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The Dimer Interfaces of Protease and Extra-Protease Domains Influence the Activation of Protease and the Specificity of GagPol Cleavage

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Activation of the human immunodeficiency virus type 1 (HIV-1) protease is an essential step in viral replication. As is the case for all retroviral proteases, enzyme activation requires the formation of protease homodimers. However, little is known about the mechanisms by which retroviral proteases become active within their precursors. Using an in vitro expression system, we have examined the determinants of activation efficiency and the order of cleavage site processing for the protease of HIV-1 within the full-length GagPol precursor. Following activation, initial cleavage occurs between the viral p2 and nucleocapsid proteins. This is followed by cleavage of a novel site located in the transframe domain. Mutational analysis of the dimer interface of the protease produced differential effects on activation and specificity. A subset of mutations produced enhanced cleavage at the amino terminus of the protease, suggesting that, in the wild-type precursor, cleavages that liberate the protease are a relatively late event. Replacement of the proline residue at position 1 of the protease dimer interface resulted in altered cleavage of distal sites and suggests that this residue functions as a *cis*-directed specificity determinant. In summary, our studies indicate that interactions within the protease dimer interface help determine the order of precursor cleavage and contribute to the formation of extended-protease intermediates. Assembly domains within GagPol outside the protease domain also influence enzyme activation.

Activation of the human immunodeficiency virus type 1 (HIV-1) protease (PR) is a critical step in the assembly of HIV-1. The structural and enzymatic proteins that comprise the virus core are initially translated as part of the Gag and GagPol polyprotein precursors. Accurate and ordered processing of these precursors is an essential step in the production of infectious viral particles (20, 24, 34, 38, 51). Processing of the precursors is accomplished by the viral protease, which is also translated as part of the GagPol precursor (19, 36). As is the case for all retroviruses, HIV-1 protease is an aspartic protease and is functional only as a dimer (30). The active site of viral protease contains two aspartic acids, each one contributed by monomers that combine to form the dimeric enzyme (35). Roughly half of the interactions that maintain the mature protease dimer occur in a region known as the dimer interface (50). This is made up of four interdigitating N- and C-terminal residues of the two monomers (residues 1 to 4 and residues 96 to 99) that form a four-stranded β-sheet (50, 52).

As all of the cleavages within GagPol are accomplished by the viral protease itself, without assistance from a cellular protease, the protease embedded within GagPol must dimerize and be active as part of the GagPol precursor (36). Consequently, the initial cleavages are carried out by the precursor-associated immature protease. This includes the cleavages that release the mature protease itself. Presumably, it is this fully processed, mature protease that is responsible for the later cleavages. Therefore, during virus assembly, the active dimeric enzyme originates as the result of the dimerization of two GagPol precursors. Once the protease domain is liberated from the precursors by cleavage at its N and C termini, a free, mature dimer, consisting of two protease monomers, is produced (45).

Despite the wealth of structural data regarding the mature protease dimer, there is little information about the structure of the immature protease dimer that is produced by dimerization of two GagPol precursors. Further, little is known about the changes that accompany the shift from precursor-associated dimer to free enzyme. Finally, the protease must dimerize within the context of precursor dimerization, and several dimerization and oligomerization domains within GagPol have been characterized (5, 7, 13–15, 21, 41, 48). However, neither the contribution of assembly domains outside the protease to enzyme activation nor the mechanism by which enzyme activation is controlled has been fully assessed.

Using an in vitro system in which the full-length GagPol precursor is expressed and cleaved by the endogenous HIV-1 protease, we characterized the initial cleavages of GagPol and the role of the dimer interface in the activation of the protease. We also compared the effect of protease dimer interface substitutions on enzyme activity in the mature, free dimer versus that found in the immature, precursor-associated enzyme. Further, we characterized interactions within the protease dimer interface that define the specificity of precursor cleavage. We found that cleavage of the GagPol precursor occurs sequentially and results in the formation of extended protease intermediates. In addition, particular residues of the dimer inter-

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face have a role in protease activation as well as the specificity of GagPol cleavages. Finally, our studies suggest that assembly domains within GagPol but outside the protease contribute to protease dimerization and activation.

MATERIALS AND METHODS

Plasmid construction and mutagenesis. Plasmid pMono expresses wild-type or mutated monomeric protease from the *tac* promoter (2) in *Escherichia coli* and was derived from plasmid P1+IQ (3). Protease sequences in P1+IQ were replaced by a linker of 5′ *Xho*I-*Xba*I-*Sal*I-*Pst*I 3′ to produce p1CVx. Wild-type or mutated protease sequences were obtained from pET-PR by PCR with primers 5′Xho (AATATA-*Xho*I-GAAGGAGATATACAT) and 3′Xba (ATAAAT-*Xba* I-CTTGGGCTGCAGGG) and were inserted into p1CVx to produce pMono. The phagemid pET-PR contains the 99-residue coding domain of the monomeric protease (HXB2 isolate [42]) inserted into the *Nde*I-*Bam*HI sites of pET24a (Novagen). The Kunkel method using single-stranded templates of pET-PR substituted with uracil was employed for site-directed mutagenesis (4, 26). Mutations were confirmed by DNA sequencing prior to transfer of the protease sequences into p1CVx.

The phagemid pGag1 was the parent construct of both pGPfs and pGPfs-PR. pGag1 contains the upstream leader, Gag, and Pol sequences from HIV-1 isolate HXB (GenBank accession no. NC 001802) (42) from the *Nar*I site (base 182) to the *Sal*I site (base 5331) inserted into the *Xba*I and *Sal*I sites of pIBI20 (International Biotechnologies) downstream of the T7 promoter of pIBI20. The frameshift mutation in pGagFS was constructed by site-directed mutagenesis of uracil-substituted single-stranded pGag1 as described above with the following oligonucleotide: GAG AGA CAG GCT AAC TTC CTC CGC GAA GAC TTG GCC TTC CTA CAA GGG. The frameshift mutation, when translated, reproduces precisely the amino acid sequence of the major GagPol Pr160 product (16, 19). pGPfs-PR was constructed from pGPfs by a D25A substitution of the catalytic aspartate of the protease. Further mutations within pGPfs and pGPfs-protease were introduced in the respective plasmids by the same method.

