The Thumb Domain of the P51-Subunit Is Essential for Activation of HIV Reverse Transcriptase†

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Received June 24, 1999; Revised Manuscript Received September 3, 1999

ABSTRACT: The biologically relevant and active form of human immunodeficiency virus reverse transcriptase is a heterodimer produced in a two-step dimerization process. Dimerization involves first the rapid association of the two subunits, followed by a slow conformational change yielding a fully active form. In the present study, we demonstrate that the interaction between the thumb domain of p51 and the RNase-H domain of p66 plays a major role in an essential conformational change required for proper folding of the primer/template and the tRNA-binding site, for maturation and for activation of heterodimeric reverse transcriptase. A synthetic peptide derived from the sequence within the thumb domain of p51, which forms the interface with the RNase-H domains of p66, binds heterodimeric reverse transcriptase with an apparent dissociation constant in the nanomolar range and selectively inhibits activation of heterodimeric reverse transcriptase with an inhibition constant of 1.2 μM. A detailed study of the mechanism of inhibition reveals that this peptide does not require dissociation of heterodimeric RT for efficient inhibition and does not affect subunit association, but interferes with the conformational change required for activation of heterodimeric reverse transcriptase, resulting in a decrease in the affinity of reverse transcriptase for the tRNA and an increase in the stability of the primer/template/reverse transcriptase complex. We have previously proposed that the dimeric nature of reverse transcriptase represents an interesting target for the design of antiviral agents. On the basis of this work, we propose that the conformational changes involved in the activation of reverse transcriptase similarly represent an important target for the design of novel antiviral compounds.

Reverse transcriptase (RT)[1] is a key enzyme in the life cycle of the human immunodeficiency virus (HIV), which is responsible for the conversion of single-stranded genomic RNA into double-stranded DNA (*1*, *2*). As such, RT constitutes one of the main targets for chemotherapy against HIV. However, most of the inhibitors that have been developed until now are only directed against the polymerase domain of HIV-1 RT, such as chain terminator nucleoside analogues or nonnucleoside inhibitors (*3*−*5*).

The biologically relevant form of HIV-1 and HIV-2 RT is a heterodimer consisting of two polypeptides of molecular mass of 66 kDa (p66) and 51 kDa (p51), p51 being derived from p66 by proteolytic cleavage of its C-terminal domain (*6*, *7*). The structure of HIV-1 RT has been solved in different states: unliganded or complexed with nonnucleoside inhibitors, with a double-stranded DNA (*8*−*12*) and, more recently, covalently trapped with template/primer DNA and deoxynucleoside triphosphate (*13*). The p66 subunit consists of five distinct subdomains, four of which, termed fingers, palm, thumb, and connection, form the polymerase domain and the additional RNase-H domain. The p51 subunit only comprises the four subdomains of the polymerase domain, with individual structures which are similar to those in the corresponding domain of p66, but with a significantly different orientation relative to one another (*8*, *9*).

We have shown previously that the formation of the active heterodimeric form of HIV-1 and HIV-2 RT occurs in a two-step process, involving a first contact between the two subunits, which yields an intermediate inactive heterodimeric RT, followed by a slow conformational change essential for complete activation of RT (*14*). The dimer interface is largely dominated by hydrophobic interactions between the two connection subdomains (*14*−*16*), involving a central tryptophan repeat motif highly conserved in HIV isolates (*14*, *17*, *18*). We have defined a synthetic peptide derived from the Trp-rich cluster which inhibits RT dimerization in vitro as well as viral production in vivo (*19*, *20*). In contrast, the precise role played by the other subdomains of RT in the dimerization process remains unclear. On the basis of the X-ray structure of HIV-1 RT, we have proposed that the conversion of the heterodimer from an inactive to an active form involves at least the thumb and the fingers domains of p51, which interact, respectively, with the RNAse H and the palm domains of p66 (*14*).

† This work was supported by the CNRS, and by grants from the ANRS "Agence Nationale pour la Recherche sur le SIDA", the ARC "Association pour la Recherche contre le Cancer" and the FRM "Fondation pour la Recherche Médicale" SIDACTION. M.C.M was supported by a fellowship from the ANRS

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[1] Abbreviations: RT, reverse transcriptase; P/T, primer/template; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; tBu, *tert*-butyl; HIV, human immunodeficiency virus; RNase-H, ribonuclease H.

10.1021/bi9914558 CCC: $18.00
Published on Web 10/29/1999

Structural analysis of HIV-1 RT has shown that the thumb domain constitutes one of the most flexible parts of HIV-1 RT and that in p66 it can adopt different positions relative to the fingers domain in the "open" or "closed" conformation of RT (*9*, *11*). The thumb domain of p66 is involved in interactions with the minor groove of the primer/template and may mediate translocation of the enzyme along the template (*13*). Moreover, cross-linking, mutagenic, and structural studies have revealed that the thumb domain of p66 is involved, at least in part, in the $tRNA_3^{Lys}$ binding site (*21*−*24*). In contrast, the role of the thumb domain of p51 remains elusive, although it has been proposed to be involved in stabilization of the heterodimer (*14*) and in the tRNA binding site (*21*).

An interesting feature of HIV-1 RT is that the dimeric form of the enzyme is absolutely required for all enzymatic activities (*25*−*28*). We have demonstrated that dimerization of RT constitutes an interesting target for AIDS chemotherapy (*19*, *20*, *25*). In the present work, to further understand the mechanism of RT activation, we have investigated the role of the thumb domain of p51 in the dimerization of HIV-1 RT. We have designed peptides derived from the sequence of the thumb domain of p51 located at the interface with the RNase H domain of p66. Using kinetic and fluorescence methods to characterize the binding of these peptides to RT as well as their effect on the dimerization process, we have shown that these peptides can block activation of RT in vitro, by preventing an essential conformational change, which folds the binding site of both the primer/template and the tRNA properly during maturation.

