Hydrolysis of a Series of Synthetic Peptide Substrates by the Human Rhinovirus 14 3C Proteinase, Cloned and Expressed in Escherichia coli

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SUMMARY

The 3C proteins of several picornaviruses, including poliovirus, foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus (EMCV), have been demonstrated to be cysteine-type proteinases, involved in the processing of the respective polyproteins expressed by the monocistronic RNA genome. Nucleotide sequencing data have indicated that the human rhinovirus 14 (HRV-14) RNA genome encodes a homologous 3C protein. The HRV-14 3C protein was purified to homogeneity from Escherichia coli expressing the cloned 3C genomic fragment. The enzyme was assayed against peptides corresponding to those residues, predicted (by nucleotide sequencing data) to occur at authentic cleavage sites within the polyprotein. The peptides representing the 1B/1C, 2A/2B, 2C/3A, 3A/3B, 3B/3C and 3C/3D cleavage sites, where proteolysis was predicted to occur at a Gln-Gly junction, were all cleaved by the 3C proteinase. The hydrolysis was shown (by reverse phase fast protein liquid chromatography and amino acid analysis) to occur specifically at the Gln-Gly bond in each of the peptides. The ready availability of such convenient substrates facilitated the further characterization of the 3C proteinase. By contrast, peptides corresponding to the predicted 2B/2C and 1C/1D cleavage sites, where the processing was presumed to occur at a Gln-Ala or Glu-Gly bond respectively, were not cleaved by the 3C proteinase. The ability of the HRV-14 3C proteinase to hydrolyse the synthetic peptides was inhibited if a Cys→Ser(146) mutation was introduced into the protein. Studies with known proteinase inhibitors substantiated the conclusion that the HRV-14 3C protein appears to be a cysteine proteinase and that the Cys residue at position 146 may be the active site nucleophile. The HRV-14 3C proteinase probably plays an important role, analogous to that implied for the poliovirus 3C proteinase, in the replication of the virus and thus represents a potential target for antiviral chemotherapy.

INTRODUCTION

The human rhinoviruses (HRVs), implicated as the major causative agents of upper respiratory tract infections collectively known as the common cold (Larson et al., 1980), belong to the largest genus of the picornavirus family. The other genera of the picornavirus family are the enteroviruses (poliovirus, echovirus, coxsackie virus), cardioviruses [encephalomyocarditis virus (EMCV), mengovirus] and aphthoviruses [foot-and-mouth disease virus (FMDV)] (Rueckert & Wimmer, 1984). The picornviruses share their genomic organization and translational strategy. The icosahedral protein particles contain a single-stranded positive-sense infectious RNA molecule of $M_r \approx 2.5 \times 10^6$ (approximately 7500 nucleotides). The RNA has a small virus-specific protein, 3B (VPg) covalently linked at its 5' end (Wimmer, 1982) and a poly(A) tail at the 3' end (Yogo & Wimmer, 1972). [When describing the picornavirus proteins,
the nomenclature adopted at the 3rd Meeting of the European Study Group on Molecular Biology of Picornaviruses (Urbino, Italy, 1983) (Rueckert & Wimmer, 1984) has been used in this paper. Where reference is made to papers that used the earlier nomenclature, the former names of the respective proteins are given in parentheses for ease of reference.] The genomic RNA acts as a monocistronic message and is initially translated to a > 200K polyprotein which is cleaved by a series of proteolytic events to yield the mature viral products, both structural and non-structural, that are essential for viral replication and the assembly of infectious viral particles (Decock & Billian, 1986; Kitamura et al., 1981; Nicklin et al., 1986; Pallansch et al., 1984; Palmenberg, 1987; Toyoda et al., 1986a).

Extensive studies have not been carried out on the processing of the rhinovirus polyprotein. By contrast, the proteolytic steps involved in the production of poliovirus proteins have been analysed in considerable detail (Nicklin et al., 1987; Palmenberg, 1981; Semler et al., 1981). The first cleavage within the poliovirus polyprotein is an autocatalytic process mediated by the poliovirus 2A protein which occurs while the polypeptide is still nascent on the ribosome. The peptide bond hydrolysed in this initial processing step is between a tyrosine and a glycine residue which connect the P1 and P2 regions (Nicklin et al., 1987; Toyoda et al., 1986a, b) (Fig. 1) whereas most of the further cleavages which take place within the capsid and non-structural protein precursors are produced by proteolysis between glutamine and glycine residues.

These Gln-Gly cleavages are carried out by the viral 3C proteinase, which is a cysteine-type enzyme mapping in the 3'-terminal region of the genome and displays a high specificity for its own polyprotein substrate (Hanecak et al., 1982, 1984; Ivanoff et al., 1986). The final poliovirus proteolytic processing event, the cleavage of the capsid 1AB (VP0) protein between an asparagine and serine residue to produce 1A (VP4) and 1B (VP2), is associated with maturation of the virion (Arnold et al., 1987).

