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Identification and Characterization of a 3C-Like Protease from Rabbit Hemorrhagic Disease Virus, a Calicivirus

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Expression studies conducted in vitro and in *Escherichia coli* led to the identification of a protease from rabbit hemorrhagic disease virus (RHDV). The gene coding for this protease was found to be located in the central part of the genome preceding the putative RNA polymerase gene. It was demonstrated that the protease specifically cuts RHDV polyprotein substrates both in *cis* and in *trans*. Site-directed mutagenesis experiments revealed that the RHDV protease closely resembles the 3C proteases of picornaviruses with respect to the amino acids directly involved in the catalytic activity as well as to the role played by histidine as part of the substrate binding pocket.

Rabbit hemorrhagic disease virus (RHDV) has been recently assigned to the *Caliciviridae* family (35, 38). The 7,437-nucleotide (nt) viral genome consists of single-stranded RNA of positive polarity and is packaged in a small icosahedral capsid of about 40 nm in diameter; in addition to the genomic RNA, an abundant subgenomic RNA of 2.2 kb in length, which is coterminal with the 3′ end of the viral genome, was detected in livers of infected animals and in purified viral particles (32, 33). The main structural component of RHDV is a single protein species of 60 kDa (VP60).

Because of the failure to grow the virus in tissue culture, studies on RHDV have relied on the experimental infection of rabbits and on in vitro methods. The genomic RNA of RHDV has been cloned as cDNA and completely sequenced. It contains a long open reading frame (ORF1) which spans 7 kb of the genome and has a coding capacity for a hypothetical polyprotein of 257 kDa (32). According to the results from sequence analysis studies and mapping experiments, the genome organization of RHDV is similar to that of other caliciviruses characterized to date (6, 20). The nonstructural genes are clustered in the 5′ region, followed by the gene for the capsid protein in the 3′ region. It is noteworthy that in the case of RHDV, ORF1 contains the nonstructural and the capsid protein genes, while in other caliciviruses, the capsid protein is encoded by a separate ORF. For RHDV, it is therefore possible that the synthesis of VP60 follows two distinct pathways: one through processing of the polyprotein precursor translated from the genomic RNA, the second by translation of the subgenomic RNA (33, 37).

As for other positive-strand RNA viruses, e.g., picornaviruses, the generation of mature RHDV proteins requires that proteolytic cleavages be effected at specific sites of the large polyprotein translated from the viral genomic RNA. For nonenveloped positive-strand RNA viruses, polyprotein processing is usually carried out by viral proteases. Computer analysis of the RHDV sequence showed that a region located immediately upstream of the putative RNA polymerase in the RHDV polyprotein may correspond to the 3C proteases of picornaviruses (32). Similar propositions were made for feline calicivirus (FCV) and Norwalk virus (6, 20, 34).

Picornavirus 3C proteases belong to a large family of trypsin-like enzymes which, according to computer predictions, share a common three-dimensional structure and a few well-conserved residues. Among these, the so-called catalytic triad, which for trypsin comprises serine as the nucleophilic group, is a common signature of the family (4). In many viral proteases, the role of serine is taken over by a cysteine residue. In the present study, in vitro transcription and translation experiments, as well as bacterial expression studies, were used to identify and characterize the RHDV protease.

MATERIALS AND METHODS

Virus purification and RNA extraction. The cDNA fragments used in this work were cloned from two independent RHDV isolates: a German isolate and an Italian one (RHDV BS89). Both isolates were completely sequenced (32, 38a).

RHDV particles were obtained from liver homogenates of experimentally infected rabbits and purified as previously described (32). Viral RNA for in vitro transcription was extracted from intact purified virions treated with micrococcal nuclease (20 min at room temperature [RT]) to remove contaminating cellular nucleic acids; micrococcal nuclease was then blocked by addition of EGTA (20 mM), and proteins were digested by incubation for 30 min at 50°C with proteinase K (150 μg/ml; Boehringer) in the presence of 0.5% sodium dodecyl sulfate (SDS). Viral RNA was extracted twice with phenol and phenol-chloroform and concentrated by ethanol precipitation (39).

Subcloning and sequencing. The RHDV cDNA plasmid clones were obtained by in vivo excision from lambda ZAPII (Stratagene) clones, isolated by hybridization to a ^{32}P-labeled cDNA probe or by expression screening with anti-RHDV monoclonal antibodies (32, 38a) as described by Sambrook et al. (39). Fractionation of restriction enzyme-digested DNA on agarose gels, DNA purification from agarose slices with a Gene Clean kit (Bio 101), and ligation into pBS KSII (Stratagene) followed by transformation of frozen *Escherichia coli* XL1-Blue competent cells (39) were performed to generate the desired subclones; in some cases, they were obtained by direct self-ligation of restriction enzyme-digested plasmid.

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Alkali-denatured plasmid DNA was sequenced by the chain termination method, using [^{35}S]dATP (Amersham) and Sequenase DNA polymerase (U.S. Biochemical) or modified T7 DNA polymerase (Pharmacia) as instructed by the manufacturer. Note that the numbering of the RHDV nucleotide and amino acid sequence throughout this report is consistent with the complete cDNA sequence (32). This also applies to the designation of restriction sites used for subcloning of cDNA into expression vectors. In those instances in which cDNA from the Italian RHDV isolate was used, some of the restriction sites may not correspond to those of the German isolate because of some sequence polymorphism between the two isolates. Any significant difference at the amino acid level is pointed out in the text.

Plasmids construction. Plasmid p672 contains an RHDV cDNA insert of 4,391 bp from the *Eco*RI site at nt 3077 to the poly(A) tract. In this insert, the first AUG is at nt 3097 to 3099 and the corresponding ORF is terminated at nt 7042 to 7044, resulting in a polypeptide of 1,315 amino acids (aa) with a calculated molecular mass of 142 kDa.

Plasmids pP7 and pP20 were subcloned from p672 by *Apa*I (position 4142) digestion followed by self-ligation (pP7) and by *Eco*RI-*Apa*I double digestion, purification of the resulting 1,065-bp fragment, and ligation into pBS-KS (Stratagene). Plasmid pMB11 was generated by digestion of p672 at the *Bgl*II site (position 4955), fill-in in the presence of Klenow polymerase (39), further digestion at the *Xho*I site present in the p672 polylinker, and insertion of the gel-purified fragment (2,532 bp) into pBS KS cut with *Eco*RV and *Xho*I. Blunt-end ligation of the *Eco*RV and the modified *Bgl*II ends creates the first ATG codon of an ORF terminating at the TGA codon at position 7042.

The pEX34B expression plasmid is a pBR322 derivative that contains the amino terminus of the MS2 polymerase driven by the leftward promoter of the lambda phage (32, 42). The protease expression plasmid pEX01 was obtained by insertion of an *Eco*RI (3077)-*Xba*I (4123) RHDV cDNA fragment that covers amino acids 1023 to 1371 into pEX34B cut with *Eco*RI and *Xba*I. Plasmid pMAL-PRT1 contained an *Eco*RI (3077)-*Sma*I (3875) RHDV cDNA fragment that covers aa 1023 to 1288 inserted via *Eco*RI linkers into the pMAL-cRI vector (New England Biolabs) linearized by *Eco*RI.

