Mutational Analysis of the Proposed FG Loop of Poliovirus Proteinase 3C Identifies Amino Acids That Are Necessary for 3CD Cleavage and Might Be Determinants of a Function Distinct from Proteolytic Activity

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Mutations were introduced into a cDNA clone of poliovirus resulting in single-amino-acid substitutions within the region of the proposed FG loop of proteinase 3C. RNAs were made by *in vitro* transcription with T7 RNA polymerase and used to transfect HeLa cells. Virus viability was assessed as indicated by cell lysis. In parallel, RNAs were translated *in vitro* by using a HeLa cell lysate, and the patterns of the processed polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Replacement of Lys-78, Arg-79, and Glu-81 had apparently no effect on virus viability and on proteolytic processing. In contrast, virus viability was abolished by mutation of Phe-83, Arg-84, Asp-85, Ile-86, and Arg-87. With respect to substitution of Phe-83, Asp-85, and Arg-87, these effects correlated with impaired processing of the 3CD cleavage site, separating 3C and 3D, and, to a lesser extent, of the P1 precursor. Replacement of Arg-84 and Ile-86, on the other hand, did not alter the processing pattern. Thus, the lethal effects in these mutant genomes may not have been caused by impaired processing. A special case was the mutant of Lys-82-Gln. Virus recovered from cells transfected with RNA carrying this mutation always contained an A-to-G transition which resulted in the replacement of glutamine for arginine. Our data suggest that residues in the proposed FG loop of proteinase 3C influence 3CD cleavage and that they are determinants of a function unrelated to proteolytic processing.

Proteinase 3C of poliovirus (3C^{pro}) plays a crucial role in the processing of the viral polypeptide that occurs during the viral replication cycle (reviewed in references 10 and 20). Recently, sequence alignments and modeling of picornavirus 3C^{pro} polypeptides led to the hypothesis that 3C^{pro} is structurally and functionally related to cellular trypsinlike serine proteinases despite having a cysteine as the active nucleophile (3, 7). Two models have been proposed that assign the amino acids of the viral enzyme to a 12-stranded bilobal trypsin-fold with certain residues forming the catalytic triad. The models differ mainly in the assignment of strands E and F and the interbarrel loop. Cys-147 and His-40 are predicted to constitute the nucleophile and base of the catalytic triad in both models, whereas Asp-85 and Glu-71 are thought to function as the catalytic acid according to Bazan and Fletterick (3) and Gorbalenya et al. (7), respectively.

Recently, we and others provided support for the Gorbalenya et al. (7) model by showing that Glu-71 is essential for proteolytic activity of 3C^{pro} of poliovirus, whereas Asp-85 is not (8, 17). This finding was surprising because Asp-85 and adjacent amino acids are highly conserved in 3C^{pro} polypeptides of different picornaviruses, a fact suggesting a specific function (7). To analyze the significance of this conserved stretch of amino acids, we introduced appropriate nucleotide exchanges in a cDNA clone, transcribed the cDNA, and checked the effect of the mutations on virus production after transfection of HeLa cells. In parallel, the transcribed RNAs were translated in a HeLa cell extract to assess the extent of proteolytic processing.

MATERIALS AND METHODS

Genetic engineering of DNA. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories. All oligonucleotides were synthesized on an Applied Biosystems apparatus. DNA manipulations were done by standard procedures (25). Polymerase chain reaction (PCR) experiments were done by using the GeneAmp DNA amplification kit on a Perkin-Elmer Cetus DNA thermal cycler, as described by Innis and Gelfand (12). The entire nucleotide sequences of all PCR products were verified by dideoxy sequencing (26).

