Molecular Analysis of the Feline Immunodeficiency Virus Protease: Generation of a Novel Form of the Protease by Autoproteolysis and Construction of Cleavage-Resistant Proteases

GARY S. LACO,1 MICHAEL C. FITZGERALD,2 GARRET M. MORRIS,1 STEPHEN B. H. KENT,3 AND JOHN H. ELDER1*

Department of Molecular Biology1 and Department of Cell Biology,2 The Scripps Research Institute, La Jolla, California 92037, and Gryphon Sciences, South San Francisco, California 940803

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The feline immunodeficiency virus (FIV) protease is essential for virion maturation and subsequent viral replication in that it cleaves the Gag and Gag/Pol polyproteins at eight sites to release the respective structural proteins and enzymes. During purification of a recombinant FIV protease (PR), we noted that it underwent autoproteolysis (autolysis) to give discrete cleavage products. These additional PR cleavage sites were defined using N-terminal amino acid sequence analysis and mass spectrometry. Protease breakdown products were also found in FIV virions and were of the same apparent molecular weights as the in vitro autolysis products.

Four primary PR autolysis sites were blocked via substitution of either the P1 amino acid with a β-branched amino acid or the P1′ amino acid with lysine. Cleavage-resistant PRs which had Km and kb values similar to those of FIV PR were constructed. An autolysis time course determined that blocking all four primary autolysis sites yielded a cleavage-resistant PR which was enzymatically stable. Concomitant with autolysis is the generation of an N-terminally truncated form of the PR (Thr6/PR) which has enhanced stability with respect to that of FIV PR. A structural basis for the Thr6/PR activity is presented, as are the possible roles of autolysis in the viral replication cycle.

Feline immunodeficiency virus (FIV) is the causative agent of an immune deficiency syndrome in cats, the disease symptoms and progression of which parallel those of human immunodeficiency virus (HIV) in humans (20). The FIV genome organization and encoded proteins are also similar to those of HIV and other lentiviruses, including a symmetrical dimeric aspartic protease (PR) (34). Although differences exist relative to the presence or absence of several small open reading frames (ORFs), both FIV and HIV express the viral structural proteins and enzymes as part of the Gag and Gag/Pol polyproteins (6, 13). The N-myristoylated Gag and Gag/Pol polyproteins and the Env glycoprotein make their way to the plasma membrane of the infected cell, where the immature virion assembles and then buds off (3, 6, 10). The monomeric PR in the Gag/Pol polyproteins dimerizes during the budding process, perhaps due to an increase in the local concentration of the enzyme (23) and/or a change in the local ionic strength (4). At this point, the active PR dimer, as part of the Gag/Pol polyprotein, then processes PR subunits out of the polyproteins, resulting in maturation of the virion and activation of several key enzymes, including reverse transcriptase (13). This allows FIV, and similarly HIV, to initiate a productive replication cycle in a newly infected cell. PR is a key component of the FIV and HIV replication cycles in that it not only regulates virion maturation and activity of viral enzymes but also, with HIV and simian immunodeficiency virus (SIV), regulates its own activity in vitro through autoproteolysis (autolysis) (17, 25). However, it is not known if autolysis of either HIV or SIV PRs occurs in vivo and whether it has a role in the virus replication cycle.

In the case of pepsin, a mammalian gastric protease, autolysis is important for removal of an N-terminal inhibitory domain from thezymogen (pepsinogen), resulting in active pepsin. Subsequently, pepsin undergoes further autolysis which inactivates the protease (8, 22). Thus, autolysis controls an inherently nonspecific protease which, if unchecked, could damage intestinal tissues after secretion and activation (16, 31).

We have studied the FIV PR to determine if it undergoes autolysis in vitro and in vivo. In this report, we describe in vitro four primary autolysis sites within FIV PR and show that FIV virions also contain PR breakdown products. These autolysis sites were blocked in vitro by specific amino acid substitutions at either the P1 or P1′ position of the cleavage sites. Blocking all four autolysis sites resulted in a PR highly resistant to autolysis and enzymatically stable. In addition, we generated by autolysis a novel truncated form of FIV PR (Thr6/PR) which is resistant to subsequent autolysis and retains greater activity at high urea concentrations than does FIV PR. The role of the FIV PR three-dimensional structure in regulating autolysis is discussed.

