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Narrow Substrate Specificity and Sensitivity toward Ligand-binding Site Mutations of Human T-cell Leukemia Virus Type 1 Protease*

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Human T-cell leukemia virus type 1 (HTLV-1) is associated with a number of human diseases; therefore, its protease is a potential target for chemotherapy. To compare the specificity of HTLV-1 protease with that of human immunodeficiency virus type 1 (HIV-1) protease, oligopeptides representing naturally occurring cleavage sites in various retroviruses were tested. The number of hydrolyzed peptides as well as the specificity constants suggested a substantially broader specificity of the HIV protease. Amino acid residues of HTLV-1 protease substrate-binding sites were replaced by equivalent ones of HIV-1 protease. Most of the single and multiple mutants had altered specificity and a dramatically reduced folding and catalytic capability, suggesting that mutations are not well tolerated in HTLV-1 protease. The catalytically most efficient mutant was that with the flap residues of HIV-1 protease. The inhibition profile of the mutants was also determined for five inhibitors used in clinical practice and inhibitor analogs of HTLV-1 cleavage sites. Except for indinavir, the HIV-1 protease inhibitors did not inhibit wild type and most of the mutant HTLV-1 proteases. The wild type HTLV-1 protease was inhibited by the reduced peptide bond-containing substrate analogs, whereas the mutants showed various degrees of weakened binding capability. Most interesting, the enzyme with HIV-1-like residues in the flap region was the most sensitive to the HIV-1 protease inhibitors and least sensitive to the HTLV-1 protease inhibitors, indicating that the flap plays an important role in defining the specificity differences of retroviral proteases.

The retroviral protease (PR)[1] is responsible for the processing of viral Gag and Gag-Pro-pol polyproteins during maturation, hence catalyzing essential steps of virion replication (for a review, see Ref. 1). Therefore, the HIV-1 PR has proved to be an effective target for antiretroviral therapy of AIDS, and various PR inhibitors are in clinical use (reviewed in Ref. 2). However, the continual emergence of viral variants that are cross-resistant to the existing inhibitors of PR indicates that there is a need for new, broad spectrum PR inhibitors (3). Mutations at more than 20 positions of the 99-residue-long HIV-1 PR have been associated with resistance (4). Residues that confer inhibitor resistance to HIV-1 PR are frequently seen in equivalent positions of other retroviral PRs, as demonstrated in case of HTLV-1 PR (5). Therefore, understanding the specificity differences of PRs may help the design of inhibitors effective against the mutant HIV-1 PR forms appearing in resistance.

HTLV-1 is a retrovirus that has been etiologically associated with a number of diseases including adult T-cell leukemia and HTLV-1-associated myelopathy (reviewed in Ref. 6). Studies indicated that blocking viral replication with azidothymidine or lamivudine could have a therapeutic effect (7). HTLV-1, together with HTLV-2, simian T-cell lymphotropic viruses, and bovine leukemia virus (BLV), is a member of the deltaretrovirus genus (8). HTLV-1 PR, like HIV-1 PR, is a homodimeric aspartyl protease, but each monomer comprises 125 residues (Fig. 1*A*). Studies on the HTLV-1 PR have been reviewed recently (9). Although HTLV-1 PR shares 28% sequence identity with HIV-1 PR, based on a molecular model of the enzyme (10), the substrate binding region is more conserved showing 45% sequence identity. Nevertheless, both the substrate specificity and inhibition profile of the two enzymes are substantially different (5, 9–12). Like all other retroviral PRs studied, the HTLV-1 PR displays a high degree of specificity, which is mediated by interactions between side chains of substrate amino acids and corresponding subsite pockets within the homodimeric enzyme. HTLV-1 PR recognizes at least eight amino acid residues of the substrate, spanning from P5 to P3′ (see Ref. 10, notation is according to Ref. 13).

Several comparative studies have been performed on wild type retroviral proteases; however, most of them were done on oligopeptide substrates representing naturally occurring cleavage sites in the same viruses or selected peptides having various amino acid substitutions (for a review see Ref. 14). In a few studies oligopeptides representing cleavage sites in other retroviruses were also probed (for example Refs. 15 and 16). However, no comprehensive studies have been performed between two proteases by using a library of oligopeptide substrates

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[1] The abbreviations used are: PR, protease; BLV, bovine leukemia virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2; HTLV-1, human T-cell leukemia virus type 1; MMLV, Moloney murine leukemia virus; RSV, Rous sarcoma virus; MMTV, mouse mammary tumor virus; SIV, simian immunodeficiency virus; MBP, maltose-binding protein; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

representing cleavage sites in several different retroviruses. Such studies may provide important knowledge about the common characteristics and differences of the PR specificity.

The substrate specificity and inhibitor susceptibility of various retroviral PRs have also been studied by introducing single or multiple mutations into the substrate-binding sites of the enzymes. Besides the HIV-1 PR, which is typically studied by introducing mutations appearing in resistance (17–20), HIV-2 PR (21), Rous sarcoma virus (RSV) PR (22, 23), murine leukemia virus (MMLV) PR (24), feline immunodeficiency virus (FIV) PR (25), and equine infectious anemia virus (EIAV) PR (26) were studied by using this approach. On the other hand, no substrate-binding site mutants of HTLV-1 or any other deltaretroviral protease have been described so far in the literature (9); the only mutant HTLV-1 PRs reported so far contained the stabilizing L40I, C90A, and C109A mutations and showed specificity and activity indistinguishable from the wild type enzyme (5).

To study the basis of substrate and inhibitor specificity of HTLV-1 PR, we used a large set of oligopeptide substrates representing naturally occurring cleavage sites of various retroviruses. All of these substrates were previously verified to be a substrate of PR of the respective retrovirus. Furthermore, the residues of the substrate-binding pockets of the enzyme were replaced with structurally equivalent residues of HIV-1 PR by site-directed mutagenesis. Because the S4-binding site appeared to be one of the major determinants of PR specificity (27), the size of this pocket was systematically changed by mutation of a key residue. Results were analyzed by using crystal structures as well as molecular models of the enzymes and by comparing the results to those obtained with mutagenesis studies on other retroviral PRs.

EXPERIMENTAL PROCEDURES

Enzyme Purification—Stabilized wild type HIV-1 and HTLV-1 PRs were prepared from inclusion bodies as described previously (5, 28). Mutant HTLV-1 PRs were purified by using the same procedure. The reversed-phase HPLC-purified enzymes were homogeneous based on SDS-PAGE. Folding of the enzymes was performed by dialysis of the HPLC fractions in large excess of 25 mM formic acid, pH 2.8, and subsequently into 50 mM sodium acetate buffer, pH 5.0, 1 mM DTT, 1 mM EDTA. Finally, the enzymes were dialyzed against 20 mM Pipes, pH 7.0, containing 1 mM EDTA, 100 mM NaCl, 10% glycerol, 5% ethylene glycol, 0.5% Nonidet P-40, and 10 mM DTT to increase the stability of the PR preparation. Amino acid composition and protein amount of the enzymes were determined by amino acid analyses with a Beckman 6300 amino acid analyzer. Active site titration for the HIV-1 PR was performed with Compound 3, a phosphinate group containing tight binding inhibitors of the enzyme (29). Active site titration of the wild type and some mutant HTLV-1 PRs was performed by using peptide KTKVL-r-VVQPK (IB268), where r represents a reduced peptide bond. Reduced peptide bond-containing inhibitors used in this study (IB268 and IB269, APQVL-r-PVMHP) were synthesized by Dr. Ivo Blaha (Ferring Leciva, Prague, Czech Republic).

