The K65R Mutation Confers Increased DNA Polymerase Processivity to HIV-1 Reverse Transcriptase*

(Received for publication, December 26, 1996, and in revised form, May 20, 1996)

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The K65R mutation in HIV-1 reverse transcriptase (RT) is associated with viral cross-resistance to 2',3'-dideoxyinosine, 2',3'-dideoxycytidine, and 2',3'-dideoxy-3'-thiacytidine. We have found that in vitro DNA synthesis by K65R RT is significantly more processive than that of wild type (wt) RT. Depending on the template/primer (T/P) used, the total incorporation of nucleotides under single processive cycle conditions was 20-50% higher with K65R RT than with wt RT. With heteropolymeric T/P, the total incorporation of dNMP by K65R and wt RT was similar under continuous DNA synthesis reaction conditions. However, under single processive cycle conditions, the rate of full-length polymerization product synthesis by K65R RT was about 2-fold higher than that by wt RT. We also found a decreased rate of T/P dissociation during K65R RT DNA synthesis, which is consistent with the increased processivity of the enzyme. We postulate that the increased processivity of the K65R RT may be a compensatory response to the decreased affinity of this mutant for certain dNTP substrates, allowing normal viral replication kinetics.

Dideoxynucleoside compounds such as 3'-azido-3'-deoxythymidine, 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyinosine (ddI) are effective inhibitors of the replication of the human immunodeficiency virus type 1 (HIV-1). These antivirals, once converted to their respective triphosphates by intracellular metabolism, may inhibit viral reverse transcriptase (RT) catalyzed proviral DNA synthesis by at least two mechanisms (1, 2). The ddNTP can act as competitive inhibitors with respect to the appropriate dNTP substrate. In addition, these inhibitors effectively terminate further DNA polymerization upon incorporation into the nascent proviral DNA chain.

Although ddN drugs may initially reduce viral load in HIV-1 infected individuals, an increased viral burden inevitably recurs despite continued therapy, due to the appearance of drug-resistant strains of HIV. Resistance to the various ddN drugs is directly correlated with mutations in the viral reverse transcriptase (3, 4).

We have previously shown that the K65R mutation in RT results in viral cross-resistance to 3TC, ddC, and ddI (5). Our recent studies with recombinant RT have shown that the K65R mutation results in a measurable change in the in vitro affinity of the mutant RT for 3TCTP, ddCTP, and ddATP (6). This altered affinity leads to a significant decrease in chain termination efficiency by each of 3TCTP, ddCTP, and ddATP when present in DNA polymerization reactions catalyzed by K65R RT (7).

However, the K65R RT also has a decreased affinity for normal dNTP substrates. Since the intracellular levels of dNTP substrates are "fixed," averaging about 3 μM (8), the K65R mutation might be expected to result in an attenuation of viral replication. In fact, the K65R mutant HIV-1 replicates with virtually identical efficiency as the wild type (wt) virus (5).

In this report, we show that DNA synthesis by recombinant K65R RT is significantly more processive than that catalyzed by the wt enzyme. Our data indicate that this increased processivity is due to a decreased rate of dissociation of template/primer. The increased processivity of the K65R mutant RT may be a compensatory response to the decreased affinity for certain dNTP substrates, enabling normal viral replication kinetics. We conclude that viral cross-resistance to 3TCTP, ddCTP, and ddATP due to the K65R mutation in RT results from the increased processivity of K65R RT coupled with decreased chain termination frequency noted with ddN antivirals.

MATERIALS AND METHODS

Recombinant wild type and K65R mutant p51 and p66 forms of HIV-1 RT were expressed in Escherichia coli transformed with the plasmids described by Gu et al. (6). The enzyme produced from these plasmids is identical in primary sequence to HIV-IIIB, except for an additional methionine residue at the N terminus that is contributed by the ATG start codon of the expression plasmid. Heterodimeric p51/p66 RT was purified from bacterial lysates using our recently described single step purification protocol (9). The K65R and wt RT preparations used in the present studies exhibited identical specific activities when assayed using poly(rA)-oligo(dT)12–18 as template/primer (T/P). The heteropolymeric RNA-dependent DNA polymerase T/P was prepared as described (6, 7, 10) using the T7 polymerase RNA transcript from AccI-linearized plasmid pHIV-PBS (10) as template and a synthetic 18-nt deoxyoligonucleotide as primer.

[3H]dNTPs, [γ-32P]ATP, [α-35S]dATP, and the DNA sequencing kit Sequenase 2.0 were from Amersham. The homopolymeric T/P poly(dC)-oligod(G)15-19, poly(dC)-oligod(G)15-19, poly(rA)-oligod(T)15-19, and poly(rU)-oligod(C)15-19 T4 polynucleotide kinase, and high purity unlabeled dNTPs were obtained from Pharmacia. All other reagents were of the highest quality available and were used without further purification.

