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HIV-1 protease inhibits its homologous reverse transcriptase by protein–protein interaction

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ABSTRACT

The reading frame of the HIV-1 *pol* gene, encoding protease (PR) and reverse transcriptase (RT), including RNase H as well as integrase, was fused to the bacterial β-galactosidase gene and overexpressed in *Escherichia coli* cells. The resulting fusion protein was cleaved autocatalytically leading to PR, RT and integrase. Immunoprecipitations of bacterial crude extracts with anti-RT antibodies precipitated both RT and PR. Co-precipitation of PR and RT was also observed with anti-PR antibodies, strongly suggesting a physical interaction between fully processed RT and PR within the bacterial cell. Physical interactions were confirmed with purified components by means of an ELISA assay. Furthermore, purified PR inhibited the DNA synthesis activity of purified RT, while its RNase H activity remained unaffected. The type of inhibition was uncompetitive with respect to poly(rA)·oligo(dT); the inhibition constant was 50–100 nM. A possible physiological significance of this type of interaction is discussed.

INTRODUCTION

The replicative enzyme of the AIDS-inducing virus HIV-1, reverse transcriptase (RT), is a heterodimer consisting of two polypeptides with molecular weights of 66 and 51 kDa (1,2). HIV-1 RT is initially formed as a $p160^{gag–pol}$ precursor protein consisting of the group-specific antigen genes (*gag*) and the open reading frame for *pol*, which encodes the enzymatic activities for protease (PR), reverse transcriptase, RNase H and integrase (IN). The $p160^{gag–pol}$ polyprotein is processed in infected cells by its intrinsic PR activity. Proteolytic processing of the Gag proteins has been extensively studied (3–7). However, there is barely any data available on the processing steps that lead to heterodimeric RT. We have inserted the HIV-1 *pol* gene, including the PR-encoding domain downstream of the *Escherichia coli lac*Z gene to follow this process in a bacterial model system. The resulting fusion protein displayed autocatalytic cleavage of HIV-1 PR, RT and IN (8). In order to follow up the time course of intracellular cleavage, we performed immunoprecipitations and subsequent Western blot experiments with antibodies directed against both PR and RT. These experiments revealed physical contacts between fully processed RT and PR. These contacts were further confirmed by an enzyme-linked immunosorbant assay (ELISA) and by demonstrating an inhibitory potential of HIV-1 PR for fully processed RT. Possibly, the inhibitory potential of PR on homologous RT might be exploited for the development of novel RT inhibitors that are based on PR-derived peptide sequences.

MATERIALS AND METHODS

Materials

Chemically synthesized PR was obtained from Bachem (Heidelberg, Germany), AMV RT and *E.coli* RNase H were from Boehringer, MMLV RT was purchased from BRL. Poly(rA) and oligo(dT) were from Pharmacia, [α-^{32}P]dTTP was from Amersham-Buchler (Braunschweig, Germany). The PR substrate His-Lys-Ala-Arg-Val-Leu-[p-NO_2-Phe]-Glu-Ala-Nle-Ser-NH_2 (corresponding to the p24–p15 junction of the Gag precursor) was obtained from Bachem (Heidelberg, Germany). All chemicals were of at least analytical grade.

Cloning and bacterial overexpression of the entire HIV-1 *pol* gene

The entire reading frame of the HIV-1 *pol* gene from nucleotide position 1445 to 5819 (9) was co-expressed in-frame with β-galactosidase and the collagen linker using the pSS20*c vector (10), yielding the vector pJS3.7 (8). HIV-1 RT was purified from *E.coli* transfected with plasmid pJS3.7 essentially as described (11).

Preparation of crude extracts of bacteria transfected with plasmid pJS3.7

pJS3.7-transfected *E.coli* strain JM109 was grown at 28°C until the beginning of the saturation phase of bacterial growth (1.5 A_{600} U/ml). Recombinant protein synthesis was induced by the addition of 8 mM IPTG. After 5 h incubation at 28°C, bacteria were harvested by centrifugation, suspended in 100 mM Tris–HCl, pH 7.6, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol (10 ml/g wet cells) and opened by sonication. Cellular debris was removed by centrifugation (30 min, 20 000 *g*). The resulting crude extract was used for immunoprecipitation experiments.