EXPERIMENTAL PROCEDURES

Materials. Acetonitrile (gradient grade) was purchased from Merck and $^{[3H]}$dTTP from Amersham Corp. $tRNA_2^{Tyr}$ was obtained from Sigma.

Enzyme Preparation. Recombinant HIV-1 $_{BH10}$ RT was expressed in *Escherichia coli* and purified as described previously (*26*). A highly homogeneous preparation of the heterodimeric form of the enzyme resulting from coexpression of 66 and 51 kDa subunits was used. Enzyme concentration was routinely determined by Bradford (*29*) using a gravimetrically prepared solution of RT as a standard.

Peptide Synthesis. Peptides were synthesized by solid-phase peptide synthesis using AEDI-Expensin resin with a 9050 pep-synthesizer (Millipore U.K.), according to the Fmoc/tBu method (*30*). Peptides were purified by semi-preparative HPLC using an acetonitrile gradient in 0.1% TFA and identified by amino acid analysis and electrospray mass spectroscopy. To increase their stability, both peptides were prepared with an acetylated-N-terminus and a cysteamide group at their C-terminus (*30*).

Oligonucleotides. A 18/36-mer oligonucleotide primer/template was routinely used with the nucleotide sequence $^{5'}$-TCCCTGTTCGGGCGCCAC-$^{3'}$ for the primer strand and $^{5'}$-TGTGGAAAATCTCATGCAGTGGCGCCCGAACAGG-GA-$^{3'}$ for the template strand, which corresponds to the sequence of the natural primer binding site from HIV-1 RT (*31*). Fluorescently labeled DNA primer was synthesized with either 6-FAM, a fluorescein derivative, at the 5′-end of the primer oligonucleotide, or a mansyl-group introduced at the penultimate nucleotide at the 3′-end of the primer oligonucleotide (*14*). Primer and template oligonucleotides were annealed by heating equimolar amounts in annealing buffer for 5 min at 90 °C, followed by cooling to room temperature over 1 h in a water bath.

RT Polymerase Assay. Polymerase activity was measured in a standard assay using poly(rA)·$(dT)_{15}$ as a primer/template as described previously (*25*). The RT preparation used presented a specific activity of about 10 000 units/mg where 1 unit of enzyme catalyzes the incorporation of 1 nmol of TMP in 10 min at 37 °C into acid insoluble material.

Fluorescence Experiments. Fluorescence experiments were performed at 25 °C using a PTI spectrofluorimeter with a band-pass of 2 and 8 nm for excitation and emission, respectively. The fluorescence emission of RT was measured in a total volume of 0.7 mL of fluorescence buffer containing 50 mM Tris-HCl (pH 8.0),10 mM $MgCl_2$, 50 mM KCl, and 1 mM DTT, and 10% glycerol added to increase the stability of the protein. Binding of peptides and of $tRNA_2^{Tyr}$ was monitored by following changes in the intrinsic Trp-fluorescence of RT. Excitation was performed at 290 nm and emission fluorescence was recorded at 340 nm. Binding of fluorescently labeled primer/template with 5′-fluorescently labeled FAM-primer/template was measured by recording fluorescence emission at 515 nm, following excitation at 492 nm. Binding of mansyl-labeled primer was monitored at 440 nm upon excitation at 350 nm. Data were analyzed with a quadratic equation using the Grafit program (Erathicus Software) as already described (*14*, *32*).

Size-Exclusion HPLC. Chromatography was performed using two HPLC columns in series (Bio-Rad TSK 250 followed by Bio-Rad TSK 125 both 7.5 × 300 mm) as previously described (*25*). Samples containing 5−10 μg of protein were applied and eluted with 200 mM potassium phosphate pH 6.8 at a flow rate of 0.8 mL/min.

RT Dimerization Kinetics. Complete dissociation of heterodimeric HIV-1 RT was achieved by 20 min of incubation in fluorescence buffer containing 17% acetonitrile. Association of the two subunits was then initiated by a 12-fold dilution of the sample in an acetonitrile-free fluorescence buffer resulting in a final concentration of 1.4% acetonitrile. All experiments were performed at 25 °C with an enzyme concentration of 20 and 2 μM for dissociation and association experiments, respectively. Establishment of the dimerization equilibrium was followed in a time-dependent manner using intrinsic fluorescence, size-exclusion HPLC, and by monitoring the polymerase activity of the enzyme (*14*, *25*).

RESULTS

Peptide Design. We have demonstrated that the dimerization mechanism of HIV-1 and HIV-2 RTs is a two-step process which corresponds to a first interaction between the two connection subdomains of p66 and p51, generating an inactive dimeric form of RT. Full activation occurs during a subsequent slow conformational change involving different structural domains of both subunits which are not yet well-defined (*14*). On the basis of the crystallographic structures of HIV-1 RT (*8*−*13*), we propose that the thumb domain of p51 could be directly involved in the maturation of RT by interacting with the RNase-H domain of p66. One of the main contacts between the two subunits corresponds to a

