting of 51% of 1.8% Noble agar, 47% of 2× Dulbecco modified Eagle medium without serum, and 2% fetal bovine serum was added to the cells, and the mixture was allowed to harden at room temperature for about 15 min. The plates were incubated at 37°C for 24 h. Then, 2.5 ml of a 0.03% neutral red solution was added to the plates. The plates were then incubated in the dark at 37°C for 2 h and aspirated. After 24 h of further incubation at 37°C in the dark, plaques were counted.

Purification of 2C, 2A^{pro}, and 3C^{pro} proteins. Poliovirus 3C^{pro} was cloned into pQE30 (Qiagen) as described earlier and purified from *Escherichia coli* M15 pREP4 strain after IPTG (isopropyl-β-D-thiogalactopyranoside) induction (63). Poliovirus 2A^{pro} was purified from *E. coli* strain BL21(DE3) harboring pET21b recombinant plasmid, and the protein was purified 3.5 h after IPTG induction. The protein was purified by using Talon resin under denaturing conditions by using a protocol similar to that described earlier (7). Poliovirus-encoded 2C protein was isolated from BL21(DE3) cells 3.5 h after IPTG induction from the supernatant fraction under nondenaturing conditions. The final eluate from the Talon resin (ClonTech) was dialyzed against buffer A (50 mM Tris [pH 7.4], 10% glycerol, 1 mM dithiothreitol [DTT], 50 mM KCl). The presence of 2C was monitored by checking RNA-binding activity by using a mobility shift assay and Western analysis with polyclonal antibody to 2C as described previously (7).

Protease reactions. The indicated amounts of purified viral 3C and 2A proteases (3C at 0.5 and 2.0 μg and 2A at ~20 ng [unless stated otherwise]) were added to in vitro-translated [^{35}S]methionine-labeled CREB or viral 3CD proteins. Reactions were incubated at 30°C for 4 h, followed by the addition of 2× SDS sample buffer to terminate the reaction. In some reactions, 3C^{pro} or 2A^{pro} was preincubated for at least 30 min at 30°C with the indicated amounts of 2C before the addition of labeled substrate to the reaction. Thrombin and enterokinase were obtained commercially (Novagen, Inc.), and cleavage reaction protocols were followed according to the manufacturer's instructions. Enterokinase was diluted to 0.06 U with dilution buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 2 mM $CaCl_2$, 50% glycerol) before use, whereas thrombin was diluted to 0.005 U with cleavage buffer (20 mM Tris-HCl [pH 8.4], 150 mM NaCl, 2.5 mM $CaCl_2$). The reactions were incubated overnight at room temperature, followed by the addition of 2× SDS-PAGE sample buffer. The samples were analyzed on 4 to 20% gradient gels.

Western blot analysis. Purified protein or reactions containing cleaved proteins were mixed with SDS sample buffer, resolved on an SDS-PAGE gel, and transferred onto nitrocellulose membrane as described earlier (7). The polyclonal antibody used for detection of 2C was a third bleed from rabbits injected with purified full-length 2C used at a 1:100 dilution.

The 48-kDa cleavage control protein used in the thrombin and enterokinase protease assays was blocked according to the manufacturer's instructions. The nitrocellulose was incubated with an S-protein alkaline phosphatase conjugate at room temperature for 30 min, and the complex was visualized by using a chromogenic substrate BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium kit (Kirkegaard and Perry Laboratories).

Immunoprecipitation. In vitro-translated, [^{35}S]methionine-labeled CAT or poliovirus 2C protein was incubated with the appropriate antibody in 1× radioimmunoprecipitation assay buffer (20 mM Tris-HCl [pH 7.5], 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 2.5 mg of bovine serum albumin [BSA]/ml) with aprotinin. Proteins were incubated for 3 h on ice; the specific polyclonal or preimmune sera appropriate for the reaction was then added, and incubation continued for an additional 2 h on ice. The anti-His monoclonal antibody was obtained from ClonTech. The immune complexes were precipitated by using preswollen protein A-Sepharose. The Sepharose-immune complex was washed extensively (five to six times) with 1× radioimmunoprecipitation assay, followed by two washes with phosphate-buffered saline and recovery of the immunoprecipitated protein complex by elution with 30 μl of 2× SDS sample buffer.

Immunodepletion assay. Immunoglobulin G (IgG) was isolated from hyperimmune rabbit sera injected with bacterially expressed glutathione *S*-transferase–2C fusion protein by using a standard protocol (Pierce Chemicals). Purified 2C (~1.5 μg of protein) was incubated with either immune IgG or preimmune sera for 3 h on ice, after which preswollen protein A-Sepharose beads (in 10 mM Tris [pH 7.4]) were added, followed by incubation overnight on ice. The protein A-Sepharose complex was pelleted by centrifugation, and the resulting supernatant were designated depleted 2C extracts. These extracts were used in the 3C^{pro} cleavage assay. For heat denaturation experiments, 2C was denatured by placement in a heating block preset at the indicated temperature for the required time. The heated samples were spun at room temperature for 5 min in a microfuge, and the supernatant was saved and used in the appropriate cleavage assay.

In vitro transcription and translation. The clones for in vitro transcription were linearized with the appropriate restriction enzymes. pCREB was linearized with HindIII, gel purified, and in vitro transcribed by using SP6 RNA polymerase. The 2C clone was linearized with AvaII before transcription by the T7 RNA polymerase. The mRNA obtained was alcohol precipitated and subsequently translated in rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). 3CD and CAT constructs were translated by using the Promega coupled transcription-translation system. In vitro-synthesized proteins were labeled with 40 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham) during translation.

In vitro RNA polymerase II transcription assays. RNA polymerase II transcription reactions were carried out by using a plasmid, pA326 (75), containing the TATA initiator promoter as a template for in vitro reactions. Transcription reaction mixtures containing 700 ng of template DNA, 100 μg of HeLa nuclear extracts, 0.5 mM each of four ribonucleoside triphosphates, 40 U of RNasin, 5 mM $MgCl_2$, and 1 mM DTT in a reaction volume of 25 μl. These reaction mixtures were incubated at 30°C for 90 min, and the reactions were terminated by the addition of 200 μl of stop solution (300 mM Tris-HCl [pH 7.4], 2 mM EDTA, 300 mM sodium acetate, 250 μg of tRNA/ml). The RNA was extracted with phenol and phenol-chloroform, followed by ethanol precipitation. The RNA was annealed with 50,000 cpm of Sp6 primer, 25 mM Tris-HCl [pH 8.3], 25 mM KCl, 5 mM $MgCl_2$, 0.25 mM spermidine, and 5 mM DTT in a reaction volume of 11 μl. The mixture was incubated for 20 min at 55°C, followed by 10 min at room temperature. This annealed mixture was analyzed by the primer extension method. The primer extension reaction contained the annealed mixture described above plus 10 U of avian myeloblastosis virus reverse transcriptase (Life Science, Inc.), 1 μl of 5× avian myeloblastosis virus reverse transcriptase buffer (250 mM Tris-HCl [pH 8.3], 50 mM $MgCl_2$, 250 mM KCl, 50 mM DTT, 2.5 mM spermidine, 1.5 mM deoxynucleoside triphosphates), and 2.8 mM sodium pyrophosphate in a 20-μl reaction volume. The reaction mixture was incubated at 42°C for 1 h. The reactions were stopped by the addition of 20 μl of stop dye (80% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue), loaded onto an 8% acrylamide–8 M urea gel, and subjected to electrophoresis.

