Autoprocessing: an essential step for the activation of HIV-1 protease

Min WAN^{*‡}, Masahiro TAKAGI[†], Boon-Nee LOH^{*}, Xing-Zhi XU^{*} and Tadayuki IMANAKA[†]

^{*}Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore and [†]Department of Biotechnology, Faculty of Engineering, Osaka University, Japan

Human immunodeficiency virus type 1 (HIV-1) expresses its structural and functional proteins within Gag-Pol precursor polyproteins. Specific proteolytic processing of the precursors by the viral protease is critical for the maturation and infectivity of viral particles. To observe the influence of autoprocessing on the activation of recombinant HIV-1 protease, we constructed different HIV-1 protease forms, with or without the Phe-Pro bond directly upstream of the protease domain, and expressed them in *Escherichia coli* systems. We found that the presence of a short upstream sequence of the protease domain, which could generate the original N-terminus of the protease by auto-

proteolysis of the Phe-Pro bond, resulted in processing of active protease, whereas for a wild-type protease extended only with the initiator methionine, the proteolytic activity was not recovered. Our results suggested that autoprocessing of the direct upstream sequence of the protease domain is an essential step for the activation of recombinant HIV-1 protease in the *E. coli* expression system. Expression of HIV-1 protease as fusion proteins revealed that the existence of a fusion portion increased the accumulation of expressed protease by affecting its homotypic dimer formation.

INTRODUCTION

The human immunodeficiency virus (HIV), like all retroviruses, expresses its structural and enzymic proteins required for the assembly of infectious viral particles. These precursor forms are designated Gag and Gag-Pol respectively. Synthesized HIV polyprotein precursors then undergo proteolytic cleavages by the viral-encoded protease to yield their mature and functional proteins [1,2]. When this step in the HIV life cycle is inhibited, only immature and non-infectious viral particles are produced [1,3–5]. Therefore, the inhibition of the HIV-1 protease has been pursued as a therapeutic target for the prevention of viral replication. Many inhibitors which can block viral maturation in tissue culture [6–8] and slow down viral replication *in vivo* [9] now exist. In spite of the emergence of resistant viral variants derived in cell cultures against protease inhibitors [10–14], development of protease inhibitors as antiviral drugs is still believed to be significant in the recombinant and convergent therapies against HIV infections [15].

The HIV-1 protease is a homologous dimeric aspartic protease containing one of the Asp-Thr-Gly (DTG) motifs at its active centre in each subunit. As each HIV encodes only one subunit of the protease in its genome, precursor dimerization is known to be necessary for the formation of the protease active site. The upstream domains of the HIV-1 [the N-terminal product from the *pol* reading frame after protease cleavage (p6*) and the nucleocapsid (NC)] have been reported to be involved in the protease activation by regulating the autoprocessing and influencing the dimerization of the protease domain in the precursor [16,17]. However, additional mechanisms to regulate protease activity, such as N-terminal pro-sequence cleavage of other aspartyl proteases in eukaryotic cells [18,19], could also be present during viral particle assembly and budding. This is because HIV Gag-Pol precursors can undergo autoprocessing

rapidly in the cytoplasm of some cells and in some *in vitro* systems such as those in *Escherichia coli* and rabbit reticulocyte lysate (RRL) [2,20–22].

In the search for HIV-1 protease inhibitors from structurally novel compounds for development as antiviral drugs, a prerequisite is to obtain an active form of recombinant HIV-1 protease. Our observations on different constructs expressed in an *E. coli* system indicated that a short upstream sequence, containing an autoprocessing site of the HIV-1 protease, is helpful in the recovery of the proteolytic activity during *in vitro* expression. Also, the recombinant HIV-1 protease expressed as fusion proteins may affect the dimerization of this enzyme. The observed increase in the expression level [23] may be a result of the reduction of the toxicity of this enzyme to the host cells.

