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Human immunodeficiency virus type 1 capsid protein is a substrate of the retroviral proteinase while integrase is resistant toward proteolysis

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Abstract

The capsid protein of human immunodeficiency virus type 1 was observed to undergo proteolytic cleavage in vitro when viral lysate was incubated in the presence of dithiothreitol at acidic pH. Purified HIV-1 capsid protein was also found to be a substrate of the viral proteinase in a pH-dependent manner; acidic pH (<7) was necessary for cleavage, and decreasing the pH toward 4 increased the degree of processing. Based on N-terminal sequencing of the cleavage products, the capsid protein was found to be cleaved at two sites, between residues 77 and 78 as well as between residues 189 and 190. Oligopeptides representing these cleavage sites were also cleaved at the expected peptide bonds. The presence of cyclophilin A decreased the degree of capsid protein processing. Unlike the capsid protein, integrase was found to be resistant toward proteolysis in good agreement with its presence in the preintegration complex.
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Keywords: HIV-1 capsid protein; Integrase; HIV-1 proteinase; Oligopeptide substrates; Enzyme kinetics

Introduction

The retroviral proteinase (PR) plays a crucial role in the late phase of the retroviral life cycle by processing of the Gag and Gag-Pol polyproteins at the boundaries of the functional domains, during viral assembly and maturation (for a review see Oroszlan and Luftig, 1990). The retroviral PR is a primary target for chemotherapy of AIDS patients, and its inhibitors have been found to have beneficial values in treatment (for a review see Eron, 2000). It was demonstrated for equine infectious anemia virus (EIAV) (Roberts et al., 1991a) and later for HIV (Welker et al., 2000) that the PR is part of the core structure, which enters the infected cells. Besides the PR, the core of HIV-1 also contains capsid (CA), nucleocapsid (NC), reverse transcriptase (RT), RNase H, integrase (IN), Vpr, and Nef (Welker et al., 2000).

During in vitro incubation of EIAV capsids, the NC was further processed into smaller fragments by the incorporated PR (Roberts and Oroszlan, 1989, Roberts et al., 1991a). Later the HIV-1 NC was found to be processed in a similar manner by HIV-1 PR (Wondrak et al., 1994). Among the other proteins of the core, the HIV-1 RT, RNAse H, and Nef have been demonstrated to be substrate of the HIV-1 PR (Tomasselli et al., 1993, Welker et al., 1996, 2000). Furthermore, the PR itself undergoes self-degradation (Mildner et al., 1994), while Vpr remains intact in the capsid (Welker et al., 2000) and it was found to be a nucleocytoplasmic shuttling protein of the preintegration complex (Sherman et al., 2001). Besides the viral components, cellular proteins were also detected in virions, including the cellular peptidyl prolyl isomerase, cyclophilin A (Cyp A), which is incorporated into the virion by binding to CA and which enhances viral infectivity (Franke et al., 1994, Thali et al., 1994). The precise function of Cyp A in HIV-1 replication is unknown.

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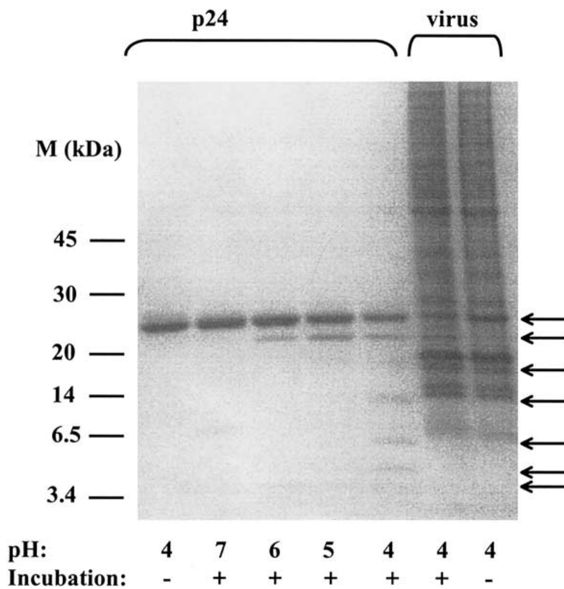

Fig. 1. pH-dependent processing of viral $p24^{CA}$ by HIV-1 PR. The $p24^{CA}$ protein purified from virus infected cells was incubated for 16 h at 37°C in 0.1 M Na-phosphate buffer containing 2.5 mM DTT at the indicated pH with HIV-1 PR. Isolated HIV-1_{HXB2} was disrupted by the addition of 0.1% Triton X-100 and 10 mM DTT and analyzed by SDS–polyacrylamide gel electrophoreses without incubation or after 16 h incubation at 37°C. M denotes the molecular weight marker. The full-size $p24^{CA}$ as well as the observed major cleavage products are indicated by arrows.

Cyp A appears to act on viral core disassembly (Steinkasserer et al., 1995, Braaten et al., 1996). Here we report whether the two remaining viral proteins of the core, CA and IN, are substrates of HIV-1 PR. The CA was found to be processed by the proteinase in a pH-dependent manner; however, integrase was resistant toward PR-mediated degradation. Since Cyp A binds to CA in virion, and alters its conformation (Dietrich et al., 2001), we have also tested the effect of Cyp A in CA processing.