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Immunoprecipitations from crude extracts

Aliquots of 200 µl crude extract were rocked with ~2 µg polyclonal antibodies against HIV-1 RT or HIV-1 PR for 1 h at 4°C. Then ~ 50 µl protein A–Sepharose (Pharmacia), equilibrated with TNE buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) and 5% bovine serum albumin were added and the suspension was incubated for another 30 min at 4°C. The protein A–Sepharose was collected by centrifugation and washed three times by suspension in and subsequent centrifugation from TNE buffer. Then the protein A–Sepharose was suspended in 50 µl Laemmli loading buffer and heated for 5 min at 95°C. For detection of RT, samples were loaded onto an 8.5% SDS–polyacrylamide gel (12); for detection of HIV-1 PR, samples were loaded on a 16% polyacrylamide–SDS–tricine gel (13).

Western blot experiments

Proteins were separated on 8.5% polyacrylamide–SDS (12) or 16% polyacrylamide–SDS–tricine gels (13) and electrically transferred to fluorotrans PVDF membranes (Pall Biosupport, Dreieich, Germany) using a JC Semi-Dry electroblotting device (Jancos, Denmark). The PVDF membrane was saturated by preincubation for 1 h with 10% heat-inactivated newborn calf serum; serum was removed by extensive washing with 10 mM sodium phosphate, pH 7.5, 130 mM NaCl (PBS). The membrane was then incubated with an appropriate dilution of the first antibody (typically 1:500) for 2 h at room temperature on a rocker. After removal of the solution that contained the antibodies, the membrane was extensively washed with PBS and incubated with a peroxidase-coupled secondary antibody (BioRad, Richmond, CA) at a 1:10 000 dilution, again for 2 h at room temperature. After extensive washing with PBS, the membrane was stained with the ECL detection reagents, according to the instructions of the manufacturer (Amersham).

Bacterial overexpression of the HIV-1 PR

HIV-1 PR can be purified from pJS3.7-transfected *E.coli* strains, however, with rather low yields (M.Böttcher, unpublished results). A better expression was achieved by inserting the reading frame of the HIV-1 protease from nucleotide position 1416 to 1919 only (9) into the pSS20*c vector (8,10). Plasmid transfection, cellular growth and induction of PR were performed essentially as described above. Again, the PR cleaved itself from the fusion product. Four grams of wet cells were suspended in 40 ml buffer A [50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.4, 250 mM NaCl, 3 mM dithiothreitol (DTT), 2 mM 2-mercaptoethanol, 0.2% (v/v) Trasylol (Bayer-Leverkusen, Germany), 0.2% (v/v) phenylmethylsulfonyl fluoride] and disrupted by sonication; cellular debris was removed by centrifugation (30 min, 20 000 *g*). HIV-1 PR was precipitated from the supernatant by 40% (saturation) ammonium sulfate. Precipitated protein was collected by centrifugation (20 min, 15 000 *g*) and redissolved in 10 ml buffer B (50 mM MES, pH 6.5, 1 mM DTT, 1 mM EDTA). The dialyzed fraction was loaded onto phosphocellulose (50 mg protein/ml phosphocellulose P11; Whatman). The column was washed with 10 column vol buffer B and then developed with a linear gradient of 0–1 M NaCl in buffer B (10 column vol). Protease eluted at ~250 mM NaCl. These fractions were collected and directly loaded onto hydroxylapatite (2 mg protein/ml column material). The column was washed with 10 column vol buffer B containing 250 mM NaCl. HIV-1 protease was found in the flowthrough and early wash fractions. The authenticity of the purified protease was measured by Western blot analysis with a polyclonal antibody against HIV-1 protease. The purity of the protease was determined on silver stained SDS–tricine–polyacrylamide gels to be better than 90%. The specific activity was 12 U/mg when the chromogenic peptide His-Lys-Ala-Arg-Val-Leu-[p-NO_2-Phe]-Glu-Ala-Nle-Ser-NH_2 served as substrate. One unit of protease is defined as the minimal amount of enzyme that catalyzes the cleavage of 1 µmol oligopeptide/min at pH 5.5 and 37°C in a buffer containing 50 mM MES, pH 5.5, 0.5 M NaCl, 3 mM EDTA, 3 mM DTT.