MATERIALS AND METHODS

Protease constructs. (i) pT7-FIV PR. The FIV 34TF10 infectious molecular clone (FIV-34TF10 [30]) was used as the template in a PCR using a positive-strand primer (5’ACATATTGGACATATGGCATATAATAAGTAGTACT ACTAC3’; nucleotides [nt] 1964 to 2005) which, when incorporated into the PCR product, added an initiation Met and Ala codon to the determined 5′ Tyr codon of the FIV PR ORF (6) as well as a 5′ NdeI restriction site. A negative-strand primer (5’ATCGAGAAAACCTTTACATTACAACTGATATAAT TTS’; complementary to nt 2306 to 2345) added a stop codon after the determined C-terminal Met codon of the PR ORF (6) in addition to a 3′ HindIII restriction site, to facilitate cloning. The resulting PCR product was digested with NdeI and HindIII and ligated into pT7-7 (29), which had been digested with NdeI and HindIII, to give pT7-FIV PR.
(ii) pT7-Thr6/PR. FIV-34TF10 was the template in a PCR with a positive-strand primer (5′TATAATACATATGCTACTACATTAGAAGGG3′; nt 1982 to 2020) which added an NdeI restriction site and an initiation Met codon 5′ to the PR ORF Thr 6 codon. The negative-strand primer was the same as that used for pT7-FIV PR. A 1-μl aliquot of each PCR was analyzed by a MALDI mass spectrometer (Ciphergen Biosysm, Inc.) for analysis with a MALDI mass spectrometer (Ciphergen Biosysm, Inc.). The mass of FIV PR and each mutant PR was analyzed by API/MS and was within ±1 Da of the calculated molecular mass (data not shown).

Protease assays. (i) Km/kcat determination. The activities of the various purified PRs were assayed by using a fluorogenic peptide substrate as described previously (7). Briefly, the [PR] was 150 nM in assay buffer (50 mM sodium citrate–100 mM sodium phosphate [pH 5.25], 0.2 M NaCl, 1 mM DTT) containing substrate at concentrations above and below the Km. Assays were done in triplicate at 37°C, and data were collected by using a JASCO FP 777 spectrophotometer.

(ii) Autolysis time course. The [PR] was either 150 nM or 15 μM in 25 mM NaPO4 (pH 7.0)–0.2 M NaCl–1 mM DTT, and PR was incubated at 37°C for 1 to 48 h. Aliquots of the PR autolysis reaction mixture were either made 50% (vol/vol) glycerol at the indicated time points (Fig. 4) and stored at −20°C or centrifuged with the cocomipropriated autolysis products and PR solubilized in 50% acetonitrile for use in MALDI analysis. The 50% glycerol stock of the PR autolysis reaction mixtures were assayed for activity in 100 μl of assay buffer (50 nM PR) with fluorogenic substrate (150 μM) at 37°C for 2 min. Under these saturating substrate conditions, the initial rate of cleavage was determined and used to estimate the amount of PR activity in the reaction. The time zero initial cleavage rate was set to 100% activity for all PR. Assays were done in triplicate and repeated.

(iii) Urea inhibition assay. The PR activity was determined as in the autolysis time course except that the assay buffer contained increasing amounts of urea (0.1 to 1.6 M). The initial cleavage rate, in the absence of urea, was set to 100% activity for all PR. Assays were done in triplicate and repeated.

Virus propagation. FIV-34TF10 (30) was propagated in Crandell feline kidney cells. Supernatants from chronically infected cells were harvested at confluency, concentrated, and purified as described previously (6).

Western blots. Purified FIV virions and FIV PR in vitro-generated autolysis products were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes (Millipore). Each lane was incubated separately with polyclonal anti-PR antibody generated in rabbits against chemically synthesized FIV PR (26). The blots were washed and then again incubated separately with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Cappel) and developed by using SuperSignal ULTRA chemiluminescent substrate (Pierce). Molecular weight standards were Mark12 (Novex).