In vitro transcription and translation. In vitro transcription with either T3 or T7 RNA polymerase was carried out as specified by the manufacturer (Stratagene), using 1 μg of plasmid DNA linearized by the appropriate restriction enzyme (*Xho*I in the cases of p672, pP7, pP20, and pMB11). In some experiments, capping of the nascent RNA chain was obtained by inclusion of P1-5′-(7-methyl)-guanosine-P3-5′-guanosine triphosphate (Boehringer) in the transcription mixture.

All clones yielded RNAs of the expected sizes; the amount of each RNA added to the translation mixture was normalized to give equimolar quantities. In vitro translation was in a volume of 25 μl, containing 12.5 μl of rabbit reticulocyte lysate (Promega), 2.5 μl of [^{35}S]methionine at 10 μCi/μl (Amersham), and variable amounts (20 to 100 ng) of template RNA. The reaction mix was incubated for 60 min at 30°C and subjected to immunoprecipitation or loaded directly on SDS-polyacrylamide gels for analysis.

Immunoprecipitation. Aliquots of the in vitro translation samples were incubated for 1 h at RT with 1 to 5 μl of anti-VP60 monoclonal antibodies (immunoglobulin G concentration, 1 mg/ml) or polyclonal rabbit serum from animals that survived RHDV infection. The protein-antibody complexes were bound to protein G-Sepharose beads (Pharmacia) by incubation for 60 min with gentle agitation, in the presence of IB buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.2% Nonidet P-40, 0.1% gelatin), and washed repeatedly with IB buffer plus 250 mM NaCl. In some cases, all incubation times were doubled and the reactions were carried out at 4°C, with no significant changes in the final results. Immunocomplexes were released by boiling for 10 min in the presence of 12 μl of 1× gel loading dye (62.5 mM Tris-HCl [pH 6.8], 1.5% SDS, 2% β-mercaptoethanol, 7% glycerol, 0.007% bromophenol blue).

Expression in *E. coli*. Cells were grown to plateau by overnight incubation at 28°C in LB medium. The next morning, the culture was diluted fivefold in prewarmed (42°C) fresh medium and further incubated for 2.5 h with continuous shaking. Bacterial cells were collected by centrifugation and washed in 100 mM NaCl–50 mM Tris-HCl (pH 8.0). The cellular pellet was resuspended in a solution (1/100 of the volume of the bacterial culture) containing 10% sucrose, 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, and 2 mg of lysozyme per ml. After incubation for 30 min at 37°C, 2 volumes of lysis mix (50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA [pH 8.0], 5% Triton X-100) was added, and the mixture was further incubated for 15 min at 0°C followed by 30 min at 37°C. Cells were finally broken by sonification in a Branson instrument. The lysate was subjected to cycles of centrifugation (20,000 × *g*) and resuspension of the pellets in increasing urea concentrations (1 and 7 M), achieving a progressive purification of the recombinant proteins which were recovered from the last pellet resuspended in 7 M urea.

Expression from plasmid pMAL-PRT1 was induced by the addition of 0.3 mM isopropythiogalactopyranoside (IPTG) to logarithmic cultures of *E. coli* XL1-Blue cells followed by incubation for 2 h at 37°C. Cells were harvested by centrifugation, washed once in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and disrupted by sonication. After three rounds of sonication for 30 s at amplitude 20 with an MSE Soniprep 150, bacterial debris was removed by centrifugation in a Kontron A8.24 rotor at 9,000 rpm for 10 min. The bacterial lysates were then subjected to ultracentrifugation (100S), and the supernatant was assayed for the presence of protease activity.

Site-directed mutagenesis. Sequential PCRs with the mutagenic oligonucleotide primers 5′-CGGTGACGGTGGGCT GCCGT-3′ (direct) and 5′-CAGCCCACCGTCACCGTGA GT-3′ (reverse) were used as described by Cormack (9) to introduce the C1212G substitution. All other mutants were constructed by the method of Kunkel et al. (25), using a Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad) as described by the manufacturer. The template DNA for the mutagenesis, uracil-containing single-stranded DNA, was obtained by superinfection of *E. coli* CJ236 (*dut ung*) carrying plasmid pCW551 or pCW552 with the filamentous phage VCS M13 (Stratagene). Plasmid pCW551 contains a *Hin*dIII (2978)-*Hin*dIII (4002) cDNA fragment which covers the complete coding region of the protease domain, inserted into the *Hin*dIII site of pTZ18U. Plasmid pCW552 contains the same cDNA fragment in the opposite orientation. Oligonucleotide primers specifying single or double base changes were used in the mutagenesis reactions at each site as follows: H1135N (5′-TCCAACACTAATACCGCCAGG-3′), C1212S (5′-ACG GGGACAGTGGGCT-3′), D1152N (5′-CACCACTAACCT GTGC-3′), D1152E and D1152G (5′-ACCCACCACT[G,A]G CTCTGCCTCGTCAAGGGTGA-3′), D1175N (5′-AGTTTG TAACTGGAAGA-3′), D1175E and D1175G (5′-CCCAGTT TGTG[G,A]GTGGAAGAAGTCTCC-3′), H1227N (5′-GTT GCCATAAACACTGGCA-3′), and H1227L (5′-TGCCATA CTCACTGGCA-3′). Nucleotides specifying base changes are underlined. A *Sac*I site was introduced with the oligonucleo-

tide used to construct the D1152E mutant. Approximately 100 ng of single-stranded DNA was mixed with 6 pmol of phosphorylated oligonucleotide and 1 μl of 10× AB (200 mM Tris-HCl [pH 7.4], 20 mM $MgCl_2$, 500 mM NaCl) in a total volume of 10 μl. The reaction mixture was heated to 65°C for 5 min and then slowly cooled to RT for 45 min. One microliter of 10× SB (300 mM Tris-HCl [pH 8.0], 80 mM $MgCl_2$, 30 mM dithiothreitol, 200 mM NaCl), 4 μl of 2.5 mM deoxynucleoside triphosphates, 1 μl of 10 mM ATP, 3 U of T4 DNA ligase, and 1.5 U of T4 DNA polymerase were added to the annealing reaction mixture, and the volume was adjusted to 20 μl with water. The reaction mixture was incubated for 5 min at 0°C, 5 min at 20°C, and 90 min at 37°C. Nine microliters of stop solution (100 mM Tris-HCl, 100 mM EDTA [pH 8.0] was added, and 15 μl of the mixture was used to transform *E. coli* XL1-Blue cells. Bacteria containing mutant plasmids were identified by DNA sequencing. The appropriate restriction fragments were then subcloned into plasmid pEX01 to yield the mutated expression plasmids. All of the subcloned fragments were sequenced to verify the presence of the desired mutation and the absence of second-site mutations.

