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Identification of the Active-Site Residues of the 3C Proteinase

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To identify the active-site residues of the 3C proteinase of foot-and-mouth disease virus (FMDV), we introduced mutations into the 3C coding region and examined the activity of mutant enzymes on various substrates. Based on alignment of FMDV 3C with other picornavirus 3C proteinases and with the trypsin family of serine proteinases, mutations were introduced at residues presumed to be part of the catalytic triad, involved in substrate binding, or present in nonconserved regions. Wild-type and mutant 3C proteins were expressed in *Escherichia coli* and tested for their ability to cleave synthetic substrates corresponding to different portions of the viral genome. Substitutions at His-46 (catalytic triad), Asp-84 (catalytic triad), or His-181 (substrate binding) produced enzymes unable to process P1, P2, or P3 substrates *in trans,* whereas a change in the conserved Asp-98 had no effect on enzyme activity. Substitution of Ser for Cys-163 (catalytic triad) yielded an enzyme that retained activity on some substrates, while a substitution of Gly at this position resulted in a completely inactive enzyme. The kinetics of *trans* processing of translation products from a transcript encoding the P1 and P2 coding regions and the 2C/3A cleavage site with wild-type 3C or a transcript encoding P1 with 3C mutants revealed that the order of cleavage was VP3-VP1, VP0-VP3, VP1-2A, 2C-3A, and 2B-2C. Mutations in 3C that resulted in a partially active enzyme were individually introduced into full-length FMDV cDNA and RNA transcripts were translated in a cell-free system and used to transfect cells. In all cases the virus that was rescued had reverted to the wild-type 3C codon.

INTRODUCTION

All picornaviral genomes are translated into a polyprotein that is processed by a number of viral encoded proteinases (Palmenberg, 1990). For foot-and-mouth disease virus (FMDV) processing into the primary cleavage products leader (L), P1, P2, and P3 occurs cotranslationally and is initiated by the L and 3C proteinases and the 2A oligopeptide. The initial processing event is the autocatalytic cleavage of L from P1 (Strebel and Beck, 1986), followed by 2A cleavage between P1 and P2 at the 2A/2B junction (Ryan *et al.,* 1991). All other cleavages are catalyzed by 3C or a 3C containing precursor (Vakharia *et al.,* 1987), except for the maturation cleavage of VP0 in the provirion to generate the capsid proteins VP4 and VP2.

Picornaviral 3C proteinases have been categorized as thiol proteinases, since iodoacetamide and *N*-ethylmaleimide inhibit their activity (Pelham, 1978), indicating that Cys may be an active-site amino acid. A comparative study of the derived amino acid sequences of these enzymes revealed a region of significant homology in the carboxyl third of the protein, including conserved Cys and His residues (Argos *et al.,* 1984). Based on this analysis, Ivanoff *et al.* (1986) constructed a clone containing the complete 3C coding region and a portion of the 3D coding region of poliovirus in an *Escherichia coli* expression system. Wild-type (WT) enzyme autocatalytically cleaved itself to produce the mature proteinase, while mutations at the conserved Cys (Cys-147) and His (His-161) residues of 3C resulted in an inactive enzyme. In an extensive computer analysis of viral and cellular proteinases, two groups independently suggested that the picornavirus 3C enzymes are structurally and evolutionarily related to the trypsin family of serine proteinases with the distinction that the active-site nucleophile Ser has been replaced by Cys (Cys-147; poliovirus numbering system) in the viral enzymes (Bazan and Fletterick, 1988; Gorbalenya *et al.,* 1989). Both groups also identified His-40 of poliovirus 3C as the base component of the putative catalytic triad. However, Bazan and Fletterick (1988) proposed that Asp-85 is the third component of the catalytic triad, whereas Gorbalenya *et al.* (1989) proposed that Glu-71 performs this function. Support for this general model was recently provided by the determination of the three-dimensional structure of rhinovirus and hepatitis A virus 3C (Mathews *et al.,* 1994; Allaire *et al.,* 1994).

In an attempt to characterize poliovirus and rhinovirus 3C, several groups have introduced mutations at specific amino acid residues and examined the mutant proteins for enzymatic activity (Baum *et al.,* 1991; Cheah *et al.,* 1990; Cordingley *et al.,* 1989; Hammerle *et al.,* 1991, 1992;

