Molecular Determinants of Multi-nucleoside Analogue Resistance in HIV-1 Reverse Transcriptases Containing a Dipeptide Insertion in the Fingers Subdomain

EFFECT OF MUTATIONS D67N AND T215Y ON REMOVAL OF THYMIDINE NUCLEOTIDE ANALOGUES FROM BLOCKED DNA PRIMERS

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Tania Matamoros‡, Sandra Franco§, Blanca M. Vázquez-Álvarez‡, Antonio Mas‡†,
Miguel Ángel Martínez‡, and Luis Menéndez-Arias‡¶

From the ‡Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, 28049 Madrid, Spain and the §Laboratori de Retrovirologia, Fundació irsiCaixa, Hospital Universitari Germans Trias i Pujol, Badalona, 08916 Barcelona, Spain

Human immunodeficiency virus type 1 isolates having dipeptide insertions in the fingers subdomain of the reverse transcriptase (RT) show high level resistance to 3′-azido-3′-deoxythymidine (AZT) and other nucleoside analogues. Insertions are usually associated with thymidine analogue resistance mutations, such as T215Y. The resistance phenotype correlates with increased ATP-dependent phosphorolytic activity, which facilitates removal of thymidine analogues from inhibitor-terminated primers. In this report, we show that substituting Thr, Ser, or Asn for Tyr-215 in a multidrug-resistant RT, bearing a Ser-Ser insertion between codons 69 and 70, leads to AZT and stavudine resensitization through the loss of the ATP-mediated removal activity. The mutation D67N, which is rarely found in insertion-containing strains, had no effect on excision and a minor influence on resistance. Substituting Tyr-215 had a larger effect than deleting the dipeptide insertion. The presence of both the insertion and mutation T215Y in the wild-type BH10 RT conferred significant ATP-mediated removal activity and moderate resistance to AZT. However, resistance levels and unblocking activities were lower than those observed with the multidrug-resistant enzyme. Removal reactions can be inhibited by the next complementary dNTP. Both Tyr-215 and the dipeptide insertion affect RT-DNA-DNA-dNTP ternary complex formation, an effect that was not detected in the presence of foscarinet. Based on crystal structures of binary and ternary complexes of HIV-1 RT, we propose that Tyr-215 exerts its function by facilitating a proper orientation of the pyrophosphate donor molecule, whereas the effects on dNTP binding are indirect and could be related to significant conformational changes occurring during polymerization.

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† Present address: Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, 08002 Barcelona, Spain.
‡ To whom correspondence should be addressed. Tel.: 34-914978477; Fax: 34-914974799; E-mail: lmenendez@cbm.uam.es.

Human immunodeficiency virus type 1 (HIV-1)\(^{1}\) reverse transcriptase (RT) replicates the viral genomic RNA to synthesize a double-stranded DNA that integrates into the host genome. The viral enzyme is multifunctional, possessing RNA- and DNA-dependent DNA polymerase, RNase H, strand transfer, and strand displacement activities (1). HIV-1 RT is a heterodimeric enzyme composed of two polypeptide chains of 66 and 51 kDa, with subdomains termed fingers, thumb, palm, and connection in both subunits and an RNase H domain in the larger subunit only.

HIV-1 RT is an important target for chemotherapeutic intervention in the control of AIDS. Antiretroviral drugs targeting the viral polymerase include nucleoside analogue inhibitors (NRTIs), acyclic nucleoside phosphonates, and non-nucleoside RT inhibitors (NNRTIs) (reviewed in Ref. 2). Inside the cell, nucleoside derivatives are converted to their active triphosphate forms by host cell kinases and are then incorporated into the HIV-1 genome by the viral RT. Because nucleoside analogues lack a 3′-OH group, their incorporation blocks elongation of the growing DNA chain. On the other hand, NNRTIs bind to an allosteric site located 10–15 Å away from the polymerase active site, distorting the geometry and/or the mobility of the polymerase catalytic site, interfering with the proper positioning of the template-primer in the nucleic acid-binding cleft of the RT or restricting the mobility of the thumb, which results in the impairment of translocation during polymerization (for reviews, see Refs. 3 and 4). Although NNRTIs resistance mutations exert their action by diminishing the ability of the enzyme to bind the inhibitor (i.e. Y181C for nevirapine, K103N for nevirapine, delavirdine, and efavirenz, etc.), NNRTI resistance mutations act either by (i) increasing discrimination against the triphosphate forms of the drugs (reviewed in Refs. 5 and 6) or (ii) increasing the ability of RT to unblock inhibitor-terminated DNA chains in the presence of physiological concentrations of pyrophosphate (PPi) or ATP (7–9).