RESULTS

Protease expression. FIV protease is expressed in vivo as part of the Gag/Pol polyprotein and, after autoprocessing out of the polyprotein, has a unit length of 116 amino acids (N-terminal tyrosine, C-terminal methionine [6]). We cloned and expressed the unit-length FIV protease with the addition of a methionine and alanine at the N terminus. This resulted in FIV PR with only an additional alanine on the N terminus with respect to the wild-type PR (Fig. 1, lane 1; Fig. 2). The N-terminal methionine was removed during expression presumably by an E. coli methionine amino peptidase (2). In the case of Thr6/PR (see below), the N-terminal methionine was removed, leaving the native threonine (Fig. 2). During the concentration of the renatured FIV PR at 5°C, it was noted that the yield of full-length enzyme decreased as a function of both time and protein concentration. The autolysis products of FIV PR accumulated as a precipitate during renaturation-concentration and were removed. The protein concentration was resulted in the PR being of >95% purity as judged by analysis of SDS-polyacrylamide gels stained with Coomassie blue (Fig. 1, lane 1).

Protease autolysis sites in vitro. With HIV type 1 (HIV-1), HIV-2, and SIV, the respective proteases have been demon-
performed to determine the mass of autolysis products (see the legend to Fig. 3). Positions of full-length FIV PR and autolysis products are indicated on the right. MW, molecular weight markers (positions are indicated in kilodaltons on the left).

Fig. 1. Coomassie blue-stained SDS-polyacrylamide gel of FIV PR and autolysis products. Lane 1, FIV PR at time zero; lane 2, soluble FIV PR after 20 h at 37°C; lane 3, precipitated autolysis products of coprecipitated FIV PR after 20 h at 37°C. Positions of full-length FIV PR and autolysis products are indicated on the right. MW, molecular weight markers (positions are indicated in kilodaltons on the left).

Autolysis-resistant proteases. To block the N-terminal and Flap autolysis sites in the FIV PR, we used a strategy similar to that used for HIV-1 PR (17, 25) in which the cleavage site P1 amino acids were substituted with b-branched amino acids that are not permitted in the HIV-1 PR S1 substrate binding pocket due to steric hindrance (Fig. 2, top) (5). However, for the FIV PR C-terminal Q83/C84 autolysis site, it was noted that HIV-1 PR Lys 70 is found at the same three-dimensional position as the FIV PR C-terminal cleavage site P1’ Cys 84 (9, 27). As a result, the C-terminal Q83/C84 cleavage site P1’ Cys was substituted with Lys, which is not found in retroviral aspartic protease S2-S2’ substrate binding pockets (Fig. 2, top) (21).

To determine the effect of blocking autolysis sites on PR activity, four mutant PRs were constructed and tested (Fig. 2, top): the Flap N55/M56 cleavage site P1 asparagine was substituted with threonine (N55T/PR); the N-terminal G5I and Flap cleavage site mutations were combined (2X/PR) and the N-terminal, Flap, and C-terminal C84K cleavage site mutations were combined (3X/PR). In addition, the PR ORF was truncated by five codons to express the N-terminally truncated form of the PR (Thr6/PR) (Fig. 2).

The FIV PR and mutant PRs were incubated (37°C, pH 7.0, 0.2 M NaCl) and tested over 16 h (Fig. 6) for activity with the fluorogenic substrate in order to quantitate remaining PR activity (Materials and Methods). FIV PR lost ~65% activity in 16 h, with accumulation of the corresponding C-terminal (A2 and A3) and Flap (A4 and A5) cleavage products as determined by SDS-PAGE (Fig. 1, lane 3) and MALDI/MS (Fig. 3A). In the case of N55T/PR, in which the Flap cleavage site was blocked, there was a loss of only ~25% activity in 16 h (Fig. 6). However, the MALDI/MS data indicated that N55T/PR cleaved at the N-terminal cleavage site, resulting in the N-terminally truncated form of the PR (Thr6/PR, autolysis product A1 [Fig. 2]), as well as at the C-terminal Q83/C84 and N88/V89 cleavage sites, giving the respective A3 and A2 autolysis products (Fig. 2 and 3). In the case of the double mutant 2X/PR, both the mutated N-terminal and Flap autolysis sites were protected, resulting in a PR which lost ~25% activity over the 16-h time course (Fig. 6). The C-terminal cleavage sites in 2X/PR were not protected (Fig. 3A). The mutant 3X/PR, which contained the three blocked autolysis sites, was highly resistant to autolysis, with no significant loss in activity after 16 h (Fig. 3A and 6). Interestingly, the C84K mutation, which blocked the Q83/C84 site, also blocked the N88/V89 site in 3X/PR (Fig. 3A). The D30N/PR active-site mutant served as a negative control in that it can bind substrate (15a) but is inactive and so cannot generate autolysis products (Table 1; Fig. 3C).

