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Lys66 residue as a determinant of high mismatch extension and misinsertion rates of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) †

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SUMMARY

A major factor contributing to the high mutation rate of HIV-1 RT is its high propensity for misincorporation. Misincorporation requires both dNTP misinsertion and the subsequent extension of the mismatched terminus thus formed. We hypothesized that Lys66 is a determinant of mismatch extension based on its position near the primer terminus. This hypothesis was tested by steady-state kinetic studies using wild type HIV-1 RT and four Lys66 substitution mutants: Lys66Arg, Lys66Ala, Lys66Asn, and Lys66Thr. Efficiency of mismatch extension (f_{ext}) was reduced for all mutants, with Lys66Ala, Lys66Asn, and Lys66Thr showing 4 to 6-fold reduction compared to wild type. Surprisingly, the non-conservative substitutions also lead to large decreases in misinsertion efficiency (f_{ins}) ranging from as low as 3-fold to values much higher than 23-fold. Thus, Lys66Arg mutant was akin to wild type, while all non-conservative mutants displayed significantly decreased efficiency for both events. Our results suggest that Lys66, much like Lys65, is a determinant of both dNTP misinsertion and mismatch extension efficiency. While Lys65 is known to contact γ -phosphate of incoming dNTP, the Lys 66 side chain is in the vicinity of primer terminus. However, our results suggest that both residues have similar influence on dNTP misinsertion and mispair extension efficiencies of HIV-1 RT. When we tested the mutants for susceptibility to selected nucleoside analog and non-nucleoside analog drugs, similar to Lys65Arg, the Lys66Ala and Lys66Asn mutants displayed mild resistance to nucleoside analog drug AZTTP.

Keywords

HIV-1; reverse transcriptase; polymerase fidelity; misinsertion efficiency; mismatch extension efficiency

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SUPPORTING INFORMATION Supporting Information Available. Figure S1. Representative Surface Plasmon Resonance binding sensorgrams showing binding of WT and Lys66Thr RT to DNA-DNA matched (A) and mismatched (B) template-primers. Figure S2. Qualitative gel showing AZT and d4T drug susceptibility profiles of WT, Lys66 and Lys65 substitution mutants. Figure S3. Qualitative gel showing TFV and EFV drug susceptibility profiles of WT, Lys66 and Lys65 substitution mutants. Table S1. Kinetic parameters of the interaction of WT, Lys66Arg, Lys66Ala, Lys66Asn and Lys66Thr with matched (A) and mismatched (B) template-primers.

Enzymes: EC 2.7.7.49, EC 2.7.7.7, EC3 .1.26.13

INTRODUCTION

HIV-1 encodes reverse transcriptase (RT), a multi-functional enzyme responsible for converting the single-stranded viral genomic RNA to a double-stranded DNA form, a process that is essential for viral replication and establishing infection. Although it has many structural similarities to other high fidelity replicative DNA polymerases, such as a structure resembling a right hand, with fingers, palm, and thumb subdomains [1] and substrate-induced structural changes [2-4], HIV-1 RT is quite error prone with an error rate in the order of 3×10^{-5} mutations/nt/cycle [5]. The relatively high error rate of HIV-1 RT in conjunction with the rapid replication of the virus in vivo leads to high genetic variation of the HIV-1 genome in patients; ultimately resulting in immune escape mutants and drug-resistant variants [6,7].

Several studies of HIV-1 RT have established a basic model for nucleotide incorporation [3,8,9]. The general mechanism of the polymerization process begins with the binding of the template-primer (t/p) to the enzyme (E) to form a binary complex (E: t/p). The incoming dNTP is subsequently bound in a loose “open” tertiary complex (E: t/p: dNTP), which undergoes a conformational change into a “closed” tertiary complex ready to undergo the chemical step (E': t/p: dNTP) [3]. A conformational change has been identified as the rate-limiting step of dNTP incorporation [8,9]. However, kinetic studies demonstrate that the extent to which the conformational change is rate limiting may differ [10], and it has been specifically suggested that in mismatch extension, conformational change may no longer be the rate limiting step [11]. During the conformational change, the p66 fingers subdomain closes down on the incoming dNTP, helping to precisely align the 3'-OH of the primer, the α -phosphate of the dNTP, and the polymerase active site for chemistry [12]. Upon formation of the new phosphodiester bond, pyrophosphate (PPi) is released leaving the t/p extended by one nucleotide, and the RT bound such that the primer 3'-end occludes the nucleotide-binding (N) site (Figure 1) [13,14]. This complex, referred to as a pre-translocation complex can be stabilized *in vitro* by the presence of the PPi analog, foscarnet. In order to extend the primer with an additional nucleotide, the RT must translocate along the t/p and reposition itself such that the primer 3'-end resides in the priming (P) site [13,14], leaving the N site [13,14] free for nucleotide binding. This post-translocation complex (Figure 1) can be stabilized in vitro using a 3'-chain-terminated primer in the presence of the next complementary nucleotide.

Discrimination to reduce error can occur either at nucleotide insertion or following insertion, by promoting removal of an incorrect nucleotide. It is well known that HIV-1 RT does not possess proofreading capability [15], thus, error prevention must occur at the point of nucleotide incorporation. The incorporation of an incorrect nucleotide in a DNA strand is the result of two processes; namely misinsertion and mismatch extension. Misinsertion is the initial insertion of a non-complementary nucleotide; mismatch extension is the subsequent extension of the mismatched terminus thus formed. HIV-1 RT can efficiently perform both misinsertion and mismatch extension events [16,17].

Examination of the HIV-1 RT-DNA-dNTP ternary structure implicates a number of amino acid residues in the formation of the dNTP-binding pocket [12]. Many of the residues lining the dNTP-binding pocket are situated in the highly flexible β 3- β 4 loop of the fingers subdomain of the p66 subunit (Figure 2). The β 3- β 4 loop plays a critical role in orienting and stabilizing the substrate for catalysis [18,19]. This loop encompasses residues 60-75, which includes Lys 70, Lys74, Lys65, Arg72 and Asp67. The three residues of the loop Arg72, Asp67 and Lys65 make contact with the α , β , and γ -phosphates of the incoming nucleotide respectively [12,20-22].

