ABSTRACT  Processing of the gag and pol gene precursor proteins of retroviruses is essential for infectivity and is directed by a viral protease that is itself included in one of these precursors. We demonstrate here that small synthetic peptides can be used as both model substrates and inhibitors to investigate the specificity and molecular parameters of the reaction. The results indicate that a peptide that extends five amino acids but not three amino acids in both directions from a known cleavage site is accurately hydrolyzed by the protease of avian sarcoma–leukosis virus. Substitutions of the amino acids to either side of the peptide bond to be cleaved affect the ability of the peptide (as well as a larger precursor protein) to serve as a substrate. The specificity is more stringent for the amino acid that will become the carboxyl end after cleavage. Some substitutions produced peptides that were not cleaved but could act as inhibitors of cleavage of a susceptible peptide. Thus, small model substrates may be used to explore both the binding and catalytic properties of these important proteases.

The dimeric RNA genomes of retroviruses are encapsidated in a symmetric icosahedral core that is surrounded by a lipoprotein envelope (1). Translation of the viral genes gag and pol yields precursor polypeptides that are cleaved to produce the mature forms of the proteins found in virions (1). Processing has been shown to be directed by a retrovirus-encoded protease (PR) in avian sarcoma–leukosis viruses (ASLV), murine leukemia virus (MuLV), and human immunodeficiency virus (HIV) (2–17). The PR and analogous domains of yeast Ty elements, Drosophila copia-like elements, cauliflower mosaic virus, and hepatitis B virus contain highly conserved amino acid sequences that are homologous with a sequence in the active site of the aspartic protease family of cellular proteins (18–20).

The sites of PR-catalyzed cleavage in several retroviral polypeptide precursors have been deduced by N- and C-terminal sequencing of mature processed products. Although some sequences occur frequently in known cleavage sites, none appears sufficiently unique to account for the specificity exhibited in the processing reaction. In this report we use small synthetic peptides as model substrates to investigate the requirements for cleavage by the ASLV PR.

Small peptides seemed appropriate to use as substrates for the retroviral PR because they are able to assume a wide variety of structural conformations, some of which are likely to resemble those that normally interact with the PR active site. It has been postulated that conformational mobility, per se, is characteristic of sites involved in protein–protein interactions (21). If so, peptides should provide better models than larger molecules of unknown structure which might be folded into a conformation not appropriate for cleavage.

The results presented here show that a decapptide composed of the cleavage site between the α and pp32 domains of the ASLV reverse transcriptase–integration polyprotein (pol) can be cleaved at the expected site by PR. Changes in the amino acids that flank the cleavage site of the peptide (and of the larger pol precursor) alter the capacity of the peptides to serve as substrates. Some peptides that are not cleaved act as inhibitors of PR.

MATERIALS AND METHODS

PR Digestion of pol Precursor Proteins Synthesized in Escherichia coli. Lysates of induced E. coli containing the pFA1-RT99 expression clone (or mutant clone) were prepared as described (22). Approximately 30 μg of purified avian myeloblastosis virus (AMV) PR was added to a 1-ml lysate prepared from 10 OD600 units of bacterial cells. The purification of AMV PR and its reaction conditions were as described previously (22). After incubation, the samples were mixed with protein gel sample buffer and boiled for 3 min. The lysates were fractionated on a NaDodeSO4/10% polyacrylamide gel and transferred to nitrocellulose, and polypeptides were detected by using an antiserum specific for a peptide in the α domain of reverse transcriptase (22).

Oligonucleotide Mutagenesis. The codons for the dipeptide cleavage site (Tyr-Pro) between α and pp32 in pFA1-RT99 were altered using oligonucleotide mutagenesis to generate a predicted Ile-Asp dipeptide cleavage site (Fig. 1A). The oligonucleotide-directed mutagenesis method described by Morinaga et al. (23) was used. The mutagenizing oligonucleotide was a 30-mer (5'-ACCTTCCAGCGATCGATTTGAAAGCGATCATCGATTTGAAAGCAATCGATCTGATTTGAAAGCGATCATCGATTTGAAAGAATCGATCTGATTTGAAAGCGATCA-3') complementary to the coding strand.