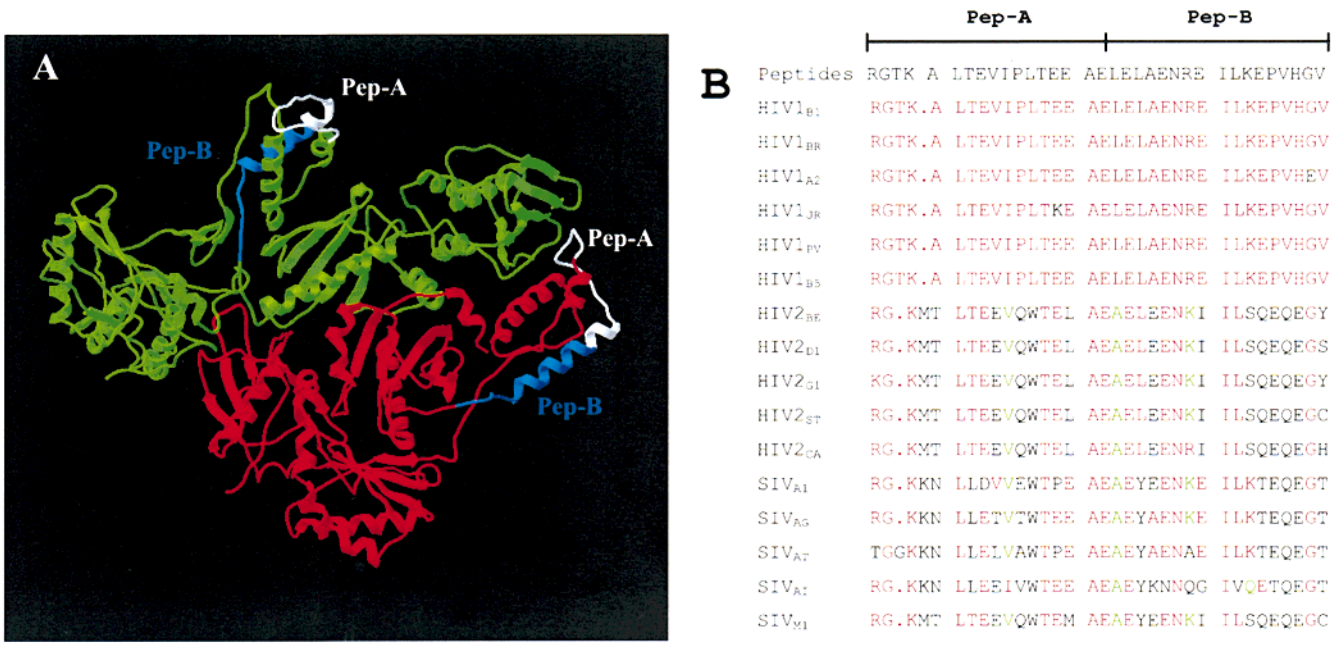

FIGURE 1: Location of Pep-A and Pep-B in the structure of heterodimeric HIV-1 RT. (A) Structure of HIV-1 RT as revealed by X-ray crystallography (8−13), p66 and p51 subunits are in green and red respectively; the structures of peptide A and peptide B are in white and blue, respectively. (B) Sequence alignments of the thumb domain region of RT (residues 284−317) in different isolates of HIV-1, HIV-2 and SIV. The position in the sequence of the peptides is referred to as in the BH10 isolate, conserved residues and conservative changes are reported in red and green, respectively.

structural motif located between amino acids 280−310 in the thumb domain of p51 and which includes two helices αI and αJ (Figure 1A). Sequence alignment of this region reveals a consensus motif which is extremely well conserved in the different isolates of HIV-1 and HIV-2, as well as in SIV (Figure 1B), suggesting that this domain is required for the integrity of RTs. Two different peptides of 17 residues derived from this region were synthesized. The first peptide (Pep-A) corresponds to the sequence between residues 284−300 (RGTKALTEVIPLTEEAE) which is the end of helix αI, the loop connecting helices αI and αJ and a part of helix αJ. The second peptide (Pep-B) derives from amino acids 301−317 (LELAENREILKEPVHGV) and corresponds to the helix αJ (Figure 1A).

Effect of the Peptides on the Stability and the Dimerization of HIV-1 RT. The effect of both peptides on the stability of heterodimeric HIV-1 RT was monitored by size-exclusion HLPC. Recombinant HIV-1 RT used in this study was fully dimeric and therefore eluted in a single peak at 13.3 min (Figure 2A). When preincubated with 100 μM of Pep-A, about 80% of RT remained dimeric and two peaks corresponding to p66 and p51 subunits eluted, respectively, at 15.7 and 16.5 min (Figure 2B). In the presence of Pep-B (100 μM), the stability of HIV-1 RT was not affected (data not shown).

To define whether the peptides derived from the thumb domain could block dimerization of HIV-1 RT, the kinetics of RT dimerization were measured in the presence of 10 μM of either peptide (Figure 3). Dissociation of RT was performed at 25 °C using 17% acetonitrile and association of the subunits was then induced by a 12-fold dilution of the sample in an acetonitrile-free buffer. At this concentration of acetonitrile, no dissociated RT could be detected (data not shown). Monomer/monomer association was monitored by both size-exclusion HPLC and maturation of RT in a

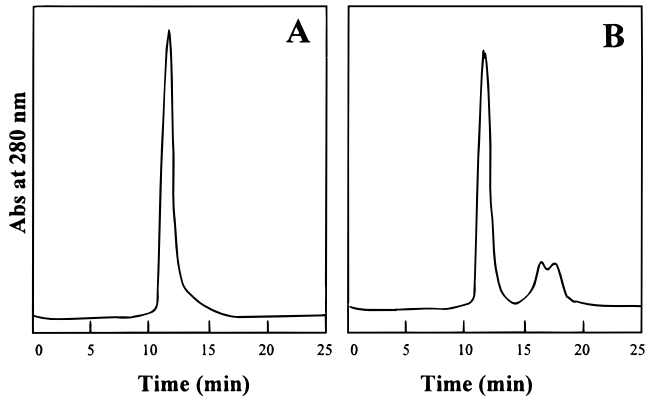

FIGURE 2: Stability of heterodimertic HIV-1 RT monitored by size-exclusion HPLC. HIV-1 RT was incubated in the absence (A) or in the presence of 100 μM of Pep-A for 30 min (B). The sample containing 5−10 μg of protein was then analyzed by size-exclusion chromatography as described in the Experimental Procedures.

polymerase activity assay. As shown in Figure 3A, dimer formation was not significantly affected by the presence of either Pep-A or Pep-B, with an association rate of 2.5×10^4 M^{-1} s^{-1}, similar to the values already reported in the absence of peptides [$k_{ass} = 2.1 \times 10^4$ M^{-1} s^{-1} (*14*)]. These results indicate that neither peptide can prevent RT dimerization.

