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A positively charged side chain at position 154 on the β8–αE loop of HIV-1 RT is required for stable ternary complex formation

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ABSTRACT

Lys154 is the only positively charged residue located in the VLPQGWK motif on the β8–αE loop at the junction of the fingers and palm subdomains of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). Some of the conserved residues in this motif are critical for RT function, while others have been shown to confer nucleoside drug resistance and fidelity to the enzyme. In order to understand the functional implication of this positively charged residue, we carried out site-directed mutagenesis at position 154 and biochemically characterized the mutant enzymes. Mutants carrying negatively charged side chains (K154D and K154E) were severely impaired in their polymerase function, while those with hydrophobic side chains (K154A and K154I) were moderately affected. Analysis of the binary complexes formed by these mutants revealed that all the mutant derivatives retained their ability to form an enzyme template primer (E–TP) binary complex similar to the wild-type enzyme. In contrast, their ability to form stable E–TP–dNTP ternary complexes varied greatly and was dependent on the nature of the side chain at position 154. The conservative Lys→Arg mutant was not affected in its ability to form a stable ternary complex, while those carrying non-polar or negatively charged side chains were significantly impaired. The apparent $K_{d\ [dNTP]}$ values for these non-conservative mutants were ~16- to 400-fold higher than the wild-type enzyme, indicating that a positively charged side chain at position 154 may be required for efficient formation of a stable ternary complex. Interestingly, all the mutant derivatives of Lys154 were completely resistant to a nucleoside analog inhibitor, 3′-dideoxy 3′-thiacytidine (3TC), implying that Lys154 may play a role in conferring 3TC sensitivity to HIV-1 RT. These findings are discussed in the context of the binary and ternary complex crystal structures of HIV-1 RT.

INTRODUCTION

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is a virally encoded enzyme essential for viral replication and establishing HIV-1 infection. This enzyme copies the single-stranded HIV-1 RNA genome into double-stranded proviral DNA which is subsequently integrated into the host genome. RT is one of the two enzyme targets against which drugs are currently employed to reduce the viral load in plasma, the other being HIV-1 protease. HIV-1 RT is a heterodimeric enzyme consisting of 66 and 51 kDa polypeptides (p66/p51). The p51 subunit lacking the C-terminal 15 kDa RNase H domain is the proteolytic cleavage product of the larger p66 subunit (1,2).

HIV-1 RT is a highly error-prone enzyme which probably contributes to the genetic diversity of HIV-1 (3–5). Various nucleoside drug resistance phenotypes have been identified in clinical isolates from patients exposed to RT inhibitors. A single natural mutation at codon 151 (Gln→Met) of HIV-1 RT confers resistance to all nucleoside inhibitors (6–8). Gln151 is a constituent of the relatively conserved VLPQGWK sequence of HIV-1 RT. This motif is a component of motif B found in all retroviral RTs and is located on the β8–αE loop at the junction of the fingers and palm subdomains of HIV-1 RT. Four of the seven residues in this sequence are highly conserved residues designated as the LPQG motif, which plays an important role in dNTP binding and in positioning the duplex DNA in the catalytic pocket of HIV-1 RT. Gln151 is an important component of the dNTP-binding pocket (9–13) and has been proposed to stabilize the side chain of Arg72, a participant in the conformational change step of the ternary complex during catalysis (10,14). Two residues preceding Q151, namely L149 and P150, have been shown to be crucial for the enzyme function (15). Gly152, the residue following Gln151, has been demonstrated to be absolutely essential for the polymerase function (13–15).

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Lys154, a constituent of the larger VLPQGWK motif B, is the only positively charged residue in this motif and has been shown to influence the processivity and fidelity of HIV-1 RT (13). In the present study, we have evaluated the significance of this positively charged residue in the catalytic function of HIV-1 RT. All the non-conservative mutant derivatives of Lys154 were moderately to severely impaired in their polymerase function and in their ability to form stable ternary complex, though they retained full wild-type binding affinity for the template primer (TP). Interestingly, all the mutant derivatives of Lys154 were resistant to (–)-β-2′,3′-dideoxy3′-thiacytidine triphosphate (3TCTP), suggesting that Lys154 may be one of the residues involved in conferring 3TC susceptibility to HIV-1 RT. These results are discussed in the context of the binary and ternary complex crystal structures of HIV-1 RT.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Promega. HPLC-purified ultrapure dNTPs, Sequenase and DNA sequencing reagents were from Roche Molecular Systems, Inc. The [α-^{32}P]dNTPs and [γ-^{32}P]ATP were obtained from PerkinElmer Life Sciences. Synthetic TPs, sequencing primers and mutagenic oligonucleotides were synthesized at the Molecular Resource Facility at the University of Medicine and Dentistry of New Jersey. All other reagents were of the highest available purity grade and were purchased from Roche Molecular Systems, Inc., Fisher or Bio-Rad.

