Amino Acid Preferences of Retroviral Proteases for Amino-Terminal Positions in a Type 1 Cleavage Site^{▽‡}

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The specificities of the proteases of 11 retroviruses were studied using a series of oligopeptides with amino acid substitutions in the P1, P3, and P4 positions of a naturally occurring type 1 cleavage site (Val-Ser-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln) in human immunodeficiency virus type 1 (HIV-1). Previously, the substrate specificity of the P2 site was studied for the same representative set of retroviral proteases, which included at least one member from each of the seven genera of the family *Retroviridae* (P. Bagossi, T. Sperka, A. Fehér, J. Kádas, G. Zahuczky, G. Miklóssy, P. Boross, and J. Tözsér, *J. Virol.* 79:4213–4218, 2005). Our enzyme set comprised the proteases of HIV-1, HIV-2, equine infectious anemia virus, avian myeloblastosis virus (AMV), Mason-Pfizer monkey virus, mouse mammary tumor virus (MMTV), Moloney murine leukemia virus, human T-lymphotropic virus type 1, bovine leukemia virus, walleye dermal sarcoma virus, and human foamy virus. Molecular models were used to interpret the similarities and differences in specificity between these retroviral proteases. The results showed that the retroviral proteases had similar preferences (Phe and Tyr) for the P1 position in this sequence context, but differences were found for the P3 and P4 positions. Importantly, the sizes of the P3 and P4 residues appear to be a major contributor for specificity. The substrate specificities correlated well with the phylogenetic tree of the retroviruses. Furthermore, while the specificities of some enzymes belonging to different genera appeared to be very similar (e.g., those of AMV and MMTV), the specificities of the primate lentiviral proteases substantially differed from that observed for a nonprimate lentiviral protease.

Infection by retroviruses, most notably human immunodeficiency virus (HIV), causes severe immunodeficiency, cancer, and anemia in humans and in many economically important vertebrates. With the exception of HIV/AIDS, relatively few studies have focused on human diseases arising from retroviral infections.

The total number of people living with HIV has continued to increase, and it is estimated to be about 33 million people in the world (36). In 2007, there were more than 2 million new infections and about 2 million people died of AIDS-related illnesses (36). Antiretroviral therapies have proved to be effective since the introduction of the combination of drugs including inhibitors of retroviral proteases (PRs). The PR of HIV is an excellent target for chemotherapy (for reviews, see references 16 and 33), since the PR activity is essential for the maturation and infectivity of the virus (as reviewed in references 14 and 21). Specificity studies of wild-type and mutant HIV PRs have provided a basis for the rational design of potent, selective inhibitors (4, 30, 38) and also may help to circumvent the problems caused by the rapidly developing resistance to the compounds used in therapy (28).

Human T-lymphotropic virus (HTLV) infection is also a global epidemic: 10 million to 20 million individuals are estimated to be carriers of the virus, and the risk of developing disease is estimated to be 5% in infected patients (15). HTLV type 1 (HTLV-1) has been etiologically associated with a number of diseases, including adult T-cell leukemia and HTLV-1-associated myelopathy (10). Studies indicate that viral replication is critical for the development of HTLV-1-associated myelopathy, and initial studies suggested that blocking replication with zidovudine had a therapeutic effect (26). HTLV PR inhibitors may also have therapeutic value for HTLV-associated diseases, similarly to the successful application of HIV PR inhibitors to treat AIDS.

Another retrovirus, which can infect people, is the human foamy virus (HFV) or prototype foamy virus. Retroviruses of the foamy virus subgroup have several unusual features (8, 23), but the PR of the HFV is also essential for viral infectivity (11). HFV holds great promise in gene therapy (20) because of its inability to cause pathogenic disease (12). However, such an application requires detailed knowledge of the replication strategy as well as the enzymes of this virus.

Retroviral PR cleavage sites are currently classified into two major groups (reviewed in reference 33). Type 1 cleavage sites have an aromatic residue and Pro, while type 2 cleavage sites have hydrophobic residues (excluding Pro) at the site of the cleavage. Other positions, especially P2 and P2' (the nomenclature is according to reference 25), also substantially contribute to the specificity and showed significant differences in terms of preference in the two types of cleavage sites.

A comparative study of divergent members of the retroviral

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FIG. 1. A schematic representation of the HIV-1 matrix/capsid substrate in the S4 to S3' subsites of HIV-1 PR. The relative size of each subsite is indicated approximately by the area enclosed by the curved line around each substrate side chain.