Decapeptides. Decapeptides were synthesized by the solid-phase method (24). Deprotection and cleavage from the resin were achieved by treatment with anhydrous hydrogen fluoride according to the procedure of Tam et al. (25). The peptides were purified by HPLC using a μBondapak C18 column (Waters Associates). The purity of the peptides was confirmed by analytical HPLC, amino acid analysis, and microsequencing.

Peptide Proteolysis by PR. Proteolytic reactions were carried out in 10 μl of 0.1 M sodium phosphate buffer (pH 6), 1.1 mM decapptide as substrate at 37°C for 20 min. Reactions were stopped by freezing in dry ice/ethanol.

Abbreviations: PR, retroviral protease; AMV, avian myeloblastosis virus; ASLV, avian sarcoma–leukosis virus; pol, retroviral reverse transcriptase and integration polyprotein; MuLV, murine leukemia virus; HIV, human immunodeficiency virus; TLE, thin-layer electrophoresis.

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Fig. 1. Immunoblot analysis of wild-type and mutant bacterially produced ASLV pol-encoded proteins after incubation with the ASLV PR. (A) The product of the E. coli expression vector pFA1-RT99, which encodes a 99-kDa pol product, p99pol, which includes the α (63-kDa), β (95-kDa), and pp32 endonuclease domains. Cleavage to generate pp32 occurs between the indicated Tyr and Pro residues. (B) A lysate was prepared as described (22) from an E. coli culture that was induced for expression of p99pol. AMV PR was added and the mixture was incubated for the indicated times. Samples were electrophoresed on a NaDodSO4/10% polyacrylamide gel and pol-specific peptides were identified by immunoblotting using an antiserum raised against a peptide from the α domain, as described previously (22). No specific cleavage was seen in samples incubated in the absence of PR, as described previously (22) (data not shown). (C) A parallel lysate was analyzed that contained the indicated mutation at the p99pol cleavage site. The mutation Tyr-Pro to Ile-Asp was introduced into pFA1-RT99 by using oligonucleotide mutagenesis.

HPLC Assay. Frozen reaction samples were diluted with 200 μl of distilled water and loaded on an LC-318 octadecyl-dimethylsilyl column (4.6 × 250 mm; Supelco, Bellefonte, PA). Elution was carried out by using a gradient of 20–60% (vol/vol) acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min⁻¹. Absorbance at 210 or 280 nm was recorded.

Thin-Layer Electrophoresis (TLE) Analysis of Decapeptide Cleavage by PR. Aliquots (2 μl out of 10-μl reaction mixtures) were spotted on TLC plates (plastic cellulose sheets Art. 5577, 20 × 20 cm; E.M. Reagents). TLC plates were subjected to 45 mA for 1 hr in pyridine/acetic acid/aceton/ distilled water (1:2:8:40, vol/vol). The plates were air dried and then sprayed with 1% triethylamine (Fierce) in acetone followed by fluorescamine (Hoffmann-La Roche) at 0.1 mg/ml in acetone. Photographs were taken under UV light.

RESULTS

Cleavage of ASLV pol-Encoded Precursor Synthesized in Bacteria. We have been investigating the mechanisms that govern the recognition and cleavage of proteins and peptides by the 15-kDa protease (PR) of ASLV. Our initial studies, using as substrate a pol precursor produced in bacteria (22), showed that the bacterially produced 99-kDa pol-encoded polypeptide (p99pol) can be cleaved very slowly by the purified protease to yield products that migrate like the 95-kDa (β) and 63-kDa (α) subunits of reverse transcriptase and the 32-kDa endonuclease, pp32 (illustrated in Fig. 1A). This cleavage is demonstrated in Fig. 1B by the disappearance of the precursor and concomitant appearance of a new band just above a serum-reactive background band at about 63 kDa. To evaluate the importance of amino acids that flank the predicted cleavage site, we prepared a second construct, in which the nucleotides coding for the Tyr-Pro sequence that flanks the α/pp32 cleavage junction were converted to Ile-Asp codons. The mutant p99pol polypeptide was resistant to cleavage by PR even after 5 hr of incubation (Fig. 1C). These wild-type and mutated pol sequences were then introduced into plasmids containing infectious viral DNA and transfected into chicken cells (26). The wild type produced infectious virus, while the mutant, containing the Ile-Asp sequence, was replication defective (R.A.K., unpublished observations). This suggested that cleavage at the α/pp32 domain junction is required for pol function. However, the alternative explanation, that the amino acid substitutions affected reverse transcriptase pol endonuclease activities, could not be ruled out.