Expression of plasmid clones and *in vitro* mutagenesis

The recombinant HIV-1 RT was expressed and purified as described before (16–19). The QuikChange™ Site-Directed Mutagenesis Kit from Stratagene was employed to generate the mutant clones using two primers corresponding to the sense and antisense strands containing the desired mutation, and ascertained by DNA sequencing.

Preparation of the HIV-1 U5-PBS RNA template

The HIV-1 genomic RNA template corresponding to the primer-binding sequence region (U5-PBS HIV-1) was prepared using an HIV-1 RNA expression clone (pHIV-PBS) as described previously (17,20). The plasmid pHIV-PBS was linearized with AccI and transcribed using T7 RNA polymerase according to the manufacturer's protocol (Gibco-BRL).

Polymerase activity assay

Polymerase activity of the wild-type and mutant enzymes was assayed on heteropolymeric U5-PBS HIV-1 RNA–DNA and homopolymeric poly(rA) templates primed with the respective complementary DNA primers (12,17). Assays were carried out in a final volume of 50 µl containing 15 nM enzyme in 50 mM Tris–HCl pH 7.8, 100 µg/ml bovine serum albumin (BSA), 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 60 mM KCl, 100 nM TP, 20 µM of each dNTP containing α-^{32}P-labeled dCTP and dGTP (0.5 µCi/nmol) with heteropolymeric template or 20 µM TTP containing [α-^{32}P]dTTP (0.5 µCi/nmol) with the homopolymeric poly(rA) template. Reactions were initiated by the addition of divalent metal ion (Mg^{2+}, 5 mM), incubated at 37°C for 3 min and terminated by the addition of ice-cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The acid-insoluble radioactive material was collected on Whatman GF/B filters, dried and counted for radioactivity in a Packard Tricarb Liquid Scintillation Counter.

Dissociation constant of enzyme–DNA binary complex

The dissociation constants (K_d) of the enzyme (E)–TP binary complexes of the wild-type HIV-1 RT and its mutant derivatives were determined as described before (18). The 33mer DNA template (5′-TGC GCG TTA TAC GGC ACT TCG GAG TGG CTA ACG-3′) primed with 5′-^{32}P-labeled dideoxy-terminated 21mer primer (5′-CGT TAG CCA CTC CGA AGT GC^{dd}C-3′) was used in these binding studies. Varying concentrations of the individual enzymes were incubated with 0.3 nM TP in a total volume of 10 µl containing 50 mM Tris–HCl pH 7.8, 5 mM $MgCl_2$ and 0.01% BSA. An equal volume of 2× gel loading dye (0.25% bromophenol blue, 20% glycerol) was added after 10 min incubation at 4°C, and the E–TP binary complexes were resolved by electrophoretic mobility shift assay (EMSA) and analyzed on a phosphorImager. The percentage of the labeled TP bound to the enzyme (E–TP binary complex) versus enzyme concentrations was plotted and the dissociation constant, $K_{d\ [DNA]}$, was determined as the enzyme concentration at which half-maximum DNA binding occurred.

Formation of stable ternary (E–TP–dNTP) complex and determination of dNTP binding affinity (apparent $K_{d\ [dNTP]}$)

The ability of HIV-1 RT and its mutant derivatives to form a stable ternary complex was evaluated as described by Tong *et al*. (21). For this purpose, the individual enzyme protein (10–50 nM) was incubated with the labeled 33/21^{ddC} TP to form the binary complex and then further incubated at 4°C for 10 min in the presence of 200 µM dGTP, a nucleotide complementary to the first template base. The incubation buffer (10 µl) contained 50 mM Tris–HCl pH 7.8, 5 mM $MgCl_2$ and 0.01% BSA. The putative ternary complexes formed were challenged by the addition of 300-fold molar excess of the unlabeled 33/21^{ddC} TP. The ternary complex species resistant to the DNA trap were resolved on a 6% native polyacrylamide gel, subjected to phosphorimaging and quantified using ImageQuant Software.

Determination of the apparent dNTP binding affinity (apparent $K_{d\ [dNTP]}$) of the wild-type enzyme and its mutant derivatives was carried out as described above except that the binary complexes were incubated in the presence of increasing concentrations of the complementary nucleotide (dGTP, 0–4000 µM) prior to being challenged by the DNA trap. The E–TP–dNTP ternary complexes resistant to the trap were resolved and quantified as above. The apparent $K_{d\ [dNTP]}$ was determined from the percentage of total E–TP binary complex converted into the stable E–TP–dNTP ternary complex as a function of dNTP concentration by fitting the data to the single-site ligand-binding equation using Sigma Plot.