A study of the complete nucleotide sequences of two human rhinoviruses, HRV-2 (Skern et al., 1985) and HRV-14 (Callahan et al., 1985; Stanway et al., 1984), indicated that both genomes were typical of the Picornaviridae family, and comparison of these two nucleotide sequences (and their predicted amino acid sequences) with those of poliovirus types 3 and 1 (Kitamura et al., 1981; Stanway et al., 1983) revealed not only a high degree of homology between the two rhinovirus serotypes but also between the two rhinoviruses and the polioviruses. Since, as described above, the cleavage sites in the polioviruses have been accurately determined, this extensive homology in the predicted amino acid sequences enabled most of the cleavage sites in HRV-2 and HRV-14 to be tentatively identified and the mature virus proteins to be mapped onto the polyprotein. In particular, comparison of the nucleotide and deduced amino acid sequences in the presumed 3C region of rhinovirus with those of poliovirus, other picornaviruses and cowpea mosaic virus (CPMV, a plant virus related in gene organization) reveals substantial homology (Argos et al., 1984; Carroll et al., 1984; Cann et al., 1983; Earle et al., 1988; Hughes et al., 1988; Jenkins et al., 1987; Lomonosoff & Shanks, 1983; Robertson et al., 1985; Skern et al., 1984, 1985; Stanway et al., 1983, 1984).

Therefore, it appears that the picornaviruses studied to date may all code for a similar 3C proteinase. The 3C proteinase in EMCV and poliovirus has been shown to play a crucial role in the processing of the viral proteins (Ivanoff et al., 1986; Semler et al., 1987; Shih & Shih, 1981; Shih et al., 1978, 1979; Ypma-Wong et al., 1987a, b) and may represent an ideal target enzyme for antiviral chemotherapy. By analogy, this is almost certainly true for the 3C proteins of all picornaviruses, including human rhinoviruses. However there have been relatively few comprehensive studies on the rhinovirus 3C protein (Libby et al., 1988). To ascertain not only whether this protein expressed similar proteolytic activity but also whether it is a suitable target for antiviral chemotherapy through the rational design of specific inhibitors, it was considered that detailed molecular characterization of this enzyme was necessary.

This information was unlikely to be obtained using conventional methods to prepare viral proteins, because of the relatively small quantities of 3C protein produced in infected cell cultures (Decock & Billian, 1986). Therefore, appropriate fragments of cDNA copies of the HRV-14 RNA genome were cloned and expressed in Escherichia coli. The 3C protein expressed by these constructs was purified and subjected to biochemical characterization (Knott et al.,
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1989). Previous investigations into structure/function relationships with 3C proteinases from other picornaviruses have been hampered by the lack of availability of convenient substrates such that most of the studies on proteolytic activity have, of necessity, been indirect, using expression in E. coli of plasmids containing various cloned fragments of the picornavirus genome (Hanecak et al., 1984; Ivanoff et al., 1986; Klump et al., 1984; Semler et al., 1987; Toyoda et al., 1986a, b) or cell-free expression systems (Parks et al., 1986; Shih & Shih, 1981; Shih et al., 1978, 1982; Vos et al., 1984, 1987; Ypma-Wong & Semler, 1987a, b).

We report on the characterization of the HRV-14 3C proteinase, which was facilitated by examination of its interaction with synthetic peptide substrates corresponding to the residues, predicted by nucleotide sequencing data, to occur at authentic sites within the HRV-14 polyprotein (Callahan et al., 1985; Stanway et al., 1984).

METHODS

Purification of 3C proteinase. The HRV-14 3C proteinase and its derivative containing the cysteine (146) to serine mutation were produced directly in E. coli. The induction and growth of the E. coli cells expressing the respective plasmids and the details of the purification protocol were as described previously (Knott et al., 1989).

Briefly, the 3C proteins were purified from the soluble extract of induced cells using a two-stage high pressure liquid chromatography (HPLC) procedure on a cation exchange column (TSK SP5PW) and a gel permeation column (TSK G3000SW). The HRV-14 3C proteins were homogeneous, as judged by SDS–PAGE, isoelectric focusing and amino acid analysis. Western blot analysis demonstrated that the purified proteins had cross-reactivity with antibodies raised against synthetic peptides corresponding to presumed antigenic sites of the HRV-14 3C protein.