MATERIALS AND METHODS

Plasmid construction

Plasmid pCris10-PR107 contains an N-terminal-extended HIV-1 protease (*PR107*) gene which refers to the protease precursor of 107 amino acid residues including the initiation codon [23]. The additional 5'-nucleotide sequence encoding the heptapeptide (M)GTVSFNF (not counting the initiator methionine) in the *PR107* gene being present within the Gag-protease-Pol polyprotein, at the p6*-protease junction, reconstituted the original Phe-Pro sequence of the N-terminal protease-processing site. Its mutant, pCris10-D33G, has a single base mutation, such that the active-site aspartic acid in the protease position 33 is changed to glycine [23]. Expression vector pGEX-KG containing the glutathione S-transferase (GST) gene and pET-8c were kind gifts from Dr. X. M. Zheng and Dr. F. W. Studier. The *PR107* gene from pCris10-PR107, flanked by *NcoI* and *HindIII* sites, was subcloned

Abbreviations used: GST, glutathione S-transferase; HIV-1, human immunodeficiency virus type 1; p6*, N-terminal product from the *pol* reading frame after protease cleavage; NC, nucleocapsid; RRL, rabbit reticulocyte lysate; IPTG, isopropyl β -D-thiogalactoside.

‡ To whom correspondence should be addressed at, Tanox Biosystems Inc., 10301 Stella Link, Houston, TX 77025-5497, U.S.A.

directly downstream of the *gst* gene in the expression vector, pGEX-KG. This in-phase GST-PR107 fusion construct was designated pGEX-PR107. The construct of pGEX-D33G was obtained using the same strategy. The following oligonucleotides were used as primers for the construction of expression vectors containing different forms of HIV-1 protease: 1. 5'-ATCCATGGCCCCTCAGATCACCTG-3' (*Nco*I site underlined); 2. 5'-AGAAGCTTATCAGAAGTTCAGAGTG-3' (*Hind*III site underlined); 3. 5'-CTCCATGGGATCCGTTTCCTTC-3' (*Nco*I site underlined); and 4. 5'-ATGGATCCTATCAGAAGTTCAG-3' (*Bam*HI site underlined).

In the construction of expression vector pGEX-PR100, the *PR100* gene, encoding the 99 amino acid residues of HIV-1 protease and the initiator methionine followed by a glycine, was obtained by PCR, using pGEX-PR107 as a template. Oligonucleotides 1 and 2 were used as primers. Oligonucleotide 1 contains the nucleotide sequence starting from the position 9 (Pro) at the 5' end of the *PR107* gene, an *Nco*I site (underlined) with double C is added to create the initiation codon and Gly to form the in-frame connection. Oligonucleotide 2 contains the sequence downstream of the protease gene and a *Hind*III site (underlined) introduced to facilitate the following cloning step. The *PR107* gene for construction of pET-PR107 was obtained by PCR using primers 3 and 4. Primer 3 has the nucleotide sequence located at the 5' end of the *PR107* gene, including an *Nco*I site (underlined), and primer 4 has the same sequence as primer 2, but with a *Bam*HI site (underlined) instead to introduce the following cloning step. Primers 1 and 4 were used to obtain *PR100* gene for the construction of pET-PR100. These DNA fragments obtained by PCR were analysed on a 1.2% agarose gel to assess their purity and were then cleaved with correspondent endonucleases (Promega, Madison, WI, U.S.A.). Thereafter, they were cloned into expression vectors previously digested using the endonucleases mentioned above.