Mutations such as M184V or M184I, which confer resistance to 2′,3′-dideoxy-3′-thiactxytidine (3TC; lamivudine) are examples of the first mechanism (10). On the other hand, RTs har-
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boring the 3'-azido-3'-deoxythymidine (AZT; zidovudine)-asso-
ciated mutations D67N and K70R in the fingers subdomain, together with the substitution T215Y, displayed an increased ATP-dependent phosphorolytic activity that facilitates the efficient removal of AZT and 2',3'-dideoxyhydro-2',3'-deoxythymidine (d4T; stavudine) from blocked DNA chains (11). Despite being detectable, the excision reaction appears to be much more efficient with other clinically relevant nucleosides, such as 2',3'-dideoxyxycytidine (dC; zalcitabine), abacavir, 3TC, 2',3'-dideoxyinosine (dI; ddI), and tenofovir (12–15).

Prolonged use of antiretroviral drugs in the clinical treat-
ment of HIV-infected patients has resulted in the emergence of
viral isolates with reduced susceptibility to multiple RT
inhibitors. Among multidrug-resistant viruses, resistance to multiple NRTIs can be achieved by three different major pathways: (i) accumulation of thymidine analogue resistance mutations (TAMs), (i.e. M41L, D67N, K70R, L210W, T215Y, and K219Q/E), which have a clinical impact on AZT, d4T, ddI, abacavir, and tenofovir resistance (16–21); (ii) selection of mutation Q151M, followed by other substitutions (A62V, V75I, F77L, and F116Y) that render viruses with significant resistance to AZT, d4T, ddI, ddC, and abacavir (22–24); and (iii) emergence of insertions of two residues (often Ser-Ser, Ser-Gly, and Ser-Ala) together with mutation T69S, in viruses containing additional amino acid substitutions associated with NRTI resistance, such as M41L, L210W, and/or T215Y. In phenotypic assays, these viruses display high level resistance to AZT and moderate levels of resistance to other NRTIs, such as d4T, ddC, and ddI (25–28).

Although the biochemical properties of the wild-type HIV-
1BH10 RT were not significantly altered upon introduction of a dipeptide (Ser-Ser) insertion, we have previously shown that eliminating the insertion in the sequence context of an HIV-1 RT bearing the drug-resistant mutations M41L, A62V, K70R, V118I, M184I, L210W, T215Y, and G333E produced a significant reduction of the ATP-dependent phosphorolytic activity that allows excision of AZT or d4T from inhibitor-terminated primers (13, 27). These data correlated with a significant re-
duction in the viral susceptibility to AZT (27). However, elimi-
nating the insertion was not sufficient to abrogate AZT resistance, because of the presence of TAMs in the insertion-containing viral isolate. In this work, we have analyzed the impact of TAMs D67N and T215Y on the ATP- and PPI-mediated removal of AZT- and d4T-monophosphate by SS RT. These two mutations were chosen based on available genotypic data showing that T215Y/F is the most frequently found mutation in viral isolates containing insertions in the fingers subdomain, whereas D67N appears very rarely in those viral strains, de-
spite being a commonly selected mutation during treatment with thymidine analogues.

**EXPERIMENTAL PROCEDURES**

**RTs—**Expression and purification of wild-type BH10, SS, and mu-
tant SS 2SS0s were as previously described (27). Other mutant RTs were generated by using the QuikChange™ site-directed mutagenesis kit (Stratagene), following the manufacturer’s instructions. Individual mutations Y215F, Y215N, Y215S, and D67N were introduced in plasmid pRT6 carrying the nuclease sequence encoding the 66-kDa subunit of SS RT (27) to generate mutant enzymes designated as SS_Y215F, SS_Y215N, SS_Y215S, and SS_D67N, respectively (see Fig. 1). On the other hand, the mutation T215Y was introduced in a pRT6 construct encoding a mutated p66 subunit of BH10 RT, which contained the T69S/D67N (21). This double mutant T69S/D67N/T215Y was designated as BH10_SSS. The mutagenic primers used were 5′-GGAGGGGAGTTACCCACCCAGAAACACATC-3′ and 5′-CTGAAAAGAAAAAACATGGTCAATGAGG-3′.

**Template-Primer Compositions and Template-Primer Preparation**—The triphosphate derivatives of NRTIs were obtained from Moravek Biochemicals (AZT-triphosphate) and Sierra Bioresearch (d4T-triphosphate). Stock solutions of dNTPs and rNTPs (100 mM) were from Amer sham Biosciences. Before use, nucleoside-triphosphates were treated with inorganic phosphatase to remove traces of PPI, as described (13). In previous studies, we used the following heteropolymers oligonucleotides (Iexotoxin) as DNA-DNA template-primer substrates: 5′-GGGCTCTTTCATTCGGT-TGACGAAATTTATAGGTTACCACTTACAGACAAAACACATC-3′ and 5′-GTGTTTTTGTGTTGGTACAAACCCACCCTC-3′ for SS_Y215N, 5′-GGTTTTTGTGTTGGTGATGAAATCAACTTACCACTTACAGACAAAACACATC-3′, and 5′-GGTTTTTGTGTTGGTACAAACCCACCCTC-3′ for BH10_SSSY. The introduced mutations were confirmed by DNA sequencing, and the appropriate MscI-KpnI inserts were then cloned in plasmids derived from pT75H (27, 29), for expression of the p51 subunits of SS or BH10 RTs. All of the RTs were purified as p66/p51 heterodimers, and the mutations were introduced in both subunits of the enzyme. The 51-kDa polypeptide was obtained with an extension of 14 amino acids at its N-terminal end, which includes 6 consecutive histidine residues to facilitate its purification by metal chelate affinity chromatography, as previously described (29).