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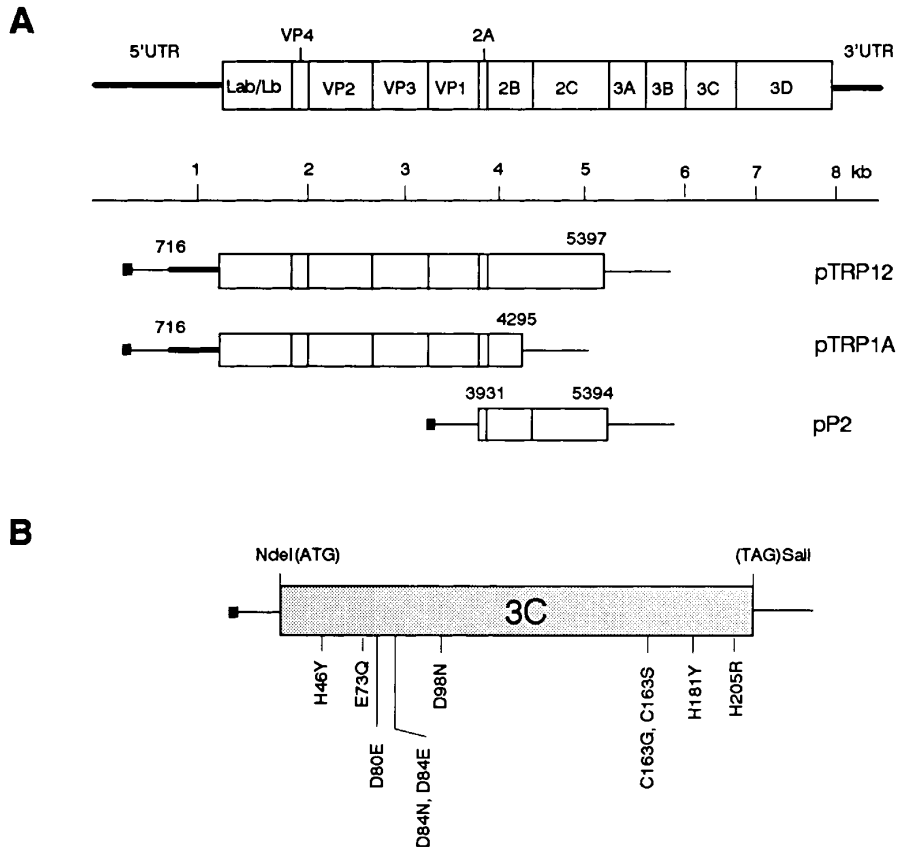

FIG. 1. Schematic representation of FMDV expression plasmids and construction of mutant 3C coding regions. The FMDV genome is shown in A and includes the 5′ and 3′ untranslated regions (UTR, thick lines) and the complete coding region (boxed) (Rueckert and Wimmer, 1984). The regions of the FMDV genome contained by the plasmids used to generate 3C substrates are indicated. B shows the individual mutations introduced into the 3C coding region. All constructs are under the control of a T7 RNA polymerase promoter (■ ———); the vector sequence is represented by thin lines.

Ivanoff *et al.,* 1986; Kean *et al.,* 1991, 1993; Lawson and Semler, 1991). We have recently reported expression of FMDV 3C in an *E. coli* system under the control of a bacteriophage T7 promoter and demonstrated biological activity on *in vitro* synthesized P1 and P2 as substrates (Bablanian and Grubman, 1993). Here we report the characterization of a series of mutant 3C proteinases with amino acid substitutions based on alignment with the active-site residues of chymotrypsin. Activity of WT and mutant enzymes was assayed on various *in vitro* synthesized substrates. Mutations that resulted in enzymes with partial processing activity were introduced into full-length FMDV cDNA and RNA transcripts translated *in vitro* and transfected into susceptible cells.

MATERIALS AND METHODS

Plasmids

E. coli plasmids pTRP12, pTRP1A, pP2 (2A, 2B, and 2C coding regions), and p3C (WT 3C coding region) were described previously (Bablanian and Grubman, 1993; Vakharia *et al.,* 1987) (Fig. 1A). The first two plasmids contain 400 bases upstream of the L coding region starting at nucleotide 716 and the L and P1 (VP0, VP3, and VP1) coding regions. pTRP12 also contains the complete P2 coding region and the first codon of the 3A sequence, while pTRP1A contains the P2 coding region up to nucleotide 4295 of 2B. In addition, both plasmids contain 20 codons from the vector pT7-7 (Tabor and Richardson, 1985) followed by an in-frame stop codon. Sequencing of both pTRP12 and pP2 at the 2A/2B junction revealed a difference from the published sequence at amino acid 16 of 2A, Arg to Gly (AGA to GGA), indicating that the 2A/2B cleavage is between Gly-Pro rather than Arg-Pro (Robertson *et al.,* 1985). Plasmid $pRMC_{35}$ is a full-length infectious clone of FMDV serotype A12 (Rieder *et al.,* 1993) and pRM-LLV2 is a full-length clone lacking the L (Lb) coding region (Piccone *et al.,* 1995).

