VIROLOGY 218, 1–13 (1996)
ARTICLE NO. 0160

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Received October 3, 1995; accepted January 26, 1996

Sequence and structure comparisons with homologous trypsin-like serine proteases have predicted the S1-specificity pocket in picornavirus 3C proteinases. In this study, we examine the putative roles of such residues in poliovirus 3C substrate recognition. Single amino acid substitutions at 3C residues Thr-142, His-161, Gly-163, Gly-164, and Ala-172 were introduced into near full-length poliovirus cDNAs, and protein processing was examined in the context of authentic 3C *cis* cleavage activity. Our data are consistent with residues Thr-142, His-161, Gly-163, and Gly-164 acting as important determinants of 3C substrate specificity and support published models of 3C protein structure. An *in vivo* analysis of mutant viruses containing individual amino acid substitutions at 3C residues Thr-142 and Ala-172 suggests that such residues are important determinants for viral RNA replication. In addition, bacterially expressed, recombinant 3CD polypeptides containing amino acid substitutions at Thr-142 and Ala-172 show altered RNA binding properties in mobility shift assays that use a synthetic RNA corresponding to the poliovirus 5′-terminal sequences.

INTRODUCTION

Picornaviruses depend on proteolysis for the production of functional gene products from a single large viral polyprotein. In the case of poliovirus, a member of the family Picornaviridae, the proteolytic cascade is initiated by cleavage of the polyprotein at the P1/P2 junction by viral-encoded proteinase 2A, which recognizes Y-G amino acid pairs (Toyoda *et al.,* 1986). The remainder of the cleavage events required for production of mature gene products are mediated specifically at Q-G amino acid pairs by viral-encoded proteinase 3C (Kitamura *et al.,* 1981; Hanecak *et al.,* 1982; for review, see Dougherty and Semler, 1993) or viral precursor polypeptides containing 3C sequences (Jore *et al.,* 1988; Ypma-Wong *et al.,* 1988a). Poliovirus 3C proteinase displays a high degree of substrate specificity which depends on recognition of Q-G cleavage sites, as well as other primary sequence and structural determinants surrounding scissile Q-G amino acid pairs (Nicklin *et al.,* 1986; Pallai *et al.,* 1989; Blair and Semler, 1990; Ypma-Wong *et al.,* 1988b).

As first recognized by Gorbalenya *et al.* (1986), picornavirus 3C proteinases have an intriguing similarity to serine proteases. Proteases can generally be divided into four classes based on active site functional groups: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. Based on these criteria, picornavirus 3C proteinases, which utilize cysteine reactive nucleophiles, might classically be defined as cysteine proteases. However, the 3C enzymes exhibit an active site geometry and overall structural topology similar to, and share sequence homology with, serine proteases rather than cysteine proteases. Two independently derived models of 3C structure and two 3C structures derived from X-ray crystallography predict a 12-strand β sheet secondary structure conformation and a bilobular tertiary structure similar to trypsin-like serine proteases (Bazan and Fletterick, 1988; Gorbalenya *et al.,* 1989; Allaire *et al.,* 1994; Matthews *et al.,* 1994). It has therefore been proposed that picornavirus 3C proteinases represent a novel class of serine-like proteinases, which utilize thiol-reactive groups in their active sites. In support of this, it was demonstrated that a serine residue could functionally substitute for the active site cysteine residue in poliovirus 3C proteinase (Lawson and Semler, 1991; Kean *et al.,* 1993).

Based on the X-ray crystal structures for two picornavirus 3C proteinases [human rhinovirus type 14 (Matthews *et al.,* 1994) and hepatitis A virus (Allaire *et al.,* 1994)], 3C sequence alignments with homologous serine proteases, and molecular modeling, residues comprising the active site of 3C have been predicted and, in part, experimentally confirmed (Ivanoff *et al.,* 1986; Hammerle *et al.,* 1991; Kean *et al.,* 1991; Lawson and Semler, 1991). Additionally, based on comparisons to homologous serine proteases, picornavirus 3C proteinase residues involved in substrate recognition have been predicted. Similar to cellular serine proteases, the location of residues com-

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0042-6822/96 $18.00

prising the S1-specificity pocket is predicted to be in the carboxyl terminus of proteinase 3C. Consistent with this, one study demonstrated that the substrate recognition domain of poliovirus 3C resides in the carboxyl-terminal one-third of the proteinase (Lawson *et al.,* 1990). More specifically, sequence alignments with homologous serine proteases have implicated residues Thr-142, His-161, and Gly-163 of picornavirus 3C proteinases as primarily responsible for substrate specificity (Bazan and Fletterick, 1988; Gorbalenya *et al.,* 1989). Molecular modeling using a trypsin–inhibitor complex structure and a rhinovirus 3C–synthetic peptide substrate structure suggests possible hydrogen bonding interactions between 3C residues Thr-142 and His-161 and the S1 bound Gln side chain of cleaved Q-G amino acid pairs (Bazan and Fletterick, 1988; Matthews *et al.,* 1994).

In this study, we directly examine the effects on proteolytic processing of single amino acid substitutions at positions which correspond to residues predicted to comprise the S1-specificity pocket of poliovirus 3C proteinase. Residues Thr-142 and His-161, which are predicted to directly interact with P1 Gln residues, were targeted for mutagenesis as well as residues Gly-163, Gly-164, and Ala-172, which are predicted to form, in part, the walls of the S1 pocket (Bazan and Fletterick, 1988). Single amino acid substitutions at these positions were introduced into near full-length poliovirus cDNAs and proteolytic processing was examined *in vitro* in the context of authentic *cis* 3C cleavage activity (Fig. 1). Our data are consistent with 3C residues Thr-142, His-161, Gly-163, and Gly-164, but not Ala-172 having a role in 3C substrate specificity. We show that mutant viruses containing single amino acid substitutions at 3C residues Thr-142 or Ala-172 exhibit significant temperature-sensitive defects in viral growth, most likely resulting from temperature-sensitive defects in viral RNA synthesis. This latter conclusion is further supported by the results of mobility shift experiments with mutated 3CD polypeptides and RNA sequences that comprise the 5′-terminal cloverleaf structure predicted for poliovirus genomic RNA.

MATERIALS AND METHODS

Construction of single amino acid substitution mutations

Synthetic oligonucleotides 20 or 21 nucleotides in length were used in the heteroduplex method of site-directed mutagenesis described by Inouye and Inouye (1987) to introduce single nucleotide substitutions in poliovirus subgenomic cDNA pT7-P3μ2 (Ypma-Wong *et al.,* 1988a) at poliovirus nucleotide 5862 (C to G transversion or C to T transition), nucleotide 5918 (C to T transition), nucleotide 5925 (G to T transversion), nucleotide 5928 (G to T transversion), or nucleotide 5952 (C to T transition or C to A transversion). Substitutions at poliovirus nucleotide 5862 resulted in plasmid pT7P3-T142S or pT7P3-T142I, and substitutions at poliovirus nucleotide 5952 resulted in plasmid pT7P3-A172V or pT7P3-A172E. Individual substitutions at poliovirus nucleotide 5918, 5925, or 5928 resulted in plasmid pT7P3-H161Y, pT7P3-G163V, or pT7P3-G164V, respectively. The resulting plasmids were digested with restriction endonucleases *Bgl*II and *Pvu*I and 3254-bp fragments containing the mutations were introduced into either subgenomic poliovirus cDNA pT7-1(P1: ΔNS) (Ypma-Wong and Semler, 1987b) or full-length poliovirus cDNA pT7-1 (Ypma-Wong and Semler, 1987a).