Assays with Oligopeptide Substrates—The PR assays were initiated by the mixing of 5 μl (8–8500 nM) of purified HTLV-1 or HIV-1 PR with 10 μl of 2× incubation buffer (0.5 M potassium phosphate buffer, pH 5.6, containing 10% glycerol, 2 mM EDTA, 10 mM dithiothreitol, 4 M NaCl) and 5 μl of 0.01–3.0 mM substrate. The synthesis and characterization of the oligopeptide substrates were described earlier (15, 16, 30–33). The substrate concentration range was selected depending on the approximate K_m values. The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by the addition of 180 μl of 1% trifluoroacetic acid, and an aliquot was injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 × 150 mm, Waters) by using an automatic injector. Substrates and the cleavage products were separated by using an increasing water/acetonitrile gradient (0–100%) in the presence of 0.05% trifluoroacetic acid. Amino acid analysis of the collected peaks was used to confirm the site of cleavage with at least one PR (typically with the PR of the same retrovirus) and to quantitate the amount of substrate cleaved; for other proteases the site of cleavage was assumed to be identical if cleavage products eluted with the same retention time and gave the same relative integration values as those identified by analysis. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis-Menten equation (or by linear fitting for k_{cat}/K_m values determined under pseudo first-order conditions) by using the Fig. P program (Fig. P Software Corp.). The standard errors of the kinetic parameters were below 20%. To test whether the noncleavable peptides representing the naturally occurring cleavage sites could bind to the enzymes, equal concentrations (0.2 mM in assay) of the HTLV-1 capsid ↓ nucleocapsid substrate and the noncleavable peptide were incubated as described above. If more than 10% inhibition was observed, the IC_{50} values for the inhibition were determined by varying the concentration of the inhibitory peptide, and the K_i values were calculated according to Williams and Morrison (34). The standard errors of the K_i values were below 15%.

To assay the HIV-1 and HTLV-1 PR inhibitors, a microtiter plate reader assay using fluorescent Dabcyl/Edans-tagged analog of the capsid ↓ nucleocapsid substrate was used (RE(Edans)TKVL ↓ VVQPK-(Dabcyl)R, where the arrow represents the cleavable bond) (35). Briefly, enzyme, substrate, and inhibitor were incubated in 250 mM phosphate buffer, pH 5.6, containing 5% glycerol, 1 mM EDTA, 5 mM DTT, 500 mM NaCl, 1% Me_2SO in 96-well microtiter plates. The increase of fluorescence was detected at 460 nm, using 355 nm excitation wavelengths in a Victor Wallace fluorimeter-luminometer and corrected with the inner filter effect. K_i values were calculated according to Williams and Morrison (34).

Mutagenesis of the HTLV-1 Protease—The clone coding for the stabilized HTLV-1 PR in a pET expression vector (5) was used as template for the mutagenesis. Mutants were generated by the Quick-Change mutagenesis protocol (Stratagene) with the appropriate oligonucleotide pairs obtained from Genosys-Sigma. Mutations were verified by DNA sequencing performed with ABI-Prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an Applied Biosystems model 373A sequencer.

Expression of the Wild Type and Mutant HTLV Proteases as MBP Fusion Proteins—HTLV-1 PR coding region and an 8-residue-long N-terminal flanking sequence of an infectious HTLV-1 clone, pCS-HTLV-1 (36), was amplified by PCR and was cloned into the EcoRI/BamHI restriction sites of pMal-c2 in-frame with the maltose-binding protein (MBP) coding sequence. The internal HTLV protease sequence (residues 12–116 of the mature PR, see Fig. 1*A*) was exchanged with the sequence of the HTLV-1 PR coding pET expression vector containing the stabilizing mutations by using PacI and EcoNI restriction sites. Ligations and transformation of DH5α cells were performed by using standard protocols (37). Individual mutations were introduced into the expression clone by using the same oligonucleotide pairs and protocol as described for the pET clones, and all vectors used for protein expression were verified by DNA sequencing. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. After expression, cells were collected by centrifugation and lysed in 50 mM Tris-HCl, pH 8.2, containing 1 mM EDTA, 1 mM DTT, 1% Triton X-100 with sonication. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane according to Towbin *et al.* (38). Immunoblots were developed using a mixture of antisera of rabbits immunized against peptides having the N-terminal and C-terminal sequences of the HTLV-1 protease, a peroxidase-conjugated anti-rabbit antibody, and an ECL detection kit (Pierce).

Molecular Modeling—The molecular model of wild type HTLV-1 PR with peptide substrate TKVL ↓ VVQP was built by using the crystal structure of the RSV S9 mutant as described previously (10). A high resolution HIV-1 PR-inhibitor complex (Protein Data Bank code 1fgc, Ref. 28), an RSV PR crystal structure (Protein Data Bank code 2rsp, Ref. 39), an HIV-2 PR crystal structure (Protein Data Bank code 2mip, Ref. 40), a simian immunodeficiency virus (SIV) PR crystal structure (Protein Data Bank code 1siv, Ref. 41), an EIAV PR crystal structure (Protein Data Bank code 1fmb, Ref. 42), and an FIV PR crystal structure (Protein Data Bank code 1fiv, Ref. 43) were used for comparison. Structural models were examined on a personal computer using AMMP (44) and RASMOL (45) or a Silicon Graphics work station using CHAIN (46) or Sybyl (Tripos Inc.).

RESULTS

Comparison of the Substrate Specificity of HTLV-1 and HIV-1 Proteases Using Oligopeptides Representing Naturally Occurring Cleavage Sites in Retroviruses—To compare the specificity of the two proteases, a large set of 50 oligopeptides representing naturally occurring cleavage sites in HIV-1,

TABLE I

Comparison of the specificity of HTLV-1 and HIV-1 proteinases with peptides representing naturally occurring cleavage sites in various viruses

The abbreviations used are as follows: CA, capsid protein; NC, nucleocapsid protein; MA, matrix protein. MPMV, Mason-Pfizer monkey virus; RT, reverse transcriptase; IN, integrase.