PR family is a promising approach not only to recognize general and specific features of the PRs but also to discover the mutational capacities of the PRs. Several of the mutations causing drug resistance of HIV type 1 (HIV-1) PR introduce residues into the substrate binding sites that are found in the equivalent positions of other retroviruses (13, 24). Retroviral PRs can recognize at least seven residues of the substrates, which bind to the enzyme in an extended conformation, as schematically shown in Fig. 1. Previously, a large set of peptides containing single amino acid substitutions in the P4-to-P3' region of the Val-Ser-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln oligopeptide, a typical type 1 substrate representing the matrix/capsid cleavage site of HIV-1 (where the arrow denotes the site of the cleavage), was used to characterize the specificity of the PRs of various retroviruses, including HIV-1, HIV-2 (32, 34), equine infectious anemia virus (EIAV) (37), Moloney murine leukemia virus (MMLV) (18), and avian myeloblastosis virus (AMV) (31). Based on these studies, the P2 position was found to be critical for determining the substrate specificity differences of retroviral PRs. Therefore, the P2 position was characterized in the first part of our study (1). Here, we have continued with studies of the specificities of the P1, P3, and P4 positions using the same PR set previously utilized for the mapping of the P2 position, comprising at least one member of each genera of the family *Retroviridae*, including HIV-1, HIV-2, EIAV, MMLV, AMV, Mason-Pfizer monkey virus (MPMV), mouse mammary tumor virus (MMTV), HTLV-1, bovine leukemia virus (BLV), HFV, and walleye dermal sarcoma virus (WDSV) PRs. Our previous (1) and present studies have the advantage that the different retroviral PRs were mapped with the same peptide series in the same reaction conditions and in the same laboratory; therefore, the results are directly comparable.

MATERIALS AND METHODS

Retroviral PRs. Chemically synthesized HIV-2 PR was purified and refolded as described previously (34). Purified MPMV PR (the shortest, 12-kDa form) was prepared as described previously (39). MMTV PR was expressed as a glutathione *S*-transferase fusion protein, processed with factor Xa, and purified

following the published procedure (19). MMLV PR was cloned with maltose binding protein and hexahistidine tags and purified as described previously (6). HIV-1 (34), HTLV-1 (13), BLV (40), and WDSV (9) PRs were purified from inclusion bodies after expression as described previously. HFV PR was cloned in fusion with MBP, and it was used in its fusion form (7). The activity of the MBP fusion form of HFV PR was similar to that of the processed form (3). The expression forms of the enzymes used in this study are summarized in Table 1. Active site titration for the HIV-1, HIV-2, EIAV, AMV, HTLV-1, BLV, and MMLV PRs was performed as described previously (6, 13, 31, 34, 37, 40). Active site titration of the MPMV PR was performed using the phenylstatine (Pst)-containing inhibitor PYVPstAMT. The purity of the PR preparations was at least 90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the number of active sites compared to the protein content (70 to 100%) suggested efficient refolding of the enzymes (data not shown).

Oligopeptides. Oligopeptides synthesized by solid-phase peptide synthesis were described previously (17, 19, 34). Stock solutions and dilutions were made in distilled water (or in 5 mM dithiothreitol for the Cys-containing peptides), and the proper peptide concentrations were determined by amino acid analysis.

PR assay. PR assays were performed at 37°C using purified retroviral PRs and chemically synthesized oligopeptides (0.4 mM) in 0.25 M potassium phosphate buffer (pH 5.6) containing 7.5% glycerol, 5 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40, and 2 M NaCl. The reaction mixtures were incubated at 37°C for 1 h or 24 h in the case of the HFV and WDSV PRs and were stopped by the addition of 9 volumes of 1% trifluoroacetic acid then injected onto a Nova-Pak C₁₈ reversed-phase chromatography column (3.9 by 150 mm; Waters Associates, Inc.) using an automatic injector. Substrates and the cleavage products were separated using acetonitrile gradient (0 to 100%) in water in the presence of 0.05% trifluoroacetic acid. The cleavage of peptides was monitored at 206 nm, and the peak areas were integrated. Amino acid analysis of the collected peaks was used to confirm the site of the cleavage with HIV-1 PR (34). For the other retroviral PRs, the cleavage products were identified by the retention time, which was found to be identical to that obtained with HIV-1 PR. Relative activities were calculated from the molar amounts of peptides cleaved per unit time at less than 20% substrate turnover by dividing the activity on a given peptide by the activity on the reference substrate, as described previously (1, 2). Measurements were performed in duplicate, and the average values were calculated. The standard error was less than 10%. The relative activities of the HIV-1 PR (34), EIAV PR (37), and AMV PR (31) have been reported previously. For WDSV PR, the

TABLE 1. Specificity constants of retroviral PRs on the Val-Ser-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln peptide