Western blot determination of the percentage of β-galactosidase cleavage in *E. coli*. Uninduced mid-log-phase (optical density at 600 nm = 0.5) cultures of *lac-E. coli* strain MC1061 carrying pMono were grown in yeast-tryptone medium to produce samples for Western blot analysis. Cells were pelleted, resuspended in 400 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (39) (optical density at 600 nm = ~0.5), and heated to 95°C for 4 min. SDS-PAGE was performed on Tris-glycine gels (7.5% polyacrylamide) (27). Separated proteins were bound to nitrocellulose by electrotransfer, blocked for 1 h with 3% bovine serum albumin in Tris-buffered saline–0.1% Tween 20, and probed with anti-β-galactosidase monoclonal antibody (Boehringer) at a 1:5,000 dilution. Following multiple washes in Tris-buffered saline–0.1% Tween 20, β-galactosidase was detected with the ECL Plus system followed by autoradiography according to the instructions of the manufacturer (Amersham Pharmacia). The extent of β-galactosidase cleavage was determined by densitometric measurement on a Molecular Dynamics Storm model 800 PhosphorImager. For scanning, the PhosphorImager was set in the blue fluorescence and chemifluorescence mode with a photomultiplier tube voltage of 700 V. The final value for percent cleavage of the β-galactosidase substrate was determined by taking the average of two or more independent inductions.

In vitro assays for the proteolytic processing of Gag. Transcription and translation of pGPfs or pGPfs-PR was performed in 50-μl reaction volumes with rabbit reticulocyte lysate (RRL) and 20 μCi of [^{35}S]cysteine (>1,000 Ci/mmol; Amersham Pharmacia Biotech), using the TNT system (Promega). For *cis* protease processing reactions, 5-μl aliquots from the pGPfs translation reaction mixtures were taken at indicated times and the reaction was stopped by the addition of 10 μl of lithium dodecyl sulfate (LDS)-PAGE loading buffer (Invitrogen).

For *trans* protease processing reactions, transcription and translation of pGPfs-PR-based constructs proceeded for 2 h at 30°C. *trans* processing of the Gag precursor derived from pGPfs-PR was performed in 50-μl reaction volumes containing 5 μl of RRL and 0.25 to 0.5 μg of purified recombinant protease in phosphate buffer (25 mM Na_2HPO_4, 25 mM NaCl, and 1 mM dithiothreitol, pH 7.0). Reactions were performed for 4 h at 30°C. Five-microliter aliquots were removed at various times, and the reaction was stopped by the addition of an equal volume of 2× LDS-PAGE loading buffer (Invitrogen). Products of the processing reaction were heated to 70°C prior to separation on NuPage Bis-Tris gradient gels (4 to 12% polyacrylamide) as recommended by the manufacturer (Invitrogen). Gels were fixed in 10% acetic acid and dried prior to performance of autoradiography.

Expression and purification of HIV PR. Recombinant wild-type HIV PR was expressed in *E. coli* and purified and refolded as described previously (17). Briefly, the cells were resuspended in buffer A (50 mM Tris-HCl buffer [pH 8.0], 25 mM NaCl, 0.2% β-mercaptoethanol), sonicated, and centrifuged. Inclusion bodies were washed first with buffer A, then with buffer A containing (consecutively) 0.1% Triton X-100, 1 M NaCl, and 1 M urea, and finally with buffer A alone. Purified inclusion bodies were solubilized by addition of buffer A containing 8 M urea at room temperature. The solution was clarified by centrifugation and loaded onto a 2.6- by 9.5-cm Q-Sepharose column equilibrated with 8 M urea in buffer A. Flowthrough fractions were collected and dialyzed against three changes of refolding buffer, which consisted of 25 mM sodium phosphate (pH 7.0), 25 mM NaCl, 0.2% β-mercaptoethanol, and 10% glycerol. The total yield of the purified protease was 5 to 10 mg/liter of *E. coli* culture. The percentage of active sites in the prepared protease was determined by active-site inhibition and titration (46) with the tight-binding inhibitor ABT-538 (17). ABT-538 inhibited cleavage of GagPol (PR negative) by 400 nM *trans*-protease (by monomer weight) at a 50% inhibitory concentration of approximately 100 nM, indicating that >50% of the protease was present in the active, dimeric form (data not shown).

RESULTS

Expression of the GagPol precursor in vitro results in protease activation and precursor cleavage. The HIV-1 Gag and Pol coding domains are overlapping; *gag* encodes the structural genes of the viral core (from the 5′ end: matrix [MA], capsid [CA], p2, nucleocapsid [NC], and p6), and *pol* encodes viral enzymes, including PR, reverse transcriptase (RT), and integrase (IN) (36). The GagPol precursor is a fusion protein that is translated as the result of a −1 frameshift near the end of the *gag* gene (19, 43).

To assess the activation of the immature protease in the context of the precursor and to characterize the determinants controlling precursor processing, we developed an HIV-1 Gag-Pol RRL expression system in which the precursor is cleaved by endogenous protease. We introduced a forced frameshift mutation in the *gag* and *pol* open reading frames (Fig. 1, pGPfs) to reproduce, exactly in sequence, the major GagPol frameshift product seen in cells infected by HIV-1 (16). This construct expresses full-length GagPol Pr160 as evidenced by SDS-PAGE (Fig. 1). Translation of the pGPfs construct containing active protease in RRL resulted in the transient appearance of the full-length Pr160 GagPol precursor at approximately 1 h into translation (Fig. 2). This was followed by the transient appearance of a 120-kDa primary intermediate before the generation of 113- and 41-kDa products (Fig. 2, 3, and 4; see also Fig. 6). Beyond 2 h of translation, there was no further accumulation of precursors or final products. A construct expressing full-length GagPol with an alanine substitution for the catalytic aspartic acid (pGPfs-PR) produced only the expected Pr160 product. This was stable over 2.5 h of translation (Fig. 2). A number of minor bands that likely represent internal initiations or premature terminations during translation were observed.