Results

Capsid protein is a pH-dependent substrate of HIV-1 PR, in vitro

Capsid protein ($p24^{CA}$) purified from eukaryotic cells infected with HIV-1 was incubated overnight with purified recombinant HIV-1 PR in buffers at various pH (Fig. 1). While no cleavage was observed at pH 7.0, decreasing the pH resulted in substantial processing of the $p24^{CA}$ protein: at pH 5.0 the largest, 22-kDa fragment appeared with very low molecular weight fragments, while at pH 4.0, three intermediate-sized bands also appeared (Fig. 1), implying that the cleavage yielding these fragments may have a lower pH optimum than the cleavage producing the 22 kDa and the very short fragments.

Isolated virus from AD293T cells infected with HIV-1_{HXB2} was disrupted by the addition of detergent (Triton X-100) and was also incubated overnight at pH 4.0 (Fig. 1). A substantial amount of the 24-kDa band disappeared, yielding cleavage products whose mobility was identical to the mobility of fragments observed with $p24^{CA}$. To verify that the cleaved protein of the viral lysate was the $p24^{CA}$ capsid protein, N-terminal sequence of the 24-kDa band as well as of the largest, 22-kDa cleavage product was determined, and both were found to be PIVQN-, the N-terminal sequence of $p24^{CA}$. This result also suggested that the cleavage yielding the largest, 22-kDa cleavage product occurred at the C-terminal part of the protein. Addition of the specific retroviral proteinase inhibitor UK 88,947 (Baboonian et al., 1991) before incubation prevented the loss of the $p24^{CA}$ protein in the viral lysate, suggesting that it was degraded by the retroviral PR, incorporated into the virion (data not shown).

Time-dependent processing of recombinant capsid protein by HIV-1 PR

To determine the cleavage sites in the capsid protein, recombinant $p24^{CA}$ protein expressed in *Escherichia coli* (r-$p24^{CA}$) was subjected to proteolysis by HIV-1 PR. At 1:20 PR:capsid ratio and pH 5.5 (which is optimal for the PR) a time-dependent processing was observed, which was prevented by the addition of 0.2 μM ritonavir, a specific PR inhibitor (Fig. 2). To identify all of the fragments obtained from $p24^{CA}$ degradation, the cleavage products were separated by SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane; then Coomassie-stained bands were cut out and subjected to N-terminal analysis (not shown). Bands were identified by their N-terminal sequence and by their apparent molecular weight (Table 1). Based on these data, two cleavage sites were identified in the $p24^{CA}$ protein, one between Ala 77 and Ala 78 of the N-terminal domain of CA, and the other between Leu 189 and Leu 190 of the C-terminal domain of CA.

Effect of cyclophilin A on the processing of capsid protein by HIV-1 PR

To study the effect of cyclophilin on the $p24^{CA}$ degradation, r-$p24^{CA}$ was preincubated with cyclophilin A at pH 4.0 (which is optimal for the N-terminal site cleavage) before addition of the PR into the reaction mixture. Binding of Cyp A to $p24^{CA}$ substantially reduced the rate of cleavage of capsid protein (Fig. 3), indicating that this chaperone was able to delay the proteolytic processing of CA by HIV-1 PR. Serum albumin or lysozyme used in the same concentration as Cyp A did not inhibit the cleavage of $p24^{CA}$, indicating that the effect of Cyp A was due to specific protein–protein interaction.

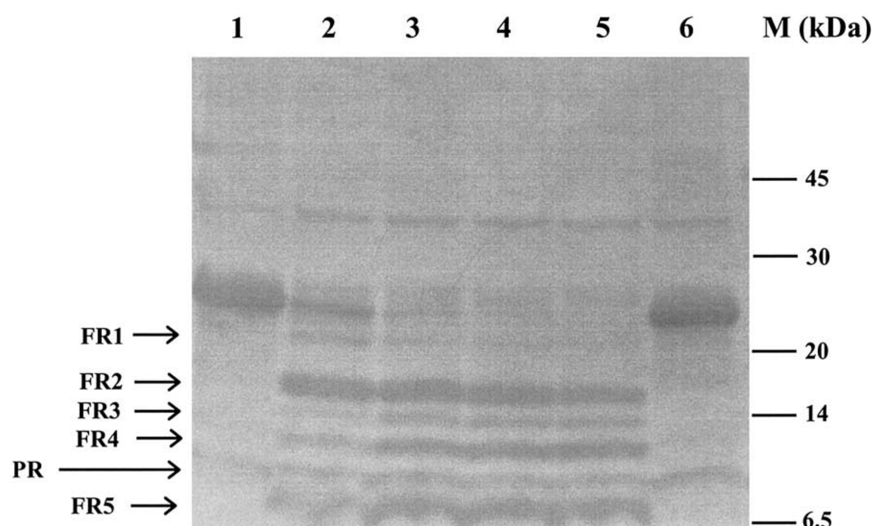

Fig. 2. Incubation of recombinant $p24^{CA}$ with HIV-1 proteinase. Recombinant $p24^{CA}$ was incubated with HIV-1 PR for the 0 (lane 1), 30 min (lane 2), 60 min (lane 3), 120 min (lane 4), and 240 min (lane 5) or for 240 min in the presence of a specific PR inhibitor, ritonavir (lane 6), and analyzed by SDS–PAGE. M denotes the molecular weight marker. FR4 migrates higher than in the case of viral capsid protein (Table 1) owing to the presence of the N-terminal 6-his tag.

