Identification and Characterization of Human Rhinovirus-14 3C Protease Deamidation Isoform*

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A purified recombinant human rhinovirus-14 3C protease preparation contained only ~50% active enzyme as titrated using specifically designed irreversible 3C protease inhibitors. Analysis of the purified 3C protein by isoelectric focusing showed differently charged 3C isoforms that had isoelectric points (pI) of 8.3 (55%) and 9.0 (45%), with the latter one being consistent with the predicted pI of the human rhinovirus-14 3C protein. Further analysis indicated that the pI 8.3 protein was the deamidated form of 3C, and it displayed ~10-fold reduced cleavage activity relative to the original 3C protease sample. Peptide mapping followed by sequence analysis revealed that a single asparagine, Asn-164, was deamidated to aspartic acid in the pI 8.3 isoform. Converting Asn-164 to Asp by site-directed mutagenesis resulted in a mutated 3C protease with extremely low activity, as seen with the pI 8.3 isoform, indicating a role of Asn-164 in substrate recognition and binding. In addition, the deamidated 3C protease was found to be present in vivo, and its abundance was related to the viral replication cycle. Moreover, mutant virus carrying Asp-164 showed reduced viability in infected cells. Taken together, our data suggest that 3C protein deamidation plays a role in the regulation of its enzymatic activity.

Human rhinovirus (HRV) infections are considered to be the most frequent causative agents of the common cold and various other upper respiratory tract infections (1). Rhinoviruses are members of the picornavirus family, which also includes the apthoviruses (foot-and-mouth disease virus), cardioviruses (encephalomyocarditis virus), enteroviruses (poliovirus and Coxsackie virus), and hepatitis A virus. All picornaviruses have a positive-sense, single-stranded RNA genome that is translated into a single polyprotein precursor. In the case of HRVs, the viral polyprotein is mainly processed by the viral proteases 2A and 3C to generate functional proteins and enzymes (2, 3). The 2A protease catalyzes the first cleavage between the structural and nonstructural proteins, whereas the 3C protease catalyzes most of the subsequent internal cleavages (2, 3).

The availability of active recombinant 3C protease greatly facilitated its biochemical characterization. Purified recombinant viral 3C protease was able to cleave different proteins and peptides at the bond formed between glutamine and glycine (4, 5). It has been found that 3C protease could regulate host cell function by cleaving important cellular proteins during infection (6, 7). In addition to its proteolytic activity, viral 3C protease has been shown to be a RNA-binding protein and may be involved in formation of the viral replication complex (8). As illustrated by its crystal structure, HRV 3C protease represents a novel class of cysteine protease that contains a cysteine as the active site nucleophile but is structurally like a serine protease (9–11). It has been considered to be an ideal target for antiviral intervention due to its essential role in viral replication and its unique protein structure. Various 3C protease inhibitors have been synthesized and evaluated in recent years (for a review, see Ref. 12).

Although extensive studies have been carried out on HRV 3C protease cleavage specificity and assay development, little is known about the regulation of viral 3C protease activity. Previous studies from our group showed that active recombinant HRV14 3C protease could be purified to homogeneity as shown by SDS-polyacrylamide gel electrophoresis and was able to cleave various synthetic peptides (13, 14). In this report, we describe the identification of a deamidated isoform of HRV14 3C protease present in the purified enzyme preparation and in the infected cells. Because the deamidated form of 3C protease displayed reduced cleavage activity as compared with the native form, we propose that 3C protease deamidation may be involved in the regulation of its cleavage activity.

**MATERIALS AND METHODS**

**Preparation and Identification of Recombinant HRV14 3C Protease—**

HRV14 3C protease was expressed in bacterial cells and purified as described previously (13). The identity of the purified HRV14 3C protease was confirmed by a combination of analyses including N-terminal amino acid sequencing, ion spray mass spectrometry, and SDS-polyacrylamide gel electrophoresis followed by Western blot using polyclonal antibodies raised in rabbits against the purified recombinant HRV14 3C protease. IEF gel electrophoresis was performed using precasted IsoGel Agarose IEF plates, pH 3–10. (FMC BioProducts) with the anode enriched with 0.5 M acetic acid, pH 2.6, and the cathode enriched with 1.0 M sodium hydroxide, pH 13. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard. Densitometric analysis of the protein isoforms was performed with the Coomassie Blue-stained IEF gels using a Bio-Rad GS-700 imaging densitometer.