In contrast, as reported in Figure 3B, the rate of activation of heterodimeric RT was dramatically reduced in the presence of pep-A (10 μM), from 0.14 h^{-1} to 0.022 h^{-1}. Moreover, after 50 h less than 20% of heterodimeric HIV-1 RT was fully active in the presence of Pep-A. The percentage of inactive RT measured was directly dependent on the concentration of Pep-A. Total inhibition of heterodimeric RT maturation was observed at a concentration of 20 μM of Pep-A (Figure 3C). Analysis of the titration curve yielded an apparent dissociation constant value of 1.2 μM for Pep-

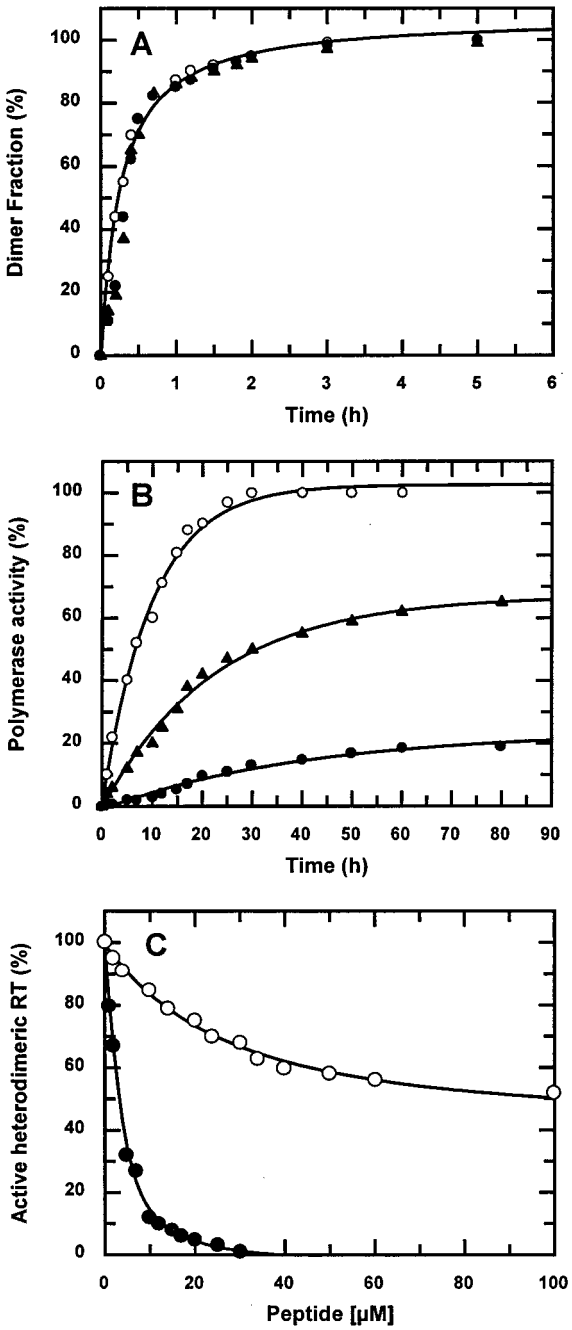

FIGURE 3: Peptide inhibition of HIV-1 RT dimerization process. HIV-1 RT (5−20 μM) was first dissociated by 17% of acetonitrile and monomer association was then initiated by a 12-fold dilution in an acetonitrile-free buffer in the absence (○) or in the presence of 10 μM Pep-A (●) or Pep-B (▲). (A) Kinetics of monomer−monomer association were monitored by size-exclusion chromatography on an aliquot fraction containing 10 μg of RT and the data were analyzed according to a second-order reaction. (B) Kinetics of formation of active heterodimeric RT were monitored by polymerase activity assay. The data were analyzed as a first-order reaction as described in the Experimental Procedures. (C) Dependence of formation of active heterodimeric RT on the concentration of Pep-A and Pep-B. RT was reassociated in the presence of increasing concentrations of Pep-A (●) or Pep-B (○). The ratio of active heterodimeric RT was determined by polymerase activity assay 50 h after dilution in an acetonitrile-free buffer, with respect to the values obtained in the absence of peptides.

A. In the presence of 20 μM Pep-B, up to 60% of heterodimeric RT was still active with an activation rate of 0.11 h^{-1}. As observed for Pep-A, inhibition of RT maturation

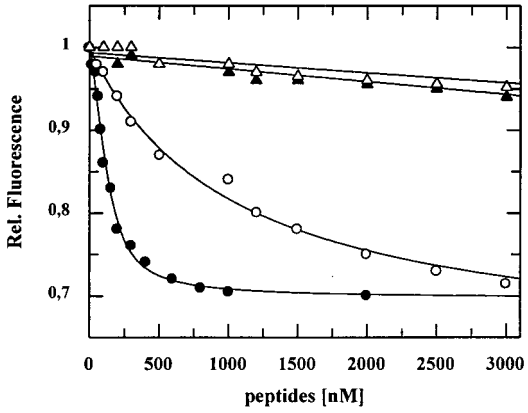

FIGURE 4: Binding titration curves of peptides A and B to HIV-1 RT. Binding of peptides A and B was measured as the quenching of intrinsic fluorescence of RT at 340 nm upon excitation at 290 nm. A fixed concentration of 40 nM of RT saturated (open symbols) or not (closed symbols) with 200 nM of primer/template, was titrated with increasing concentrations of Pep-A (○,●) and peptide B (△,▲). Curves were fitted with a quadratic equation and best fits yield K_d values of 90 nM and 1200 nM, respectively for Pep-A in the absence or in the presence of primer/template.

was also dependent on the concentration of Pep-B (Figure 3C), but we were unable to obtain complete inhibition of RT maturation. Even at the highest concentration used (300 μM), 40% of the dimeric form of RT was still active. The apparent K_d value for Pep-B was estimated to 54 μM. From these data, we concluded that both peptides were inhibiting maturation of RT. Moreover, no additive effect was observed when an equimolar mix of Pep-A and Pep-B was used, indicating that the two peptides target the same binding domain on RT (data not shown).