Plasmids. A hybrid cDNA clone of polioviruses type 1 (Mahoney) and type 2 (Sabin) used as a template to generate RNA for transfection and translation was constructed as follows. First, an *HpaI* site was introduced at bp 5747 of the cDNA of poliovirus type 1 (Mahoney) by PCR-driven mutagenesis (11), using the oligonucleotides 5'-CTAGTGTTAA CGATCAA-3' and 5'-TTGATCGTTAACAAGTAG-3' as mutagenic primers (named *HpaI* primers 1 and 2, respectively) and oligonucleotides 5'-CCCACGCTCCACCTGGTG-3' (bp 5553 to 5570) and 5'-CGAAGGTCTCATCC-3' (bp 6013 to 5999) as outer primers. The template was plasmid pBS⁺KS⁺3CD, which consists of part of the 5'-nontranslated region of encephalomyocarditis virus (13), the entire region of 3CD, and downstream sequences to the *EcoRI* site derived from pPV1-5 (27) cloned into the *EcoRI* site of pBS⁺KS⁺ (Stratagene, La Jolla, Calif.). The PCR product was cut with *BglII* and *NarI* and inserted into pBS⁺KS⁺3CD that was cut similarly.

A clone harboring the new *HpaI* site was cut with *BglII* and *EcoRI*. The 1,900-bp fragment was isolated and inserted

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into pT7XLD cut similarly (22). The resulting plasmid was named pT7XLD/HpaI. The sequence between the *Bgl*III and *Hpa*I sites encoding amino acid residues 56 to 103 of 3C^{pro} was replaced by the homologous sequence of poliovirus type 2 (Sabin), which was generated by PCR with the oligonucleotide 5'-GAGGTGGAGATCTTGGACGCCAAAGCCAAA GCCCTT-3' (*Bgl*III primer, bp 5597 to 5623) and the *Hpa*I primer 1 mentioned above. The procaryotic expression vector for 3C^{pro} (Sabin), pMN35 (23), was used as a template. The resulting plasmid was named pTH/3C^{pro}wt. All plasmids harboring a particular mutant in 3C^{pro} were named according to the scheme pTH/3C^{pro}ABC, where A stands for the residue replaced, B for the position in 3C^{pro} (the N-terminal residue of 3C^{pro} numbered 1), and C for the residue introduced. The one-letter code is used to identify amino acids.

Site-directed mutagenesis. Mutagenesis of residues in 3C^{pro} by using the expression vector pMN35 (23) was done as follows. Replacement of Asp-85 by Asn has been described previously (8). Residue Glu-81 was exchanged for Ser in the same way by using the oligonucleotide 5'-GAGGAAGCTCGA AGTTCAGAGATATCAGGCCTCACATTC-3', which also introduces a new *Stu*I site at bp 5696 without changing the amino acid sequence. Replacement of residue Arg-84 by Ser was done by PCR with the oligonucleotides 5'-GTGAGGC CTGATATCGCTGAACCTTCTC-3' and 5'-GGGAGACCAC AACGG-3' as mutagenic primer and counterprimer, respectively. This counterprimer binds to bp 63 to 49 upstream of the initiation codon of 3C^{pro} in pMN35. The PCR product was cut with *Nco*I and *Stu*I and inserted into pMN35 (3C^{pro}E81S) cut similarly.

Transfer of these mutants from pMN35 into the full-length clone was done by PCR with *Hpa*I primer 1 and *Bgl*III primer and the appropriate species of pMN35 harboring the particular mutant as a template. The PCR fragments were cut with *Bgl*III and *Hpa*I and inserted in pTH/3C^{pro}wt cut similarly, thus generating plasmids pTH/3C^{pro}D85N, pTH/3C^{pro}E81S, and pTH/3C^{pro}R84S.