In vitro transcription and translation of poliovirus single amino acid substitution mutations

Subgenomic or full-length plasmids pT7-P3μ2, pT7-1, or pT7-1(P1: ΔNS), or derivatives thereof, containing the above mutations in 3C sequences, were linearized with restriction endonuclease *Sac*I, while subgenomic cDNA pT7-P1 (Ypma-Wong *et al.,* 1988b) was linearized with restriction endonuclease *Hin*dIII. Linearized cDNAs were transcribed using bacteriophage T7 RNA polymerase as described previously (Ypma-Wong *et al.,* 1988b; Lawson *et al.,* 1990). RNAs transcribed from subgenomic cDNAs were translated *in vitro* in a rabbit reticulocyte lysate system supplemented with HeLa S10 cell extract (Ypma-Wong and Semler, 1987a). Where indicated, *in vitro* translation reactions were incubated either with an extract from PV1-infected cells (a source of proteinases 3C and 3CD) at 30% of the reaction volume or with an equal volume of *in vitro* translation reactions programmed with RNAs derived from pT7-P3μ2 cDNA constructs for 3 hr prior to analysis on SDS–polyacrylamide gels. RNAs transcribed from full-length poliovirus cDNAs were used to transfect HeLa cell monolayers without purification (see below).

Transfection of RNAs derived from full-length poliovirus cDNAs pT71-T142S and pT71-A172V and mutant virus stock preparation

RNAs derived from plasmids [pT7-1(T142S) or pT7-1(A172V)] harboring full-length poliovirus cDNAs were used to transfect HeLa cell monolayers at 33° as described previously (Vaheri and Pagano, 1965; Blair and Semler, 1991). Mutant virus containing the T142S lesion in 3C was designated Se1-3C-30, and virus with the A172V 3C lesion was designated Se1-3C-31. Virus stocks were prepared as follows: liquid overlays from transfected monolayers were harvested 2 to 3 days after transfection and used to infect 60-mm plates of cell monolayers at 33°, mutant virus plaques were picked and plaque purified, and viral stocks were expanded by two serial passages through HeLa cell monolayers at 33°. Single amino acid substitution mutations were confirmed by sequencing of mutant viral RNA prepared by Nonidet P40 lysis of virus-infected HeLa cells (Campos and Villarreal, 1982). A synthetic 20-nucleotide oligo primer corresponding to poliovirus nucleotides 6001–6020 was hy-

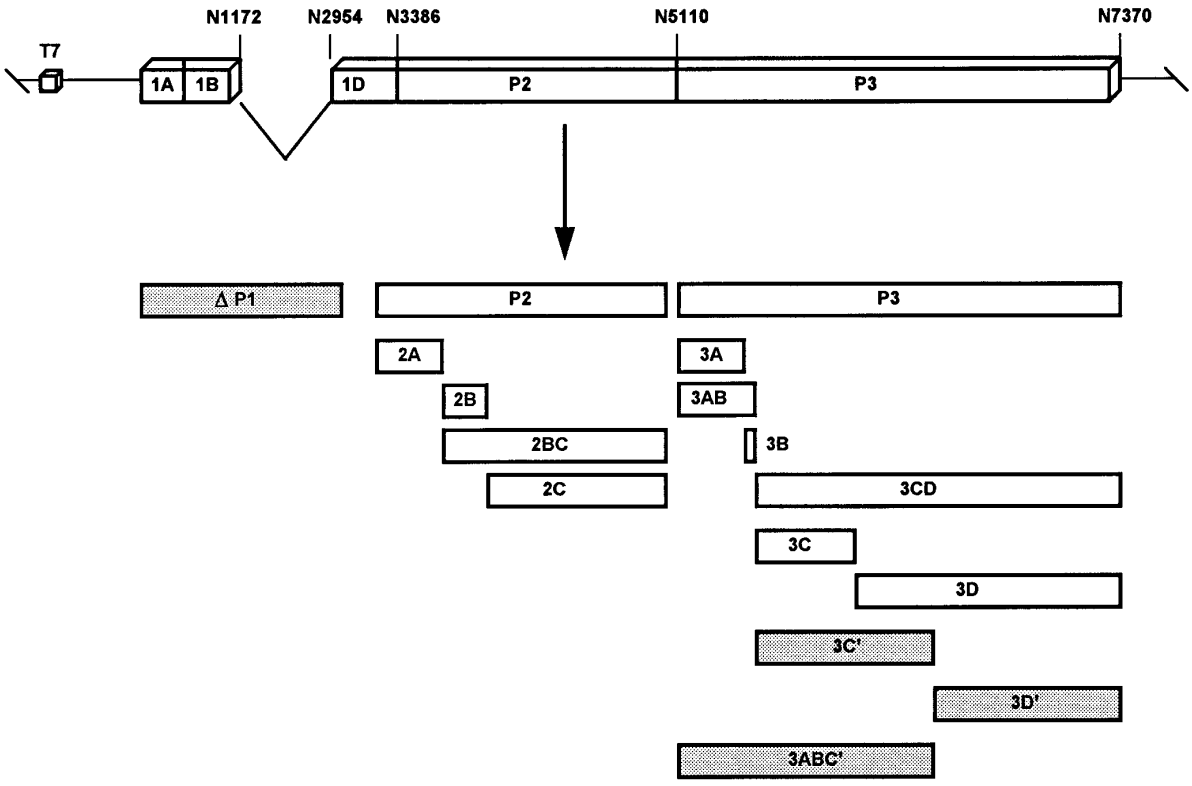

FIG. 1. Diagram of expected precursor polypeptides and resulting cleavage products derived from *in vitro* translation of mRNAs derived from subgenomic poliovirus cDNA pT7-1(P1: ΔNS). 3C- and 3CD-mediated cleavage products are represented by open rectangles, and 2A-mediated cleavage products are represented by shaded rectangles. The box with "T7" above it denotes the promoter for bacteriophage T7 RNA polymerase. This promoter element is followed by sequences encoding the 5′ noncoding region of the poliovirus genome. Numbers above the boxed representation of the viral polyprotein correspond to poliovirus (type 1, Mahoney) nucleotides. ΔP1 denotes the truncated polypeptide corresponding to an in-frame deletion of the capsid precursor, with the genomic deletion spanning the N1172 to N2954 sequences in the viral genome.

bridized to isolated RNA and extended using [α-^{32}P]-dATP, dideoxynucleotides, and reverse transcriptase (Sanger *et al.,* 1977; Hamlyn *et al.,* 1978).

[^{35}S]Methionine pulse-labeling of cells infected with PV1, Se1-3C-30, or Se1-3C-31

[^{35}S]Methionine pulse-labeling of cells infected with PV1, Se1-3C-30, or Se1-3C-31 was carried out at 33° as described previously (Blair *et al.,* 1990) with the exception of labeling times. Cells infected with PV1 or Se1-3C-31 were labeled with [^{35}S]methionine 4, 6, 8, and 10 hr post-infection, while cells infected with Se1-3C-30 were labeled with [^{35}S]methionine 4 and 6 hr postinfection. Infected and [^{35}S]methionine-labeled cells were incubated for 1 hr at either 33 or 39° prior to harvest.

Viral growth analysis

The kinetics of mutant virus growth were examined at 33 and 39° as described (Blair *et al.,* 1990). Cells were infected with PV1, Se1-3C-30, or Se1-3C-31 at 33 or 39° and cells and supernatants harvested 2, 4, 6, 8, and 10 hr after infection at 33° and 2, 3, 4, 5, and 6 hr, after infection at 39°. PFU (plaque-forming units) per cell were determined using plaque assays.

Analysis of viral RNA synthesis

HeLa cell monolayers were infected at a multiplicity of infection of 30 at 33 or 39°. Total cytoplasmic RNA was harvested from infected cells 3, 6, 9, and 12 hr after infection or from mock-infected cells after 6 hr incubation at 33 or 39° by Nonidet P40 lysis. Seven micrograms of total cytoplasmic RNA was immobilized on nitrocellulose filters with a vacuum slot-blot apparatus and probed with a poliovirus plus-strand-specific, synthetic oligonucleotide that had been 5′-end-labeled with [γ-^{32}P]ATP (Dildine and Semler, 1989).

RNA electrophoretic mobility shift analysis with partially purified recombinant 3CD proteins

Histidine-tagged recombinant 3CD proteins containing the μ10 mutation (Semler *et al.,* 1987) or single amino acid substitutions T142I, T142S, and A172V were separately expressed in *Escherichia coli* BL21(DE3) cells and purified from the insoluble fraction using a nickel affinity column. Partially purified fractions were dialyzed overnight at 4° against RNA binding buffer [5 m*M* HEPES, pH 7.9, 25 m*M* KCl, 2.5 m*M* MgCl2, 20 m*M* DTT, and 3.8% (v/v) glycerol]. Total protein content was determined with

the Bio-Rad protein assay, and 5 μg of total protein was resolved on a 12.5% polyacrylamide–SDS gel, which was subsequently stained with Coomassie brilliant blue and scanned with an LKB laser densitometer to determine 3CD content.