**Nucleoside Polynucleotides and Template-Primers**—Triphosphate derivatives of NRTIs were obtained from Moravek Biochemicals (AZT-triphosphate) and Sierra Bioresearch (d4T-triphosphate). Stock solutions of dNTPs and rNTPs (100 mM) were from Amer sham Biosciences. Before use, nucleoside-triphosphates were treated with inorganic phosphatase to remove traces of PPI, as described (13). In previous studies, we used the following heteropolymers oligonucleotides (Iexotoxin) as DNA-DNA template-primer substrates: 5′-GGGCTCTTTCATTCGGTGACGAAATTTATAGGTTACCACTTACAGACAAAACACATC-3′ and 5′-GTGTTTTTGTGTTGGTGATGAAATCAACTTACCACTTACAGACAAAACACATC-3′, and 5′-GGTTTTTGTGTTGGTACAAACCCACCCTC-3′ for SS_Y215N.

**Template-Primer Binding Affinity**—The incorporation of the appropriate nucleotides into the nascent DNA chain was measured using the high-resolution DNA gel electrophoresis method described by Sambrook et al. (30). Briefly, the phosphorylated template-primer was incubated with 32P-labeled d4T-terminated 25-mer (D38/25PGAd4T) (0.3 nM) was incubated for 30 min at 37 °C in the presence of the corresponding time course. The reactions were initiated by adding an equal amount of dATP and T4 polynucleotide kinase (Promega) in the presence of all four rNTPs (100 mM). The newly formed 3′-phosphorylated molecules were then annealed to gel-purified templates in a solution containing 150 mM NaCl and 150 mM magnesium acetate, as described (30). The template-primer molar ratio was adjusted to 1:1.

**Chain Terminator Removal Assays**—RT-catalyzed DNA rescue reac-
tions were performed with D38/25PGA DNA duplexes as previously described (13). Briefly, the phosphorylated template-primer (30 nM) was preincubated at 37 °C for 10 min in the presence of the corresponding time course. The reactions were initiated by adding an equal amount of dATP, ATP, and T4 polynucleotide kinase (Promega) in the presence of all four rNTPs (100 mM). The newly formed 3′-phosphorylated molecules were then annealed to gel-purified templates in a solution containing 150 mM NaCl and 150 mM magnesium acetate, as described (30). The template-primer molar ratio was adjusted to 1:1.

**Dissociation Constants (Kd)**—The dissociation constants of wild-type HIV-1 RT were measured as described (13). Briefly, the phosphorylated template-primer (30 nM) was preincubated at 37 °C for 10 min in the presence of the corresponding time course. The reactions were initiated by adding an equal amount of dATP, ATP, and T4 polynucleotide kinase (Promega) in the presence of all four rNTPs (100 mM). The newly formed 3′-phosphorylated molecules were then annealed to gel-purified templates in a solution containing 150 mM NaCl and 150 mM magnesium acetate, as described (30). The template-primer molar ratio was adjusted to 1:1.

**Stable Ternary Complex Formation Assays and Determination of dNTP Binding Affinity (Apparent Kd[dNTP])**—The HIV-1 RT-
variants to form a stable ternary complex was assessed as described (32, 33). For this purpose, RTs (20 nm) were incubated with the labeled D38/25PGAd4T template-primer (0.3 nm) to form the binary complex under the conditions described above and then further incubated at 37 °C in the presence of an excess of dNTP (200 μM dATP, in our assay conditions) to obtain a stable ternary complex. The putative ternary complexes formed were challenged with unlabeled D38/25PGAd4T template-primer at a final concentration of 500 nm. The ternary complex species resistant to the DNA trap were resolved on a 6% native polyacrylamide gel, subjected to phosphorimaging, and quantified using the Tina software.

The apparent dNTP binding affinity (apparent K_d) of the variant RTs was determined as described above, except that the binary complexes were incubated in the presence of increasing concentrations of the complementary nucleotide (dATP, 0–4000 μM) prior to being challenged with the DNA trap. In some experiments, ternary complexes were obtained in the presence of 200 μM fosfornac to monitor the effect of the PPi analogue in the apparent K_d. The RT-DNA-DNTP complexes resistant to the trap were resolved and quantified as indicated above. To determine the apparent K_d, the percentage of total RT-DNA-DNA-DNTP complex converted into stable RT-DNA-DNA-dNTP ternary complex as a function of the concentration of dNTP was fitted to the single-site ligand-binding equation.