The precise location of the primary and secondary processing sites was determined by placing blocking mutations at individual cleavage sites; it has been demonstrated that substitution of an Ile for the P1 residue of a substrate severely inhibits cleavage (6, 38, 47). We inserted blocking mutations into two known protease-processing sites, p2/NC (M377I) and NC/p1 (N432I) (18, 53). In addition, we substituted an Ile at a novel Phe/Leu site previously reported by Almog et al. (1),

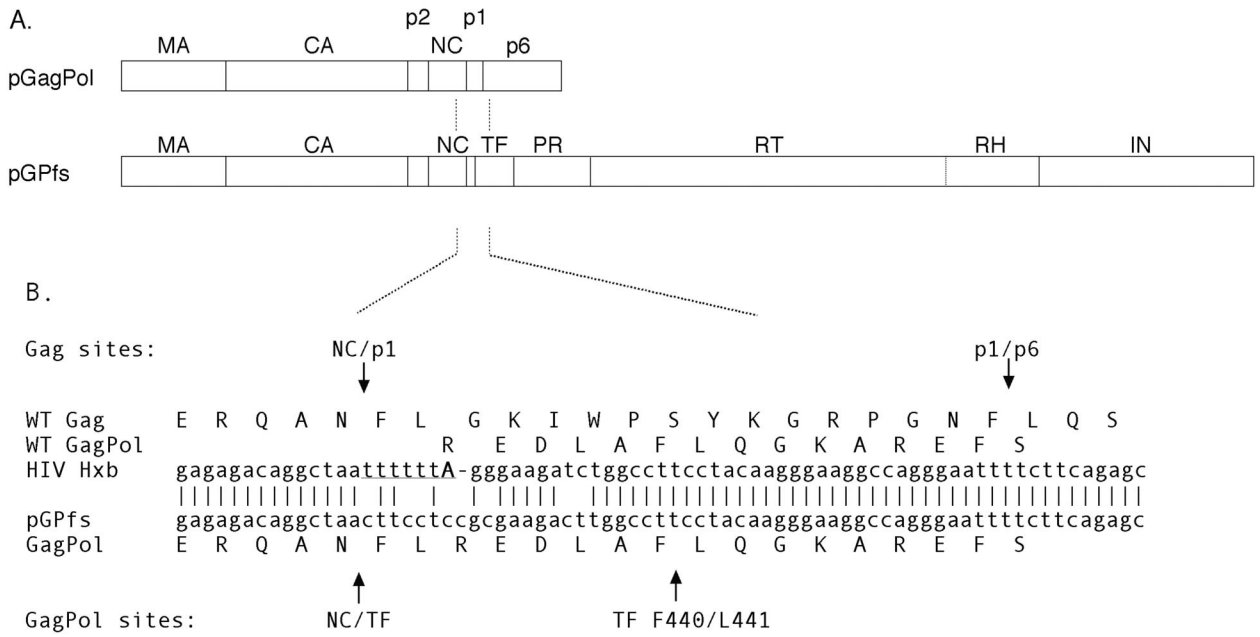

FIG. 1. Schematic of the GagPol processing sites and the forced frameshift mutation in pGPfs. (A) Organization of the processing sites in the HIV-1 Gag and GagPol precursor. The Gag and GagPol precursors of HIV-1 are represented as boxes with processing sites as vertical lines. Processed components are given with their accepted nomenclature (28). (B) Sequence of the wild-type (WT) HIV in the area of translational frameshift is shown above with the 7-nucleotide heptanucleotide sequence required for translational frameshifting underlined (43). The exact site of frameshifting in the virus is variable with 70% of GagPol product containing Leu as the second residue of the TF domain (16). Below is the forced frameshift mutation of pGPfs. pGPfs expresses the major GagPol product in exact sequence. Additional translationally silent substitutions were inserted in the frameshift sequence in pGPfs to improve translational readthrough. The locations of the Gag NC/p1 (53) and pl/p6 (18) sites and the GagPol NC/TF and TF F440/L441 sites are marked with arrows.