For mobility shift analysis, radiolabeled RNA transcripts representing the first 108 nt of the poliovirus genome were generated using T7 RNA polymerase (Pharmacia) in *in vitro* transcriptions of a *Dde*I-linearized pT7-5′NCR plasmid (Haller and Semler, 1992) in the presence of 100 μCi of [α-^{32}P]UTP (Amersham). Different molar amounts (0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 pmol) of 3CD protein from each partially purified preparation were preincubated in the presence of a micrococcal-nuclease-treated HeLa cellular extract (Andino *et al.,* 1993), 2 mg/ml *E. coli* tRNA, 1.5 m*M* ATP, and 13 U RNasin (Promega) in a total volume of 14 μl for 10 min at 30°. Following preincubation, 1μl of 10 fmol/μl ^{32}P-labeled RNA was added, and incubation was continued for 10 min at 30°. Four microliters of RNA loading dye (1.25% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol) was then added and the complexes were resolved at 4° on a native 5% polyacrylamide gel containing 5% glycerol. Following electrophoresis, the gel was dried and exposed to Kodak XAR 5 film.

Proteolytic processing kinetics of partially purified, mutated 3CD

For analysis of the proteolytic processing capabilities of the recombinant 3CD proteins containing individual amino acid substitutions, 15 μl of an *in vitro* translation reaction mixture (derived from translation of a transcript synthesized from a linearized pT7-P1 plasmid) was incubated at 30° with 3 pmol of the respective mutated 3CD protein and 3 μl of 10$\times$ cleavage buffer (0.2 *M* HEPES 7.4, 1.5 *M* KOAc, 10 m*M* EDTA, and 10 m*M* DTT) in a total volume of 30 μl. Aliquots of 10 μl were removed from each cleavage reaction after 30, 60, and 120 min of incubation and mixed with 40 μl of Laemmli sample buffer. These mixtures were subsequently boiled 3 min and cleavage products were resolved by electrophoresis on a 12.5% polyacrylamide–SDS gel. The gel was subjected to fluorography, dried, and exposed to X-ray film.

RESULTS

Cleavage of P2–P3 precursor polypeptides by 3C proteinases containing single amino acid substitutions in the putative S1-specificity pocket

Structural analysis of 3C proteinases and sequence comparisons with homologous trypsin-like serine proteases have predicted residues of picornavirus 3C proteinases which may comprise the S1-specificity pocket. In this study, the importance of such residues for 3C substrate specificity was examined in the context of authentic 3C *cis* cleavage activity. Conservative and nonconservative single amino acid substitutions were introduced at 3C residues 142 (Thr to Ser, Thr to Ile) and 161 (His to Tyr), which are predicted to directly interact with the Gln residue of cleaved Q-G amino acid pairs. Single amino acid substitutions were also introduced at 3C residues 163 (Gly to Val), 164 (Gly to Val), and 172 (Ala to Val, Ala to Glu), which are predicted to form the walls of the 3C S1-specificity pocket. These substitution mutations were introduced into a near full-length poliovirus cDNA pT7-1(P1: ΔNS) (Fig. 1) to construct mutations T142S, T142I, H161Y, G163V, G164V, A172V, and A172E (Table 1). As shown in Fig. 2A, conservative amino acid substitutions at positions 142 (Thr to Ser) (lane 4) and 172 (Ala to Val) (lane 8) had no effect on 3C-mediated proteolytic processing of P2 and P3 precursor polypeptides. In addition, a nonconservative substitution at position 172 (Ala to Glu) (Fig. 2A, lane 9) affected 3C activity, although it did not completely abrogate 3C-mediated proteolytic cleavage. All of the authentic P2 and P3 cleavage products were generated in *in vitro* translation reactions programmed with RNAs derived from pT7ΔNS-A172E (Fig. 2A, lane 9). However, a nonconservative amino acid substitution at position 142 (Thr to Ile) (Fig. 2A, lane 5) and amino acid substitutions at residue 161 (His to Tyr) (Fig. 2A, lane 10) and residue 163 (Gly to Val) (Fig. 2A, lane 6) resulted in the elimination of authentic 3C activity on P2 and P3 substrates. This is demonstrated by the accumulation of higher molecular weight precursor polypeptides (migrating more slowly than P3 polypeptides during polyacrylamide gel electrophoresis) and the production of primarily those cleavage products derived from 2A-mediated cleavage of precursor polypeptides at Y-G amino acid pairs (e.g., 3D′; refer to Fig. 1). A single amino acid substitution at residue 164 (Gly to Val) significantly affected 3C proteolytic activity, but authentic cleavage events at the 2C/3A and 2A/2B cleavage sites were detected (Fig. 2A, lane 7). This is apparent from the production of precursors P2, 2BC, and 3ABC′ and the production of 2A in *in vitro* translation reactions programmed with RNAs derived from mutation pT7ΔNS-G164V. These

TABLE 1

Summary of 3C Substitution Mutations

Mutation	Virus	*In vitro* cleavage P2/P3	*In vitro* cleavage P1
Thr142 to Ser	*Se1-3C-30*	+++	++
Thr142 to Ile	NT	–	–
Gly163 to Val	NT	–	–
Gly164 to Val	NT	+/–	–
Ala172 to Val	*Se1-3C-31*	+++	++
Ala172 to Glu	NT	+	–
His161 to Tyr	NT	–	–

Note. +++, wild-type levels of cleavage; ++, slight defects in cleavage; +, significant defects in cleavage; –, no detectable cleavage; NT, virus recovery not tested.