Several residues in the $\beta 3$ - $\beta 4$ loop are implicated in drug resistance as primary mutations, but only a few including Leu74, Val75 and Lys65 have been identified as playing a role in misinsertion and mismatch extension. Leu74Val mutations have shown up to a 6-fold reduction in misinsertion frequency, and a 15-26-fold reduction in mismatch extension frequency [23]. Matamoros et al. showed that the mutation Val75Ile increased the mismatch extension fidelity 3-fold while showing no apparent effect on misinsertion fidelity. The authors show that the increased fidelity is due to a decrease in nucleotide affinity in the presence of mismatched t/ps [24]. Early work from our lab investigating the influence of Lys65Arg mutations on overall mutation rates and error specificity indicated a qualitative defect in both misinsertion and mismatch extension as seen by a reduction in extension products on the gel based minus dNTP assay [18].

Further investigation into the influence of mutations at Lys65 show that a Lys65Arg mutation conserves polymerase activity similar to wild type, whereas a non-conservative substitution (i.e. Lys65Ala) results in reduced RT catalytic efficiency (k_{cat}/K_m) [25] as well as decreased affinity for dNTP [26]. The increased fidelity of the Lys65Ala mutation appears to be directly due to a cumulative effect of a reduction in k_{pol} and increased dNTP K_d in both misinsertion and mismatch extension [26]. Although mutagenesis studies have investigated the role of a number of $\beta 3$ - $\beta 4$ residues in fidelity [25,27], a role for Lys66 in RT fidelity has not been investigated so far.

In the primary sequence of RT, Lys66 is in a cluster of three lysines in the fingers subdomain. Unlike Lys64, which points away from the polymerase active site in the three dimensional structure of a ternary complex Lys66 lies adjacent to the phosphate of the penultimate primer base/nucleotide (Figure 2) [12]. This conformation is strictly seen in the presence of template-primer and dNTP, while in the absence of bound dNTP, the fingers subdomain is further from the active site [28,29]. Due to its location in the vicinity of the dNTP-binding pocket, we hypothesized that Lys66 could play a role in mismatch extension. The significance of this residue is further supported by its broad conservation among reverse transcriptases of retroviruses and other retroelements [30].

Likewise, Lys66 is conserved in patient isolates. Mutations identified among patient isolates at this position include: Lys66Arg, Lys66Asn, Lys66Thr, and Lys66Glu. These mutations are not known to confer drug-resistance but rather occur at low frequency in both drug naïve and drug-resistant isolates [31,32].

In the present study, we have investigated the potential role of Lys66 residue in RT fidelity by creating substitutions Lys66Arg, Lys66Ala, Lys66Asn, and Lys66Thr and testing them for both misinsertion and mismatch extension efficiencies. Our results show not only that the Lys66 substitutions display decreased mismatch extension efficiency, but are also poor at dNTP misinsertion. In addition, drug susceptibility tests indicated that substitutions at position 66 confer low resistance to nucleotide reverse transcriptase inhibitor (NRTIs), AZTTP. Furthermore, using site-specific hydroxyl radical footprinting, we show that these substitutions do not alter the positioning of HIV-RT on the t/p but rather, similarly to the wild-type, their positioning on the t/p substrate is affected in the presence of mismatched primer terminus.

RESULTS

Mismatch extension Fidelity

In order to examine the effect of the substitutions at Lys66 on the ability of RT to extend from a mismatched terminus, single nucleotide incorporation assays were performed. Lys66Ala was used as a non-conservative substitution in addition to the three substitutions

(Arg, Asn and Thr) observed in clinical isolates [31,32]. Recombinant HIV-1 RT WT and Lys66 substitution mutants were used to extend either a matched (T:A) or mismatched (G:A) terminus by incorporation of the next complementary nucleotide (dATP). A broad range of nucleotide concentrations was tested (Figure 3). Steady-state kinetic parameters were calculated using this single nucleotide insertion assay, with reaction conditions adjusted such that less than 30% primer extension was observed [33].

When studying extension from matched terminus, the catalytic efficiency (k_{cat}/K_m) of extension was comparable for the WT and the Lys66Arg enzymes (Table 1A). While the non-conservative mutants (Lys66Ala, Lys66Asn and Lys66Thr) exhibited slightly lower extension efficiencies (up to 2-fold), the data suggests that substitutions at position 66 have a minimal effect on the efficiency of extension from a matched terminus (Table 1A). However, the presence of a mismatch (G:A) at the primer terminus resulted in a greater decrease in k_{cat}/K_m especially for the non-conservative mutants, which showed reductions of up to 12-fold when compared to WT (Table 1B). Thus, Lys66 substitutions hamper the innate ability of the enzyme to extend from a mismatched terminus. This effect appears to be due to both an increase in substrate K_m and a decrease in the overall k_{cat} of the enzyme (Table 1B).

In order to better elucidate the overall effect on mismatch extension fidelity, these data were expressed as mismatch extension efficiencies (f_{ext}), defined as $[(k_{cat}/K_m)_{incorrect} / (k_{cat}/K_m)_{correct}]$. The Lys66Arg substitution displayed a mismatch extension efficiency that was comparable to WT, whereas the reduction in f_{ext} for the non-conservative mutants (Lys66Ala, Lys66Asn, Lys66Thr) was 4-6-fold (Table 1B).

Misinsertion Fidelity

The efficiency of insertion of each nucleotide was measured using single-nucleotide insertion assays. Figure 4 illustrates the results from an assay in which primer was extended in the presence of the correct (dATP) or each incorrect nucleotide by the wild type and mutant RTs.