Expression of different forms of recombinant HIV-1 protease and its mutant D33G

The constructs were expressed in the *E. coli* strain DH5 α for pGEX-PR107, pGEX-PR100 and pGEX-D33G or in *E. coli* strain BL21(DE3) for pET-PR107 and pET-PR100. One clone of each strain was inoculated into 5 ml of the medium [Luria-Bertani medium for DH5 α and NZCYM for BL21(DE3)] containing 100 μ g/ml ampicillin (Sigma, U.S.A.) and cultured at 37 °C. The overnight cultures were then transferred into 50 ml of fresh medium the next morning (10 times dilution) and grown for 2 h. The cultures were induced with isopropyl β -D-thiogalactoside (IPTG; Sigma, U.S.A.) at a final concentration of 1 mM and grown for another 2 h. The cells were then harvested by centrifugation. Cell pellets from 1 ml of culture were used for the quantification of the HIV-1 protease by immunoblotting and the pellets from 25 ml of the culture were used for the HPLC enzyme activity assay, upon sonication to release the HIV-1 protease.

Protease assay

The proteolytic activity of HIV-1 protease was measured according to the method we established in our laboratory [24].

Analysis of proteins

Proteins were analysed using SDS/12%-PAGE. An appropriate amount of protein was mixed with 3 $\times$ loading buffer, boiled for 3 min and used for electrophoresis. Western blots were carried out using a specific antiserum raised toward the flap region of the protease, obtained after immunization of rabbits with the syn-

thetic peptide EMSLPGRWKPKMIGGI. Detection of bound antibody to the nitrocellulose membranes was done using monoclonal IgG conjugated with alkaline phosphatase (Sigma, U.S.A.). The reaction was developed with the substrate 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) converted *in situ* into a dense blue compound by immunolocalized alkaline phosphatase. Protein sequence was analysed using a model 470A protein sequencer equipped with a phenylthiohydantoin amino acid analyser (Applied Biosystems) in accordance with the manufacturer's instructions.

RESULTS

Overexpression of different forms of recombinant HIV-1 protease in *E. coli*

For the study on autoprocessing, four constructs containing different recombinant forms of the protease domain were designed to express fusion proteins. Control construct was designed to encode a protease domain containing a mutation which replaced the catalytic Asp residue with a Gly (D33G) fused with GST (Figure 1). To observe the specific effect of autoprocessing of the Phe-Pro bond on the activation of recombinant HIV-1 protease, a stop codon was placed directly at the C-terminus of the protease-coding region.

The pGEX-PR107 contains an N-terminal-extended HIV-1 protease gene (*PR107*) fused to the *gst* gene under the control of the *tac* promoter. The design of the *PR107* gene was aimed at the recovery of the original Phe-Pro sequence of the N-terminal protease-processing site which is present at the p6*-protease junction within the Gag-protease-Pol precursor. *E. coli* DH5 α containing pGEX-PR107 was grown and induced with IPTG. The cell pellet was lysed and boiled with SDS/PAGE loading

Figure 1 Different forms of recombinant protease used for the analysis of Phe-Pro bond autoprocessing

Open boxes represent fusion portion (GST), sequence from multiple cloning sites (MCS) and protease N-terminal extended sequences; hatched boxes represent protease domain. The numbers indicate the amino acid residues at the start or stop positions of each domain. Construct PR107 contains an N-terminal-extended HIV-1 protease gene (*PR107*) which refers to the protease precursor of 107 amino acids with an initiation codon. The design of the *PR107* gene was aimed at the recovery of the original Phe-Pro sequence of the N-terminal protease processing site. The construct of PR100 contains a complete DNA sequence of an HIV-1 protease subunit. Instead of the N-terminal-extended sequence with a Phe-Pro bond in PR107, it has an initiator codon with double C sequence to form Met-Gly at its N-terminus. Both constructs PR107 and PR100 were under the control of bacteriophage T7 promoter in the pET expression vector. Construct GST-PR107 contains a *PR107* gene fused with *GST* gene under the control of *tac* promoter. GST-PR100 has a *PR100* gene fused with *GST* in the same pGEX-KG expression vector. GST-D33G is similar to GST-PR107, the only difference is the mutation in its active centre, Asp $\rightarrow$ Gly.