Kinetic measurement of the HIV-1 RT DNA synthesizing activity

RT activity was measured with 10–100 nM 3′-OH-termini of $(dT)_{20}$, hybridized in a 1:5 (nucleotide:nucleotide) molar ratio to poly(rA) in 100 µl 50 mM Tris–HCl, pH 8.0, 80 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µM [α-^{32}P]dTTP (256 c.p.m./pmol) and an amount of enzymes that ensured initial rate conditions. After 5 and 10 min incubation at 37°C (if not stated otherwise), portions of 10 µl were spotted onto GF34 filters (Schleicher & Schüll, Dassel, Germany) and passed into ice-cold 10% trichloroacetic acid. After 5 min, filters were passed to a suction device and were washed 10 times with 2 ml 1 M HCl (each) and five times with 95% ethanol, dried and counted in a 1211 Minibeta (LKB, Bromma, Sweden) liquid scintillation counter.

Measurement of RNase H activity

Homopolymeric poly(dT) (Pharmacia) was filled in with *E.coli* RNA polymerase (Boehringer) in the presence of [^{3}H]rATP (Amersham) (14). A sample of 100 µl reaction buffer contained 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.5 mM [^{3}H]rATP (2 µCi/mM), 0.1 mg/ml poly(dT), 0.1 mg/ml BSA and 20 ng RNA polymerase. The mixture was incubated for 5 min at 37°C, then the RNA polymerase was inactivated by heating for 10 min at 65°C. Proteins were removed by phenolization. The DNA–RNA hybrid was collected by precipitation with ethanol and dissolved in 100 µl sterile 10 mM Tris–HCl, pH 7.8, 1 mM EDTA. RNase H activity was measured in 50 µl 20 mM Tris–HCl, pH 7.3, 75 mM KCl, 5 mM $MnCl_2$, 4 mM DTT, 10 µM [^{3}H]poly(rA)·poly(dT) (98 c.p.m./pmol). The reaction was started by addition of 1 U/50 µl *E.coli* RNase H or 10 U/50 µl HIV-1 RT. After 0, 10, 30 and 60 min incubation at 37°C, portions of 10 µl were spotted onto GF34 filters (Schleicher & Schüll, Dassel, Germany) and passed into ice-cold 10% trichloroacetic acid. Acid-insoluble radioactivity was measured as described above.

RESULTS

Overexpression of the β-galactosidase–*pol* gene fusion protein and HIV-1 PR; purification of recombinant proteins

The open reading frame of the HIV-1 *pol* gene, including the protease domain, was fused to the bacterial *lac*Z gene (8). A spacer region was inserted between the *lac*Z and the *pol* genes that contained part of the mammalian collagen gene (10). The spacer was intended to allow independent folding of both enzymatic domains, i.e. β-galactosidase (β-gal) and the Pol polyprotein consisting of PR, RT and IN. From this construct, the overexpressed RT was purified to near homogeneity as described elsewhere (11).

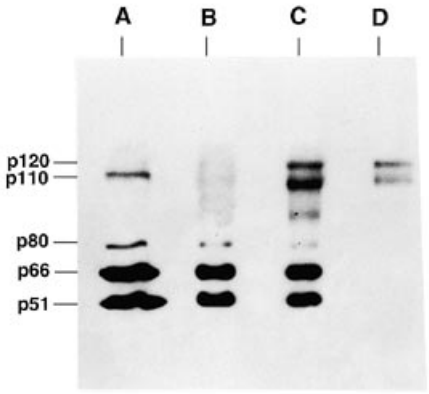

Figure 1. Immunoprecipitation of PR–RT complexes developed with a monoclonal antibody against HIV-1 RT. Bacterial cells that overexpressed the *lacZ–pol* gene fusion product were opened and cleared by centrifugation. An aliquot of 10 µl crude extract was directly loaded onto lane A. Lane B displays crude extract that has been immunoprecipitated with a polyclonal antibody against HIV-1 RT. Lane C displays crude extract immunoprecipitated with a polyclonal antibody against HIV-1 PR. Lane D shows purified RT that was immunoprecipitated with a polyclonal antibody against HIV-1 PR. After gel electrophoresis and blotting, the PVDF membrane was incubated with a monoclonal antibody against HIV-1 RT and a secondary anti-mouse antibody, coupled to peroxidase. Protein detection was achieved by enhanced chemoluminescence (Amersham).