Site-directed mutagenesis

Mutant 3C coding regions were synthesized by overlap extension PCR (Ho *et al.,* 1989) using standard techniques. Each mutant coding region had a unique *Nde*I

restriction site including an in-frame start codon (ATG) at the 5′ end and a termination codon (TAG) followed by a *Sal*I restriction site at the 3′ end. The mutated PCR products were digested with *Nde*I and *Sal*I and ligated into *Nde*I, *Sal*I digested pT7-7 vector. Plasmids containing the mutant 3C coding regions were sequenced through the amplified region by the dideoxy chain termination method (Sanger *et al.,* 1977) using Sequenase Version 2.0 (U.S. Biochemicals). There were differences in the WT and all mutant coding regions compared to the published sequence at residues 150, Ser to Gly (AGC to GGC), and 196, Lys to Arg (AAG to AGG) (Robertson *et al.,* 1985). The differences were confirmed to be errors in the original sequence, since the original plasmid (pT268) was resequenced. Mutant 3C plasmids are designated by the single-letter code for the WT amino acid followed by codon number and mutated amino acid (e.g., His at position 46 to Tyr is H46Y).

Construction of full-length cDNA clones with mutations in the 3C gene

To facilitate construction of full-length cDNAs with either the D80E or the D84E mutation, a unique *Nde*I site was created in pRM-LLV2 in the 3C coding region upstream of the mutations (pRM-LLV2/N). p3C-D80E and p3C-D84E were amplified using a primer containing the unique *Nde*I site and a second primer at the 3′ end of the 3C coding region, inserted into pCR (Invitrogen), and the amplified regions completely sequenced. These latter plasmids were digested with *Nde*I and *Eco*RV (unique site downstream of codon 84) and inserted into similarly digested pRM-LLV2/N. The clones were digested to remove the appropriate fragment and this fragment was inserted into similarly digested $pRMC_{35}$. The full-length clones, pRM-3CD80E and pRM-3CD84E, were checked by restriction enzyme analysis and sequenced through the inserted mutation.

To construct a full-length cDNA containing the C163S mutation, a region encompassing codon 163 was amplified by overlap extension PCR to contain the Ser-163 mutation, inserted into a subgenomic clone, and the amplified region was completely sequenced. The subgenomic clone was digested with restriction enzymes that cut at unique sites in the genome surrounding codon 163 and the appropriate fragment inserted into similarly digested $pRMC_{35}$. The full-length cDNA containing the 3C mutation, pRM-3CC163S, was checked as above.

In vitro transcription and translation

Transcripts from *Sal*I linearized plasmid pP2 were synthesized using T7 RNA polymerase and the mMESSAGE, mMACHINE capping kit (Ambion). Transcripts from *Eco*RV linearized plasmids pTRP1A and pTRP12, which contain the majority of the FMDV internal ribosome entry site, and *Not*I linearized plasmids pRM-3CD80E, pRM-3CD84E, and pRM-3CC163S were synthesized with the T7 MEGAscript or T7 RiboMAX kits (Ambion and Promega, respectively). Approximately 0.5–1 μg of RNA from transcription reactions or 1 μg of FMD virion RNA was used in a rabbit reticulocyte lysate *in vitro* translation system as previously described (Bablanian and Grubman, 1993; Grubman and Baxt, 1982; Vakharia *et al.,* 1987).

E. coli expression products and assay of enzyme activity

WT and mutant p3C plasmids were expressed in BL21 (DE3) cells and pellet fractions were prepared and stored at −70° (Bablanian and Grubman, 1993). [^{35}S]Methionine *in vitro* translation products were mixed with an equal volume of 0.1 *M* unlabeled methionine and incubated overnight, or as indicated in the figure legend, at 30° with 1 volume of resuspended pellet fraction from induced BL21 (DE3) cells containing pT7-7, WT, or mutant p3C plasmids or unlabeled FMDV-infected or mock-infected bovine kidney cell (LF-BK) postmitochondrial cytoplasmic extracts (S10). Products were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The amount of *E. coli* WT and mutant 3C used in the cleavage assays was quantitated by SDS–PAGE and Coomassie blue staining (Bablanian and Grubman, 1993) and essentially equivalent amounts of all *E. coli* expressed enzymes were used.

Transfection and characterization of 3C mutant viruses

RNA transcripts were transfected into baby hamster kidney cells (BHK-21) using Lipofectin or electroporation (Rieder *et al.,* 1993; Mason *et al.,* 1994). After electroporation, cells were radiolabeled with [^{35}S]methionine and examined for virus assembly and viral protein synthesis. Supernatants from cells transfected by either procedure (Pass 1) were used to produce virus stocks. RNA extracted from Pass 2 or Pass 3 virus was used as a template for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Superscript, Gibco BRL) and random hexamers. The cDNA corresponding to the 3C coding region was amplified with the appropriate primers by PCR and the PCR products were directly sequenced with the Sequenase PCR Product Sequencing kit (USB/Amersham).