Synthesis of peptides corresponding to the putative cleavage sites in the viral polyprotein. Eight peptides, each spanning a putative (Gln-Gly; Gin-Ala; Glu-Gly) cleavage site in the rhinovirus polyprotein were synthesized (Fig. 1). Occasional changes were made in the naturally occurring (predicted) sequence, for example by substitution of a tyrosine residue at the C terminus of peptide IV in order to facilitate u.v. detection of the parent peptide and its degradation products. In all cases where such a strategy was employed, the replacement residues were distant from the predicted P1-P2 scissile peptide bond. The changes are indicated in the sequences in Fig. 1 by inclusion of the naturally occurring residues in parentheses (Lys) following the residue, Tyr, which replaced it (e.g. in peptide IV). A derivative of peptide VII was also synthesized in which the putative Gln-Gly scissile peptide bond was altered to Gtu-Gly. The sequence of this peptide, peptide VII (Q-*E) was thus Arg-Pro-Val-Val-Glu-Gly-Pro-Asn-Thr. Two further peptides were prepared. Peptide IX had the sequence Thr-Leu-Glu-Ser-Gln-Thr-Val-Glu-Arg-Leu and peptide X had the sequence Gly-Gly-Asn-Gly-Arg-Gln-Thr-Val-Glu-Arg-Leu-Lys, corresponding to residues 162 to 174 of the HRV-14 3C protein except that the native Ala at position 166 was replaced by Arg in the synthetic peptide.

Peptides were synthesized using the Merrifield solid-phase technique on phenylacetamidomethyl polystyrene resins (Applied Biosystems). Synthesis was carried out with an Applied Biosystems Model 430A peptide synthesizer utilizing ABI chemicals, the manufacturer's standard protected symmetric anhydride programmes and a double coupling protocol. A portion of each synthetic peptide was removed after the addition of the glycine alanine in the case of peptide III) to provide a sample of the C-terminal fragment expected from enzymatic hydrolysis at the respective Gln-Gly (or Ala) or Glu-Gly peptide bonds. After removal of the peptides from the resins with 90% anhydrous HF, 10% anisole, the samples were extracted with 10 to 50% acetic acid, diluted with water, and lyophilized to dryness. Reversed phase HPLC was performed on each peptide [Waters C18/ßBondapak in Radial Pak cartridges, 10 to 90% acetonitrile gradients with 0-1% trifluoroacetic acid (TFA)-water as solvent (alanine in the case of peptide III)]. Occasional changes were made in the naturally occurring (predicted) sequence, for example by substitution of a tyrosine residue at the C terminus of peptide IV in order to facilitate u.v. detection of the parent proteinase.

Monitoring peptide bond cleavage. Samples of each of the potential peptide substrates were incubated with recombinant HRV-14 3C proteinase under a variety of experimental conditions. Aliquots were withdrawn at several time points and TFA was added to give a final concentration of 0.1% (v/v). The samples were centrifuged at 5000 g for 5 min and portions of each supernatant were analysed by reverse phase chromatography using either C18 ßBondapak (Waters) or more commonly PepRPC fast protein liquid chromatography (FPLC) columns (Pharmacia). The flow rate was 1 ml/min with Buffer A (A) consisting of H2O containing 0.1% (v/v) TFA and Buffer B (B) consisting of 100% acetonitrile, 0.1% (v/v) TFA. The gradients used were 0 to 10 min with 100% A; 10 to 15 min with 0 to 15% B; 15 to 40 min with 15 to 50% B; 40 to 45 min with 50 to 100% B; 45 to 50 min with 100% B; 50 to 55 min with 100% B plus 100% A. Detection was at a wavelength of 214 nm.
Identification of the cleavage products generated. After prolonged incubation of potential substrate peptides with proteinase, newly generated peaks were collected in acid-cleansed test tubes and then lyophilized. Samples (50 to 100 pmol) were acid-hydrolysed on the PICO-TAG work station for 20 h at 105°C and analysed using the Millipore-Waters Corporation PICO-TAG column connected to Millipore-Waters Corporation HPLC equipment. α-Amylo backdrop acid (ABA) was incorporated into the samples as an internal standard.

Monitoring hydrolysis of p-nitroanilide and p-nitrophenyl ester containing peptides. Various p-nitroanilide and p-nitrophenyl ester-linked peptides and amino acids were dissolved in DMSO at a concentration of 10 to 50 mM. Aliquots of these preparations diluted with the required buffer were added to flat-bottomed microtitre plate wells and incubated at 37°C with 3C proteinase which had been preincubated for 10 min at 37°C in the presence of 5 mM-L-cysteine, 1 mM-EDTA, 5 mM-2-mercaptoethanol and 10% (v/v) glycerol. Colour development was monitored at 405 nm using a Titre-Tek Multiskan plate reader.