Cleavage of oligopeptides representing the processing sites in the capsid protein by HIV-1 PR

Oligopeptides were synthesized, representing the cleavage sites at the identified sites in CA of HIV-1_{HXB2}, and assayed as substrates of HIV-1 PR. At assay conditions we previously found to be optimal for HIV-1 PR (pH 5.6, 2 M NaCl) (Tözsér et al., 1991), the peptide representing the C-terminal cleavage site appeared to be a somewhat better substrate (Table 2). When the kinetic parameters were determined under conditions similar to that of the in vitro protein cleavages at pH 4.0, the other substrate was much better hydrolyzed (Table 2).

Integrase is not a substrate of HIV-1 PR

Recombinant IN of HIV-1 was incubated with HIV-1 PR under the same conditions as used for the capsid cleavage. During the 4 h incubation, the integrase protein remained intact (Fig. 4).

Discussion

We have identified two cleavage sites in the HIV-1 $p24^{CA}$, between residues 77 and 78 as well as between residues 189 and 190. The cleavage was strongly pH-dependent. No processing was observed at pH 7.2, decreasing the pH to 6 already yielded a detectable level of $p24^{CA}$ cleavage at the C-terminal domain, while the cleavage of the N-terminal domain was observed only at lower pH. Oligopeptides representing these cleavage sites were also cleaved at the expected peptide bonds. On the other hand, integrase was found to be resistant toward proteolysis, in good agreement with its presence in the preintegration complex.

Based on the crystal structures of $p24^{CA}$, the cleavage site of the C-terminal domain is in helix 9, where Leu 189 is a part of the dimer interface (Berthet-Colominas et al., 1999), while the cleavage site of the N-terminal domain is in helix 4, which leads to the Cyp A binding loop (Berthet-Colominas et al., 1999). These structural elements are in-

Table 1
Analysis of full size $p24^{CA}$ (from HIV_{IIIB}) and its fragments separated by SDS–polyacrylamide gel electrophoresis

Band	Residues	N-terminal sequence of the protein	Molecular weight (kDa)	
			Observed	Calculated
p24	1–231	P-I-V-Q-N-	26.0	25.6
FR1	1–189	P-I-V-Q-N-	22.0	21.3
FR2	78–231	A-E-X-D-R-	18.0	17.2
FR3	78–189	A-E-X-D-R-	13.0	12.9
PR	(HIV-1 PR)	P-Q-V-T-L-	11.0	10.8
FR4	1–77	Not determined	7.0	8.4
FR5	190–231	L-V-Q-N-A-	5.0	4.3

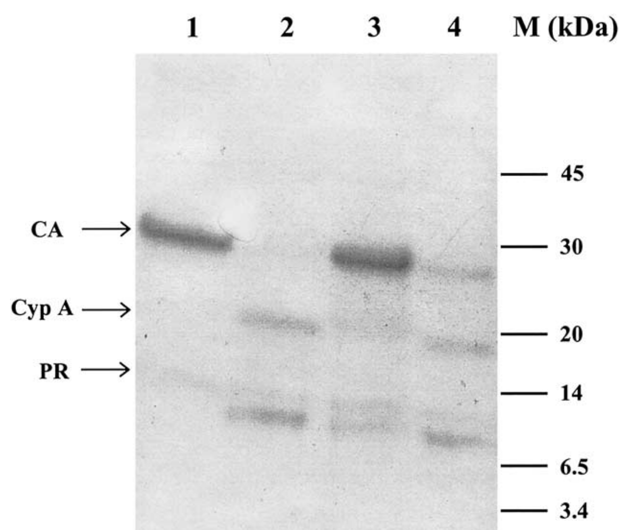

Fig. 3. Effect of cyclophilin A on HIV-1 PR-catalyzed processing of p24[CA]. Recombinant p24[CA] protein alone (lanes 1 and 2) or complexed with Cyp A (lanes 3 and 4) was incubated with (lanes 2 and 4) or without HIV-1 PR (lanes 1 and 3) for 30 min at 37°C; then the reaction mixture was analyzed by SDS–PAGE. M denotes the molecular weight marker.

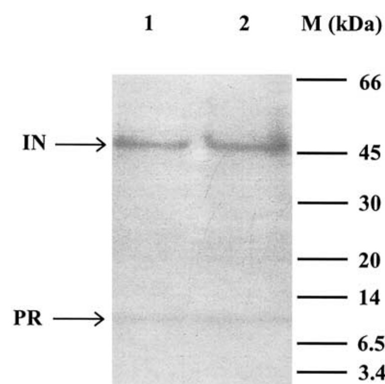

Fig. 4. Incubation of and p32[IN] HIV-1 integrase with HIV-1 proteinase. Recombinant p32[IN] was incubated with (lane 1) or without (lane 2) HIV-1 PR for 4 h; then the reaction mixture was analyzed by SDS–PAGE. M denotes the molecular weight marker.

compatible with the structural requirement for the PR-mediated hydrolysis, since the substrate should bind into the enzyme in an extended conformation (for a review, see Wlodawer and Erickson, 1993). Similar discrepancies were also found with other protein substrates of HIV-1, in which the cleavage site regions were found to be part of alpha helices (Tomasselli and Heinrickson, 1994). However, the crystals for determination for the p24[CA] structure were grown at pH 7.5, at which the protein appeared to be resistant toward proteolysis. The structure and higher organization of p24[CA] is very sensitive to the pH; below 6.6 the multimeric capsid forms disassemble into monomers, and its structure assumes a “molten globule” state (Misselwitz et al., 1995; Ehrlich et al., 2001) in which the local structure around the cleavage sites may be altered to allow productive binding to the PR.