No.	Virus site[a]	Substrate sequence	HTLV-1 PR k_{cat}/K_m	HIV-1 PR k_{cat}/K_m
			$mM^{-1}\ s^{-1}$	
1	HIV-1 MA ↓ CA	VSQNY ↓ PIVQ	0	45.3[b]
2	HIV-1 CA ↓ p2	KARVL ↓ AEAMS	0	90.0[b]
3	HIV-1 p2 ↓ NC	TATIM ↓ MQRGN	0	74.0[b]
4	HIV-1 NC ↓ p1	ERQAN ↓ FLGKI	0	1.0[c]
5	HIV-1 p1 ↓ p6	RPGNF ↓ LQSRP	0	0.6[b]
6	HIV-1 in p6	DKELY ↓ PLTSL	0.02[d]	0.02[b]
7	HIV-1 TF ↓ PR	VSFNF ↓ PQITL	0	6.9[b]
8	HIV-1 PR ↓ RT	AETF ↓ YVDGAA	0	10.0[b]
9	HIV-1 RT ↓ RH	CTLNF ↓ PISP	0	24.1[b]
10	HIV-1 RT ↓ IN	IRKIL ↓ FLDG	8.4[d]	202.0[b]
11	HIV-2 MA ↓ CA	KGGNY ↓ PVQHV	0	0.03
12	HIV-2 CA ↓ p2	KARLM ↓ AEALK	0	2.3[b]
13	HIV-2 p2 ↓ NC	IPFAA ↓ AQQRK	2.6	1.1[b]
14	HIV-2 NC ↓ p6	KPRNF ↓ PVAQV	0	0.6[b]
15	HIV-2 TF ↓ PR	RGLAA ↓ PQFSL	0	14.6[b]
16	HIV-2 PR ↓ RT	MSLNL ↓ PVAKV	0	110.0[b]
17	HIV-2 RT ↓ IN	IRQVL ↓ FLEKI	113.6	58.0[b]
18	EIAV MA ↓ CA	PSEEY ↓ PIMID	0.7	15.2
19	EIAV CA ↓ X	QKMML ↓ LAKAL	0	11.8
20	EIAV NC ↓ p9	QKQTF ↓ PIQQK	<0.01	0
21	EIAV PR ↓ RT	AKLVL ↓ AQLSK	0	13.4
22	EIAV RT ↓ UT	KEEIM ↓ LAYQG	<0.01	18.3
23	RSV MA ↓ p2A	GTSCY ↓ CHGTA	0	2.5[e]
24	RSV p2B ↓ p10	PPYVG ↓ SGLYP	0	0[f]
25	RSV p10 ↓ CA	PVVAM ↓ PVVIK	0.1	0
26	RSV CA ↓ p3	IAAAM ↓ SSAIQ	0	0
27	RSV p3 ↓ NC	IQPLIM ↓ AVVNR	>100	318[e]
28	RSV NC ↓ PR	PPAVS ↓ LAMTM	0	0.13[e]
29	RSV PR′ ↓ RT	RATVL ↓ TVALH	0.3	1.9[e]
30	RSV RT ↓ IN	TFQAY ↓ PLREA	0	0.18[d]
31	MMTV MA ↓ pp21	SDLVL ↓ LSAEARR[g]	0	6.9
32	MMTV pp21 ↓ p3	DSKAF ↓ LADTW	0	7.5
33	MMTV p3 ↓ p8	DELIL ↓ PVKRK	2.6	1.5
34	MMTV p8 ↓ n	PPVGFAG ↓ AMA	0	<0.01[h]
35	MMTV n ↓ CA	LTFTF ↓ PVVFMRR[g]	0.01	0.9
36	MPMV p12 ↓ CA	PKDIF ↓ PVTET	0.2	0.2
37	BLV MA ↓ CA	PPAIL ↓ PIISE	164.5[i]	0.3
38	BLV CA ↓ NC	KQPAIL ↓ VHTPG	0	0
39	BLV TF ↓ PR	ELECL ↓ LSIPL	0	8.5
40	BLV PR ↓ p13	PPMVG ↓ VLDAP	0.7[i]	0.04
41	MuLV MA ↓ p12	PRSSLY ↓ PALTP	0	0.2
42	MuLV p12 ↓ CA	TSQAF ↓ PLRAG	0	8.7
43	MuLV CA ↓ NC	MSKLL ↓ ATVV	0[j]	0
44	MuLV NC ↓ PR	TQTSLL ↓ TLDDQ	0	0
45	MuLV PR ↓ RT	PLQVL ↓ TLNIERR[f]	0.1	0
46	MuLV RT ↓ IN	TSTLL ↓ IENSS	0	0[k]
47	HTLV-1 MA ↓ CA	APQVL ↓ PVMHP	85.2[d]	16.1[d]
48	HTLV-1 CA ↓ NC	KTKVL ↓ VVQPK	150.6[d]	14.3[d]
49	HTLV-1 TF1 ↓ PR	DPASIL ↓ PVIP	3.8[d]	0
50	HTLV-1 PR ↓ Px	KGPPVIL ↓ PIQAP	288.3[d]	8.4[d]

[a] Nomenclature of the retroviral proteins is according to Leis *et al*. (58).
[b] Taken from Ref. 30.
[c] Taken from Ref. 20.
[d] Taken from Ref. 5.
[e] Taken from Ref. 32.
[f] This peptide inhibited HIV-1 PR with a K_i of 20 μM.
[g] Residues that were added to the cleavage site sequences to enhance the solubility of the peptides are underlined.
[h] This peptide inhibited HIV-1 PR with a K_i of 14 μM.
[i] Taken from Ref. 33.
[j] This peptide inhibited HTLV-1 PR with a K_i of 61 μM.
[k] This peptide inhibited HIV-1 PR with a K_i of 12 μM.

HIV-2, EIAV, RSV, MMTV, MMLV, BLV, and HTLV-1 were used. The selected peptides were characterized previously and were found to be hydrolyzed by the respective protease coded within the same virus (15, 16, 30–33). Only a few peptides were cleaved by appreciable kinetics with both HIV and HTLV-1 proteases. Although most PR cleavage sites contain hydrophobic residues at P1 and P1′, with one exception a common characteristic of those peptides, which were substrates for both enzymes, is having hydrophobic β-branched residue (Val or Ile) at P2 and also a hydrophobic β-branched residue or Leu at P2′ positions. These are typical arrangements for the naturally occurring HTLV-1 cleavage sites and to a lesser extent for the BLV cleavage sites (see Table I). Three peptides were hydrolyzed only by the HTLV-1 PR but not by HIV-1 PR. On the other hand, HIV-1 PR was able to cleave many peptides, which were not substrates of the HTLV-1 PR. In most cases the lack of hydrolysis by the proteases could be attributed to the inefficient binding of the substrates, because only one noncleaved

peptide showed inhibitory effect on HTLV-1 PR and three on HIV-1 PR, with K_i values being in the micromolar range (see legend of Table I). These results indicated a substantially broader specificity of the HIV-1 PR.

Mutations Introduced to the HTLV-1 Protease—The sequence alignment of HTLV-1 and HIV-1 PRs based on the multiple alignments of PRs with known structures is shown in Fig. 1*A* (10). The kinetic data have been analyzed using a molecular model of HTLV-1 PR (10) and a crystal structure of the HIV-1 PR-inhibitor complex (28). The substrate-binding site of PR is comprised mainly by residues located at three regions: the active site region, the flap region that closes down on the bound ligand, and the C-terminal region at the shoulder of the substrate-binding site (Fig. 1*B*). The active site region is very conserved in retroviral PRs; it contains only one amino acid substitution between HTLV-1 and HIV-1 PRs; the residue corresponding to HTLV-1 PR Met-37 is Asp in HIV-1 PR. The same residue differs in other retroviral PRs. Unlike the active site region, most of the residues of the flap and C-terminal regions are different in the two PRs (Fig. 1*A*). In this study, 15 HTLV-1 PR mutants were generated by replacing key residues of the substrate-binding pockets with the structurally equivalent residues of HIV-1 PR. These mutations included M37D, V56I, L57G, A59I, F67Q, N96T, N97P, and W98V (Fig. 1 and Table II). Because the residue corresponding to Met-37 is an important determinant of specificity in PRs (27), hydrophobic residues of various sizes (Ala, Val, and Ile) as well as the drug-resistant mutation Asn of HIV-1 PR were introduced at this position (Table II). Three mutants with combined substitutions at the flap and C-terminal regions were also tested. The mutant PRs were purified to homogeneity from inclusion bodies and folded as described previously for wild type PRs (5, 28).