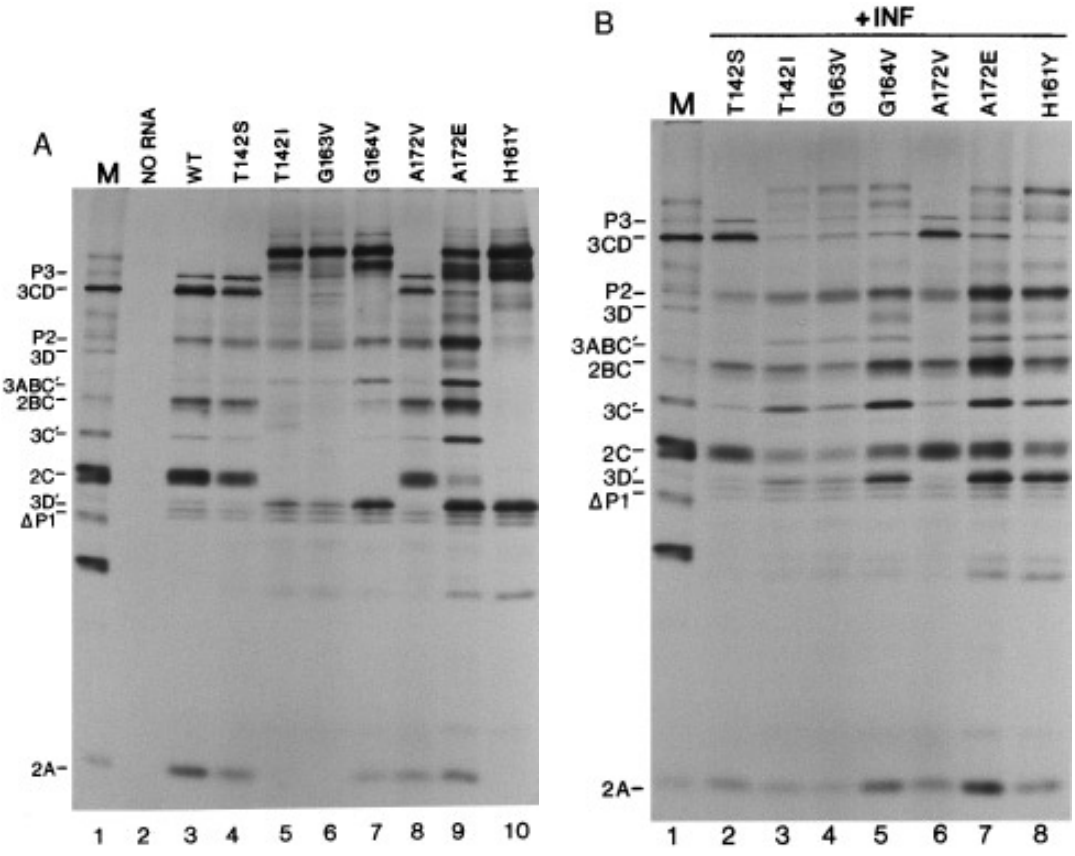

FIG. 2. *In vitro* analysis of the cleavage activities of 3C proteinases containing single amino acid substitutions in the putative S1-specificity pocket. (A) *In vitro* translations of mRNAs derived from near full-length poliovirus cDNAs containing mutations in 3C. RNAs transcribed from poliovirus cDNAs pT7-1 (P1: ΔNS) (WT), pT7ΔNS-T142S (T142S), pT7ΔNS-T142I (T142I), pT7ΔNS-G163V (G163V), pT7ΔNS-G164V (G164V), pT7ΔNS-A172V (A172V), pT7ΔNS-A172E (A172E), and pT7ΔNS-H161Y (H161Y) were translated as described under Materials and Methods. *In vitro* synthesized polypeptides were diluted in Laemmli sample buffer and analyzed on SDS–polyacrylamide gels. No RNA, translation reactions were incubated in the absence of RNA. (B) *Trans* cleavage of *in vitro* synthesized precursor polypeptides containing single amino acid substitutions in 3C sequences by exogenous wild-type 3C proteinase. *In vitro* translation reactions from (A) were incubated in the presence of an extract from PV1-infected HeLa cells for 3 hr prior to analysis on SDS–polyacrylamide gels. M, marker lane of an extract of [^{35}S]methionine-labeled, PV1-infected cells.

data demonstrate that nonconservative amino acid substitutions at residues Thr-142, His-161, Gly-163, and Gly-164 significantly affect 3C-mediated protein processing of P2 and P3 precursor polypeptides, while conservative or nonconservative substitutions at residue Ala-172 do not abrogate 3C activity. These data, therefore, are consistent with residues Thr-142, His-161, Gly-163, and Gly-164 having functional roles in 3C substrate specificity.

To determine if the observed defects in 3C-mediated protein processing resulted from gross 3C structural alterations or 3C protein misfolding, P2–P3 precursor polypeptide substrates containing the above mutations in proteinase 3C were tested for their ability to serve as substrates for exogenous wild-type 3C activity. The *in vitro* translation reactions described in Fig. 2A were incubated in the presence of an extract from PV1-infected cells (a source of wild-type poliovirus 3C and 3CD proteinases) prior to analysis on SDS–polyacrylamide gels. As shown in Fig. 2B, *in vitro* synthesized P2–P3 precursors containing the above-described mutations in 3C were cleaved detectably (although to varying degrees; compare 3CD and 2C levels shown in lanes 3, 4, 5, 7, and 8) by exogenous wild-type proteinase 3C activity. Within the population of mutated polyproteins produced upon *in vitro* translation of RNAs harboring the different 3C mutations, a significant fraction was cleaved into authentic protein products, indicating that defects in 3C-mediated protein processing by the mutated 3C enzymes were not a result of gross structural alterations in the 3C-containing proteins.

Cleavage of P1 precursors by 3CD proteinases containing single amino acid substitutions in the putative S1-specificity pocket

The amino acid substitution mutations in 3C proteinase described above were incorporated into poliovirus subgenomic cDNA pT7-P3μ2, which encodes poliovirus proteinase 3CD sequences as well as 3B and a portion of 3A. Such subgenomic cDNAs were transcribed and translated *in vitro* either in the presence of L-methionine (Fig. 3A) or [^{35}S]methionine (Fig. 3B). As shown in Fig. 3B, polypeptides translated in the presence of [^{35}S]-

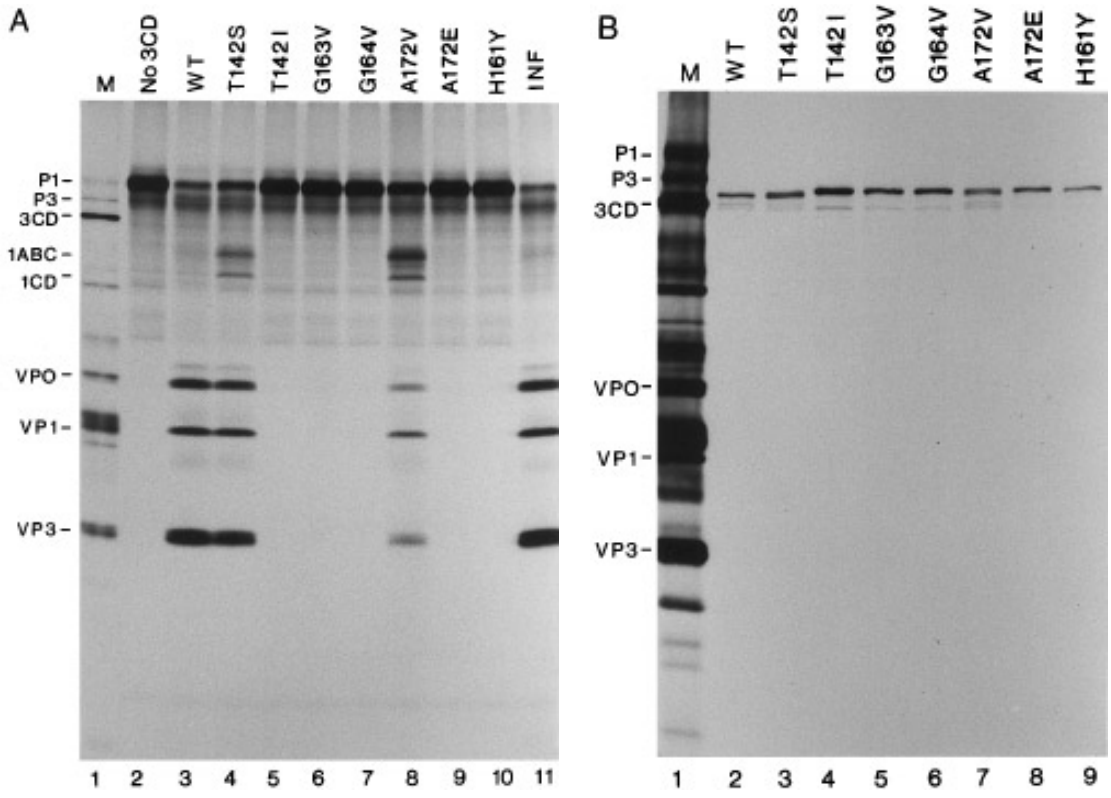

FIG. 3. *In vitro* analysis of the P1 polypeptide cleavage activities of 3CD proteinases containing single amino acid substitutions in 3C sequences. (A) RNAs derived from poliovirus subgenomic cDNA pT7-P1 were translated *in vitro* in the presence of [^{35}S]methionine. These translation reactions were incubated with an equal volume of *in vitro* 3CD translation mixtures carried out in the presence of L-methionine and programmed with RNAs derived from poliovirus subgenomic cDNAs pT7-P3μ2 (WT), pT7P3-T142S, pT7P3-T142I, pT7P3-G163V, pT7P3-G164V, pT7P3-A172V, pT7P3-A172E, or pT7P3-H161Y or incubated with an extract of PV1-infected HeLa cells for 3 hr at 30°. Cleavage reactions were then diluted in Laemmli sample buffer and analyzed on SDS–polyacrylamide gels. No 3CD, *in vitro* translated P1 precursors were incubated in the absence of *in vitro* synthesized 3CD or an extract from PV1-infected cells prior to analysis. (B) *In vitro* 3CD translation reactions, similar to those used for the P1 cleavage reactions in (A), carried out in the presence of [^{35}S]methionine. M, marker lane of extracts from [^{35}S]methionine-labeled PV1-infected cells.