Substitution of the following residues in 3C^{pro} was done by insertion of the respective PCR fragment into pTH/3C^{pro}E81S cut with *Bgl*III and *Stu*I. PCR was done with the particular mutagenic oligonucleotide and the *Bgl*III primer as the counterprimer. pTH/3C^{pro}E81S was used as a template: pTH/3C^{pro}K78Q, 5'-GTGAGGCCTGATATCTCTGAACCTTCTC GTTCCTTTGTAGTGAATTATGG-3'; pTH/3C^{pro}R79Q, 5'-GTGAGGCCTGATATCTCTGAACCTTCTCTCGTTCTGCTT TAGTGAATTATGG-3'; pTH/3C^{pro}K82Q, 5'-GTGAGGC CTGATATCTCTGAATTGCTCGTTCTCTTAG-3'; pTH/3C^{pro}F83A, 5'-ATGTGAGGCCTGATATCTCTGGCCTTCT CGTTCTCTTAG-3'; pTH/3C^{pro}R84K, 5'-TGTGAGGCCT GATATCTTGAACCTTCTCGTTCTC-3'; pTH/3C^{pro}D85S, 5'-TGTGAGGCCTGATGCTTCTGAACCTTCTCGTTCC-3'; pTH/3C^{pro}I86A, 5'-ATGTGAGGCCTGGCATCTCTGAAC TTCTCGTTCC-3'.

The following two mutations were made by insertion of the respective PCR fragment into pTH/3C^{pro}E81S cut with *Bgl*III and *Hpa*I. PCR was done by the method of Higuchi et al. (11) by using the appropriate mutagenic primers and *Bgl*III primer and *Hpa*I primer 1 as outer primers. The template was either pTH/3C^{pro}E81S or pTH/3C^{pro}wt: pTH/3C^{pro}R87Q, 5'-GTGTGGCTGGATATCTCTGAACCTTC-3' and 5'-GATATCCAGCCACATTCCCAACC-3'; pTH/3C^{pro}R87 K, 5'-GTGGCTTGATATCTCTGAACCTTCTCGT-3' and 5'-GTTTCAGAGATATCAAGCCACACATTCCCAACC-3'.

In vitro transcription. In vitro transcription was done by using T7 RNA polymerase as described previously (27).

Transcripts were extracted with phenol-chloroform-isoamyl alcohol (24:24:1), precipitated with ethanol, washed once, dissolved in water, and stored at -80°C until further use. The approximate concentration of RNA was estimated by staining with ethidium bromide after agarose gel electrophoresis.

In vitro translation. In vitro translation was done by using a HeLa cell extract. Label was [³⁵S]methionine (Amersham). The detailed conditions and the preparation of the extract have been described elsewhere (21). Briefly, 50 to 200 ng of RNA was translated in a volume of 12.5 µl at 30°C for 15 h. Aliquots (4 µl) were analyzed on a 15% polyacrylamide gel (19). Gels were fixed, fluorographed with En³Hance (New England Nuclear), dried, and exposed at -70°C on XRP films (Kodak).

Transfection and plaque assay. Subconfluent HeLa R19 cells were transfected with 100 to 200 ng of RNA per 3.5-cm plate by the DEAE-dextran method as described previously (27). After a primary incubation of 48 h, cells plus medium were frozen and thawed three times. Cell debris was pelleted by centrifugation in an Eppendorf tube for 10 min. Fresh cells were infected with 200 µl of supernatant (referred to as first stock), incubated under 1.5% agar for 48 h, and stained with crystal violet. Mutants were considered nonviable if there was no cell lysis after the second incubation.

Preparation of virus stocks and determination of RNA sequences. First, stocks were serially diluted and used to infect HeLa cell monolayers. After 48 to 60 h under 1.5% agar, isolated plaques were picked and expanded to working stocks through one or two passages in HeLa cells.

HeLa cells were infected with stock virus at a multiplicity of infection of 20. After 6 to 8 h, cytoplasmic extracts were prepared as described previously (5). RNA sequences were determined by using an RNA-sequencing kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer's protocol, using ³⁵S-dATP and the *Hpa*I primer 1 mentioned above.

In some cases, direct sequencing of viral RNA proved difficult because of band compressions. This was overcome by reverse transcription and amplification by PCR as described below. The order of addition of primers was reversed. Thereafter, the sequences of the PCR products were determined directly or after subcloning into pBS⁺SK⁺ (Stratagene, La Jolla, Calif.) with the *Cla*I and *Hind*III sites. In both cases, the Sequenase sequencing kit (U.S. Biochemical, Cleveland, Ohio) was used with ³⁵S-dATP as the labeled deoxynucleotide and the *Bgl*III primer mentioned above. To exclude artifacts caused by misincorporation during PCR, we determined the sequence of the products derived from at least two different amplifications of the same RNA batch.