Recombinant Virus and Drug Susceptibility Tests—The assays were performed as previously described (27, 34). Briefly, full-length RT-coding sequence DNA was amplified from plasmids carrying the different RTs using primers IN5 (5'-AAATTCCCAATTCCATTAGGAA-CTGTACCA-3') and IN3 (5'-TCTATTCCATCAAAATGGATTTTCTGATTCC-3'). The PCR products were then transfected into an RT-deficient HXB2-D clone in SupT1 cells (35). When the HIV-1 p24 antigen concentration in the cultures surpassed 20 ng/ml, the supernatants were harvested. Progeny virus was propagated and titrated in MT-4 cells. The nucleotide sequence of the RT-coding region of the progeny virus was checked for possible reversions or additional mutations. The SupT1 and MT-4 cells and the deleted HXB2-D clone were obtained from the AIDS Reagent Program (Medical Research Council). HIV-1 drug susceptibility profiles were obtained after infecting 35000 MT-4 cells with 100 50% tissue culture infective doses of virus, at a multiplicity of infection of 0.003, by exposing the HIV-1-infected cultures to various concentrations of each drug (5-fold dilutions). After MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was determined by a tetrazolium-based colorimetric method as described (27, 36).

RESULTS

Mutations Associated with Insertions in the Fingers Subdomain of HIV-1 RT—Although with a relatively low prevalence (0.5–2.7%), several studies have identified heavily treated patients carrying HIV-1 isolates with RTs containing an insertion in the fingers subdomain plus additional resistance mutations, including TAMs (37–41). We have done an extensive survey of the literature to generate a database containing 200 sequences of RTs having insertions of one or two amino acids between codons 69 and 70 (supplemental table). The most frequently found insertions were Ser-Gly, Ser-Ser, and Ser-Ala, which represented approximately two-thirds of the total number of sequences reported. Interestingly, TAMs frequently associated with insertions include M41L, K70R, L210W, and T215Y/F. An aromatic residue at position 215 was observed in 90% of all isolates and appeared in all strains having a Ser-Ser insertion. On the other hand, the classical AZT resistance mutation D67N was never found in consensus sequences of insertion-containing RTs from clinical samples, although it has been occasionally observed in clones derived from viral populations (42).

The SS RT is a good model to study the effect of those mutations, because this polymerase contains both the insertion and the T215Y mutation while having wild-type Asp at position 67 (Fig. 1). We constructed mutants having the SS RT background where Tyr-215 was replaced by Thr (SS_Y215T), Ser (SS_Y215S), or Asn (SS_Y215N) or where Asp-67 was replaced by Asn (SS_D67N). The amino acid substitution Y215T involves two nucleotide changes at codon 215, whereas Y215S and Y215N involve just one nucleotide change and are potential intermediates during a possible reversion of Tyr to wild-type Thr. In addition, both the insertion and T215Y were introduced in the sequence context of the wild-type BH10 RT (mutant BH10_SSSY) to determine whether both changes were sufficient to confer resistance to thymidine analogues. The properties of all mutants on primer unblocking, dNTP inhibition, and DNA chain elongation, as well as their ability to form stable ternary complexes were compared with those of reference RTs (i.e. SS and BH10 RTs), as well as mutant SS_22OS, which lacks the didepsipeptide insert while maintaining the SS RT sequence background.

Removal of AZT- and d4T-Monophosphate from Blocked DNA Primers—The ability of RTs to rescue AZT- and d4T-terminated primers was assessed by using the template-primer shown in Fig. 2A. These experiments were carried out in two steps. First, the HIV-1 RT was incubated with the template-primer in the presence of the triphosphoramidate form of the inhibitor. Then unblocking and extension reactions leading to the accumulation of a 38-nucleotide product were carried out by adding dNTPs in the presence of physiological concentrations of ATP or PPi (3.2 mM and 200 μM, respectively). These reactions can be inhibited by the next complementary dNTP (dATP in our assay conditions), and therefore time courses were carried out in the presence of low dATP concentrations (i.e. 1 μM). Non-complementary dNTPs were included at 100 μM to facilitate full extension of primers after removal of chain-terminating nucleotides. At these concentrations, inhibition of the rescue reaction because of potential mispairs between the template T and dGTP, dTMP, or dCTP was found to be negligible.