Assay of RT DNA Polymerase Activity—HIV-1 RT DNA polymerase activity was determined in a fixed time assay. Briefly, reaction mixtures (20–100 μl of total volume) contained 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, variable amounts of purified recombinant p51/p66 heterodimeric wt or K65R RT, and tem-
and K65R RT catalyzed similar amounts of dNMP incorporation under continuous polymerization conditions in the absence of heparin with all of the T/P used (Table I). In contrast, under single processive cycle conditions in the presence of heparin, dNMP incorporation catalyzed by the K65R RT was approximately 20–40% greater than that by the wt enzyme with all T/P except poly(rC)-oligo(dG)_{12–18}, as shown by the K65R/wt ratios presented in Table I. Control experiments showed that addition of the heparin trap prior to initiation of DNA synthesis resulted in less than 2% incorporation of dNMP compared to that under normal assay conditions (data not shown).

Polymerase Product Distributions Under Continuous and Single Processive Cycle DNA Synthesis by wt and K65R RT—With an oligodeoxynucleotide primer, reverse transcription on the RNA template derived from pHIV-PBS produces several smaller polymerization products in addition to the expected full-length product (7, 10). Continuous polymerization conditions allow rebinding of RT to a partially extended primer, enabling subsequent elongation of the extended primer. Under these conditions, both wt and K65R RT gave similar polymerization product profiles (Fig. 1A). The rates of full-length product formation by the two enzymes were identical (Fig. 2A). In contrast, under single processive cycle reaction conditions, which prevent rebinding to and re-extension of primer, the synthesis of full-length product by K65R RT was 2-fold greater than that of wt RT (Fig. 1B and Fig. 2B).

Analysis of T/P Dissociation from wt and K65R RT—Although the studies described above showed differences in DNA polymerase processivity between wt and K65R RT, the mechanism underlying these differences was unclear. One possibility was that in T/P dissociation from RT. A greater tendency for dissociation of the T/P from wt RT either before or after limited primer extension might result in an apparent decreased processivity of DNA synthesis.

We tested this with a "template challenge" assay (11), in which we examined the inhibition of RT-catalyzed polymerization on one homopolymeric T/P following the addition of another non-complementary T/P. As shown in Fig. 3, incorporation of [\textsuperscript{3}H]dCTP into poly(rI)-oligo(dC)_{12–18} catalyzed by wt RT and by K65R RT did not stop immediately upon addition of excess poly(dC)-oligo(dG)_{12–18}, but rather decreased in a time-dependent manner. This decrease in activity followed first-
**FIG. 1.** Time course of RNA-dependent DNA polymerization by wt and K65R RT under continuous polymerization and single processive cycle conditions. Reaction mixtures contained the pHIV-PBS RNA transcript annealed with 5'-32P-18-nt DNA oligonucleotide primer as TP (65 nM), RT p51/p66 heterodimer (6 nM), and 50 μM each of dATP, dCTP, dGTP, and dTTP. Reactions were carried out at 37 °C, and aliquots were removed, quenched, and analyzed by PAGE as described under "Materials and Methods." A, polymerization product distribution by wt and K65R RT under continuous polymerization conditions (in the absence of poly(rA)-oligo(dT)12-18 trap). B, polymerization product distribution by wt and K65R RT under single processive cycle conditions (in the presence of 50 μg/ml poly(rA)-oligo(dT)12-18 trap). The exposure time for this section of the autoradiogram was 3-fold longer than that shown in A, in order to compensate for the decreased polymerization product formation in the presence of the trap. It should be noted that the time indicated as 0 in fact corresponds to about 0.5 min, the minimum time required for quenching of the reaction after addition of dNTPs. M illustrates sequencing reactions carried out on pHIV-PBS, used as size markers to aid in identification of the various polymerization products shown in A and B.

**FIG. 2.** Variation in formation of full-length polymerization product with time by wt (▼) and K65R (●) RT under (A) continuous and (B) single processive cycle conditions. The amount of n+173 nt full-length product was assessed by densitometry of exposed autoradiographic films, similar to those presented in Fig. 1.
order kinetics (Fig. 3, inset), yielding rate constants of 1.2 min$^{-1}$ for wt RT and 0.8 min$^{-1}$ for K65R RT.