All of the enzymes tested (WT and Lys66 substitution mutants) efficiently inserted the correct nucleotide with similar efficiencies with the most impaired RT being Lys66Asn, leading to a ~3 times decrease in k_{cat}/K_m . (Figure 4). Under conditions where dGTP is inserted opposite a template T resulting in a G:T mispair, all of the enzymes show similar efficiencies of misinsertion except Lys66Ala. The Lys66Ala effect is dramatic in this instance resulting in a 23-fold decrease in k_{cat}/K_m . Interestingly, WT, Lys66Arg, and Lys66Asn display increased G:T mispair catalytic efficiency (k_{cat}/K_m) compared to A:T. These experiments were repeated using a template with a different sequence, in which an additional nucleotide is inserted immediately 5' to the repetitive CCCC (5'CCCC3' to 5'ACCCC3') sequence. The addition of the insulating nucleotide allowed for the same percentage of extension forming a G:T mismatch suggesting that the mismatch was formed by misincorporation and not by template slippage. Additionally, insertion of the nucleotide abolished +2, +3, +4 extension products, which are a result of canonical G:C extension following G:T mismatch formation in the original template, further suggesting that the observed multiple extension events were most likely due to +1 product elongation and unlikely due to template slippage events (Data not shown). However, it is difficult to rule out the contribution of template slippage entirely. Under conditions where a C:T mispair was formed, WT and Lys66Arg behaved similarly while the non-conservative mutants were severely impaired with a considerable increase seen in K_m . Unexpectedly, there was a dramatic effect of the substitutions on the ability of the RT to form a T:T mismatch. While WT and Lys66Arg retain the ability to misinsert dTTP complementary to a template thymidine base, the non-conservative substitution mutants are inept, resulting in a 6-8 fold

reduction in misinsertion efficiency as compared to WT. Although the mutants show an effect on C:T and T:T mispairs, this reaction is already a lower efficiency reaction as demonstrated by the low k_{cat}/K_m values. For example, the k_{cat}/K_m values for incorporation of A opposite T and C opposite T were 0.200 ± 0.031 and 0.033 ± 0.013 for WT RT and 0.137 ± 0.030 and 0.005 ± 0.0043 for Lys66Ala mutant respectively. As previously demonstrated for RT and many other polymerases, there is a preference for certain mispairs: G:T mispairs being more favorable than C:T or T:T mispairs. This preference is unaffected by the Lys66 substitutions. Representation of this data in terms of misinsertion efficiencies (f_{ins}) demonstrated that WT and Lys66Arg had a similar misinsertion fidelity, while the nonconservative substitutions at position 66 caused enhanced fidelity with an up to 23-fold reduction in misinsertion efficiency (Table 2).

Site-specific Hydroxyl Radical Footprinting

Differential positioning of the RT on the t/p can be a potential explanation for the different fidelity exhibited by the Lys66 substituted enzymes on matched and mismatched primers. We have previously shown that a mismatch at the primer 3'-terminus is sufficient to cause the repositioning of the RT on a t/p [26]. To investigate whether Lys66 substitutions also cause such repositioning upon encountering a mismatched primer terminus, site-specific footprinting was carried out with all five enzymes. Previously, Gotte et al. have shown that the addition of Fe^{2+} to an extension reaction results in the generation of hydroxyl radicals in the vicinity of the RNaseH domain of HIV-1 RT. These radicals result in cleavage of the template in a site-specific manner and thus allow one to deduce the positioning of the polymerase active site with respect to the primer terminus.

In order to determine the relative positions of the RT on the template, we used a ladder generated by potassium permanganate cleavage, compared to the positions of the site-specific cleavage with five different primers (A, B, C, D, E) (Figure 5A). Primer B and D are used to determine the position of the RT on a matched terminus. In the presence of foscarnet, cleavage occurs at position -18, corresponding to pre-translocation (Figure 5A, arrow a). In the presence of the next complementary nucleotide, and utilizing a primer terminated with a ddNMP to prevent further extension, cleavage occurs at -17, corresponding to post-translocation (Figure 5A, arrow b). Primers C and E are used to determine the position of RT on a mismatched terminus. In the presence of the next complementary nucleotide and a ddNMP-terminated primer, cleavage occurs at -19 (Figure 5A, arrow c). This corresponds to a position where cleavage occurred on a matched t/p shortened by a single nucleotide at the 3'-end as shown in primer pair A.

To characterize the positioning of the WT and the Lys66 substitution mutants on the matched and mismatched template-primers, we performed our reactions in the presence of primer B or C respectively. Experimental control reactions show that template cleavage in these reactions is contingent on the presence of both the enzyme and Fe^{2+} (Figure 5B) as no cleavage products are observed in their absence. These data were quantified by densitometry and are represented as percentage of enzyme present at position -17, -18 or -19 (Figure 5C). Under conditions where the enzyme encounters a matched primer terminus, ~60% of the enzymes is in the post-translocation (-17) position, with ~30% at the (-18) position and ~10% at the (-19) position. These results are similar for WT and the substitution mutants. However, the situation is quite different when the enzymes encounter a mismatched terminus. Whereas, previously the majority of the enzyme was trapped at the post-translocation (-17) position, now an equal amount is positioned at an additional pre-translocation state, which we have termed the -2 state (corresponding to the polymerase active site) (Figure 1 and 5).

Template-primer binding kinetics

The catalytic and binding properties of the purified wild type (WT) and the mutant enzymes were characterized. To measure the kinetics of binding between the enzyme and the t/p substrate, we performed Surface Plasmon Resonance (SPR) experiments using the wild-type and four mutant RTs (Lys66Arg, Lys66Ala, Lys66Asn, and Lys66Thr) with matched or mismatched template/primers. The purified enzymes were allowed to interact with the immobilized t/p at concentrations ranging from 0nM-200nM to generate binding sensorgrams such as those shown in supplementary Figure S1.