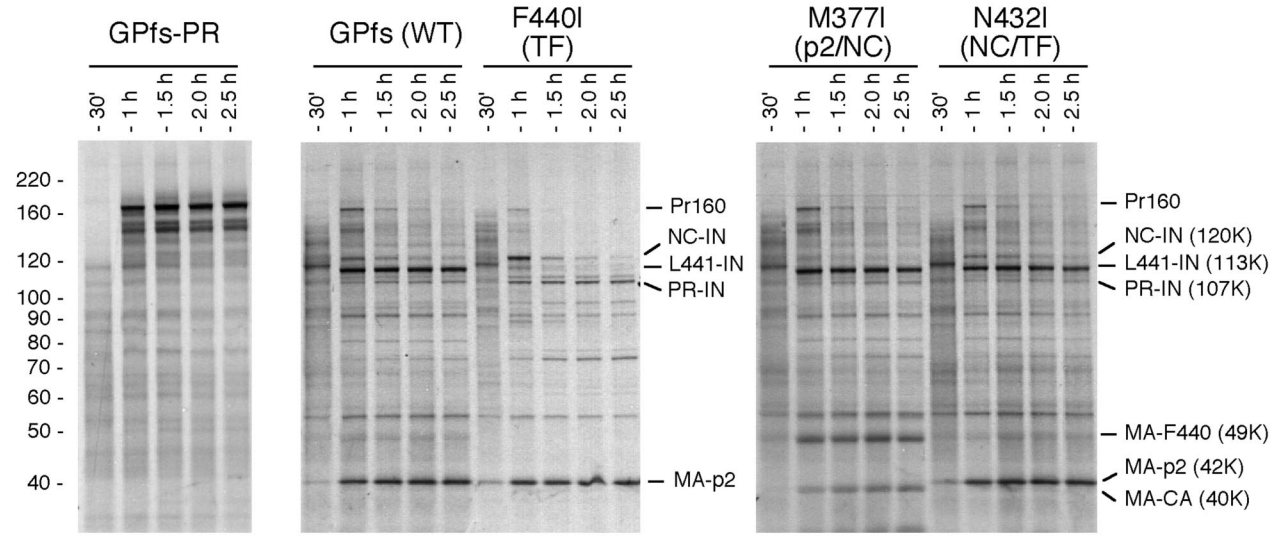

FIG. 2. The kinetics of protease activation and the identification of initial cleavages in the GagPol precursor. The full-length GagPol precursor was generated by in vitro transcription and translation in RRL. Aliquots were removed at the indicated time and separated by SDS-PAGE. Wild-type pGPfs is shown on the left. The effect of inhibiting cleavage at the p2/NC, NC/TF, and TF F440/L441 sites with blocking P1 Ile substitutions is shown. The composition and calculated molecular mass of the products based on published sequence are shown on the right. Products are presented in abbreviated form by the N-terminal and C-terminal domains only. Times in minutes (′) and hours (h) are shown above the gels. Kilodaltons are shown on left.

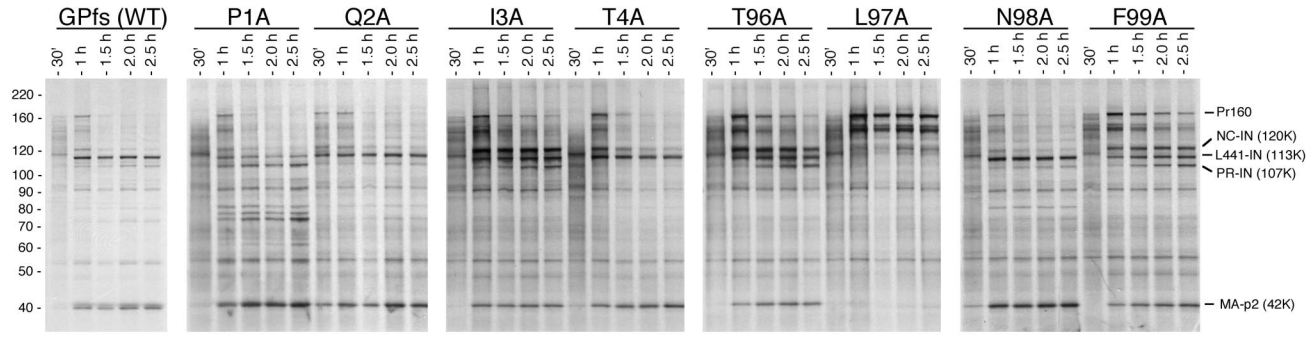

FIG. 3. Effect of single alanine substitutions of the dimer interface on protease activation and specificity within the GagPol precursor. pGPfs (left) or pGPfs containing single alanine substitutions of the eight-dimer interface residues of protease was translated in RRL and separated by SDS-PAGE. Molecular mass markers are shown on the left, and the composition of generated products with estimated molecular mass is shown on the right. Products are abbreviated to the N- and C-terminal domains only. Times in minutes (′) and hours (h) are shown above the gels. Kilodaltons are shown on left. WT, wild type.

located 8 residues downstream from NC in the transframe (TF) domain of GagPol (F440I) (Fig. 1).

We found that initial cleavage of wild-type GagPol occurred at the p2/NC site, generating the 42-kDa MA-CA-p2 intermediate and the 120-kDa NC-TF-PR-RT-IN product. The 120-kDa product is further cleaved at TF F440/L441 to generate the 113-kDa TF L441-PR-RT-IN product. Blocking the initial p2/NC site (M377I) resulted in the appearance of alternative products composed of MA-CA (40-kDa) and MA-CA-p2-NC-TF F440 (49-kDa) in addition to the TF L441-PR-RT-IN (113-kDa) product (Fig. 2). Likewise, an F440I substitution prevented accumulation of the secondary 113-kDa product (TF-PR-RT-IN) and extended the presence of the initial 120-kDa product (NC-TF-PR-RT-IN). The absence of the 49-kDa product (indicative of cleavage at the TF F440/L441 site) and the predominance of the 42-kDa product (indicative of cleavage at p2/NC) during early cleavage of wild-type GagPol are consistent with primary cleavage of the p2/NC site (Fig. 2, GPfs, 1-h time point).

Blocking the NC/TF site had no noticeable effect on the generation of products, suggesting that this site is not among those cleaved. The identity of the cleaved sites was confirmed by excising two residues flanking the scissile bond (P1-P1′) of either site, making the site unrecognizable by the protease. The cleavage pattern obtained with these constructs was the same as that which we observed when we were using the Ile cleavage site-blocking mutations (data not shown). Of note, initial cleavage at the p2/NC site has been observed in Gag processing in HIV-infected cells (38, 44, 51). This result is also consistent with studies of Gag *trans* processing in which the p2/NC site was cleaved most rapidly (8, 12, 25, 38).

When either the p2/NC or the TF F440/L441 site was blocked, increased cleavage of alternative neighboring sites was observed. For example, blocking the p2/NC site produced a novel 40-kDa product (MA-CA), indicating alternate site selection of the CA/p2 site, which is not normally cleaved in this assay. Similarly, blocking cleavage of the TF F440/L441 site resulted in increased amounts of the 107-kDa PR-RT-IN

