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Mutations That Alter the Activity of the Rous Sarcoma Virus Protease*

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Mutations designed by analysis of the Rous sarcoma virus (RSV) and human immunodeficiency virus (HIV)-1 protease (PR) crystal structures were introduced into 1) the substrate binding pocket, 2) the substrate enclosing "flaps," and 3) surface loops of RSV PR. Each mutant PR was expressed in *Escherichia coli*. Changes in activity were detected by following cleavage of a truncated (NC-PR) precursor polypeptide in *E. coli* and cleavage of synthetic peptide substrates representing RSV and HIV-1 PR cleavage sites *in vitro*. Mutations in the substrate binding pocket exchanged amino acid residues located close to the substrate in the HIV-1 PR for structurally equivalent residues in the RSV PR. Changing histidine 65 to *glycine* (H65*G*) gave an inactive enzyme, while a double mutant R105*P*,G106*V*, as well as the triple mutant, H65*G*,R105*P*,G106*V*, produced enzymes which showed significant activity toward a substrate that represented a HIV-1 cleavage site. Mutating the catalytic aspartate (D37S) or an adjacent conserved alanine to threonine (A40T), produced inactive enzymes. In contrast, the substitution A40S was active, but showed a reduced rate of catalysis. Mutations in the flaps of conserved glycines (G69L, G70L) produced inactive PRs. Two extended RSV PR surface loops were shortened to the size found in HIV-1 PR and resulted in drastically reduced activity. These results have confirmed some of the basic predictions made from structural models but have also revealed unexpected roles and interactions in the protein.

Recent interest in the retroviral aspartic protease stems from its essential role in retroviral replication and its potential as a therapeutic target to block HIV[1] infection (for reviews see Refs. 1 and 2). The PR is responsible *in vivo* for processing viral *gag* and *gag-pol* precursor polypeptides, whose products are required for the production of mature infectious virus particles (3–5). To do so, the PR must specifically cleave 7–9 unique sequences, depending upon the virus. At present, it is not clear how the PR recognizes such a diverse group of substrates. The minimum sequence required for cleavage, defined by structural and biochemical studies, is 7 amino acids (P4–P3′); inspection of these various sequences (6) does not reveal a consensus. The only obvious recurrent feature is that amino acids at or near the cleavage sites tend to be hydrophobic.

The RSV and HIV-1 PRs recognize different substrates and exhibit very different catalytic rates (7–9); the HIV-1 PR is approximately 10-fold more active than the RSV enzyme. To begin to understand the relationship between PR structure and function, we have compared the high resolution crystallographic structures of the RSV (10, 11) and HIV-1 (12–19) PRs for similarities and differences that might be related to specificity. This information has been used to select targets for site-directed mutational analysis, which for this report includes the surface loops, the substrate-enclosing flap region, and the active site of the RSV PR. Some mutational analyses of the RSV and HIV PRs have been reported previously (20–23). Substitution of one or both of the catalytic aspartate residue(s) has always resulted in an inactive enzyme. In addition, Loeb *et al.* (20) have identified three regions of the HIV-1 PR that are very sensitive to amino acid substitution. These regions include the amino acids surrounding the catalytic triad, the flaps, and a region in HIV-1 PR corresponding to *T74* to *R87*. All of these regions are expected to include amino acids directly involved in substrate binding.

Despite differences in molecular size and sequence, the three-dimensional structures of the two PRs resemble each other, as well as cellular aspartic proteases such as pepsin (24), rhizopuspepsin (25), and endothiapepsin (26). The retroviral PRs are unique, however, since they are only half as

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[1] The abbreviations used are: HIV, human immunodeficiency virus; RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; PR, retroviral protease; NC, nucleocapsid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. All amino acids and numbers placed in *italics* refer to the sequence of the HIV-1 PR.

large as the cellular enzymes and must dimerize to form a catalytically active enzyme. The structural alignment of the HIV and RSV enzymes (shown in Fig. 1*A*) reveals 31 identical residues and 9 additional conservative amino acid substitutions. Since the HIV-1 PR has 99 residues, compared to 124 for the RSV PR, parts of the latter structure do not correspond to the former. Most of these differences are localized to two surface loops and the flaps which are smaller in the HIV-1 PR (Fig. 1*B*).

The structural similarity between the two PRs is especially evident around the active site where 44 C_α backbone atoms superimpose within a 0.5-Å root mean square deviation (11). This similarity allowed us to use structural models for HIV-1 PR complexed to substrate-based peptide inhibitors (14–19) to predict amino acid residues in the RSV PR substrate binding pocket. Thirteen residues were observed to be in close proximity to a substrate-based inhibitor in HIV-1 PR and are indicated by *bold letters* in Fig. 1*A*. Nine of these residues were identical in the RSV PR. Three of these residues were sufficiently different to be candidates that may discriminate between substrates of the two PRs. To test this hypothesis, amino acids found in the HIV PR were introduced into comparable structural positions in the RSV enzyme (Fig. 1*B*). Analysis of the activity of these altered RSV PRs on peptide substrates representing known RSV and HIV-1 cleavage sites verified that the candidate residues do influence selection of substrate by PR.

EXPERIMENTAL PROCEDURES

Bacterial Cells and DNA Constructs—The expression vectors used for the mutagenesis, pPR and pNC-PR (Fig. 2), were as previously described (21). They contain, respectively, the RSV protease and the nucleocapsid-protease fusion protein (precursor p23). The viral genes are under the control of a temperature-sensitive λ *cI* repressor. Two strains of *Escherichia coli*, AR68 and MC1061, were used for cloning and expression. In MC1061 cells, the repressor is supplied in *trans* from the plasmid pRK248cIts, while in AR68 cells, the repressor is supplied by an integrated copy of the λ genome containing *cI857*. The construction of plasmid pPR-GGGG-PR and details for purification of linked PR dimers are as previously described (28). The plasmid pPR-GGGG-PRΔ(A59-M73) was created by oligodeoxynucleotide-directed mutagenesis of the parent vector pPR-GGGG-PR. The plasmid pPR-GGGG-PR(HIV) was constructed by substituting the distal DNA subunit of PR from pPR-GGGG-PR with a DNA fragment encoding the HIV-1 PR from the vector pPROT/RT (a gift from Stuart LeGrice, Case Western Reserve University).

Bacterial Cell Growth and Induction—The bacteria were grown at 30 °C in M9 medium containing 0.5% glucose and 0.5% Casamino acids or in YT media. Expression from the P_L promoter was induced by shifting the culture to 42 °C at midlog phase. Cells were collected 3 h postinduction, or as indicated, and protein was analyzed by polyacrylamide gel electrophoresis (27) and immunoblotting (21, 28) using antisera directed against NC or PR.