Materials

Chromogenic peptides and other peptides. Dinitrophenyl (DNP)-Gln-Gly-Ile-Ala-Gly-Gln-p-Arg and DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-p-Arg were from the Peptide Institute (Osaka, Japan). The dipeptides Glu-Gly and Gln-Gly were from Serva (Heidelberg, F.R.G.). The serum thymic factor (pGlu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn) and Fibrinopeptide A (Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg) were from Sigma. Other chromogenic peptides obtained from either Sigma or the Peptide Institute were Ac-t-Asp-p-nitroanilide (NA), N-Succ-t-Tyr-t-Leu-t-Val-p-NA, N-Ac-t-Ala-t-Ala-t-Ala-p-NA, N-Succ-t-Ala-t-Ala-t-Pro-t-Phe-p-NA, N-Succ-t-Ala-t-Ala-t-Pro-t-Leu-p-NA, N-Succ-t-Ala-t-Ala-t-Pro-t-Leu-p-NA, N-Succ-t-Ala-t-Ala-t-Pro-t-Leu-p-NA, N-Succ-t-Ala-t-Ala-t-Pro-t-Leu-p-NA, Ac-Cbz-L-Gly-L-Leu-p-NA, N-Succ-t-Ala-t-Ala-t-Ala-p-NA, N-Asp-Val-p-nitrophenyl ester (NPE), N-tert-butoxycarbonyl (N-t-BOC)-benzyl-L-Glu-p-NPE, N-t-BOC-L-Gln-p-NPE, N-t-BOC-L-Asp-p-NPE and N-t-BOC-L-Gly-p-NPE.

Proteinase inhibitors. Amastatin, antipain, bestatin, chymostatin, E-64, elastatin, leupeptin and phosphoramidon were all obtained from the Peptide Institute, α-2-macroglobulin (from bovine plasma), and trypsin inhibitor (from chicken egg white) were all obtained from Boehringer Mannheim. Aprotinin (from bovine lung), bromelain inhibitor (from pineapple stem), p-chloromercuriphenyl-sulphonic acid, cystatin (from egg white), epirestatin, iodoacetamide, N-ethylmaleimide, 4-amidophenyl-methane sulphonyl fluoride (APMSF), PMSF, 2-nitro-4-carboxyphenyl-N, N-diphenyl carbamate (NCDC), N-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were all obtained from Sigma.

Two synthetic chymostatin analogues containing a phenylalanine aldehyde (Z-Arg-Val-Phe-CHO) or a semicarbazone (Z-Arg-Leu-Phe-CN=NH-CONH2) reactive function respectively (Place et al., 1987) were generous gifts from Dr R. J. Beynon, University of Liverpool, U.K. Recombinant human cystatin C (Abrahamson et al., 1989) was kindly provided by Dr M. Abrahamson, Malmö General Hospital, Sweden.

RESULTS

Analysis of the cleavage site in the various peptide substrates

Samples of each of the synthetic peptides I to VIII (Fig. 1) corresponding to the putative polyprotein cleavage sites were incubated at neutral pH with the homogeneous 3C proteinase and the mixtures were analysed by reverse-phase HPLC or FPLC. As internal controls, with the appearance of only two novel peaks, one of which (in every case) corresponded exactly with the Gln-Gly residues within the respective sequences. No novel peptides were generated when any of the peptides were incubated with buffer alone, with proteinase pretreated at 100°C for 10 min or with the mutant [Cys(146)-Ser] proteinase (D. S. Montgomery, D. C. Orr, C. W. Dykes & A. N. Hobden, unpublished results; Knott et al., 1989).

By contrast, peptides III and V were not cleaved by the 3C proteinase, even upon prolonged incubation (26 h) under the same conditions used to achieve significant hydrolysis of the other synthetic substrates. Furthermore, preincubation of peptides III or V (120 μg) with proteinase (0.5 μg) for 2 or 16 h prior to addition of peptide VII (120 μg with continuation of the incubation) showed no inhibition of the cleavage of peptide VII. Thus, it would appear that recombinant...
rhinovirus 3C proteinase did not (to any significant extent) recognize the sequences of peptide III (the putative 2B/2C junction) or V (the predicted 1C/1D junction, Fig. 1).

Peptide VIII (Q→E) in which the Gln-Gly scissile peptide bond was altered to a Glu-Gly linkage was also not cleaved by the viral 3C proteinase. Peptides IX and X, both containing the Gln-Gly pair in their respective sequences (see Methods), also were not hydrolysed by the enzyme.
Table 1. *Kinetic constants for the hydrolysis of eight synthetic substrates by recombinant HRV-14 3C proteinase*