Figure 2 Immunoblotting detection of different forms of recombinant HIV-1 protease and its active-centre-mutated form, D33G

Protein samples from bacteria containing pGEX-PR107 (lane 1, not induced; lane 2, induced), pGEX-PR100 (lane 3, induced), pGEX-D33G (lane 4, induced), pET-PR107 (lane 5, induced) and pET-PR100 (lane 6, induced) were used. The anti-(HIV-protease) serum specifically reacted with 11 kDa protease (lanes 2, 5 and 6), 36 kDa GST-PR100 fusion protein (lane 3) and 37 kDa GST-D33G (lane 4). The 37 kDa GST-PR107 fusion protein in lane 2 is hardly detectable.

buffer, and then analysed by immunoblotting as shown in Figure 2 [lanes 1 (uninduced) and 2 (induced)]. As compared with the uninduced cell lysate, the induced cells showed the expression of an 11 kDa protein which strongly reacted with specific anti-(HIV protease) serum. The predicted size of the fusion protein of the GST domain and protease precursor (PR107) should be 37 kDa. This result indicated that the full-length fusion product was expressed. However, the HIV-1 protease was rapidly autoprocessed to its mature form, despite only one subunit of the dimeric protease being attached to the GST. Hence, the fusion product (37 kDa) was only faintly visible (Figure 2, lane 2).

Clone pGEX-PR100 contains the nucleotide sequence of a complete subunit of HIV-1 protease (encoding 99 amino acid residues) with an initiator methionine. Upon expression, a major product of 36 kDa from the cell lysate of clone pGEX-PR100 was observed by immunoblotting (Figure 2, lane 3). In contrast

to clone pGEX-PR107, fusion protein from clone pGEX-PR100 was the major product (> 90%).

In order to further confirm the size of the GST-protease fusion proteins, the mutated *PR107* gene, *D33G*, was subcloned into pGEX-KG. *D33G* gene is a single base mutant of the *PR107* gene, in which the active-site aspartic acid in the protease position 33 is changed to glycine. It has been proven that the proteolytic activity was abolished by this substitution [23]. The immunoblotting of the cell lysate of clone pGEX-D33G after induction by IPTG showed the presence of a 37 kDa product (Figure 2, lane 4), which is the size corresponding to the fusion protein of the GST domain and protease precursor (PR107).

Both *PR107* and *PR100* genes have also been constructed in an expression vector pET-8c under the control of bacteriophage T7 promoter. The position of the expressed products from clones pET-PR107 and pET-PR100, which strongly reacted with HIV-1 protease-specific antisera, was found to be similar to clone pGEX-PR107 (Figure 2, lanes 5 and 6). However, the product band from clone pET-PR100 was much more intense than that from pET-PR107.

Specific proteolytic activity of different recombinant HIV-1 proteases

The results from immunoblotting analysis essentially confirmed that the autoprocessing of the protease from the full-length fusion protein of GST-PR107 has occurred and that no detectable free protease was observed from overexpression of GST-PR100 fusion protein. The sample from clone pGEX-PR100 showed a 36 kDa band which reacted with the HIV-1 protease antibody and migrated slightly faster than the band from inactive mutant pGEX-D33G fusion protein (37 kDa). It is reasonable because pGEX-PR100 was 7-amino-acid residues less than pGEX-D33G in its sequence. To date, it has been difficult to study the intermolecular mechanism of protease processing from the polyprotein. However, the proteolytic activity of this recombinant enzyme expressed by different constructions could be a good indicator of the requirements for the autoprocessing. Overall, both *PR107* and *PR100* genes contain an entire HIV-1 protease coding region. The only difference is that there is an extra nucleotide sequence at the 5' end of the protease gene in the *PR107* gene. This gene design was aimed at the recovery of the original Phe-Pro sequence of the N-terminal protease processing site. Table 1 shows the proteolytic activity of recombinant HIV-1 proteases from the cell lysates on its synthetic peptide substrate determined by HPLC assay and the relative amount of expressed proteases determined by quantitative immunoblotting. The substrate has the sequence SQNYPIV, corresponding to the HIV-1 protease-specific cleavage site between p17 and p24 in Gag polyprotein. The proteolytic activity from the cell lysates was 383 units/l for the clone pGEX-PR107 and 70.8 units/l for the clone pET-PR107, whereas an activity of only 10.3 units/l for the clone pGEX-PR100 was determined. No activity from the cell lysates of clones pET-PR100 and pGEX-D33G has been detected. However, the amount of expressed protease protein detected by quantitative immunoblotting showed only approx. 3-fold differences among all of the constructs. It is understandable that the proteolytic activity was completely abolished in the clone pGEX-D33G because of the change of Asp-33 → Gly in its active centre; however, a similar outcome was also observed in clones containing *PR100* gene. Considering the immunoblotting analysis of the overexpression of this recombinant HIV-1 protease from different constructions (Figure 2), the loss of proteolytic activity in the cell lysate of clones pGEX-PR100 and pET-PR100 seems to be related to the autoprocessing of this enzyme.