HIV-1 PR cleaves the Gag and Gag-Pol proteins at various positions during viral maturation. The amino acid residues in the P4–P3′ region of these sites, which are recognized by the PR, are listed in Table 3. Most of these sites contain hydrophobic residues at the P2–P2′ region. Although only one viral HIV-1 PR maturation cleavage site, the CA ↓ p2 site, contains charged residue at this region, a P2′ glutamate (Louis et al., 2000), this residue is highly conserved in HIV-1 (Korber et al., 1998). The cleavage at this site is accelerated by lower pH, and this was suggested to play a regulatory role in the viral protein processing (Pettit et al., 1994). While the sequence of the cleavage site at the C-terminal domain of p24 fits well into the other Gag and Gag-Pol sequences, the cleavage site in the N-terminal domain of p24 contains Glu residue at both the P2 and the P2′ positions (Table 3). Crystallographic structures of HIV-1 PR have been reported with inhibitors or products having P2′ Glu residues (Tong et al., 1993; Weber et al., 1997). In these structures the P2′ Glu showed two weak hydrogen bond interactions with the amides of Asp 29 and Asp 30 of the enzyme. The proximity of the Glu side chain to the side chain of Asp 30 suggested that they share a proton (Weber et al., 1997). The presence of glutamate at P2′ position of various cleavage sites was found to be responsible for the pH effect (Boross et al., 1999). The presence of two glutamates in this p24 cleavage site may enhance such a pH effect, explaining why lowering the pH substantially enhanced the cleavage in the N-terminal do-

Table 2
Cleavage of oligopeptides representing the cleavage sites within p24[CA] protein of HIV_{HXB2} by HIV-1 proteinase

Peptide[a]	Assay conditions[b]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1. INEEA- ↓ -AEWDR	A	0.617 ± 0.035	0.20 ± 0.01	0.32
	B	<0.1	N.D.	7.6[c]
2. MTETL- ↓ -LVENR	A	0.224 ± 0.049	0.14 ± 0.01	0.61
	B	0.731 ± 0.110	0.43 ± 0.05	0.59

[a] Scissile bond is marked by an arrow.
[b] A; measured in phosphate buffer, pH 5.6, containing 2 M NaCl; B, measured in phosphate buffer, pH 4.0 in the absence of salt.
[c] Increase of the substrate concentration above the apparent K_m value resulted in a decrease of the measurable activity. Therefore the k_{cat}/K_m value was determined in a competitive assay by using substrate 2.

Table 3
Comparison of the HIV-1 Gag and Gag-Pol sites cleaved by the HIV-1 PR during maturation with the cleavage sites within $p24^{CA}$ protein of HIV_{HXB2}

Site of cleavage	P4	P3	P2	P1		P1′	P2′	P3′
MA ↓ CA	Ser-	Gln-	Asn-	Tyr-	↓	-Pro-	Ile-	Val[a]
CA ↓ p2	Ala-	Arg-	Val-	Leu-	↓	-Ala-	Glu-	Ala
p2 ↓ NC	Ala-	Thr-	Ile-	Met-	↓	-Met-	Gln-	Arg
NC ↓ p1	Arg-	Gln-	Ala-	Asn-	↓	-Phe-	Leu-	Gly
p1 ↓ p6	Pro-	Gly-	Asn-	Phe-	↓	-Leu-	Gln-	Ser
in p6	Lys-	Glu-	Leu-	Tyr-	↓	-Pro-	Leu-	Thr
TFP ↓ $p6^{Pol}$	Asp-	Leu-	Ala-	Phe-	↓	-Leu-	Gln-	Gly
$p6^{Pol}$ ↓ PR	Ser-	Phe-	Asn-	Phe-	↓	-Pro-	Gln-	Val
PR ↓ RT	Thr-	Leu-	Asn-	Phe-	↓	-Pro-	Ile-	Ser
p66 ↓ p51	Ala-	Glu-	Thr-	Phe-	↓	-Tyr-	Val-	Asp
RT ↓ IN	Arg-	Lys-	Val-	Leu-	↓	-Phe-	Leu-	Asp
In CA (1)	Asn-	Glu-	Glu-	Ala-	↓	-Ala-	Glu-	Trp
In CA (2)	Thr-	Glu-	Thr-	Leu-	↓	-Leu-	Val-	Glu

[a] Cleavage site sequences are given as in Louis et al. (2000), but for the HXB2 isolate. TFP, transframe protein, p66 and p51, are the two forms of the RT subunits in the functional dimer.

main of CA. Unlike the P2 and P2′ glutamates, Glu at P3 and P3′ in the peptide representing the cleavage site in the C-terminal domain did not result in such an effect.