Detection of minus-strand synthesis by reverse transcription and PCR. At 18 h after transfection, total cytoplasmic RNA was prepared by lysis with Nonidet P-40 (5). Reverse transcription and then PCR were done as described previously (15), using avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). First primer was 5'-CCCACGC TCCACCTGGTG-3' (bp 5553 to 5570), and second primer was 5'-GCACTGGGTTCAAGCTTGG-3' (bp 6072 to 6054). A 15-µl aliquot was analyzed by agarose gel electrophoresis.

RESULTS

The plasmid used for most studies described here is shown in Fig. 1. The poliovirus-related sequences are mostly related to poliovirus type 1 (Mahoney), with the exception of

FIG. 1. Scheme of the plasmid pTH/3C^{pro}wt. pTH/3C^{pro}wt is a modified cDNA clone of poliovirus type 1 (Mahoney) which was used to generate RNA for transfection and in vitro translation. pTH/3C^{pro}wt is different in having a unique *Hpa*I site at bp 5747. Additionally, the sequence between the *Bgl*II and the *Hpa*I sites has been replaced by the homologous sequence of poliovirus type 2 (Sabin). The P1, P2, and P3 regions of the polyprotein are indicated. Numbers in italics give the positions of restriction sites used in the construction of the plasmid. Other numbers mark the region encoding 3C^{pro} within the polyprotein as well as the sequence where mutations have been introduced. This sequence is given in Table 1. Note that the *Stu*I site is only present in some of the mutants. For further details, see Materials and Methods.

bp 5601 to 5747 (amino acids 56 to 103 of 3C^{pro}), which are derived from poliovirus type 2 (Sabin) (23). The replacement of this segment resulted in the exchange of some bases relative to poliovirus type 1 (Mahoney), but it did not alter the amino acid sequence. This construct offered the advantage that mutations previously introduced into this segment (8) could easily be introduced into pTH/3C^{pro}wt. Furthermore, the Sabin-specific sequences could be used as a genetic marker in identification of a particular virus preparation.

Plasmids were transcribed in vitro with T7 polymerase, and RNAs were used to transfect HeLa cells. The progression of infection was judged by inspection of the cells for lysis. Table 1 shows the results. Replacement of Lys-78, Arg-79, and Lys-82 by glutamine and of Glu-81 by serine resulted in viable virus. In contrast, replacement of Phe-83 by alanine, Arg-84 or Asp-85 by serine, Ile-86 by alanine, and Arg-87 by glutamine abolished virus viability com-

pletely, whereas mutation of Asp-85 to asparagine resulted in recovery of virus only after prolonged incubation. It is noteworthy that replacement of the positively charged Arg-87 with lysine resulted in viable virus, whereas the same conservative exchange in position 84 was lethal.

To assess the effects of the mutations on proteolytic processing of the polyprotein, we translated T7 transcripts in a HeLa cell extract. The analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 2. In comparison with the pattern of proteins obtained from infected cells, all proteins derived from the polyprotein could be detected, with the exception of products of the so-called maturation cleavage yielding VP2 and VP4. This cleavage event takes place during or after particle assembly and may be autocatalytic (9).

RNAs derived from plasmids pTH/3C^{pro}R79Q, pTH/3C^{pro}K82Q, and pTH/3C^{pro}E81S (Fig. 2A, lanes 7 and 8, and 2B, lane 4) showed processing patterns that were similar to those of RNA derived from pT7XLD/*Hpa*I or pTH/3C^{pro}wt or of viral RNA (Fig. 2A, lanes 3, 4, and 5). These findings are consistent with the result presented above, showing that these mutations did not affect virus viability. A slightly altered pattern of processing was seen with mutant Lys-78-Gln, which showed reduced production of 3C' and 3D' and also slightly less 3D^{pol} (Fig. 2A, lane 6). We have previously shown that the relative ratios of 3D^{pol}, 3C', and 3D' influence poliovirus replication only a little (reviewed in reference 10). It is therefore not unusual that the mutant Lys-78-Gln is viable.