Analysis of the protease mutants. Samples of the 7 M urea fractions from inclusion bodies of *E. coli* cells harboring the wild-type (pEX01) or mutant expression plasmids were mixed with loading buffer (62.5 mM Tris-HCl [pH 6.8], 6 M urea, 2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.02% phenol red), boiled for 5 min, and separated on an SDS–12% polyacrylamide gel (26). Each sample contained about 7 μg of protein except for the extract from *E. coli* 537 cells without expression plasmid, which contained 1.5 μg of protein. After electrophoresis, the gel was stained with Coomassie blue (0.25% coomassie blue G250, 50% methanol, 10% acetic acid) for 3 h and destained in 30% methanol–7% acetic acid. Alternatively, electrophoretic transfer to nitrocellulose (Schleicher & Schuell) was performed as described by Towbin et al. (43) for 12 h at 4°C and 400-mA constant current in a Hoefer Transphor apparatus. The nitrocellulose was incubated over night at RT in phosphate-buffered saline (PBS) containing 2.5% low-fat milk powder and 0.02% sodium azide. The next day, rabbit antiserum was added at a dilution of 1:10,000 and allowed to bind for 1 h at RT. The membrane was washed three times with PBS containing 0.05% Tween 20 (PBST), and horseradish peroxidase-conjugated anti-rabbit antibody (Dianova, Hamburg, Germany) was added at a dilution of 1:50,000. After 1 h at RT, the nitrocellulose was washed three times with PBST and incubated with ECL substrate as instructed by the supplier (Amersham). The membrane was then exposed to Kodak XAR5omat film to detect specific binding.

Alignment of amino acid sequences. The amino acid sequences of the protease domains of RHDV (32 [accession number M67473]), FCV (6 [accession number M86379]), Norwalk virus (20 [accession number M87661]), and Southampton virus (27 [accession number L07418]) and the sequence of the mature 3C proteases of poliovirus type 2 (28 [accession number M12197]) and human rhinovirus 14 (41 [accession numbers K02121 and X01087]) were aligned with the program Pileup from release 7.3 of the University of Wisconsin Genetics Computer Group software package (11). The program was run with a gap weight setting of 2 and a gap length weight setting of 0.1. The sequence of Southampton virus (27) was deleted from the final alignment, and the glycine residues at positions 148 and 149, respectively, in the rhinovirus 14 and poliovirus type 2 sequences (the second glycine after the cysteine of the catalytic triad) were shifted one position to the left in accordance with the alignment of Gorbalenya et al. (14).

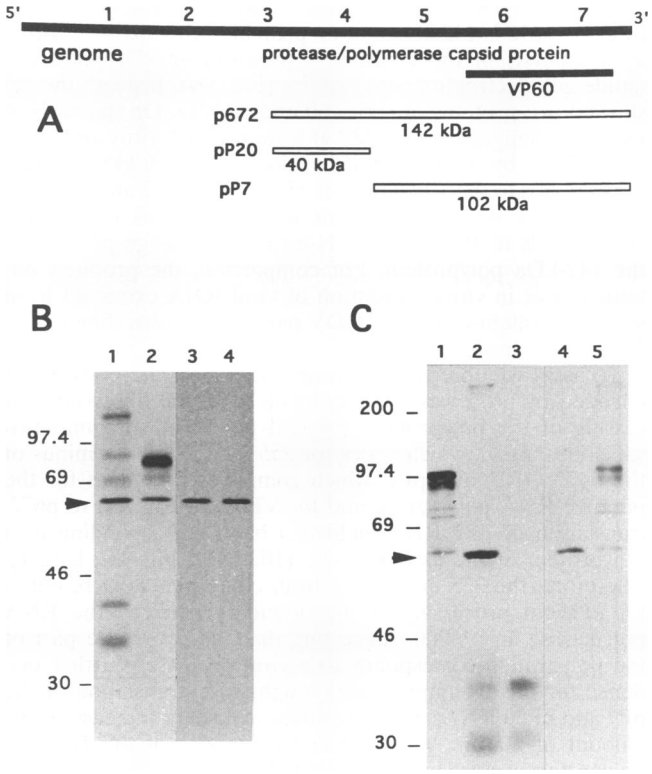

FIG. 1. In vitro translation. (A) Diagram of the RHDV genome (numbers indicate the size in kilobases) showing the positions of the protease, RNA polymerase, and capsid protein (VP60) genes; the capsid protein is also encoded by a subgenomic RNA. The slash between the protease and polymerase genes indicates that the boundary between them has not been determined. The clones used in this experiment are aligned to the RHDV genome according to the cDNA insert that they contain; the molecular sizes of the corresponding polypeptides are also shown. (B) Translation products of purified viral RNA before (lane 1) and after (lane 3) immunoprecipitation with an anti-VP60 monoclonal antibody. Lanes 2 and 4, p672 RNA translation products before and after immunoprecipitation, respectively. (C) Translation products of clone pP7 (lane 1) and pP20 (lane 3) and of cotranslation of pP7 and pP20 (lane 2). Lanes 4 and 5, immunoprecipitation by an anti-VP60 monoclonal antibody of the proteins from the translation mixtures shown in lanes 2 and 1, respectively. The sizes in kilodaltons of molecular weight markers are indicated. The arrowheads point to the positions of the capsid protein VP60.

RESULTS

Autocatalytic cleavage of the RHDV polyprotein. Computer analysis of the RHDV ORF1 sequence revealed that a region of the polyprotein adjacent to the putative RNA polymerase gene has a limited degree of homology to known proteases of other positive-strand RNA viruses (32). To demonstrate that a functional protease is encoded in the respective genomic region, in vitro translation experiments with RNAs transcribed in vitro from RHDV cDNA clones were performed.

A representative result obtained after translation of such RNAs is shown in Fig. 1. Transcription by T7 RNA polymerase of clone p672 linearized by digestion with *Xho*I yielded an RNA molecule coding for a polypeptide of 1,315 residues starting at aa 1030 of the RHDV ORF1 and extending to the stop codon of ORF1. The polypeptide includes the putative RNA polymerase and the complete capsid protein VP60 (Fig. 1A).

The calculated molecular mass of the polyprotein encoded by clone p672 is 142 kDa, but no protein of this size was visible after analysis of the translation products by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1B). Instead, the gel showed two proteins of about 60 and 80 kDa. On the basis of its exact comigration with VP60 from purified virus and of its recognition by a monoclonal antibody, the 60-kDa protein corresponds to the VP60 protein (Fig. 1B, lanes 2 and 4) (38a). The protein with an apparent molecular mass of 80 kDa corresponds to the expected N-terminal cleavage product of the 142-kDa polyprotein. For comparison, the products obtained after in vitro translation of viral RNA extracted from sucrose gradient-purified RHDV particles are also shown (Fig. 1B, lanes 1 and 3).

To assay whether the N-terminal region of the polyprotein encoded by p672 was actually involved in the observed processing of the polyprotein, plasmid p672 was split into two subclones: pP20, which codes for 355 aa at the N terminus of the p672 ORF, and pP7, which comprises the genes for the putative RNA polymerase and for VP60. In contrast to p672, translation of pP7 RNA yielded a band corresponding to a polyprotein of the expected size (102 kDa; Fig. 1C, lane 1). Therefore, the 355 aa missing from clone pP7 were required for efficient processing at the boundary between the RNA polymerase and VP60, suggesting that the respective part of the polyprotein corresponds to a viral protease. Further evidence for this assumption was sought by cotranslation of the pP7 and pP20 RNAs, which resulted in a sharp increase of the amount of mature VP60, while the amount of the 102-kDa polyprotein was reduced in parallel (Fig. 1C, lane 2). Additional bands are visible in Fig. 1C; those present in samples containing the pP7 RNA translation products (lanes 1 and 5) are probably due to a low degree of initiation from internal AUG codons, a common phenomenon during in vitro translation. Translation of the pP20 RNA (lane 3) yielded a protein with the expected molecular mass of 40 kDa and an additional 30-kDa band, probably a product of processing at the N terminus of the protease domain (see also experiments below). The 40-kDa band visible in the cotranslation sample (lane 2) may represent the polypeptide encoded by pP20 or the putative N-terminal cleavage product (42 kDa) of the 102-kDa protein from pP7, or both. However, the latter must be unstable because of the discrepancy in the intensity of the VP60 and the 40-kDa bands. Identical results were obtained by independent in vitro translation of pP7 and pP20 followed by mixing of the translation products and further incubation for 60 min at 30°C (data not shown).