Table 1. Polymerase activity of the wild-type HIV-1 RT and Lys154 mutant derivatives

Enzyme	Poly(rA)·$(dT)_{18}$	49/17mer template–primer	U5-PBS RNA/17mer template–primer
Wild type	100 (74)	100 (33)	100 (26)
K154A	65 ± 5.8	68 ± 6.1	73 ± 7.9
K154D	27 ± 3.2	24 ± 2.9	29 ± 3.7
K154E	9 ± 0.8	16 ± 1.9	20 ± 3.5
K154I	68 ± 6	58 ± 6.1	50 ± 5.7
K154R	117 ±10	110 ± 9.6	131 ± 12.5

The polymerase activities of the wild-type HIV-1 RT and its mutant derivatives were determined on three different template–primers as described in Materials and Methods. The values represent percentage activity of the wild-type enzyme and are the mean values ± SD of three independent experiments. Values shown in parentheses are total picomoles of acid-insoluble dNMP incorporated into the primer DNA by wild-type HIV-1 RT in 3 min at 37°C.

RESULTS

Construction and purification of Lys154 mutants

Five different mutants of Lys154 were generated by site-directed mutagenesis as described before (16). The positively charged Lys154 was replaced either by non-polar uncharged alanine or isoleucine, by negatively charged aspartate and glutamate or by the positively charged guanidino side chain of arginine. The mutant enzymes were purified to near homogeneity (>98% purity) and stored at –80°C in 50% glycerol containing 50 mM HEPES pH 7.0, 1 mM DTT and 100 mM NaCl.

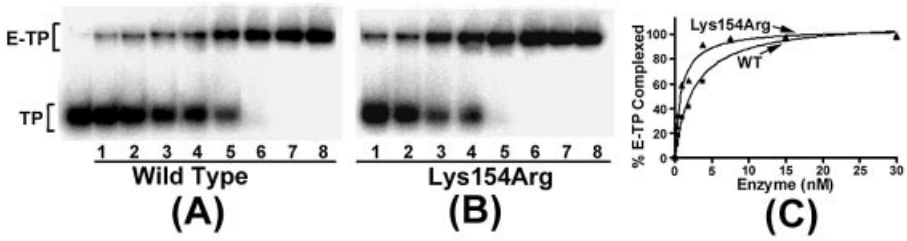
Impact of substitution at position 154 on the DNA polymerase function of the enzymes

Heteropolymeric RNA and DNA as well as homopolymeric poly(rA) templates were used to examine the impact of these mutations on the DNA polymerase activity of the enzyme. The results shown in Table 1 are expressed as percentage polymerase activity relative to the wild-type enzyme. The conservative Lys→Arg substitution at position 154 slightly enhanced the polymerase activity of the enzyme, while two mutant derivatives carrying a non-polar hydrophobic side chain (K154A and K154I) retained ~50–73% polymerase activity on all templates. In contrast, mutants with a negatively charged side chain (Lys→Asp and Lys→Glu) were impaired, exhibiting 9–29% polymerase activity.

Formation of E–TP binary and E–TP–dNTP ternary complexes

These results suggest that a positively charged side chain at position 154 may not be crucial for the polymerase function of the enzyme since Lys→Ala and Lys→Ile substitution retained 50–70% polymerase activity. In contrast, a negatively charged side chain at this position seems to be unfavorable for polymerase function of the enzyme. Since, in the crystal structure of HIV-1 RT–DNA–dNTP ternary complex, Lys154 is seen interacting with the DNA phosphate backbone (22), it may be reasonable to infer that a negative charge at this position may interfere with the DNA-binding function of the enzyme. To investigate this possibility, the $K_{d\ (DNA)}$ in the E–TP binary complex for the wild-type enzyme and its mutant derivatives was determined by EMSA. The results of this analysis are shown as an inset table in Figure 1 along with representative data obtained with the wild-type and K154R mutant enzymes. Interestingly, all the conservative and non-conservative mutant derivatives of Lys154 had no influence on the formation of E–TP binary complex. In fact, a slightly enhanced DNA binding affinity was noted for some of the mutants.