Table 1 Comparison of the expression level of different forms of recombinant HIV-1 protease and their proteolytic activities

Abbreviation: N.D., not detectable.

Construct	HIV-1 protease activity* (units/l)	Relative amount of protease expressed†
pGEX-PR107	383	3.23
pGEX-PR100	10.3	2.08
pGEX-D33G	N.D.	1.69
pET-PR107	70.8	1.0‡
pET-PR100	N.D.	3.39

* Proteolytic activity of HIV-1 protease on its synthetic peptide substrate measured by HPLC assay.

† The relative amount of protein of interest detected by immunoblotting using densitometry.

‡ The intensity of the protease band from pET-PR107 was used as the standard for comparison in this assay.

pGEX-PR107	Pro-Gln-Ile-Thr-Leu-Trp-Gln...
pET-PR107	Pro-Gln-Ile-Thr-Leu-Trp-Gln...
pET-PR100	Met-Gly-Pro-Gln-Ile-Thr-Leu...

Figure 3 The N-terminal sequences of recombinant HIV-1 protease from different clones

The evidence of autoprocessing of protease fusion protein and its effect on the proteolytic activity

In order to reveal the relationship between autoprocessing and proteolytic activity of the recombinant HIV-1 protease in the overexpression, the proteins of HIV-1 protease expressed by clones pGEX-PR107, pET-PR107 and pET-PR100 were isolated and purified to homogeneity [24]. The purified HIV-1 proteases from clones pGEX-PR107, pET-PR107 and pET-PR100 were subjected to N-terminal sequence analysis. Figure 3 shows their N-terminal sequences determined, up to seven residues, by Edman degradation. The protease isolated from clones pGEX-PR107 and pET-PR107 matched the known sequence of wild-type HIV-1 protease that started with Pro-Gln-Ile. The protease isolated from clone pET-PR100 showed the predicted N-terminal sequence that started with Met-Gly-Pro-Gln-Ile. This result clearly indicates that wild-type HIV-1 protease expressed as a fusion protein will be capable of specifically cleaving itself and recovering its proteolytic activity only if its natural processing site, the Phe-Pro bond, is present at the N-terminal of the protease. It also explains why the protein expressed by pGEX-PR100 was a full-length fusion protein instead of an auto-processed protease and why protease expressed by pET-PR100 does not exhibit proteolytic activity.

DISCUSSION

Different clones containing the HIV-1 protease gene have been used to identify the need of autoproteolysis for the activation of expressed recombinant HIV-1 protease. Our experiment demonstrates that the directly upstream sequence of HIV-1 protease, GTVSFNF, which offers a Phe-Pro bond for its autoprocessing to generate the N-terminus of the protease, is essential for the activity of this enzyme. This result indicates that the mechanism of activation of the retroviral protease may be similar to that of the other members of the aspartyl protease family, such as pepsin [19]. In contrast to the clones containing *PR107* gene, the protease expressed from the constructs with *PR100* gene, which have an entire protease gene and an intact active centre, have been shown to be like the clone with *D33G* gene, in its failure to cleave synthetic peptide substrate and autoprocess its mature form from the fusion protein. The ability of the protease to cleave the peptide substrate could not be recovered, even when a proper peptide, SFNFPQIT, a natural N-terminal cleavage sequence of the protease, was added to the cell lysate containing large amounts of PR100 protein (results not shown). The proteolytic activity of the HIV-1 protease can be abolished by the mutations at the Asp position in the active centre [1,23]. However, this phenomenon has also been observed in the protease which was expressed by clones containing the complete and correct