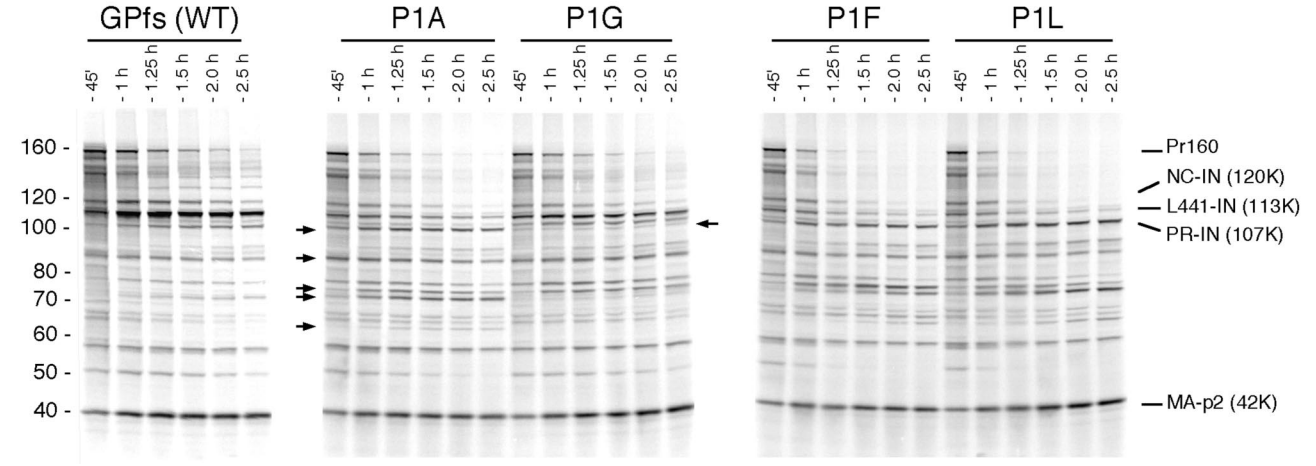

FIG. 4. The effect of proline 1 substitutions on the specificity of GagPol cleavage. Proline 1 of the protease domain was replaced by alanine, glycine, phenylalanine, or leucine in the full-length GagPol precursor GPfs prior to translation in RRL and separation by SDS-PAGE. The substitutions resulted in the generation of novel cleavage products (right-facing arrows). Only the P1G substitution inhibited cleavage at the TF/PR site (loss of 107-kDa intermediate; left-facing arrow). Times in minutes (′) and hours (h) are shown above the gels. Kilodaltons are shown on left. WT, wild type.

intermediate compared to those of wild-type GagPol, indicating enhanced cleavage at the N terminus of the protease.

Individual residues of the dimer interface play differential roles in protease activation. Given the relationship between protease dimerization within GagPol and enzyme activation, we assessed the role of individual dimer interface residues in protease activation. We performed alanine-scanning mutagenesis on residues 1 to 4 and 96 to 99 of the protease dimer interface in the GagPol expression system and examined the impact of these substitutions on enzyme activation and specificity. These results were later compared with data obtained by substitution of alanines for dimer interface residues in the mature 99-amino-acid protease. As shown in Fig. 3, individual alanine substitutions had differential effects on activation. Substituting alanine for residue 2, 4, or 98 (Gln, Thr, or Asn, respectively) had little effect on protease activation compared to wild-type GagPol (GPfs). Complete cleavage of the Pr160 precursor occurred roughly 1.5 h into the translation reaction. The L97A substitution resulted in the most severe defect in activation, as there was no observed cleavage of the Gag precursor during the 2.5-h assay. The I3A, T96A, and F99A substitutions resulted in reduced activity and incomplete cleavage of the precursor. Decreased protease activation in these three mutants resulted in increased amounts of the initial 120-kDa NC-TF-PR-RT-IN product, which is typically cleaved further to the 113-kDa TF F441-PR-RT-IN product in wild-type GPfs. In addition, increased amounts of the 107-kDa PR-RT-IN intermediate were seen, indicative of increased cleavage of the TF/PR site. However, none of the mutations tested increased the rate of cleavage of the PR/RT site, as the 97-kDa RT-IN product remained minor.

The P1A substitution resulted in a protease that appeared to be fully active, as judged by the disappearance of the Pr160 precursor, but with altered specificity toward distal sites. Products in the range of 75 to 95 kDa were generated in addition to the typical 120-kDa and 113-kDa intermediates formed from initial and secondary cleavage of the p2/NC and TF F440/L441 sites, respectively. There was also enhanced cleavage at the TF/PR site at the amino terminus of the protease.

We compared these results with the effect of the same alanine substitutions in the context of the mature 99-amino-acid protease molecule. For these studies, we modified an *E. coli* expression system developed by Gluzman and coworkers (3). The vector (pMono) expresses HIV protease and the β-galactosidase gene. A cleavage cassette consisting of a decapeptide corresponding to an HIV protease cleavage site was inserted into the β-galactosidase open reading frame. In this system, enzyme activity may be assessed via Western blotting as the percent cleavage of full-length β-galactosidase.

Again, single alanine substitutions in the monomeric protease produced differential effects on activity (Table 1). As in the full-length-GagPol activation assay, we found that P1A, Q2A, and T4A substitutions had no effect on protease activity. Introducing an alanine at position 3, 97, or 99 completely inhibited protease activity. The N98A mutation showed an intermediate phenotype with 68% cleavage of the substrate. Our results suggest that there are significant differences between the effects of identical mutations on the activity of the GagPol protease and the results obtained with the mature protease. Overall, mutations had less of an effect on enzyme activity when present in the precursor form. I3A and F99A were inactive in the processed protease and intermediate in the GagPol-processing system. Similarly, increases in enzyme activity were seen when the T96A and N98A substitutions were expressed in the full-length construct. This comparison suggests that sequences within GagPol can compensate, to some degree, for detrimental mutations in the dimer interface.

TABLE 1. Effect of single alanine substitutions of the interface residues on the activity of processed PR on cleavage of a β-galactosidase substrate in *E. coli*

Mutation	% Cleavage
Wild type	96
D25A[a]	0
P1A	92
Q2A	96
I3A	0
T4A	96
T96A	14
L97A	0
N98A	68
F99A	0

[a] D25A is a replacement of the catalytic aspartate and serves as a negative control.