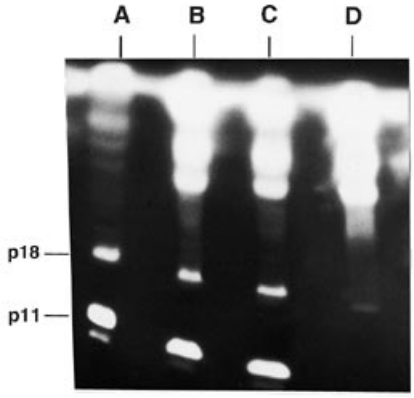

Figure 2. Immunoprecipitation of PR–RT complexes developed with a polyclonal antibody against HIV-1 PR. Bacterial cells overexpressing the *lacZ–pol* gene fusion product were opened and cleared by centrifugation. An aliquot of 10 µl crude extract was directly loaded onto lane A. Lane B displays crude extract that has been immunoprecipitated with a polyclonal antibody against HIV-1 RT. Lane C displays crude extract immunoprecipitated with a polyclonal antibody against HIV-1 PR. Lane D shows purified PR that was precipitated with a polyclonal antibody against HIV-1 RT. After gel electrophoresis and blotting, the PVDF membrane was incubated with a polyclonal antibody against HIV-1 PR and a secondary anti-rabbit antibody, coupled to peroxidase. Proteins were detected by the ECL technique.

The specific activity of RT was 1.1 µmol dTMP incorporation/min/mg when measured on poly(rA)·$(dT)_{20}$.

Similarly, HIV-1 PR was overproduced in *E.coli* as a C-terminal fusion protein with β-gal without the rest of the *pol* gene reading frame. Again, the enzyme released itself autocatalytically from the fusion product. Protease was purified from *E.coli* crude extracts to >90% homogeneity by ammonium sulfate precipitation and chromatography on phosphocellulose and hydroxylapatite (see Materials and Methods). The purified protease displayed a specific activity of 12 µmol/min/mg when measured with the synthetic peptide substrate His-Lys-Ala-Arg-Val-Leu-[p-NO_2-Phe]-Glu-Ala-Nle-Ser-NH_2; it was free from detectable amounts of non-specific protease activities, nucleases and dNTPases (data not shown).

Immunoprecipitation of bacterial crude extracts containing simultaneously expressed RT and PR

Bacteria that overexpressed the entire *pol* gene reading frame as a C-terminal fusion with β-gal were lysed and the crude cellular extract was treated with polyclonal antibodies directed against either HIV-1 RT or PR. Antigen–antibody complexes were isolated by affinity absorption to protein A–Sepharose and subsequent elution with an SDS-containing buffer. After gel electrophoresis under denaturing conditions and transfer to a PVDF membrane, the immunoprecipitated proteins were probed with a monoclonal antibody directed against HIV-1 RT. Crude extract without immunoprecipitation contained p66 and p51 of heterodimeric RT, another band at ~110 kDa that most likely represented incompletely cleaved p11-PR/p66-RT/p32-IN and a fourth band of ~80 kDa that most likely represented p51-RT/IN (Fig. 1, lane A). Crude extract treated with a polyclonal antibody against HIV-1 RT displayed a very similar pattern to crude extract without immunoprecipitation, the PR-containing band at ~110 kDa, however, was barely visible (Fig. 1, lane B). Surprisingly, a polyclonal antibody against HIV-1 PR also precipitated heterodimeric RT and two further bands at ~120 kDa (most likely consisting of part of the collagen spacer, PR, RT and IN) (Fig. 1, lane C). In contrast, immunoprecipitation of purified RT with a polyclonal antibody against HIV-1 PR revealed only the two PR-containing bands at 120 and 110 kDa (Fig. 1, lane D) that were not visible in Coomassie stained preparations of purified RT. These results suggest the existence of a complex between RT and PR in crude bacterial extracts.