Mutagenesis—Site-directed oligodeoxynucleotide mutagenesis was used as previously described (21) to introduce amino acid changes (substitutions and/or deletions) into the protease coding region of pPR and pNC-PR. Oligodeoxynucleotides used for mutagenesis are shown in Fig. 3. The oligodeoxynucleotides were mixed with linearized pPR and pNC-PR (*Pvu*I) and gapped DNA from the expression vectors (pPR: *Bgl*II and *Bss*HII; pNC-PR: *Bss*HII and *Bam*HI). To construct pNC-PR (H65*G*,R105*P*,G106*V*), the mutant plasmid pNC-PR (H65*G*) was digested with *Eco*RI and *Bgl*II, and the fragment containing the H65*G* substitution was subcloned into *Eco*RI/*Bgl*II-digested and purified pNC-PR (R105*P*,G106*V*). The same strategy was used to construct pPR (H65*G*,R105*P*,G106*V*) from pPR (H65*G*) and pPR (R105*P*,G106*V*).

Preparation of Retroviral Proteases—Inclusion bodies were prepared from *E. coli* induced for expression of the RSV PR as previously described (21). The cells were disrupted with EDTA and lysozyme followed by treatment with Triton X-100 or sonication. The pellet was collected and washed by sequential suspension and centrifugation (6–8 times), first with 25% sucrose, 10 mM 2-mercaptoethanol and 25 mM sodium phosphate, pH 7.5, then with 1–2 M urea. The inclusion bodies were solubilized in 8 M urea (with 150 mM 2-mercaptoethanol), and the protease was refolded by dialysis (0.5–1 mg of protein/ml) at 23 °C against a decreasing concentration of urea (4, 2, and 1 M for 2 h each). The final dialysis (against 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, and 10 mM 2-mercaptoethanol) was overnight. The clarified soluble fraction containing active PR was stored at 4 °C. The preparations were at least 95% pure as determined by SDS-polyacrylamide gel electrophoresis with Coomassie staining (28). Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) and bovine serum albumin as a standard. In some experiments, wild type and mutant PR subunits were mixed in 8 M urea and renatured together.

Purified HIV-1 PR was a generous gift from Dr. C. Z. Giam (Dept. of Medicine, Case Western Reserve University). Purified HIV-2 PR was generously provided by S. LeGrice (Dept. of Medicine, Case Western Reserve University). AMV PR, prepared from viral particles by the procedure of Alexander *et al.* (29), was obtained from Molecular Genetic Resources (Tampa, FL). The RSV and AMV PRs, which differ in primary sequence by two amino acids, are enzymatically indistinguishable and have been used interchangeably in these studies.

The efficiency of refolding of the bacterial derived RSV PR varied from preparation to preparation. Therefore, the specific activity of all refolded enzymes was assessed by the method of Tomasselli *et al.* (30), which involves titrating PR concentrations against a nanomolar or stoichiometric inhibitor and extrapolating to the inhibitor concentration that yields complete loss in activity. The inhibitor used was PPCV(phe-statine)AMTM (31). The RSV recombinant PRs refolded with an efficiency that varied between 20 and 40%, while the HIV PR refolded with close to 100% efficiency.

Peptides—Peptides were synthesized and purified as previously described (28). Lyophilized preparations were dissolved in 1 mM 2-mercaptoethanol. Concentrations were determined by amino acid composition analysis. In addition to the amino acids assumed to interact with the protease, all peptides contained an N-terminal Pro (to prevent reaction with fluorescamine), and 2 C-terminal arginines (to improve solubility). The peptides used in the present study are listed in Table I.

Proteolytic Assay—The assay was based on the reaction of fluorescamine (Pierce) with primary amines produced at the P1′ position after cleavage (21). The assay mixture (25 μl) contained 2.4 M NaCl (1 M for the HIV PRs), 0.1 M sodium phosphate (pH 5.9), 0.2 mM 2-mercaptoethanol, 50–300 nM protease (active dimers according to titration), and 10–400 μM peptide. The reaction was started by adding the protease and stopped by adding 175 μl of 0.5 M sodium borate (pH 8.5). The incubation time (37 °C) varied from 1 to 45 min. With HIV-1 PR, incubation times longer than 8 min were avoided, as this enzyme had a half-life of approximately 20 min when incubated in the absence of substrate (presumably due to autocatalysis).

The amount of cleaved peptide was determined by adding 20 μl of 0.05% (w/v) fluorescamine, mixing vigorously, and measuring the net increase in fluorescence intensity at excitation and emission wavelengths of 386 and 477 nm, respectively, using a Perkin-Elmer LS-5B Spectrofluorometer with both excitation and emission slits set at 10 nm. Fluorescence was converted to nanomoles of product formed using a standard curve obtained by reacting fluorescamine with the peptide FQAY(CH_2N)PLR. This peptide was used as a standard since

TABLE I
Peptides used as PR substrates

Virus	Location of cleavage site	Peptide sequence[a]
RSV	*gag* MA-p2	PTSCY-HCGTRR
	gag CA-NCb	PLIM-AVVNRR
	gag NC-PR	PPAVS-LAMTMRR
	pol PR-RT	PATVL-TVALRR
	pol IN-p4	PLFA-GISDRR
HIV-1	*gag* CA-NCa	PARVL-AEAMRR
	gag CA-NCb	PATIM-MQRERR
	gag NC-p6a	PGNF-LQSRR
	gag NC-p6b	PRQAN-FLGKRR
	pol RT-IN	PRKIL-FLDGRR

[a] Hyphens indicate the scissile bond. In addition to the P4-P3′ amino acids assumed to interact with the PR, N-terminal prolines were added to avoid reactivity with fluorescamine and C-terminal arginines to improve solubility.

it was found that the reaction of fluorescamine with short peptides was somewhat different from the reaction with free amino acids or proteins. The presence of high concentrations of peptides quenched the fluorescence readings. Measurements made in the presence of 200 μM peptide or above were therefore corrected for quenching: 7.5% for 200 μM and proportionally more at higher peptide concentrations. The extent of quenching was found to be approximately the same with all substrates used in this study. All assays were performed in triplicate using several independent PR preparations. The standard deviation was less than 10%. Correct cleavage of selected substrates was verified by HPLC separation of products and amino acid composition analysis. Two of the HIV-1 peptides (Table I) contain lysine residues with amino groups. The pK_a values of these groups are approximately 10.5 so that at pH 8.5 they are mostly protonated and therefore do not react efficiently with the fluorescamine. However, an increase in the background fluorescence of these substrates of 2- to 3-fold over peptides without lysine residues was noted. Nonetheless, activity measurements reported here were at least 10-fold above background fluorescence in each case.

Kinetic Analysis—For each substrate peptide, 5–7 different concentrations were tested and the reaction was stopped before 20% of the substrate was cleaved. Within that limit, the reaction was shown to be linear with time, implying that the turnover could be used as a measure of initial velocity. Kinetic parameters were determined by fitting the velocity *versus* substrate concentration data to the Michaelis-Menten equation using the program NFIT (Island Products, Galveston, TX). The R^2 values were greater than 0.99. Standard deviations for the K_m values were less than 20% and for the k_{cat} values less than 10%.