**Inhibitor Titration of Active HRV 3C Protease—LY387838**

(Boc-E(t-Bu)VLFLVQ-O-Me) is an irreversible pepstatid 3C protease inhibitor prepared as described previously (15). Purified 3C protease (150 μl at a concentration of 0.2 mg/ml) was pre-incubated with LY387838 (concentration range: 0–0.9 nmol) in a total volume of 900 μl containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 3% Me2SO. After 30 min at room temperature, the remaining 3C protease activity was determined by a colorimetric assay, as described previously, using peptide EALFQ-p-nitroanilide (250 μM) as a substrate (14).

**Preparation and Isolation of Deamidated HRV14 3C Protease—**

The purified 3C protease preparation containing the two differently charged
3C isoforms was further fractionated onto a Mono S HR 5/5 column (Pharmacia Biotech Inc.). After washing the column with Buffer A (25 mM Tris, 1 mM EDTA, and 5 mM dithiothreitol, pH 8.0), the bound protein was eluted with a linear gradient of 0–500 mM KCl in Buffer A. To obtain a large amount of the deamidated HRV 3C isoform, we performed chemical deamidation of 3C protein as described previously (16). Briefly, purified 3C protease (5 mg) was incubated at 37 °C for 72 h in the presence of 100 mM ammonium bicarbonate, pH 9.0. After centrifugation at 16,000 × g for 10 min, the clarified 3C protein was then dialyzed against Buffer A. The converted pI 8.3 isoform was confirmed by IEF gel analysis of the treated 3C protein sample.

**Measurement of Native and Deamidated 3C Protease Activity**—The fluorogenic peptide (aminobenzoic acid)-Thr-Leu-Phe-Gln-Gly-Pro-Val-Phe(p-nitro)-Lys has been shown to be cleaved between glutamine and glycine by HRV14 3C protease (13). The cleavage reaction was performed at 30 °C for 60 min in a 400-μl mix containing 50 mM Heps, pH 7.5, 150 mM NaCl, 1 mM EDTA, 20 μM peptide substrate, and 0.3 μM 3C protease. The reaction was started with the addition of the enzyme and monitored by the fluorescence signal increase using a Perkin-Elmer LS50B luminescence spectrophotometer at an excitation of 340 nm and an emission of 415 nm. In some cases, 3C protease activity was determined using the chromogenic peptide substrate EALFQ-p-nitroanilide as described previously (14).

**Identification of the 3C Protein Deamidation Site**—Peptide mapping of native and deamidated 3C protein using trypsin was performed at 37 °C for 18 h in 100 mM Tris-HCl, pH 8.5, at an enzyme:substrate ratio of 1:20 (w/w). Digestion of viral 3C with V8 protease (Roche Molecular Biochemicals) was performed for 18 h at 25 °C in 25 mM ammonium bicarbonate, pH 7.8, with an enzyme:substrate ratio of 1:25 (w/w). Reactions containing the cleaved 3C proteins were directly applied to a reverse-phase HPLC for peptide separation. Peptide products were eluted by an acetonitrile gradient of 10–60% in 20 min and 60–90% in 6 min in 0.1% trifluoro-acetic acid with a flow rate of 1 ml/min using a C18 column (4.6 × 250 mm; 5-mm particle size). Separated peptides were then subjected to N-terminal amino acid sequencing.

**HRV14 Infection of Cultured Cells**—Confluent monolayers of cloned H-1 HeLa cells were infected with HRV14 (a gift from R. Rueckert, University of Wisconsin, Madison, WI) at a multiplicity of infection of 100 plaque-forming units/cell. After an adsorption period of 30 min at 37 °C, the infected cells were incubated at 35 °C for 6, 8, 12, and 20 h before harvesting. The collected cell pellets were washed twice with cold phosphate-buffered saline, resuspended in 200 μl of phosphate-buffered saline and 0.5% Nonidet P-40 containing protease inhibitors, incubated on ice for 15 min, and spun for 5 min at 1,000 × g. 10 μg of the crude supernatant extract from each sample was loaded onto IEF gels, and the level of 3C protein was determined by Western blot. To determine virus plaque formation, transfected cells were incubated at 35 °C for 48–72 h, fixed, and stained with crystal violet, and the numbers of plaques formed were counted (17). The transfections were done in duplicate and repeated four times. As controls, mock-infected cells were treated as described above, but excluded the addition of virus.