In the early phase of viral replication, the viral capsid structure enters the cell. Based on compositional studies of EIAV and HIV-1 capsids (Roberts et al., 1991b, Welker et al., 2000), they contain the viral RNA covered by nucleocapsid protein, capsid protein, and at least the replication enzymes (RT, RNaseH, IN, and PR). Reverse transcription occurs in this highly organized structure; then a preintegration complex (PIC) is formed, containing the viral DNA as well as integrase (Farnet and Haseltine, 1991), although other proteins such as MA and Vpr were also detected in PIC (Miller et al., 1997). So far the HIV-1 RT and RNase H (Tomasselli et al., 1993), the NC protein (Wondrak et al., 1994), and the PR itself (Mildner et al., 1994) were described as substrate of the HIV-1 proteinase. Here we report the CA protein is also a substrate of HIV-1 PR. Its processing is strongly dependent from a pH-induced conformational change. Lowering the pH was also found to be required for the processing of RNaseH (Tomasselli et al., 1993) and could remove the coordinated zinc from the NC protein (Mely et al., 1996), which is a prerequisite of its processing (Wondrak et al., 1994).

Although it has been shown previously that HIV-1 is able to enter the cells by clathrin-coated vesicles (Grewe et al., 1990), it has been demonstrated only recently that endocytic entry of HIV-1 into cells can lead to viral integration and gene expression (Fackler and Peterlin, 2000). In fact, the vesicular pathway could be the predominant route for certain virus isolates in infecting $CD4^-$ cells (Fackler and Peterlin, 2000). Chloroquine, a weak base capable of increasing the pH of acidic vesicles, was found to be able to inhibit HIV-1 replication, by reducing the gp120 production of the infected cells (Tsai et al., 1990). Chloroquine may also block the early phase of replication of HIV-1 entering the cell through receptor-mediated endocytosis.

Since the virus entering the cells contains the active protease, its activity could be boosted by the acidic pH of the endosomes, and protease-mediated cleavage of viral proteins, including NC, and CA may be important for the preparation of proper preintegration complex. The different trafficking route of HIV-1 depending on the presence of receptor and virus isolate (Fackler and Peterlin, 2000) may be responsible at least in part for the conflicting results obtained with PR inhibitors to study the role of PR in the early phase of viral life cycle. Several research groups including ours found an inhibitory effect on replication (Baboonian et al., 1991; Nagy et al., 1994; Panther et al., 1999; Goto et al., 2001), while others did not observe such an effect (Jacobsen et al., 1992; Kaplan et al., 1996; Uchida et al., 1997).

Materials and methods

Purification of the HIV-1 capsid protein

Purification of viral $p24^{CA}$ protein from cells infected with HIV-1 was performed as described previously (Veronese et al., 1988). For the expression of recombinant capsid protein (r-$p24^{CA}$), the plasmid bearing the CA protein from HIV_{IIIB} isolate with an N-terminal 6-his tag was obtained from Dr. Carol Carter (Department of Molecular Genetics and Microbiology, S.U.N.Y. Stony Brook, USA). Expression of the recombinant protein in BL21(DE3) *E. coli* cells (Novagen) was induced by the addition of 0.4 mM IPTG to the cultures and incubation at 37°C for 3 h. Bacterial cells were suspended in 10 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, and 1 mM PMSF, and then disrupted by sonication. The insoluble fraction was collected by centrifugation and resuspended in 20 mM phosphate buffer, pH 7.5, containing 500 mM NaCl, 0.1% Triton X-100, and 8 M urea. After sonication, the solution was clarified by filtration and then applied to the Ni-NTA Superflow affinity resin (Qiagen) equilibrated with the same buffer. After extensive consecutive washing with a buffer having the same composition as the loading buffer, but with pH 6.0 and containing 500 mM LiCl, the recombinant protein bound to the resin was eluted with 50 mM Na-acetate, pH 5.0, containing 8 M urea, 500 mM NaCl, 10% glycerol, 1% Triton X-100, and 5 mM β-mercaptoethanol. The purified protein was dialyzed overnight at 4°C, against 25 mM formic acid, lyophilized, and reconstituted in assay buffer. Protein concentrations were determined by the Bradford spectrophotometric method (Bio-Rad).

Purification of HIV-1 integrase

HIV-1 pINSD.His.Sol plasmid coding for a soluble integrase mutant (Jenkins et al., 1996) was obtained through

the AIDS Research and Reference Program. Integrase was expressed in BL21(DE3) *E. coli* cells by addition of 0.4 mM IPTG to the culture and incubation at 37°C for 3 h. Bacterial cells were suspended in 10 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 0.1% Triton X-100, and treated with 100 μg/ml DNAse I (Sigma) for 30 min at 37°C in the presence of 10 mM $MgCl_2$. The insoluble fraction was collected by centrifugation and solubilized in 20 mM phosphate buffer, pH 7.5, containing 500 mM NaCl, 0.1% Triton X-100, and 8 M urea. The solution was clarified by filtration and applied to nickel-charged ProBond resin (Invitrogen) equilibrated with the same buffer. After extensive consecutive washings with the loading buffer, protein was eluted by gradually decreasing the pH to 5.0. Fractions containing the recombinant protein (as determined by SDS–PAGE) were combined and dialyzed overnight at 4°C, against 25 mM formic acid, lyophilized, and reconstituted in assay buffer.