These results suggest that the polar or non-polar nature of the side chain at position 154 has no influence on the formation of E–TP binary complex. In the crystal structure of E–TP binary and E–TP–dNTP ternary complexes, the side chain conformation of Lys154 is dissimilar (22–24). In the binary complex, it mainly interacts with Asp86 and Gln85 which are located away from the TP-binding track (23,24) while, in the ternary complex, it interacts directly with the penultimate template nucleotide in the duplex region (22). Therefore, the reduction in the polymerase activity observed with some of the mutant derivatives of Lys154 may be due to



Enzyme	WT	K154A	K154I	K154D	K154E	K154R
$K_{d(DNA)}$	2.5± 0.17	3.4± 0.21	3.8± 0.20	1.2± 0.07	1.6± 0.10	0.8± 0.1

Figure 1. Gel mobility shift assay for analysis of DNA binding affinity. The 33mer DNA primed with 5′-^{32}P-labeled ddC-terminated 21mer primer was incubated with varying concentrations of the wild-type HIV-1 RT or its mutant derivatives (0–40 nM) at 0°C for 10 min. The mixture was electrophoresed under non-denaturing conditions on a 6% polyacrylamide gel, followed by phosphorImager analysis. (**A** and **B**) The gel shift pattern obtained with the wild-type enzyme and K154R mutant, respectively. Lanes 1–8 indicate the formation of E–TP complex in the presence of protein concentrations corresponding to 0.19, 0.38, 0.75, 1.50, 3.75, 7.50, 15 and 30 nM, respectively. The positions of the free template–primer (TP) and enzyme-bound template–primer (E–TP binary complex) are shown on the left. The percentage of E–TP complex formed as a function of enzyme concentration (**C**) was plotted to determine the $K_{d\ [DNA]}$ values of the individual enzymes. The values shown in the inset table are the mean values ± SD of three independent experiments.

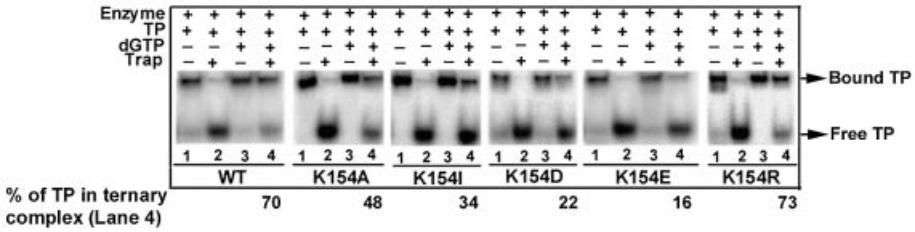
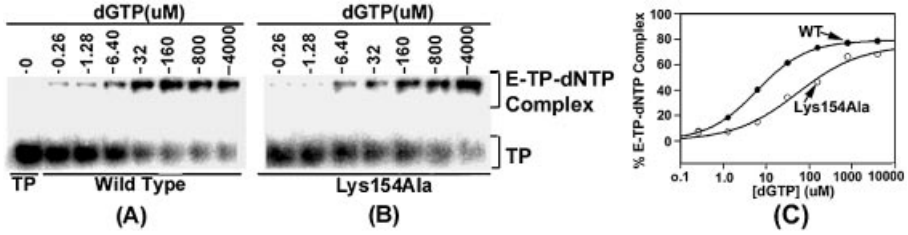


Figure 2. Analysis of the ternary complex formed by the wild-type HIV-1 RT and Lys154 mutants. The binary (lane 1) and ternary complex (lane 3) formed by the individual mutant enzyme was challenged by addition of the DNA trap, and the labeled TP bound in the stable ternary complex was analyzed on a non-denaturing polyacrylamide gel. Lanes 2 and 4 represent the extent of dissociation of the TP in the binary and ternary complexes, respectively. The numbers at the bottom indicate the percentage of undissociated TP in the ternary complex.



Enzyme	WT	K154A	K154I	K154D	K154E	K154R
Apparent $K_{d\ (dNTP)}$	12	199	475	>4000	>4000	10

Figure 3. Determination of apparent dNTP binding affinity in the ternary complex. The apparent dNTP binding affinity ($K_{d\ [dNTP]}$) of the wild-type and mutant enzymes was determined by incubating the individual E–TP binary complex at varying concentrations of dNTP followed by gel shift analysis. For this purpose, the concentration of individual enzyme was such that all the TP molecules are bound to the enzyme in the binary complex. (**A** and **B**) Ternary complex formation by the wild-type and K154R mutant, respectively, at increasing concentrations of dNTP. The positions of the template–primer (TP) in free form and associated in the ternary complex are indicated on the right. The apparent dNTP binding affinity ($K_{d[dNTP]}$), in the ternary complex for the wild-type enzyme and K154R mutant (**C**) was determined by quantifying the percentage E–TP–dNTP complex formed as a function of dGTP concentration and fitting the data to the equation for single-site ligand binding using Sigma Plot.