**Mutant Virus and 3C Protein Preparation**—Site-directed mutagenesis was performed at the 3C deamidation site by combinatorial polymerase chain reaction mutagenesis (18). The mutation of Asn-164 to Asp reached when the sample was subjected to IEF gel electrophoresis, two bands could be recognized by the polyclonal antibodies against 3C protein (data not shown). Amino acid sequence analysis of the treated 3C protein sample revealed an identical 15-residue sequence at the N terminus, consistent with the predicted 3C protein sequence. Further analysis by ion spray mass spectrometry yielded the expected molecular weight of 19,998 (data not shown). Taken together, these data indicated that purified viral 3C protein existed in two differently charged forms with very similar molecular mass. The calculated pI of the recombinant 3C protein is 9.0; therefore, we assumed that the pI 9.0 isoform was the native one. The pI 8.3 isoform showed decreased pI, and we reasoned that it was due to either a loss of positively charged groups or a gain of negatively charged amino acids in the protein. Considering the fact that

vinylogous glutamine ester were designed and synthesized as described previously (15). These Michael acceptors were found to inhibit HRV14 viral 3C protease selectively by forming stable 1:1 enzyme-inhibitor adducts (15). We used LY387838 (Fig. 1A), one of the most potent Michael acceptors against 3C protease, to titrate the active 3C enzyme present in the preparation. Incremental addition of LY387838 to 1.5 nmol of purified 3C protein preparation resulted in proportional decreases of 3C cleavage activity. A complete loss of the enzyme activity was reached when −0.7 nmol of LY387838 was added into the 3C protein sample (Fig. 1B). Because the inhibitor binds 3C protease irreversibly in a 1:1 ratio, these results indicated that only ~47% of the purified 3C protein was active. Interestingly, when the sample was subjected to IEF gel electrophoresis, two bands were identified, 55% of which had a pI of 8.3, and the remaining 45% had a pI of 9.0 (Fig. 2, lane 2).

To further elucidate the identities of the proteins present in the 3C preparation, several experiments were performed. Western blot analysis indicated that both bands separated by IEF gels could be recognized by the polyclonal antibodies against 3C protein (data not shown). Amino acid sequence analysis of the two proteins revealed an identical 15-residue sequence at the N terminus, consistent with the predicted 3C protein sequence. Further analysis by ion spray mass spectrometry yielded the expected molecular weight of 19,998 (data not shown). Taken together, these data indicated that purified viral 3C protein existed in two differently charged forms with very similar molecular mass. The calculated pI of the recombinant 3C protein is 9.0; therefore, we assumed that the pI 9.0 isoform was the native one. The pI 8.3 isoform showed decreased pI, and we reasoned that it was due to either a loss of positively charged groups or a gain of negatively charged amino acids in the protein. Considering the fact that

**RESULTS**

**Identification of Different HRV 3C Protease Isoforms**—HRV14 3C protease has been found to cleave the viral polyprotein precursor mainly at the Gln-Gly bond (4, 5). On the basis of the 3C protease cleavage specificity, peptidyl derivatives of...
the two isoforms had very similar molecular weight, we thought that the pl 8.3 isoform was generated from deamidation at the amide group of certain asparagine and/or glutamine residues of the native 3C protein because deamidation could convert an uncharged amino acid to a negatively charged residue with only a 1 Da mass difference.

To verify whether the pl 8.3 isoform was caused by deamidation, purified recombinant 3C protein sample was treated with ammonium bicarbonate to promote deamidation chemically as described previously (16). Analysis of the treated sample by IEF gels indicated that the pl 9.0 isoform disappeared along with an increase of the pl 8.3 isoform, which was shown as the major band covering over 90% of the total protein on the gels (data not shown). These data strongly suggested that the pl 8.3 isoform was generated from deamidation at certain residues of the HRV14 3C protease.