To confirm these results, we repeated the immunoprecipitation experiments and developed the corresponding Western blots with a polyclonal antibody against HIV-1 PR. When crude extract of the *pol* gene-overexpressing strain was directly loaded onto an SDS–tricine gel, electrophoresed, blotted onto a membrane and subsequently probed with a polyclonal antibody against PR, fully processed p11-PR showed up (Fig. 2, lane A). There was another band at ~18 kDa that has also been observed with other expression systems (15). There were some further and rather faint bands that most likely represented partially processed precursor molecules containing HIV-1 PR (Fig. 2, lane A), probably the same precursors as mentioned above. Bacterial extract was also immunoprecipitated with a polyclonal antibody against HIV-1 RT and subsequently probed with polyclonal antibody against HIV-1 PR. Again, this procedure revealed p11-PR and p18-PR, strongly indicating physical interactions between PR and RT (Fig. 2, lane B). PR was precipitated, as expected, from crude extracts with a polyclonal antibody directed against PR (Fig. 2, lane C), whereas PR-directed antibodies probed only a minor amount of p18 when purified PR was precipitated with anti-RT antibodies, and p11-PR was not detected (Fig. 2, lane D). In all these cases, the light and heavy chains of the antibody as well as some unreduced antibody were also stained, because the precipitating antibodies and the probing secondary anti-PR antibody were both elicited in rabbits.

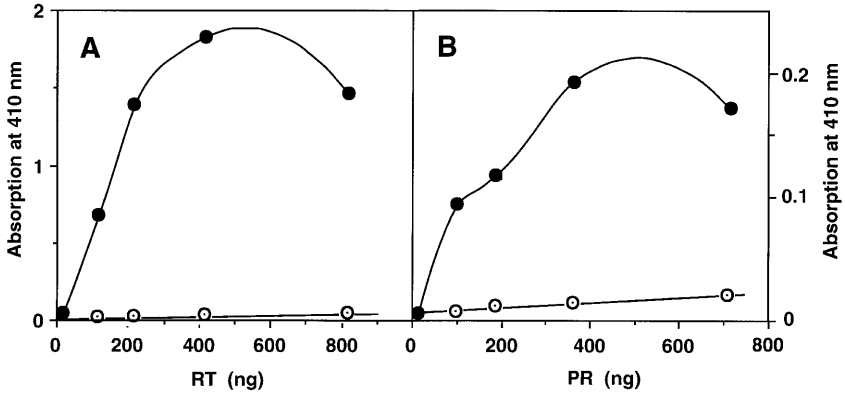

Figure 3. Binding of purified HIV-1 PR to purified HIV-1 RT. Purified HIV PR (**A**) or p66/p51 RT (**B**) were immobilized on ELISA plates (1 µg/well). Blocking of reactive sites and washing procedures were as described by Dornreiter*et al.* (16). Immobilized HIV proteins were detected by incubation with 1µg purified monoclonal antibody directed against RT (A) or PR (B), rabbit anti-mouse immunoglobulin and a chromogenic substrate (16). The amount of HIV proteins bound to immobilized RT or PR was quantified spectrophotometrically (closed circles). Background values of control wells without PR and without RT are shown as open circles.

Physical interactions between HIV-1 RT and HIV-1 PR

The immunoprecipitation studies suggested a contact between HIV-1 RT and PR, which, however, might have been mediated by another component present in crude bacterial extracts. To exclude this possibility, complex formation was measured with highly purified preparations of HIV-1 RT and PR (>90% homogeneity each) using an ELISA (16). HIV-1 PR was bound to the solid phase of microtiter plates and remaining protein binding sites were saturated with bovine serum albumin. Then, varying amounts of HIV-1 RT were added to the PR-loaded wells of the plate. Purified monoclonal antibody against HIV-1 RT and a peroxidase-conjugated secondary antibody were used to detect RT bound to immobilized HIV-1 PR. The amount of complex formation depended on the concentration of RT added, demonstrating that RT can directly bind to purified PR (Fig. 3A). Essentially the same result was obtained when the plates were loaded with HIV-1 RT and subsequent binding of PR was probed with an antibody against HIV-1 PR (Fig. 3B). In control assays without PR or RT bound to the plates, both antibodies gave signals of <5% of those measurements where PR or RT was present. These results strongly suggest physical contacts between PR and RT and seem to exclude the possibility that contact formation was mediated by another component present in crude extracts.