In the presence of ATP, AZT removal reactions catalyzed by SS and mutant SS_D67N RTs showed the highest efficiency, whereas SS RT mutants lacking an aromatic side chain at position 215 (i.e. mutants SS_Y215T, SS_Y215N, and SS_Y215S) were devoid of ATP-dependent phosphorolytic activity (Fig. 2B). Substituting Tyr-215 had a larger effect than
deleting the two serines forming the insertion. However, the presence of the insertion together with an aromatic residue at position 215, as in mutant BH10_SSSY, was not sufficient to confer the high ATP-dependent phosphorolytic activity displayed by the SS_RT. The large differences observed between mutant enzymes in ATP-dependent phosphorolysis using AZT-terminated primers were not detected in phosphorolytic reactions when PPi was used as the donor (Fig. 2C). All of the enzymes showed AZT-monophosphate removal rates that were within a 2-fold range of each other.

The time courses for d4T removal in the presence of ATP were similar to those obtained with AZT-terminated primers for all tested RTs (Fig. 3A). D67N had no effect on the removal reaction, whereas eliminating the aromatic side chain of Tyr-215 had a deleterious effect of the unblocking activity. Interestingly, some differences were also detected between the enzymes when analyzing PPi-mediated removal of d4T monophosphate from chain-terminated primers. Excision rates were approximately three times higher for SS RT than for mutants SS_Y215T, SS_Y215S, and SS_Y215N (Fig. 3B).

Primer Unblocking Inhibition by the Next Complementary dNTP and Formation of Stable Ternary Complexes—As previously shown, removal reactions can be inhibited by the next complementary nucleotide (13, 27). AZT removal reactions catalyzed by BH10_SSSY, SS, SS_2S0S, and SS_D67N were highly resistant to dNTP inhibition (IC50 > 0.4 mM), but unblocking of d4T-terminated primers was >20-fold more sensitive to inhibition by the next complementary dNTP (Table I). Under our assay conditions, the pyrophosphate donor (i.e. ATP or PPi) or the specific mutation introduced in the recombinant RT had no significant influence on the IC50 values for the next complementary dNTP. However, RTs having a dipeptide insertion between codons 69 and 70 and the T215Y mutation were shown to be >5-fold less sensitive to inhibition by the next complementary dNTP in ATP-dependent removal reactions, using deoxyxadenosine-terminated primers (43). In addition, it has been reported that for AZT- and ddT-terminated primers, the concentration of the next complementary dNTP required to shift the RT from the pretranslocational to the post-translocational stage was remarkably larger for mutant M41L/T69SSS/T215Y than for the wild-type HIV-1 RT (44). In an attempt to clarify why our assay failed to detect differences in RT sensitivity to dNTP inhibition, we analyzed how mutations could impact dNTP binding by looking at ternary complex formation.

Ternary complexes formed by the RT, a d4T-terminated template-primer, and the next complementary dNTP, using wild-type and mutant RTs, were analyzed by electrophoretic mobility retardation assays. For this purpose, each mutant enzyme was first allowed to form binary complexes with 32P-labeled d4T-terminated template-primers and then supplemented with the next complementary dNTP to form the ternary complex. All of the tested RTs showed normal DNA polymerase activity (data not shown), and the equilibrium dissociation constants (Kd) of the corresponding binary complexes ranged between 1.6 and 7.4 nM (Fig. 4).

Binding of dNTP to the enzyme is an ordered mechanism that occurs only after DNA binding, and the amount of labeled template-primer that remains bound to the RT in the presence of high dNTP concentration and an excess of DNA trap represents the level of stable ternary complex that is formed. This is
concentration of dATP, and the data were fitted to a hyperbola to obtain the IC50 for each enzyme. Reported values were obtained from two to three cases, the incubation times were within the linear range of the corresponding time course. The percentage of inhibition was plotted against the

2. All of the dNTPs in the assays were supplied at 100

ably higher than the IC50 values obtained in rescue assays carried out in the presence of ATP or PPi (Table I). This discrepancy could be due to the presence of the PPi donor in the reaction. Analyzing ternary complex formation in the presence of ATP or PPi is difficult, because these metabolites are substrates of the phosphorolytic reaction. To circumvent this limitation, we used sodium foscarnet, a PPi analogue. Foscarnet is a relatively weak inhibitor of the replication of recombinant viruses containing wild-type BH10 and SS RTs (27). The foscarnet IC50 values for the recombinant BH10 and SS RTs in primer rescue assays using d4T-terminated primers were 99.8 ± 5.2 and 98.7 ± 5.2 μM, respectively. Foscarnet is expected to bind at the dNTP-binding site, but unlike in the presence of dNTPs, ternary complexes of RT-DNA-foscarnet are rather unstable even at concentrations of the inhibitor above 200 μM (data not shown) (45). Interestingly, when RT-DNA-dNTP ternary complexes were formed in the presence of 200 μM foscarnet, all of the studied RTs displayed similar apparent Kd[dNTP] values (Fig. 6 and Table II).