The WT enzyme exhibits a high affinity for both matched and mismatched t/ps with equilibrium dissociation constants (K_D) of 1nM and 1.94nM respectively (supplementary Table S1). The dissociation constant on matched t/p is well within the range of previously published K_D s [9,16,34]. Interestingly, a conservative substitution at position 66 (Lys66Arg) affected the affinity of the enzyme for the matched t/p (6nM) and mismatched (8nM) 6- and 8-fold respectively, while a non-conservative Lys66Ala alteration had minimal effects. Under conditions where the enzyme contains a Lys66Asn or Lys66Thr substitution at position 66, the affinity is decreased 5-49 fold for a matched and 10-32 fold for a mismatched t/p.

Drug Resistance Profiles

In an attempt to study the role of Lys66 in drug susceptibility, qualitative gels, and quantitative filter-based drug resistance assays were performed using the various enzymes (WT, Lys66Arg, Lys66Ala, Lys66Asn, Lys66Thr, Lys65Ala and Lys65Arg) in the presence of 3 nucleoside reverse transcriptase inhibitors (NRTIs) and 1 non-nucleoside reverse transcriptase inhibitors (NNRTIs). The resistance profiles of the mutants to 3'-Azido-3'-Deoxythymidine (AZTTP), and Efavirenz were specifically examined because the small percentage of mutations seen at position 66 occur in patients on regimens which include the aforementioned inhibitors [31,32].

Recombinant HIV-1 RT WT, Lys66 and Lys65 substitution mutants were used to extend a long template-primer in the presence of all four dNTPs and a range of inhibitor concentrations (Supplementary Figures S2 and S3). In the presence of 3'-Azido-3'-Deoxythymidine-5'-Triphosphate (AZTTP), Lys66Arg and WT appear to have a similar resistance profile to the WT enzyme, as less full-length product is seen even at 1 μ M AZTTP (Supplementary Figures S2). In contrast, the non-conservative substitution mutants specifically Lys66Ala and Lys66Asn allow for more full length product formation even at the highest concentration of AZTTP (4 μ M) (Supplementary Figures S2). The qualitative data is supported by drug susceptibility measurements (IC_{50} s) obtained from our quantitative filter-based assays (Table 3). The data from these experiments shows that no difference in susceptibility is seen between the WT and Lys66Arg RTs (185nM vs. 250nM), while Lys66Ala and Lys66Asn exhibit IC_{50} s that are twice as high (559 and 563 respectively) resulting in a 3-fold decrease in susceptibility (Table 3).

Extension in the presence of 2',3'-Didehydro-3'-deoxy-thymidine-5'-triphosphate (d4TTP) shows once again that Lys66Arg allows for full length product extension similar to the WT enzyme (Supplementary Figures S2). However, in this instance the non-conservative mutants show an intermediate resistance profile when compared to WT and Lys65Arg (Supplementary Figures S2). This is confirmed with the quantitative assays, where the IC_{50} s of the non-conservation mutants (7.91, 8.64 and 6.84 μ M) show ~2-fold decrease in susceptibility, while the Lys65 mutants show a 3-fold decrease as compared to WT (Table 3).

Interestingly, in the presence of Tenofovir diphosphate (TFV-DP), even though the three non-conservative Lys66 substitutions exhibit slightly higher levels of full length product at the highest inhibitor concentration as compared to WT or Lys66Arg (Supplementary Figures S3), there does not appear to be a large difference in the IC_{50} s obtained from the filter-based assays (Table 3). A larger difference is seen between the Lys65 substitution mutants and the rest of the enzymes, with the Lys65 mutants showing 2 to 6 fold more resistance to TFV-DP (Table 3).

Lastly, no apparent differences are observed for the WT, the Lys66 substitution mutants or the two Lys65 mutants in the presence of Efavirenz (EFV) with the exception of Lys65Ala. Analysis of the IC_{50} values shows that the Lys65Ala mutant is the most susceptible to the inhibitor with an IC_{50} of 960nM (2-fold increase in susceptibility), while the Lys66 substitution mutants are more similar to the Lys65Arg mutant (Table 3).

DISCUSSION

In this report, we have shown that Lys66, located in the $\beta 3$ - $\beta 4$ loop of HIV-1 RT, contributes to mismatch extension fidelity of HIV-1 RT. Perturbing this residue by mutation decreases mismatch extension efficiency. Interestingly, in addition to mismatch extension fidelity, Lys66 also appears to be important for dNTP insertion fidelity. We also show that substitutions at this position have little effect on the positioning of the enzyme on the substrate t/p.

In the event that a mispair is created, the enzyme may extend past the mispair, dissociate from the substrate or remain bound as an inactive complex, in which case the enzyme would no longer contribute to the rate of the reaction. Analysis of the t/p binding kinetic parameters k_a (association rate) and k_d (dissociation rate) shows that t/p binding does not correlate with mismatch discrimination. A “bulkier” (Arg) or polar uncharged (Asn,Thr) residue shows decreased k_a and increased k_d for a net slight decrease in affinity (5-50 times increase in K_D). Furthermore, the effect on the K_D for matched and mismatched t/p follow similar trends such that no significant differences are seen in affinity of the RT for a matched or mismatched t/p. As seen before, a mismatch has little effect on WT t/p binding [32] [27]. Thus, WT RT does not discriminate against a mismatch based on t/p binding and the Lys66 mutations that do increase discrimination do not do so through binding. Although substitutions at position 66 do alter template/primer binding, they do not discriminate against the mismatched terminus; rather, they lead to an overall reduction in affinity for the DNA substrate. This was unexpected, as given the proximity of the Lys66 residue to the primer strand, we had hypothesized that discrimination would likely be through altered interaction with the template primer. Furthermore, substituting the Lys66 with Arg, Ala, Asn and Thr residues into the structure of a ternary complex of HIV-1 RT showed that non-conservative substitutions caused a greater distortion in the active site geometry than the conservative substitutions, and particularly change the relative position and proximity of the side-chain of residue 66 and the phosphate on the penultimate nucleotide (Figure 6).

Data from our single nucleotide extension assays show that while WT and Lys66Arg are efficient in extending from a mismatched primer terminus, the non-conservative mutants show 5-6-fold reduction in mismatch extension efficiency (f_{ext}). Analysis of catalytic efficiency (f_{ext}) allows one to delineate the influence of the substitution specifically on the mismatch extension, as the role of the mutation in normal catalysis is compensated for. Hence, this reduction in efficiency implicates Lys66 explicitly in mismatch extension fidelity.