The processing patterns of mutations that have been shown to affect virus viability could be assigned to one of three categories: first, a pattern that was deficient in proper 3CD cleavage while all the other cleavage events appeared to be normal; second, a pattern that showed impaired 3CD cleavage as well as impaired processing of P1 precursor; and third, a pattern that was virtually identical to that of wild type. Mutation of Arg-87 for glutamine or lysine and Asp-85 for asparagine belong to category 1 in that, relative to the 3CD band, there were hardly any 3D or 3C bands visible (Fig. 2B, lanes 2, 3, and 5). The deficiency in processing resides almost exclusively in the aberrant stability of 3CD, an observation suggesting that the amount of free 3C^{pro} could be drastically diminished without affecting processing of most Q-G cleavage events. Replacement of Phe-83 for alanine and Asp-85 for serine resulted in processing patterns of category 2 (Fig. 2B, lanes 6 and 7). The processing of 3CD as well as that of the P1 precursor was impaired. It is interesting that a serine in position 85 had a stronger effect on processing than an asparagine.

All other mutations produced patterns related to the wild-type phenotype (category 3). This is particularly interesting with respect to the Arg-84-Lys (Fig. 2B, lane 1) and Ile-86-Ala (Fig. 2B, lane 8) mutations that were lethal for viral replication. Therefore, proteolytic activity seemed not to be affected by these mutations introduced, and thus, their lethal effects could not be correlated with impaired cleavage events obtained by translation in vitro. The lethal Arg-84-Ser mutation (Fig. 2A, lane 9) was nearly wild type in processing, although interestingly, there was less cleavage of 3CD to 3C' and 3D', similar to the viable mutant Lys-78-Gln. These findings suggest that the lethal mutation of category 3 affected a function of 3C^{pro} distinct from proteolytic processing. We therefore analyzed whether the RNA containing the Arg-84-Ser mutation would replicate after transfection. This was done by reverse transcription with a minus-strand-specific primer and then by PCR. A control experiment with

TABLE 1. Effect of mutations in the proposed FG loop of 3C^{pro} on recovery of virus and proteolytic processing of the P1 precursor and the 3CD cleavage site

Plasmid ^a	Sequence ^b												Processing ^c	Recovery ^d
pTH/3C ^{pro} wt	AAG K ₇₈	AGG R	AAC N ₈₀	GAG E	AAG K ₈₂	TTC F	AGA R ₈₄	GAT D	ATC I ₈₆	AGG R	CCA P ₈₈		Normal	Yes
K78Q	CAA	—	—	—	—	—	—	—	—	—	CCT		Normal	Yes
R79Q	—	CAG	—	—	—	—	—	—	—	—	CCT		Normal	Yes
E81S	—	—	—	TCG	—	—	—	—	—	—	CCT		Normal	Yes
K82Q	—	—	—	—	CAA	—	—	—	—	—	CCT		Normal	Yes ^e
F83A	—	—	—	—	—	GCC	—	—	—	—	CCT		Impaired	No
R84S	—	—	—	—	—	—	AGC	—	—	—	CCT		Normal	No
R84K	—	—	—	—	—	—	AAG	—	—	—	CCT		Normal	No
D85N	—	—	—	—	—	—	—	AAT	ATT	—	—		Impaired	Yes ^f
D85S	—	—	—	—	—	—	—	AGC	—	—	CCT		Impaired	No
I86A	—	—	—	—	—	—	—	—	GCC	—	CCT		Normal	No
R87Q	—	—	—	—	—	—	—	—	—	CAG	—		Impaired	No
R87K	—	—	—	—	—	—	—	—	—	AAG	—		Impaired	Yes ^f

^a Plasmids were named according to the amino acid exchange introduced in 3C^{pro}. For nomenclature, see Materials and Methods.

^b The DNA sequence of the wild-type plasmid from bp 5669 to 5701 is shown. The deduced amino acid sequence corresponds to residues 78 to 88 of 3C^{pro}. For each particular mutant plasmid, the changes introduced at the DNA level are given. Some plasmids have additional but silent mutations that created new restriction sites used for cloning.