RESULTS

Construction of 3C mutants

Mutations were introduced individually at amino acid residues 46(H46Y), 73(E73Q), 80(D80E), 84(D84E and D84N), 98(D98N), 163(C163S and C163G), and 181(H181Y)

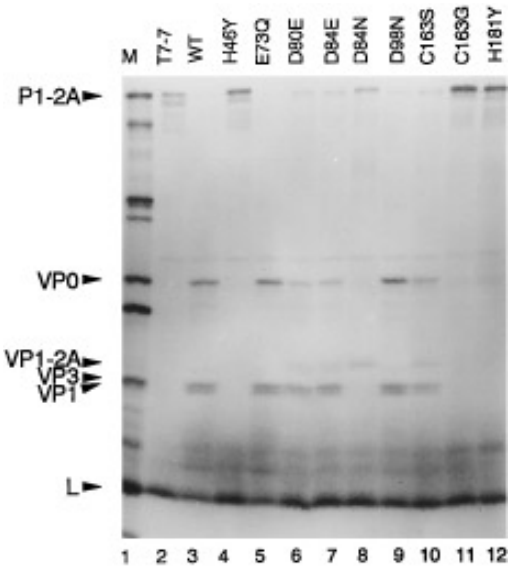

FIG. 2. Processing of radiolabeled translation products from pTRP1A transcripts by WT and mutant 3C proteinases expressed in *E. coli.* [^{35}S]Methionine-labeled translation products synthesized from pTRP1A transcripts were mixed with the pellet fraction of lysates from transformed *E. coli* expressing WT or mutant 3C proteinases, incubated overnight at 30°, and analyzed by SDS–PAGE on a 15% gel. Lanes 2–12, translation products mixed with pT7-7, WT, H46Y, E73Q, D80E, D84E, D84N, D98N, C163S, C163G, and H181Y lysates, respectively; lane M, cell-free translation programmed with FMD virion RNA as marker.

in the 3C coding region by overlap extension PCR. The mutant coding regions were cloned into the vector pT7-7 and transformed into HB101 cells. Plasmids containing the 3C coding region with the expected mutation were transformed into BL21 (DE3) cells, induced with IPTG, treated with rifampicin, and radiolabeled with [^{35}S]-methionine. All the mutant 3C proteins comigrated with the WT 3C and as determined by Coomassie blue staining each comprised about 5% of the total cell protein (data not shown). An additional mutant derived during construction of WT 3C had a change at amino acid residue 205 (H205R) and was also utilized in the following studies. Figure 1B shows the position of the mutations introduced in the 3C coding region.

Activity of 3C mutants on P1 substrate

To examine the effect of different mutations in 3C on processing of P1, radiolabeled substrate derived from translation of pTRP1A (Fig. 1A) was incubated with unlabeled lysates from induced *E. coli* cells containing the vector pT7-7, WT 3C, and each of the 3C mutants. A lysate from induced cells containing pT7-7 had no effect on P1-2A, whereas WT 3C completely processed P1-2A into structural proteins VP0, VP3, and VP1 (Fig. 2, lanes 2 and 3). An S10 extract from FMDV-infected LF-BK cells also completely processed P1-2A, while an extract from mock-infected LF-BK cells had no effect on the substrate (data not shown; Bablanian and Grubman, 1993). Substitutions at residues 46 (H46Y) and 181 (H181Y) completely eliminated 3C activity (lanes 4 and 12), while mutants E73Q, D98N, and H205R showed WT cleavage activity (lanes 5, 9, and data not shown). Mutants D80E, D84E, and C163S had almost WT activity on P1-2A, but did not process the VP1/2A cleavage site efficiently (lanes 6, 7, and 10). If Asp-84 was mutated to Asn (D84N) only the VP3/VP1 site was cleaved (lane 8). Mutation of Cys-163 to Gly (C163G) resulted in a completely inactive enzyme (lane 11).

Activity of 3C mutants on P2 substrates

Transcripts synthesized from *Sal*I linearized pP2 (Fig. 1A) with T7 RNA polymerase were translated in a cell-free system and radiolabeled translation products incubated with the various lysates from induced *E. coli* (Fig. 3). WT and mutants E73Q, D98N, and H205R completely processed P2 into 2C, 2A-2B, and 2B (lanes 3, 5, 9, and data not shown), while 3C mutants H46Y, D84N, C163G, and H181Y were completely inactive (lanes 4, 8, 11, and 12). Mutants D80E, D84E, and C163S retained approximately 5–10% WT activity on P2 as measured by the presence of 2C (lanes 6, 7, and 10). The kinetics of pro-