Cleavage of Peptides by PR. We have used synthetic peptides, rather than large precursor polypeptides, as substrates to directly investigate the sequence requirements for cleavage by ASLV PR. Fig. 2A shows that the ASLV PR can cleave a decapptide in place of the sequence at the α/pp32 cleavage site in the ASLV pol precursor (see Table 1, peptide 1). Two peptide products, distinct from the starting material, were resolved by HPLC. Amino acid composition analysis of these peptides, referred to as P1 and P2, verified that they contained equimolar amounts of Pro, Leu, Arg, Glu, and Ala (P1) and Thr, Phe, Gln, Ala, and Tyr (P2). These results indicate that cleavage of the peptide occurred between Tyr and Pro residues. Fig. 2Ba shows the kinetics of cleavage of this decapptide. These data provided a baseline for comparing rates of cleavage of five additional decapetides, which varied by a single amino acid to either side of the cleavage site (Table 1, peptides 2–6). Fig. 2Bc shows that replacement of Tyr by the aromatic amino acid Phe, yielded a good substrate, but replacement by another hydrophobic residue, Ile, did not. This is consistent with the lack of cleavage of the Ile-Asp sequence observed in bacterially produced p99pol (Fig. 1). A fourth decapptide in which Tyr was replaced with the neutral or weakly hydrophilic Ala residue was also not cleaved. Thus, there appears to be a strong requirement for an aromatic residue at position −1 for cleavage of this decapptide. Replacing the Pro at position +1 with a neutral Gly or an acidic Asp (Fig. 2Bb) reduced the efficiency of cleavage by 80–90% but did not abolish it. Thus, in this α/pp32-derived decapptide, there seems to be less specificity for the amino acid at the +1 position than at −1. Estimates of the relative activity of the PR on each of the peptides tested are summarized in Table 1. Similar values were obtained in other experiments in which 10 times the amount of enzyme was used. The minimal turnover number of ASLV PR cleavage of the Tyr-Pro decapptide, estimated from the data shown in Fig. 2B, is 1–3 min⁻¹.

A Convenient Assay for Cleavage of Peptides. The results obtained with HPLC were confirmed qualitatively by a simpler TLE assay (Fig. 3). The peptide products were
visualized under a UV lamp after treatment of the plates with fluorescamine. Under these conditions, peptides fluoresce unless there is an N-terminal Pro, which produces chromophores that absorb UV light and thus quench the fluorescence (27, 28). The results in Fig. 3 show that PR digestion of synthetic peptides containing Tyr-Pro and Phe-Pro at the cleavage sites both yield two products, one that fluoresces and one that absorbs UV light. These results confirm that a peptide with an N-terminal Pro was produced by PR cleavage. Digestion of peptides containing Tyr-Gly or Tyr-Asp at the same site yields two products, both of which yield fluorescent spots. The identities of products detected on TLE plates were verified by chromatography with products isolated by HPLC (not shown). Thus, TLE is a quick, convenient, and sensitive method to detect peptide hydrolysis.