Activities of the Mutant HTLV-1 Proteases against a Peptide Substrate and Their Autoprocessing Capabilities from MBP Fusion Protein—The peptide representing the capsid ↓ nucleocapsid cleavage site of HTLV-1 was shown to be a good substrate of both wild type HTLV-1 and HIV PRs (Ref. 5, Table I and Table II). Eight of 15 HTLV-1 PR mutants were able to hydrolyze this substrate, although mostly with dramatically reduced kinetic efficiency as compared with the two wild type enzymes (Table II). However, the K_m values obtained with some mutants were similar to those obtained with the wild type enzymes, whereas some other mutants showed moderately increased values. Because many of the mutants showed very low activity and loss of inhibitor potency, active site titration was possible only for M37V, M37I, and the mutant having HIV-1 PR-like flap (Table II). Comparison of the active protein content with the total amount of protein suggested that the folding efficiency of these mutants is much lower as compared with the wild type enzymes. These results also suggest that the lack of activity of some mutants may be the consequence of the inability to refold properly.

To further verify the low activity/improper refolding capability of the mutants, they were also expressed as MBP fusion proteins, from which the wild type enzyme is capable of self-processing itself out (Fig. 3). An advantage of expressing a protein in fusion with MBP is that it is very effective to promote the solubility of polypeptides to which it is fused, compared with other commonly used proteins like glutathione *S*-transferase and thioredoxin (47, 48). All of the mutants that appeared to be inactive after folding from inclusion bodies using the pET expression system were also expressed in fusion with MBP. Unlike the wild type enzyme as well as the mutant having HIV-1 PR-like flap, none of those mutants were capable of self-processing during expression (Fig. 3).

Mutations Affecting Mainly the S4/S2-binding Sites of HTLV-1 Protease—The S4 subsite is close to the surface of the enzyme and is partly exposed to solvent for all retroviral PRs. In contrast, the S2 subsites are relatively small and formed by mostly hydrophobic residues and consequently would be expected to accommodate smaller hydrophobic P2 residues. However, whereas HIV-1 PR preferred more polar amino acids at P2 (and P2′), all other PRs including HTLV-1 PR preferred hydrophobic residues (10, 27, 49).

Residues forming the S4-binding sites of the HTLV-1 and HIV-1 PRs include Met-37/Asp-30, Ser-55/Met-46, Val-56/Ile-47, Leu-57/Gly-48, and Val-92/Leu-76 with very distal contributions from Phe-67/Gln-58 and the Pro-7 to Ala-8 insertion in HTLV-1 PR (Fig. 2, Ref. 10). Asp-30 of HIV-1 PR and structurally equivalent residues of other PRs were shown to be important determinants for the different specificities of the PRs at S4 and S2 (23, 24). In another example, HIV PR mutant Asn-30, harboring a mutation appearing in nelfinavir resistance, showed specificity changes compared with wild type enzyme (19) accompanied by small structural changes in PR-substrate interactions (28). Therefore, HTLV-1 PR Met-37 was substituted by Asp, Asn, Ala, Val, and Ile. Most interesting, the mutants containing Asp-37 or Asn-37 were inactive on all substrates tested (not shown). They were also not able to self-process themselves from MBP fusion proteins (Fig. 3). Substituting the smaller Ala in this position also rendered the enzyme inactive on most of the substrates (not shown), except for the substrate with Phe at P1, which was the best substrate for the wild type enzyme (10), suggesting that proper hydrophobic interactions at this position of HTLV-1 PR are required for efficient catalysis. The other mutants with conserved substitutions (Val or Ile) at residue 37 still showed substantially reduced k_{cat} values without an apparent change of K_m values as shown for the wild type substrate (Table I). The specificity of these mutants was also substantially altered. Both preferred P4 Ile as compared with the P4 Val preference for the wild type HTLV-1 PR (Fig. 4*A*), similar to the HIV-1 PR. However, they also preferred Leu over Val at this position, which is not seen with either of the wild type PRs. Val-56 of HTLV-1 is part of the flap region, and Ile is the equivalent residue in HIV-1 PR. The V56I mutant was a very inefficient enzyme (Table I), but it hydrolyzed the P4 Leu substituted peptide with a substantially increased rate (Fig. 4*A*), suggesting that the larger side chain of Leu at P4 was able to make better hydrophobic interactions with V56I than the other smaller side chains of Val, Thr, and Ile at P4. The order of P4 side chain preference of the mutant having HIV-1 PR-like flap resembled that of the wild type HIV-1 PR, except it did not hydrolyze well with the substrate having Ala at this position (Fig. 4*A*).

The S2 subsite shows differences in residues Met-37/Asp-30, Val-56/Ile-47, Ala-59/Ile-50, and Val-92/Leu-76 for HTLV-1 and HIV-1 PRs (Fig. 2) (10). These differences result in a somewhat larger, more hydrophobic S2 subsite in HTLV-1 PR as compared with HIV-1 PR. Most interesting, both M37V and M37I mutations changed the Val → Leu → Ile P2 preference to Ile → Val → Leu, the same as observed for HIV-1 PR; however, they were not able to hydrolyze the substrate containing bulky aromatic Phe at this position (Fig. 4*B*). Introduction of β-branched residues into this position may restrict the size of the side chain, which can fit into this subsite. Similar to the findings obtained with the P4-modified substrate, the mutant having the HIV-1 PR-like flap showed a P2 specificity more similar to that of HIV-1 PR than HTLV-1 PR, but this mutant also did not tolerate Phe at this position, likely due the two β-branched residues introduced into the sequence (Fig. 4*B*). These results suggested that the flap region is important not only for the enzyme activity (27) but also for the specificity differences of the two wild type PRs.

A

Structural alignment of the HTLV-1 and HIV-1 proteinases

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          1                  10                  20                  30
HTLV-1    P V I P L D PA R R P V I K A Q V D T Q T S H P K T I E A L

HIV-1     P Q I T L W    Q R P L V T I K I G          G Q L K E A L
          1                   10                        20

                          40                  50      54 54'          60
HTLV-1    L D T GA D M T V L P I A L F S S N T P L K N T1 T2 S V L G A G
          -----------------                             ------------
          active site region                            flap region
HIV-1     L D T GA D D T V L E E    M S   L P G R W K P K   M I G G I G
                    30                        40                  50

                          70                  80                  90
HTLV-1    G Q T Q D H F K L T S L P V L I R L P F R T T P I V L T S C L

HIV-1     G F I K V R Q Y D   Q I L I E I C          G H   K A I G T V
                         60                             70

                        100                 110                 120       124
HTLV-1    V D T K NN W A I I G R D A L Q Q C Q G V L Y L P E A K G P P V I L
          ------------------
          C-terminal region
HIV-1     L V G P T P V N I I G R N L L T Q I G C T L N F
                80                  90                  99
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B