The Lys66Arg substitution did not alter the misinsertion fidelity, while the non-conservative mutants showed a reduced ability to misinsert. G:T mispairs are purine:pyrimidine mispairs and are more efficiently formed, compared to C:T or T:T mispairs, as they are similar to A:T base pairs. All the enzymes were relatively efficient in G:T mispair formation while the C:T and T:T were less tolerated.

Taken together, the data suggests that the Lys66 residue not only facilitates the rather high wild type levels of mismatch extensions but also misinsertions. It is likely that the effects on misinsertion and mispair extension are due to local, active site distortions, demonstrated by the increases in distance of the residue side chain to the primer (Figure 6) for the substitution mutants.

Although there are subtle, and statistically significant, differences between the positions of the wild-type and non-conservative Lys66 substituted enzymes on the mismatched primer terminus, these differences do not account for the differences in fidelity that were observed. The extent to which the enzyme is found in a -19(-2) additional pre-translocation position differs significantly for WT and the Lys66Ala, Asn and Thr (Figure 5C, Right panel). In the context of a mismatched t/p more of the WT enzyme shifts backwards to a -19 (-2) position compared to the non-conservative mutants.. This suggests that upon encountering an incorrect base pair at the primer 3'-terminus the enzyme could potentially realign its active site by sliding backwards on the template-primer. Likewise there is bias in the percent of complexes found at -17 on the mismatched t/p, with more of the non-conservative mutant complexes found at this position. Interestingly, although the enzymes in complexes at -17 are positioned such that they can readily accept an incoming nucleotide, this increase in post-translocation complexes does not correlate with an increase in extension from a mismatched t/p for Lys66Ala, Asn and Thr. This discrepancy between t/p positioning and mismatch extension efficiency was also described previously for a Lys65Ala mutant [26].

HIV-1 RT has evolved the ability to extend mismatches and misinsertions efficiently. All the amino acid residues that are responsible for this property have not been delineated. For dNTP misinsertion, a number of residues that line the dNTP binding pocket, including Lys65, Thr115 and Met184, have been shown to impact RT fidelity [26,35,36]. Residues affecting the fidelity of RT are not limited to those situated in the polymerase active site. Several substitutions at residues that do not directly line the active site, but yet impinge upon it via interaction with template or primer, such as Met230Ile (in the primer grip) [37] and Glu89Gly (at -2 position on the template) [23,38] have also been shown to bring about increases in fidelity. Similarly, as seen from our data, substitutions at the Lys66 residue, which appears poised to contact the penultimate nucleotide at the primer 3'-terminus led to enhanced fidelity of misinsertion. In addition to the impact on mispair extension fidelity that we predicted based on Lys66 proximity to primer terminus, the Lys66 substitutions also enhanced the fidelity of misinsertion.

Previously, through substitution of Lys65, which contacts the gamma-phosphate of the incoming nucleotide, it was demonstrated that this residue also has a role in both aspects of fidelity. Although the Lys65 and Lys66 residues are adjacent to each other in the $\beta 3$ - $\beta 4$ loop, the similarity of the effect of substitutions of these residues on fidelity was unexpected, as their orientation as shown in the crystal structure is quite different from each other.

In an attempt to investigate whether the similarity between Lys66 and Lys65 would extend to drug susceptibilities we performed both gel based and filter-based reverse transcriptase assays. In addition, Lys65Arg has also been identified as the primary mutation selected for by Tenofovir (TFV) [40] and has been shown to confer cross-resistance to other inhibitors

including d4T in both cell-based [41] and cell-free assays [42]. Here we showed that the non-conservative Lys66 mutants we examined show phenotypes that are quite different from the WT but not as profound as resistance profiles seen with the Lys65 mutants. While the Lys65Arg mutation has been widely studied in drug resistance, the drug resistance effects of mutations at position 66 have not been tested. It is however of interest that mutations at position 66 (which rarely occur in the virus) lead to decreases in drug susceptibility suggesting that the high conservation of this residue is important for viral fitness. The differences in mutation frequencies observed when substitutions of wild type residue are created at positions 65 and 66 may reflect the degree of resistance conferred by these substitutions; thus, although the Lys65 substitutions have a greater effect on enzymatic activity than the Lys66 substitutions, they also lead to higher levels of drug resistance.

CONCLUSION

The Lys66 substitutions increased the fidelity of misinsertion and mispair extension events by increasing the sensitivity to other factors that disturb active site geometry such as mismatches, rather than by affecting t/p binding. When a mismatched primer terminus or a mispaired dNTP is encountered at the active site, the wild type or Lys66Arg mutant enzymes would efficiently proceed to incorporate these errors, while Ala/Asn/Thr mutants would be inefficient. The data presented show that the Lys66 mutations do not grossly alter the kinetic constants, except when the active site is disrupted either by a wrong nucleotide in the N-site or mismatched primer terminus. Therefore, our results suggest that the HIV-1 RT has evolved the Lys66 residue and maintained it as it continues to evolve, to maintain a high mutation rate as multiple Lys66 substitutions appear to all decrease the efficiency of biochemical events that are necessary for mutation. Our results also suggest that, despite the fact that Lys66 residue orientation at the active site is distinct from that of Lys65, its impact on polymerase fidelity is similar to that of Lys65.

EXPERIMENTAL PROCEDURES

Materials

IPTG, restriction and DNA modification enzymes (T4 polynucleotide kinase, BsmBI) were obtained from New England Biolabs (NEB, Ipswich, MA, USA). Deoxynucleotides (dATP, dGTP, dCTP, and dTTP) were purchased from CLP (Mercury Ultra-pure nucleotides, San Diego, CA, USA). All other chemicals were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA). FPLC columns were obtained from GE Healthcare (Waukesha, WI, USA). Integrated DNA Technologies (IDT, Coralville, IA, USA) synthesized all oligonucleotides.