Identification of the 3C Protein Deamidation Site—HRV14 3C protease contained 182 amino acids along with 12 asparagine and 7 glutamine residues. To identify the 3C deamidation sites, ammonium bicarbonate-treated and untreated 3C protein samples were digested with trypsin or V8 protease, and the resulting peptides were separated by reverse-phase HPLC. As seen in Fig. 3, the HPLC elution profiles of the tryptic peptides generated from both treated and untreated 3C protein were similar, except that peak patterns eluted at ~19.7 min from the two samples were different. Amino acid sequencing data showed that peak 36 of the treated 3C protein corresponded to amino acid residues 156–166 (Ile-Phe-Gly-Ile-His-Gly-Gly-Asn-Gly-Arg); however, the predicted asparagine (Asn-164) was found to be its deamidated form, Asp-164 (Table I). In contrast, the corresponding peptide from the untreated 3C protease eluted as a doublet peak (peaks 30 and 31 in Fig. 3) with retention times of 19.6–19.8 min. Amino acid sequencing of peak 30 identified the expected Asn-164, but the peptide from peak 31 was found to contain only Asp-164 (Table I).

The molecular mass of these peptides was examined by mass spectrometry (Table I). The molecular mass of the peptides in peaks 30, 31, and 36 was 1126.6 and 1127.3 Da, respectively, consistent with the mass change resulting from one asparagine deamidation to aspartic acid. These data suggested that Asn-164 was deamidated in the 3C sample treated with ammonium bicarbonate, and it was also present in the untreated 3C protease sample (Table I). Using trypsin and Staphylococcus aureus V8 protease digestion, we were able to identify 17 of the 19 total potential deamidation sites in 3C protein, with two exceptions for Asn-80 and Gln-182. Of the 17 residues examined, Asn-164 was the only one found to be deamidated.

Cleavage Activity of Different 3C Isoforms—Considering that only half of the 3C protein present in the preparation was active as shown by the inhibitor titration, we decided to separate the two isoforms, the pl 8.3 and pl 9.0 forms, to examine their protease activity. Separation was performed by fractionating the mixed 3C protein on an ion-exchange column as described under “Materials and Methods.” Through which two peaks were eluted from the column. Analysis by IEF gels revealed that the first peak was free of the pl 9.0 protein, and it contained only the pl 8.3 form (Fig. 2, lane 3); however, the
second peak contained both isoforms (Fig. 2, lane 4). As seen in Fig. 4A, the purified pI 8.3 isoform showed ~10-fold reduced cleavage activity against a peptide substrate as compared with the original enzyme preparation containing both isoforms, whereas the second peak sample showed a very similar activity to the original 3C protease. Numerous attempts to isolate just the pI 9.0 form proved difficult, always contaminating with a small amount of the pI 8.3 isoform (Fig. 2, lane 4).

Examination of the chemically deamidated 3C sample for peptide cleavage activity was also performed. It showed approximately 10-fold reduced cleavage activity as compared with the enzyme treated in the absence of the chemical (data not shown). In addition, CD spectra indicated that the secondary structure of the chemically deamidated 3C protease was very similar to that of the native 3C protease (data not shown), suggesting that the reduced activity of the treated 3C sample was not the result of a denatured 3C protein. Furthermore, we generated the mutant 3C protein containing Asp-164 using site-directed mutagenesis. Analysis of the purified recombinant Asp-164 mutant 3C protein on IEF gels indicated that the native pI 9.0 form disappeared, and the mutant protein appeared as a single band with a pI of approximately 8.3 (data not shown). This mutant 3C protease showed reduced protease activity as compared with the wild type 3C protein (Fig. 4B).

Presence of the HRV14 3C Deamidation Isoform in Vivo—To determine whether or not the HRV14 3C protease was possibly deamidated in vivo, we infected HeLa cells with HRV14 and examined the extracts at different time points after infection via IEF gel separation followed by Western blot analysis. The 3C protease was detected at 6 h after infection, mostly in the native pI 9.0 protein form (Fig. 5, lane 1). However, the pI 8.3 isoform started to show at 8 h and reached approximately equal intensity compared with the pI 9.0 isoform at 12 h after infection (Fig. 5, lane 3). These results suggested that HRV14 3C protease existed as two isoforms in vivo (native and deamidated), consistent with the in vitro data.

Next, we constructed a mutant HRV14 virus carrying the Asp-164 mutation to examine the potential effect of the deamidation of 3C on viral replication. This single mutation was introduced into the HRV14 cDNA clone, transcribed into RNA, and then transfected into HeLa cells as described. The plaques formed by the wild type and mutant viruses were compared. It was found that the mutant virus showed ~71% reduction in viral plaque formation as compared with the wild type, and the mutant plaques were smaller than the wild type ones. These results indicated that the viability of the virus was directly linked to the 3C function and support our finding that Asn-164 might play an important role for regulation of 3C protease activity in vivo.