PR	Source	Reference	k_{cat}/K_m (mM ⁻¹ s ⁻¹) ^a
HIV-1	Recombinant	34	45.3 ± 9.1
HIV-2	Chemically synthesized ^b	34	34.3 ± 6.2
EIAV	Purified from virions ^b	37	11.1 ± 2.0
AMV	Purified from virions ^b	31	0.015 ± 0.003
MPMV	Recombinant p12, self-processed from fusion protein	39	0.41 ± 0.02
MMTV	Recombinant, processed from glutathione <i>S</i> -transferase fusion protein	19	ND ^c
MMLV	Recombinant, processed from MBP fusion protein ^d	6	0.48 ± 0.04
HTLV-1	Recombinant, stabilized from autoprolysis	13	0
BLV	Recombinant, self-processed from MBP fusion protein	40	0.061 ± 0.005
WDSV	Recombinant, processed from a fusion form	9	ND
HFV	Recombinant, MBP fusion protein	7	ND

^a Values are given as means ± standard deviations.

^b The enzyme has catalytic efficiency and specificity identical to those of a recombinant form (our unpublished results).

^c ND, not determined. Due to the lack of a sufficiently potent inhibitor, it was not possible to measure active site titration and estimate the catalytic efficiency of the enzyme.

^d The enzyme has catalytic efficiency and specificity identical to those of enzymes isolated from virions (5).

FIG. 2. Comparison of the relative activities obtained for selected Val-Ser-Gln-Asn-Xaa ↓ Pro-Ile-Val-Gln peptides for retroviral PRs.

efficient substrates were also hydrolyzed using 1 h of incubation time with a more concentrated enzyme stock, and the relative activities obtained for these substrates were within the experimental error of the values obtained using a 24-h incubation (data not shown).

Molecular modeling. The sequence alignment of the PRs used in this study and the construction of the molecular models were described previously (1). A model of VSQNY ↓ PIVQ oligopeptide was docked into the substrate binding site of each retroviral PR, and the minimization and analysis procedure were applied as described previously (1) with the Sybyl program package (Tripos Inc., St. Louis, MO) run on the Silicon Graphics Fuel computer graphics system. Sequence identity and similarity percentage values for the full PR sequences as well as for the set of residues involved in the substrate binding (18 to 20 residues of the PRs) are provided in Table S1 in the supplemental material. Cavities were calculated from the minimized structures containing Gly at the P1, P3, or P4 position using the SiteID module of Sybyl. It should be noted that the calculated cavity volumes are comparable only for a given subsite and not suitable for comparison with different subsites, as a consequence of the cavity-finding algorithm of the SiteID program and the diverse solvent accessibilities of the binding sites (e.g., S1 is fully buried while S4 is opened to the surface). The volume of the amino acid residues was retrieved from the literature (41).

RESULTS

Hydrolysis of the peptide representing the matrix/capsid cleavage site of HIV-1 by retroviral PRs. The unmodified peptide representing the matrix/capsid cleavage site of HIV-1 was hydrolyzed by the studied PRs with various efficiencies, as shown in Table 1. The source of the PRs is also provided in Table 1. We were able to perform active site titration to calculate the true catalytic constants for most of the PRs. For three enzymes (the MMTV, WDSV, and HFV PRs), this was not possible due to the lack of sufficiently potent inhibitors (data not shown). Furthermore, the HTLV-1 and HFV PRs were not able to hydrolyze the unmodified peptide. HFV PR was also unable to hydrolyze the substituted peptides except for the one containing P3-Val; therefore, this enzyme was omitted from further analysis. Our previous studies established that the relative activities measured under the given conditions correlated well with the specificity constants (k_{cat}/K_m) (31).

Substrate specificity of the S1 subsite of retroviral PRs. The results obtained with representative P1-modified substrates are visualized in Fig. 2, and the complete set of relative activity values is provided in Table S2 in the supplemental material.