Binding of the Peptides to the Dimeric Form of RT. To further understand the maturation of heterodimeric HIV-1 RT, we investigated if the peptides described above could interact with the dimeric form of HIV-1 RT. As both peptides are devoid of tryptophan (Trp) residues, their binding to RT was monitored using the intrinsic Trp-fluorescence of RT. The binding of Pep-A to the dimeric form of HIV-1 RT induced a 30% quenching of the intrinsic fluorescence of RT. Binding titration curves revealed that only one molecule of Pep-A bound heterodimeric RT with a very high affinity of 90 ± 8 nM (Figure 4). When RT was first saturated with a DNA primer/template (18/36 mer), the affinity of RT for Pep-A was reduced to a value of 1.2 ± 0.3 μM. In contrast, no significant changes in the fluorescence of RT were observed when the concentration of Pep-B was increased up to 100 μM, suggesting that Pep-B could not bind dimeric RT. Alternatively, binding of Pep-B might not induce any changes in the fluorescence of RT. This is, however, unlikely, as RT contains 37 Trp residues distributed throughout its sequence.

Effect of Pep-A on the Affinity of HIV-1 RT for the Primer/Template. To determine if Pep-A and DNA share a common binding site on RT, we next analyzed the effect of Pep-A on the affinity of RT for P/T. The affinity of RT for P/T was monitored by equilibrium fluorescence using a fluorescently labeled DNA-P/T (18/36-mer) labeled at the 5′-end of the primer with the fluorescein derivative 6-FAM. In the absence of peptides, binding of FAM oligonucleotides to HIV-1 RT led to a 35% quenching of the fluorescence of the FAM group (Figure 5A) and a K_d value of 2.5 ± 0.2

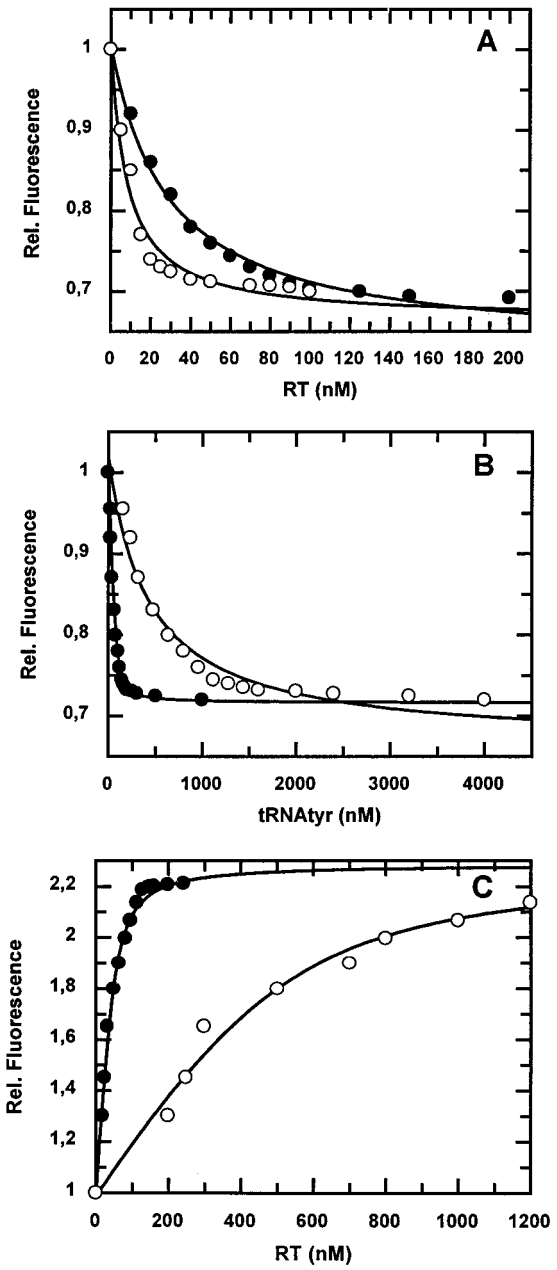

FIGURE 5: Effect of Pep-A on the binding of single- and double-stranded DNA and $tRNA_2^{Tyr}$ to RT. (A) Binding double-stranded DNA to RT was monitored by following the quenching of fluorescence of a 6-FAM group linked to the 5′-end of the primer. A fixed concentration of double-stranded DNA (50 nM) was titrated with increasing concentrations of RT previously incubated (○) or not (●) with 10 μM of Pep-A. Fluorescence emission was measured at 550 nm upon excitation at 480 nm. (B) Binding of $tRNA_2^{Tyr}$ to RT was following by quenching of intrinsic fluorescence of RT. A fixed concentration of RT (40 nM) was titrated with increasing concentrations of $tRNA_2^{Tyr}$ in the presence (○) or in the absence (●) of Pep-A (10 μM). Fluorescence changes were recorded at 340 nm, upon excitation at 290 nm. (C) Binding of single-stranded DNA was monitored by following the increase in fluorescence of a mansyl-group linked to the 3′-end of the primer. A fixed concentration of single-stranded DNA (50 nM) was titrated with increasing concentrations of RT previously incubated with 10 μM of Pep-A (○) or in the absence of Pep-A (●). Fluorescence emission was measured at 440 nm upon excitation at 350 nm. Data were fitted as described in the experimental procedures and best fits are reported below.

nM. The presence of a saturating concentration of Pep-A (20 μM) increased the affinity of RT for the P/T 5-fold, with a K_d value of 0.51 ± 0.1 nM. In contrast, the presence of Pep-B did not modify the binding of double-stranded DNA (data not shown).