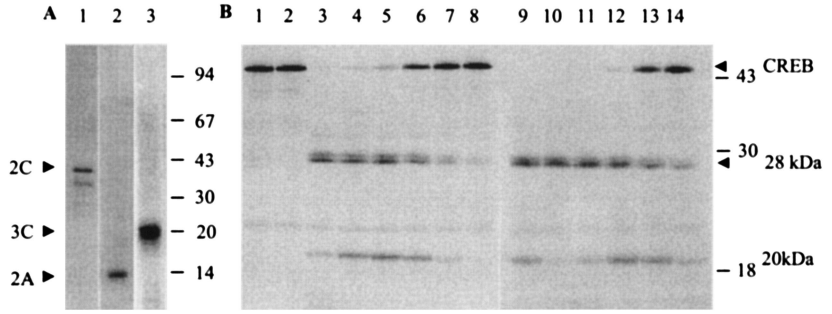

FIG. 2. Poliovirus $3C^{pro}$ activity is inhibited by the 2C polypeptide. (A) Totals of 300 ng of 2C (lane 1) and 2A (lane 2) and 800 ng of 3C (lane 3) were analyzed by SDS-PAGE, followed by silver staining as described in Materials and Methods. (B) Totals of 500 ng (lanes 3 to 8) and 2 μg (lanes 9 to 14) of $3C^{pro}$ was preincubated with buffer (lane 1), 500 ng of 2C (lane 2), or various amounts of purified 2C prior to labeled substrate addition (1 μl of in vitro-translated, [^{35}S]methionine-labeled CREB) as described in Materials and Methods. Reactions were terminated by the addition of 2× SDS sample buffer (1:1 [vol/vol]), and proteins were resolved by SDS–14% PAGE, followed by autoradiography. The numbers on the left show the position and approximate molecular mass (in kilodaltons) of the marker proteins. The arrowheads on the right indicate the migration of full-length and the proteolyzed CREB products (~28 and ~20 kDa). Lanes 4 through 8 contain 50, 125, 250, 375, or 500 ng of 2C, respectively. Lanes 10 through 14 contain 50, 125, 250, 375, or 500 ng of 2C, respectively.

RESULTS

2C inhibits $3C^{pro}$-mediated cleavage of transcription factor CREB in vitro. In order to gain further understanding into the function of 2C, its amino acid sequence was analyzed extensively. The main goals were (i) to find distantly related proteins of known functions and (ii) to align related sequences to the 2C protein in order to identify possible motifs or domains of interest. Initial profile search was performed by using a nonredundant protein database containing entries from the SWISS-PROT sequence data bank at the European Molecular Biology Laboratory (EMBL) (6), the protein identification resource of the National Biomedical Research Foundation, and the Gert peptide database (6, 64). Protein sequences with a Z score higher than 6.0 were then characterized further by reading their annotations in the database by using local alignment programs. Table 1 shows pairwise local alignments between poliovirus 2C and various protease inhibitors. Four potential protease inhibitory domains were scattered throughout the entire 2C sequence. The longest serpin motif identified by the computer program was present in the 2C central domain (amino acids 100 to 235), which also included a shorter motif (amino acids 210 to 227) with a high degree of homology (Table 1 and Fig. 1). Two other serpin motifs were located at the N- and C-terminal regions of 2C spanning amino acid residues 35 to 106 and 264 to 323, respectively (Table 1).

To verify experimentally whether the 2C polypeptide indeed contains protease inhibitory activity, the 2C protein was expressed in bacteria and purified under nondenaturing conditions. Bacterially expressed, poliovirus $3C^{pro}$ and $2A^{pro}$ were also purified to near homogeneity (63, 73). Figure 2A shows analysis of purified $3C^{pro}$, 2C and $2A^{pro}$ by SDS-PAGE, followed by silver staining. Purified preparations of both $2A^{pro}$ and $3C^{pro}$ showed single polypeptides with the appropriate molecular weights. The highly purified 2C protein consisted of mainly two polypeptides: the full-length 2C protein and a truncated form of 2C. Both polypeptides were found previously to specifically react with anti-2C (7). The transcription factor cyclic AMP-responsive element binding protein (CREB),

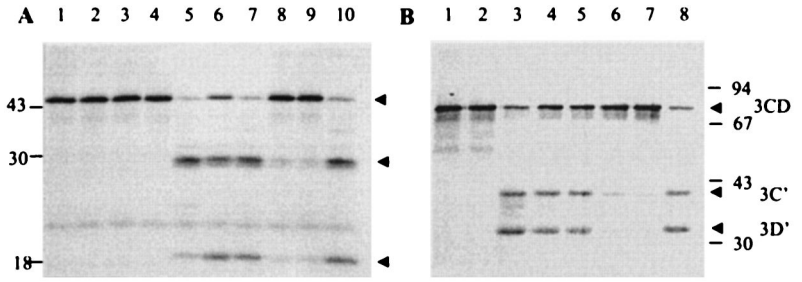

FIG. 3. 2BC inhibits $3C^{pro}$ activity. (A) In vitro-translated, [^{35}S]methionine-labeled CREB was incubated with buffer (lane 1) or 500 ng each of bacterially expressed, affinity-purified 2B (lanes 2 and 6), $3D^{pol}$ (lanes 3 and 7), 2BC (lanes 4 and 9), and 2C (lane 8) in the absence (lanes 1 to 4) or presence (lanes 5 to 9) of 500 ng of $3C^{pro}$. Lane 10 is the same as lane 5 except that 500 ng of an extract from bacteria harboring the plasmid without the 2C insert was used. (B) $2A^{pro}$ activity is inhibited by 2C. Various amounts of 2C were preincubated with 20 ng of purified $2A^{pro}$ before addition to the reactions containing [^{35}S]methionine-labeled 3CD. Reactions were terminated by the addition of 2x SDS sample buffer and proteins resolved on a 14% SDS polyacrylamide gel. All reactions contain labeled [^{35}S] methionine-labeled 3CD. Lane 1, control reaction with labeled 3CD as substrate plus buffer; lane 2, 500 ng of 2C; lane 3, 20 ng of $2A^{pro}$; lanes 4 to 7, reactions containing $2A^{pro}$ (20 ng) preincubated with 125 ng (lane 4), 250 ng (lane 5), 375 ng (lane 6), 500 ng (lane 7) of 2C, or 500 ng of heat-inactivated 2C (lane 8) before the addition of labeled 3CD substrate. The arrowheads indicate full-length 3CD and proteolyzed products 3C′ and 3D′, respectively. Positions of the migration of molecular markers are indicated on the right (in kilodaltons).