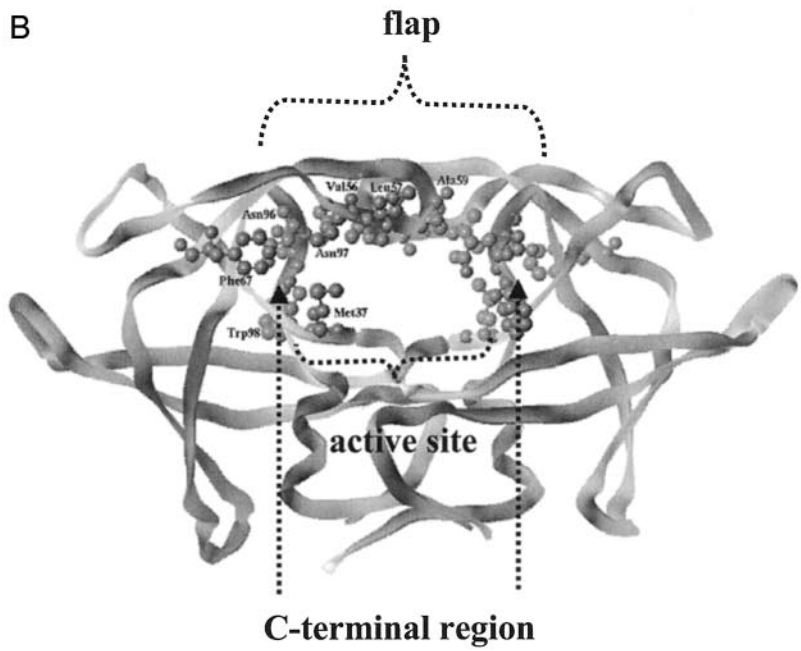


FIG. 1. *A*, alignment of HTLV-1 and HIV-1 PR sequences. HTLV-1 PR T1 and T2 represent different positions of Thr-54 for modeling subunit 1 and 2, respectively (10). Residues of the PRs that are involved in the substrate binding are shown in *boldface*. Residues mutated to stabilize the enzymes from autoproteolysis are *underlined*. HTLV-1 and HIV-1 proteases used in this study contained three mutations (L40I, C90A, and C119A) and five mutations (Q7K, L33I, L63I, C67A, and C95A), respectively. Previous studies demonstrated that the specificity of these mutated enzymes was identical to the specificity of nonstabilized forms (5, 19). *B*, ribbon model of HTLV-1 PR. Position of the residues that were mutated in this study is indicated by a *ball-and-stick* representation. Residues are labeled only for the first monomer.

TABLE II

Description of the mutant enzymes, affected substrate-binding sites, and kinetic parameters for the hydrolysis of the HTLV-1 capsid ↓ nucleocapsid cleavage site peptide (KTKVL ↓ VVQPK)

Enzyme	Affected site or region[a]	Folding efficiency[b]	K_m	k_{cat}	k_{cat}/K_m
		%	mM	s^{-1}	$mM^{-1}\ s^{-1}$
HTLV-1 PR (wt)[c]		100	0.063	10.0	158.7
HIV-1 PR (wt)[c]		100	0.16	3.6	22.5
HTLV-1 PR (M37D)	S4, S2	ND[d]	Not hydrolyzed[e]		
HTLV-1 PR (M37N)	S4, S2	ND	Not hydrolyzed		
HTLV-1 PR (M37A)	S4, S2	ND	Not hydrolyzed		
HTLV-1 PR (M37V)	S4, S2	63	0.040	0.11	2.78
HTLV-1 PR (M37I)	S4, S2	4	0.060	0.17	2.90
HTLV-1 PR (V56I)	S4, S2	ND	ND	< 0.001	ND
HTLV-1 PR (L57G)	S4, S3, S2	ND	Not hydrolyzed		
HTLV-1 PR (A59I)	S3, S2, S1	ND	0.438	(0.021)	(0.05)
HTLV-1 PR (F67Q)	S4	ND	Not hydrolyzed		
HTLV-1 PR (N96T)	S3, S1	ND	0.301	(0.007)	(0.02)
HTLV-1 PR (N97P)	S3, S1	ND	0.127	(0.002)	(0.02)
HTLV-1 PR (W98V)	S3, S1	ND	0.200	(0.002)	(0.01)
HTLV-1 PR (V56I/L57G/A59I)	All	24	0.155	1.68	10.87
HTLV-1 PR (N96T/N97P/W98V)	S3, S1	ND	Not hydrolyzed		
HTLV-1 PR (V56I/L57G/A59I/N96T/N97P/W98V)	All	ND	Not hydrolyzed		

[a] Note that due to the symmetrical homodimeric nature of the PR, the respective primed binding sites (like S4′, S2′, etc.) are also affected by the mutation(s), but these sites were not probed in this study.

[b] Folding efficiency was calculated from the ratio of active enzyme determined by using active site titration and total protein amount. For enzymes with low detectable activity, it was not possible to determine this ratio, for these mutants the catalytic constants calculated from the protein content are given in parentheses.

[c] These data are taken from Ref. 10.

[d] Not determined.

[e] No hydrolysis was observed when incubated with 0.4 μM PR for 20 h at 37 °C.

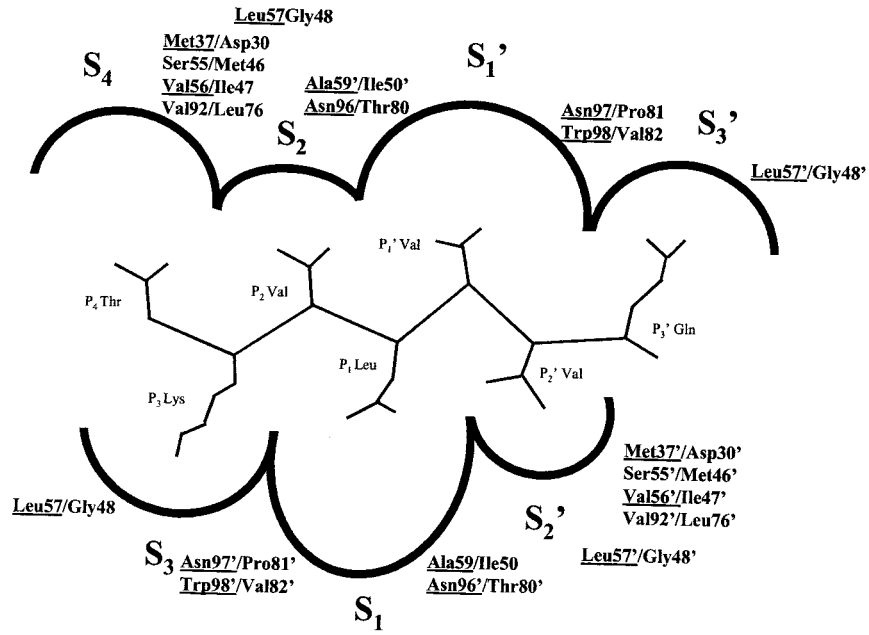


FIG. 2. **A schematic representation of the HTLV-1 capsid ↓ nucleocapsid substrate in the S4-S3′ subsites of PR.** The relative size of each subsite is indicated approximately by the area enclosed by the *curved line* around each substrate side chain. PR residues forming the subsites are shown only for those that differ between the two PRs as HTLV-1/HIV-1 residues. Residues of HTLV-1 that were mutated in this study are *underlined*.