Effect of Pep-A on the Binding of $tRNA_2^{Tyr}$ and of Single-Stranded DNA to RT. Intrinsic fluorescence of HIV-1 RT was used as a probe to monitor the effect of Pep-A on the binding of $tRNA_2^{Tyr}$ to RT (Figure 5B). Binding of $tRNA_2^{Tyr}$ induced a 25% quenching of the intrinsic fluorescence of RT, with a K_d value of 17 ± 5 nM, as already reported (*33*). When RT was first saturated with Pep-A (20 μM), the affinity for $tRNA_2^{Tyr}$ was strongly reduced to a $K_d = 470 \pm 24$ nM. Moreover, Pep-A did not bind $tRNA_2^{Tyr}$ directly (data not shown). These results reveal that binding of Pep-A to RT induces a marked change in the conformation of RT, which modifies the binding site of the tRNA.

The binding of a single-stranded DNA oligonucleotide (19−mer) to RT was monitored by extrinsic fluorescence, using a mansyl group linked to the penultimate base of the primer oligonucleotide (*14*). The binding of mansyl oligonucleotide to RT resulted in a 2.2-fold increase in the fluorescence of the mansyl group (Figure 5C). In the absence of Pep-A, the K_d value of RT for single-stranded DNA was calculated to 9.6 ± 0.5 nM; in the presence of a saturating concentration of Pep-A (20 μM), this value was increased to 112 ± 10 nM. The effect of Pep-A on the binding of single-stranded DNA is therefore similar to that observed for binding of $tRNA_2^{Tyr}$ and suggests that binding of Pep-A to RT induces a marked conformational change which increases the selectivity of RT with respect to the nature of the oligonucleotide, favoring binding of double-stranded DNA.

Inhibition of the Polymerase Activity of HIV-1 RT by Pep-A. As reported in Figure 6A, Pep-A inhibited the polymerase activity of RT in a dose-dependent manner. Approximately 50% of polymerase activity was inhibited for a peptide concentration of 30 μM, without inducing any dissociation of the heterodimer. The Pep-A-mediated mechanism of RT inhibition was investigated in more detail, by steady-state kinetics and by varying the concentration of both P/T DNA and Pep-A (Figure 6B). For these experiments, reactions were initiated by addition of RT to a mixture containing both P/T and Pep-A. Dixon plot analysis revealed that Pep-A acts as a noncompetitive inhibitor of RT with respect to P/T, and the K_i value for inhibition of HIV-1 RT by Pep-A was extrapolated to 35 μM, a value higher than that obtained from the dimerization kinetics. These results indicate that Pep-A binds at a site other than at the P/T-binding site and suggests that Pep-A induces conformational changes in RT which produce a more stable RT/PT complex maintained in an inactive conformation.

DISCUSSION

We have recently shown that the formation of active HIV-1 and HIV-2 RTs occurs in a two-step mechanism initiated by a rapid monomer/monomer interaction generating an inactive intermediate heterodimer, followed by a slow conformational change which renders RT fully active (*14*, *34*). The absence of polymerase activity in the intermediate dimer implies that the correct organization of the catalytic site is not yet achieved after the first monomer/monomer association and that further conformational changes are

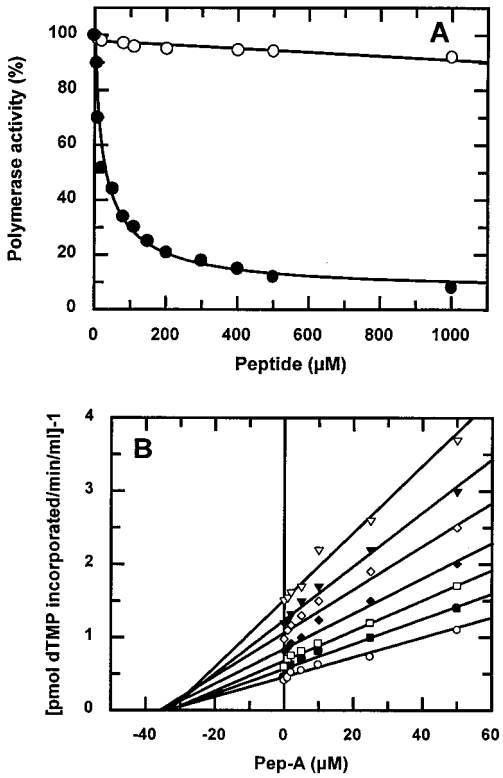

FIGURE 6: Inhibition of the polymerase activity of HIV-1 RT by Pep-A. (A) concentration dependence of the HIV-1 RT polymerase activity by Pep-A (●) and Pep-B (○). (B) Dixon plot of the inhibition of HIV-1 RT by Pep-A. The concentration of poly(rA)·$(dT)_{15}$ used were 0.1 μM (▽), 0.2 μM (▼), 0.5 μM (◇), 0.8 μM (◆), 1.2 μM (□), 3 μM (■), 5 μM (●), 10 μM (○). Reactions were initiated by addition of RT to a mixture containing both poly(rA)·$(dT)_{15}$ and Pep-A.

required for full maturation of RT. In this work, we demonstrate that the thumb domain of p51 plays a major role in the maturation of heterodimeric HIV-1 RT and that peptides derived from the sequence of this domain at the interface with the RNase-H domain of p66 can selectively inhibit activation of HIV-1 RT. A detailed study of the mechanism of inhibition reveals that such peptides inhibit the polymerase activity of RT by modulating binding of both the DNA primer/template and the tRNA to the enzyme.