The competence of the poly(dC)-oligo(dG)$_{12-18}$ challenge to inhibit polymerization on poly(rI)-oligo(dC)$_{12-18}$ was tested in control experiments in which both this T/P and poly(rI)-oligo(dC)$_{12-18}$ were added simultaneously at the beginning of the experiment. Under these conditions, no [3H]dCMP was incorporated into acid-precipitable material during the subsequent incubation period (data not shown).

**DISCUSSION**

The K65R mutation in the RT gene of HIV-1 confers cross-resistance to ddI, ddC, and 3TC (5). We have previously shown that recombinant K65R RT shows decreased sensitivity to certain ddNTP inhibitors in vitro (6). This resistance is due in part to decreased chain termination in the presence of ddI, ddC, and 3TC (7). The K65R RT also shows a decreased affinity for deoxynucleotide substrates (6), with a mean $K_m$ for the four dNTPs of 3.3 $\mu$M compared to 1.4 $\mu$M for the wt enzyme (6). Intracellular levels of dNTPs are obviously dependent on cell type, but average 3 $\mu$M (8). The K65R mutation might therefore be expected to attenuate viral replication. In fact, virus with the K65R mutation in RT replicates with identical efficacy as wild type (wt) virus (5).

We have now shown that K65R RT has an increased DNA polymerase processivity in vitro compared to wt enzyme. This increased processivity may be a compensatory mechanism, allowing the drug-resistant K65R mutant virus to overcome the inherent disadvantage of decreased affinity for dNTP substrate, thereby maintaining normal replication capability.

The processivity of a DNA polymerase refers to the number of nucleotides added to the nascent DNA chain before the enzyme dissociates from the T/P. Although HIV-1 RT DNA polymerase activity is essentially processive, pausing occurs during the transcription process and results in a variety of smaller polymerization products in addition to the expected full-length product (12–15). The overall processivity of polymerization is an obvious function of dNTP concentration (16).

Under our in vitro single processive cycle reaction conditions, K65R RT consistently synthesized more full-length product compared to wt RT, at all dNTP levels used (data not shown).

A major factor in transcriptional pausing is dissociation of RT from the RTz T/P complex (17). In our template/primer challenge experiments, the rate constant for the loss of DNA polymerase activity upon addition of competitor T/P is a direct measure of the rate of dissociation of the T/P complementary to the dNTP used in the experiment. Although the apparent $K_m$ for the T/P poly(rI)-oligo(dC)$_{12-18}$ is essentially identical for wt and K65R mutant RT, our data imply that T/P dissociation from K65R RT is about 50% less than that from wt enzyme. This is in good agreement with the difference in polymerization processivity between K65R and wt RT with poly(rI)-oligo(dC)$_{12-18}$ as T/P (Table I). The increased DNA polymerase processivity shown by the K65R mutant may thus be due to a decreased rate of T/P dissociation.

It is interesting to note that another ddC/3TC-resistant mutant RT, M184V, shows decreased processivity compared to wt enzyme (18), in contrast to the increased processivity of our ddC/3TC/ddI cross-resistant K65R mutant. The M184V mutation is located in the "palm" subdomain of RT, and may interact with the ribose of the terminal primer nucleotide of the T/P.

![Fig. 3. Effect of the addition of poly(dC)-oligo(dG)$_{12-18}$ on poly(rI)-oligo(dC)$_{12-18}$ directed DNA synthesis by wt and K65R RT.](image-url)
In contrast, the K65R mutation occurs in the β3-β4 loop, a highly flexible segment of the “fingers” subdomain of RT that may be involved in dNTP/ddNTP binding (6,20–22), although others have presented compelling evidence that this segment may be primarily involved in T/P interaction (23). The enhanced processivity of K65R RT appears to support the latter conjecture. However, the observation that RT polymerase processivity increases after the third or fourth dNMP residue incorporated (12, 13, 24) is also consistent with a direct correlation between dNTP binding and processivity. Thus, the precise functional role of this region of RT remains equivocal. Further studies are necessary to clarify the function of this RT fingers substructure.

Finally, recent studies have indicated that the D67N/K70R/T215Y/K219Q multiple mutations in RT that are associated with high level resistance to 3′-azido-3′-deoxythymidine confer increased processivity to the mutant RT (25). It is presently unclear how these data relate to our findings for the K65R enzyme, since the mutations for 3′-azido-3′-deoxythymidine-resistance occur in two distinct regions of RT. The combination of these mutations might be expected to result in very different functional effects than a single change in any one subdomain of RT. Nonetheless, two of these mutations, D67N/K70R, occur in the same segment as K65R. It would be interesting to determine whether these mutations are the major determinants for the increased processivity of the 3′-azido-3′-deoxythymidine-resistant mutant RT.

Acknowledgment—We thank Dr. Neerja Kaushik for critical review of the manuscript.

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