Mutations Affecting the S3/S1-binding Sites of HTLV-1 Protease—The S3 subsite is usually large and is similar to S4 in being partly exposed to solvent at the surface of the enzyme. Consequently, the P3 side chain may be positioned either to interact with the more polar residues of the PR surface or to interact with the hydrophobic internal residues of the enzyme. Several P3-substituted substrates were hydrolyzed by both wild type PRs, indicating that this site is not restrictive, but although HTLV-1 PR preferred the original Lys-containing substrate, HIV-1 PR preferred larger hydrophobic residues (Fig. 5*A*) (10). Analysis of the HIV-1 PR crystal structure and the model structure for HTLV-1 PR suggested that about half of the residues participating in the S3-P3 interactions are identical in both enzymes (10). The S3 subsites differ in residues Leu-57/Gly-48 and Ala-59/Ile-50 from the flap, whereas Asn-96′/Thr-80′, Asn-97′/Pro-81′, and Trp-98′/Val-82′ residues from the other subunit form one side of the subsite. Wild type HTLV-1 PR was less sensitive to P3 changes (Fig. 5*A*) (10), whereas Leu-57 and Trp-98′ were predicted to interact favorably with hydrophobic residues, Asn-96′ and Asn-97′ can potentially interact with polar P3 residues, including Lys of the unmodified substrate. Both L57G and A59I flap mutants were very inefficient enzymes on the unsubstituted peptide (Table I). Leu-57 is predicted to interact favorably with larger hydrophobic P3 residues in the wild type HTLV-1 PR, and this interaction appears to be important for efficient hydrolysis. Although P3 substitutions did not substantially improve the hydrolysis for the L57G mutant, surprisingly the P3 Ala-substituted peptide was the best substrate for this enzyme (not shown). Unlike the L57G mutant, introduction of larger hydrophobic residues

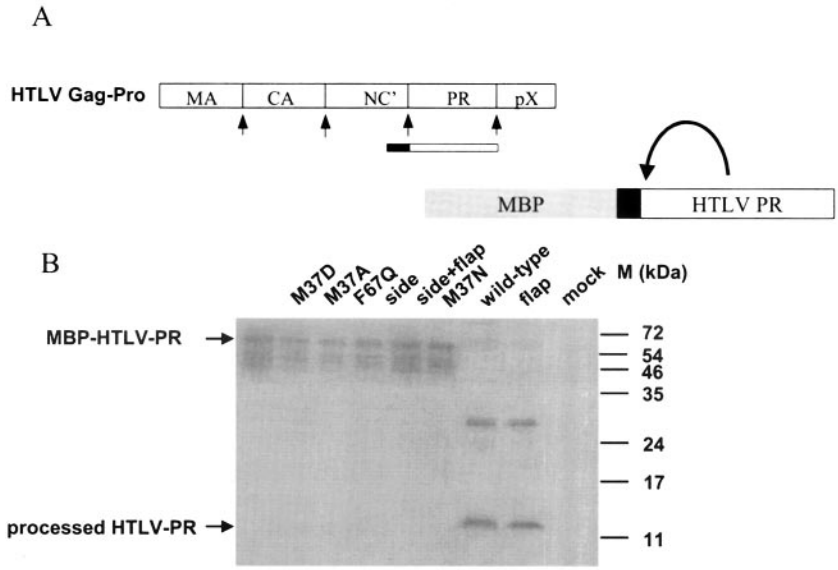


FIG. 3. **Expression of the HTLV-1 protease mutants as MBP fusion proteins.** *A*, schematic representations of the fusion proteins used in this study. The 8-residue N-terminal flanking sequence allows the protease to process itself out of the fusion protein. *B*, self-processing capability of mutant HTLV-1 proteases. Fusion proteins were expressed in *E. coli* cells, and the self-processing capability of the proteases was analyzed by immunoblotting using anti-HTLV-1 PR antisera. Mutations in the HTLV-1 PR coding regions are indicated *above the lanes*. *Side*, N96T/N97P/W98V; *flap*, V56I/L57G/A59I; *side+flap*, V56I/L57G/A59I/N96T/N97P/W98V. As a control, pMal-c2 vector expressing only MBP was used for transformation and expression (labeled as *mock*).

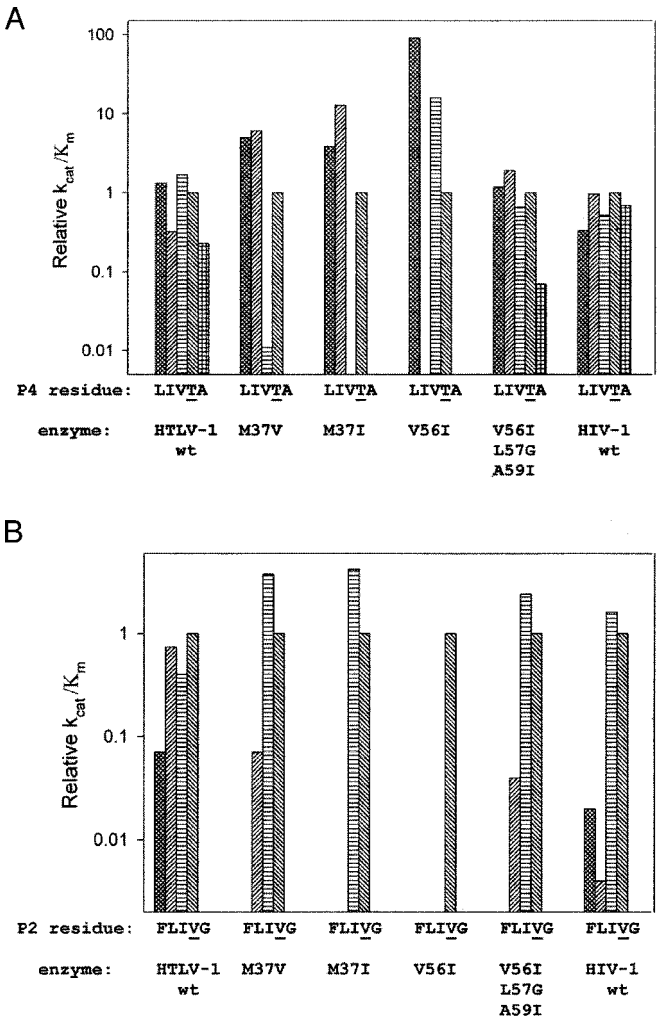


FIG. 4. **Relative specificity constants of the wild type and mutant HTLV-1 proteases for peptides having P4 (*A*) and P2 (*B*) substitutions in the HTLV-1 capsid ↓ nucleocapsid substrate sequence.** Original P4 residue (Thr) of the substrate (KTKVL ↓ VVQPK) is *underlined*. Values are calculated as k_{cat}/K_m values relative to the unmodified substrate. The detection limit of the relative k_{cat}/K_m values for the wild type PRs was 0.001. Because of the low activity of the mutants, the detection limit of the relative k_{cat}/K_m values for M37V, M37I, V56I, and the triple mutant were 0.01, 0.7, 0.7, and 0.01, respectively.

into P3 dramatically improved the specificity constants for the A59I mutant; the Ile side chain in this position was predicted to make more hydrophobic contacts with hydrophobic P3 side chains as compared with the original Ala residue. The A59I mutant showed a preference for Phe at P3, consistent with additional favorable hydrophobic interactions. Substitution of Asn-96 to Thr and Asn-97 to Pro increased the relative preference toward hydrophobic P3 residues over Lys at P3 (Fig. 5*A*). Unexpectedly, the W98V mutant did not have a preference toward larger hydrophobic residues, it rather preferred smaller hydrophobic ones (Fig. 5*A*).