Immunoblotting—Proteins from uninduced *E. coli* extracts or from *E. coli* induced to express NC-PR fusion protein were separated on SDS-18% polyacrylamide gels (28), visualized, and transferred to membranes as previously described (28). The membranes were incubated with precleared rabbit anti-RSV PR serum (diluted 1/5000), washed, and incubated with biotinylated goat anti-rabbit antibody followed by the Vectastain Elite reagent, and the peroxidase reaction was visualized using Indophane blue. Where indicated, the membranes were incubated with precleared rabbit anti-RSV NC or PR serum in phosphate-buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin for 1 h at 37 °C. After washing twice with phosphate-buffered saline containing 0.1% Tween 20, the membranes were incubated with 20–50 μCi of ^{125}I-labeled protein G (Amersham) in phosphate-buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin for 1 h at room temperature. The membranes were then washed three times as above, air-dried, and exposed to Kodak X-OMAT AR-5 x-ray film at −70 °C with an intensifying screen for 10–20 h.

Molecular Modeling—The crystal structures of the native RSV PR (11) and HIV-1 PR, as well as of the complexes of the latter with inhibitor(s) (14–19), were examined on an Evans and Sutherland PS390 computer graphics system using the program FRODO (32). The residues forming the subsites were determined directly for the HIV-1 PR complexed with inhibitors, and the corresponding residues in RSV PR were obtained from the structural and sequence alignments. The structural superimposition of the C_α atoms from the subunits of RSV and HIV-1 PRs (33, 34) was used to deduce the residues to be deleted in the extended surface loops of RSV PR and in the flaps.

RESULTS

Rationale for PR Mutations

The superimposed crystal structures of HIV-1 and RSV PRs were compared in order to locate regions that would be expected to influence substrate binding and catalysis or to have other interactions with the polyprotein precursors or other mature viral proteins. The active site residues of the two PRs are found in virtually identical conformations. Much of their subunit structure is very similar as well, despite the fact that the two enzymes differ in specificity and rates of catalysis. Their role in regulation of the viral life cycle may also differ since the RSV PR is present in both the *gag* and *gag-pol* precursors and thus may have other functions in addition to proteolytic cleavage of precursors. Based upon this structural analysis, a series of mutations in (*a*) the active site, (*b*) the substrate binding pocket, and (*c*) on the protein surface have been designed to explore structure/function relationships of the PR and its role in viral replication. Fig. 1*B* shows the location of the altered amino acids and structures. It should be noted that the retroviral PR is a homodimer. Thus, when mutations are introduced into the monomeric subunit, they are *de facto* double mutations in the native PR. This is true except for the mutations placed into a PR in which the subunits are covalently linked.

Active Site Mutants—Several types of active site mutations have been constructed. These include substituting serines for the catalytic aspartic acid residues (D37) to prove that these residues are essential for catalysis. Serine was chosen since it was not expected to drastically alter the structure of the catalytic site. Serine and threonine were also substituted for alanine 40. This alanine residue is highly conserved in all retroviral PRs. In the closely related nonviral aspartic proteases, serine and threonine are conserved in the corresponding positions. It has been suggested that this difference may account for the lower pH optimum of the cellular enzymes (35, 36).

An important feature of the active site is the flap structure, an extended surface loop that lies over the substrate binding region (Fig. 1*B*). It is present in all aspartic proteases and may affect substrate binding and/or catalysis. In retroviral PRs there are two flaps, one per subunit in the dimer, in contrast to the single flap of nonviral aspartic proteases. However, the flap sizes are not conserved. For instance, the RSV PR flaps contain three more amino acids than the HIV-1 PR flaps. RSV PR mutants were constructed which contained either a single flap or which substituted the smaller HIV-1 flaps for those found in the RSV PR. The flaps are also characterized by a series of conserved glycines, which are presumed to provide flexibility to the loop conformation. These residues may also have a steric role in binding substrate. In order to test the importance of these residues, glycine 69 and 70 were mutated to bulkier leucine residues.

RSV Specificity Mutants—The substrate binding region of the retroviral protease was initially defined by modeling a peptide substrate based on the position of the inhibitor in the co-crystal structure of rhizopuspepsin with a reduced peptide inhibitor (25, 33). This was later substantiated by examination of the crystal structures of HIV-1 PR with inhibitors (14–19). This analysis indicated that the peptide backbones of the inhibitors were in the same extended conformations (19). This observation suggested that hydrogen bond interactions with the backbone of substrates were similar for all aspartyl proteases with the exception that the single flap of the nonviral aspartyl proteases makes only 2 to 3 contacts with the backbone of the inhibitor while the flaps of the retroviral PR make at least 6 contacts (42). Thus, substrate specificity may arise through interactions between the amino acid side chains from residues of the substrate and enzyme. Superposition of the dimers of HIV-1 and RSV PR and examination of the HIV-1 PR inhibitor binding regions showed that there were at least 13 amino acid residues in the substrate binding pocket sufficiently close to an inhibitor (14–18) to allow side chain interactions. A comparison of these 13 HIV-1 residues with those found in comparable positions in the RSV PR indicated that 9 were identical (Fig. 1*A*). The 4 which were different were I44-*V32*, H65-*G48*, R105-*P81*, and G106-*V82*, in RSV and HIV-1 PRs, respectively. As the first of these involved two closely related amino acids, the other 3 residues were chosen as targets for mutagenesis. Three RSV PR constructs were made, one containing H65*G*, one contain-