loss of these interactions, which may impact dNTP binding in the ternary complex. To explore this possibility, we analyzed the effect of these mutations on the formation of stable E–TP–dNTP ternary complexes by gel shift assay. For this purpose, each mutant enzyme was first allowed to form binary complexes with ^{32}P-labeled dideoxy-terminated TP and then supplemented with the next correct dNTP to form the ternary complexes. When these complexes were challenged with excess TP (DNA trap), the E–TP binary complexes are completely competed out, while E–TP–dNTP ternary complexes are stable and resistant to this competition. The results shown in Figure 2 indicate that the E–TP binary complexes formed by each of the mutant enzymes (lane 1) were fully competed out by the DNA trap (lane 2), while a portion of TP bound in E–TP–dNTP ternary complexes remained stable and resistant to the trap (lane 4). The percentage of labeled TP retained in the ternary complexes by each mutant enzyme following the trap challenge essentially correlates with their polymerase activity profile. For instance, K154A and K154I mutants displaying 32–52% reduction in their abilities to form ternary complex also exhibited 27–50% reduction in their polymerase activity compared with the wild-type enzyme. Similarly, 69–80% reduction in the formation of stable ternary complex observed with K154D and K154E mutants correlates well with 71–90% reduction in their polymerase activity.

Apparent dNTP binding affinity of wild-type HIV-1 RT and Lys154 mutants

The results depicted in Figures 2 and 3 were obtained with 33/21^{ddC} TP carrying dC as the first template base accepting dGTP as the first correct dNTP. As shown in Figure 2, substitutions at position 154 had no influence on the formation of E–TP binary complex but seem to have major impact on stabilizing the E–TP–dGTP ternary complexes. Similar patterns were also obtained with dATP and dTTP when the corresponding first template base was dT and dA, respectively (data not shown). Since Lys154 of HIV-1 RT is not seen interacting directly with dNTP in the crystal structure of the ternary complex, its influence on stabilizing the dNTP in the ternary complex was particularly interesting. Although the side chain of Lys154 does not interact with DNA in the binary complex (23,24), it establishes direct contact with the duplex region of the DNA in the ternary complex (22). This interaction between the positively charged Lys154 and negatively charged phosphate group of DNA may be required to stabilize the latter in the ternary complex. The negatively charged side chain at this position (K154D and K154E) may destabilize the TP in the ternary complex due to charge repulsion. We therefore assert that the negative impact of K154D and K154E mutants on the formation of stable ternary

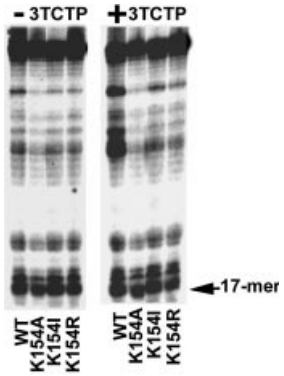


Figure 4. Sensitivity of the Lys154 mutants to 3TCTP. The U5-PBS 49mer DNA template primed with 5′-^{32}P-labeled 17mer PBS primer was used to analyze the extent of DNA synthesis carried out by the Lys154 mutant derivatives in the presence or absence of 3TCTP. The concentration of each mutant enzyme was normalized to contain polymerase activity equal to the wild-type enzyme. Reactions were carried in the standard reaction mixture containing 10 μM dNTP and 2 μM 3TCTP in a final volume of 5 μl. Following 5 min incubation at 37°C, reactions were terminated by Sanger's gel loading dye and the products were analyzed by denaturing 8% polyacrylamide–8 M urea gel electrophoresis followed by phosphorImaging. The position of the 17mer primer is indicated on the right. The data obtained with two less active mutants derivatives (K154D and K154E) are not shown.

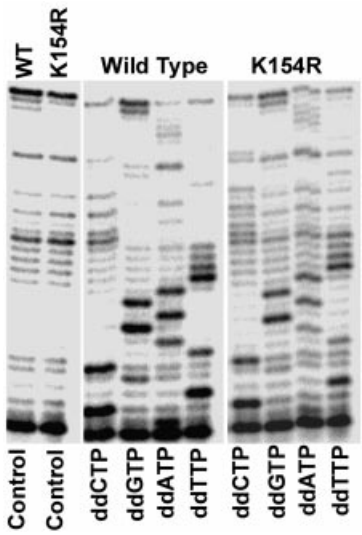


Figure 5. Sensitivity of the K154R mutant towards ddNTPs. The U5-PBS 49mer DNA template primed with 5′-^{32}P-labeled 17mer PBS primer was used to analyze the extent of DNA synthesis carried out by the wild-type HIV-1 RT and its K154R mutant in the presence of individual ddNTPs. The ratio of dNTP to ddNTP was 2:1 and the reactions were carried out for 3 min at 37°C. Control represents reaction in the absence of ddNTP.