shown to be directly cleaved by $3C^{pro}$ (74), was used as a $3C^{pro}$ substrate in an in vitro cleavage assay. In vitro-translated [^{35}S]methionine-labeled CREB was incubated with buffer (lane 1), 2C (lane 2), $3C^{pro}$ (lane 3), or increasing concentrations of 2C in the presence of a constant amount of $3C^{pro}$ (lanes 4 to 8) (Fig. 2B). The following lanes in Fig. 2 (lanes 9 to 14) show a similar experiment except that a fourfold-higher amount of $3C^{pro}$ was used in this experiment compared to those shown in lanes 1 to 8. Incubation of the labeled CREB with 2C alone had no significant effect on the CREB protein (lane 2) compared to the control (lane 1). Proteolytic cleavage of CREB by $3C^{pro}$ into two major fragments (28 and 20 kDa) was apparent at both concentrations of $3C^{pro}$ (lanes 3 and 9, Fig. 2B). We have shown previously that the two fragments of CREB are generated by $3C^{pro}$-mediated cleavage of the Q-G pair at position 172 to 173 of CREB (74). The 28-kDa proteolytic fragment of CREB migrated as a doublet. The precise reason for this is not known. However, $3C^{pro}$-mediated cleavage of an additional Q-G bond between amino acids 187 and 188 of CREB could be responsible for the production of the doublet. Increasing the concentration of 2C in the reaction clearly inhibited the cleavage of CREB by $3C^{pro}$, as evidenced by the reduction of both the proteolytic fragments concomitant with the appearance of full-length CREB (lanes 4 to 8 and lanes 10 to 14). Quantification of the full-length CREB polypeptide showed >90% inhibition of $3C^{pro}$-mediated cleavage of CREB at the highest concentration of 2C (lane 8). The inhibition of CREB cleavage at higher concentrations of $3C^{pro}$ was significantly lower than that observed at the lower concentration of the protease (compare lanes 6 and 12). We then tested a few other viral nonstructural proteins for their ability to block $3C^{pro}$-mediated cleavage of CREB. As presented in Fig. 3A, both 2C and 2BC were able to almost totally block the proteolytic cleavage of CREB by $3C^{pro}$ (lanes 5, 8, and 9). The viral RNA polymerase, $3D^{pol}$, however, had no detectable effect in blocking $3C^{pro}$ activity (lane 7). The viral protein 2B showed low but detectable activity compared to the control (lanes 5 and 6). Thus, 2C and 2BC were the major polypeptides that efficiently blocked $3C^{pro}$ activity among the viral proteins tested. An extract prepared from bacteria that harbored the plasmid lacking the 2C insert did not inhibit $3C^{pro}$ activity significantly (lane 10). To rule out the possibility that 2C-mediated inhibition of $3C^{pro}$ activity was specific to cellular transcription factor CREB, two other transcription factors, TBP (TATA-binding protein) and an RNA polymerase I factor, SL-1, were tested in this assay. The results were very similar to that observed with CREB; the $3C^{pro}$-mediated cleavage of both TBP and SL-1 was almost completely blocked by 2C, suggesting the protease inhibitory effect of 2C was not limited to CREB (data not shown). These results imply that 2C's protease inhibitory effect is most probably mediated through $3C^{pro}$ rather than through the protease substrates.

2C inhibits 2A ^{pro}-catalyzed cleavage of poliovirus 3CD precursor polypeptide. To examine whether 2C is also capable of inhibiting the other poliovirus-encoded protease $2A^{pro}$, in vitro-translated [^{35}S]methionine-labeled 3CD precursor was used as a substrate. Viral protease $2A^{pro}$ cleaves a Tyr-Gly bond within the polymerase sequence of 3CD to generate 3C′ (~37 kDa) and 3D′ (~34 kDa) (43, 67). The 3C′ contains the entire 3C, as well as part of the 3D sequence. As can be seen in Fig. 3B, incubation of 3CD with bacterially expressed, purified $2A^{pro}$ resulted in the cleavage of 3CD to two polypeptides that migrated at ca. 33 and 39 kDa (compare lanes 1 and 3). The molecular masses of these polypeptides are consistent with those of 3C′ and 3D′ observed in poliovirus-infected cells (38). Addition of 2C inhibited cleavage of 3CD in a dose-dependent manner (lanes 4 to 7), and the generation of both 3C′and 3D′ was almost totally blocked at the highest concentration of 2C (lane 7). As a negative control, we used heat-inactivated 2C, which had lost its ability to block $2A^{pro}$ activity (lane 8). Also, affinity-purified proteins from bacteria harboring the plasmid without the 2C insert was unable to block both $3C^{pro}$ and $2A^{pro}$ activities (data not shown). Moreover, $2A^{pro}$-mediated cleavage of the TATA-binding protein, TBP at the 34th Y-G bond (73) was also inhibited by 2C (data not shown). These results suggest that 2C is capable of inhibiting $2A^{pro}$ activity in vitro.

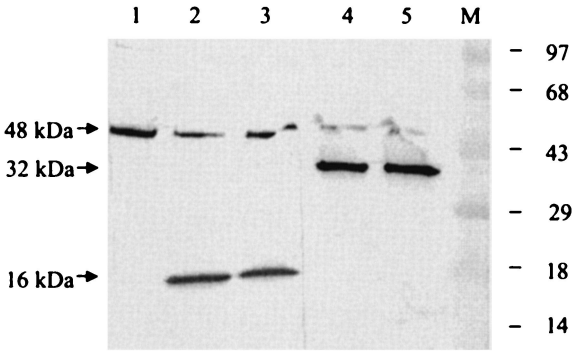

FIG. 4. Effect of 2C protein on cellular proteases. Two eukaryotic proteases, thrombin, and enterokinase were used in this experiment. The activity of the proteases was monitored by Western blot analysis after cleavage of a control protein of 48 kDa supplied by the manufacturer (Novagen, Inc.) in the presence or absence of 2C polypeptide as detailed in Materials and Methods. Lane 1, control protein plus buffer; lane 2, control protein in the presence of 0.06 U of enterokinase; lane 3, same as lane 2 but also containing 500 ng of purified 2C; lane 4, control protein and 0.005 U of thrombin; lane 5, same as lane 4 but also containing 500 ng of purified 2C. The numbers on the right indicate the positions of the prestained molecular weight markers (lane M). The migrations of the 32- and 16-kDa cleaved products and the full-length protein are marked by arrows.

2C does not inhibit cellular enterokinase and thrombin activities. To determine whether 2C inhibits cellular protease activity, we initially examined nonspecific cleavage of bovine serum albumin by trypsin. We found no inhibitory activity of 2C against trypsin (data not shown). We also examined the effect of 2C on two other cellular proteases: a nonserine protease (enterokinase) and a serine protease (thrombin). In the protease assay, the control peptide (~48 kDa), provided by the manufacturer (Novagen), is cleaved by enterokinase and thrombin to 16- and 32-kDa fragments, respectively. As can be seen in Fig. 4, neither the enterokinase nor thrombin catalyzed cleavage of the control peptide can be inhibited by the amount of 2C (Fig. 4, lanes 2 and 3 and lanes 4 and 5) sufficient to almost totally inhibit cleavage of CREB (Fig. 2). It should be noted that the same 2C preparation was used for both experiments (shown in Fig. 2 and 4) and that these experiments were performed concurrently. Thus, 2C does not appear to inhibit at least three cellular proteases.