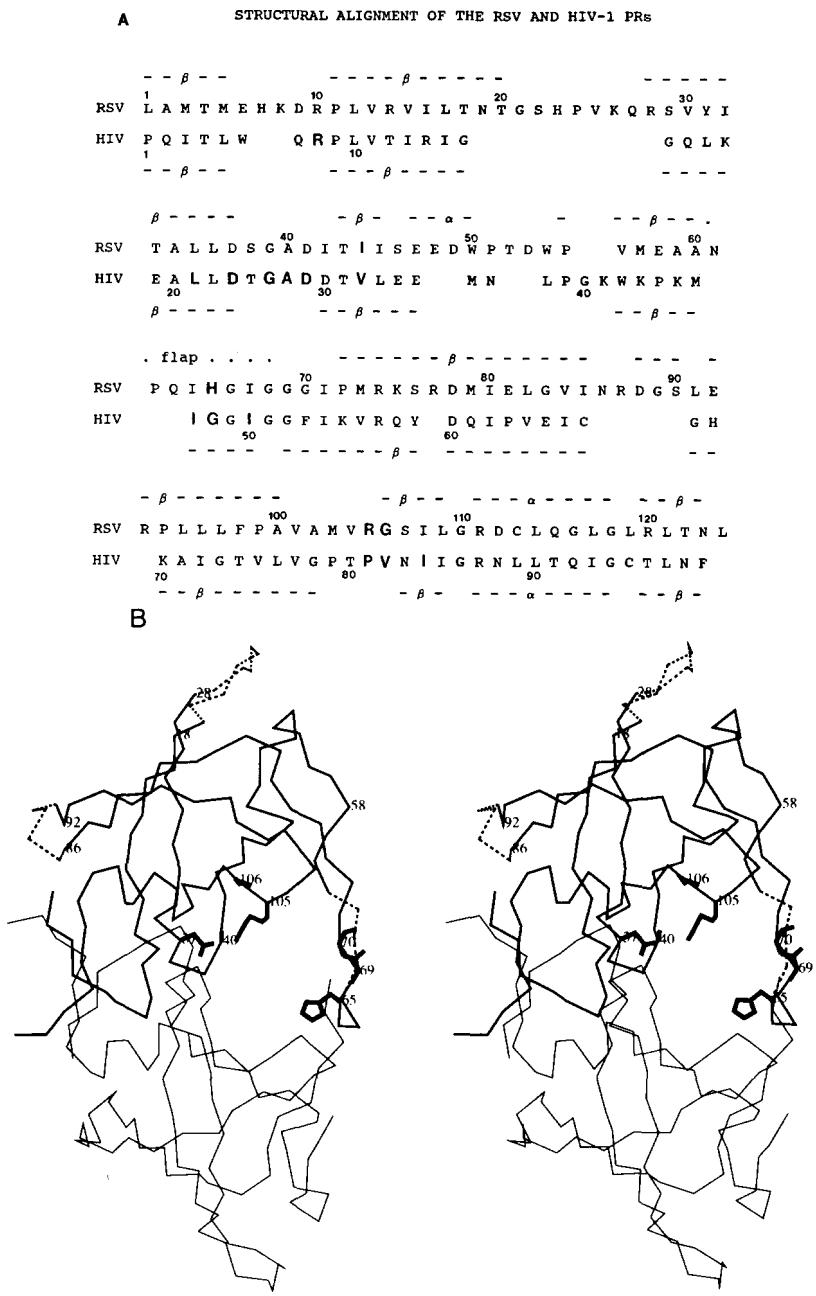

FIG. 1. **Structure and sequence comparison of the RSV and HIV-1 PRs.** *A*, structural alignment of the RSV and HIV-1 PRs. The numbers of amino acids for the RSV and HIV-1 PRs are indicated. Secondary structure elements (α helices and β structure) are indicated above and below the sequences for the RSV and HIV-1 PRs, respectively. Thirteen amino acid residues of HIV-1 PR with side chains in close proximity to a substrate-based inhibitor are shown in *bold letters*. Of these, four are different in RSV (also shown in *bold letters*). *B*, tracing of the C_α backbone of the RSV PR showing sites of mutations. The two subunits of the RSV PR are drawn in the *medium line (top)* and *thin line (bottom)*. Residues belonging to the flap were modeled in the top subunit only. This modeling was for illustrative purposes only and may not represent the true structure. Mutations and deletions discussed in this paper are identified in the top subunit only. The deletions are shown in *dashed lines*. Amino acid substitution mutations next to the deletions are not shown separately. Side chains of the mutated residues, or main chain atoms for glycines, are shown in *thick lines*. More detail about the mutations is provided in Fig. 3.

ing R105*P* and G106*V*, and one containing all three substitutions.

Surface Loops—The other obvious structural difference between the two enzymes is that the RSV PR has two extended surface loops from residues 19 to 28 (loop A) and from 86 to 90 (loop B), compared to shorter loops present in HIV PR. Many other retroviral PRs show the shorter loops characteristic of HIV-1 PR at these two regions (37). Exceptions are bovine leukemia virus and human T-cell leukemia virus, where loop A is longer than in the HIV-1 PR but shorter than in the RSV PR, while loop B has the same length as the RSV PR. Therefore, one might predict that deletion of the "extra" residues in the RSV surface loops, which lie far from the active site, might have little effect on catalysis but could affect viral replication via the loss of interactions with other domains of the polyprotein. To test this hypothesis, shorter loops were introduced into the RSV PR. In one set of constructs, T18*G*Δ(N19-R28)S29*G* and Δ(N86-L91)E92*G*, resi-

dues were removed and substitutions were made to include glycine at the predicted turn regions as occurs in HIV-1 PR.

Preparation of Mutant PR and PR-containing Precursor Fragments

Fig. 2 shows a map of the region in RSV DNA that contains the PR coding sequences. The indicated fragments were cloned in an *E. coli* plasmid vector downstream from the bacteriophage λ P_L promoter and an ATG initiator codon (21). The pPR clone expresses a protein that is predicted to be identical with the viral PR with the exception of the initiator methionine (which is often removed in bacterial cells). The pNC-PR expresses a protein that contains PR upstream from the NC sequences as normally occurs in the viral *gag* precursor protein. The pPR-GGGG-PR clone expresses a PR dimer linked by 4 glycine residues. The plasmid expression clones were subjected to site-directed mutagenesis, as described under "Experimental Procedures," to introduce the mutations listed in Fig. 3.

Expression and Analysis of Mutant PRs in Bacterial Extracts

We have shown previously that the NC-PR protein produced by the clone pNC-PR described in Fig. 2 processes itself in *E. coli* to form mature NC and PR products (38). Fig. 4 shows a comparison of products observed in extracts of bacteria that express NC-PR precursor fragments with different catalytic site mutations. The viral proteins were detected by immunoblot analysis using an anti-PR serum. Extracts of cells that express the wild type precursor fragment contained both NC-PR and PR, as expected. The same two proteins were detected in the extracts from cells expressing the NC-PR(A40S) mutant, although in this case there appeared to be

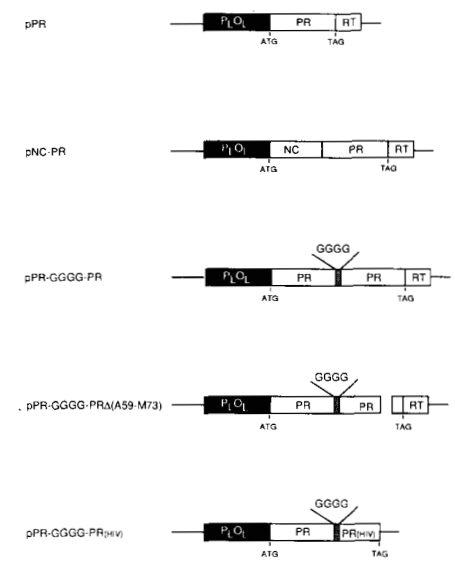

FIG. 2. **Molecular clones of PR and NC-PR.** pPR and pNC-PR are *E. coli* expression clones described previously (21) that contain the viral PR or nucleocapsid and protease (NC-PR) genes, respectively. The numbers correspond to the Rous sarcoma virus sequence. P_LO_L is the leftward promoter derived from λ phage DNA. *pPR-GGGG-PR* is an *E. coli* expression clone described previously (28) that contains a covalently linked PR dimer. *GGGG (hatched box)* indicates a 4-glycine linker that fuses the two PR subunits. *pPR-GGGG-PRΔ(A59-M73)* is an expression plasmid that contains a covalently linked PR with a truncated flap in the indicated subunit. *pPR-GGGG-PR(HIV)* is an expression plasmid containing a covalently linked PR dimer with one RSV and one HIV subunit. The initiation codon provided by the expression vector is indicated (ATG). The genetically engineered amber stop codon (TAG) is shown at the end of the PR domain.

less of the PR product made. Free PR was not detected in cells expressing the other mutant proteins, including A40T and D37S. In these cases, there was some antibody-reactive protein in intermediate positions that probably reflects digestion by bacterial proteases. We also note that the mutant NC-PR proteins migrate slightly differently from the wild type protein in this gel system.