None of the substrates were cleavable by the HTLV-1 and HFV PRs. For the other enzymes, the activities were calculated relative to those obtained with the original P1 Tyr-containing substrate. The S1 binding site of the PRs appears to be hydrophobic. The HIV-1, HIV-2, EIAV, AMV, MMLV, and WDSV PRs shared similar specificity, showing the preference order of Phe > Tyr > Leu $\approx$ Met > Ala, although the individual values for relative activity showed some variation. The MPMV and MMTV PRs were more selective for the large aromatic residues. On the other hand, the PR of BLV showed the highest preference for Leu at P1, followed by Phe or Met. Therefore, the S1 site is large, hydrophobic, and well conserved among retroviral PRs. These results are in agreement with predictions from the molecular models that a bulky hydrophobic P1 side chain will fill the S1 subsite to obtain efficient cleavage. However, there are some fine specificity distinctions in terms of whether the enzymes would also favor (or tolerate) smaller residues at this position. The HIV-1, HIV-2, EIAV, AMV, MMLV, and WDSV PRs tolerated the smaller Leu and Met residues substantially better than the MPMV and MMTV PRs (Fig. 2; see also Table S2 in the supplemental material). Interestingly, in the case of HIV-1 PR, a similar preference order of Phe $\gg$ Met $\approx$ Tyr $\approx$ Leu was observed using another substrate set (Lys-Thr-Lys-Val-Xaa ↓ Val-Val-Gln-Pro-Lys) based on the HTLV-1 CA/NC (typical type 2) cleavage site (35), while the HTLV-1 and BLV PRs preferred Phe > Leu > Tyr > Ala and Tyr $\approx$ Phe > Leu $\approx$ Met, respectively (27). The strong sequence context dependence of the preference at a given site was recognized earlier (reviewed in reference 33).

Substrate specificity of the S3 subsite of retroviral PRs. Unlike the S1 subsite, various residues were preferred when the S3 binding sites were mapped for different retroviral PRs (Fig. 3). The complete set of relative activity values is provided in Table S3 in the supplemental material. The HIV and EIAV PRs preferred the original polar Gln at P3 (Fig. 3), but the second-best residue was hydrophobic (Val for the HIV-1 and HIV-2 PRs and Phe for the EIAV PR). Smaller residues (Gly and Ala) were also well tolerated. A pronounced preference for the large hydrophobic residues Phe and Leu characterized

FIG. 3. Comparison of the relative activities obtained for selected Val-Ser-Xaa-Asn-Tyr ↓ Pro-Ile-Val-Gln peptides for retroviral PRs.

the AMV, MPMV, and MMTV PRs. In fact, MMTV PR did not hydrolyze the substrates with Gly or Ala at P3. Also, the MPMV PR was unusual in recognizing Asp at P3 in this substrate. On the other hand, the small Ala and the polar Lys were preferred by the BLV PR and Gly by the WDSV PR (Fig. 3). The size of the residue appears to be the main specificity determinant at this position, since, with the exception of the WDSV PR, the size of the average volume of the two most-preferred P3 residues correlated well with the mean cavity volume of the S3 subsites of the enzymes (Fig. 4), as previously established for the P2 position (1). In the exceptional case of WDSV PR, the S3 subsite binding pocket was predicted to be

relatively large and less suitable for small residues at P3. However, the uncertainty of the model of WDSV PR may contribute to this discrepancy.

Substrate specificity of the S4 subsite of retroviral PRs. Various residues were found to fit well in the S4 sites of the PRs, similarly to the S3 sites, as shown in Fig. 5. The activities were calculated relative to that of the P4 Val-containing substrate instead of the original Ser-containing one, since the former was also a substrate of the HTLV-1 PR. The complete data are provided in Table S4 in the supplemental material. In the case of the primate lentiviral PRs, like the HIV-1 and HIV-2 PRs, the polar Ser as well as the smaller Gly or Ala

FIG. 4. Mean cavity volume of the S3 subsites of various retroviral PRs versus the average volume of the two P3 residues for which the measured relative activity was the highest.

FIG. 5. Comparison of the relative activities obtained for selected Val-Xaa-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln peptides for retroviral PRs. Activities were calculated relative to that of the P4-Val-containing substrate instead of the original Ser-containing one to allow the inclusion of the HTLV-1 PR results.

residues are strongly preferred. EIAV PR prefers the hydrophobic residues Val, Leu, Phe, and Pro. The AMV, MPMV, and MMTV PRs preferred medium-sized or large hydrophobic residues such as Ile, Leu, and Phe (Fig. 5). In contrast, the BLV and WDSV PRs preferred small hydrophobic residues (Ala and Pro) at P4, while MMLV PR preferred medium-sized polar residues (Ser, Thr, and Asp) (Fig. 5). However, the S4 subsite lies at the PR surface and lacks the well-defined pockets of the more internal subsites. For many of these PRs, a correlation was observed between the size of the average vol-

ume of the best two residues and the mean cavity volume of the S4 subsites of the enzymes, similarly to that of the S3 subsites. However, the HIV-1 and MMLV PRs did not fit this correlation for S4 (Fig. 6), which could be due to the less-well-defined S4 subsites of these PRs suggested by the molecular models.