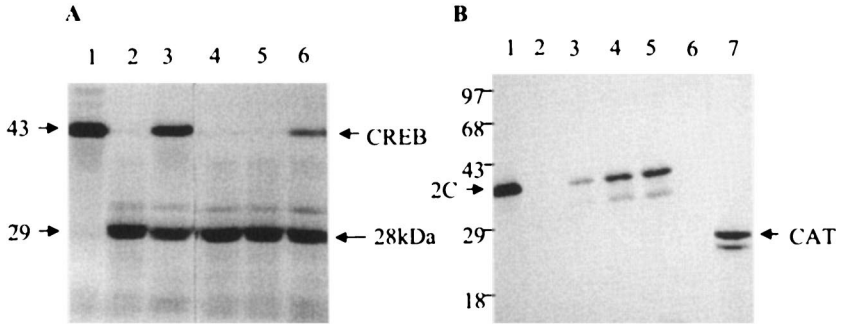

FIG. 5. The protease inhibitory activity of 2C is depleted specifically by 2C antibody. (A) A 1.5-μg portion of 2C was incubated with 3 (lane 4) or 4.5 (lane 5) μg of anti-2C IgG or 4.5 μg (lane 6) of preimmune IgG for 2 h at 4°C. The antigen-antibody complex was removed after incubation with protein A-Sepharose, and the supernatants were assayed for 2C-mediated inhibition of $3C^{pro}$ activity as described in Materials and Methods. [^{35}S]methionine-labeled CREB was incubated with 2C (lane 1), 3C (lane 2), or both 2C and 3C (lane 3) and analyzed directly. Anti-2C depleted supernatants from reactions containing 3 μg of anti-2C IgG (lane 4), 4.5 μg of anti-2C IgG (lane 5), and 4.5 μg of preimmune IgG (lane 7) were assayed for inhibition of $3C^{pro}$ activity. (B) Analysis of physical interaction between $3C^{pro}$ and 2C. A fixed amount (200,000 cpm) of in vitro-translated [^{35}S]methionine-labeled 2C was incubated with various amounts of unlabeled, purified His-tagged $3C^{pro}$ as described in Materials and Methods. An antibody directed against the His epitope was used to immunoprecipitate the 2C-3C complex. Lane 1, [^{35}S]methionine-labeled 2C protein after immunoprecipitation with anti-2C IgG; lanes 2 to 5, [^{35}S]methionine-labeled 2C was preincubated in the presence of 5 μg of BSA (lane 2) or 1.25 (lane 3), 2.5 (lane 4), or 5 (lane 5) μg of unlabeled His-$3C^{pro}$, and the 2C-3C complex was immunoprecipitated with anti-His. [^{35}S]methionine-labeled CAT was preincubated with 5 μg of unlabeled His-$3C^{pro}$ and immunoprecipitated with anti-His (lane 6) or anti-CAT (lane 7) antibodies. The arrows indicate positions of full-length 2C and CAT, and the numbers to the left correspond to the position and approximate molecular masses (in kilodaltons) of marker proteins.

The protease inhibitory activity is depleted by anti-2C antibody. The ability of 2C to inhibit $3C^{pro}$-mediated cleavage of CREB was completely blocked by heating the protein at 80°C, suggesting that the protease inhibitory activity associated with 2C was not due to a contaminating small molecule in the preparation (Fig. 3B). To determine whether the 2C polypeptide was indeed responsible for the protease inhibitory activity, the purified 2C preparation was incubated with anti-2C IgG or preimmune IgG. After incubation, protein A-Sepharose was added to the reaction, and the protein A-bound antigen-antibody complex was removed by centrifugation. The remaining supernatants were then assayed for inhibition of $3C^{pro}$catalyzed cleavage of CREB. As can be seen in Fig. 5A, depletion of 2C by anti-2C leads to almost total inhibition of 2C protease inhibitory activity compared to the reaction treated with preimmune IgG (compare lanes 4 and 5 with lane 6). Some nonspecific inhibition of 2C protease inhibitory activity was observed with the preimmune IgG (lane 6) compared to the untreated sample (lane 3). This is due to nonspecific binding of 2C to protein A-Sepharose (data not shown). In this experiment both the 20-kDa proteolyzed product of CREB and the 18-kDa marker were not detected because they ran off the gel.

2C can form a complex with $3C^{pro}$ in vitro. To determine whether 2C physically interacts with $3C^{pro}$ to bring about inhibition of $3C^{pro}$ activity, in vitro-translated [^{35}S]methionine-labeled 2C was incubated with purified, unlabeled His-tagged $3C^{pro}$, and the resulting complex was immunoprecipitated with anti-His antibody. The ability of anti-His antibody to specifically coimmunoprecipitate labeled 2C would indicate physical association between His-3C and 2C. Lane 1 of Fig. 5B shows analysis of in vitro-translated [^{35}S]methionine-labeled 2C after immunoprecipitation with anti-2C. Immunoprecipitation with anti-His monoclonal antibody of reactions where a constant amount of labeled 2C was incubated with increasing amounts of unlabeled, His-tagged $3C^{pro}$ showed coprecipitation of 2C (lanes 3 to 5). In fact, the intensity of the 2C protein immu-

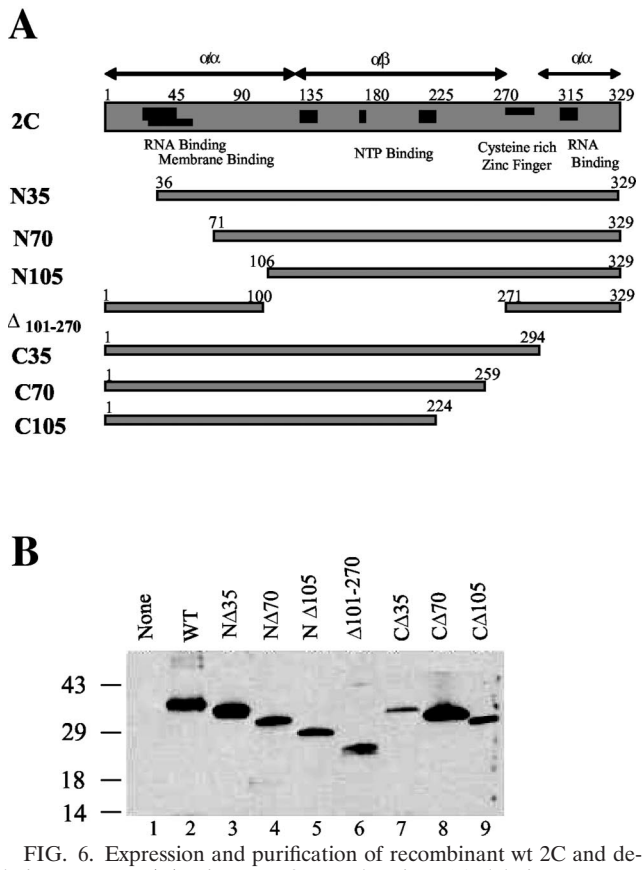

FIG. 6. Expression and purification of recombinant wt 2C and deletion mutants. (A) Diagram of wt and various 2C deletion mutants; (B) V5-tagged wt 2C or various 2C mutants were expressed in *E. coli* and purified by affinity chromatography. The figure shows a Western blot of wt 2C (lane 2) and deletion mutants NΔ35 (lane 3), NΔ70 (lane 4), NΔ105 (lane 5), Δ101-270 (lane 6), CΔ35 (lane 7), CΔ70 (lane 8), or CΔ105 (lane 9) with anti-V5 tag antibody. In lane 1, proteins purified thorough an affinity column from bacterial extracts without the 2C plasmid were analyzed.

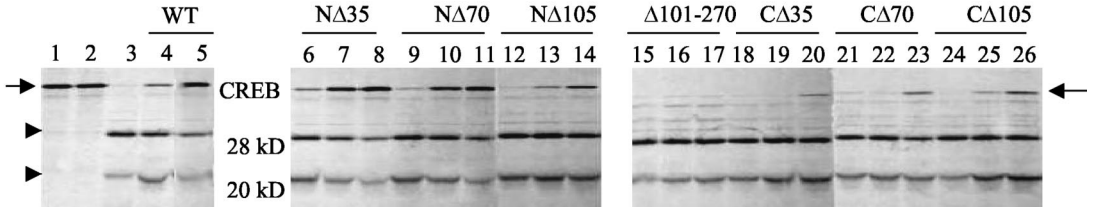

FIG. 7. Effect of 2C mutations on its 3C inhibitory activity. First, 2 μg of $3C^{pro}$ was preincubated with various amounts of purified wt or mutant 2C polypeptides as indicated. The preincubated proteins were then added to reactions containing [^{35}S]methionine-labeled CREB. Reactions were terminated by the addition of 2× SDS sample buffer (1:1 [vol/vol]), and proteins were resolved on 14% SDS-acrylamide gels. The arrow and arrowheads on the left show the migration of full-length CREB and the proteolyzed CREB products (28 and 20 kDa), respectively. All lanes contain [^{35}S]methionine-labeled CREB, and all lanes except lanes 1 and 2 contain $3C^{pro}$. Lanes 1 and 2, CREB alone and CREB incubated with buffer, respectively; lane 3, same as lane 2 except $3C^{pro}$ was added instead of buffer; lanes 4 and 5, same as lane 3 except that 0.25 and 1.0 μg of wt 2C was included; lanes 6, 9, 12, 15, 18, 21, and 24, same as lane 3 except 0.25 μg of various mutant 2C proteins (as indicated at the top) was added; lanes 7, 10, 13, 16, 19, 22, and 25, same as lane 3 except 0.5 μg of various 2C mutants were added; lanes 8, 11, 14, 17, 20, 23, and 26, same as lane 3 except 1.0 μg of various 2C mutants was added.