methionine from mRNAs derived from wild-type pT7-P3μ2 (or mutated derivatives) were produced in similar quantities, indicating that any possible differences in P1 cleavage could not be attributed to different levels of 3C-containing polypeptides in cleavage reactions. Parallel *in vitro* translation reactions carried out in the presence of unlabeled methionine were incubated with [^{35}S]-methionine-labeled P1 substrates, and the cleavage products were analyzed on SDS–polyacrylamide gels (Fig. 3A). P1 precursors incubated in the presence of *in vitro* synthesized 3CD proteinases containing single amino acid substitution mutations Thr-142 to Ile, His-161 to Tyr, Gly-163 to Val, Gly-164 to Val, and Ala-172 to Glu remained uncleaved (Fig. 3A). P1 precursors incubated in the presence of 3CD proteinases containing substitution mutations Thr-142 to Ser (Fig. 3A, lane 4) or Ala-172 to Val (Fig. 3A, lane 8) were cleaved at slightly lower efficiencies than P1 precursors incubated in the presence of *in vitro* synthesized wild-type 3CD proteinase (Fig. 3A, lane 3) or an extract from PV1-infected cells (Fig. 3A, lane 11). From these data, it is apparent that although 3C proteinase containing an Ala-172 to Glu substitution cleaves P2 and P3 precursor polypeptides, proteinase 3CD containing the same substitution in 3C sequences is unable to cleave P1 substrates *in trans.*

[^{35}S]Methionine labeling of cells infected with PV1, Se1-3C-30, or Se1-3C-31

As summarized in Table 1, two of the seven 3C single amino acid substitution mutations described in this study (T142S and A172V) exhibited readily detectable proteolytic cleavage activity *in vitro* on poliovirus P1, P2, and P3 substrates. Therefore, such mutations were incorporated into full-length poliovirus cDNAs. RNAs derived from full-length poliovirus cDNAs encoding amino acid substitution mutations at 3C residues 142 (Thr to Ser) or 172 (Ala to Val) were used to transfect cell monolayers at 33°, and mutant viruses Se1-3C-30 and Se1-3C-31 were recovered (Table 1), which displayed small plaque phenotypes. To examine viral-specific protein processing *in vivo,* cells were infected with PV1, Se1-3C-30, or Se1-3C-31 at 33°, pulse-labeled with [^{35}S]methionine at various times after infection, and incubated at 33 or 39° for 1 hr. Cells were subsequently harvested, extracts were sus-

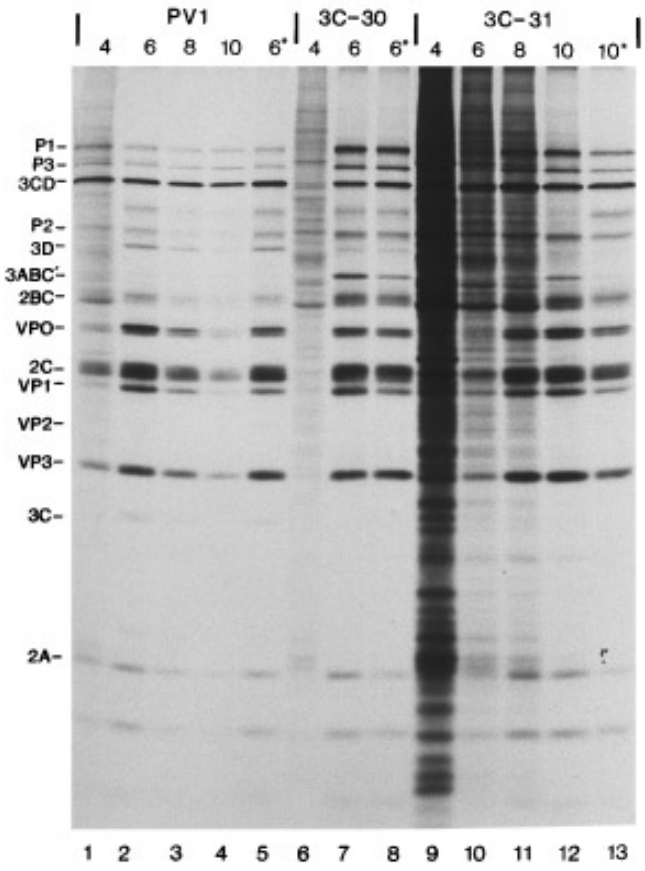

FIG. 4. [^{35}S]Methionine pulse-labeling analysis of cells infected with PV1, Se1-3C-30, or Se1-3C-31. Cells were infected with PV1, Se1-3C-30 (3C-30), or Se1-3C-31 (3C-31) at 33°, pulse-labeled with [^{35}S]-methionine at various times after infection, and incubated at either 33 or 39° (denoted by asterisks) for 1 hr. Viral-specific polypeptides were diluted in Laemmli sample buffer and analyzed on SDS–polyacrylamide gels. Numbers above the lanes represent hours postinfection cultures were pulsed with [^{35}S]methionine.

pended in Laemmli sample buffer, and viral-specific proteins were analyzed on SDS–polyacrylamide gels. In cells infected with Se1-3C-30 (T142S) or Se1-3C-31 (A172V) at 33° or infected at 33° and shifted to 39° following the pulse with [^{35}S]methionine, slight defects in P1 and P3 processing were observed. This was demonstrated by a slight accumulation of P1, P3, and polypeptide 3ABC′ (resulting from 3C cleavage at the P2/P3 junction and 2A cleavage at a Y-G amino pair in 3D sequences) and a slightly decreased production of 3C and 3D (P3 cleavage products) in cells infected with mutant virus Se1-3C-30 or Se1-3C-31 when compared to protein production of wild type (Fig. 4). The pulse-labeling analysis also revealed a slight delay in the inhibition of host-cell protein synthesis (when compared to that of wild type) in cells infected with Se1-3C-30 and a significant delay in cells infected with Se1-3C-31 (Fig. 4). Such delays in host-cell shut-off most likely result from a delayed production of viral proteins involved in the inhibition of host-cell protein synthesis, possibly as a result of a defect in mutant virus RNA synthesis (discussed below).

One-step growth analysis of cells infected with PV1, Se1-3C-30, or Se1-3C-31

To examine the kinetics of Se1-3C-30 and Se1-3C-31 virus growth, cells were infected with PV1, Se1-3C-30, or Se1-3C-31 at 33 or 39°, and at various times after infection, virus production was analyzed. As shown in Fig. 5A, mutant viruses Se1-3C-30 and Se1-3C-31 exhibited significant delays in virus growth at 33° when compared to wild type. In addition, mutant viruses Se1-3C-30 and Se1-3C-31 displayed >3 log unit and >2 log unit decreases, respectively, in virus yield 10 hr postinfection at 33° when compared to that of wild type (Fig. 5A). At 39° very little mutant virus production was observed in cells infected with either Se1-3C-30 or Se1-3C-31 (Fig. 5B). The slight defects in P1 and P3 processing observed in cells infected with Se1-3C-30 or Se1-3C-31 may contribute to, but do not seem to account for, such severe temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 viral growth. It is possible, therefore, that the temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 viral growth may result from defects in 3C or 3CD functional activity other than protein processing.