**Inhibition by Peptides That Are Not Cleaved.** Using the TLE assay, we next asked whether the decapetides that are resistant to ASLV PR digestion are inhibitors of the enzyme. Various amounts of decapetides containing the Ala-Pro and Ile-Pro sequences were added to a reaction mixture with a constant amount of the Tyr-Pro substrate. The results (Fig. 3B) showed that proteolysis was inhibited by approximately

**Figure 2.** Cleavage of decapetides by PR. (A) Typical reverse-phase HPLC analysis of PR-generated products. Ten micrograms of the Tyr-Pro decapetide (8.3 nmol) was incubated at 37°C for 20 min in 10 μl with 0 μg (a), 1 μg (b), or 20 μg (c) of purified AMV PR. Reactions were stopped by freezing in dry ice, then the mixtures were diluted with distilled water to 200 μl and loaded on an HPLC column. (B) Comparison of the cleavage rates of six decapetides that varied in amino acid composition at the cleavage sites. Samples (25 nmol) of decapetide substrates were incubated with 7 pmol of PR at 37°C in 5 μl of 0.1 M sodium phosphate buffer, pH 6. Samples were taken at the indicated times and analyzed on HPLC. At the latest time point, less than 3% of the substrate was hydrolyzed. The minimum turnover number, estimated from the last three time points in a, was in the range 1-3 min⁻¹. (a) Time course of cleavage of Tyr-Pro decapetide as expressed by the production of the Tyr C-terminal (P2; o) and the Pro N-terminal (P1; ●) pentapeptides. (b) Production of C-terminal Tyr pentapeptides (P2) derived from Tyr-Pro (●), Tyr-Asp (○), and Tyr-Gly (□) decapetides. (c) Production of pentapeptides containing an N-terminal Pro (P1) derived from Tyr-Pro (●), Phe-Pro (□), Ala-Pro (○), or Ile-Pro (□). The absorbances of P1 and P2 at 210 nm differ, resulting in different slopes. The percentages of pentapeptide released are equimolar when correction for the difference in absorbance is included.

**Table 1.** Relative cleavage rates and inhibitory properties of synthetic peptides

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Sequence</th>
<th>Relative cleavage rate</th>
<th>Inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-Thr-Phe-Glu-Ala-Tyr-Pro-Leu-Arg-Glu-Ala-OH</td>
<td>1.00</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>Thr-Phe-Glu-Ala-Pro-Leu-Arg-Glu-Ala</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Thr-Phe-Glu-Ala-Phe-Pro-Leu-Arg-Glu-Ala</td>
<td>0.76</td>
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<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Thr-Phe-Glu-Ala-Tyr-Glu-Leu-Arg-Glu-Ala</td>
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<td>Yes</td>
</tr>
<tr>
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<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Ac-Glu-Ala-Tyr-Pro-Leu-Arg-NH₂</td>
<td>0</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The arrow indicates the PR cleavage site. The relative cleavage rates were calculated from the HPLC data of Fig. 2B. The ability of the peptides to inhibit the cleavage of peptide 1 (Tyr-Pro) was determined qualitatively on TLE as shown in Fig. 3. Ac, acetyl; 0, little or no cleavage (limit of detection ≤ 0.02).
half in the presence of an equal amount of the Ile-Pro decapeptide but not appreciably affected by as much as 20-fold excess of the Ala-Pro decapeptide. The subtlety of the difference between these two peptides suggests a highly specific binding mode for the Ile-containing one. While this specificity might conceivably reflect differences in conformational potential of the two peptides, it seems more likely, considering the flexibility of small peptides, that it reflects a better fit of the Ile side chain into the Tyr 'pocket.' Table 1 also summarizes results of an analysis with a hexapeptide that contains the Tyr-Pro sequence (Table 1, peptide 7). This molecule was not cleaved and was only a weak inhibitor of cleavage of the analogous decapeptide (data not shown). This suggests that, for the peptides tested, the minimal size for cleavage is probably longer than six amino acids. Nonetheless, in preliminary experiments (not included here) we have found that another hexapeptide corresponding to a sequence in HIV that also includes a Tyr-Pro processing site is a relatively poor substrate for ASLV PR but almost as potent an inhibitor of cleavage of the ASLV-derived Tyr-Pro decapeptides as the Ile-Pro ASLV decapeptide. This shows that binding capacity and Tyr-Pro are not sufficient for catalysis.