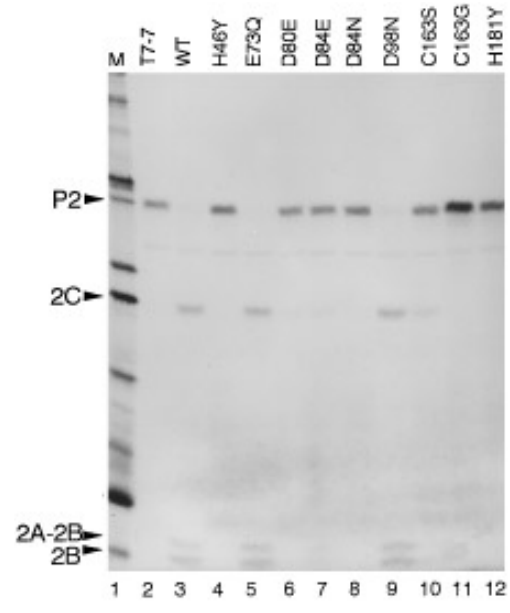

FIG. 3. Processing of radiolabeled translation products from pP2 transcripts by WT and mutant 3C proteinases expressed in *E. coli.* [^{35}S]Methionine-labeled translation products synthesized from pP2 transcripts were mixed with the pellet fraction of lysates from transformed *E. coli* expressing WT or mutant 3C proteinases, incubated overnight at 30°, and analyzed by SDS–PAGE on a 15% gel. Lanes 2–12, translation products mixed with pT7-7, WT, H46Y, E73Q, D80E, D84E, D84N, D98N, C163S, C163G, and H181Y lysates, respectively; lane M, cell-free translation programmed with FMD virion RNA as marker.

cessing of P2 by WT 3C revealed that 2A-2B and 2B appeared at the same time and in the same ratio as after an overnight incubation (data not shown).

Activity of 3C mutants on P1, P2, and 2C-3A substrates

Plasmid pTRP12 (Fig. 1A) provides a source of P1 and P2 substrates as well as the 2C/3A cleavage site. Translation of transcripts from pTRP12 resulted in synthesis of P1-2A and P2-3A′. Protein P2-3A′ migrates slightly slower than P2, since it contains the first amino acid of 3A (isoleucine) and 20 amino acids from the vector before an in-frame stop codon. WT 3C and mutants E73Q, D98N, and H205R completely processed the substrates, at all 3C cleavage sites, into VP0, VP3, VP1, 2C, and 2B (Fig. 4, lanes 3, 5, 9, and data not shown), whereas mutants H46Y, C163G, and H181Y were completely inactive on all P1-2A, P2, and 2C/3A cleavage sites (lanes 4, 11, and 12). Mutant D84N partially processed the 2C/3A and VP3/VP1 sites, but was inactive at all other sites (lane 8). Mutants D80E, D84E, and C163S had partial activity on P1-2A and at the 2C/3A cleavage site to produce P2, but, as shown previously, did not efficiently cleave at the VP1/2A cleavage site or within P2 (lanes 6, 7, and 10).

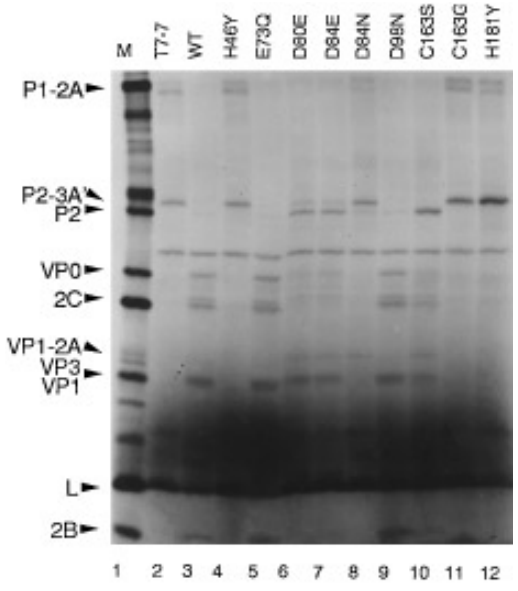

FIG. 4. Processing of radiolabeled translation products from pTRP12 transcripts by WT and mutant 3C proteinases expressed in *E. coli.* [^{35}S]Methionine-labeled translation products from pTRP12 transcripts were mixed with the pellet fraction of lysates from transformed *E. coli* expressing WT or mutant 3C proteinases, incubated overnight at 30°, and analyzed by SDS–PAGE on a 15% gel. Lanes 2–12, translation products mixed with pT7-7, WT, H46Y, E73Q, D80E, D84E, D84N, D98N, C163S, C163G, and H181Y lysates, respectively; lane M, cell-free translation programmed with FMD virion RNA as marker. The major band immediately above VP0 in lanes 2–12 is an endogenous reticulocyte protein.