noprecipitated with anti-His antibody increased almost linearly with increasing concentrations of unlabeled His-$3C^{pro}$ present during preincubation with 2C (lanes 3, 4, and 5). However, the [^{35}S]methionine-labeled 2C protein preincubated with the equivalent amount of BSA comparable to that of His-$3C^{pro}$ used in lane 5 could not be immunoprecipitated by anti-His antibody (lane 2). An unrelated protein (^{35}S-labeled CAT), when preincubated with $3C^{pro}$, was not coimmunoprecipitated by anti-His antibody (lane 6), although the labeled CAT protein from the same reaction can be immunoprecipitated by anti-CAT antibody (lane 7). Immunoprecipitation of [^{35}S]methionine-labeled 2C by anti-2C antibody did not reveal any significant difference in the amount of 2C between samples analyzed in lanes 2 to 5 (data not shown). In this experiment, ca. 20% of the labeled 2C was immunoprecipitated by the anti-His antibody at the highest concentration of His-$3C^{pro}$ (data not shown). These results suggest that the 2C polypeptide is capable of forming a complex with the viral protease $3C^{pro}$ in vitro.

2C deletion mutations affect its ability to inhibit $3C^{pro}$. In order to determine which region(s) of 2C might be involved in the inhibition of $3C^{pro}$ activity, various deletion mutants of 2C were constructed (Fig. 6A). Both the wt and various mutant 2C polypeptides (V5-tagged) were expressed in bacteria and purified by affinity chromatography (Fig. 6B).

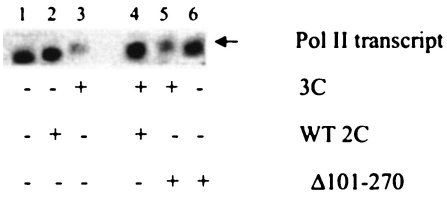

FIG. 8. Reversal of $3C^{pro}$-mediated transcription inhibition by 2C in vitro in HeLa cell extract. In vitro RNA polymerase II-catalyzed transcription was performed in HeLa nuclear extracts as described in Materials and Methods. RNA polymerase II transcription was carried out in the presence of 100 μg of HeLa nuclear extract plus 2C buffer (lane 1), 2.5 μg of 2C (lane 2), 1 μg of $3C^{pro}$ (lane 3), 2.5 μg of wt 2C and 1 μg of $3C^{pro}$ (lane 4), 2.5 μg of mutant (Δ101-270) 2C and 1 μg of $3C^{pro}$ (lane 5), or 2.5 μg of mutant (Δ101-270) 2C (lane 6). The arrow indicates the appropriate transcript.

When CREB was incubated with $3C^{pro}$ in the presence of increasing amounts of 2C protein, various mutants exhibited distinctively different levels of inhibitory capability (Fig. 7). The mutants NΔ35 (lanes 6 to 8) and NΔ70 (lanes 9 to 11) showed no significant difference in the protease inhibitory ability compared to the wt 2C (lanes 4 and 5). The remaining amino-terminal deletion mutant NΔ105 (lanes 12 to 14) showed ca. 55% inhibitory activity compared to the wt protein (lanes 4 to 5). In contrast, the carboxyl-terminal deletions demonstrated only 25% (CΔ35, lanes 18 to 20), 27% (CΔ70, lanes 21 to 23), and 39% (CΔ105, lanes 24 to 26) of the inhibitory activity of the wt protein. The most dramatic decrease in $3C^{pro}$-inhibitory activity was observed in the mutant containing the central deletion (mutant Δ101-270; lanes 15 to 17). The inhibitory activity for this mutant compared to wt 2C was only 3%. These results suggest that the central domain of 2C, which includes two separate but overlapping serpin motifs (Table 1) is important for $3C^{pro}$-inhibitory activity, although the C-terminal domain also plays a role in protease inhibition.

2C reverses $3C^{pro}$-mediated inhibition of RNA polymerase II-mediated transcription in vitro. Poliovirus shuts off the host cell transcription catalyzed by all three cellular RNA polymerases (20). We have shown previously that transcription shutoff by poliovirus can be recapitulated in vitro by $3C^{pro}$ treatment of uninfected cell extracts and is due to $3C^{pro}$-mediated cleavage of TBP (20, 75). To verify the $3C^{pro}$-inhibitory activity of 2C in an independent assay that does not involve the use of reticulocyte lysates, we tested whether purified 2C would inactivate 3C's ability to shutoff pol II transcription in HeLa cell extracts. Purified $3C^{pro}$ was preincubated with purified 2C or equivalent amount of the Δ101-270 2C mutant. These mixtures were then added to HeLa cell extracts, and polymerase II transcription was carried out. As can be seen in Fig. 8, $3C^{pro}$ inhibited transcription from the TATA-initiator promoter significantly (compare lane 3 with lane 1). The $3C^{pro}$-mediated inhibition of transcription can be completely restored by wt 2C (lane 4) but not by the Δ101-270 2C mutant (lane 5), which is defective in blocking $3C^{pro}$ activity. The mutant protein alone did not alter transcription significantly compared to the control (lanes 1 and 6). These results are consistent with our previous

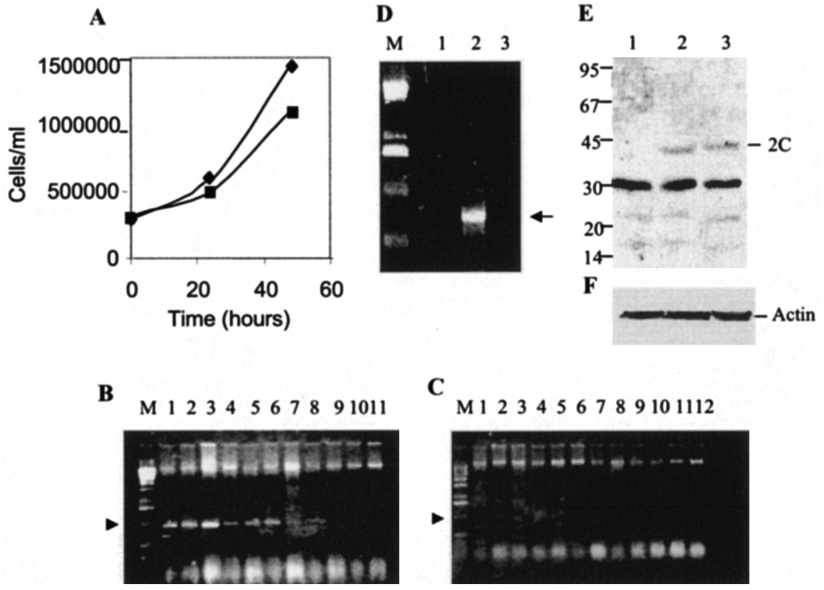

FIG. 9. Inducible expression of 2C in HeLa cells. A HeLa cell line that expresses 2C when induced by doxycycline was prepared as described in Materials and Methods. (A) Rate of cell growth for the 2C cell line (E2 [■]) versus that of the control line with vector alone (9E [◆]) for up to 48 h. (B) Genomic DNA was used to detect 2C sequence from various antibiotic-resistant colonies transformed with a vector containing the 2C sequence by PCR with appropriate primers. The arrowhead indicates the position of the appropriate PCR product. (C) Genomic DNA from various colonies transformed with the vector alone was analyzed by PCR. (D) Total RNA isolated from E2 (lane 2) and 9E (lane 3) cells after 10 h of induction with doxycycline and from HeLa cells (lane 1) was analyzed to detect 2C RNA with appropriate primers. The arrowhead indicates the position of the RT-PCR product. (E) Fifty micrograms of total protein from the E2 cell line (lanes 2 and 3, duplicate) or 9E (lane 1) were used for Western blot with a polyclonal antibody to 2C.

finding that TBP cleavage by $3C^{pro}$ is inhibited by 2C in reticulocyte lysates (data not shown).