Plasticity of the PR Substrate Binding Pocket

A similar analysis was carried out with mutant PRs containing substitutions and/or deletions in the flap region of the RSV PR. This region appears to be very sensitive to mutation as indicated by the results from bacterial lysates shown in Fig. 5. Substitutions at positions 65, 69, and 70, exchange of the HIV-1 PR flaps for those in RSV, or removal of one of the RSV flaps (pPR-GGGG-PRΔ(A59-M73) in Fig. 2) produced inactive PRs. Also, a mosaic PR (pPR-GGGG-PR(HIV) in Fig. 2) containing one HIV-1 and one RSV subunit was inactive (data not shown).

The enzymatic studies of the RSV PR(H65*G*) mutant were of particular interest. When substituted singly, *glycine* at position 65 produces an RSV enzyme which was inactive on the RSV NC-PR precursor. However, if this mutation was combined with two other RSV to HIV-1 PR amino acid exchanges, R105*P* and G106*V*, activity on this substrate was restored (Fig. 6). The RSV PR mutant containing only R105*P*,G106*V* was active. These results indicate that unfavorable conformational changes in the flaps introduced by H65*G* can be compensated by additional changes in the substrate binding pocket.

Purification of Mutant PRs

While analysis of cleavage of the NC-PR precursor in bacterial extracts provides a rapid screen for mutations that change PR activity, accurate quantitation of these effects is not possible. To facilitate such studies, selected PR mutants were expressed as the free enzyme and purified from bacterial extracts. With the exception of HIV-2 PR, all of the proteins were recovered from the inclusion body fraction. The PRs were solubilized by suspension in urea and renatured by sequential dialysis. A RSV wild type PR prepared by this procedure had the same substrate preference as the PR purified from AMV. However, the efficiency of refolding varied from preparation to preparation. In order to correct for differences in estimates of the specific activities of the different PR preparations, renatured PRs were titrated with a substrate-based inhibitor, and the activities were related to the number of active dimers as described under "Experimental Procedures." The titration experiments indicated that with the better bacterial-derived preparations 30–40% of the protein was folded correctly.

Activity of PR Mutants

A comparison of the activity with an NC-PR peptide substrate of the purified RSV PRs containing substitutions at positions 37, 40, 65, and 69 and 70 or deletions of surface loops is summarized in Table II. Of the 7 mutant proteins analyzed, only the A40S protein had significant activity, consistent with the immunoblot analysis shown in Fig. 4. Its activity was 23% that of the wild type, a difference attributable to a decrease in k_{cat}; there was no detectable change in its K_m for binding substrate (data not shown). Despite the change in catalytic rate, the relative activity of this mutant on various RSV-based substrates followed that of the wild type enzyme (data not shown). However, the A40S PR ap-

1. Active site and binding pocket mutations:

Oligonucleotide	Mutation	Restriction Enzyme #
5' ATCACCGCGCTGTTGAGCTCTGGAGCGGACATC 3'	D37S	*Sac* I
5' CTGTTGGACTCTGGATCCGACATCACTATTATT 3'	A40S	*BamH* I
5' GCTGTTGGACTCTGGTACCGACATCACTATTATT 3'	A40T	*Kpn* I
5' CCGCAGTAGCTATGGTACCAGTGAGTATCCTAGGAAGA 3'	R105P,G106V	*Kpn* I

2. Flap mutations:

Oligonucleotide	Mutation	Restriction Enzyme
5' TGGAGGCCGCGAACCCCCAGATCGGTGGGATAGGAGGGGGA 3'	H65G	*Bstx* I
5' ATCCATGGGATAGGATTATTAATTCCCATGCGAAAA 3'	G69L,G70L	*Ase* I
5' TTGGCCAGTGATGGAGATAGGGGGGATAGGAGGGGG 3'	A59I,A60G,Δ(N61-H65)	*Nco* I*
5' CGATTGGCCAGTGATGCCTAAGATGATCGGGGGGATAGGAGGGGGGA 3'	E58P,A59K,A60M,Δ(N61-Q63)H65G	*Nco* I*
5' TGATGGAGCGAAAATCCCGGGACATGATAGAGTTGG 3'	Δ(A59-M73)	*Sma* I

3. Surface loop mutations:

Oligonucleotide	Mutation	Restriction Enzyme
5' TAGGGTCATTCTGACTAGTGTGTATATCACCGCGC 3'	Δ(N19-R28)	*Spe* I
5' CCTTGGTTAGGGTCATCCTAGGCGGCGTGTATATCACCGCGC 3'	T18G,Δ(N19-R28)S29G	*Avr* II
5' GTTGGGGGTTATTGAGAGGCCTCTGCTCCTCTTCCCCG 3'	Δ(N86-L91)	*Stu* I
5' AGAGTTGGGGGTTATTGGACGTCCCCTGCTCCTCTTCC 3'	Δ(N86-L91)E92G	*Aat* II

A new restriction site was introduced in each oligonucleotide (see undelined nucleotides) to allow srceening of mutant PR-containing plasmids.

* Loss of the original *Nco* I restriction site.

FIG. 3. **RSV PR mutations.** Oligodeoxynucleotides used to place mutations into the pPR and pNC-PR plasmids by site-directed mutagenesis are as indicated. New restriction enzyme sites (*underlined*) were introduced to facilitate identification of mutated clones. Mutations are listed by the amino acid and position number of the RSV PR followed by the substituted amino acid. Deletion mutations are indicated by Δ followed by the position number of the first and last deleted amino acids.

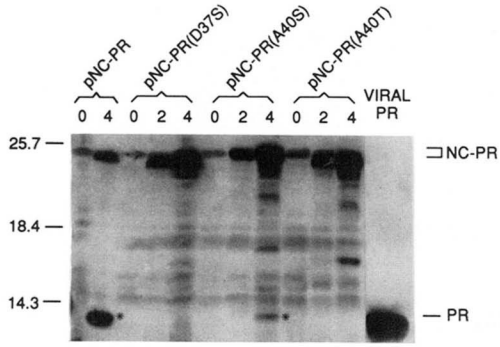

FIG. 4. **Effect of active site mutations on PR activity.** Immunoblotting of cell lysates from induced bacterial cultures expressing NC-PR and NC-PR mutant proteins containing NC-PR, NC-PR(D37S), and NC-PR(A40S or A40T). *E. coli* were grown at 30 °C in minimal medium to an OD_{600} of 0.8 and then at 42 °C for 2 or 4 h to induce expression of the cloned DNAs. Samples of bacteria from uninduced and induced cultures were fractionated on an 18% polyacrylamide-SDS gel. AMV PR was included as a marker. After electrophoresis, proteins were transferred to an Immobilon membrane and PR was detected using rabbit anti-PR serum and peroxidase staining as described under "Experimental Procedures." The *numbers* above the *lanes* indicate time (h) of incubation at the induction temperature. * denotes position of PR where applicable.

peared to be slightly more active than wild type at pH values below 5.5 (Fig. 7).