Taken together, the pattern observed after expression of the p672 RNA and the ability of the pP20 translation product to cleave the polyprotein when expressed as a separate polypeptide indicate that proteolysis occurs via an RHDV-encoded enzyme which is able to cut substrates in *trans*. Cleavage of the p672 polyprotein is extremely rapid, especially in comparison with substrates that do not include the protease domain (data not shown), suggesting that the protease is functional in *cis* as well. For final proof of autocatalytic cleavage, dilution-independent proteolysis will have to be demonstrated.

Expression of the RHDV protease in *E. coli*. A different line of work was aimed at the expression of the putative protease in *E. coli*. In-frame insertion of foreign sequences in plasmid pEX34B normally result in synthesis of a fusion protein in which the N-terminal domain is constituted by an 11-kDa fragment of the bacteriophage MS2 RNA polymerase. Plasmid pEX01, a pEX34B derivative containing a fragment of the RHDV cDNA from nt 3077 to 4123 (Fig. 2A), was transfected in *E. coli*, and its expression was induced by a temperature shift. Instead of the expected fusion protein with a calculated molecular mass of 50.9 kDa, two proteins of about 21 and 30 kDa were found in the 7 M urea fraction (Fig. 2B, lane 3). Since the molecular masses of the two proteins account for the size of the 50.9-kDa fusion protein, Western blot (immunoblot) experiments were performed to confirm their identity. The 21-kDa polypeptide is the N-terminal fragment, as demonstrated by specific reaction with an anti-MS2 polymerase serum (Fig. 2B, lanes 1 and 2). In lane 2, a faint band of about 38 kDa is also visible and may constitute an alternative cleavage product of the 50.9-kDa protein; it was not further studied because of its very low amount. The reactivity of the 30-kDa product with a polyclonal serum raised against a polypeptide corresponding to the RHDV ORF1 from aa 1172 to 1331 indicated that it derives from the C-terminal region of the pEX01 construct (data not shown). Taking into account the fact that the pEX01 insert includes the entire cDNA sequence of pP20, which could restore processing of the RHDV polyprotein after in vitro translation, one can conclude that the 30-kDa polypeptide contains the protease domain and is sufficient for specific proteolytic cleavage.

The ability of the bacterially expressed protease to specifically cut polyprotein substrates in *trans* was determined by using crude lysates of *E. coli* XL1-Blue cells harboring plasmid pMAL-PRT1 (Fig. 2A) as the source of proteolytic activity. This expression system yielded about 60% of the protease in a soluble form, as demonstrated by Western blotting of the cytoplasmic and insoluble fractions after cell lysis (data not shown). The substrate for the protease was synthesized from pMB11 (Fig. 2A), a plasmid containing a portion of the RHDV ORF1 from amino acids 1650 to 2344 that includes 116 C-terminal amino acids of the putative RNA polymerase and the entire VP60 sequence but no protease domain. The in vitro-labeled 73.7-kDa precursor from pMB11 was cut at the junction between the RNA polymerase and the capsid protein only upon addition of the bacterial lysate expressing the protease (Fig. 2C). As a result of this cut, the precursor band disappeared and a band corresponding to VP60 became detectable (Fig. 2C, lane 2); the other product of the proteolytic reaction, an N-terminal peptide of about 13 kDa, was also detected (data not shown). Processing of the 73.7-kDa protein was not due to experimental artifacts, as demonstrated by incubation with extract of bacteria (Fig. 2C, lane 3) that harbor a pMAL-PRT1 expressing an inactive protease (by mutation of Cys-1212 to Gly; see below).

It should be noted that the processing observed after expression of pEX01 occurred at the N terminus of the protease domain, while the in vitro translation experiments as well as cleavage in *trans* by the pMAL-PRT1 product demonstrated cleavage at the boundary between the putative RNA polymerase and VP60.

Mutagenesis of the RHDV protease. Although scarcely similar with respect to primary sequence, the large family of trypsin-like proteases, to which picornavirus 3C proteases belong, can be modeled to reveal common structural features (3, 14). Four similarity boxes have been identified in these proteins (4) (Fig. 3); the amino acids constituting the so-called catalytic triad, in the case of picornavirus proteases His, Asp/Glu, and Cys, are distributed in the first three boxes, while the fourth box is thought to participate in the structure of the substrate binding pocket. The key role of these amino acids has been demonstrated in several instances by mutagenesis studies (8, 15, 22). The sequence of the RHDV protease region was aligned with the putative protease sequences of two caliciviruses (FCV and Norwalk virus) and with two well-characterized proteases from poliovirus and human rhinovirus (Fig. 3).