DISCUSSION

Previous studies have shown that a synthetic dodecapeptide, corresponding to the sequence at a cleavage site on the gag precursor, can be accurately hydrolyzed by partially purified AMV PR (29). Our results show that a decamer that contains the sequence of a known cleavage site in pol is also processed correctly. Thus, in both cases, the minimum requirement for cleavage resides near the cleavage site. We have also shown that changing the two amino acids involved in the pol target peptide bond dramatically alters the ability of a susceptible peptide as well as a large protein substrate (p99pol) to be cleaved.

A consensus cleavage sequence for retroviral proteases has been proposed on the basis of comparison of the amino acids adjacent to known sites of human T-cell leukemia virus I and MuLV retroviral precursor proteins (30). Recently Pearl and Taylor (31) have noted a strongly conserved pattern encompassing amino acids −3 to +1. Our own computer analyses (using methods described in refs. 32–34) of the sequences at 27 protease cleavage sites from various retroviruses, including all of the known sites in ASLV and HIV, also show a generally hydrophobic pattern. However, no common secondary structure is evident in these sites within or between proteins from different viruses. Certain dipeptide sequences do appear frequently at the cleavage site. For example, Tyr-Pro is found in gag and pol cleavage sites in avian, human, and murine viruses. In fact, the dipeptide sequences Tyr-Pro and Phe-Pro comprise ~25% of the cleavage sites examined. However, this dipeptide sequence is represented only once among the known ASLV cleavage sites. And, as we have shown, even though a small peptide serves as a substrate, its recognition is complex.

The estimated turnover number for ASLV PR cleavage of the Tyr-Pro decapeptide is rather low. It has been observed previously that this enzyme also cleaves larger protein substrates inefficiently. We note that in ASLV the protease domain is included in the gag precursor and is therefore present in the virion in amounts equal to the viral structural proteins. The molar ratio of enzyme to target sites is thus on the order of 1:6. In mammalian viruses, where the protease is expressed as a part of the gag-pol precursor, this ratio would be about 1/10th as high. It will be of interest to determine if the turnover numbers of mammalian virus PRs are proportionally higher.

Earlier reports have shown that the ASLV enzyme can process the MuLV (15–17) and feline leukemia virus (35) gag polypeptide precursors to products that migrate like the authentic virion components. Our preliminary results (not included here) suggest that the ASLV PR can also hydrolyze two peptides that contain HIV cleavage sites (Val-Ser-Gln-Asn-Tyr↓Pro-Ile-Val-Gln-Asn and Tyr-Cys-Thr-Leu-Asn-Phe↓Pro-Ile-Ser-Pro-Ile). Because of this apparent cross-specificity, the readily available ASLV protein may serve as a useful screen for general inhibitors that affect other PRs. Our results suggest that binding of a peptide to protease is independent of cleavage. Thus, it seems possible that a peptide derivative or peptide mimic with high binding affinity (but no substrate activity) could be an effective inhibitor of this reaction. The recent report of PR inhibition by the aspartic protease inhibitor pepstatin supports this prediction (36). Such inhibitors might have therapeutic value for the treatment of retrovirus-induced disease, if interference with cell protease function can be avoided.

The synthetic peptides will provide an extremely useful tool to study the requirements for cleavage of a protein by the purified PR as described above. However, the PRs are synthesized in cells as part of larger precursor polypeptides, in which form they are incorporated into budding virions. A preliminary analysis of a bacterially produced precursor polypeptide suggests that ASLV PR can act autocatalytically to release itself from such a precursor (M.K. and R.A.K., unpublished data). The question of whether peptide analogues or mimetics are effective inhibitors of such autocatalysis is of importance for evaluating their potential as antiviral agents. A comparison of the kinetic parameters of the autocatalysis and the reaction of the mature PR on peptide substrates should provide further insight into the mechanism of this important class of enzymes.
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