No activity was detected with the purified D37S, A40T, H65*G*, and G69L, G70L PRs (Table II). When the denatured D37S, A40T and G69L, G70L proteins were mixed with equimolar or twice equimolar amounts of denatured wild type AMV PR, respectively, and refolded as described under "Experimental Procedures," the amount of active wild type PR activity recovered was as predicted for a binomial distribution of the various dimer forms (Table III). This indicates that the mutant subunits were able to form stoichiometric complexes with those of wild type protein. Therefore, we conclude that their lack of activity is not due to incorrect folding.

Activity of PR Mutants with Modified Surface Loops

Mutant PRs with one or two of the surface loops shortened were also purified. If one of the loops was shortened, then the mutant exhibited only 2 to 3% of the activity associated with the wild type RSV PR (Table II). If both of the loops were shortened, no activity was detected. When introduced into an infectious virus DNA clone, the amount of residual activity found in these "loopless" PRs was insufficient to support normal processing of *gag* precursor polypeptides *in vivo*, and infectious progeny virions were thus not obtained (data not shown).

Mutations of Residues Involved in Substrate Recognition

Purification of RSV PR mutants, R105*P*,G106*V* and H65*G*,R105*P*,G106*V*, allowed us to test their activities on both RSV and HIV peptide substrates (Table I). The H65*G* was inactive on all substrates tested (data not shown). In contrast, the R105*P*,G106*V* enzyme had the same activity as the wild type PR on all RSV substrates (Table IV). In addition, it was indistinguishable from wild type in its requirements for pH and temperature and in its salt dependency (data not shown). The R105*P*,G106*V* mutant differed significantly from wild type with at least one HIV-1 peptide substrate (RT-IN), for which it exhibited a 10-fold increased activity (Table IV). The combined mutant (H65*G*,R105*P*,G106*V*) had about 25% of the activity of the

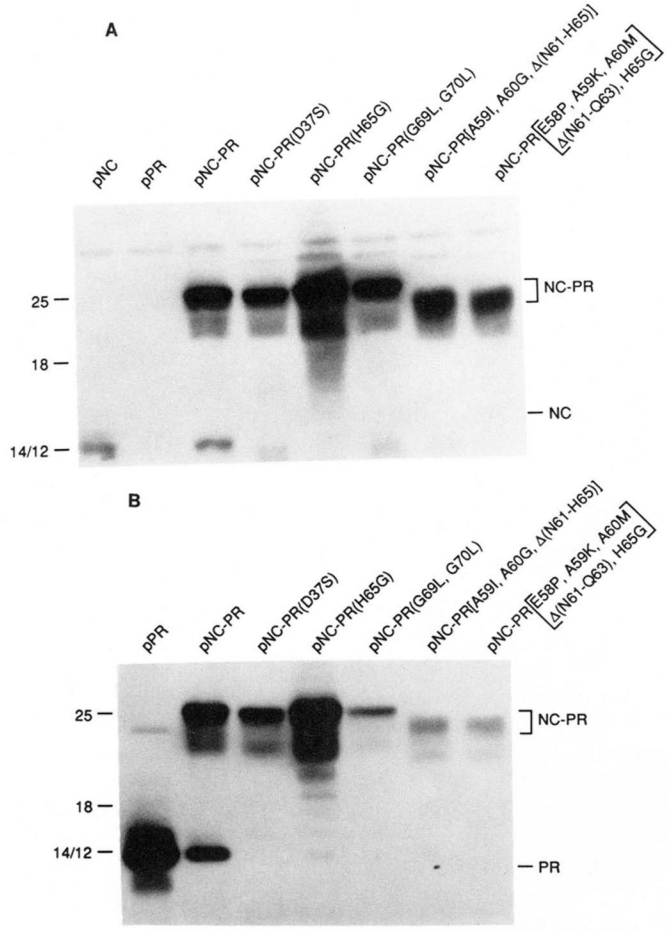

FIG. 5. **Effect of flap mutations on PR activity.** Immunoblot analysis of NC-PR proteins containing flap mutations in the PR and expressed in *E. coli*. Cell lysates were fractionated on a SDS-15% polyacrylamide gel, and the virus-related proteins were detected with rabbit antiserum directed against the NC (*panel A*) or the PR (*panel B*) with ^{125}I-labeled protein G as described under "Experimental Procedures." Molecular size markers in kilodaltons are indicated on the *left*. The positions of NC-PR and PR are indicated on the *right*.

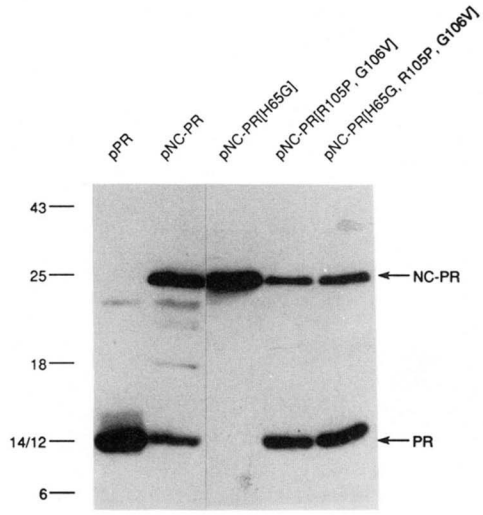

FIG. 6. **Effect of substrate binding pocket mutations on PR activity.** Immunoblot analysis of NC-PR proteins containing substrate binding pocket mutations and expressed in *E. coli*. Cell lysates were analyzed as described in the legend to Fig. 5 except that only anti-PR serum was used to detect proteins.

TABLE II

Activities of RSV proteases with active site or structural mutations

PR mutation	Activity[a]
	% recombinant wild type
D37S	<0.1
A40S	23
A40T	<0.1
G69L,G70L	<0.1
H65*G*	<0.1
ΔLoop A	3
ΔLoop B	2

[a] Activity was determined as described under "Experimental Procedures" using mutated RSV PRs as indicated and the RSV NC-PR peptide substrate. Activity of the wild type PR is 575 nmol of peptide cleaved/min/mg of enzyme.

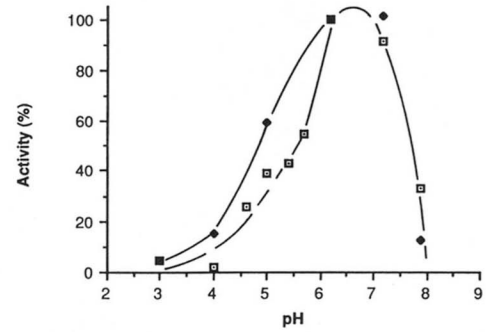

FIG. 7. **The pH dependence of AMV and A40S RSV PR.** AMV PR (46 ng) or RSV PR(A40S) (150 ng) was incubated with the NC-PR peptide substrate using 100 mM citrate for pH 3–7 and 100 mM HEPES for pH 7.9 instead of sodium phosphate buffer. The rate of cleavage was determined as described under "Experimental Procedures." 100% activity for the AMV and RSV A40S PRs is defined as 584 and 81 pmol of peptide cleaved, respectively, in 30 min. ⊡, AMV PR; ■, RSV PR(A40S).