The proline at position 1 of the protease serves as a determinant of specificity for the activated protease. The substitution of alanine for proline at position 1 of the protease produced enhanced cleavage of the amino terminus of the enzyme. In addition, this substitution uniquely altered the specificity of the order of GagPol cleavage without greatly affecting overall enzyme activity. We introduced other substitutions at position 1 (Gly, Phe, and Leu) to examine the influence of this residue on enzyme activation and specificity. All substitutions tested resulted in similarly altered specificity without greatly affecting the overall activity of the protease. Altered specificity resulted in decreased amounts of the 113-kDa TF L441-PR-RT-IN product and increased amounts of other products in the range of 60 to 95 kDa. Although the degree of altered specificity varied with each substitution, the generation of products is similar for the different substitution mutants (Fig. 4, right-facing arrows). P1A, P1F, and P1L also increased relative amounts of the 107-kDa PR-IN product, indicating enhanced cleavage at the amino terminus of the protease (TF/PR). The P1G substitution differed only in that it inhibited cleavage at the TF/PR (Fig. 4, left-facing arrow). These results suggest that Pro 1, as a component of activated GagPol protease, functions as a specificity determinant for the cleavage of distal sites in addition to influencing the rate of cleavage at the TF/PR site.

Mutations in the protease dimer interface alter the pattern of cleavage of the GagPol precursor when purified protease is added in *trans*. The processing of the GagPol precursor was also evaluated with purified protease provided in *trans*. Full-length GagPol (pGPfs) was modified by a D25A substitution at the protease active site (pGPfs-PR), and the kinetics of *trans* processing was monitored over time by the addition of purified recombinant wild-type protease.

Precursor processing of wild-type Gag Pol by *trans*-protease occurred in an order similar to that observed in *cis* processing. Initial cleavage occurred rapidly at the p2/NC site (Fig. 5,

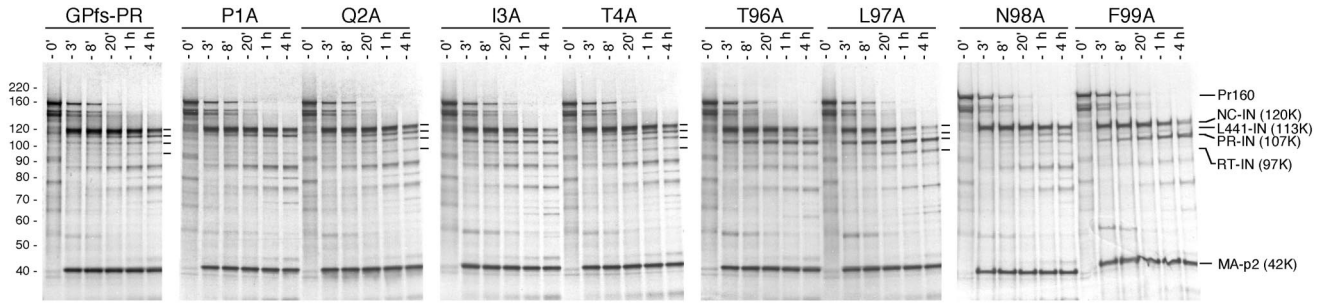

FIG. 5. Effect of alanine substitutions of the protease dimer interface residues on the processing of PR-negative GagPol by *trans* protease. Pr160 produced from the GPfs-PR construct contains a D25A catalytic aspartate substitution of protease that renders the intrinsic protease inactive. Replacement by alanine of the GagPol protease dimer interface residues 1 to 4 and 96 to 99 was evaluated for its effect on processing by wild-type protease provided in *trans*. Reactions were performed at pH 7, and purified wild-type protease was added at the 0-min time point. Times in minutes (′) and hours (h) are shown above the gels. Kilodaltons are shown on left.

120-kDa product) followed sequentially by cleavage at the TF F440/L441 site (113-kDa product). Cleavage at these sites was confirmed by the disappearance of the appropriate intermediates upon introduction of the M377I and F440I blocking mutations (data not shown). As reported above with the expression of the pGPfs construct, little processing was observed at the sites at the N and C termini of the protease itself when purified enzyme was added in *trans* (Fig. 5, 107- and 97-kDa products).

Residues 1 to 4 and 96 to 99 were individually replaced with alanines in the pGPfs-PR construct, and the effect on processing was assessed. The majority of the mutations increased cleavage of the sites flanking the protease and decreased cleavage of the TF F440/L441 site. Increased cleavage at the amino terminus of the protease was most pronounced with replacement by alanine of the isoleucine normally found at position 3 of the protease and with the carboxy-terminal T96A, L97A, and F99A substitutions. Enhanced cleavage of the protease carboxy terminus was most pronounced with the I3A and L97A substitutions. The Q2A and T4A substitutions showed little effect on *trans*-processing kinetics. As expected, the Phe-to-Ala substitution at position 99 prevented processing at the carboxy terminus of the protease; this result is likely due to the unfavorable effect of an alanine at the P1 position of the substrate.

trans processing of the constructs containing substitutions at position 1 of the protease differed dramatically from the results obtained when the precursor was processed by the endogenous GagPol protease. For the P1A mutant, we observed an altered pattern of processing when the precursor was cleaved by the endogenous protease within GagPol (Fig. 3 and 4). In contrast, adding purified protease in *trans* to the pGPfs-PR construct containing the P1A substitution produced a cleavage pattern largely indistinguishable from that of the wild-type GagPol (Fig. 4). Further, cleavage patterns similar to the parental pGPfs-PR construct were seen when the protease was added in *trans* to the P1G, P1F, or P1L substitution (data not shown). It is important that, because a wild-type cleavage pattern was observed when the purified protease was delivered in *trans*, it is unlikely that the altered pattern of pGPfs cleavage obtained with the mutant P1A (Fig. 3) is due to a global disordering of the precursor structure.

Mutation of the amino acids immediately N and C terminal to the protease suggests that these flanking residues do not play a role in enzyme activation. Our alanine-scanning comparisons between the mature protease and the GagPol-associated protease suggest that, when the protease is embedded within GagPol, there are alternate domains outside the protease that contribute to enzyme dimerization and activation. It is possible that the protease dimer interface itself (i.e., residues 1 to 4 and 96 to 99) extends beyond the amino and carboxy termini of the protease into the adjoining GagPol coding domains. To define the borders of the protease dimer interface, we produced GagPol constructs in which the five amino acids N terminal or the five amino acids C terminal to the protease domain were replaced with alanine.