complex may be due to their destabilizing effect on the TP rather than to interference in the dNTP binding *per se*. In this scenario, apparent dNTP binding affinity in the ternary complex may be greatly affected due to unstable transition of binary complexes into ternary complexes. This postulation was explored by determining the apparent dNTP binding affinity of individual mutant derivatives of Lys154. The results are shown as an inset table in Figure 3. Representative data depicting the ternary complex formation as a function of varying concentration of dGTP by the wild-type enzyme and K154A mutant are shown in Figure 3. A 16- to 320-fold reduction in the apparent dGTP binding affinity was noted with those mutants carrying uncharged or negatively charged side chains, suggesting that the reduced polymerase activity observed with these mutants may be attributable to unstable ternary complex formation. Since binding affinity of the mutant enzymes for dNTP and the extent of their ternary complex formation are intrinsically linked, a correlation of these parameters with their polymerase activity is clearly established. The conserved Lys→Arg mutant carrying a positively charged guanidino side chain exhibited apparent dNTP binding affinity similar to the wild-type enzyme.

TP binding affinity in the presence and absence of dNTP

The binding of TP and dNTP to HIV-1 RT follows an ordered mechanism where dNTP binding occurs only after the binding of TP to the enzyme (25–27). It is expected that improper binding/orientation of the TP may also affect the binding of dNTP to the E–TP binary complex. It is therefore unlikely that dNTP binding in the ternary complex may influence the affinity of the enzyme for the TP. In order to rule out this possibility, we have determined DNA binding affinity of the wild-type and mutant enzymes in the presence or absence of dNTP. Under both these conditions, no significant differences in their $K_{d\ (DNA)}$ were noted (data not shown).

Sensitivity to nucleotide analogs

Since Lys154 is in the vicinity of Gln151 and Glu89, which are involved in conferring sensitivity to various nucleoside analogs (6–8,28), we also examined the sensitivity of Lys154 mutants to 3TCTP, an inhibitor of HIV-1 RT. Interestingly, all the mutant derivatives of Lys154 were resistant to 3TCTP, suggesting that Lys154 may be one of the residues involved in conferring 3TC susceptibility to RT (Fig. 4). The 3TCTP-resistant phenotype observed with the conservative K154R mutant was unexpected. We therefore examined the sensitivity of the K154R mutant to other nucleotide analogs and found that it is comparatively less sensitive to all the four ddNTPs (Fig. 5). So far, no naturally occurring drug-resistant mutation has been reported at this conserved position. This residue lies in the vicinity of Glu89 and Gln151, mutant derivatives of which have been shown to exhibit cross resistance phenotype to dNTP analogs (7,29).

DISCUSSION

Lys154 located on the β8–αE loop at the junction of the fingers and palm subdomains of HIV-1 RT is the lone positively charged residue in the reasonably conserved VLPQGWK motif. The last four residues of this motif, including Lys154, are constituents of the 'template grip' region of HIV-1 RT (22). Other constituents of the grip region are Asp76, Arg78, Glu89, Gly93 and Ile94 (22). Two charged residues of this grip region (Asp76 and Arg78) interact directly with the single-stranded region of the template (15,30), and their mutant derivatives have been shown to alter DNA binding affinity of the enzyme (31). Glu89 interacts with the penultimate template nucleotide in the duplex region of the TP in both binary and ternary complexes (22,23) and has been shown to be involved in both fidelity and sensitivity to several nucleoside analogs (28,32,33). Another important