Virus production and in vitro capsid protein processing in virus lysate

pMM4XB2 plasmid containing an infectious proviral clone of HIV-1_{HXB2} was a kind gift of Dr. Ronald Swanstrom (Department of Biochemistry and Biophysics, UNC Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC). The plasmid (6 μg) was transfected onto 80% confluent AD293T cell monolayers in a 9-cm petri dish by the calcium-phosphate method (Graham and van der Eb, 1973). Cells were maintained in DMEM containing 10% fetal calf serum. Supernatant containing virus particles was collected after 4 days, filtered through a 0.45-μm filter (Millipore), and purified by centrifugation through a 20% sucrose pad. Viral pellet was resuspended in 0.1% Triton X-100 containing 10 mM dithiothreitol. The pH of the disrupted virus solution was 4.0. The p24 content of the viral lysate was measured according to Popovic et al. (1984). An aliquot of the disrupted virus was incubated for 16 h at 37°C in the presence or absence of 50 μM HIV-1 proteinase inhibitor UK 88,947 (Baboonian et al., 1991). Ten to twelve micrograms of viral p24 were used per lane for SDS–PAGE.

Incubation of viral CA and IN proteins by HIV-1 proteinase

To determine the effect of pH on capsid protein degradation, $p24^{CA}$ isolated from virus-infected cells (4.3 μM final concentration) was incubated in 100 mM potassium phosphate, 2.5 mM DTT, pH 4.0 with recombinant HIV-1 PR (0.7 μM final concentration) (Louis et al., 1991). After 16 h incubation reactions were stopped by the addition of equal volume of 2× tricine–SDS sample buffer (Novex). The samples were analyzed using 16 or 10–20% gradient SDS–polyacrylamide tricine-buffered gel (Novex). Molecular weight of the fragments was estimated using Rainbow molecular weight markers (Amersham).

For the cleavage of r-$p24^{CA}$ as well as r-$p32^{IN}$, recombinant protein (20 and 8 μM final concentration, respectively) was incubated with recombinant HIV-1 PR (1 μM final concentration) in 100 mM sodium acetate buffer, pH 5.5, containing 1 mM DTT, 1 mM EDTA, and 150 mM NaCl. To test the effect of cyclophilin A on the capsid cleavage, 43 μM Cyp A (Sigma) was preincubated with the capsid protein for 1 h at room temperature. As nonspecific controls, bovine serum albumin and lysozyme (both from Sigma) were used in 43 μM concentration, in the same way as Cyp A. After incubation for various times at 37°C, reactions were stopped by the addition of an equal volume of 2× tricine–SDS sample buffer, and the samples were analyzed using 16 or 10–20% gradient SDS–polyacrylamide tricine-buffered gels (Novex).

Protein sequencing

Fragments of the CA protein cleaved by PR were separated by SDS–PAGE and transferred to PVDF membrane (ProBlot, Applied Biosystems) according to Towbin et al., (1979). N-terminal amino acid sequence analysis was carried out by stepwise Edman degradation in a model 470A gas-phase sequencer (Applied Biosystems) or in a model 910 protein sequencer (Knauer).

Oligopeptide synthesis and characterization

Oligopeptides were synthesized by solid-phase peptide synthesis on a model 430A automated peptide synthesizer (Applied Biosystems) using 9-fluorenyl-methyloxycarbonyl chemistry and were purified by reversed-phase HPLC. Amino acid composition of the peptides was determined with a Beckman 6300 amino acid analyzer. Stock solutions and dilutions were made in distilled water, and the peptide concentrations were determined by amino acid analysis.

Enzyme assay with synthetic peptide substrates

The assay was performed either in 0.1 M phosphate buffer, pH 5.6, 2 M NaCl (Buffer A) or in 0.1 M phosphate buffer, pH 4.0 (Buffer B), as described previously (Tözsér et al., 1992; Ishima et al., 2001). Briefly, the reaction mixture was incubated at 37°C for 1 h, and the reaction was stopped by the addition of guanidine–HCl (6 M final concentration). The solution was acidified by the addition of 1% trifluoroacetic acid (TFA), and an aliquot was injected onto a Nova-Pak C_{18} reversed-phase HPLC column (3.9 × 150 mm). Substrates and the cleavage products were separated using an increasing water-acetonitrile gradient (0–100%) in the presence of 0.05% TFA. Composition of the cleavage products was determined by amino acid analysis. Kinetic parameters were determined at less than 20% substrate turnover by fitting the data to the Michaelis–Menten equation by using the Fig. P program (Fig. P Software Corp., Durham, NC). The range of substrate concentration was 0.02–2.0

mM, depending on the approximate K_m values. Active-site titration was performed for the HIV-1 PR with compound 3 in Grobelny et al. (1990).

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References

Baboonian, C., Dalgleish, A., Bountiff, L., Gross, J., Oroszlan, S., Rickett, G., Smith-Burchnell, C., Troke, P., Merson, J., 1991. HIV-1 proteinase is required for synthesis of pro-viral DNA. Biochem. Biophys. Res. Commun. 179, 17–24.

Berthet-Colominas, C., Monaco, S., Novelli, A., Sibai, G., Mallet, F., Cusack, S., 1999. Head-to-tail dimers and interdomain flexibility revealed by the crystal structure of HIV-1 capsid protein (p24) complexed with a monoclonal antibody Fab. EMBO J. 18, 1124–1136.

Boross, P., Bagossi, P., Copeland, T.D., Oroszlan, S., Louis, J.M., Tözsér, J., 1999. Effect of substrate residues on the P2′ preference of retroviral proteinases. Eur. J. Biochem. 264, 921–929.