^c As estimated from the patterns of the processed polyproteins after in vitro translation; see Fig. 2. Note that mutants pTH/3C^{pro}K78Q and pTH/3C^{pro}R84S exhibit slightly less production of 3C' and 3D', as estimated by scanning (see text).

^d Yes or no means there was or was no recovery, respectively, of virus after transfection of HeLa cells with RNA made from a particular plasmid. For details, see Materials and Methods. Additionally, temperature sensitivity was checked by incubating cells after transfection at either 39°C (viable mutants) or 32°C (nonviable mutants). No temperature sensitivity was detected.

^e Virus recovered had an A-to-G transition at bp 5682 resulting in a replacement of glutamine by arginine at residue 82 of 3C^{pro}.

^f Both viruses recovered had an A-to-G transition (at bp 5690 in pTH/3C^{pro}D85N and 5697 in pTH/3C^{pro}R87K), restoring the wild-type residue. In both cases, virus could only be recovered after prolonged incubation of transfected cells (>72 h) when the supernatant of cell debris became infectious (see Materials and Methods). With respect to the other lethal mutations, this prolonged incubation did not result in recovery of virus.

cells transfected with wild-type RNA resulted in the appearance of a band with the expected size which depended on the presence or absence of reverse transcriptase (Fig. 3, lanes 3 and 2, respectively). No band was obtained with the Arg-84-Ser RNA, an observation suggesting that this RNA is unable to replicate (Fig. 3, lane 4).

Finally, we investigated whether the mutants that produced viable virus had retained their genotype. For this purpose, virus stocks were grown as described above. RNAs were isolated and sequenced. Viruses derived from plasmids pTH/3C^{pro}K78Q, pTH/3C^{pro}R79Q, and pTH/3C^{pro}E81S exhibited the particular mutation introduced at DNA level (sequences not shown). Furthermore, these mutant viruses showed a plaque morphology similar to that of wild type (Fig. 4A, plates 1 to 4). Virus recovered after transfection with RNA derived from plasmids pTH/3C^{pro}D85N and pTH/3C^{pro}R87K had an A-to-G transition at bp 5690 and 5697, respectively. These changes restored the wild-type amino acid, and concomitantly, the plaque morphology of these isolates was wild type (Fig. 4A, plates 5 and 6).

A different situation was found with pTH/3C^{pro}K82Q. The first stock of the mutant virus showed mixed plaque sizes (Fig. 4A, plate 8), whereas on expansion, all plaques were of near-wild-type size (Fig. 4A, plate 7). Direct sequencing of RNA isolated from virus from plate 8 was unsuccessful, due to compression of bands precisely at the mutated site. However, the sequence of this isolate was obtained from two independently derived PCR fragments, and it showed an A-to-G transition changing the presumptive Gln residue at position 82 to an arginine (Fig. 4B). We therefore conclude that the virus isolated from plate 8 is a pseudorevertant. Three different isolates, after independent transfections with RNA of pTH/3C^{pro}K82Q, showed an Arg residue at position 82, an observation suggesting that there is strong selective pressure to restore a positive charge at this site.

Figure 5 summarizes our results. Bold-face capital letters mark residues that strongly affect virus viability when mutated. Residue Lys-82 has been included in the segment of amino acids because a glutamine residue introduced at this position resulted in a transient small/mixed-plaque phenotype and exerted a strong selective pressure to restore a positive charge. Residues that might be determinants of a function(s) beside proteolytic activity and that are involved in 3CD and P1 cleavage are marked by arrows. Residues that could be changed without any apparent effect are shown in lowercase letters.

DISCUSSION

The experiments presented above were performed to shed light on the function of the proposed FG loop of 3C^{pro} which contains a sequence of amino acids (KRNEKFRDIR) that is highly conserved in 3C^{pro} of enteroviruses and rhinoviruses. We took advantage of the availability of an in vitro translation system of poliovirus RNA developed recently that offered an opportunity to analyze effects of mutations in full-length cDNA clones on proteolytic processing and to compare this with the viability of the construct.