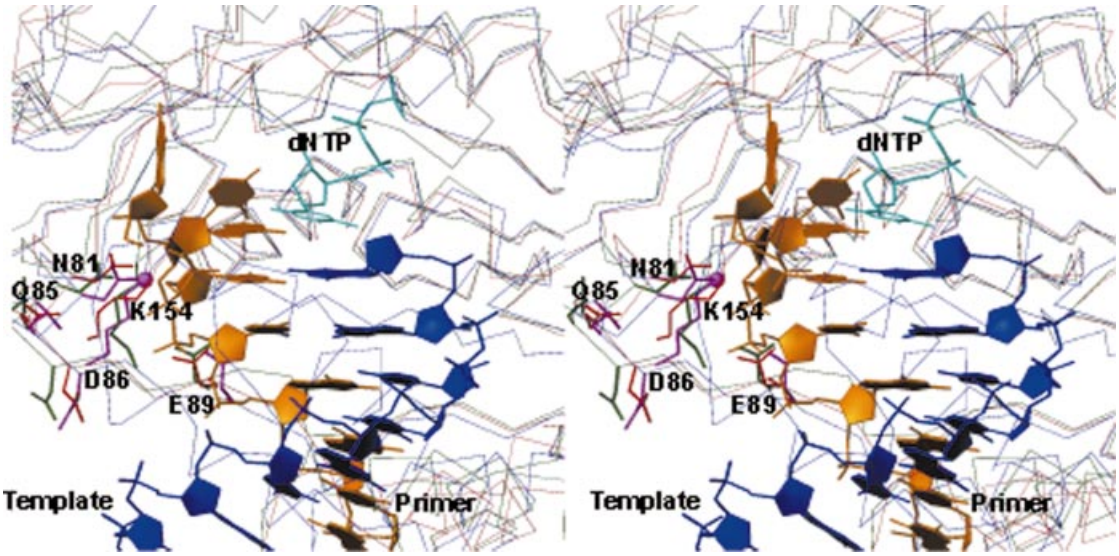


Figure 6. Stereo view of the superimposed crystal structures of HIV-1 RT showing the side chain conformations of Lys154 in the apo, binary and ternary complex structure. The 3D structures of HIV-1 RT in apo form, E–TP binary complex and E–TP–dNTP ternary complex were obtained from PDB files 1QE1, 1HYS and 1RTD, respectively. Superposition of the above three forms of the crystal structures, showing the relative orientation of Lys154, was carried out by fitting the 38 Cα atoms from the palm subdomain from β-strands 6, 9 and 10. The template–primer shown is from the ternary complex structure of HIV-1 RT (PDB file 1RTD). The Cα trace (thin line) as well as the side chains of surrounding residues in apo, binary and ternary complex structures are shown in magenta, red and green, respectively. The primer is colored in magenta, while the template is shown in blue. As seen in the structure, the side chain of Lys154 displays different interactions due to subtle changes in its conformation in the three crystal structures.

residue in the VLPQGWK motif is Gln151, which is also located on the β8–αE loop and has been studied extensively. This residue interacts with both the template and the incoming dNTP substrate (10,11,34) and has been implicated in conferring drug sensitivity to HIV (6–8). In the 3D crystal structure of the RT–DNA binary complex (23,24), Lys154 does not make any direct or indirect contact with the bound DNA and therefore may not be involved in the formation of E–TP binary complex. Our gel shift analysis data confirmed this premise as none of the Lys154 mutant derivatives showed any reduction in their DNA binding affinity. However, their polymerase activity varied significantly depending upon the nature of their side chain at position 154. The hydrophobic side chain of alanine or isoleucine at this position allowed the enzyme to retain 50–70% of the wild-type activity, while mutants carrying negatively charged side chains were significantly impaired in their polymerase activity, retaining only 9–29% of the wild-type activity (Table 1). These results suggest that Lys154 may have a pertinent role only after the formation of E–TP binary complexes. This premise was examined by monitoring the next step of dNTP binding to E–TP binary complexes of individual Lys154 mutant derivatives. Since proper positioning of the TP during the transition of binary complex to ternary complex is a prelude to the formation of stable and productive ternary complexes, a subtle change in the position of the TP will greatly impact the stability of the E–TP–dNTP ternary complex. It was observed that all the mutant derivatives of Lys154, except K154R, are significantly affected in their ability to form stable ternary complexes (Fig. 2). This effect was more pronounced with mutant derivatives carrying a negatively charged side chain (K154D and K154E) at this position. The apparent dNTP binding affinity was significantly decreased in the case of all non-conservative mutants of Lys154, suggesting the possibility of an improper positioning of TP at their active site (Fig. 3). This implies that Lys154 may specifically be involved only after the initial TP binding step.

To understand these interactions at the structural level, we examined the apo, binary and ternary complex crystal structures of HIV-1 RT. It was observed that Lys154 adapts distinctly different conformations in these structures (Fig. 6). In the ternary complex, the side chain of Lys154 (green) interacts with the phosphate atom of the penultimate template nucleotide in the duplex region from the primer terminus. It is also within interacting distance of Glu89, which contacts the sugar moiety of the same template nucleotide. These interactions of Lys154 are not seen in the crystal structure of HIV-1 RT bound to TP (23,35). Instead, in the E–TP binary complex, Lys154 (red) forms a salt bridge with the side chain of Asp86 and hydrogen-bonds with the main chain oxygen of Asp86 and Gln85, both of which are away from the TP-binding track and are located at the subunit interface. Similar interactions of Lys154 (magenta) are also seen in the apo-crystal structure (24) of HIV-1 RT. Our results with the mutant derivatives of Lys154 pertaining to the formation of E–TP binary and E–TP–dNTP ternary complexes are in agreement with the interactions seen in the corresponding crystal structures. These observations suggest that Lys154 participates in the formation of a closed ternary complex by maintaining proper positioning of the TP either by itself or in conjunction with Glu89. A drastic reduction in the stable ternary complex formation observed with K154D and K154E mutants could be a direct consequence of repulsion and loss of interaction with the side chain of Glu89.