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>Sequence</th>
<th>$V_{\text{max}}$ (µmol/min/mg)</th>
<th>$K_{\text{m}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>D-S-L-E-T-L-F-Q-G-P-V-Y-K</td>
<td>4.3</td>
<td>1.2</td>
</tr>
<tr>
<td>II</td>
<td>E-A-I-A-E-E-Q-G-L-S-D-Y-I-T</td>
<td>0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>III</td>
<td>V-P-Y-I-E-R-Q-A-N-D-G-W-F-R-K</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>R-S-K-S-i-V-P-Q-G-I-P-T-T-T-Y</td>
<td>&lt;0.05</td>
<td>NM†</td>
</tr>
<tr>
<td>VI</td>
<td>K-L-F-A-Q-T-G-Q-G-P-Y-S-G-N-P</td>
<td>7.0</td>
<td>2.6</td>
</tr>
<tr>
<td>VII</td>
<td>Y-R-P-V-V-V-Q-G-P-N-T-E-F</td>
<td>11.5</td>
<td>0.7</td>
</tr>
<tr>
<td>VIII</td>
<td>K-Q-Y-F-V-E-K-Q-G-Q-V-I-A-R</td>
<td>&lt;0.1</td>
<td>NM</td>
</tr>
</tbody>
</table>

* Each peptide was incubated at 35 °C with HRV-14 3C proteinase in 6 mM-citric acid, 187 mM-Na$_2$HPO$_4$ buffer pH 7.6, containing 10% (v/v) glycerol, 5 mM-DTT and 2 mM-EDTA. At appropriate times, aliquots were removed and reaction rates ($V$) at each concentration of substrate were determined from these time courses using reverse-phase FPLC. The area of the newly generated peak corresponding to the carboxy-terminal fragment was measured in each case and converted into nmol of product, using a calibration graph. Best values for $K_{\text{m}}$ and $V_{\text{max}}$ were obtained by computerized curve-fitting as described previously (Jupp et al., 1988).

† NM, Not measurable.

The recombinant HRV-14 proteinase was also tested for its ability to attack a variety of chromogenic peptides containing p-nitroanilide or p-nitrophenyl esters as the carboxy-terminal reporter group and a number of random peptides which chanced to contain the dipeptide sequences Gln-Gly or Glu-Gly. Hydrolysis was not observed for any of these peptides.

Quantification of peptide cleavage by 3C proteinase

Cleavage rates for each of the peptides were obtained by monitoring the appearance of the carboxy-terminal cleavage fragment. By applying increasing amounts (nanograms) of each of the C-terminal peptide fragments onto the reverse-phase column and integrating the peak area from each trace, calibration graphs relating peak area to nanograms loaded were constructed. Having previously identified the products of the proteolytic reactions, it was thus feasible to quantify the extent of cleavage in any of the reactions through measuring the area of the carboxy-terminal fragments generated. Recoveries were generally in excess of 90% of the amount of intact substrate peptide introduced initially into the incubations. The generation of the carboxy-terminal fragment was thus a reliable indicator of the extent of substrate cleavage so that by removal of several samples at different times of incubation, initial rates of hydrolysis for each of the peptides I, II, IV, VI, VII and VIII were determined. The cleavage rates of the peptides were linear with respect to time.

Determination of kinetic parameters ($K_{\text{m}}$ and $V_{\text{max}}$)

The kinetic parameters, $K_{\text{m}}$ and $V_{\text{max}}$, for the cleavage of these six peptides were determined (Table 1). From these data, it is clear that although all six peptides were cleaved by the proteinase, peptides I, VI and VII (all containing the scissile peptide bond within the sequence -Gln-Gly-Pro-) were much better substrates than II, IV or VIII. Consequently peptide VII was selected as a good substrate upon which to base further biochemical characterization of the 3C proteinase.

pH optimum and the effects of divalent cations, temperature and thiol compounds

The rate of cleavage of peptide VII at a variety of pH values was investigated. From this, it appeared that the proteinase was stable at alkaline pH values and had a pH optimum in the range of 7.6 to 8.0. The temperature for maximum cleavage of peptide VII was ascertained to be in the region of 35 °C (data not shown).

At concentrations up to 50 mM, neither Ca$^{2+}$ nor Mg$^{2+}$ had any effect on the proteolytic activity against peptide VII. The cleavage rate of peptide VII was also assayed in the presence of various concentrations of the reducing agents, dithiothreitol (DTT) and L-cysteine. In
Hydrolysis by recombinant HRV proteinase

Table 2. Effect of proteinase inhibitors on the hydrolysis of peptide VII by HRV-14 3C enzyme

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Concentration of compound (μg/ml)</th>
<th>Inhibition of peptide VII cleavage† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chloromercuriphenyl sulphonic acid</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>E64</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>85</td>
</tr>
<tr>
<td>Antipain</td>
<td>100</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Elastatinal</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>TPCK</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>TLCK</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Egg white cystatin</td>
<td>178</td>
<td>0</td>
</tr>
<tr>
<td>Human cystatin C</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>α-2-Macroglobulin</td>
<td>178</td>
<td>0</td>
</tr>
</tbody>
</table>

* Aliquots of 3C proteinase (0.5 μg) were preincubated with the indicated concentrations of each modifier for 15 min at 35 °C before determination of the residual proteolytic activity using Peptide VII as the substrate.
† The inhibition of cleavage was calculated as the decrease in the carboxy terminus peptide fragment generated in the presence of each modifier expressed as a percentage of that obtained in the respective control samples (incubated with buffer alone or with DMSO or ethanol where required).