Viability of Recombinant HIV-1 Variants and Resistance to Nucleoside Analogue Inhibitors—All enzymes described were found to be viable after transfecting cells with a recombinant infectious HIV-1 clone harboring the corresponding RT. All

illustrated in Fig. 5A, which shows the higher stability of the ternary complex versus the binary complex using wild-type BH10 RT as a model. In the presence of over 1500-fold molar excess of DNA trap, the RT-DNA-DNA complex was competed out, whereas a significant amount of ternary complex was resistant to competition with DNA trap. The results shown in Fig. 5B, reveal that the RT-DNA-DNA complexes formed by each of the mutant enzymes (lane 2) were fully competed out by the DNA trap (lane 2), whereas a portion of d4T-terminated template-primer bound in RT-DNA-dNTP ternary complexes remains stable and resistant to the trap (lane 4). This portion was roughly similar for all tested RTs.

Ternary complex formation was tested on the labeled D38/25PGA42T template-primer as a function of dATP, the next complementary nucleotide (Fig. 6). The apparent Kd[dNTP] values for SS, SS_S250S, and SS_Y215S were >5-fold higher than for the wild-type BH10 RT and somewhat higher than for mutants BH10_SSSY, SS_Y215T, and SS_Y215S (Table II). For most enzymes, the apparent Kd[dNTP] values were remarkably higher than the IC50 values obtained in rescue assays carried out in the presence of ATP or PPi (Table I). This discrepancy could be due to the presence of the PPi donor in the reaction. Analyzing ternary complex formation in the presence of ATP or PPi is difficult, because these metabolites are substrates of the phosphorolytic reaction. To circumvent this limitation, we used sodium foscarnet, a PPi analogue. Foscarnet is a relatively weak inhibitor of the replication of recombinant viruses containing wild-type BH10 and SS RTs (27). The foscarnet IC50 values for the recombinant BH10 and SS RTs in primer rescue assays using d4T-terminated primers were 99.8 ± 7.2 and 98.7 ± 5.2 μM, respectively. Foscarnet is expected to bind at the dNTP-binding site, but unlike in the presence of dNTPs, ternary complexes of RT-DNA-foscarnet are rather unstable even at concentrations of the inhibitor above 200 μM (data not shown) (45). Interestingly, when RT-DNA-dNTP ternary complexes were formed in the presence of 200 μM foscarnet, all of the studied RTs displayed similar apparent Kd[dNTP] values (Fig. 6 and Table II).

Viability of Recombinant HIV-1 Variants and Resistance to Nucleoside Analogue Inhibitors—All enzymes described were found to be viable after transfecting cells with a recombinant infectious HIV-1 clone harboring the corresponding RT. All
viruses recovered from transfections were able to infect and replicate in SupT1 cells. Recombinant HIV-1 clones were assayed to measure their susceptibility to nucleoside analogues, including AZT and d4T (Table III). SS RT showed high level resistance to AZT and 3TC and moderate resistance to d4T, dDI, and dDC. Deleting the two-serine insertion or introducing mutations at codons 67 and 215 did not produce large effects on resistance to 3TC, dDC, or dDI. The mutant SS_D67N showed a resistance profile that was very similar to the one displayed by SS RT. However, replacing Tyr-215 with Thr, Ser, or Asn in the multidrug-resistant SS RT led to virus sensitive to both thymidine analogues. On the other hand, introducing the T215Y mutation together with the insertion T69SSS within the wild-type BH10 RT sequence background produce a significant increase in resistance to AZT and particularly to d4T. These data are in good agreement with the results of the biochemical assays, indicating that Tyr-215 is a very important residue in the acquisition of resistance to thymidine analogues displayed by multidrug-resistant insertion-containing RTs.

**Discussion**

The presence of a dipeptide insertion at codons 69–70 in combination with amino acid substitutions related to drug resistance (including TAMs) in the HIV-1 RT has been reported by several groups. These viral isolates display high level resistance to AZT and moderate to low level resistance to other NRTIs in phenotypic assays. The insertion by itself does not have a major impact on resistance in the absence of drug-resistant mutations but contributes to AZT resistance in the presence of TAMs and other mutations (27, 46). A good correlation between the results of phenotypic assays and the ATP-dependent phosphorolytic activity displayed by the recombinant RTs has been observed. This activity is higher with AZT-terminated primers, followed by d4T-terminated primers and very low (albeit detectable) for primers terminated with other NRTIs, including cytidine analogues (13) and tenofovir (47).