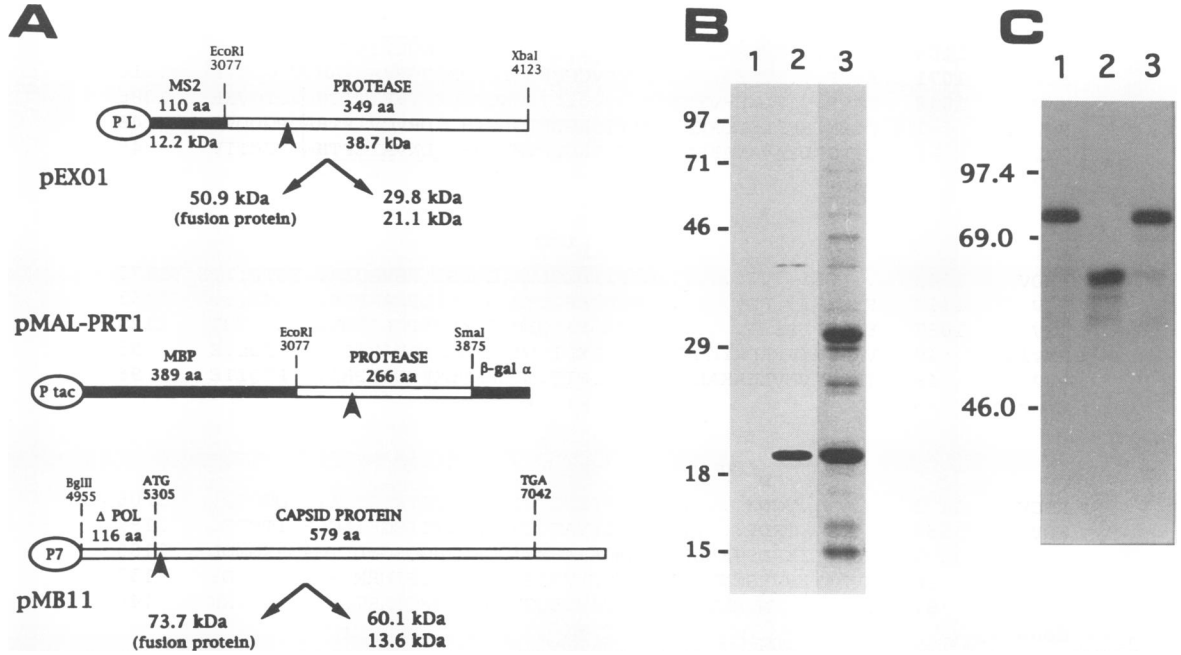

FIG. 2. (A) Diagram of the plasmids used for expression of the RHDV protease in *E. coli* (pEX01; pMAL-PRT1) and for in vitro translation of a labeled polyprotein substrate (pMB11). Open bars, RHDV cDNA fragments; filled bars, vector sequences. Some restriction sites used for subcloning and their corresponding positions on the RHDV sequence (32) are shown. In plasmid pEX01, transcription from the lambda left promoter (PL) directs the synthesis of a fusion protein comprising 110 aa from vector sequences and 349 aa (38.7 kDa) from the RHDV ORF1. In plasmid pMAL-PRT1, the fusion protein includes 389 residues of the maltose-binding protein (MBP), 266 residues from the RHDV ORF1, and a 9-kDa α fragment of β-galactosidase (β-gal); in this case, transcription starts from an hybrid tryptophan-*lacZ* promoter (P tac). Plasmid pMB11 codes for a 73.7-kDa polyprotein spanning the carboxy-terminal part of the RHDV RNA polymerase and the entire capsid protein gene from nt 5305 to 5307 (corresponding to the translation start codon of the subgenomic RNA) to the stop codon at nt 7042 to 7044 of the RHDV cDNA sequence. In vitro transcription is driven by the T7 promoter; translation starts at an AUG codon near the *Bgl*II site. The arrowheads point to the approximate positions of the protease target sites. The sizes of the expected fusion protein and of the products after proteolytic cuts are also reported for pEX01 and pMB11. (B) Seven molar urea protein fraction from bacteria harboring plasmid pEX01. Lanes 1 and 2, Western blot with anti-MS2 polyclonal serum from uninduced bacteria (lane 1) and from bacteria induced by a temperature shift to 42°C (lane 2); lane 3, same as lane 2 but stained by Coomassie blue. The sizes (in kilodaltons) of molecular weight markers are shown. (C) Processing in *trans*. One microliter of in vitro-translated polyprotein from pMB11 was incubated for 60 min at 37°C in the absence (lane 1) or presence (lane 2) of 5 μl of a bacterial extract containing pMAL-PRT1. Lane 3, same as lane 2 but with a mutant RHDV protease expressed from a pMAL-PRT1 derivative.

Only a few residues, mostly clustered within the similarity boxes, are well conserved between the calicivirus and the picornavirus sequences. On the basis of their conservation and relative positions along the sequence, residues His-1135, Asp-1152, Asp-1175, Cys-1212, and His-1227, were selected for replacement by oligonucleotide-specific site-directed mutagenesis, thus generating a set of single amino acid substitution mutants.

We took advantage of the ability of the protease to cut the fusion protein derived from expression of pEX01 in *E. coli* to compare the activity of the wild-type enzyme versus a set of pEX01 derivatives carrying the desired mutation. The recombinant proteins were purified from inclusion bodies, separated by gel electrophoresis, and stained by Coomassie blue (Fig. 4). A replicate of this gel was also analyzed by Western blotting to confirm the identity of each product (data not shown).

The first amino acid to be replaced was Cys-1212 because Cys distinguishes the viral 3C-like proteases from other members of the trypsin family, which contain Ser as one of the catalytic residues. Replacement of Cys-1212 with Gly completely abolished proteolytic activity in *E. coli* (Fig. 4, lane 4). On the other hand, replacement with Ser, which maintains the nucleophilic character of Cys but does not have similar structural functions such as the ability to form disulfide bridges, yielded a protease with relatively high activity corresponding to about one-half of the activity of the wild-type protease (lane 3); these data and the good degree of conservation of the amino acids immediately surrounding Cys-1212 strongly suggest that Cys-1212 is an essential constituent of the catalytic site of the RHDV protease.

The trypsin-like proteases characterized so far invariably include His in the catalytic triad. We mutated His-1135 because its position and the sequence context made it a more likely candidate than two other His residues, at positions 1116 and 1124, respectively. Replacement of His-1135 by Asn (Fig. 4, lane 5) demonstrated that this residue is crucial for proteolytic activity.

Two amino acids that could fulfill the role of the acidic residue of the catalytic triad were identified: Asp-1152 and Asp-1175. Both residues were mutated independently to Gly (different size and different chemistry with respect to Asp), Asn (similar size but different chemistry), and Glu (different size but similar chemistry). The Asp-to-Gly mutants completely lost activity in both cases (Fig. 4, lanes 8 and 11). However, the results of the other substitutions were strikingly different according to which position was involved: the Asp-to-Glu mutation yielded a protease with close to wild-type activity when the mutation was at position 1152, but very little activity

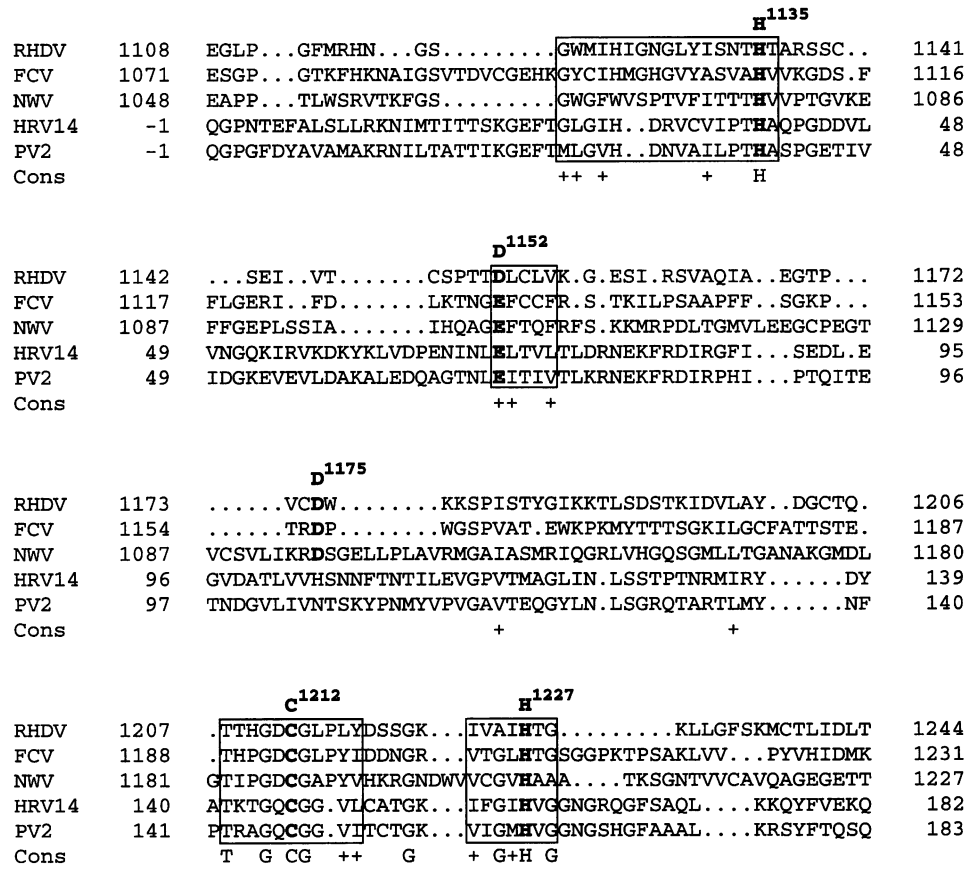

FIG. 3. Alignment of the RHDV protease region (aa 108 through 1244 of ORF1) with similar sequences of FCV (6) and Norwalk virus (NWV) (27) and the 3C proteases of poliovirus type 2 (PV2) (28) and human rhinovirus 14 (HRV14) (41). The letters above the alignment indicate the amino acids that were mutated in this study. Four similarity boxes highlight structural motifs common to viral and cellular proteases as described by Bazan and Fletterick (4). The settings used to run the analysis programs and any modification of the original output are described in Materials and Methods. Cons., consensus.