Generating Site-directed Lys66 Mutants

The Lys66 mutants were constructed using cassette mutagenesis. BsmB I restriction sites flanking codons 49 to 69 were introduced into the pRT expression plasmid by polymerase chain reaction (PCR) amplification as described [43,44]. Following cleavage of this intermediate construct with BsmB I, a double stranded DNA adapter, designed to restore the deleted sequence as well as to include a change in the triplet codon at position 66 (AAA) to either CGC (Lys66Arg), GCG (Lys66Ala), AAC (Lys66Asn) or ACC (Lys66Thr), was ligated into the BsmB I-digested plasmid. The mutations were verified by performing polymerase chain reaction (PCR) and by sequencing in both directions, confirming that only the desired substitutions had been introduced.

Protein Expression and Purification

Mutations were made in the p66 subunit. The p66 and p51 subunits were over-expressed separately in DH5 α , F'IQ. pRIL (codon plus) *E. coli* strain, subsequently combined and the heterodimers purified as published before [19]. The specific RT activity was tested using a Poly (rA) Oligo (dT) activity assay as previously described [45]. All purified enzymes were additionally tested for nuclease contamination.

Single Nucleotide Mismatch Extension Assay

Gel purified oligonucleotides (IDT DNA) 5'-CAGGGTCTCCCGATCCCGGACGAGCCCCCGGT-3' (matched) and 5'-CAGGGTCTCCCGATCCCGGACGAGCCCCCGG-3' (mismatched) were 5'-end labeled with γ -[³²P] using T4 polynucleotide kinase (PNK) and annealed to the template 3'-ACGTCACAGAGGGCTAGGGCCTGCTCGGGGGCCATCCCC-5'. Extension from either t/p pair was performed in the presence of 10nM RT, 100nM primer-template, 50mM Tris-HCl (pH 8.0), 50mM KCl, 6mM MgCl₂, 1mM DTT and 0.1 mg/ml BSA and varying concentrations of dATP (the next complementary nucleotide). The concentrations used were 10, 20, 100, 200 and 1000nM for matched t/p and 2, 10, 50, 200 and 800 μ M for the mismatched t/p. The reactions were allowed to proceed for 10 minutes (allowing for no more than 30% substrate utilization) and stopped with a mixture of 95% formamide, 15mM EDTA, and 0.2mg/ml bromophenol blue. The products were analyzed on a 12% denaturing polyacrylamide gel. The data are fit to a Michaelis-Menten equation in GraphPad Prism to generate kinetic constants. Only data points that did not exceed 30% substrate utilization were used for the determination of the kinetic constants.

Single Nucleotide Misinsertion Assays

Gel purified oligonucleotides (IDT DNA) 5'-CAGGGTCTCCCGATCCCGGACGAGCCCCCGGT-3' (matched) were 5'-end labeled with γ -[³²P] using T4 polynucleotide kinase (PNK) and annealed to the template (3'-ACGTCACAGAGGGCTAGGGCCTGCTCGGGGGCCATCCCC-5') by heating to 80°C followed by slow cooling. Extension was performed in the presence of 10nM RT, 100nM matched template-primer 50mM Tris-HCl (pH 8.0), 50mM KCl, 6mM MgCl₂, 1mM DTT and 0.1 mg/ml BSA. The reactions were either run in the presence of the correct (dATP) or incorrect (dGTP, dCTP, or dTTP) nucleotide. The concentrations used were 10, 100, 200, 750, 1000, 2000, and 3000 μ M of nucleotide. The reactions were allowed to proceed for 2, 5 or 10 minutes (allowing for no more than 30% substrate utilization). The products were treated under the same conditions as mentioned above for mispair extension assays. Extension products are quantitated by including +1, +2 and +3 bands as a percentage of the total in the lane using Image Quant. The data are fit to a Michaelis-Menten equation in GraphPad Prism to generate kinetic constants. Only data points that did not exceed 30% substrate utilization were used for the determination of the kinetic constants.

Site-specific Hydroxyl Radical Footprinting

The positioning of the RT on the t/p was detected by a technique initially described by Gotte et al. [46], and performed as previously described [26]. Briefly, 2.5nM of t/p was incubated for 5 minutes in a reaction mix containing 40nM enzyme, 50mM Tris-HCl (pH 8.0), 50mM KCl, 6mM MgCl₂, 5mM DTT and 0.1 mg/ml BSA. The reactions were subsequently incubated in the presence of foscarnet or dATP for 5 minutes, after which, 33 μ M Fe(NH₄)₂ was added to initiate the cleavage reaction. All incubations were performed at 37°C. The cleavage reactions were stopped after 2 minutes using a stop mix of 95% formamide, 15mM EDTA, and 0.2mg/ml bromophenol blue. The products were separated on a 15% denaturing polyacrylamide gel.

Template-Primer Binding (dissociation constant experiments)

Binding experiments for each of the enzymes with the matched and mismatched t/p was performed on a Biacore 3000 (GE Healthcare). Briefly, 500nM of biotinylated template was annealed to 2.5 μ M oligonucleotide. The annealing reactions were allowed to proceed for 3 minutes at 80°C, and then cooled to below 30°C. The streptavidin-pretreated carboxymethylated dextran sensor surface (Biacore SA chip) was pre-conditioned with three consecutive 1-minute injections of a solution containing 1M NaCl. Different concentrations (0, 0.06, 0.08, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 12, 20, 50, 100, 120, 200nM) of RT were injected onto the matched (ggt) or mismatched (ggg) t/p. The binding of RT was detected as changes in the response units. A blank of running buffer alone was included to control for background readings. Analysis was performed using the BIAevaluation program. The data were fit to a 1:1 Langmuir model where analyte A binds to analyte B to form complex AB with the rate of AB formation measured as an association rate constant (k_a) and the disassembly of AB measured as the off-rate constant (k_d).