Inhibition of p66/p51 reverse transcriptase by purified HIV-1 protease

The observed interactions between HIV-1 PR and fully processed p66/p51 RT suggested some functional interactions as well. To study functional interactions we analyzed both RT activity in the presence of increasing concentrations of PR as well as PR activity in the presence of increasing concentrations of purified RT. PR activity was marginally inhibited (<1.5-fold) in the presence of a 20-fold molar excess of RT when it was measured with the synthetic undecamer peptide substrate (data not shown). In contrast, the DNA synthesizing activity of HIV-1 RT was markedly inhibited by purified PR (Table 1). Inhibition of RT activity was also observed in the presence of inactive PR, where the aspartate residue at position 25 was replaced by alanine (D25A) (Table 1). Furthermore, incubation of 10 nM RT with 200 nM PR for up to 2 h did not lead to further degradation of fully processed RT (data not shown). Hence, the inhibitory potential of PR was independent of its proteolytic activity. Inhibition of RT activity was also observed with chemically synthesized HIV-1 PR (Table 1), rendering it unlikely that the inhibitory activity was co-purified from PR-overproducing bacteria.

Table 1. Inhibition of RTs by HIV-1 PR

System	PR activity (%)	RT inhibition (%)
HIV-1 RT		
Recombinant HIV-1 protease	100	70
Mutant HIV-1 protease (D25A)	0	72
Synthetic HIV-1 protease	11	50
SIV_{agm} RT		
Recombinant HIV-1 protease	100	70
MMLV RT		
Recombinant HIV-1 protease	100	0
Synthetic HIV-1 protease	11	0
AMV RT		
Recombinant HIV-1 protease	100	0

A 70% inhibition was obtained with 180 nM PR and 1.7 nM RT per assay. A 100-fold molar excess of PR over RT was necessary for experimental reasons. A linear time course of nucleotide incorporation requires nanomolar concentrations of RT; since DNA synthesis is inhibited with a K_i value of 100 nM (see Results), a 70% inhibition can only be achieved with ~200 nM PR. HIV-1 PR containing the active site mutation D25A was produced by site-directed mutagenesis (30).

To achieve measurable amounts of RT inhibition under Michaelis–Menten conditions, i.e. 1.7 nM RT/assay, a molar excess of PR over RT was necessary (Table 1). The inhibitory potential of PR versus RT was quantified using a steady-state kinetic approach, in which the PR to RT ratio was varied systematically (Fig. 4A). This analysis revealed that HIV-1 PR inhibited p66/p51 RT with an inhibition constant (K_i) of 100 nM (Fig. 4B). The type of inhibition was uncompetitive with respect to poly(rA)·oligo(dT). Concerning the dNTP substrates, a more complex inhibitory pattern was observed: half-maximal inhibition

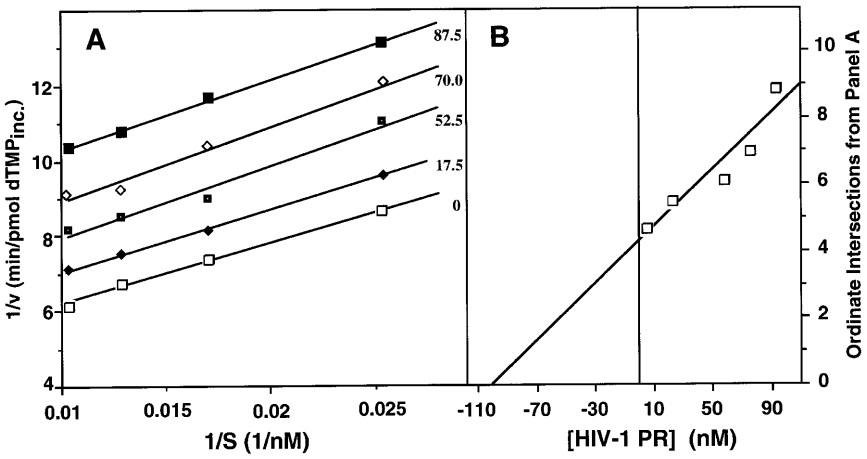

Figure 4. HIV-1 PR inhibits the DNA synthesizing activity of heterodimeric HIV-1 RT. RT activity was measured with 1.25 nM purified p66/p51 RT as described in Materials and Methods. The concentrations of PR added (nM) were as indicated at the corresponding lines of the Lineweaver–Burk diagram (**A**). The K_i value was evaluated by replotting the intersections of the straight lines with the ordinate of the Lineweaver–Burk diagram over the inhibitor concentration present in each experiment (**B**). All kinetic measurements were done at least in triplicate.