2C expression in HeLa cells affects proteolytic processing of poliovirus precursor polypeptides. To determine whether the 2C polypeptide is capable of regulating proteolytic processing of poliovirus precursor polypeptides in vivo, we wished to make a cell line that expresses the 2C protein. After repeated attempts we were unable to obtain any cells that constitutively express 2C. We, therefore, used the Tet On/Off system to make a cell line, which expresses 2C in an inducible manner. Stable cell lines were selected by using G418 and hygromycin B. 2C is expressed in these cell lines in the presence of doxycycline (1 to 3 μg/ml). Genomic DNA PCR assay confirmed the presence of the 2C DNA sequence in the 2C cell lines but not in the control cell line (Fig. 9B and C). Clonally selected cell lines that contain 2C DNA (Fig. 9B, lane 2) and vector alone (Fig. 9C, lane 9) were selected for further studies. RT-PCR of total RNA from doxycycline-treated cells confirmed the presence of 2C RNA only in the 2C expressing cell line but not in the vector alone cells or in normal HeLa cells (Fig. 9D, lanes 1 to 3). The 2C protein could be detected in the cell line (called E2) but not in the vector control line (called 9E) by Western analysis with a polyclonal antibody to 2C (Fig. 9E, lanes 1 to 3). The level of the 2C protein in E2 cells was relatively low. The cells that expressed higher levels of 2C after induction grew poorly, suggesting that higher levels of 2C expression was detrimental to cell growth (data not shown). However, the E2 line expressing relatively low levels of the 2C protein grew efficiently, and the growth rate of these cells was comparable to that of the control cell line (Fig. 9A). We also did not find any significant difference in the overall protein synthesis between the 2C and vector control cells (data not shown). A nonspecific protein, which migrated slightly above the 30-kDa marker, was found to cross-react with the 2C antibody and was present in both cell lines with or without the 2C insert. In addition, small amounts of two other cellular polypeptides of 23 and 18 kDa were also found to cross-react with the 2C antibody nonspecifically.

To examine whether inducible expression of 2C has any effect on proteolytic processing of viral proteins, the 2C cell line (E2) and vector control cell line (9E) were grown overnight (10 h) in the presence of doxycycline (2 μg/ml) and then infected with poliovirus. The infection was allowed to continue for 3 h. At the end of this time, 5 μg of actinomycin D/ml was added to the cells. After 15 min, the cells were pulsed with [^{35}S]methionine for 10 min, followed by a chase with 100 mM cold methionine. Samples of approximately equal number of cells were obtained every 20 min after the chase and analyzed by SDS-PAGE. As can be seen in Fig. 10, the processing of the P1 precursor was relatively fast and essentially complete by 40 min in vector control cells (lanes 1 to 3). This processing event generated VP0, VP1, and VP3. In these cells, very little P3 precursor was detected at zero time of chase (lane 1); however, relatively high amounts of 3CD were present at zero time, indicating that the cleavage at the 3B-3C junction occurs relatively quickly. The 3C and 3D polypeptides were detectable after 20 to 40 min of chase (lanes 2 and 3), and the intensity of these bands increased slightly after 40 to 80 min of chase (lanes 3 to 5). Although P1 processing appeared to be normal with the appearance of VP0, VP1, and VP3 in the 2C-expressing cells, there was a significant difference in the processing patterns of the 2C and vector control cells (lanes 6 to 10). First, after 10 min of labeling (no chase), the intensity of viral pre-

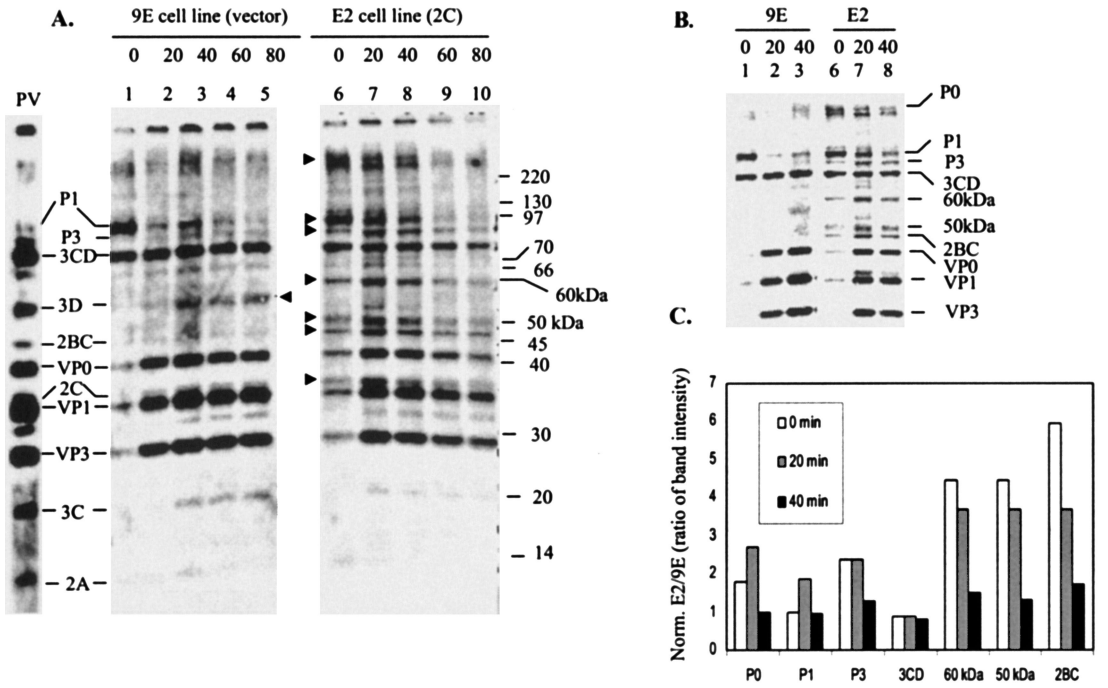

FIG. 10. Proteolytic processing of viral precursor polypeptides in stable HeLa cell line that express 2C in an inducible fashion. (A) Pulse-chase analysis of protein processing in poliovirus-infected HeLa cells expressing the 2C polypeptide. HeLa cells lines 9E (with vector alone, lanes 1 to 5) and E2 (2C expressing cells, lanes 6 to 10) were infected at a multiplicity of infection of 10 with type 1 poliovirus as described in Materials and Methods. Cells were labeled with [^{35}S]methionine at 3 h postinfection. The numbers above the lanes indicate times in minutes after the chase with unlabeled methionine. The lane marked PV shows the migration of poliovirus proteins from HeLa cells infected with poliovirus for 5 h. The migration of molecular markers is indicated on the right (in kilodaltons). The arrowheads pointing right indicate precursor and mature viral proteins that are either present exclusively or in higher amounts in the E2 cell line compared to the control 9E cell line. The arrowhead pointing left indicates the position of migration of 3D. (B) A lower exposure of lanes 1 to 3 and 6 to 8 from Fig. 10A is shown. (C) Quantification of viral precursor proteins from E2 and 9E cells. The viral precursor polypeptides P0, P1, P3, 3CD, 2BC, 60 kDa, and 50 kDa were quantified by densitometric scanning as described in Materials and Methods. The numerical values were normalized with respect to total cellular proteins from virus-infected E2 and 9E cells. The ratio of the numerical values for E2 to 9E cells for each polypeptide at 0, 20, and 40 min of chase are shown.