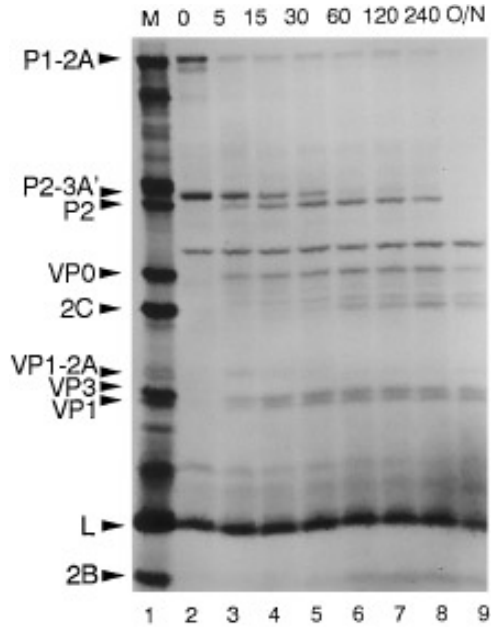

FIG. 5. Kinetics of processing of radiolabeled translation products from pTRP12 transcripts by WT 3C proteinase expressed in *E. coli.* [^{35}S]Methionine-labeled translation products from pTRP12 transcripts were incubated for varying periods of time at 30° with the pellet fraction of a lysate from transformed *E. coli* expressing WT 3C proteinase and analyzed by SDS–PAGE on a 15% gel. Lanes 2–9, translation products mixed with WT 3C and incubated for 0, 5, 15, 30, 60, 120, and 240 min and overnight, respectively; lane M, cell-free translation programmed with FMD virion RNA as marker. The major band immediately above VP0 in lanes 2–9 is an endogenous reticulocyte protein.

Order of processing of P1, P2, and 2C/3A substrates

To determine the order of processing of primary products P1, P2, and at the 2C/3A cleavage site, translation products from pTRP12 transcripts were incubated for varying periods of time with WT 3C (Fig. 5). Within 5 min of addition of WT 3C to the translation products, P1-2A is almost completely processed to VP0, VP3, and VP1 (lane 3). Some VP1-2A is still present at 5 min, but is completely processed by 15–30 min (lanes 4 and 5). Processing of P1-2A is just as rapid when incubation is at 25° (data not shown). Processing at the 2C/3A site starts within 5 min, as demonstrated by the decrease in amount of P2-3A′ and the appearance of P2, and is essentially complete by 60 min (lanes 3–6). The amount of P2 increases until about 60 min and decreases with longer incubation (lanes 3–9). 2C and 2B do not appear until about 30–60 min (lanes 5 and 6) and the amount of these two proteins increases with time (lanes 5–9).

Addition of 3C mutants D84E or C163S to translation products of pTRP1A resulted in processing of P1-2A, but at a reduced rate as compared to processing by WT 3C (data not shown). The initial processing event occurred at the VP3/VP1 cleavage site, resulting in the appearance of VP0-VP3 and VP1-2A. Mutant D84E processed the

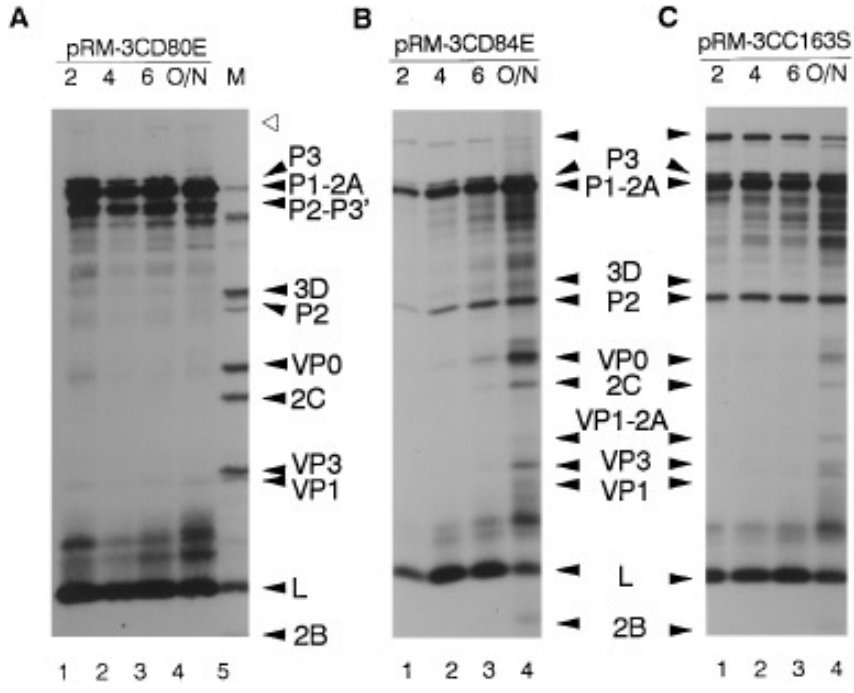

FIG. 6. *In vitro* translation of full-length 3C mutant transcripts pRM-3CD80E (A), pRM-3CD84E (B), and pRM-3CC163S (C). The above transcripts were translated for 2 hr, 4 hr, 6 hr, or overnight (lanes 1–4, respectively) and analyzed by SDS–PAGE on a 15% gel. Lane M in A is a cell-free translation programmed with FMD virion RNA as marker.