Analysis of viral RNA synthesis in cells infected with PV1, Se1-3C-30, or Se1-3C-31

To determine if the observed severe temperature-sensitive defects in mutant virus growth resulted from defects in viral RNA synthesis, viral RNA production was measured in cells infected with wild-type or mutant virus. As described under Materials and Methods, cells were infected with wild-type or mutant virus at 33 or 39°, and total cytoplasmic RNA was harvested at different times after infection, immobilized on nitrocellulose filters, and hybridized to a poliovirus plus-strand-specific, [γ-^{32}P]-ATP-labeled oligonucleotide probe. Such an analysis showed significant delays in mutant viral RNA synthesis and a five- or fourfold decrease in maximum viral RNA production in cells infected with Se1-3C-30 or Se1-3C-31, respectively, at 33° when compared to that of wild type (Fig. 6). In cells infected with Se1-3C-30 or Se1-3C-31 at 39°, very little viral RNA synthesis was detected (Fig. 6). The observed temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 viral RNA synthesis appear to correlate with the observed temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 virus growth and the observed mutant virus host-cell shut-off phenotypes. Consistent with these conclusions, our preliminary results from temperature shift experiments showed that both mutant viruses displayed significant decreases in the rate of RNA synthesis (as measured by [^{3}H]uridine incorporation) after a shift from the permissive temperature (33°) to the nonpermissive temperature (39°) (Nguyen, Blair, and Semler, unpublished data). It is likely, therefore, that the temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 virus growth result primarily from

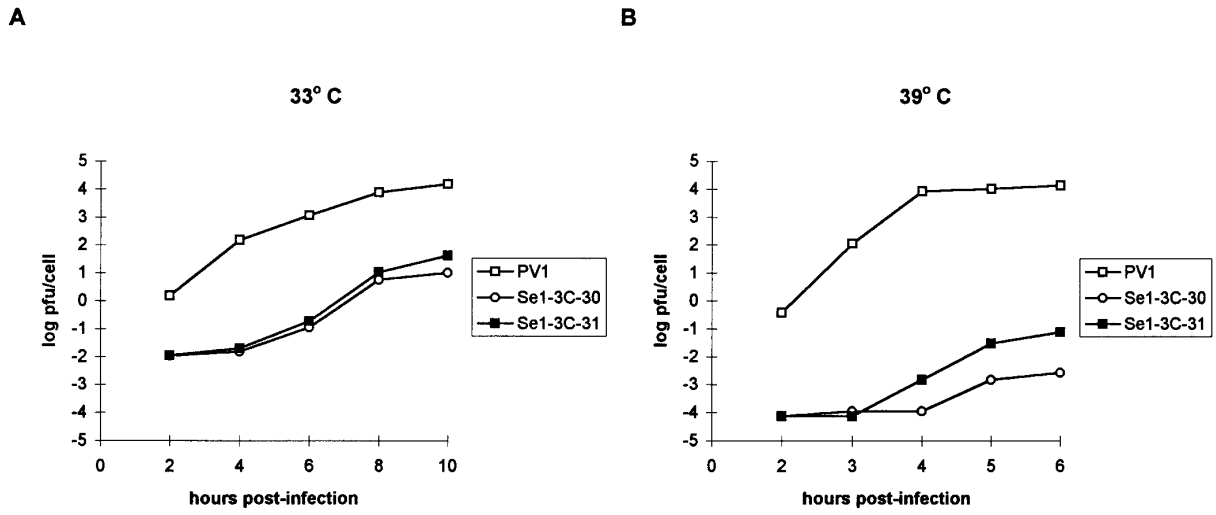

FIG. 5. The kinetics of virus growth in cells infected with PV1, Se1-3C-30, or Se1-3C-31. Cells were infected with PV1 (□), Se1-3C-30 (○), or Se1-3C-31 (■) at 33° (A) or 39° (B). Cells and supernatants were harvested at various times after infection and PFU per cell were determined.

defects in RNA replication functions associated with the mutant virus-encoded 3C or 3CD proteinases.

Expression of recombinant 3CD polypeptides in *E. coli*

To demonstrate that the processing and RNA replication properties ascribed to the mutated polyproteins (described above) were specific for the mature 3CD protein, we cloned the DNA corresponding to the 3C mutations T142I, T142S, A172V, and μ10 into a bacterial expression plasmid that produces proteins with a poly-histidine "tag" at their amino terminus (Lilius *et al.,* 1991; Parsley, Li, Nguyen, and Semler, unpublished data). It should be noted that the μ10 construct contains an inserted codon that introduces a serine residue between the P3 and P2 residues upstream of the 3C–3D cleavage site (Semler *et al.,* 1987). This insertion results in a 3CD protein that is nearly resistant to cleavage at the 3C–3D junction, possibly as a result of changing the P4 amino acid from threonine to glutamine (Charini *et al.,* 1994). However, the processing activity produced by the μ10 3CD is equivalent to that of wild-type 3CD (Ypma-Wong *et al.,* 1988a) and will be used as the wild-type equivalent in the experiments described below. In addition, the construct harboring the T142I lesion was included as a negative control for protein processing activity.

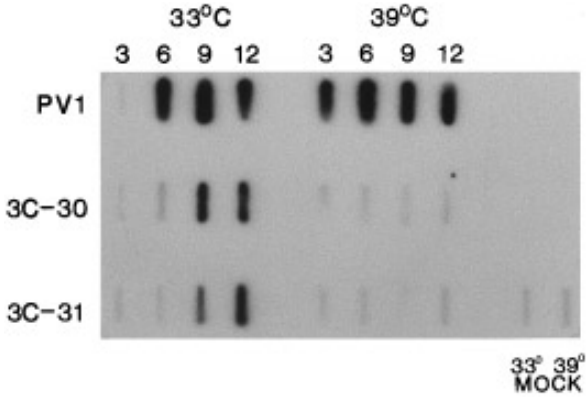

FIG. 6. Analysis of viral RNA synthesis in cells infected with PV1, Se1-3C-30, or Se1-3C-31. Cells were infected with PV1, Se1-3C-30, or Se1-3C-31 at 33 or 39°. Total cytoplasmic RNA was harvested at 3, 6, 9, or 12 hr after infection, immobilized on nitrocellulose filters, and hybridized to an oligonucleotide complementary to poliovirus nucleotides 2622 to 2642, labeled with [γ-^{32}P]ATP and polynucleotide kinase. Numbers above slots represent hours postinfection that RNA was harvested. Mock infected cells were harvested after 6 hr incubations at 33 or 39°.

Following induction of bacteria harboring the different recombinant plasmids and crude fractionation of the extracts from bacterial lysates, the genetically altered forms of 3CD were subjected to nickel affinity chromatography (Smith *et al.,* 1988; Walker *et al.,* 1994). Using this approach, all of the recombinant mutated 3CD polypeptides were purified to at least ~30% homogeneity (Parsley, Nguyen, and Semler, unpublished data). As described below, these enzymes were then analyzed for the ability to proteolytically cleave the poliovirus P1 precursor and to form a ribonucleoprotein complex with the first ~100 nucleotides of viral RNA.

Biochemical analysis of mutated 3CD polypeptides

In order to compare the proteolytic processing efficiencies of the purified, mutated 3CD proteins, equal amounts of each mutated recombinant protein were incubated with a source of P1 substrate in an *in vitro* cleavage assay (Ypma-Wong *et al.,* 1988b). Aliquots from the cleavage reaction mixtures were removed after 30, 60, and 120 min of incubation at 30° and resolved by electrophoresis on a SDS–polyacrylamide gel (Fig. 7). Results from this analysis correlated with the above-described processing abilities of the *in vitro* synthesized proteinases.