Protease enzymatic activity. The kinetic parameters $K_{\text{m}}$ and $k_{\text{cat}}$ were determined for the FIV PR and mutant PRs. The values obtained for the autolysis-resistant mutants were similar to those for FIV PR, with one exception: Thr6/PR had an increase in $k_{\text{cat}}$ (Table 1) and was resistant to autolysis (Fig. 6). To test if the Thr6/PR increase in $k_{\text{cat}}$ was due to an increase in the PR dimer/monomer ratio, FIV PR, Thr6/PR, and 3X/PR were assayed for activity in increasing concentrations of urea. Thr6/PR retained four times more activity than FIV PR and 3X/PR when 1.2 M urea was included in the assay buffer (Fig. 6).

DISCUSSION

The FIV protease is activated during budding of immature virions, resulting in the maturation of infectious virus, and may play other roles in post-virion maturation events (1, 24). In this study, we have demonstrated that the FIV PR undergoes au-
tolysis in vitro. To confirm that the autolysis that we observed with the PR was not due to expression in *E. coli* or of high in vitro concentrations of enzyme, we performed three experiments to address these issues. First, we showed that an inactive mutant PR (D30N/PR) expressed and purified under identical conditions did not undergo autolysis (Fig. 3C). Thus, FIV PR autolysis is not due to a contaminating *E. coli* protease. Second, we analyzed purified FIV virions by Western blot analysis to determine if similar autolysis products could be identified during normal virus expression. The results, shown in Fig. 5, demonstrated that PR breakdown products of the same apparent molecular weights as autolysis products generated in vitro were present in virions. Third, we performed autolysis reactions with PR at concentrations ranging from 15 μM to 150 nM. The results show that the same autolysis products were generated regardless of concentration (Fig. 3A and B), supporting the conclusion that PR autolysis involves specific recognition of high-affinity cleavage sites.

Autolysis occurs at four primary sites in FIV PR: (i) near the N terminus (G5/T6), (ii) in the Flap (N55/M56), (iii) in the C terminus (Q83/C84), and (iv) in the C terminus (N88/V89) (Fig. 2 and 4A). Cleavage in the Flap and C terminus results in...
inactivation of the PR (autolysis products precipitate [Fig. 1, lane 3]) and may be important in degradation of the PR in vivo, while cleavage near the N terminus (which removes five native amino acids from the FIV PR) results in a truncated Thr6/PR (Fig. 2 and 3A) which is resistant to autolysis (Fig. 6) and retains four times more activity than FIV PR in 1.2 M urea (Fig. 7). These findings are consistent with Thr6/PR having increased dimer stability and likely explain the increased observed in $k_{cat}$ (Table 1).

Analysis of the autolysis-resistant PRs determined that autolysis sites were blocked with the respective P1 or P1′ amino acid substitutions. The N55/M56 Flap cleavage site was blocked in N55T/PR, though autolysis was predictably not blocked at the unmodified N-terminal and C-terminal cleavage sites (Fig. 3A). In addition, Thr6/PR (autolysis product A1 [Fig. 2]) accumulated in the N55T/PR autolysis reaction, suggesting that blocking the Flap cleavage site made the N-terminal cleavage site more favorable (Fig. 3A). The accumulation of Thr6/PR with a higher $k_{cat}$ in the N55T/PR autolysis reaction may explain why even though the N55T/PR is breaking down (Fig. 3A), the reaction retains significantly more activity over the 16-h autolysis time course than FIV PR (Fig. 6). Blocking all four primary autolysis sites in 3X/PR results in an enzyme which is similar in kinetics and stability in urea to FIV PR (Table 1; Fig. 7) but is highly resistant to autolysis (Fig. 3A and 6). Since evolution could have yielded a PR resistant to autolysis, with retention of wild-type activity, these results imply that the autolysis sites may have been positively selected for in the PR for activity regulation.