The absence of any thiol reducing agents, no cleavage of the peptide substrate was observed. Maximum activation of hydrolysis was achieved between approximately 5 and 20 mM-DTT whereas, when l-cysteine was used as the reductant, concentrations as high as 50 mM were still unable to stimulate maximum activation of hydrolysis.

Effects of general proteinase inhibitors on the cleavage of peptide VII by the 3C proteinase

A range of known inhibitors of serine and cysteine proteinases were studied for their effect on the activity of the 3C proteinase towards peptide VII (Table 2). If DMSO or ethanol were required for the solubilization of the inhibitor, these solvents were added at the required level to the respective controls. N-Ethylmaleimide, iodoacetamide and p-chloromercuriphenyl sulphonate inactivated the enzyme completely (Table 2) and since these compounds have been shown to be inhibitors of a wide range of cysteine proteinases including ficin, papain and stem bromelain (Lienér & Friedenson, 1970; Murachi, 1970; Rich, 1986), it would thus appear that the rhinovirus 3C proteinase does indeed belong to the cysteine proteinase family. TLCK and TPCK, which are also known to inhibit cysteine proteinases non-specifically by alkylation of the sulphydryl group of the enzyme (Rich, 1986), were also seen to have some inhibitory effect on the viral proteinase activity. Of the two peptide aldehyde inhibitors known to inhibit cysteine proteinases, antipain and leupeptin (Rich, 1986), only leupeptin was seen to have any activity against the 3C proteinase. E64, an epoxysuccinyl peptide shown to inactivate rapidly several cysteine proteinases, including ficin, papain, and cathepsin B and stem bromelains (Barrett et al., 1982; Elliott & Liu, 1970; Rich, 1986), did not inhibit the viral proteinase. However, another peptide aldehyde, chymostatin, an inhibitor of chymotrypsin-like enzymes (Place et al., 1987; Powers & Harper, 1986b), was found to be a competitive inhibitor of the HRV 3C proteinase, albeit not a particularly good one. Two synthetic chymotrypsin analogues, Z-Arg-Val-PheH and Z-Arg-Leu-PheSc (Place et al., 1987), were also tested, but were found to possess no inhibitory activity. Other serine proteinase inhibitors (NCDC, aprotinin, elastatinal, APMSF and PMSF) (Powers & Harper, 1986b) and metalloproteinase inhibitors (bestatin, amastatin, epiamastatin and phosphoramidon) (Powers & Harper, 1986a) also all failed to show any activity against the HRV-14 3C proteinase.
None of the high Mr protein inhibitors of cysteine proteinases (Abrahamson et al., 1987; Barrett et al., 1986) that were tested at relatively high concentrations, i.e. αt-macroglobulin human cystatin C or egg white cystatin, showed any inhibitory activity against the HRV-14 3C proteinase.

DISCUSSION

The difficulties of acquiring protease from natural sources and the lack of availability of convenient substrates have hampered detailed investigations into structure/function relationships in the picornavirus proteases (2A and 3C). While the present work was in progress however, one report did appear documenting the cloning and expression of an active HRV-14 3C protease. The construct used was such that the authors were able to demonstrate activity in the protease only when it was associated with the membrane fraction of E. coli extracts; attempts to obtain soluble protease by detergent extraction and other means were unsuccessful (Libby et al., 1988). However, in the present study, a large proportion of the 3C protein expressed from the cloned HRV-14 genome cDNA fragments was present in the soluble fraction (Knott et al., 1989).