coding sequence of HIV-1 protease in our experiment. Several groups have concluded that the sequence upstream of the HIV-1 protease including p6* and NC domains of the Gag precursor could influence the protease autoprocessing, thereby affecting its maturation [1,16,17,25]. Also mutation of Phe-Pro at the N-terminus of the protease domain will lower the proteolytic activity of the protease in comparison to the wild-type [22]. Therefore, multiple mechanisms of regulation of this enzyme maturation must be present during new HIV particle assembly and budding. In view of our results, the directly upstream sequence of the protease domain shows positive regulation for the recovery of the HIV-1 protease proteolytic activity in *in vitro* expression systems. A similar activation process in mammalian aspartic proteases could also be involved in the activation of HIV-1 protease; that is activation with the intramolecular cleavage of the peptide bond of the inactive precursor being accompanied by some spectacular conformation changes [17].

From the HIV life cycle, the protease is believed to be functional only within the confines of assembling and budding, where it is essential for the processing of the Gag and Gag-Pol precursors into the structural proteins and enzymes of the mature, infectious virus [1,26]. Its activity appears to be uniquely regulated by the concentration of Gag-Pol polyprotein, upstream domains and autoproteolysis [4,16,17,20,22]. The activation mechanisms of this enzyme could be simplified during the expression *in vitro*, when the concentration of expressed subunit of the protease is sufficiently high and occurrence of dimerization is greater. It was observed that production of this enzyme either by expression in *E. coli* or *Saccharomyces cerevisiae* did not yield a satisfactory level in the previous experiment [24,27–36]. Recent evidence suggests that the HIV-1 protease can exhibit activity not only to the viral precursor proteins, but also to the host cell proteins such as calmodulin, troponin, pro-interleukin 1 β , actin and NF- κ B precursor protein [37–39]. Such low stringency in substrate specificity of HIV-1 protease could be a causative factor for the low expression level. Our observation upon quantitative immunoblotting and proteolysis of peptide substrate indicated that the amount of HIV-1 protease expressed by pGEX-PR107 was much higher than that expressed by pET-PR107 although the T7 system was designed for more exclusive expression of cloned genes under the control of a strong bacteriophage T7 promoter. This difference could also be observed in cell density during the induction (results not shown). The causes of these phenomena could be understood as the influences of dimerization of expressed protease on its activity. As each clone contains only one unit of protease gene, it will meet with great steric interference during the formation of appropriate dimer, especially when it is expressed with a large fusion moiety such as GST. Therefore, the protease activity is relatively lower in the host cells and this is helpful for the accumulation of protease. However, the PR107 protein expressed by pET-PR107 will be easily activated because of less steric interference on the dimerization. The active protease could then be involved in destruction of host cell function, thereby lowering cell density and thus less expressed protease accumulation has been observed.

In conclusion, the possible factors involved in the regulation of HIV-1 protease expression are not fully understood, although various mechanisms have been proposed. With increased recognitions of the importance of proteases and protease inhibitors in the physiopathology of human diseases, cloning and expression of active protease thus becomes critical in the search for protease inhibitors as potentially therapeutic tools to treat human diseases. Our design of an HIV-1 protease expression system, a small upstream sequence of the protease containing its autoprocessing site to generate the natural N-terminus of the protease and

expressed as fusion protein, may be a useful consideration for obtaining active enzyme in higher yield.

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