VP0/VP3 and VP1/2A cleavage sites much more rapidly than C163S.

Effect of mutant 3C proteins on viral replication

Since mutations at residues 80 (D80E), 84 (D84E), and 163 (C163S) resulted in enzymes with partial cleavage activity, we individually introduced these mutations into a genome-length cDNA clone to determine their effect on viral replication. *In vitro* translation of transcripts derived from the three mutant clones revealed that there was only partial protein processing as compared to translation of WT RNA (Fig. 6). Primary cleavage products P3, P1-2A, P2, and L were present in the translation of transcripts pRM-3CD84E and pRM-3CC163S (Figs. 6B and 6C, lane 1), but the 3C catalyzed secondary cleavages to yield mature structural and nonstructural proteins did not occur. In addition, a larger protein (indicated by an arrowhead), which was immunoprecipitated with sera specific for P2 or P3 polypeptides (data not shown), was present. Longer incubation of the pRM-3CD84E and pRM-3CC163S translation reactions showed that the mutant 3C could gradually process the primary products as well as the larger precursor to VP0, VP1-2A, VP3, VP1, and less efficiently to 3D, 2C, and 2B (Figs. 6B and 6C, lanes 2–4). In both cases processing was also examined by addition of WT *E. coli* expressed 3C to the 2-hr translation products. Processing within P1-2A was rapid, while processing within P2 and P3 was extremely inefficient. We were not able to follow the order of processing within P3 (data not shown). Translation reactions from pRM-3CD80E derived transcripts contained P3, P1-2A, and L, but only small amounts of P2 were present (Fig. 6A). In addition, there were small amounts of two high-molecular-weight proteins (indicated by an open arrowhead), as well as a major protein migrating slightly faster than P1-2A (P2-P3′). Immunoprecipitation of the 4-hr translation products of pRM-3CD80E transcripts indicated that P2-P3′ contained 2B, 2C, and 3C (data not shown). Furthermore, the translation products were correctly processed by *E. coli* expressed WT 3C (not shown).

Transcripts derived from the three full-length mutant cDNA clones were transfected into BHK-21 cells by Lipofectin, but no plaques were detected by plaque assay. To examine for the presence of viral protein synthesis in transfected cells, we used electroporation, which we have previously shown to be a highly efficient method of transfection (Mason *et al.,* 1994). Examination of radiolabeled culture fluids and cell lysates showed that viral protein synthesis did not occur. The supernatants from transfected cells were used to infect BHK cells and plaque-forming virus was obtained with the three different mutant full-length cDNA clones. RNA extracted from second- and third-passage virus stocks was used as a template for reverse transcriptase–PCR and in all cases the mutant codons had reverted to the WT codon.

TABLE 1

Characterization of 3C Mutants

Mutants	Codon change	Processing			Virus recovery
		P1[a]	P2[a]	2C-3A′ [a]	
Wild type		++++	++++	++++	Yes
H46Y	CAT → TAT	−	−	−	ND[b]
E73Q	GAG → CAG	++++	++++	++++	ND
D80E	GAC → GAG	+++	+	+++	Reverted
D84E	GAC → GAA	+++	+	+++	Reverted
D84N	GAC → AAC	+/−	−	+	ND
D98N	GAT → AAT	++++	++++	++++	ND
C163S	TGT → AGT	+++	+	++++	Reverted
C163G	TGT → GGT	−	−	−	ND
H181Y	CAC → TAC	−	−	−	ND
H205R	CAC → CGC	++++	++++	++++	ND

[a] Each assay represents the average of at least three independent experiments (++++, WT activity; −, no activity).
[b] Not determined; this mutation was not transferred to a full-length cDNA.

DISCUSSION

To identify amino acid residues which are important in FMDV 3C proteinase activity, we characterized the activity of a series of single-site mutants. The residues chosen for modification were based on the models of Bazan and Fletterick (1988) and Gorbalenya *et al.* (1989), which suggested that the picornavirus 3C enzymes are structurally and evolutionarily related to the trypsin family of serine proteinases. The WT and mutant enzymes expressed in *E. coli* were assayed for *trans* cleavage activity on a number of *in vitro* radiolabeled substrates generated from transcripts representing different portions of the genome. Our results agree with the general model proposed by both groups and recently supported utilizing a similar mutagenesis strategy for poliovirus and rhinovirus 3C (Cheah *et al.,* 1990; Hammerle *et al.,* 1991, 1992; Kean *et al.,* 1991, 1993; Lawson and Semler, 1991).