Gel Based Resistance Assays

Gel purified oligonucleotide (Invitrogen/Life Technologies, Grand Island, NY, USA) VP229: 5'-CGC TTT CAG GTC CCT GTT CGG GCG CCC AC-3' was 5'-end labeled with γ -[³²P] using T4 polynucleotide kinase (PNK) and annealed to the template PBS_A: 5'-TTT AGT CAG TGT GGA AAA TCT CTA GCA GTG GGC GCC CGA ACA GGG ACC TGA AAG CG -3' by heating to 80°C followed by slow cooling. Extension reactions were performed in the presence of 1nM γ -[³²P] 5'-end-labeled template/primer, 10nM of enzyme, 50mM Tris-HCl (pH 8.0), 50mM KCl, 6mM MgCl₂, 1mM DTT, 0.1 mg/ml BSA, 50 μ M of each nucleotide not analogous to the inhibitor, 20 μ M of the analogous nucleotide and 0, 1 μ M, and 4 μ M 3'-Azido-3'-Deoxythymidine-5'-Triphosphate (AZTTP), 2',3'-Didehydro-3'-deoxy-thymidine-5'-triphosphate (d4TTP) and Tenofovir diphosphate (TFV-DP), 32nM and 20 μ M Efavirenz (EFV). The reactions were run for 10 minutes at 37°C and stopped with a solution containing 95% formamide, 15mM EDTA, and 0.2mg/ml bromophenol blue. The products were separated on an 8% denaturing polyacrylamide gel.

Filter-based Reverse Transcriptase Assay

Gel purified oligonucleotide (Invitrogen/Life Technologies) VP229 (8 nmol) was annealed to template PBS_A (16 nmol) by heating to 80°C. The reverse transcriptase assays were performed in the presence of annealed t/p (PBS_A.VP229), 6.25nM of enzyme, 50mM Tris-HCl (pH 8.0), 50mM KCl, 6mM MgCl₂, 1mM DTT, 0.1 mg/ml BSA and various concentrations of inhibitor. The reactions were allowed to proceed for 5 minutes and stopped by spotting onto DEAE (Diethylaminoethyl) paper followed by phosphoimaging. Spot intensities were determined by ImageQuant software (GE Healthcare). The IC₅₀s were determined from plots of % activity vs. inhibitor concentration using GraphPad Prism 4.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

HIV	human immunodeficiency virus
RT	reverse transcriptase
RNase H	ribonuclease H
dNTP	deoxynucleotide triphosphate
t/p	template primer
WT	wild type

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Figure 1.

Graphical representation of the positioning of reverse transcriptase on matched and mismatched primer templates. Cleavage positions in the RNaseH active site are highlighted with stars, with the black star indicating the precise position of cleavage for each complex. The positioning of the P (priming) and N (nucleotide binding) sites of the polymerase active site [13,14], relative to the template-primer are also highlighted as green and pink rectangles respectively. The RT forms two complexes with matched primer termini: pre- and post-translocation. Binding to a mismatched primer results in an additional complex (–2).

Figure 2. Positioning of Lys66Arg in the $\beta 3$ - $\beta 4$ loop relative to the primer in the dNTP binding site. The distance between the Lys66 and the phosphate backbone of the penultimate base pair (bp) is labeled with a red line and is measured using the distances tool under structural analysis menu of Chimera [47]. The ultimate and penultimate nucleotides are labeled respectively. The incoming dNTP is represented as a mesh surface, while the $\beta 3$ - $\beta 4$ strand is shown in light gray. The relative positions of atoms are obtained from 1rtd [12].

Figure 3.

Single nucleotide mismatch extension assays for WT, Lys66Arg, Lys66Ala, Lys66Asn or Lys66Thr. The reactions were performed in the presence of a 100nM γ -[32 P] 5'-end-labeled mismatched primer annealed to a template, 10nM of enzyme and 0-800 μ M dATP. The reactions were ran for 2, 5, or 10 minutes and stopped with a solution containing 95% formamide, 15mM EDTA, and 0.2mg/ml bromophenol blue.

Figure 4.

Gel to illustrate single nucleotide misinsertion assays in the presence of 10nM enzyme (WT, Lys66Arg, Lys66Ala, Lys66Asn or Lys66Thr) and 100nM of matched t/p. Enz is used to designate which of the enzymes are used in each reaction and dNTP labels the four nucleotides. P represents the unextended primer and p + 1 designates the primer extended by one nucleotide and so forth. The reactions were performed in the presence of 10nM of enzyme and 0-1mM dATP, dGTP, dCTP and dTTP. The reactions were ran for 10 minutes and stopped with a solution containing EDTA.

Figure 5.

Site-specific RNase H directed hydroxyl radical footprinting. (A) various primers (A-E) were separately annealed to the template to help distinguish cleavage positions. Pre- and post-translocation positions are designated by arrows a and b respectively. (B) Representative footprinting gel showing cleavage positions for each enzyme bound to a matched (T:A) or mismatched (G:A) primer-template. No cleavage products are observed in the absence of enzyme or Fe^{2+} . (C) Graphical representation of the positioning of wild-type and mutant RTs on either a matched (T:A) or mismatched (G:A) template, shown as positions relative to the 3' primer terminus. $P < 0.025$ represents the statistical significance between the percentage of wild type and Lys66Asn complexes at position -19. $P < 0.004$ represents the significance between Lys66Arg and Lys66Ala complexes at position 19. $P < 0.02$ represents the statistical significance between the percentage of wild type and Lys66Ala complexes at position 17.

Figure 6.

Mapping of relative positions of Lys66 substitution mutants with respect to the phosphate of the penultimate bp. Molecular models of residue 66 in comparison to the conservative and non-conservative substitution mutants using the 3⁰ crystal structure by Huang et al [12]. The β 3- β 4 strands are shown in yellow, while the incoming dNTP is shown as a mesh surface. The primer strand and the template strand are both labeled while the nucleotides in both strands are color-coded: dA is blue, dG is green and dC is pink. Each of the non-conservative mutations has been introduced into position 66 of model 1rtid using the command line swapaa and the Dunbrack rotamer library [48]. Distances (represented by red lines) were measured from the indicated atom of the residue at position 66 to the phosphate on the primer strand using the distances tool under structural analysis menu of Chimera [47].