T215Y is the most frequent mutation associated with dipeptide insertions in the RT-coding region. HIV clones containing the insertion together with mutation T215Y in the sequence context of wild-type BH10 RT had reduced susceptibility to AZT and d4T, although AZT resistance was not as high as with the SS RT that contains additional TAMs such as M41L or L210W. These observations are broadly in agreement with phenotypic data reported by others (26, 28) and correlate with the ATP-dependent phosphorolytic activities displayed by the mutant RTs on AZT- and d4T-terminated primers. Although the presence of a Ser-Ser insertion together with an aromatic side chain at position 215 is sufficient to confer some resistance, it is clear that further changes are needed to achieve the high level resistance displayed by the SS RT. Previously reported evidence revealed that T215Y alone produced a very small increase (<3-fold) in the ATP-dependent phosphorolytic activity on primers terminated with AZT (48, 49). Therefore, the question of whether Tyr-215 is a requirement for maintaining ATP-dependent phosphorolytic activity on primers blocked with thymidine analogues was addressed by substituting Thr, Ser, and Asn for Tyr-215 in the SS RT. The mutant enzymes obtained were unable to remove AZT-monophosphate and d4T-

**Fig. 4.** Gel mobility shift assays for the analysis of DNA binding affinity. The 38-mer DNA primed with 5' ^32P-labeled d4T-terminated 28-mer primer (D38/PG25A4^4T) was incubated with varying concentrations of the corresponding RT. A 0.3 nM concentration of D38/PG25A4^4T was incubated with different concentrations of enzyme at 37 °C for 10 min. Lanes 1–7 show the formation of the binary complex in the presence of RT concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.625 nM, respectively. C, control in the absence of enzyme. The dissociation constants of the RT-DNA complexes were: 3.4 ± 0.9 nM for wild-type BH10 RT, 6.3 ± 2.1 nM for BH10_SSSY, 7.4 ± 2.3 nM for SS, 1.6 ± 0.6 nM for SS_S250S, and 2.7 ± 1.3 nM for SS_Y215T.

**Fig. 5.** Effect of DNA trap concentration on the formation of RT-DNA-DNA and RT-DNA-DNA-dNTP complexes. A, binary (left panel) and ternary complexes (right panel) of the wild-type BH10 RT were formed by incubating 20 nM of RT with 0.3 nM radiolabeled D38/25PGA^4T at 37 °C in the absence or in the presence of complementary dNTP (dATP, 200 μM). Lanes 1–7 represent DNA trap concentrations of 0, 50, 75, 100, 200, 300, and 500 nM, respectively. B, analysis of the ternary complex formed by derivatives of BH10 and SS RTs. The binary (lane 1) and ternary complex (lane 3) formed by the individual mutant enzymes as described for A were challenged by the addition of DNA trap at 500 nM, and the labeled template-primer bound in the stable ternary complex was analyzed on a nondenaturing polyacrylamide gel. Lanes 2 and 4 represent the extent of dissociation of the template-primer in the binary and ternary complex, respectively.
monophosphate from inhibitor-terminated primers, and the corresponding mutant viruses were all susceptible to inhibition by thymidine analogues. Interestingly, the PPI-mediated removal activity of primers terminated with d4T, conferred by Tyr-215 mutants was also reduced in comparison with the parental SS RT. Although the physiological relevance of PPI is not clear, all of these data suggest that these mutations have a significant effect in the optimal alignment of the chain terminated primer relative to the PPI donor.

HIV clones having mutations Y215T, Y215S, or Y215N within the SS RT retained low level resistance to ddI and ddC and high level resistance to 3TC in phenotypic assays. High level resistance to 3TC is expected because of the presence of M184I in all SS-derived clones, but SS RT lacks drug resistance-specific mutations for ddI and ddC (i.e. K65R, L74V). It has been shown that primers terminated with ddC and ddA (the active metabolite of ddI) are substrates of the ATP-dependent phosphorolytic activity catalyzed by AZT-resistant RTs carrying the T215Y/F mutation. However, the efficiency of the removal reaction was very low in comparison with thymidine analogues (11–15). For ddA-terminated primers, it has been shown that ATP-dependent removal reactions catalyzed by RTs having mutations M41L/T69SSS/T215Y and M41L/T69SSS/L210W/R211K/L214F/T215Y were 10- and 18-fold more efficient than the wild-type enzyme, whereas the insertion alone had no effect on the unblocking reaction (43). Despite these large differences that point to M41L and T215Y as key determinants for ATP-dependent phosphorolytic activity, none of the recombinant viruses generated with those enzymes showed significant resistance to ddI in phenotypic assays (26). Taken together, these data suggest that the contribution of the ATP-dependent phosphorolytic activity to the low level resistance to ddI observed with SS RT is likely to be small and is probably due to the complex arrangement of drug resistance mutations found in the multidrug-resistant RT.