cursor proteins, such as the P1, P3, 2BC, 60-kDa, and 50-kDa (just above 2BC) proteins and the large polyprotein (P0) migrating more slowly than the 220-kDa marker, was higher than in vector control cells (compare lane 6 with lane 1). Also, the intensity of the P3 precursor increased significantly after 20 min chase compared to that at zero time, suggesting delayed processing of the P0 precursor in the infected E2 cells (lanes 6 and 7, Fig. 10). This increase in P3 can be correlated with the decrease in the intensity of the P0 precursor. In general, the levels of VP0, VP1, VP3, 3D^{pol}, and 3C^{pro} were lower in 2C-expressing cells than in control cells lacking the 2C insert. In particular, there was a striking difference in the amount of 3D^{pol} between the E2 and 9E cells (compare lanes 3 to 5 with lanes 8 to 10). Significant amounts of viral precursors remained even after 40 min of chase in E2 cells, which could be seen clearly in a lower exposure of the gel (compare lanes 7 and 8 with lanes 2 and 3, Fig. 10B). The overall level of the 2C polypeptide was higher in E2 cells than in 9E cells. This was true even at the zero time of chase (compare lanes 1 and 6, Fig. 10A), which could be due to residual translation from the 2C mRNA expressed in these cells from the vector encoding the 2C sequence. This is possibly due to incomplete shutoff of existing mRNAs at 3 h postinfection. The possibility that higher amounts of 2C is made from viral precursors in E2 cells seems unlikely since a significant amount 2BC, a precursor of 2C, is found accumulated in E2 cells (Fig. 10A, lane 7). However, generation of 2C from a larger precursor, e.g., 2BC-3ABCD, cannot be ruled out.

To quantify the difference in the processing of viral precursor polypeptides between E2 and 9E cells, the intensities of individual polypeptides were measured and expressed as E2 to 9E ratio for each polypeptide after normalizing for total protein in infected extracts. After a 20-min chase, the 2BC, 50-kDa, and 60-kDa polypeptides showed the highest level of difference with values close to 3.7-fold between E2 and 9E cells (Fig. 10C). The levels of P0 and P3 were >2-fold in E2 cells compared to 9E cells, whereas the P1 level was ~1.8-fold higher in E2 cells. The 3CD ratio did not change significantly over the period of 40-min chase, although the ratio was <1. These results suggest that viral protein processing is delayed or inhibited in the 2C-expressing cells compared to cells containing the vector alone. Total incorporation of labeled methionine into virus-specific proteins, as determined by densitometric scanning of individual viral proteins, was not significantly different between the 9E and E2 cells (data not shown). This suggests that synthesis of the viral polyprotein during the pulse was not significantly affected by 2C expression. Despite the accumulation of a number of viral precursor polypeptides and a reduced level of mature proteins, the virus titer in E2 cells was reduced only by ~2-fold compared to the 9E cells (data

not shown). This is most probably due to the fact that poliovirus proteins are synthesized in far excess than required for efficient viral RNA replication and packaging. These results suggest that processing of viral precursor proteins is significantly affected in HeLa cells that express 2C.

DISCUSSION

Both genetic and biochemical studies implicate poliovirus 2C polypeptide in a number of key processes during viral infection. These processes include the initiation and elongation of viral RNA synthesis, membrane proliferation, and vesicle induction, and possibly virion assembly. The 2C polypeptide also contains ATPase and GTPase activities and is capable of binding cellular membranes and viral RNA. Here we describe a new activity associated with the 2C polypeptide, namely, its ability to negatively regulate viral protease $3C^{pro}$.

Various lines of evidence strongly suggest that 2C indeed contains protease inhibitory activity. Initial computer analysis of the 2C amino acid sequence identified domains of considerable homology to protease inhibitors with Z (local alignment) scores ranging from 22 to 68 for various serpin motifs scattered throughout the 2C protein (Table 1). Experimental evidence included the demonstration that bacterially expressed, affinity purified 2C protein was capable of inhibiting both viral proteases $3C^{pro}$ and $2A^{pro}$. An *E. coli* extract derived from cells harboring the plasmid lacking the 2C insert did not have the protease inhibitory activity. The protease inhibitory activity was heat labile, suggesting that it was not a small molecule or that the inhibition was due to a component of the buffer. An antibody directed against the 2C protein depleted the protease inhibitory activity associated with 2C. Deletion analyses showed that the central as well as C-terminal amino acid sequences were important for 2C's ability to inhibit $3C^{pro}$ activity. Finally, poliovirus infection of HeLa cells that express 2C in an inducible fashion resulted in accumulation of a number of precursor polypeptides compared to those containing the vector alone in a pulse-chase experiment, suggesting a role of the 2C polypeptide in the regulation of poliovirus protein processing. Although the in vivo results implicate 2C in the regulation of viral protein processing, we cannot rule out the possibility that cellular changes induced by 2C could also contribute to the incomplete protein processing observed in 2C-expressing cells. The in vitro results, on the other hand, strongly suggest a role of the 2C protein in the regulation of viral proteases.

Our choice of using transcription factors as $3C^{pro}$ substrates for the detection of 2C protease inhibitory activity in vitro was based on the availability of these substrates in our laboratory and on the fact that the $3C^{pro}$ cleavage specificity was found to be similar, if not identical, for both viral precursors and transcription factors (20). Both an accessible Q-G pair and an aliphatic amino acid at position P4 were shown to be required for the efficient cleavage of TBP and CREB by $3C^{pro}$, which closely parallels the requirement for cleavage of viral precursors by this protease (reference 20 and data not shown). Thus, although CREB was used as a substrate in the in vitro protease assay, we anticipated similar results with viral precursor proteins. Indeed, our in vivo data of viral protein processing in 2C-expressing cells are clearly consistent with the idea that 2C is capable of regulating viral protein processing by $3C^{pro}$ in poliovirus-infected cells.

The experiment demonstrating 2C's ability to block $2A^{pro}$ activity utilized 3CD as the substrate. Since our immunoprecipitation experiment suggested a physical interaction between 2C and $3C^{pro}$ (Fig. 5), the possibility that 2C-mediated inhibition of 3CD cleavage (by $2A^{pro}$) could be due to 3CD-2C interaction rather than through inhibition of $2A^{pro}$ could not be ruled out. This is highly unlikely, however, since this experiment was performed under conditions that did not favor 3CD (or 3C)-2C interaction. First, $2A^{pro}$ was preincubated with highly concentrated 2C for 2 h in a small volume. The [^{35}S]methionine-labeled 3CD substrate was then added to these reactions so that 2C is diluted at least 15 to 20 times in the final reaction. Under these conditions, the concentration of 2C in the final reaction (~13 ng/ml) was not sufficient to inhibit $3C^{pro}$ (and possibly 3CD) activity. Moreover, $2A^{pro}$-mediated cleavage of TBP at the 34th tyrosine-glycine site (19, 71) was also inhibited by 2C (data not shown).