Braaten, D., Franke, E.K., Luban, J., 1996. Cyclophilin A is required for early step in the life cycle of human immunodeficiency virus type 1 before initiation of reverse transcription. J. Virol. 70, 3551–3560.

Dietrich, L., Ehrlich, L.S., LaGrassa, T.J., Ebbets-Reed, D., Carter, C., 2001. Structural consequences of cyclophilin A binding on maturational refolding in human immunodeficiency virus type 1 capsid protein. J. Virol. 75, 4721–4733.

Ehrlich, L.S., Liu, T., Scarlata, S., Chu, B., Carter, C.A., 2001. HIV-1 capsid protein forms spherical (immature-like) and tubular (mature-like) particles in vitro: structure switching by pH-induced conformational changes. Biophys. J. 81, 586–594.

Eron, J.J., 2000. HIV-1 protease inhibitors. Clin. Infect. Dis. 30 (Suppl. 2), S160–S170.

Fackler, O.T., Peterlin, B.M., 2000. Endocytic entry of HIV-1. Curr. Biol. 10, 1005–1008.

Farnet, C.M., Haseltine, W.A., 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J. Virol. 65, 1910–1915.

Franke, E.K., Yuan, H.E., Luban, J., 1994. Specific incorporation of cyclophylin A into HIV-1 virions. Nature 372, 359–362.

Goto, T., Nakano, T., Kohno, T., Morimatsu, S., Morita, C., Hong, W., Kiso, Y., Nakai, M., Sano, K., 2001. Targets of a protease inhibitor, KNI-272, in HIV-1-infected cells. J. Med. Virol. 63, 203–209.

Graham, F.L., van der Eb, A.J., 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

Grewe, C., Beck, A., Gelderblom, H.R., 1990. HIV: early virus-cell interactions. J. Acquir. Immune Defic. Syndr. 3, 965–974.

Grobelny, D., Wondrak, E.M., Galardy, R.E., Oroszlan, S., 1990. Selective phosphinate transition-state analogue inhibitors of the protease of human immunodeficiency virus. Biochem. Biophys. Res. Commun. 169, 1111–1116.

Ishima, R., Ghirlando, R., Tözsér, J., Gronenborn, A.M., Torchia, D.A., Louis, J.M., 2001. Folded monomer of HIV-1 protease. J. Biol. Chem. 276, 49110–49116.

Jacobsen, H., Ahlborn-Laake, J., Gugel, R., Mous, J., 1992. Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. J. Virol. 66, 5087–5092.

Jenkins, T.M., Engelman, A., Ghirlando, R., Craigie, R., 1996. A soluble active mutant of HIV-1 integrase: involvement of both the core and carboxyl-terminal domains in multimerization. J. Biol. Chem. 271, 7712–7718.

Kaplan, A.H., Manchester, M., Smith, T., Yang, Y.L., Swanstrom, R., 1996. Conditional human immunodeficiency virus type 1 protease mutants show no role for the viral protease early in virus replication. J. Virol. 70, 5840–5844.

Korber, B., Kuiken, C., Foley, B., Hahn, B., McCuthan, F., Mellors, J., Sodroski, J., (Eds.), 1998. Human Retroviruses and AIDS, Los Alamos, NM.

Louis, J.M., McDonald, R., Nashed, N., Wondrak, E.M., Jerina, D., Oroszlan, S., Mora, P., 1991. Autoprocessing of the HIV-1 protease using purified wild-type and mutated fusion proteins expressed at high levels in *Escherichia coli.* Eur. J. Biochem. 199, 361–369.

Louis, J.M., Weber, I.T., Tözsér, J., Clore, G.M., Gronenborn, A.M., 2000. HIV-1 protease: maturation, enzyme specificity and drug resistance. Adv. Pharmacol. 49, 111–146.

Mely, Y., De Rocquigny, H., Morellet, N., Roques, B.P., Gerad, D., 1996. Zinc binding to the HIV-1 nucleocapsid protein: a thermodynamic investigation by fluorescence spectroscopy. Biochemistry 35, 5175–5182.

Mildner, A.M., Rothrock, D.J., Leone, J.W., Bannow, C.A., Lull, J.M., Reardon, I.M., Sarcich, J.L., Howe, J., Tomich, C.S., Smith, C.W., Heinrickson, R.L., Tomasselli, A.G., 1994. The HIV-1 protease as enzyme and substrate: mutagenesis of autolysis sites and generation of a stable mutant with retained kinetic properties. Biochemistry 33, 9405–9413.

Miller, M.D., Farnet, C.M., Bushman, F.D., 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J. Virol. 71, 5382–5390.

Misselwitz, R., Hausdorf, G., Welfle, K., Hohne, W.E., Welfle, H., 1995. Conformation and stability of recombinant HIV-1 capsid protein p24 (rp24). Biochim. Biophys. Acta 1250, 9–18.

Nagy, K., Young, M., Baboonian, C., Merson, J., Whittle, P., Oroszlan, S., 1994. Antiviral activity of human immunodeficiency virus type 1 protease inhibitors in a single cycle of infection: evidence for a role of protease in the early phase. J. Virol. 68, 757–765.

Oroszlan, S., Luftig, R.B., 1990. Retroviral proteinases. Curr. Top. Microbiol. Immunol. 157, 153–185.