Table 1

Single Nucleotide Extension Assay

A. Steady-state extension kinetics from a matched template primer						
dNTP	RT	k_{cat} (min ⁻¹) ± S.E	K_{m} (nM) ± S.E	$k_{\text{cat}}/K_{\text{m}}$ (nM ⁻¹ min ⁻¹) ± S.E		
dATP	WT	6.88±0.29	34.51±6.12	0.20±0.03		
	K66R	14.21±1.21	54.80±18.31	0.26±0.07		
	K66A	6.95±0.40	50.61±11.58	0.14±0.03		
	K66N	6.33±0.84	85.35±22.27	0.08±0.02		
	K66T	14.51±0.64	86.56±13.78	0.17±0.02		
B. Steady-state extension kinetics from a mismatched template primer						
dNTP	RT	k_{cat} (min ⁻¹) ± S.E	K_{m} (μM) ± S.E	$k_{\text{cat}}/K_{\text{m}}$ (μM ⁻¹ min ⁻¹) ± S.E	f_{ext} *	Δf_{ext}
dATP	WT	2.74±0.43	116.6±60.1	0.024±0.009	0.12	
	K66R	2.99±0.75	124.5±100.8	0.024±0.015	0.09	1
	K66A	0.87±0.18	326.4±163.6	0.003±0.0009	0.02	6
	K66N	0.80±0.23	412.8±266.9	0.002±0.0007	0.03	4
	K66T	1.37±0.39	414.6±263.0	0.003±0.001	0.02	6

* f_{ext} is calculated as $(k_{\text{cat}}/K_{\text{m}})_{\text{incorrect}}/(k_{\text{cat}}/K_{\text{m}})_{\text{correct}}$

Δf_{ext} represents the fold change as compared to Wild-Type

Data shown are mean and standard deviation values from at least 3 independent experiments

Kinetic constants were calculated using GraphPad Prism TM

Table 2
Steady-state kinetics of nucleotide misinsertion by wild-type and Lys66 substitution mutants

dNTP	RT	k_{cat} (min^{-1}) $\pm$ S.E	K_m (μM) $\pm$ S.E	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$) $\pm$ S.E	f_{ins}^*	Δf_{ins}
dGTP	WT	5.52 $\pm$ 0.57	12.65 $\pm$ 7.80	0.46 $\pm$ 0.25	2.32	
	K66R	8.30 $\pm$ 0.77	14.20 $\pm$ 8.07	0.58 $\pm$ 0.30	2.93	1
	K66A	1.52 $\pm$ 0.22	74.38 $\pm$ 43.64	0.02 $\pm$ 0.01	0.10	23
	K66N	1.52 $\pm$ 0.08	4.31 $\pm$ 1.95	0.35 $\pm$ 0.15	1.77	1
dCTP	K66T	2.83 $\pm$ 0.20	15.41 $\pm$ 6.69	0.18 $\pm$ 0.07	0.92	3
	WT	3.30 $\pm$ 0.49	100.7 $\pm$ 51.89	0.03 $\pm$ 0.01	0.16	1
	K66R	6.52 $\pm$ 1.29	212.7 $\pm$ 116.70	0.03 $\pm$ 0.01	0.15	1
	K66A	1.79 $\pm$ 0.65	351.1 $\pm$ 326.5	0.005 $\pm$ 0.003	0.03	6
dTTP	K66N	6.92 $\pm$ 2.32	1529 $\pm$ 1091	0.007 $\pm$ 0.002	0.02	8
	K66T	8.01 $\pm$ 1.53	1618 $\pm$ 656.4	0.005 $\pm$ 0.001	0.02	8
	WT	1.59 $\pm$ 0.35	71.89 $\pm$ 63.30	0.010 $\pm$ 0.003	0.05	1
	K66R	12.60 $\pm$ 1.78	888.3 $\pm$ 234.7	0.014 $\pm$ 0.002	0.07	1
	K66A	2.17 $\pm$ 1.22	603.5 $\pm$ 729.4	0.004 $\pm$ 0.003	0.02	3
	K66N	11.36 $\pm$ 2.22	1623 $\pm$ 659.7	0.007 $\pm$ 0.002	0.04	1
	K66T	11.48 $\pm$ 2.4	1615 $\pm$ 704.0	0.007 $\pm$ 0.002	0.04	1

* f_{ins} is calculated as (kcat/ Km)_{incorrect} / (kcat/ Km)_{correct}
 Δf_{ins} represents the fold change as compared to Wild-Type (Table 1A)
 Data shown are mean and standard deviation values from at least 3 independent experiments

Table 3IC₅₀s of recombinant RTs for NRTIs and NNRTI by filter-based assay

RT	Drug susceptibility (IC ₅₀)			
	AZT (nM)	d4T (μM)	TFV (nM)	EFV (nM)
WT	185±1.11	4.52±1.16	950.3±1.49	2067±1.07
K66R	250.4±1.22 (1)	4.87±1.23 (1)	1056±1.22 (1.1)	2005±1.14 (1)
K66A	559.3±1.10 (3)	7.91±1.22 (1.7)	1635±1.16 (1.7)	1669±1.08 (1.2)
K66N	563.1±1.08 (3)	8.64±1.23 (1.9)	1065±1.21 (1.1)	1870±1.08 (1.1)
K66T	218.9±1.28 (1.2)	6.84±1.18 (1.5)	1222±1.16 (1.3)	1255±1.07 (1.6)
K65A	372.5±1.17 (2)	15.70±1.17 (3.5)	5663±1.23 (5.9)	960.3±1.05 (2)
K65R	453.9±1.44 (2.4)	16.68±1.34 (3.7)	2531±1.16 (2.6)	1281±1.05 (1.6)

Data shown are mean and standard deviation values from 3 independent experiments.

The values in parentheses represent the fold resistance.