The results presented in Fig. 10 strongly suggest a role of protein 2C in the regulation of proteolytic processing of viral precursor polypeptides. The processing of viral precursors was significantly slower in 2C-expressing cells than in those containing the vector without the 2C insert. The processing defect was evidenced by accumulation of significant amounts of P0, P1, P3, and 2BC. Increased accumulation of these precursors was accompanied by decreased production of mature polypeptides VP0, VP1, VP2, 3D, and 3C in E2 cells compared to 9E cells. We also detected two viral proteins with approximate molecular masses of 60 and 50 kDa that accumulated in significant amounts in E2 cells. The 60-kDa polypeptide appears to be a 3D-related protein, as it can be immunoprecipitated by an anti-3D antibody (data not shown). A similar protein of ~60 kDa (referred to as protein 4a) was described earlier as an unstable precursor to 3D in the membrane-associated replication complex (25). Interestingly, accumulation of a very similar 3D-related 60-kDa viral protein was detected in cells infected with a mutant poliovirus containing a point mutation (Val to Ala) at position 54 of $3C^{pro}$ (22). The 3C mutant virus was deficient in the production of $3D^{pol}$ and showed a proteolytic defect leading to accumulation of the 60-kDa polypeptide. Despite this processing defect, however, the mutant virus grew to near-wt titers (22). The processing defect imparted either by 2C expression or mutation in the 3C protein may be explained by structural changes in the protease as a result of mutation or interaction with the 2C polypeptide. However, in E2 cells the effect on viral protein processing was more profound, as evidenced by the accumulation of number of precursors rather than just the 60-kDa polypeptide. The nature of the 50-kDa polypeptide detected in E2 cells is not known at present, and its identification will require further investigation.

The results presented in Fig. 10 also suggest that proteolytic processing of P2 and P3 precursors were more affected than that of P1 in 2C-expressing cells. Although these results may suggest differential regulation of 3C versus 3CD proteolytic activity by the 2C polypeptide, these results must be considered tentative until confirmed by direct analysis of the effect of 2C in proteolytic processing of the P1 precursor by the recombinant 3CD protein. In addition, it would be interesting to determine whether 2C is capable of interacting directly with 3CD.

The protease inhibitory activity associated with the 2C protein appears to be specific with respect to viral proteases. Although both poliovirus $3C^{pro}$ and $2A^{pro}$ activities were inhibited by 2C in a dose-dependent manner in vitro, three different cellular proteases—thrombin, enterokinase, and trypsin—were not inhibited by 2C (Fig. 4 and data not shown). These observations, along with the reduced processing of viral precursors in E2 cells, raise the possibility that 2C may be involved in modulating viral protease activities in viral RNA replication The most likely target for this function would be the precursor $3CD^{pro}$ (or $3C^{pro}$). The role of $3CD^{pro}$ as the major protease in the processing of both structural and nonstructural precursors is becoming increasingly apparent (48). Moreover, $3C^{pro}$-related proteolysis could be stimulated by the genome-linked protein (VP_g) and its precursor 3AB in vitro (44). The precursor polypeptides $3CD^{pro}$ and 3AB bind to the 5′ positive strand cloverleaf structure along with the cellular protein, PCBP2 (4, 47, 72). We have shown that 2C (and 2BC) polypeptides interact with the 3′-terminal sequences in the minus-strand RNA, which is complementary to the plus-strand 5′ cloverleaf (7). The physical proximity of 2C to both 3CD and 3AB in the replication complex may provide 2C with the opportunity to control the proteolysis of 3AB into 3A and VP_g by $3CD^{pro}$ (or $3C^{pro}$) (36). Alternatively, 2C could interact with free $3CD^{pro}$ (or $3C^{pro}$) in the cytoplasm. Recent evidence also suggests that the $3D^{pol}$-catalyzed uridylylation of VP_g is stimulated by 3CD (51). Thus, prolonging the half-lives of both 3AB and 3CD by 2C/2BC may help initiation of RNA synthesis (possibly through 3AB uridylylation).

Although prior expression of 2C in HeLa cells results in inefficient processing of viral precursors during viral infection, how 2C exerts its protease inhibitory function in polyprotein processing during a normal infection is not known. The magnitude of 2C-induced inhibition of processing observed in vivo was much lower than that seen in vitro. In fact, the virus titer in E2 cells was reduced only twofold compared to the 9E cells. The regulation of viral proteases by 2C in infected cells must be precise and presumably temporal to ensure proper processing of viral precursor polypeptides, thereby ensuring efficient viral replication. The 2C/2BC polypeptides interact with intracellular membranes, virus-specific RNAs and possibly other viral and/or cellular polypeptides. Thus, the availability of free 2C (or 2BC) in poliovirus-infected cells and when and how they interact with viral proteases or their precursors will be important factors in the regulation of viral proteases.

Studies utilizing deletion mutants of 2C revealed that several regions of the protein might play a role in the regulation of $3C^{pro}$ activity (Fig. 10). This observation is consistent with the fact that many known serine protease inhibitors are characterized by having several repeats of serpin motifs (76). A deletion of the central region of the 2C protein (mutant Δ101-270) demonstrated the most dramatic loss of $3C^{pro}$ inhibitory activity. This region includes the NTP-binding domain (42, 58). The central region also includes two separate but overlapping serpin motifs (Table 1), which could explain the almost total loss of protease inhibitory activity of the Δ101-270 mutant. Alternatively, the overall structure of the protein may have been be altered by this fairly large deletion. The deletion of the C-terminal region also results in significantly decreased protease inhibitory activity of 2C. This can be seen in all three C-terminal deletion mutants including the C-terminal Δ35 mutant. This region of 2C containing a putative alpha helix motif, as well as an arginine-rich RNA-binding motif (49, 57), also contains a 54-amino-acid serpin motif (Table 1). Future studies involving more defined mutations should shed more light on the protease inhibitory activity of the 2C serpin motifs.

How does 2C negatively regulate $3C^{pro}$ activity? We believe that the protease inhibition occurs through physical interaction of 2C with $3C^{pro}$. Indeed, results presented here (Fig. 8) indicate physical association between the two molecules. It is interesting to point out in this context that serpins generally act by binding to the active site of the protease. This binding is characterized by having a slow kinetics. The binding of serpin results in the formation of an SDS-stable protease-serpin covalent complex that eventually leads to cleavage of the serpin itself. However, another class of serpins bind but do not form a covalent complex with the protease. There is also evidence that binding of some serpins to the target protease leads to distortion of the active site of the protease and consequent loss of its catalytic activity (55). Further studies utilizing the full-length protein, as well as individual serpin domains, will be required to delineate the mechanism by which 2C inhibits $3C^{pro}$ activity.

The best-studied viral serpin is crmA, a poxvirus protein, that inhibits both cysteine and serine proteases involved in the regulation of host inflammatory and apoptotic processes (35). Although not classified as a serpin, the antiapoptotic protein, p35, of baculovirus has been shown to inhibit cellular caspases (17). Interestingly, only specific caspases such as caspase-1, -3, -6, -7, and -8 were inhibited by p35; 12 unrelated serine or cysteine proteases were not affected (77). Although three cellular proteases tested (thrombin, enterokinase, and trypsin) were not inhibited by 2C, it remains to be seen whether 2C is able to block other cellular proteases involved in apoptosis and inflammatory response. Cells infected with poliovirus undergo many changes, including both induction and inhibition of apoptosis (1, 68). Inducible expression of $3C^{pro}$ and $2A^{pro}$ leads to apoptotic cell death (9, 27). It will be interesting to determine whether 2C plays any role in the inhibition of apoptosis in poliovirus-infected cells.

In summary, we report a previously unknown activity associated with the poliovirus-encoded 2C polypeptide. We believe that the protease inhibitory activity of 2C (or 2BC) plays a key role in viral replication and pathogenesis of this important group of human pathogens. Future studies will elucidate the exact sequences or domains of the 2C protein required for the protease inhibitory activity and will include molecular genetic analyses to probe the role of these regions in the virus life cycle.

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