was retained after introduction of the same change at position 1175 (compare lanes 7 and 10). In contrast, the substitution of Asp by Asn, while giving a nonfunctional protease if position 1152 was involved, yielded a nearly normal activity for the position 1175 mutant (lanes 6 and 9). These data indicate that an acidic residue is required at position 1152 but not at position 1175.

Some amino acids in the proximity of the active nucleophile (Cys or Ser) of the trypsin-like proteases are known to participate in the substrate binding pocket (box 4 in Fig. 3). In particular, a His residue is invariably present within this box in all viral proteases that cleave after Glu or Gln. In the case of the RHDV protease, the conserved His is probably represented by His-1227. Mutagenesis of this amino acid to either Asn or Leu (lanes 12 and 13) led to complete loss of proteolytic activity. This finding is consistent with the notion that His-1227 is directly involved in substrate binding. However, it cannot be ruled out that protease inactivation by the His-1227 mutation is the consequence of a change in protein structure.

DISCUSSION

The genome organization of RHDV implies that mature viral proteins are generated through specific proteolytic cuts of a large precursor. In other plus-strand RNA viruses, processing is usually mediated by one or more highly specific proteases. Therefore, the existence of a protease gene in the genome of RHDV was suspected. Amino acid sequence alignments provided the first indication for a protease that exhibits similarity to 3C proteases of picornaviruses and is encoded in the central part of the genome in RHDV and other caliciviruses (20, 27, 32, 34).

In this report, we demonstrate that the predicted protease of RHDV is functional and is able to cut polyprotein substrates in *cis* as well as in *trans*. The location of the protease gene in front of the putative RNA polymerase gene is similar to that found for 3C proteases of picornaviruses and related proteases of several plus-strand RNA viruses of plants (12, 24). By this criterion, caliciviruses are members of the supergroup I of plus-strand RNA viruses, which includes picorna-, como-, nepo-, and potyviruses.

A large number of viral proteases, including 3C and 2A proteases of picornaviruses, are thought to be homologous to the trypsin family of serine proteases. This conclusion is based on molecular modeling studies and on amino acid sequence alignments that identify conserved residues proposed to correspond to the catalytic triad of cellular serine proteases (3, 14) and has been recently supported by resolution of the crystal structure of the hepatitis A virus 3C protease (1). According to the alignment presented here, the putative catalytic residues identified in picornavirus proteases 3C and 2A (15, 18, 22, 40,

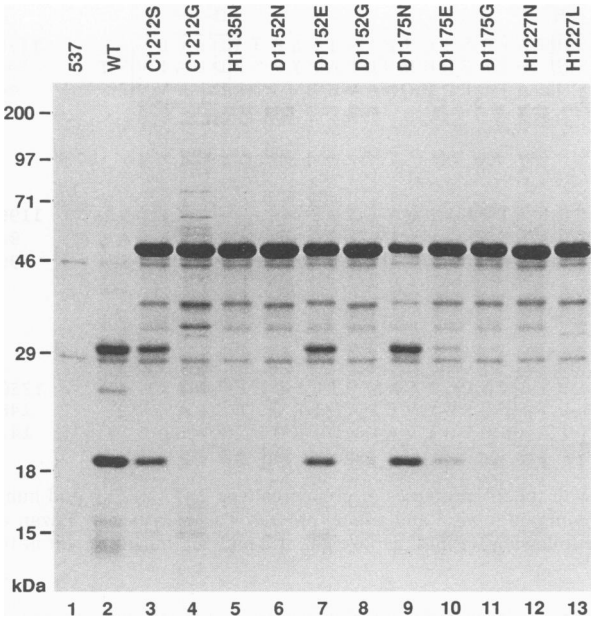

FIG. 4. SDS-PAGE of 7 M urea protein fractions from bacteria harboring pEX01 (wild-type protease) or its derivatives after site-directed mutagenesis. The gel was stained with Coomassie blue. Lane 1, *E. coli* 537 cells without expression vector; lane 2, wild-type (wt) protease construct (pEX01); lanes 3 through 13, expression of fusion proteins with mutant proteases. The single amino acid substitutions are indicated above the lanes. The sizes (in kilodaltons) of prestained molecular weight markers are also shown.

46) are conserved in the calicivirus proteases. Thus, the calicivirus proteases belong to the same family of trypsin-related proteases as 3C and 2A proteases.

The prediction of His-1135, Asp-1152, and Cys-1212 as constituents of the catalytic triad of the RHDV protease is strongly supported by the mutagenesis analysis. Cys-1212 very likely represents the nucleophilic residue because substitution by Gly abolished the proteolytic activity, whereas substitution by Ser, which acts as the nucleophile in trypsin and other proteases, yielded a mutant protein with a good degree of activity. Replacement of Cys by Ser has been performed in several other viral proteases. In some cases, activity was observed after in vitro translation of the mutant protease (13, 29, 30), but no activity was detected after expression in *E. coli* (8, 15, 19) and in assays for cleavage in *trans* of peptides or synthetic polyprotein fragments as substrates (15). This is the first report of a Cys-to-Ser mutant protease that retains activity after expression in *E. coli*.

The position relative to the N terminus and the sequence context of His-1135 made it the most likely candidate to be homologous to His-40 (Fig. 3, box 1) and to His-20 (Fig. 5) of 3C and 2A picornavirus proteases, respectively, a prediction supported by the functional inactivation of the enzyme obtained after mutation of His-1135 to Asn.

Finally, Asp-1152 most likely represents the acidic residue of the putative catalytic triad, because of three substitutions at position 1152, only Glu had no effect on protease activity, while both Gly and Asn behaved as null mutations. Similar results were obtained with poliovirus 3C protease and tobacco etch virus 49-kDa protease (13, 15). The homology of 3C proteases with cellular serine proteases has been suggested by two independent models that differ in the prediction of the acidic amino acid of the catalytic triad: Glu-71 in the poliovirus 3C protease according to Gorbalenya et al. (14) and Asp-85 according to Bazan and Fletterick (3). No conserved acidic residue is present in the RHDV and FCV proteases at the position corresponding to Asp-85 of the poliovirus 3C protease. Our alignment and the mutagenesis data are thus consistent with the model of Gorbalenya et al. (14). A similar conclusion has been reached for the 3C protease of poliovirus (15), the 3C-like protease of grapevine fanleaf nepovirus (30), and the 24-kDa protease of cowpea mosaic virus (10).