The S1 subsite shows differences in Ala-59/Ile-50, Asn-96′/Thr-80′, Asn-97′/Pro-81′, and Trp-98′/Val-82′ in HIV-1 and HTLV-1 PRs (10). Despite these sequence differences, the structure and the specificity at the S1 subsite of the two PRs appears to be similar (10). The S1 subsite is a relatively large and hydrophobic internal pocket in the enzyme. The best substrate for both enzymes had Phe at P1 (10). Unexpectedly, A59I, N97P, and W98V mutants preferred P1 Met instead of the P1 Phe preferred by both wild type PRs (Fig. 5*B*). These substitutions introduced β-branched side chains, or conformational restraints in case of Pro, which may restrict the fit of the large bulky Phe P1 side chain within the subsite.

The specificity of the mutant having HIV-1 PR-like flap resembled the specificity of HIV-1 PR on both P3 and P1; however, it also had some unique features, for example the best P3 substitution was Leu and the mutant did not favor Phe at P1 or P1′.

Inhibition Profile of the Wild type and Mutant HTLV-1 Proteases—Several potent inhibitors that target HIV-1 PR are used in clinical practice. These include indinavir, saquinavir, nelfinavir, ritonavir, and amprenavir. We have tested these inhibitors and two reduced peptide-containing HTLV-1 inhibitors on the wild type and the mutant HTLV-1 PRs. The assays were performed in a high-throughput microtiter plate fluorescent assay system (35). Except for indinavir, the HIV-1 PR inhibitors were not effective against the wild type HTLV-1 PR (Fig. 6). We have previously tested several inhibitors of HIV-1 PR, including saquinavir, as inhibitors of the HTLV-1 PR by using the very labor-intensive and time-consuming HPLC assay. Only compound 3 inhibited the PR up to 10 μM (5). Four of the HIV-1 PR inhibitors used in clinical practice were also tested on HTLV-1 Gag processing *in vitro* and did not show any

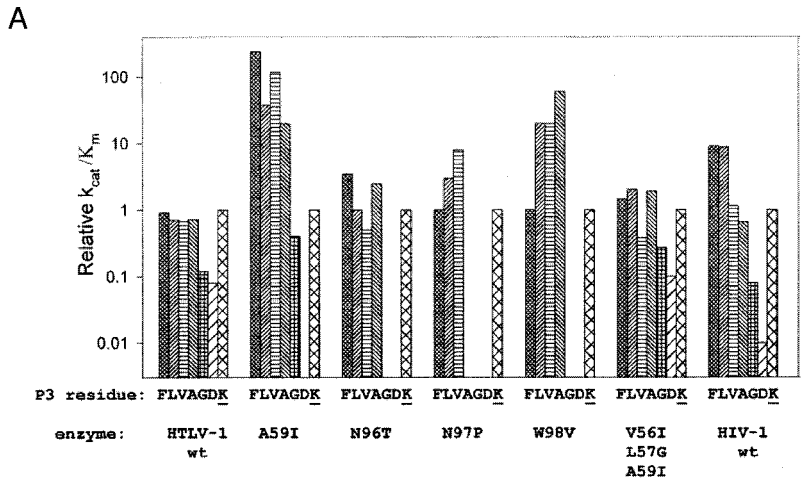
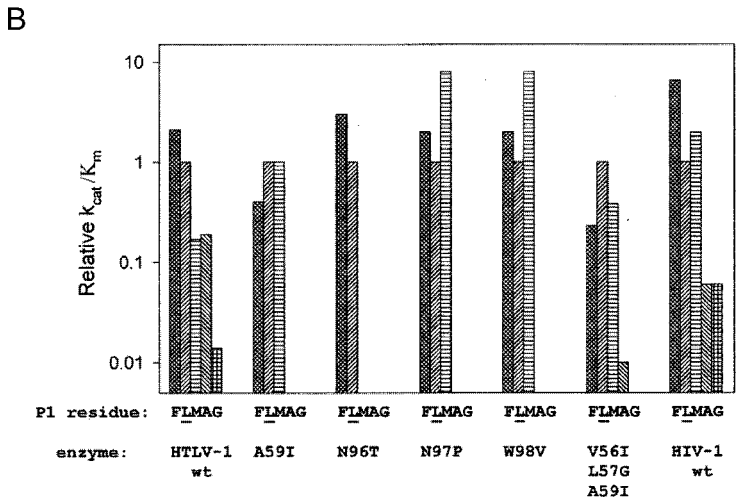


FIG. 5. **Relative specificity constants of the wild type and mutant HTLV-1 proteases for peptides having P3 (*A*) and P1 (*B*) substitutions in the HTLV-1 capsid ↓ nucleocapsid substrate sequence.** Original P3 residue (Lys) of the substrate (KTKVL ↓ VVQPK) is *underlined*. Values are calculated as k_{cat}/K_m values relative to the unmodified substrate. The detection limit of the relative k_{cat}/K_m values for the wild type PRs was 0.001. Because of the low activity of the mutants, the detection limit of the relative k_{cat}/K_m values for A59I, N96T, N97P, W98V, and the triple mutant were 0.3, 0.7, 0.7, 0.7, and 0.01, respectively.



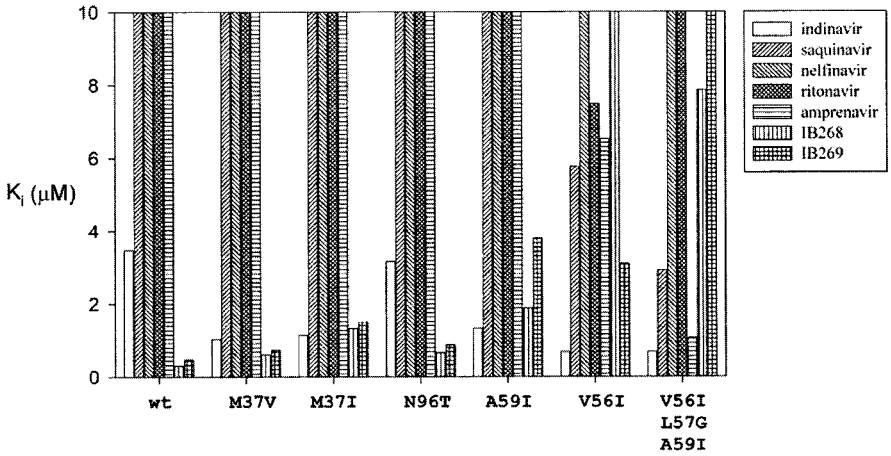


FIG. 6. **Inhibition of wild type and mutant HTLV-1 proteases by clinically used HIV-1 PR inhibitors as well as HTLV-1 cleavage site analogs.** A fluorescent substrate having the sequence of the HTLV-1 capsid/nucleocapsid cleavage site was used to measure the inhibition of the proteases. K_i values were determined according to Williams and Morrison (34).

effect (50). These results demonstrate the large differences in specificity of the two enzymes. The most potent inhibitor of the HTLV-1 PR in our previous studies was a statine-containing inhibitor, based on the matrix ↓ capsid cleavage site sequence of HTLV-1. In contrast, the statine-containing peptide based on the capsid ↓ nucleocapsid cleavage site sequence did not inhibit the enzyme (5). Here we have tested two reduced peptide bond-containing inhibitors of the HTLV-1 PR, IB268 and IB269, with the sequences of the HTLV-1 capsid ↓ nucleocapsid and matrix ↓ capsid cleavage sites, respectively. Our previous studies on HIV-1 and HIV-2 PRs indicated that reduced peptide bond-containing substrate analogs act as potent inhibitors of the retroviral proteinases in the presence of high salt (30). IB268 and IB269 were potent inhibitors of the wild type HTLV-1 PR in the HPLC assay, with K_i values below 50 nM (35). They were also the best inhibitors of the wild type HTLV-1 PR in the fluorometric assay, which was performed in a substantially lower ionic strength (Fig. 6).