Since purified FIV virions contain PR breakdown products similar in mass to the in vitro-generated autolysis products (Fig. 5), we conclude that PR autolysis may play a role in regulation of PR activity in vivo. However, since the N-terminal G5/T6 autolysis site is cleaved slowly to generate Thr6/PR, it may represent a remnant cleavage site that had been used to cleave the N terminus of the PR out of the Gag/Pol polypro-
the corresponding C-terminal oxygen (COO\(^{-}\)) of cleavage.

In the case of pepsin, a mammalian gastric protease, the inactive zymogen (pepsinogen) is secreted from the cells lining the stomach. An acidic environment in the gut then activates the zymogen to autoprocess near the N terminus to give the unit-length pepsin (8, 11, 22). While autolysis results in degradation of pepsin in vitro (8, 22), it may also be important in removing the inherently nonspecific protease from the gut and so minimizing degradation of the cells lining the stomach (16, 31). Similar to pepsinogen, viral aspartyl proteases are also activated due to a change in the local environment, resulting in the autoprocessing of the PRs out of a polyprotein (4, 23).

Subsequently, the unit-length HIV-1, HIV-2, and SIV PRs have been shown to undergo autolysis, initially near the N terminus, resulting in inactivation of the PRs in vitro (17, 25). In the case of FIV PR, cleavage near the extended N terminus increases the activity and stability of the PR (Table 1; Fig. 6).

The remaining autolysis sites in FIV PR are located in β sheet/hairpins/β sheets (β hairpins), perhaps making them accessible in the monomer. Several autolysis sites in the HIV-1, HIV-2, and SIV PRs are also in β hairpins (25).

With FIV, roles of the PR after virion maturation have not been described. However, HIV-1 PR has been shown to degrade inhibitor-resistant forms of HIV-1 reverse transcriptase in mature virions, resulting in decreased viral infectivity (18). HIV-1 PR can also partially degrade the Nef protein in virions (33). While equine infectious anemia virus PR can degrade the nucleocapsid protein in virions in vitro, this could affect initiation of reverse transcription in vivo (19). Together, these results indicate that retroviral PRs can partially degrade key viral proteins in the virion and suggest that inactivation of the PR in vivo through autolysis may limit further degradation of the mature viral proteins. The fact that FIV virions contain PR breakdown products lends support to autolysis playing a role in PR inactivation in vivo.

During virion entry and initiation of the replication cycle in a newly infected cell, the continued presence of 100 to 200 PRs/virion within the cell may ultimately have an adverse effect on virus replication. The ability of retroviral PRs to undergo autolysis could be important in removing the PR activity so that de novo-synthesized Gag and Gag/Pol polyproteins are not prematurely processed by the exogenous PR from infecting virus but are targeted to the plasma membrane for virion assembly (12, 14, 15). The role of autolysis in inactivation of FIV PR can now be tested ex vivo by expressing autolysis-resistant PRs, using recombinant FIVs in cell cultures.

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**FIG. 6.** FIV PR and mutant PR activities at indicated time points during the autolysis assay.

**FIG. 7.** Effect of urea concentration on FIV PR and mutant PR initial rates of cleavage.

**TABLE 1. FIV PR and mutant PR activities**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Mean ± SD</th>
<th>( k_{\text{cat}} )</th>
<th>( K_{m} )</th>
<th>( k_{\text{cat}}/K_{m} )</th>
</tr>
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<tbody>
<tr>
<td>FIV PR</td>
<td>0.38 ± 0.02</td>
<td>33 ± 5</td>
<td>0.0115 ± 0.0010</td>
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<tr>
<td>N5ST/PR</td>
<td>0.36 ± 0.05</td>
<td>49 ± 10</td>
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<tr>
<td>2X/PR</td>
<td>0.34 ± 0.04</td>
<td>45 ± 6</td>
<td>0.0075 ± 0.0005</td>
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<tr>
<td>3X/PR</td>
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<td>53 ± 12</td>
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</tr>
<tr>
<td>Thr6/PR</td>
<td>0.60 ± 0.05</td>
<td>37 ± 4</td>
<td>0.0162 ± 0.0005</td>
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<tr>
<td>D30N/PR</td>
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\( * \) ND, not detected.