The dimer interface of HIV-1 RT is mainly formed by an interaction between the connection domains of p51 and p66 (*8*−*13*), including the Trp-repeat motif (residues 389−407 in HIV-1_{BH10} RT), which is essential for monomer/monomer association (*14*−*17*, *19*−*20*). Two other interface contacts have also been identified in the structure of RT, one between the thumb domain of p51 and the RNase H domain of p66 and the other between the fingers domain of p51 and the palm domain of p66. The detailed molecular interpretation of dimer organization based on both the X-ray structure of HIV-1 RT (*8*−*11*) and the prediction of the secondary structure of HIV-2 RT (*14*) reveals that the stacking of the thumb domain of p51 onto the RNase H domain of p66 is crucial for both maturation and stabilization of heterodimeric RT. Here, we show that two peptides derived from the thumb domain, corresponding to residues 284−300 (Pep-A) and 301−316 (Pep-B), are able to interfere with the maturation of HIV-1 RT in vitro. That these two peptides do not significantly affect the stability of dimeric RT or reduce the rate of monomer/monomer association, but do indeed block activation of heterodimeric RT, confirms the existence of a second conformational step in the dimerization process. This conformational change is essential for RT activation and clearly involves the thumb domain of p51.

The most dramatic effects were observed with Pep-A, which contains the end of helix αI and the loop connecting helices αI and αJ (Figure 1A). Pep-A presents an inhibition constant of 1.2 μM and completely inhibits maturation of HIV-1 RT at a concentration of 20 μM. Pep-B, which corresponds to the helix αJ, is less efficient than Pep-A and does not inhibit preformed mature heterodimeric RT. Finally, peptides derived from residues 265−280, corresponding to the helix αI, did not alter the dimerization or the activation of RT (data not shown). Taking together these results with the lack of additive effect of Pep-B on Pep-A-induced inhibition of RT maturation, we suggest that the main area involved in this interaction is covered by Pep-A and, therefore, corresponds to the end of helix αI and the beginning of the loop connecting helices αI and αJ (residues 284−300). Several other studies have also proposed that this loop (residues 282−310) is buried in the dimer interface and involved in hydrophobic protein/protein interactions, which are essential for dimer formation and the integrity of RNase-H activity (*15*−*17*, *35*).

Heterodimeric RT is extremely stable, with a dissociation constant in the nanomolar range. Given that the energy required for dimer formation is extremely high (12 kcal/mol), attempts to induce its dissociation with small molecules have been unsuccessful. In the present work, we demonstrate that targeting the conformational reorganization of the maturation step does not require RT dissociation for inhibition and can be reverted by addition of a short peptide. That Pep-A should inhibit mature preformed heterodimeric RT suggests that this short peptide can reverse the conformation of RT induced during maturation and maintain the RT/PT complex in an inactive conformation. In contrast, preliminary dissociation of RT is required for the binding of Pep-B. Pep-A exhibits high affinity for heterodimeric RT (90 nM) and acts as a noncompetitive inhibitor with respect to the P/T. A detailed study reveals that binding of such a short peptide to RT induces dramatic changes in the ability of the enzyme to bind nucleic acids, increasing its affinity for the P/T and preventing binding of the $tRNA_2^{Tyr}$. The inhibition constant of Pep-A estimated from the maturation kinetics (1.2 μM) is 20-fold lower than the value obtained in polymerase assays with the mature preformed heterodimer ($K_i = 35$ μM). This difference can be attributed to the need of Pep-A to reverse the conformational changes required for activation of RT by competing with its corresponding sequence in the thumb domain of p51.

From these results, a molecular mechanism can be proposed to describe RT maturation as a process, which involves the proper folding of both the P/T and the tRNA-binding sites. This conformational change most likely involves both the thumb of p51 and the RNase H subunits and suggests that the stacking of the thumb of p51 onto the RNAse H of p66 is essential for both activity and maturation of RT, by modifying the conformation of the catalytic site of RT. Both X-ray structural and biochemical data have proven that the thumb domain of RT is mainly involved in the P/T binding site and is essential for polymerase activity

(*13*, *21*−*24*). Monitoring the in vitro interactions between the tRNA and RT using different biochemical and structural approaches have shown that both p51 and p66 interact with the tRNA, (*21*−*24*) and that both the tRNA and the PT bind partially the same or at least overlapping sites (*33*, *37*). Cross-linking of the primer oligoribonucleotide $tRNA_3$lys with RT has revealed that residues "241−244" in β-sheet 14 of the thumb domain of p66 interact with the tRNA (*23*). Mutation of residues Lys^{249}, Arg^{307}, and Lys^{311} within the thumb domain of p66 reduces the affinity of RT for the tRNA and reveals a crevice in this domain which interacts with the tRNA anti-codon loop (*21*−*23*). As Pep-A was initially derived from sequence in the thumb domain of p51, which interacts with RNase H domain, we propose that this inhibition targets conformational changes arising from interaction between these two domains. Pep-A may as such indirectly induce conformational change in the thumb domain of p66. However, we cannot at this point exclude that Pep-A may directly affect changes arising from interactions between the thumb and the fingers domain of p66.

We have already discussed novel inhibitors of both HIV-1 and HIV-2 RT which efficiently target monomer/monomer association. On the basis of our results, we now conclude that maturation of RT also constitutes an interesting target for the design of new inhibitors of HIV RT. Such inhibitory peptides block maturation of RT in vivo, do not require RT dissociation, and are efficient on both the isolated subunits and dimeric RT, thus bypassing the problem of the high stability of HIV RTs. The complete in vivo mechanism of RT formation and activation, has not yet been fully elucidated, but may require a first association of the p66/p66 homodimer, followed by the proteolytic cleavage of one of the RNase-H domains by HIV protease (*6*, *7*). Interactions between the thumb domain of p51 and the RNase-H domain of p66 would then occur and produce mature heterodimeric RT. The peptides described in this study might potentially interfere with such processing of homodimeric p66/p66 into heterodimeric p66/p51. Our results provide a structural basis for the design of new anti-RT compounds which target the activation step of RT during viral formation.