Residues Phe-83, Asp-85, and Arg-87 seem to be necessary for proper cleavage of 3CD and, to a lesser extent, of the P1 precursor. The effects of replacement of these sites on virus viability can be explained by an insufficient amount of active polymerase owing to the fact that 3D has polymerase activity while 3CD does not (reviewed in reference 24).

A positive charge at position 87 seems to be essential for virus viability. However, lysine was not an adequate substitute since, in several experiments, virus recovered after prolonged incubation of the transfected cells always had the wild-type arginine restored. Therefore, characteristics of the side chain of arginine other than the positive charge must

FIG. 2. In vitro translation and proteolytic processing of full-length transcripts. cDNA clones harboring wild-type or mutant sequences were transcribed with T7 RNA polymerase. RNAs were translated in vitro with a HeLa cell extract and analyzed by gel electrophoresis. Fluorograms of parallel translations are shown in panels A and B. A lysate of HeLa cells infected with poliovirus type 1 (Mahoney), labeled with [35 S]methionine, was used as a marker. At the top of each lane, the particular mutation in 3C^{pro} is indicated. Additionally, RNA derived from pT7XLD/HpaI was translated (panel A, lane 3). +, virus recovered; -, no virus recovered; ·, only revertants recovered after transfection with full-length transcripts. For further details, see Materials and Methods.

play an important role. With respect to Asp-85, our data confirm the observation that substitution of this residue by glutamate resulted in an impairment of 3CD processing accompanied by a small-plaque phenotype (17). This mutation also impaired the processing of P2 to a lesser extent. The observed difference may be explained by the residue introduced. We also confirm that there is a high selective pressure to restore an aspartate at position 85 of 3C^{pro} (17) as indicated by the reversion found in virus derived from plasmid pTH/3C^{pro}D85N. This reversion was not seen when two bases of the codon were changed to yield a serine in position 85. Unexpectedly, this mutation caused a stronger

effect on the processing pattern than the Asp-85-Asn change, as indicated by the large amount of unprocessed P1 precursor observed in cell-free translation. We do not know at present why no revertants were recovered after transfection of RNA derived from plasmid pTH/3C^{pro}D85S, but the effect on processing must have been augmented by inhibition of RNA replication and the requirement for double substitutions in codon 85 of this 3C^{pro} mutant to regain the wild-type amino acid.

Polypeptide 3CD has been implicated in the processing of the capsid precursor P1 (14, 23, 28). The surprising lack of P1 processing of the Asp-85-Ser mutant indicates either that Asp-85 is directly involved in 3CD-P1 interaction or that a serine in this position distorts the structure of the loop or the entire 3CD moiety such that 3CD can no longer recognize and process its substrate P1. This indicates that the size of the residue at position 85 is probably important. A similar consideration holds for the Phe-83-Ala mutation.

In vitro translation of RNAs containing Asp-85-Asn, Arg-87-Gln, and Arg-87-Lys mutations in 3C^{pro} also show that the amount of free 3C^{pro} can be substantially reduced without severe impairment of the overall processing patterns. A similar phenomenon was observed by others. For example, exchange of Val-54 for alanine and Ile-74 for threonine also inhibited 3CD cleavage (6, 16). The latter diminished to some extent processing of the P2 precursor, also.

Mutations of Lys-78-Gln, Arg-79-Gln, and Glu-81-Ser neither affected the in vitro processing pattern nor influenced virus viability. Although the viruses recovered after trans-

FIG. 3. Detection of minus-strand RNA synthesis by reverse transcription and PCR. A Polaroid photograph of the reaction products is shown after agarose gel electrophoresis and staining with ethidium bromide. Lanes: 1, PHI-X DNA digested with *BstEII*; 2 and 3, cytoplasmic RNA of cells transfected with RNA derived from pTH/3C^{pro}wt without and with reverse transcriptase added, respectively; 4 and 5, cytoplasmic RNA of cells transfected with RNA derived from pTH/3C^{pro}R84S and of mock-infected cells, respectively, and processed as in lane 2; 6, plasmid DNA pTH/3C^{pro}E81S used for PCR. The numbers at the left give the lengths of some fragments of the PHI-X marker.