Detailed characterization of the homogeneous rhinovirus 3C protease was accomplished by examining its action on a series of peptides, representing putative processing sites within the HRV-14 polyprotein, which were designed to fulfil all of the primary sequence requirements of 3C recognition and cleavage (Callahan et al., 1985; Stanway et al., 1984). All of these peptides (Fig. 1) containing Gln-Gly as the putative cleavage site were hydrolysed specifically at this peptide bond by the preparations of the purified 3C protease. However, in the case of peptide VII (Q→E), substitution of Gln-Gly with a Glu-Gly sequence rendered this peptide resistant to proteolytic attack by the 3C protease. Furthermore, when the HRV-14 3C protease was assayed against a variety of other peptides, chosen because they contained a Gln-Gly sequence in their structure, no cleavage of any of the peptides was detected. This suggests that the HRV-14 3C protease not only has a stringent specificity for Gln in subsite P1 and Gly in P2 [the nomenclature of amino acid residues at the cleavage sites is according to Berger & Schechter (1970)] in peptide substrates but also appears to require further structural features to be met as well. In the serum thymic factor, fibrinopeptide A, and in the various chromogenic peptides examined as potential substrates, the Gln-Gly dipeptide was flanked by four or less amino acids as opposed to the six upstream and five downstream residues in the standard peptide (peptide VII). The possibility cannot be excluded that these former peptides were not cleaved simply because they were not held in the active site in the correct conformation owing to the lack of flanking residues. However, since two further peptides (peptides IX and X; see Methods; each containing the target Gln-Gly dipeptide flanked by seven or five residues respectively upstream with six downstream) were not hydrolysed at all by the protease, this would seem unlikely. Some form of recognition, either in linear or spatial terms, would appear to be operative. However examination of the primary sequences flanking the putative HRV-14 cleavage sites reveals no apparent homology or motif (Callahan et al., 1985; Stanway et al., 1984), except perhaps a preference for an amino acid with a hydrophobic group (Ala, Ile or Val) in position P4. The frequency of occupation of this site by a hydrophobic residue in the cleavage sites of other picornaviruses is also apparent (Palmenberg et al., 1987; Pallansch et al., 1984; Parks & Palmenberg, 1987; Vos et al., 1987). Three of the HRV-14 cleavage sites are flanked on one side by a proline residue (2C/3A, 3A/3B and 3B/3C sites) and the corresponding peptides which were synthesized (I, VI and VII) were by far the most rapidly processed of the HRV-14 3C peptide substrates. Whether the rapid rate of cleavage of these peptides may be attributed to sequence recognition with the proline residue specifically occupying the P2 subsite, or whether the presence of the proline residue in this position may predispose the peptide to a conformation suitable for recognition and cleavage, is clearly worthy of further investigation.

In the EMCV polyprotein, cleavage by the viral 3C protease takes place at seven Gln-Gly (or Ser) sequences that are flanked by proline residues (Palmenberg, 1987) whereas four other Gln-Gly (or Ser) sequences which happen to exist but which do not have an adjacent proline residue are not processing sites. By contrast, in poliovirus, only three of the processed Gln-Gly
pairs are flanked by prolines (Pallansch et al., 1984), and experiments on the processing of CPMV and EMCV capsid protein precursor molecules altered by site-directed mutagenesis have suggested that exact proximity of a proline residue to a cleavage site is not essential for processing (Parks & Palmenberg, 1987; Vos et al., 1987). Future experiments using derivatives of the substrate peptide and mutated and cloned polyprotein precursor molecules which have been shown to be processed by the HRV-14 3C proteinase (D. S. Montgomery, D. C. Orr, C. W. Dykes & A. N. Hobden, unpublished results), should provide further information on the requirements for particular residues to occupy specific P or P' sites in order to maintain effective cleavage.

Peptides III and V were not hydrolysed upon incubation with 3C proteinase. Since preincubation of either of these peptides with 3C did not affect activity towards peptide VII, neither would appear to be able to bind to the active site of the proteinase. The potential cleavage sites within these two peptides were Gln-Ala and Glu-Gly respectively. The Gln-Ala (2A/2B) cleavage site was chosen as being the most likely processing point in the predicted region of the 2A/2B boundary (Stanway et al., 1984) although there are no reports of a Gln-Ala site being the natural cleavage junction for any picornavirus 3C proteinase. However Parks & Palmenberg (1987) showed that when the P Gly residue at the poliovirus 1C/1D processing junction was replaced by Ala, efficient processing still occurred. Peptide V was taken to represent the HRV-14 1C/1D cleavage site although in the region of the predicted boundary, the sequence Gln-Thr occurs twice and Gln-Gly once. It is not known with certainty which (if either) of these sequences is recognized for processing. Stanway et al. (1984) concluded that one of the Gln-Thr sites was used, based on the fact that this is the 1C/1D cleavage point in FMDV. Other workers favour the Glu-Gly site to be the one processed (Callahan et al., 1985) due to the number of cleavage events occurring at this type of junction in FMDV (Klump et al., 1984; Palmenberg, 1987; Robertson et al., 1985). Thus it would be interesting to examine synthetic peptides representing the possible Gln-Thr sites and assess these as potential substrates. Another area for future studies would be to ascertain whether peptides III and V are cleaved if the Gln-Ala or Glu-Gly dipeptides respectively are replaced with Gln-Gly.