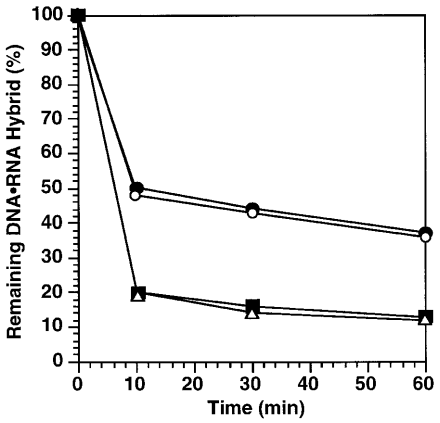

Figure 5. HIV-1 PR has no effect on the RNase H activity of heterodimeric HIV-1 RT. RNase H activity was measured with 1 nM RNase H from *E.coli* (● with PR, ○ without PR) or 10 nM purified p66/p51 RT (■ with PR, Δ without PR) as described in Materials and Methods. PR (200 nM) was added and degradation of [^{3}H]poly(rA)·oligo(dT) was measured after 10, 30 and 60 min.

was again achieved at a PR concentration of 100 nM in reaction mixtures with 10 µM dTTP, whereas in reaction mixtures with 100 µM dTTP only 50 nM PR was necessary for half-maximal inhibition (data not shown).

We also analyzed a potential inhibitory effect of PR on the RNase H activity of HIV-1 RT. As shown in Figure 5, there was neither a measurable inhibition of the activity of commercially available RNase H from *E.coli* (open and closed circles) nor of the RNase H activity associated with p66/p51 RT (triangles and squares). Since RNase H activity was not affected by the presence of PR, we conclude that complex formation does not inhibit the binding of RT to its nucleic acid substrate.

Specificity of the interactions between RT and PR

To study the specificity of the PR-induced RT inhibition, a similar set of experiments was repeated with recombinant RT from the simian immunodeficiency virus of the African green monkey (SIV$_{agm}$ 3) (17). SIV$_{agm}$ RT was inhibited by HIV-1 PR non-competitively with respect to poly(rA)·(dT)$_{20}$ and with a similar K_i value (120 nM) as HIV-1 RT. ELISA assays were repeated with immobilized HIV-1 PR and SIV$_{agm}$ RT by exploiting the cross-reactivity of a polyclonal anti-HIV-1 RT antibody with SIV$_{agm}$ RT. Very similar results were obtained as with the homologous PR–RT interaction (data not shown). In addition, commercially available RTs from avian myeloblastosis virus (AMV) and murine Moloney leukemia virus (MMLV) were included in these studies. In contrast to the findings with RTs from HIV-1 and SIV$_{agm}$, the AMV- and MMLV-derived RTs were not inhibited by HIV-1 PR (Table 1). The conservation of PR inhibition of RT between the human virus and the 62% homologous RT from the monkey-derived virus, but its absence between the differently organized avian and murine retroviruses, may point to a physiological function of this type of interaction.

DISCUSSION

Immunoprecipitation experiments with antibodies directed against HIV-1 RT as well as HIV-1 PR revealed co-precipitation of both enzymes in bacterial extracts expressing both viral activities. This strongly suggests complex formation between PR and RT in bacterial extracts. This type of complex formation might have been mediated by other components present in crude extracts, such as nucleic acids, proteins or peptides. However, complexes were also formed with purified proteins, as shown by immunoprecipitations (M.Böttcher, unpublished results) and ELISA assays. Moreover, the DNA synthesizing activity of purified RT was inhibited by purified PR. Therefore, it is reasonable to conclude that complex formation between PR and RT was caused by direct protein–protein interactions.