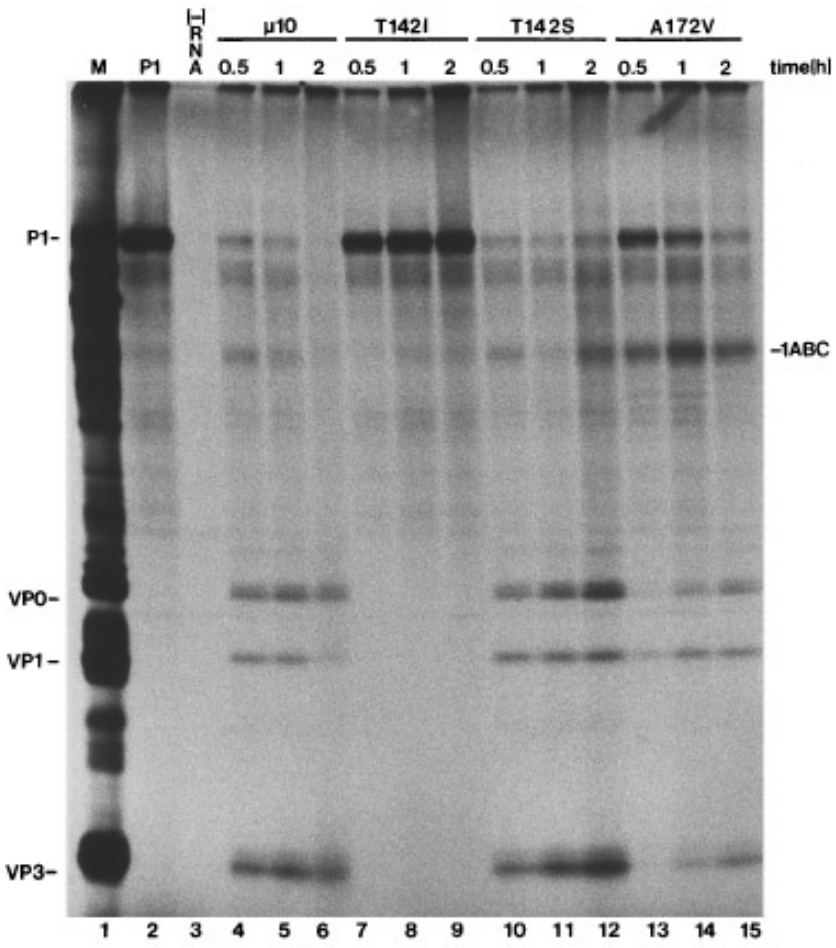

FIG. 7. Cleavage of *in vitro* translated P1 precursors by partially purified recombinant 3CD polypeptides. As described under Materials and Methods, 15 μl of *in vitro* P1 translation mixture was incubated at 30° with 3 pmol of the indicated purified, recombinant 3CD protein in a total volume of 30 μl. Aliquots of 10 μl were removed after 30 min (lanes 4, 7, 10, and 13), 60 min (lanes 5, 8, 11, and 14), and 120 min (lanes 6, 9, 12, and 15) of incubation and subjected to electrophoresis on a 12.5% polyacrylamide–SDS gel. Lane 1 is a PV1 protein marker. Lane 2 is a P1 translation mix incubated in the absence of 3CD. Lane 3 is a control translation reaction incubated in the absence of a poliovirus-specific transcript.

Both T142S and A172V had P1 processing efficiencies that were just slightly reduced compared to those of the wild-type enzyme (as indicated by the accumulation of a 1ABC cleavage product), and T142I exhibited no proteolytic activity.

RNA mobility shift assays were then performed using increasing amounts of each partially purified recombinant 3CD harboring the individual amino acid substitutions. Two major ribonucleoprotein complexes were identified when a labeled probe representing the 5′ terminal ~100 nt was incubated with extract from uninfected HeLa cells and increasing amounts of μ10 3CD (equivalent to wild-type 3CD). As shown in Fig. 8, complex I may represent the viral RNA interacting with a reported 36-kDa HeLa cell protein and probably corresponds to a complex called "RNP-A" by Andino *et al.* (1993). Complex II is most likely the result of interactions of the viral RNA with both the HeLa protein(s) and 3CD and corresponds to the "RNP-B" complex described by Andino and colleagues (Andino *et al.,* 1993). The results of our experiments (shown in lanes 9–28 in Fig. 8) indicated that the mutated 3CD polypeptides exhibited altered RNA binding properties (as evidenced by RNP complex formation) relative to wild-type 3CD (shown as μ10 in Fig. 8). The T142I mutation displayed the greatest effect on binding, and the T142S mutation had the least effect. The recombinant 3CD polypeptide harboring the T142S mutation was able to bind to the RNA to form a complex with an electrophoretic mobility similar to that of wild-type 3CD (complex II, visible in lanes 5–8 and 19–22 in Fig. 8). However, formation of this complex required a higher concentration of the mutated protein (relative to wild type) and was preceded by the formation of a faster mobility complex (complex IIa) at lower concentrations of the T142S 3CD not seen in the corresponding wild-type lanes. At present, we do not know the basis of the difference between complexes II and IIa but it may be related to conformational differences in 3CD resulting in a qualitatively altered ribonucleoprotein complex. The A172V polypeptide was also able to form the faster mobility complex (IIa), although only at the highest concentrations of 3CD employed in the assay (Fig. 8, lanes 27

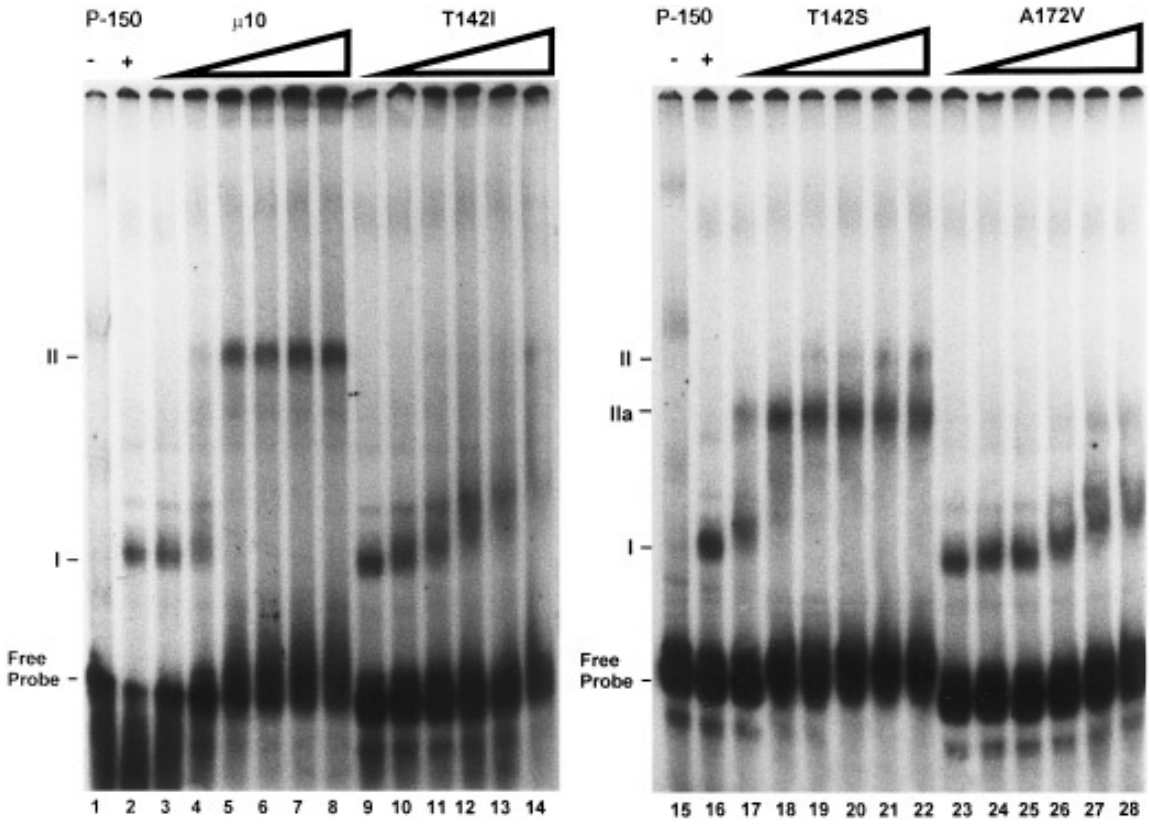

FIG. 8. RNA electrophoretic mobility shift assay with increasing amounts of partially purified, recombinant 3CD. Micrococcal-nuclease-treated HeLa cellular extract (P150 fraction), binding buffer (5 m*M* HEPES, pH 7.9, 25 m*M* KCl, 2.5 m*M* MgCl, 20 m*M* DTT, and 3.8% glycerol), 2 mg/ml *E. coli* tRNA, 1.5 m*M* ATP, and 13 U RNasin were preincubated in the absence (lanes 2 and 16) or presence (lanes 3–14 and 17–28) of increasing amounts [0.1 pmol (7.4 ng), 0.5 pmol (37 ng), 1.0 pmol (74 ng), 2.0 pmol (148 ng), 3.0 pmol (222 ng), and 4.0 pmol (296 ng), lanes 3–8, 9–14, 17–22, and 23–28, respectively) of partially purified, recombinant 3CD polypeptides for 10 min at 30°. Following preincubation, 10 fmol of ^{32}P-labeled RNA representing the first 108 nt of poliovirus RNA was added and incubation was continued for an additional 10 min. Complexes were resolved on a native 5% polyacrylamide gel at 4°. Complex I, complex II, and complex IIa are described in the text.

and 28). However, under the conditions of this assay, no formation of complex II was observed for A172V. These RNA binding data are consistent with our *in vivo* analysis of temperature-sensitive viruses containing the T142S and A172V mutations and correlate the ability of the 3CD proteinase to bind to viral RNA sequences with the ability of the virus to replicate its RNA.