It would appear that the HRV-14 3C protein is a cysteine proteinase. In order to generate activity towards the synthetic peptide substrates, the presence of a thiol compound (DTT or L-cysteine) was required. Furthermore, N-ethylmaleimide, iodoacetic acid, TPCK, TLCK and p-chloromercuriphenylsulphonic acid, all known to be inhibitors of a wide range of cysteine proteinases (Rich, 1986), were all found to block the action of the 3C proteinase efficiently. In addition, specific replacement of the cysteine residue at position 146 with serine by site-directed mutagenesis (Knott et al., 1989; D. S. Montgomery, D. C. Orr, C. W. Dykes & A. N. Hobden, unpublished results) resulted in a total loss of activity towards the peptide substrates. This identifies Cys (146) as a residue that may participate in the catalytic mechanism of the enzyme, as shown previously for the poliovirus 3C proteinase (Ivanoff et al., 1986). Apart from this Cys residue (in a -Gly-X-Cys- sequence) which is conserved together with a His residue (in position 160 for HRV-14) in the picornavirus proteinases, there is no further homology detectable between the 3C enzymes and papain or other members of the cysteine proteinase family (Berger & Schechter, 1970; Kamphuis et al., 1985; Polgar & Halasz, 1982). Therefore, it may well be that the picornavirus 3C proteinases represent a subclass of cysteine proteinases that are mechanistically similar to but homologous with papain. A further reflection of this distinction may lie in the value of the pH optimum observed for the HRV-14 3C proteinase (between pH 7.6 and 8.0), whereas that for papain and other archetypal cysteine proteinases is more commonly in the acid region below pH 7.0. The different nature of the active sites is substantiated by the observations that the HRV-14 3C proteinase was poorly susceptible to inhibition by the peptide aldehyde inhibitors (antipain and leupeptin). The competitive inhibition observed by chymostatin was also a somewhat unexpected finding since this peptide is considered primarily as an inhibitor of chymotrypsin-like serine proteinases. Similarly, a2-macroglobulin, human cystatin C and egg white cystatin, all very effective inhibitors of archetypal cysteine proteinases, did not act against the HRV-14 3C proteinase even at very high concentrations. Chicken cystatin and a2-macroglobulin have been suggested (Korant et al., 1986a, b) to have inhibitory activity towards...
the poliovirus 3C proteinase. Unfortunately however, only binding assays were performed in these studies, not direct inhibition measurements. In a plaque reduction assay, where the uptake of these high M, protein inhibitors might be problematic, chicken cystatin had a minimal antiviral effect whereas the α2-macroglobulin was ineffectual (Korant et al., 1986a, b).

Small peptides would appear to be appropriate to use as substrates since they may be able to assume a variety of structural conformations including those required for interaction with the active site of the proteinase. The substrate peptides utilized in this study permitted the rapid analysis of the proteolytic activity of the cloned HRV-14 3C protein and its demonstration as a member of the cysteine proteinase family. The peptide cleavage data coupled with observations on the 3C proteinase-mediated processing of cloned viral precursor polypeptides (D. S. Montgomery, D. C. Orr, C. W. Dykes & A. N. Hobden unpublished results) indicate that the 3C region of the HRV-14 genome represents the core sequence responsible for proteolytic processing (at Gln-Gly) of the precursor polypeptide. Using the in vitro transcription translation system with the poliovirus proteinase, Ypma-Wong et al. (1988) demonstrated that although an intact 3C coding region was sufficient to support cleavage of the expressed polypeptide between 2A/2B, 2B/2C and 2C/3A, the processing of the P1 region (cleavagies between the structural proteins 1B, 1C and 1D) was more efficiently carried out by the proteinase precursor, 3CD. They suggested that this effect may have arisen from the D sequence of 3CD making additional specific contacts with the polypeptide that stabilize the precursor–proteinase complex (Ypma-Wong et al., 1988). Such studies may begin to establish the nature of the active enzyme within the infected cells as well as the order of processing of the precursor, but parallel investigations have yet to be carried out on the rhinovirus proteinase.

The demonstration that certain synthetic peptides are cleaved under defined conditions by the HRV-14 3C proteinase now makes it feasible to undertake further studies to assess the significance of the primary sequence surrounding 3C proteinase cleavage sites through the production of new generations of peptide substrates with systematic substitutions in individual subsites. By carrying out such studies in conjunction with experiments on the processing of rhinovirus precursor polypeptides, valuable information would be obtained on the significance of secondary and tertiary protein determinants in the recognition and interaction of the proteolytic enzyme with its substrates. Interpretation of such data would be greatly facilitated by determination of the three-dimensional structure of the viral proteinase by X-ray crystallography. Such an approach may facilitate the rational design of specific inhibitors of the rhinovirus proteinase which would prevent the 3C proteinase-mediated processing of viral precursor polyproteins essential for replication whilst having no effect on other host cell proteinases.

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REFERENCES


Hydrolysis by recombinant HRV proteinase