While PR was inhibitory for the polymerase activity of RT, we did not observe a significant effect of up to 125 nM RT on the proteolytic activity of 125 nM PR. These experiments were carried out with a synthetic oligopeptide (His-Lys-Ala-Arg-Val-Leu-[p-NO_2-Phe]-Glu-Ala-Nle-Ser-NH_2) as substrate. However, with a substrate bearing greater resemblance to a natural one, HIV-1 RT has been reported to stimulate the activity of HIV-1 PR. Stimulation of PR was most pronounced at the cleavage site between RT and IN (18,19). These findings also support the view

of complex formation between RT and PR. Moreover, optimal stimulation of PR by RT required >125 nM each enzyme (18), indicating a binding constant very similar to that observed in our study, i.e. 100 nM.

It is not yet known whether inhibition of the DNA synthesizing activity of RT by its homologous PR is of biological significance. On first sight this seems questionable, because, despite the obvious presence of co-packaged PR, partial reverse transcription of the viral RNA may occur in the virion before infection (20–23). On the other hand, a biological function of RT–PR complex formation might be deduced from the observation that HIV-1 RT stimulates PR (18,19) and, as shown here, PR inhibits RT from both HIV-1 as well as the closely related SIV_{agm}, while RTs from the differently organized retroviruses AMV and MMLV were not inhibited. The latter result suggests some evolutionary conservation of this type of inhibition, which might point to a physiological function. A physiological role of this type of inhibition is also supported by stoichiometric considerations in connection with the measured inhibition constant: with 30–100 molecules of both RT and PR present per virion (24,25) and a diameter of ~100 nm for a virus particle, an inner virion concentration of 100–300 µM of each enzyme would result. This is three orders of magnitude higher than the K_i value determined here and sufficient to postulate a complete inhibition of HIV-1 RT activity in the budding virion. Complete inhibition of RT activity in circulating virions seems to be of advantage, at least until all the co-packaged dNTPs have been lost by diffusion. With 20–40 µM dNTPs present in T lymphoblastoid cell lines (26), one can calculate that only ~25–50 dNTP molecules are encapsulated within a virion. With active RT, these dNTPs should become immediately incorporated into DNA, because all the other components necessary for reverse transcription, such as primer tRNA, genomic RNA and Mg^{2+}, are also present. After a short period of DNA synthesis reverse transcription would stop because of depletion of the dNTPs. Even if one considers the presence of 30–100 nM dNTPs in blood or semen (23), DNA synthesis in circulating virions would be at least 50- to 100-fold slower than in infected cells (based on a K_m value for dNTPs of 2.5 µM; 27). It is very likely that stalled or severely slowed down DNA synthesis should nevertheless activate the endonucleolytic activity of RNase H, which in turn would degrade genomic RNA opposite the incomplete plus strand. Hence, premature and incomplete DNA synthesis might be dangerous for the virus and might result in loss of both genomic information and viral infectivity. Upon infection of a novel target cell (as well as upon lysis of virions under experimental conditions), dilution effects should provoke a dissociation of PR–RT complexes, releasing active RT for a novel cycle of reverse transcription.

The measured inhibition constant for PR–RT interactions of 50–100 nM comes close to the K_i value of 40 nM that has been reported for RT inhibition by azidothymidine triphosphate (27,28). Therefore, PR–RT interactions might be exploited to inhibit RT activity *in vivo*, e.g. by expressing HIV-1 PR in human $CD4^+$ lymphocytes. Indeed, overexpression of PR multimers in $CD4^+$ lymphocytes inhibits infection by HIV-1 virions (29). Although it is not yet known at which stage of the infection cycle this type of inhibition occurs, the finding of complex formation between RT and PR and, moreover, the inhibitory effect of PR upon the polymerizing activity of RT, might help explain this unexpected effect. Perhaps the observed type of inhibition can also be exploited in a more direct manner, such as for the design of novel peptide inhibitors that mimic the inhibitory domain(s) of the protease. For this it will be necessary to map the domains of RT and PR that interact with each other. A better characterization of PR–RT interactions might help to develop strategies for further improving the observed inhibitory potential of PR for RT.

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