Previous alignments of the calicivirus protease domains and 3C proteases of picornaviruses identified the conserved cysteine and a conserved histidine that is thought to be part of the substrate binding pocket (20, 32, 34) but not the conserved histidine and acidic amino acid of the catalytic triad. The failure to identify the latter amino acids can be explained by the low overall homology as well as by the shorter size of the RHDV protease domain. In RHDV, the spacing between His-1135 and Asp-1152 (17 aa) is very close to the distance between His-20 and Asp-38 of the poliovirus 2A protease (18 aa) but considerably shorter than the distance between His-40 and Glu-71 (31 aa) of the poliovirus 3C protease. The distance between Asp-1152 and Cys-1212 (60 aa) is even shorter than the distance between the corresponding Asp-38 and Cys-109 (71 aa) of the 2A protease; the homologous residues of 3C protease (Glu-71 and Cys-147) are separated by 76 residues. Therefore, the size and possibly the structure of the RHDV protease are more similar to those of 2A proteases (Fig. 5) than to those of 3C proteases. On the other hand, the protease domain of Norwalk virus approaches 3C proteases in size. Our expression studies showed that the protease retains activity if the putative polymerase domain is removed. It should be noted, however, that we never detected processing at the protease-polymerase boundary, an event observed for picornaviruses when similar constructs were used (17, 36). To clarify this issue further, functional studies and size estimation of the protease in infected cells are needed.

A survey of the 3C-like protease target sites shows that the P1 position is usually occupied by Gln or Glu, a requirement attributed to the interaction with a conserved His in the substrate binding pocket. A notable exception is represented by the 3C-like proteases of nepoviruses, in which His is replaced by Leu, and it has been shown that these proteases cleave after Arg, Cys, or Gly (5, 31). Similarly, 2A proteases which cleave after Tyr, Ala, Thr, or Val (16, 23, 44) lack the conserved histidine and instead contain a valine, leucine, or isoleucine at the corresponding position within the binding pocket. For RHDV, the putative binding pocket contains a His at the corresponding position (His-1227); substitution with either Leu or Asn abolished the activity of the enzyme, suggesting that His-1227 is indeed involved in binding to the substrate and that the RHDV protease should cut after Gln or Glu. In FCV, a related calicivirus, N-terminal sequence determination of the capsid protein which is generated by a proteolytic processing supports the prediction that the P1 target of the protease is either Gln or Glu (7). According to these considerations, the dipeptide Glu-Gly at positions 1108 and 1109 was chosen as the N-terminal boundary of the RHDV protease domain in the alignments reported here. Similarly, two candidate cleavage sites exist at the polymerase-VP60 boundary: Glu-Gly at positions 1767 and 1768 and Gln-Gly at positions 1775 and 1776; because of their proximity to each other, it was not possible to discriminate between them on the basis of the relative molecular weights of the proteolytic products.

Recently Parra et al. (37) performed partial sequencing of cyanogen bromide-derived peptides from purified RHDV and

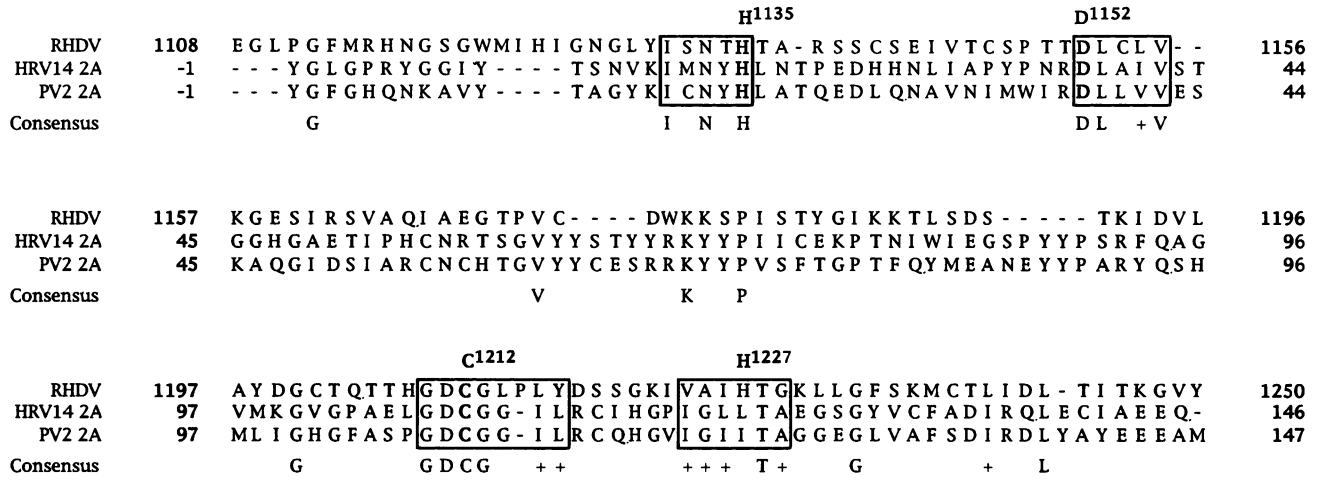

FIG. 5. Alignment of the RHDV protease region (aa 1093 through 1251) with the 2A proteases of poliovirus type 2 (PV2) (28) and human rhinovirus 14 (HRV14) (41). Four similarity boxes highlight structural motifs common to viral and animal proteases as described by Bazan and Fletterick (3). The sequences were aligned with the program Pileup, using the following settings: gap weight at 2 and gap length weight at 0.1.

suggested that the VP60 incorporated into virions originates from translation of subgenomic but not genomic RNA. The experiments reported here demonstrate that at least in vitro, the capsid protein can also be generated by a protease mediated cut of the ORF1 product at the polymerase-VP60 boundary. The function of this additional pathway for expression of the RHDV capsid protein remains to be established.