In both models, FMDV 3C residues His-46 and Cys-163 (equivalent to poliovirus 3C His-40 and Cys-147, respectively) are implicated as part of the catalytic triad and His-181 (equivalent to poliovirus His-161) as part of the binding pocket. Our results support the essential role of these residues in the function of 3C (Table 1). The models differ as to the third member of the catalytic triad. Our data support the prediction of Gorbalenya *et al.* (1989), since mutation of Asp-84 to Asn (D84N) results in an almost inactive enzyme, whereas the same mutation at Asp-98 (D98N) has essentially no effect on *trans* cleavage activity. Additional support for this model was recently provided by determination of the X-ray crystal structure of the rhinovirus 3C proteinase (Mathews *et al.,* 1994). However, the X-ray crystal structure of the hepatitis A virus 3C suggests that the active site is only a dyad of Cys/His residues (Allaire *et al.,* 1994).

We made additional mutations at residues that are not conserved among picornavirus 3C enzymes. Mutation at Glu-73, proposed by Gorbalenya *et al.* (1989) to be part of the loop connecting β-strands E and F to Gln (E73Q), has no effect on enzyme activity. However, a change in Asp-80, also predicted to be part of the loop connecting β-strands E and F, but closer to β-strand F, to Glu (D80E), results in an enzyme that has reduced activity at the VP1/2A and 2C/3A sites and significantly reduced activity at the 2B/2C site. Mutant H205R, which has an alteration in a residue present in an α-helix region close to the C-terminus of the protein, was indistinguishable from WT in all cleavage assays examined.

We found that substitution of Ser for the Cys residue (C163S), proposed to be the catalytic nucleophile, did not result in a completely inactive enzyme. Instead this enzyme retained activity on some of the substrates assayed. These results are in agreement with those of Lawson and Semler (1991) and Kean *et al.* (1993) for poliovirus 3C proteinase. The FMDV 3C C163S mutant retained activity on the structural protein precursor P1-2A, although the rate of cleavage was significantly reduced. The mutant completely processed the 2C/3A site, but showed minimal activity at the 2B/2C cleavage site. In contrast, the equivalent poliovirus 3C mutant (C147S) showed minimal activity on P1, but displayed WT activity on P2. Presumably, as suggested by Lawson and Semler (1991), the ability of the C163S mutant to retain partial *trans* proteolytic activity may be explained by the ability of the Ser hydroxyl group to substitute for the Cys sulfhydryl group as a nucleophile. In support of this, substitution of Gly for Cys-163 (C163G) in FMDV 3C or Ala for Cys-147 in poliovirus 3C resulted in a completely inactive enzyme (Figs. 2–4; Lawson and Semler, 1991).

Addition of *E. coli* expressed WT 3C to a system containing P1-2A and P2 precursors demonstrated that pro-

cessing of P1-2A was essentially complete prior to cleavage within P2. Processing of P2 synthesized from pTRP12 derived transcripts resulted in the appearance of 2C and 2B, whereas 2C, 2A-2B, and 2B appeared upon processing of P2 synthesized from pP2 derived transcripts. The open reading frame of the latter template codes for an amino-terminal Met directly followed by 2A. These results suggest that 2A lacking upstream amino-terminal residues cannot efficiently process at the 2A/2B junction. Ryan *et al.* (1991) demonstrated that a 19-amino-acid sequence spanning the 2A region mediated the 2A/2B cleavage. More recently, Ryan and Drew (1994) placed FMDV 2A or truncated 2A regions between two reporter genes and demonstrated that autoproteolytic processing at the C-terminus of 2A occurred and cleavage required a minimum of 13 residues of 2A. Our results suggest that in addition to the 13 residues of 2A, amino acids N-terminal to 2A are also required for efficient cleavage, although these residues apparently can vary. Perhaps the function of these residues is to properly orient 2A so that it can efficiently cleave at its C-terminus.

Mutations in 3C that resulted in enzymes that retained partial P1-2A and 2C/3A *trans* cleavage activity were introduced into full-length cDNA clones. By *in vitro* translation of transcripts derived from mutants pRM-3CD84E and pRM-3CC163S, 3C processing initially occurred only at the 2C/3A cleavage site, although processing at additional 3C sites occurred during longer incubations of the translation reactions. Translation of transcripts derived from mutant pRM-3CD80E resulted in processing at only the 3C/3D site, yielding a P2-P3′ precursor. No additional processing within P1, P2, or P3 occurred with the mutant enzyme, although further processing with exogenous WT enzyme was possible. All three altered residues are critical for FMDV replication, since viral proteins were undetectable in cells transfected by high-efficiency electroporation and in each case virus rescued from transfection had reverted to the WT amino acid. Kean *et al.* (1991) obtained identical results with similar poliovirus 3C mutants.

Our results with FMDV agree with the general model for picornavirus 3C proteinases. It is interesting to note, however, that while the scissile bond cleaved by other picornavirus 3C enzymes is relatively conserved that for FMDV is highly variable. Furthermore, there does not appear to be any consensus sequence around the FMDV 3C cleavage sites. Identification of residues required for the construction of efficient 3C cleavage sites will be useful in future work.

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