FIG. 4. Plaque morphologies of wild-type and mutant viruses and sequence of virus derived from plasmid pTH/3C^{pro}K82Q. (A) Plaque morphologies of stocks of wild-type virus derived from plasmid pTH/3C^{pro}wt (plate 1) as well as of mutant viruses derived from plasmids pTH/3C^{pro}K78Q (plate 2), pTH/3C^{pro}R79Q (plate 3), pTH/3C^{pro}E81S (plate 4), pTH/3C^{pro}D85N (plate 5), and pTH/3C^{pro}R87K (plate 6). In addition, the plaque morphologies of the first stock of virus derived from pTH/3C^{pro}K82Q (plate 8) and the virus after expansion (plate 7) are shown. Note that viruses have retained the particular mutation, with the exception of viruses derived from pTH/3C^{pro}D85N and pTH/3C^{pro}R87K, which had restored the wild-type residue, and virus derived from pTH/3C^{pro}K82Q. (B) Sequencing ladder derived from RNA of this virus. This ladder was generated by sequencing a subcloned PCR product derived from RNA. The nucleotide sequence from codons 80 to 84 of 3C^{pro} and the deduced amino acid sequence are given at the right. Codon 82 apparently has an A-to-G transition resulting in a exchange of Gln-82 for arginine.

fection had retained the mutant genotypes, we cannot exclude the possibility that second-site mutations have occurred. This is unlikely, however, since cell lysis of the transfected monolayers occurred at the same time as seen in wild-type transfections and because the isolated viruses showed uniformly wild-type plaque morphology in the first passage.

An intriguing result is that replacement of residues Arg-84 and Ile-86 abolished viral viability without an apparent effect on proteolytic processing. The Lys-82-Gln mutation falls into the same category. The difference between the mutations in Arg-84 and Ile-86, on the one hand, and the Lys-82 mutation, on the other hand, is that the latter must have permitted RNA replication because revertants to the wild-type phenotype were readily isolated. Altogether, these findings are consistent with the proposal that 3C^{pro} has determinants for a function(s) distinct from its proteolytic activity, most likely in RNA replication (1, 2).

Figure 5 summarizes our results. Residues that are impor-

FIG. 5. Summary of results. Residues that could be replaced without any apparent effect on virus viability are shown in lower-case letters. Boldface capital letters mark residues that strongly affected virus viability when mutated. Arrows point to residues that are involved in processing of 3CD and the P1 precursor and that might be determinants of a function(s) of 3C^{pro} distinct from proteolytic activity. Note the periodicity of 2.

tant for virus viability are given in bold-face capital letters. Residues Lys-82, Arg-84, and Ile-86 might be determinants of a function of 3C^{pro} distinct from proteolytic processing, which supposedly is a protein-RNA interaction during initiation of plus-strand synthesis (1, 2). It has been suggested recently that arginine residues are specifically involved in protein-RNA interaction as deduced from the interaction of a synthetic peptide with an RNA stem-loop structure of human immunodeficiency virus (4). Arg-84 in 3C^{pro} or 3CD may therefore be involved in RNA recognition.

Finally, we want to emphasize the observed periodicity of two of the residues involved in 3CD cleavage and those that might be involved in a second function. This suggests a functional relationship that remains to be elucidated. Furthermore, if this functionally determined periodicity is reflecting a structural element, it is likely that these amino acids are part of an extended β -strand, at least in 3CD or other larger precursors of 3C^{pro}. This arrangement might be a prerequisite for interaction with residues of one of the conserved motifs that have been recently detected in RNA polymerases of plus-strand RNA viruses in order to ensure proper cleavage of 3CD (18). Future experiments will focus on the detailed analysis of the structural prerequisites, the molecular events leading to cleavage of 3CD, and their implications for the structure of 3C^{pro} as a single protein.

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