TABLE III

Renaturation mixing experiments with RSV and mutant PR subunits

PR added[a]	Ratio of wild type to mutant subunits	PR activity[b]
		nmol/90 min
Wild type (WT)		2.1
PR(A40T)		0.0
PR(G69L,G70L)		0.0
PR(D37S)		0.0
WT + PR(A40T)	1:1	0.42
WT + PR(A40T)	1:2	0.27
WT + PR(G69L,G70L)	1:1	0.77
WT + PR(G69L,G70L)	1:2	0.29
WT + PR(D37S)	1:1	0.41
WT + PR(D37S)	1:2	0.21

[a] Wild-type RSV and mutant PR subunits were solubilized from the inclusion body fraction with 8 M urea and renatured either alone or in mixtures of wild type and mutant subunits (25–45 μg/ml) as described under "Experimental Procedures." The molar ratio of the wild type to the mutant subunits is as indicated.

[b] The activity of the PR preparations containing in each case 0.6 ng of wild type subunit and variable amounts of the mutant subunits, as indicated, were assayed for cleavage of the NC-PR peptide substrate as described under "Experimental Procedures."

wild type on RSV substrates, but was more active than the wild type on the HIV-1 RT-IN substrate. If the amount of activity observed with the wild type and mutant enzymes for the HIV-1 RT-IN substrate is normalized to that of the RSV NC-PR substrate, then the R105*P*,G106*V* and the H65*G*,R105*P*,G106*V* PRs had 10- and 20-fold increases in activity toward the HIV-1 substrate, respectively. Specific cleavage of peptides was independently verified by HPLC

TABLE IV

Activity of wild type and mutant enzymes on peptide substrates containing RSV and HIV-1 cleavage sites

Substrate	Protease activity[a]				
	AMV	RSV (R105*P*, G106*V*)	RSV (H65*G*,R105*P*, G106*V*)	HIV-1	HIV-2
RSV-based substrates[b]					
MA-p2	0.7	0.8	0.2	6.5	4.2
CA-NCb	7.9	6.4	1.7	13.0	10.9
NC-PR	14.9	15.1	4.3	41.0	25.4
PR-RT	12.0	10.7	3.0	7.3	3.4
IN-p4	2.6	2.9	1.2	3.0	2.2
HIV-1-based substrates[b]					
CA-NCa	0.6	0.4	0.2	114.5	64.8
CA-NCb	0.2	0.2	0.1	281.1	195.0
NC-p6a	0.0	0.0	0.0	151.7	121.8
NC-p6b	0.0	0.0	0.0	51.0	52.7
RT-IN	0.5	4.7	2.8	124.5	166.4

[a] The PR activity was determined by the fluorescamine reaction as described under "Experimental Procedures." The activity is the initial rate of turnover in the presence of 100 μM substrate measured as molecules of product formed per active enzyme dimer per min.

[b] The sequences are listed in Table I.

TABLE V

Kinetic measurements with NC-PR (RSV) and RT-IN (HIV-1) peptide substrates

Kinetic values were obtained from Michaelis-Menten plots of the initial velocity *versus* substrate concentration. Activity was determined using the fluorescamine assay as described under "Experimental Procedures."

Source of PR	Substrate	K_m	k_{cat}	k_{cat}/K_m
		μM	min^{-1}	$\mu M^{-1}\ min^{-1}$
AMV	RSV NC-PR	49	21.6	0.45
RSV(R105*P*,G106*V*)		45	19.9	0.44
RSV(H65*G*,R105*P*,G106*V*)		77	6.1	0.08
HIV-1		16	43.8	2.77
HIV-2		23	29.7	1.29
AMV	HIV-1 RT-IN	98	1.3	0.01
RSV(R105*P*,G106*V*)		100	11.6	0.12
RSV(H65*G*,R105*P*,G106*V*)		95	5.5	0.06
HIV-1		37	170.1	4.65
HIV-2		42	221.4	5.27

separation of the products and amino acid composition analysis (data not shown). Little or no activity for the other four HIV-1 substrates was detected with wild type or mutant PRs (Table IV).

As a control, each of the peptide substrates was incubated with either the purified HIV-1 or HIV-2 PRs. Each HIV-1 substrate was rapidly cleaved by both HIV enzymes with rates that were 10- to 20-fold greater than that observed with the RSV PR for its homologous substrates. While the RSV and AMV PRs generally cleaved HIV substrates very inefficiently, the HIV PRs cleaved the RSV substrates with rates comparable to that of the RSV enzyme. The best RSV substrate for the AMV PR (the NC-PR peptide) was also the best RSV substrate for the HIV enzymes.

Kinetic Analysis of Activity on the RSV NC-PR and the HIV-1 RT-IN Substrates

To determine the nature of the differences in activity between the mutant and wild type enzymes, a steady state kinetic analysis was carried out using the RSV NC-PR and the HIV-1 RT-IN peptide substrates (Table V). The wild type AMV and HIV PRs had K_m values for their respective homologous substrates in the 16–50 μM range. Interestingly, the HIV PRs bound the RSV substrate more efficiently than its own homologous substrate. This is in contrast to the AMV PRs which bound the heterologous substrate less efficiently.

The binding affinities of the RSV R105*P*,G106*V* and H65*G*,R105*P*,G106*V* mutants for the HIV-1 RT-IN substrate were the same as wild type PR. Thus, the increased activity observed with the mutant PRs for this substrate is solely an effect of k_{cat}. A comparison of the efficiency (k_{cat}/K_m) of the R105*P*,G106*V* and the H65*G*,R105*P*,G106*V* PRs for the RSV NC-PR or the HIV-1 RT-IN peptide substrates indicated that the mutants were 12- and 6-fold, respectively, more efficient on the HIV substrate than wild type PR. If one normalizes to the amount of activity that is observed with the RSV NC-PR peptide substrate, then the relative efficiencies calculated for the R105*P*,G106*V* and the H65*G*,R105*P*,G106*V* PRs are 12- and 34-fold, respectively, more efficient than wild type. These results indicate that introduction of amino acids found in the substrate binding pockets of HIV-1 PR into the RSV PR confers increased relative specificity for an HIV-1 substrate.

DISCUSSION

The results of our mutational studies verify that the amino acids in and around the catalytic site triad and in the flap region of the RSV PR are essential for enzymatic activity. Consistent with previous studies on avian and human retroviruses (20–23), substitution of the catalytic aspartate (D37) results in an inactive PR. However, the D37S mutant may be useful for biochemical and structural studies of PR since its overall structure should not be altered. This prediction is supported by our observation that when refolded together, D37S PR subunits can form stoichiometric complexes with wild type PR subunits. Nonconservative substitutions, such as D37I, cause conformational distortion of the PR (20). Craven *et al.* (41) also have shown that release of RT from a *gag-pol* precursor polypeptide containing PR mutations by exogenously added PR occurs efficiently when the mutation in the precursor is D37S but not when it is D37I. Again, this suggests that the latter mutation causes distortions in the polypeptide structure.