DISCUSSION

Retroviral PRs were expressed in various forms or purified from viruses for the specificity studies, as summarized in Table

FIG. 6. Mean cavity volume of the S4 subsites of various retroviral PRs versus the average volume of the two P4 residues for which the measured relative activity was the highest.

FIG. 7. Phylogenetic tree and schematic representation of the preferred residues of the S4 to S1 subsites of representative retroviral PRs. The phylogenetic tree contains Rous sarcoma virus (RSV) PR, but measurements were performed using AMV PR. These two PRs differ only in two residues that do not influence the specificity. The size of the oval objects representing the substrate amino acid side chains approximates the size of the most-preferred residues. Gray objects represent sites that prefer predominantly hydrophobic residues, while white objects represent sites that do not discriminate based on hydrophobicity. Dashed lines for the P4 subsites indicate that these pockets are less defined than the other ones, due to their proximity to the protein surface.

1. Despite the various sources, the folding efficiency of the enzymes was very good (70 to 100%) where it was possible to perform the calculation based on active site titration (data not shown). Furthermore, the recombinant enzymes for the AMV, EIAV, and MMLV PRs were found to have activity and specificity identical to the activity and specificity of recombinant enzymes purified from virions (42; our unpublished results). As shown in Table 1, the unmodified peptide representing the matrix/capsid cleavage site of HIV-1 was hydrolyzed by the studied PRs with various efficiencies. Nevertheless, the specificity constants obtained for the HIV-2, EIAV, AMV, and BLV PRs were within the range of those obtained with peptides representing their naturally occurring cleavage sites (27, 31). We have mapped the specificity of retroviral PRs using a series of oligopeptides having amino acid substitutions in the P1, P3, and P4 positions. HFV PR was unable to hydrolyze the peptides except for the one containing the P3-Val, unlike the case of the P2-modified substrates where many of the substitutions formed substrates of the enzyme (1). Therefore, we omitted this enzyme from the further analysis. These results, together with the previously published analysis of the S2 subsite, provide a basis for characterization of the specificity of the amino-terminal subsites (S4 to S1) of orthoretroviral PRs (excluding the HFV PR) using a type 1 cleavage site substrate,

having Tyr and Pro at the site of the cleavage. A schematic representation of the specificities is provided in Fig. 7. The alpharetroviral AMV and betaretroviral MMTV PRs appear to share very similar specificity. All their substrate binding sites are hydrophobic and large, except for the small S2 pocket (Fig. 7). The deltaretroviral BLV PR has large hydrophobic S1 and S2 pockets and smaller S3 and S4 subsites, and only the S3 subsite has some polar character. The gammaretroviral MMLV PR has a similar specificity, except that the S4 site appears to be small and hydrophilic. On the other hand, the epsilonretroviral WDSW PR has hydrophobic S4 and S1 subsites and somewhat hydrophilic S2 and S3 subsites. Interestingly, the specificity of the lentiviral PRs can be subdivided into two groups. The primate lentiviral HIV-1 and HIV-2 PRs have a substantially different specificity relative to that of the EIAV PR, both in the hydrophobicity of the S2 and S4 subsites as well as the size of the S4 subsite. The important conclusion is that the specificity patterns of the subsites agree with the evolutionary relationship among the PRs as represented by the phylogenetic tree (Fig. 7). For example, EIAV, AMV, and MMTV share very similar specificity at the S1, S2 (1), and S4 subsites. It is of interest to note that while the degree of sequence identity and similarity of retroviral PR sequences is fairly low, the residues forming the substrate binding sites are substantially more conserved (see Table S1 in the supplemental ma-

terial), a likely consequence of the selective evolutionary pressure to maintain these residues compared to those that are less critical for the structure and activity of the enzyme.

HIV-1 PR clinical inhibitors typically only weakly inhibit, if at all, the other retroviral PRs, with the exception of MMLV PR, which was inhibited by an HIV-1 PR inhibitor (5). So there is an apparent contradiction between the relatively conserved substrate specificity among retroviral PRs and an almost complete lack of inhibition by HIV-1 PR inhibitors. This contradiction might be due to the fact that the clinical inhibitors are typically rigid molecules, while substrates are more flexible, and capable of adapting to altered substrate binding sites in different retroviral PRs. The same phenomenon is observed in drug resistant HIV-1 PR variants (22, 29). The development of resistance toward the drugs designed against HIV-1 PR is one of the main problems with PR inhibitor therapy for AIDS. Many of the mutations occurring in drug resistance introduce amino acids that can be found at the equivalent position in other retroviral PRs. Therefore, characterization of the similarities and differences of the specificities of these enzymes may help to design broad-spectrum inhibitors against HIV-1 PR.

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