ACKNOWLEDGMENT

We thank Roger Goody for helpful discussions and for providing *E. coli* strains that expressed HIV-1 RT.

REFERENCES

1. Baltimore, D. (1970) *Nature 226*, 1209−1211.
2. Temin, H. M., and Mizutani, S. (1970) *Nature 226*, 1211−1213.
3. De Clercq, E. (1992) *AIDS Res. Hum. Retro. 8*, 119−134.
4. Mitsuya, H., Yarchoan, R., and Broder, S. (1990) *Science 249*, 1533−1544.
5. Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J. C., Shih, C. K., Eckner, K., Hattox, S., Adams, J., Rosehthal, A. S., Faanes, R., Eckner, R. J., Koup, R. A., and Sullivan, J. L. (1990) *Science 250*, 1411−1413.
6. Di Marzo Veronese, F., Copeland, T. D., De Vico, A. L., Rahman, R., Oroszlan, S., Gallo, M., and Sarngadharan, M. G. (1986) *Science 231*, 1289−1291.
7. Lightfoote, M. M., Colligan, J. E., Folks, T. M., Fauci, A. S., Martin, M. A., and Venkatesan, S. (1986) *J. Virol. 60*, 771−775.
8. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science 256*, 1783−1790.
9. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6320−6324.
10. Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., and Stuart, D., (1995) *Nat. Struct. Biol. 2*, 303−308.
11. Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D., and Stammers, D. (1995) *Nat. Struct. Biol. 2*, 293−302.
12. Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C., and Harrison, S. C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1222−1226.
13. Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998) *Science 282*, 1669−1675.
14. Divita, G., Rittinger, K., Geourjon, C., Deleage, G., and Goody, R. S. (1995) *J. Mol. Biol. 245*, 508−521.
15. Wang, J., Smerdon, S. J., Jäger, J., Kohlstaedt, L. A., Rice, P. A., Friedman, J. M., and Steitz, T. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7242−7246.
16. Becerra, S. P., Kumar, A., Lewis, M. S., Widen, S. G., Abbotts, J., Karawya, E. M., Hughes, S. H., Shiloach, J., and Wilson, S. H. (1991) *Biochemistry 30*, 11707−11719.
17. Baillon, J. G., Nashed, N. T., Kumar, A., Wilson, S. H., and Jerina, D. M. (1991) *New Biol. 3*, 1015−1019.
18. Debyser, Z., and De Clercq, E. (1996) *Protein Sci. 5*, 278−298.
19. Divita, G, Restle, T., Goody, R. S., Chermann, J. C., and Baillon, J. G. (1994) *J. Biol. Chem. 269*, 13080−13083.
20. Divita, G., Baillon, J. G., Rittinger, K., Chermann, J. C., and Goody, R. S. (1995) *J. Biol. Chem. 270*, 28642−28646.
21. Mishima, Y., and Steitz, J. A. (1995) *EMBO J. 14*, 2679−2687.
22. Arts, E. J., Miller, J. T., Ehresmann, B., and Le Grice, S. J. (1998) *J. Biol. Chem. 273*, 14523−14532.
23. Dufour, E., Reinbolt, J., Castroviejo, M., Ehresmann, B., Litvak, S., Tarrago-Litvak, L., and Andreola, M.-L. (1999) *J. Mol. Biol. 285*, 1339−1346.
24. Isel, C., Westhof, E., Massire, C, Le Grice, S. F. J., Ehresmann, B., Ehresmann, C., and Marquet, R. (1999) *EMBO J. 18*, 1038−1048.
25. Restle, T., Muller, B., and Goody, R. S. (1990) *J. Biol. Chem. 265*, 8986−8988.
26. Muller, B., Restle, T., Weiss, S., Gautel, M., Sczakiel, G., and Goody, R. S. (1989) *J. Biol. Chem. 264*, 13975−13978.
27. Restle, T., Müller, B., and Goody, R. S. (1992) *FEBS Lett. 300*, 97−100.
28. Müller, B., Restle, T., Kühnel, H., and Goody, R. S. (1991) *J. Biol. Chem. 266*, 14709−14713.
29. Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
30. Mery, J., Granier, C., Juin, M., and Brugidou, J. (1993) *Int. J. Pept. Protein Res. 42*, 44−52.
31. Wain-Hobson, S., Sonigi, P., Danos, O., Cole, S., and Alison, M. (1985) *Cell 40*, 9−17.
32. Müller, B., Restle, T., Reinstein, J., and Goody, R. S. (1991) *Biochemistry 30*.
33. Thrall, S. H., Reinstein, J., Wöhrl, B. M., and Goody, R. S. (1996) *Biochemistry*, *35*, 4609−4618.
34. Divita, G., Rittinger, K., Restle, T., Immendorfer, U., and Goody, R. S. (1995) *Biochemistry 34*, 16337−16346.
35. Goel, R., Beard, W. A., Kumar, A., Casas-Finet, J. R., Strub, M. P., Stahl, S. J., Lewis, M. S., Bebenek, K., Becerra, P., Kunkel, T. A., and Wilson, S. H. (1993) *Biochemistry 32*, 13012−13018.
36. Jacques, P. S., Wöhrl, B. M., Howard, K. J., and Le Grice, S. (1994) *J. Biol. Chem. 269*, 1388−1393.
37. Wöhrl, B. M., Krebs, R., Thrall, S. H., Le Grice, S. J. F., Scheidig, A., and Goody, R. S. (1997) *J. Biol. Chem. 272*, 17581−17587.

BI9914558