These substitutions had little effect on the kinetics or specificity of activation compared to wild-type GPfs (Fig. 6). This results suggests that the side chains of the five residues immediately flanking the protease in GagPol have little influence on activation of the protease and initial cleavage of the precursor. As expected, the N-terminal substitutions prevented cleavage at the TF/PR site, as the 107-kDa PR-RT-IN minor product was absent in the TF 5A/PR mutant (Fig. 6, center, double arrow). Similarly, replacing the five residues immediately downstream of the protease with alanine (PR/5A RT) showed little effect on protease activation. The 97-kDa RT/IN product was not observed in either the wild-type GPfs or the PR/5A RT constructs, indicating that the PR/RT site is not cleaved in either context.

DISCUSSION

Dimerization and activation of the viral protease within a precursor are a phenomenon that is conserved across all retroviruses. However, little is known about the role that precursor domains outside the protease play in promoting enzyme activation. Further, although precursor processing appears to be a carefully controlled, ordered process, the forces that maintain the order of processing remain poorly defined (8, 25, 31, 32, 55).

We sought to address these questions by examining the spontaneous processing of a full-length GagPol precursor by the endogenous protease. Unlike studies that rely on truncated forms of the precursor, our use of the full-length precursor is likelier to recapitulate the mechanisms of protease activation that occur in vivo. Overall, our studies suggest new insights into

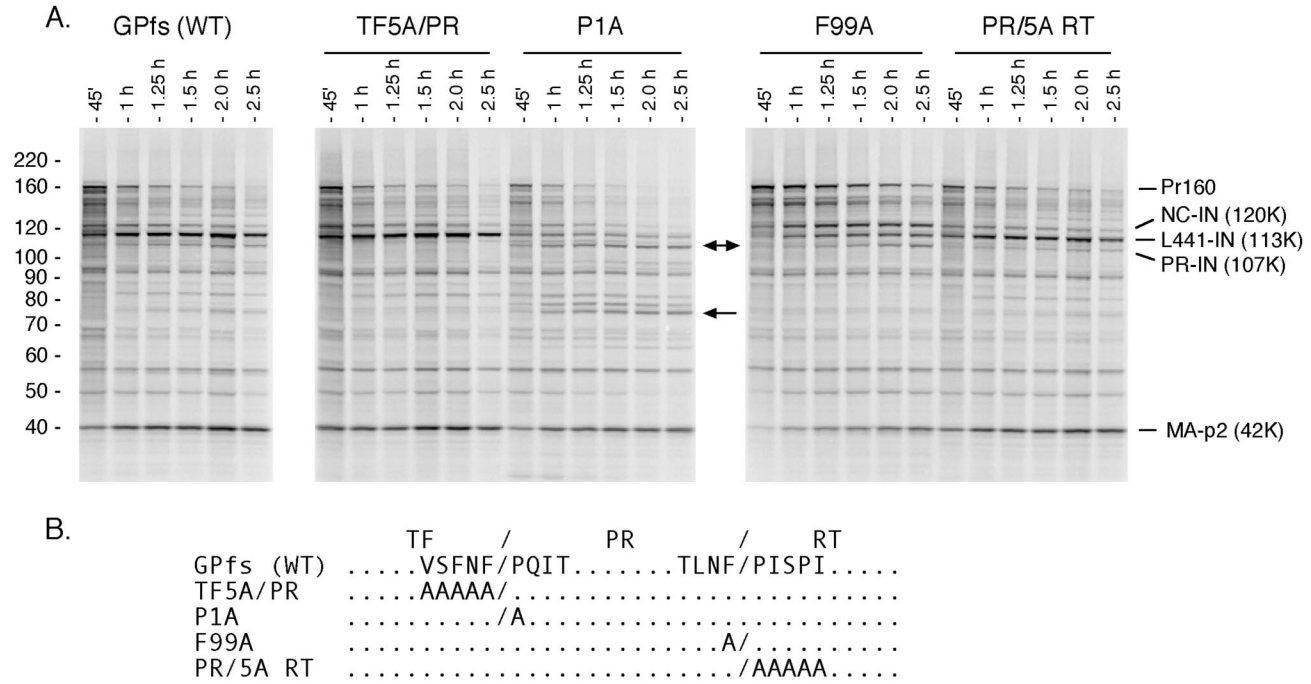

FIG. 6. Effect of multiple-alanine replacement of the residues flanking protease on the activation of the protease within GagPol. (A) An SDS-PAGE gel showing the effect of alanine replacement of the five residues flanking the protease domain on activation of protease and processing of Pr160 GagPol in RRL. Activation and cleavage of the wild-type (WT) GagPol are shown on the left (FS-WT). The P1A substitution results in a active protease with altered specificity of cleavages as shown by the generation of novel products (left arrow). The F99A substitution results in a less active PR. Both the P1A and F99A substitutions enhance cleavage at the TF/PR domain (double arrow). Replacement of the five residues flanking either the N- or C-terminal scissile bond (TF 5A/PR and PR/5A RT mutation) has little effect on either activation or specificity other than preventing cleavage of the mutated processing site. Times in minutes (′) and hours (h) are shown above the gels. Kilodaltons are shown on left. (B) Schematic of the representative mutations.

the interaction between protease activation and the control of precursor processing. Specifically, these data indicate that the forces promoting the initial dimerization and activation of the unprocessed protease include contributions from sequences in GagPol outside the protease. Further, interactions within the protease dimer interface appear to play a role in defining ordered processing of the GagPol precursor. We found that initial cleavage occurs at the p2/NC site, which has also been reported by others to be the first cleaved site within the Gag precursor (34, 38, 51). We found that secondary cleavage likely occurs at the TF F440/L441 site located 8 residues downstream of the NC/TF site. Cleavage at this site was previously noted by others using extended protease miniprecursors in vitro (32, 40), and processing intermediates suggesting utilization of this site have been seen in infected cells (1, 9, 29).