A puzzling aspect of these results is that all the mutant derivatives of Lys154 are completely resistant to 3TCTP, a nucleoside inhibitor of HIV-1 RT. In the ternary complex, the conformation of the side chain of Lys154 is within interacting

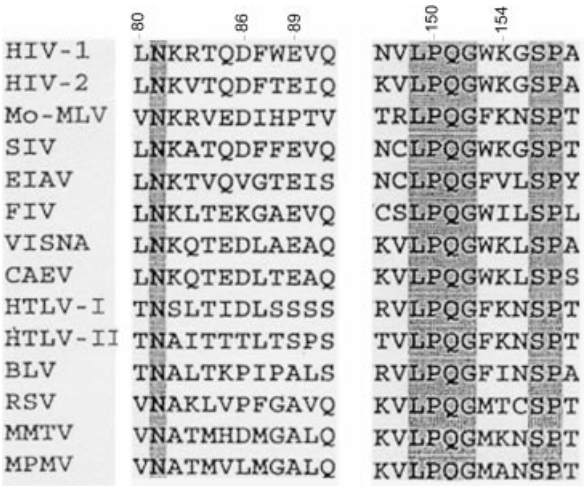


Figure 7. Comparison of amino acid sequences of HIV-1 RT and other retroviral RTs. Invariant amino acids are shaded. The numbers represent the position of corresponding amino acids in HIV-1 RT. Mo-MLV, Moloney murine leukemia virus; SIV, simian immunodeficiency virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; VISNA, visna virus; CAEV, caprine arthritis encephalitis virus; HTLV, human T-cell leukemia virus; BLV, bovine leukemia virus; RSV, Rous sarcoma virus; MMTV, mouse mammary tumor virus; MPMV, Mason–Pfitzer monkey virus.

distance from Glu89, in addition to interacting with the phosphate atom of the penultimate template nucleotide in the duplex region of DNA (Fig. 6, green). Salt bridge interactions may occur between amino acid side chains with opposite charges when they are within 4 Å apart (36,37). In the crystal structure of the ternary complex, the distance between N2 (Lys154) and OE2 (Glu89) is 3.65 Å, suggesting the possibility of such an interaction between them. A systematic conformation search of the side chain of Lys154 and Glu89 revealed that in one of the conformations, both the side chains face each other. Since salt bridge interactions are not local but long-range interactions, such interaction may be significant in stabilizing the side chain of Glu89. Since Glu89 mutants have been shown to confer resistance to ddNTPs (28,38), we postulate that the 3TCTP resistance phenotype of Lys154 mutants may be via Glu89 due to loss of interaction between their side chains.

Why is a conservative K154R mutant not sensitive to 3TCTP? Similarly to Lys154, it should also be able to interact with and stabilize the side chain of Glu89. In order to explain the observed 3TCTP-resistant phenotype of the K154R mutant, we carried out mutant modeling of Lys154 in the ternary complex crystal structure using the Look Modeling package. Unlike Lys154, which assumes two distinct orientations in the binary and ternary complexes, the side chain conformation of Arg154 is similar in both the binary and ternary complexes. In our modeling studies, the guanidino group of Arg154 directly interacts with the phosphate atom of the penultimate template base in the duplex region and also makes a hydrogen bond with Asp86 in both binary and ternary complexes. However, unlike Lys154, it does not seem to interact with and stabilize the side chain of Glu89, which may be one of the factors contributing to its 3TCTP-resistant phenotype.

Amino acid sequence alignment of RTs from different retroviruses shows that Lys154 is fairly well conserved (Fig. 7). In RTs from HIV-1, HIV-2, simian immunodeficiency virus, visna virus, caprine arthritis encephalitis virus, Moloney murine leukemia virus, human T-cell leukemia virus I and II, and mouse mammary tumor virus, Lys154 is absolutely conserved, whereas in others it is replaced by smaller side chain such as valine, isoleucine, threonine and alanine. Interestingly, of the two residues proposed to be involved in salt bridge interactions with Lys154 (Asp86 and Glu89), Asp86 is always present along with Lys154, except in HTLV-II which has threonine in place of aspartate. In contrast, the concurrent presence of Glu89 is observed only in a few retroviral RTs including HIV-1. Thus, the salt bridge formation property between Lys154, Asp86 and Glu89 seems rather confined to this group of RTs. This phenomenon appears to have functional significance since in HIV-1 RT, the E89G mutation has been noted to confer nucleoside drug-resistant phenotypes (28,38) while in other RTs which do not have glutamate at the equivalent position, natural nucleoside resistance is observed.

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