**DISCUSSION**

Determination of the concentration of active protease present in a protein sample is very important for studying protease cleavage activity or inhibition kinetics. This is often done using specific burst titrants, for example, irreversible protease inhibitors. Many cysteine proteases can be titrated using readily available E-64, an epoxysuccinyl derivative that specifically inhibits papain-like cysteine proteases (20). However, the viral cysteine protease 3C has been found to be insensitive to E-64 (5, 14), perhaps due to the unique active site conformation and protein architecture of HRV 3C. Recently, we developed a series of irreversible peptidic inhibitors targeting the HRV14 3C protease (15). These compounds, mimicking the 3C peptide substrate sequences, have been shown to be potent 3C protease inhibitors exhibiting specific antiviral activity in cell culture assays (15). Because these compounds could rapidly form 1:1 covalent complexes with the enzyme, we have explored the possibility of using these inhibitors for titration of active 3C protease present in the sample or preparation. Our data clearly show that these Michael acceptors, such as LY387838, can be used as active site titrants for rhinovirus 3C protease. These results, in combination with the IEF gel analysis data, led to the identification of the deamidated 3C protease isoform present in the recombinant protein preparation and in the HRV-infected cells.

It is worthy to note that the sequence flanking the 3C deamidation site is very conserved among different HRV serotypes as well as polioviruses. Table II shows the amino acid sequence alignment across the deamidated site Asn-164 of HRV14 3C protein among the other HRV serotypes. As revealed by the 3C NMR model formed between the protease and a peptide substrate (14), this region is involved in substrate binding, especially the interaction with the hydrophobic P4 amino acid present in the substrate. Deamidation of Asn-164 would convert the uncharged asparagine to a hydrophilic Asp residue, which would result in a loss of the efficient hydrophobic interaction between the protease and its substrate and could explain the reduced peptide cleavage activity associated with the Asp-164 mutant 3C protease.

Regulation of enzymatic activity by post-translational mod-

**TABLE I**

Amino acid sequence of isolated peptides derived from HRV14 3C protein

| HPLC peak | Source | Amino acid sequence | M<sub>r</sub> |
|-----------|--------|---------------------|-------------|
| 30        | Native | Ile-Phe-Gly-Ile-His-Val-Gly-Gly-Asn-Gly | 1126.3 |
| 31        | Native | Ile-Phe-Gly-Ile-His-Val-Gly-Asp-Gly | 1127.3 |
| 36        | Deamidated | Ile-Phe-Gly-Ile-His-Val-Gly-Asp-Gly | 1127.3 |

**TABLE II**

Sequence alignments of HRV 3C protease flanking Asn-164

| Serotype | Partial 3C amino acid sequence |
|----------|--------------------------------|
| HRV14    | -IRYDYATKlGVCQCCGVLATGK1G1SHVGGNGRQGFSAOLKQKQVFEKQ |
| HRV89    | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV9     | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV16    | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV1B    | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV1A    | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV85    | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
| HRV2     | -LYKYKTVAGCGVLYVGDSAMLKLQKQVFEKQ |
Existence of different 3C protease isoforms in HRV14-infected HeLa cells. IEF gel separation and Western blot analysis of the infected HeLa cell lysates were performed as described under “Materials and Methods.” Protein extracts from HRV14-infected HeLa cells at 6, 8, and 12 h after infection are shown in lanes 1, 2, and 3, respectively. Lane 4 is the extract from the control mock-infected HeLa cells. Isoelectric points of the standard proteins are shown on the left of the gel.

Figure 5. Cleavage activity of deamidated 3C protease. A, cleavage activity of the 3C isoforms. The purified recombinant 3C protein sample was further fractionated on a Mono S column, and the separated 3C isoforms (0.3 μM) were assayed for cleavage activity against the fluorescent peptide substrate as described under “Materials and Methods.” The enzymatic activity of the control 3C protease pre-Mono S column (○), the pl 8.3 isoform in the first peak (□), and the second peak sample containing both isoforms after the Mono S column (△) is shown. B, proteolytic activity of mutant Asp-164 3C protein (●) as compared with the wild type enzyme (○). 3C protease (0.2 μM) activity was assayed using the colorimetric peptide EALFQ-p-nitroanilide as the substrate under the conditions described previously (14). Absorbance (A405nm) of the released yellow-colored p-nitroaniline was recorded by a spectrophotometer.

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