Most of the HTLV-1 PR mutants were not inhibited by the HIV-1 PR inhibitors; only the enzyme having an HIV-1 PR-like flap showed some degree of sensitivity toward some of the HIV-1 PR inhibitors, although it showed decreased affinity toward the HTLV-1 PR inhibitors (Fig. 6). The large effect of the V56I mutation on inhibitor susceptibility is in good agreement with previous findings (4) that the corresponding I47V mutation appears in drug resistance *in vitro*. This residue seems to be important for defining the differences in inhibitor susceptibility of the HIV-1 and HTLV-1 PRs.

DISCUSSION

The specificity of HTLV-1 and HIV-1 proteases was compared by using oligopeptides representing maturation cleavage sites in various retroviruses. Based on the results, the specificity of HTLV-1 PR appears to be substantially narrower as compared with the specificity of HIV-1 PR. Cleavage site sequences of HTLV-1 PR appear to be more "ancient" than those in HIV-1; distantly related retro-elements, like Ty3 or Ty1 which are evolutionarily much more conserved than infectious retroviruses, also contain β-branched residues at P2 and P2′, although the P1 and P1′ residues are typically smaller and less hydrophobic than the residues in HTLV-1 and HIV-1 cleavage sites (51, 52). Although the highest specificity constants were obtained with the RSV p3 ↓ nucleocapsid protein cleavage site peptide (Table I), this peptide behaved anomalously. The increase of the substrate concentration caused a decrease in activity (data not shown), likely due to nonproductive binding, and therefore we have selected the capsid ↓ nucleocapsid cleavage site (KTKVL ↓ VVQPK) for developing a fluorescent assay for both proteinases. This peptide was also found to be a substrate for the HIV-2, EIAV, and BLV proteases[2]; therefore, it may also serve as a starting sequence for a general retroviral PR assay.

To characterize further the specificity of HTLV-1 PR, mutations were introduced into its ligand-binding sites. Many of the mutants we have generated were either inactive or possessed scarcely detectable activity and loss of capability to self-process from an MBP fusion protein. Based on active site titration, the folding efficiency of the active mutants is much lower as compared with the wild type enzymes. In the case of HIV-1 PR, similar to the wild type HTLV-1 PR, close to 100% folding efficiency was observed (5, 22). Many HIV-1 PR forms with drug-resistant mutations had a similar folding efficiency and also comparable catalytic efficiency to that of wild type PR (17–20). However, in the case of some single mutants of HIV-1 PR, we observed substantial reduction (40–90%) in folding efficiency[2] (20), suggesting that although the wild type sequence appears to be optimal, even single mutations may have substantial effect on the protease folding, which may influence the viability of the mutant viruses harboring these mutant protease sequences.

The activity of four single mutants harboring HIV-like residues (M37D, M37N, L57G, and F67Q) was undetectable in the assays. Mutation to Asp of the residue corresponding to HIV-1 PR Met-37 of murine leukemia virus PR (His) and RSV PR (Ile) resulted in enzymes that showed specificity constants close to those of the respective wild type PRs (23, 24). On the other hand, mutation to Asp of the corresponding Ile residue of FIV PR resulted in an inactive mutant (25). The side chain of the corresponding HIV-1 Asp residue in HIV-1 PR crystal structures points toward the solvent, but it turns inward in crystal structures with P2′ Glu residues and interacts with that side chain (53). This Asp residue is also part of one of the regions involved in the cooperative folding and stability of HIV-1 PR (54). There is a hydrophobic cluster in the S4/S4′-binding site of all retroviral PRs with known crystal structure (HIV-1, HIV-2, SIV, EIAV, FIV, and RSV PRs). It seems that the residue in retroviral PRs corresponding to Met-37 of HTLV-1 PR can be changed without any deleterious effect if the cluster is composed of at least 4 side chains as is the case for HIV-1, HIV-2, SIV, EIAV, and RSV PRs. However, if this hydrophobic cluster is formed by only 3 residues as in FIV PR and HTLV-1 PRs, the hydrophobic nature of Met-37 of HTLV-1 PR or the corresponding Ile of FIV PR may be crucial for stabilizing the structure or for the folding pathway.

Leu-57 is part of the flap. Mutation of the corresponding Ile of FIV PR and EIAV (25, 26), as well as His of avian myeloblastosis virus/RSV PR (22), also resulted in inactive enzymes, although the HIV-1 PR was able to accommodate Ile, His, and various other amino acid residues in place of Gly at this position (55), and its mutation to Val occurs in saquinavir and indinavir resistance (4). Phe-67 in HTLV-1 was predicted to contribute to the S4-binding pocket (10). The corresponding Gln residue is not subject to natural variations (except that it is replaced by Glu in some cases) and to resistant mutations (4), indicating that it may be a structurally important residue.

When residues of the feline immunodeficiency virus (FIV PR) or murine leukemia virus PR were changed to the equivalent residues of HIV-1 PR, many of the mutants had catalytic efficiency close to that of the wild type enzyme (24, 25). Although no folding efficiency data were reported for these mutants, the catalytic efficiency implied that their folding capability was not substantially less than that of the respective wild type enzymes. Whereas most of the RSV PR mutants as well as the wild type enzyme refolded with similar efficiencies (18–30%), some mutants showed even higher catalytic activity than the wild type RSV PR (23). Based on our results, the specificity of HTLV-1 PR is narrower than that of HIV-1 PR, and both the folding pathway and the catalytic efficiency of HTLV-1 PR is apparently much more sensitive toward mutations than those of other retroviral PRs, especially HIV-1 PR. In this aspect it is important to note that after infection HTLV-1 typically replicates by cell division and not by producing exogenous virions, omitting the error-prone reverse transcription step. Therefore, HTLV-1 is much more conserved than HIV-1 (56). As a consequence, the HTLV-1 PR has not undergone the rapid evolution that was able to optimize the HIV-1 PR for both high catalytic efficiency as well as flexibility in tolerating mutations under selective pressure.

The most active mutant was the one containing the complete flap substitution, although the enzymes bearing the individual L57G and A59I mutants were very inefficient enzymes. Our previous studies (27) indicated the importance of the flap region for catalytic activity of HIV PR, but it did not appear to have a major role in determining substrate specificity. A comparative mutational study on HIV-1 and HIV-2 PRs also suggested that the C-terminal region was important for the differential activity of the enzymes (21). The mechanism of resistance due to mutation of Gly-48 of the HIV-1 PR flap was suggested to be an alteration of the flap mobility instead of changing the enzyme-ligand interactions (57). The results presented here suggest that the flap region has an important role in determining the differences in both substrate specificity and inhibitor susceptibility of the HTLV-1 and HIV-1 proteases.

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[2] J. Tözsér, unpublished data.

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