The catalytic aspartate (D37) residues are part of a rigid structure, held in place by an intricate network of hydrogen bonds which are formed with amino acids 38 and 39, as well as with the 37–39 active site triad from the other subunit. This unique structure is highly conserved in all aspartic proteases (1). In retroviral PRs, an alanine residue always follows the active site triad. In related cellular proteases, serine is found at this position in the amino domain, whereas threonine is predominantly present in the carboxyl domain of the enzyme. The latter is infrequently substituted by serine and in one case, that of human renin, by alanine (39). Sielecki *et al.* (35) suggested that the presence of alanine rather than serine at this position may account for the higher pH optimum of human renin compared to other cellular aspartyl proteases, since serine or threonine, but not alanine of renin could form a hydrogen bond with the catalytic aspartate. Mantafounis and Pitts (36) have made the substitution T218A in chymosin B and shown that this change shifted the pH optimum for activity toward neutral values. Whether a similar situation exists in the retroviral PR is not clear. When serine is substituted for alanine in the RSV PR, an enzyme is produced which is less active than wild type and its pH for maximum activity appears to be unchanged. However, this mutant PR does appear to retain a greater percentage of its activity at acid pH ranges compared to wild type. In contrast, threonine substitution at position 40 results in an inactive enzyme. Molecular modeling of the relevant structures indicates that

the A40S substitution can take place without steric hindrance while that of the A40T cannot (21). Thus the latter mutation is expected to cause some movement or rearrangement in the backbone atoms near the catalytic site which, presumably, is unfavorable for binding or catalysis. Loeb *et al.* (20) have introduced 4 amino acid substitutions at the same conserved alanine in the HIV-1 PR. In contrast to the results described here, none of these substitutions, including serine, produced an active enzyme. The difference between our results and those of Loeb *et al.* (20) may reflect inherent differences between the two PRs or may be due to their use of a less sensitive assay, which followed precursor cleavage in bacterial extracts.

One of the interesting regions of the PR molecule contains the flaps. Residues in this region interact with the peptide substrates and may exclude water molecules from the catalytic site. Unfortunately, structural information about the flaps in the RSV PR is incomplete due to disorder in the crystal structure (11). The structure of the flaps in HIV-1 PR is known and they have been shown to move as much as 7 Å and to change orientation upon substrate binding (14–16). Comparison of the amino acid sequence of the flaps of RSV to that in the HIV-1 PR shows that those in RSV contain 3 additional residues (37). Loeb *et al.* (20) have reported that amino acid substitutions in the HIV-1 flap region usually inactivate the PR. Our mutational analysis yielded similar results. Replacement of the longer RSV flaps with the shorter ones from HIV-1, removal of one of the two RSV flaps (data not shown), construction of a mosaic enzyme with RSV and HIV-1 PR subunits, and replacing glycines at positions 69 and 70 with leucines or histidine at position 65 with *glycine*, all produced inactive enzymes. This last result was not predicted since substitutions at *glycine 48* (the structurally identical position in HIV-1 PR to RSV PR H65) with histidine, or 4 other amino acids, produced active enzymes (20).

Avian retroviral PRs have two extended external loops, one of which is larger than any comparable loop found in mammalian retroviral PRs. The presence of these loops in the RSV PR may serve a possible structural role in virion core assembly since, unlike HIV or Moloney leukemia virus, the avian retrovirus PR is synthesized as part of both *gag* and *gag-pol* precursors and can be purified from virions in amounts equal to that of the capsid structural proteins. Thus, we hypothesized that removal of the surface loops of RSV may leave an enzymatically active protein which may exhibit defects in formation of mature virus particles. The analysis presented here indicates that the function of these structures is more complex than first supposed. When the surface loops were shortened individually, a nearly inactive enzyme resulted. When the mutation was placed into an infectious virus DNA clone, the level of residual PR activity was insufficient to support processing of viral precursor polypeptides *in vivo* (data not shown). It should be noted that these constructs contained substitutions that placed amino acids found at the turns of the smaller HIV-1 PR surface loops. Without these substitutions, the singly deleted surface loop mutants were completely inactive. Thus, while surface loops of RSV PR can be removed and a partially active PR obtained, the enzymes are very defective. There are several possible explanations for these results. First, Δ(N86-L91)E92*G* removes E92 which forms a salt bridge with the N terminus of the second subunit and is thus important for stabilizing the dimer structure of the PR. Elimination of a salt bridge in the linked dimers reduces catalytic activity (28). Second, amino acids in loop A form several hydrogen bond interactions with residues at the base of the flap and removal of these may also reduce activity. Third, removal of the surface loops exposes amino acids normally buried beneath the surface, and this could create a distortion of the polypeptide backbone which might interfere with catalytic activity. Finally, there may be some problem with folding of this bacterially derived mutant PR. Thus, new constructs which introduce more limited alterations into the surface loops will have to be analyzed before any conclusion of a structural role for these loops can be made.

Several amino acids found in the HIV-1 PR in close proximity to a bound peptide-based inhibitor were exchanged for amino acids in comparable positions in the RSV PR. These mutations were designed to identify amino acid residues that may be important determinants for substrate specificity. Three constructs were made, RSV PR(H65*G*), RSV PR(R105*P*,G106*V*), and RSV PR(H65*G*,R105*P*, G106*V*). The H65*G* exchange produced an inactive enzyme. *Glycine 48* in the HIV-1 PR is involved in forming hydrogen bonds to the peptide backbone of the inhibitor. The substitution of *glycine* for H65 in RSV PR may have induced conformational changes in the flaps which interfere with interactions with substrate.

In contrast, the RSV PR(R105*P*,G106*V*) was active. This is significant since the amino acids found in the comparable structural positions of HIV-1 PR, *P81* and *V82*, respectively, are singly very sensitive to amino acid substitution (20). Four amino acid substitutions for *P81*, including arginine, resulted in inactive enzymes. Some substitutions of HIV-1 *V82*, however, appeared to be tolerated, although substitution of glycine, the amino acid found in RSV PR, was not (20). These results highlight the need to make multiple amino acid substitutions in the substrate binding pocket to understand their significance. This is illustrated again with the striking observation that combining the H65*G* substitution, which inactivated the RSV PR, with the 105 and 106 substitutions partly restored activity to the RSV PR.

Examination of the substrate specificity of the RSV PR(R105*P*,G106*V*) and RSV PR(H65*G*,R105*P*,G106*V*) indicated that there was a significant increase in activity toward the HIV-1 RT-IN peptide substrate with both enzymes. A steady state kinetic analysis of these PRs indicated that the increase was due solely to an increase in catalytic rate; there was no appreciable effect on the binding of substrate. From these results, we conclude that the substitution of HIV amino acid residues found in positions comparable to RSV PR 105 and 106 and at 105, 106, and 65 imparts the ability to cleave a HIV substrate. It should be noted, however, that four other HIV-1 substrates were either not cleaved or were cleaved at low rates by the wild type and the mutant RSV PRs. The reason for inducing only partial HIV-1 PR-like behavior in the RSV PR mutants is provided by an analysis of NC-PR cleavage site substrates with single amino acid substitutions at the P4 to P3′ positions in the accompanying manuscript (40).

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Note Added in Proof—Ido *et al.* (Ido, E., He-ping, H., Kezdy, F. J., and Tang, J. (1991) *J. Biol. Chem.* **266,** 24359–24366) have reported that the A285 substitution in HIV-1 PR results in an active enzyme with a lower p*K* value. These results are consistent with the A40S mutant presented here.

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