Our data show that protease variants with inactivating mutations of the dimer interface are compensated, to some degree, when expressed as part of the GagPol precursor (Fig. 3). This suggests that there are extra-protease sequences that are important in enzyme activation and can compensate for dimer interface substitutions, although the location and nature of these domains remain obscure. It should be noted that the two processing systems are not completely analogous and that the comparison is somewhat artificial. However, these findings are supported by several studies of protease-containing abbreviated N- or C-terminal extensions into GagPol that show altered dimerization kinetics or activity (8, 10, 31, 33, 37, 41, 49, 55). Several assembly and oligomerization domains have been identified within the Gag precursor, and all of the proteins encoded within the *pol* gene have been demonstrated to multimerize (5, 7, 14, 15, 41, 48). Any or all of these sequences may play a role in promoting precursor dimerization and protease activation. The importance of these sequences in supporting protease activation suggests that they may provide attractive targets for drug development.

The introduction of alanine substitutions immediately adjacent to the protease had little effect on enzyme activation (Fig. 6). Therefore, it appears that the dimer interface within protease is sufficient to allow for enzyme activation within GagPol and does not extend into the immediately flanking GagPol sequences. This suggests that the additional dimerization domains in GagPol are distinct from the protease dimer interface.

Although GagPol sequences outside the protease do appear to play a role in enzyme activation, our data suggest that interactions within the protease dimer interface are important in directing the order of precursor cleavage by the precursor-associated protease. An alanine replacement of the Pro at position 1 of the protease appeared to alter the order of GagPol cleavage (Fig. 4). Similar results were obtained with other substitutions at this position. To our knowledge, this is the first report of a determinant of the order of precursor cleavage located within the protease itself.

The altered pattern of cleavage seen with substitutions at position 1 was observed when the precursor was processed by

the endogenous protease. In contrast, when purified protease was added in *trans* to a protease-deficient construct containing these same substitutions, a cleavage pattern similar to the parental construct was seen (Fig. 5). The observation that the order of cleavage is dependent on interactions within the protease only when the precursor is cleaved by its endogenous protease supports the idea that these initial cleavages are intramolecular (i.e., that the dimerized protease domains within a GagPol dimer cleave processing sites in the two precursors that make up the dimer).

This conclusion is supported by results reported by others. Several groups have examined the processing of constructs containing the protease with extensions into GagPol of various lengths. Wondrak et al. (54) evaluated the in vitro processing of a construct in which the protease linked to short native sequences is fused at its N terminus to the maltose binding protein. They demonstrated that the initial cleavage flanking the protease is intramolecular and occurs at the N terminus. This produces a protease with a C-terminal extension whose activity is similar to that of the mature enzyme. Intramolecular processing at the N terminus of the protease is also suggested by the studies of Co et al. (10) and Louis et al. (33), in which processing of a protease construct containing a short extension of native flanking sequences was examined in vitro. Our studies extend these observations through the use of the full-length GagPol precursor. This is particularly relevant, given our data suggesting that extra-protease sequences within GagPol appear to help support dimer formation and enzyme activation. In addition, our experiments include explicit comparisons between processing with endogenous protease and processing with protease added in *trans*, as well as the evaluation of the processing phenotype of precursors containing mutants in the protease dimer interface.

It is unclear why there is no additional processing of GagPol beyond the cleavage sites noted above. As noted above, our data suggest that these initial cleavages are intramolecular. Because the efficiency of intermolecular cleavages is likely limited by substrate concentration, we may not observe subsequent processing events due to inadequate concentration of substrate. We estimate that the concentration of GagPol precursor produced by in vitro transcription and translation in RRL is approximately 1 nM, a concentration similar to the reported K_d of processed protease. Thus, there may be differences in dimerization efficiency between full-length and intermediate precursors that affect subsequent processing in vitro in addition to substrate concentration.

Structural studies demonstrate that the two terminal residues in the protease (Pro 1 and Phe 99) are in close proximity to each other, which likely presents a stereochemical obstacle to cleaving the sites flanking the protease (11, 35, 50, 52). Several of the mutations that are predicted to disrupt the interaction between Pro 1 and Phe 99 also promote cleavage at the amino terminus of the protease (Fig. 4, P1F and P1L). It is possible that this altered cleavage pattern is due to a generalized increase in enzyme activity with these substitutions. However, this seems unlikely, since these mutations produce relatively significant decreases in enzyme activity (i.e., 20 to 60%) in our *E. coli*-processing assay (data not shown). We were particularly surprised to find that an alanine substitution at this position (P1A) also enhanced cleavage, as peptide studies suggest that this mutation would result in a less efficiently cleaved site. Of note, cleavage at this site was also promoted by mutations in the carboxy-terminal residues of the dimer interface (Fig. 3). In this setting, therefore, the context of the site may play a more important role than the primary sequence in determining the efficiency of cleavage. Further, our analysis of the order of GagPol processing demonstrates that cleavage at either end of the protease is a relatively late event. Overall, these observations suggest that the virus has evolved a structural arrangement at the dimer interface that delays the release of the protease from the precursor.

Taken together, our studies indicate that the release of the viral protease from the precursor is a relatively late event in particle assembly and suggest that, at least for the initial cleavages, precursor processing is an intramolecular event. Initial intramolecular precursor processing and a delay in the release of mature PR until after the release of the viral particle from the membrane of the host cell are consistent with efficient viral assembly for a number of reasons. First, studies by Kräusslich and coworkers using a very active tethered dimer system (22, 23) demonstrate that PR activation before particle release results in high levels of cellular toxicity and minimal particle production. Second, intramolecular cleavages would be helpful in the lower substrate concentrations present at the cell membrane before particle formation. Specifically, a delay in release of mature PR/PR dimers capable of *trans* cleavage until after budding allows *trans* cleavages to occur in a setting of high (1 μM) substrate concentration. Finally, delay prevents the cleavage of precursor before GagPol reaches the membrane. Because the membrane-targeting signal for the GagPol is present at the precursor's N terminus, early cleavage may prevent all of the components of the precursor from reaching the point at which the particle is assembled. The significance of these findings for virus assembly and replication is presently being explored.

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