Panther, L.A., Coombs, R.W., Aung, S.A., dela Rosa, C., Gretch, D., Corey, L., 1999. Unintegrated HIV-1 circular 2-LTR proviral DNA as a marker of recently infected cells: relative effect of recombinant CD4, zidovudine and saquinavir in vitro. J. Med. Virol. 58, 165–173.

Pettit, S.C., Moody, M.D., Wehbie, R.S., Kaplan, A.H., Nantermet, P.V., Klein, C.A., Swanstrom, R., 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J. Virol. 68, 8017–8027.

Popovic, M., Sarngadharan, M.G., Read, E., Gallo, R.C., 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.

Roberts, M.M., Copeland, T.D., Oroszlan, S., 1991a. In situ processing of a retroviral nucleocapsid protein by the viral proteinase. Protein Eng. 4, 695–700.

Roberts, M.M., Oroszlan, S., 1989. The preparation and biochemical characterization of intact capsids of equine infectious anemia virus. Biochem. Biophys. Res. Commun. 160, 486–494.

Roberts, M.M., Volker, E., Copeland, T.D., Nagashima, K., Cassel, M.B., Briggs, C.J., Oroszlan, S., 1991b. Regulated proteolytic processing within mature retroviral capsids, in: Kumar, A. (Ed.), Advances in Molecular Biology and Targeted Treatment for AIDS, Plenum Press, New York, pp. 273–280.

Sherman, M.P., de Noroncha, C.M., Heusch, M.I., Greene, S., Greene, W.C., 2001. Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. J. Virol. 75, 1522–1532.

Steinkasserer, A., Harrison, R., Billich, A., Hammersmid, F., Werner, G., Wolff, B., Peichl, P., Palfi, G., Schnitzel, W., Mlynar, E., Rosenwirth, B., 1995. Mode of action of SDZ NIM 811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus 1 (HIV-1): interference with early and late events in HIV-1 replication. J. Virol. 69, 814–824.

Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C., Sodorski, J., Göttlinger, H.G., 1994. Functional association of cyclophilin A with HIV-1 virions. Nature 372, 363–365.

Tomasselli, A.G., Heinrickson, R.L., 1994. Specificity of retroviral proteases: an analysis of viral and nonviral protein substrates. Methods Enzymol. 241, 279–301.

Tomasselli, A.G., Sardich, J.L., Barrett, L.J., Reardon, I.M., Howe, W.J., Evans, D.B., Sharma, S.K., Heinrickson, R.L., 1993. Human immunodeficiency virus type-1 reverse transcriptase and ribonuclease H as substrates of the viral protease. Protein Sci. 2, 2167–2176.

Tong, L., Pav, S., Pargellis, C., Do, F., Lamerre, D., Anderson, P.C., 1993. Crystal structure of human immunodeficiency virus (HIV) type 2 protease in complex with a reduced amide inhibitor and comparison with HIV-1 protease structures. Proc. Natl. Acad. Sci. USA 90, 8387–8391.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Tözsér, J., Bláha, I., Copeland, T.D., Wondrak, E.M., Oroszlan, S., 1991. Comparison of the HIV-1 and HIV-2 proteinases using oligopeptide substrates representing cleavage sites in Gag and Gag-Pol polyproteins. FEBS Lett. 281, 77–80.

Tözsér, J., Weber, I.T., Gustchina, A., Bláha, I., Copeland, T.D., Louis, J.M., Oroszlan, S., 1992. Kinetic and modeling studies of S3-S3′ subsites of HIV proteinases. Biochemistry 31, 4793–4800.

Tsai, W.P., Nara, P.L., Kung, H.F., Oroszlan, S., 1990. Inhibition of human immunodeficiency virus infectivity by chloroquine. AIDS Res. Hum. Retroviruses 6, 481–489.

Uchida, H., Maeda, Y., Mitsuya, H., 1997. HIV-1 protease does not play a critical role in the early phase of HIV-1 infection. Antiviral Res. 36, 107–113.

Veronese, F.D., Copeland, T.D., Oroszlan, S., Gallo, R.C., Sarngadharan, M.G., 1988. Biochemical and immunological analysis of human immunodeficiency virus gag gene products p17 and p24. J. Virol. 62, 795–801.

Weber, I.T., Wu, J., Adomat, J., Harrison, R.W., Kimmel, A.R., Wondrak, E.M., Louis, J.M., 1997. Crystallographic analysis of human immunodeficiency virus 1 protease with an analog of the conserved CA-p2 substrate - interactions with frequently occurring glutamic acid residue at P2′ position of substrates. Eur. J. Biochem. 249, 523–539.

Welker, R., Hohenberg, H., Tessmer, U., Huckhagel, C., Krausslich, H.G., 2000. Biochemical and structural analysis of isolated mature cores of human immunodeficiency virus type 1. J. Virol. 74, 1168–1177.

Welker, R., Kottler, H., Kalbitzer, H.R., Krausslich, H.G., 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology 219, 228–236.

Wlodawer, A., Erickson, J.W., 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. 62, 543–585.

Wondrak, E.M., Sakaguchi, K., Rice, W.G., Kun, E., Kimmel, A.R., Louis, J.M., 1994. Removal of zinc is required for processing of the mature nucleocapsid protein of human immunodeficiency virus, type 1, by the viral protease. J. Biol. Chem. 269, 21948–21950.