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REFERENCES

1. **Allaire, M., M. M. Chernaia, B. A. Malcolm, and M. N. G. James.** 1994. Picornaviral 3C cysteine proteinase have a fold similar to chymotrypsin-like serine proteinases. Nature (London) **369:**72–76.
2. **Baum, E. Z., G. A. Bebernitz, O. Palant, T. Mueller, and S. J. Plotch.** 1991. Purification, properties, and mutagenesis of poliovirus 3C protease. Virology **185:**140–150.
3. **Bazan, J. F., and R. J. Fletterick.** 1988. Viral cysteine proteases are homologous to the trypsin-like family of serine protease: structural and functional implications. Proc. Natl. Acad. Sci. USA **85:**7872–7876.
4. **Bazan, J. F., and R. J. Fletterick.** 1990. Structural and catalytic models of trypsin-like viral proteases. Semin. Virol. **1:**311–322.
5. **Blok, V. C., J. Wardell, C. A. Jolly, A. Manoukian, D. J. Robinson, M. L. Edwards, and M. A. Mayo.** 1992. The nucleotide sequence of RNA-2 of raspberry ringspot nepovirus. J. Gen. Virol. **73:**2189–2194.
6. **Carter, M. J., I. D. Milton, J. Meanger, M. Bennet, R. M. Gaskell, and P. C. Turner.** 1992. The complete nucleotide sequence of a feline calicivirus. Virology **190:**443–448.
7. **Carter, M. J., I. D. Milton, P. C. Turner, J. Meanger, M. Bennet, and R. M. Gaskell.** 1992. Identification and sequence determination of the capsid protein gene of feline calicivirus. Arch. Virol. **122:**223–235.
8. **Cheah, K.-C., L. E.-C. Leong, and A. G. Porter.** 1990. Site-directed mutagenesis suggests close functional relationship between a human rhinovirus 3C cysteine protease and cellular trypsin-like serine proteases. J. Biol. Chem. **265:**7180–7187.
9. **Cormack, B.** 1991. Mutagenesis by the polymerase chain reaction, p. 8.5.1–8.5.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene-Wiley, New York.
10. **Dessens, J. T., and G. P. Lomonossoff.** 1991. Mutational analysis of the putative catalytic triad of the cowpea mosaic virus 24 K protease. Virology **184:**738–746.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**6587–6601.
12. **Dolja, V. V., and J. C. Carrington.** 1992. Evolution of positive-strand RNA viruses. Semin. Virol. **3:**315–326.
13. **Dougherty, W. G., T. D. Parks, S. M. Cary, J. F. Bazan, and R. J. Fletterick.** 1989. Characterization of the catalytic residues of the tobacco etch virus 49-kDa protease. Virology **172:**302–310.
14. **Gorbalenya, A. E., A. P. Donchenko, B. M. Blinov, and E. V. Koonin.** 1989. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. FEBS Lett. **243:**103–114.
15. **Haemmerle, T., C. U. T. Hellen, and E. Wimmer.** 1991. Site-directed mutagenesis of the putative catalytic triad of polyovirus 3C proteinase. J. Biol. Chem. **266:**5412–5416.
16. **Harris, K. S., C. U. T. Hellen, and E. Wimmer.** 1990. Proteolytic processing in the replication of picornaviruses. Semin. Virol. **1:**323–333.
17. **Harris, K. S., S. R. Reddigari, M. J. H. Nicklin, T. Haemmerle, and E. Wimmer.** 1992. Purification and characterization of poliovirus polypeptide 3CD, a proteinase and a precursor for RNA polymerase. J. Virol. **66:**7481–7489.
18. **Hellen, C. U. T., M. Faecke, H.-G. Kraeusslich, C.-K. Lee, and E. Wimmer.** 1991. Characterization of poliovirus 2A proteinase by mutational analysis: residues required for autocatalytic activity are essential for induction of cleavage of eukaryotic initiation factor 4F polypeptide p220. J. Virol. **65:**4226–4231.
19. **Ivanoff, L. A., T. Towatari, J. Ray, B. D. Korant, and S. R. Petteway.** 1986. Expression and site-specific mutagenesis of the poliovirus 3C protease in Escherichia coli. Proc. Natl. Acad. Sci. USA **83:**5392–5396.
20. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk Virus. Virology **195:**51–61.
21. **Jore, J., B. De Geus, R. J. Jackson, P. H. Pouwels, and B. E. Enger-Valk.** 1988. Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 in vitro. J. Gen. Virol. **69:**1627–1636.
22. **Kean, K. M., N. L. Teterina, D. Marc, and M. Girard.** 1991. Analysis of putative active site residues of the Poliovirus 3C protease. Virology **181:**609–619.
23. **Kowalski, H., I. Maurer-Fogy, M. Zom, H. Mischak, E. Kuechler, and D. Blaas.** 1987. Cleavage site between VP1 and P2A of human

rhinovirus is different in serotypes 2 and 14. J. Gen. Virol. **68**:3197–3200.
24. **Kraeusslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
25. **Kunkel, T. A., J. D. Roberts, and T. A. Zakour.** 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection, p. 367–392. *In* R. Wu and L. Grossman (ed.), Recombinant DNA, vol. 154. Academic Press, San Diego, Calif.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
27. **Lambden, P. R., O. E. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round structured (Norwalk-like) virus. Science **259**:516–519.
28. **LaMonica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.
29. **Lawson, M. A., and B. L. Semler.** 1991. Poliovirus thiol proteinase 3C can utilize a serine nucleophile within the putative catalytic triad. Proc. Natl. Acad. Sci. USA **88**:9919–9923.
30. **Margis, R., and L. Pinck.** 1992. Effects of site-directed mutagenesis on the presumed catalytic triad and substrate-binding pocket of grapevine fanleaf nepovirus 24-kDa proteinase. Virology **190**: 884–888.
31. **Margis, R., C. Ritzenthaler, C. Reinbolt, and L. Pinck.** 1993. Genome organization of grapevine fanleaf nepovirus RNA2 deduced from the 122K polyprotein P2 vitro cleavage products. J. Gen. Virol. **74**:1919–1926.
32. **Meyers, G., C. Wirblich, and H.-J. Thiel.** 1991. Rabbit hemorrhagic disease virus—molecular cloning and nucleotide sequencing of a calicivirus genome. Virology **184**:664–676.
33. **Meyers, G., C. Wirblich, and H.-J. Thiel.** 1991. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. Virology **184**:677–686.
34. **Neill, J. D.** 1990. Nucleotide sequence of a region of the feline calicivirus genome which encodes picornavirus-like RNA-dependent RNA polymerase, cysteine protease and 2C polypeptides. Nucleic Acids Res. **17**:145–160.
35. **Ohlinger, V. F., B. Haas, G. Meyers, F. Weiland, and H.-J. Thiel.** 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. J. Virol. **64**:284–294.
36. **Parks, G. D., J. C. Baker, and A. C. Palmenberg.** 1989. Proteolytic cleavage of encephalomyocarditis virus capsid region substrates by precursors to the 3C enzyme. J. Virol. **63**:1054–1058.
37. **Parra, F., J. A. Boga, M. A. Marin, and R. Casais.** 1993. The amino terminal sequence of VP60 from rabbit hemorrhagic disease virus supports its putative subgenomic origin. Virus Res. **27**:219–228.
38. **Parra, F., and M. Prieto.** 1990. Purification of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. **64**:4013–4015.
38a. **Rossi, C., et al.** Unpublished data.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sommergruber, W., M. Zorn, D. Blaas, F. Fessl, P. Volkmann, I. Maurer-Fogy, P. Pallai, V. Merluzzi, M. Matteo, T. Skem, and E. Kuechler.** 1989. Polypeptide 2A of human rhinovirus type 2: identification of a protease and characterization by mutational analysis. Virology **169**:68–77.
41. **Stanway, G., P. J. Hughes, R. C. Mountford, P. D. Minor, and J. W. Almond.** 1984. The complete nucleotide sequence of a common cold virus: human rhinovirus 14. Nucleic Acids Res. **12**:7859–7875.
42. **Strebel, K., E. Beck, K. Strohmaier, and H. Schaller.** 1986. Characterization of foot-and-mouth disease virus gene products with antisera against bacterially synthesized fusion proteins. J. Virol. **57**:983–991.
43. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
44. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45**:761–770.
45. **Ypma-Wong, M. F., P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semler.** 1988. Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology **166**:265–270.
46. **Yu, S. F., and R. E. Lloyd.** 1991. Identification of essential amino acid residues in the functional activity of poliovirus 2A protease. Virology **182**:615–625.