DISCUSSION

Based on sequence alignments with homologous trypsin-like serine proteases, molecular modeling, and recently solved structures of two different picornavirus 3C enzymes, residues comprising the 3C proteinase S1-specificity pocket have been predicted. These residues include Thr-142, His-161, and Gly-163 as the major determinants of 3C substrate specificity, as well as residues Gly-164 and Ala-172 as putatively located on the walls of the S1 pocket (Bazan and Fletterick, 1988; Matthews *et al.,* 1994). The mutational analysis described in this study was designed to examine the functional roles of such residues. A functionally conservative amino acid substitution at 3C residue 142 (Thr to Ser) had little affect on 3C activity *in vitro,* while a functionally nonconservative substitution at residue 142, or single amino acid substitutions at 3C residues 161 (His to Tyr) or 163 (Gly to Val), abolished 3C activity *in vitro* (Fig. 2A). Consistent with these data, a previous study demonstrated that a His to Gly amino acid substitution at 3C residue 161 abolished 3C-specific activity on viral polyproteins *in vitro* (Lawson and Semler, 1991). Similarly, a single amino acid substitution at 3C residue 164 (Gly to Val) significantly reduced 3C-mediated cleavage of P2 and P3 precursors *in vitro.* These data are consistent with residues Thr-142, His-161, Gly-163, and Gly-164 having a role in 3C substrate specificity as putative components of the S1 pocket. It is interesting that identical amino acid substitutions (Gly to Val) introduced independently at adjacent Gly residues (163 and 164) resulted in differential 3C cleavage activities. Possibly, the relative positioning of the substituted Val residues is such that at position 163, the Val side chain blocks access of the recognized Gln substrate residue to the S1 pocket, while at position 164 the Val side chain only partially limits the accessibility of the recognized Gln residue to the S1 pocket. It is also interesting that a single amino acid substitution mutation

at Gly-163 (conserved primarily among enteroviruses and rhinoviruses) has a greater effect on 3C activity than a substitution mutation at Gly-164 (more highly conserved among picornaviruses). This latter observation suggests that Gly-163 may have a role in genus-specific substrate recognition (Lawson *et al.,* 1990).

Despite the conservation of Ala-172 among enterovirus and rhinovirus 3C proteinases, Ala-172 does not seem to have a direct role in poliovirus P2 and P3 substrate recognition. In this study, conservative and nonconservative amino acid substitutions at Ala-172 did not eliminate 3C recognition of poliovirus P2 and P3 substrates in the context of the viral polyprotein (Fig. 2A). However, in a separate study, an Ala to Pro amino acid substitution in bacterially expressed 3C proteinase abolished *trans* cleavage activity on synthetic peptides containing poliovirus 2C–3AB sequences (Baum *et al.,* 1991). This latter study suggested that Ala-172 may be important for 3C proteolytic activity. These apparently conflicting results may be explained by the different chemical properties of the substituted residues in both studies. The Ala-172 to Pro substitution in the latter study most likely alters 3C protein structure more severely than the Ala-172 to Glu substitution in this study. Additionally, in the latter study, only 3C *trans* cleavage activity was examined and not 3C *cis* cleavage activity in the context of the viral polyprotein. Analysis of 3C *trans* proteolytic activity is often less sensitive than analysis of 3C *cis* cleavage activity (Lawson and Semler, 1991). In the present study, the nonconservative Ala-172 to Glu substitution disrupted proteinase 3CD *trans* cleavage of P1 precursors. Possibly, nonconservative amino acid substitutions at 3C residue Ala-172 affect proteinase 3C or 3CD domains required for *trans* P1 cleavage activity (Hammerle *et al.,* 1992) or for 3CD interaction with a cellular factor required for efficient P1 processing (Blair *et al.,* 1993). Alternatively, *trans* cleavage activity, particularly on the P1 precursor, may be more sensitive to 3C or 3CD structural perturbations resulting from nonconservative amino acid substitutions.

Two of the seven 3C single amino acid substitution mutations described in this study (Thr-142 to Ser, Ala-172 to Val) exhibited near wild-type cleavage activities on poliovirus P1, P2, and P3 precursor polypeptides *in vitro.* We were able to recover mutant viruses Se1-3C-30 and Se1-3C-31, which encode Thr-142 to Ser and Ala-172 to Val single amino acid substitutions, respectively. Mutant viruses Se1-3C-30 and Se1-3C-31 displayed severe temperature-sensitive defects in viral growth at 39°. Analysis of protein processing in cells infected with mutant virus Se1-3C-30 or Se1-3C-31 showed slight defects in P1 and P3 processing compared to that of wild-type. Such slight defects in 3C-mediated protein processing may contribute to, but do not seem to be the primary cause for, the severe temperature-sensitive defects observed in Se1-3C-30 or Se1-3C-31 viral growth. Unlike mutant virus growth, the slight protein processing defects observed in cells infected with Se1-3C-30 or Se1-3C-31 at 33 or 39° seem to be temperature-independent. Additionally, analysis of viral-specific RNA synthesis in cells infected with Se1-3C-30 or Se1-3C-31 showed significant temperature-sensitive defects in RNA production, which correlated with the observed temperature-sensitive defects in mutant virus growth. Significant levels of viral polymerase 3D were present at 33 and 39°. Therefore, the observed temperature-sensitive defects in Se1-3C-30 and Se1-3C-31 virus growth most likely result primarily from defects in 3C or 3CD replicase functions rather than in proteolytic activity.

Previous studies have reported a functional role for 3C or 3CD in viral RNA replication (Dewalt and Semler, 1989; Andino *et al.,* 1990a, 1993: Xiang *et al.,* 1995). Initial genetic experiments revealed the suppression of mutations in the poliovirus 5′ NCR which affect viral RNA replication by second-site reversion mutations in 3C sequences (Andino *et al.,* 1990b). Subsequently, electrophoretic mobility shift assays were used to demonstrate the binding of 3C, as part of 3CD, to a stem–loop structure in the 5′ NCR of poliovirus plus-strand RNA (the 5′ cloverleaf or stem–loop I) in the presence of a 36-kDa cellular factor(s) (Andino *et al.,* 1990a, 1993; Harris *et al.,* 1994) or purified viral protein 3AB (Xiang *et al.,* 1995). Mutations in stem–loop structures within the first ~100 nt of the poliovirus 5′ NCR and mutations in the 3C domain of protein 3CD affect *in vitro* binding of 3CD to viral RNA and plus-stranded RNA synthesis in infected or transfected cells (Andino *et al.,* 1990a, 1993). In this latter study, the authors suggested that 3C residues Asp-32, Arg-84, Asp-85, Thr-154, Gly-155, Lys-156, and Arg-176 may comprise a putative RNA binding domain in poliovirus polypeptide 3CD. Results from mutagenesis and structural studies on the human rhinovirus 3C polypeptide are consistent with the above data, although additional residues are probably involved in forming an RNA binding domain (Leong *et al.,* 1993; Matthews *et al.,* 1994: Walker *et al.,* 1995). Indeed, the 3C residues mutated in the present study (Thr-142 and Ala-172) may exert their effects on RNA binding through formation of such a domain rather than via direct interaction with RNA, as might be expected of properly positioned basic amino acid residues. Uncovering the extent of overlap between such putative RNA binding domains and polyprotein substrate binding domains involved in proteolytic cleavage will require a three-dimensional structural analysis of 3CD, along with additional functional studies using variant forms of 3CD containing lesions at proposed RNA and protein interfaces.

ACKNOWLEDGMENTS

We thank Michelle Tabb for help with plasmid constructions. We also thank Richard Kuhn and Louis Leong for helpful comments on the manuscript. This work was supported by Public Health Service Grant AI22693 from the National Institutes of Health. W.S.B. and T.B.P. were predoctoral trainees of the Public Health Service (Grants AI07319 and GM07311, respectively).

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