The efficiency of nucleoside analogue removal under physiological conditions depends on the rate of the reaction but also on the ability of dNTPs to inhibit it. HIV-1 RT bound to a chain-terminated template-primer and an incoming dNTP complementary to the next nucleotide on the template strand forms a stable ternary complex. A crystal structure of a ternary complex containing a dideoxyguanosine-terminated DNA primer complexed with a DNA template and dTTP is available (50) (Fig. 7). Under these conditions, the excision reaction is blocked. The concentrations of dNTP required to inhibit the unblocking reaction have been determined for a number of RTs displaying ATP-dependent phosphorolytic activity (i.e. D67N/K70R/T215F/K219Q, M41L/T215Y, M41L/T69SSS/T215Y, etc.) (9, 11, 13, 43). Although the dNTP concentration required to inhibit AZT-monophosphate removal was rather high (IC50 40 to >250 μM), the IC50 values obtained with d4T-, ddT-, and ddA-terminated primers were usually below 25 μM. These differences have been attributed to the presence of a bulky substituent at the 3’ position of the ribose in the AZT-terminated primers that sterically hinders the dNTP-binding site (termed the N site), reducing its accessibility by dNTPs (52). Under our assay conditions none of the mutations tested had a significant effect on the IC50 values for AZT and d4T removal. Furthermore, the presence of ATP or PPI had no effect on the inhibition by the next complementary dNTP.

The formation of ternary complexes has been monitored for wild-type BH10 RT and mutant M41L/T69SSS/T215Y with chemical footprinting techniques in the presence of increasing concentrations of the next complementary nucleotide and DNA-DNA complexes having AZT-terminating primers (44). The IC50 values obtained were 22 μM for the wild-type RT and 570 μM for the mutant containing the insertion. Higher IC50s were also observed with the mutant using DNA-DNA complexes having a ddT-terminated primer. However, large differences were not detected in our assays measuring RT suscepti-

### Table II

| Enzymes |  | Apparent Kd (dNTP) μM |
|---------|  |                      |
|         | Without foscarnet | With foscarnet |
| BH10    | 18.8 ± 8.5        | 10.8 ± 8.0       |
| BH10_SSSY | 87.9 ± 12.2     | 23.5 ± 9.2       |
| SS      | 106.3 ± 25.8      | 13.4 ± 5.6       |
| SS_250S | 129.3 ± 31.8      | 10.7 ± 2.5       |
| SS_Y215T | 58.4 ± 7.7       | 22.4 ± 12.2      |
| SS_Y215S | 81.4 ± 13.9     | 26.9 ± 7.5       |
| SS_Y215N | 109.4 ± 32.8    | 39.7 ± 10.5      |

Fig. 6. Determination of the apparent dNTP binding affinity in the ternary complex by gel mobility shift assays. The apparent Kd(dNTP) of the studied RTs was determined by incubating RT-DNA-DNA complexes containing a primer terminated with d4T in the presence of different concentrations of dNTP, and subjecting them to gel shift analysis. The positions of free DNA-DNA template-primer and bound in the ternary complex are shown on the right. Lanes 1–7 represent dATP concentrations of 4000, 800, 160, 32, 6.4, 1.28, and 0 μM, respectively. Lane C stands for a control reaction carried out without enzyme and without dNTP. The gels shown were obtained in the absence (left panel) or in the presence of 200 μM foscarnet (right panel).
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The IC50 values represent the means of two to five tests, each one performed six times. The fold increase in IC50 relative to wild-type HXB2 virus control carrying the RT sequence of BH10 is shown in parentheses. The reported values for BH10, BH10_T69SSS, SS, and SS_2S0S RTs were taken from Ref. 27.

| RTs          | AZT (μM) | d4T (nM) | d4C (nM) | 3TC (nM) | ddl (nM) |
|--------------|----------|----------|----------|----------|----------|
| BH10         | 6.6 × 10⁻³ | 0.24     | 0.23     | 0.56     | 1.26     |
| BH10_T69SSS  | 2.2 × 10⁻³ (0.3) | 0.54 (2.2) | 0.14 (0.6) | 1.84 (3.3) | 1.91 (1.5) |
| BH10_SSSS    | 0.043 (6.5) | 2.09 (8.7) | 1.56 (6.8) | 4.6 (8.2) | 2.09 (1.7) |
| SS           | 5.17 (786) | 2.23 (9.1) | 1.10 (4.7) | >20 (≥35) | 13.4 (10.6) |
| SS_2S0S      | 0.94 (143) | 5.94 (24.2) | 2.88 (12.2) | >20 (≥35) | 11.3 (8.9) |
| SS_D67N      | 3.54 (536) | 2.62 (10.9) | 2.65 (11.5) | >20 (≥35) | 9.54 (7.8) |
| SS_Y215T     | 6.6 × 10⁻³ (1) | 0.49 (2.1) | 2.41 (10.5) | >20 (≥35) | 11.6 (9.2) |
| SS_Y215S     | 0.019 (2.9) | 0.32 (1.3) | 1.49 (6.5) | >20 (≥35) | 6.31 (5.0) |
| SS_Y215N     | 5.8 × 10⁻³ (0.9) | 0.29 (1.2) | 1.29 (5.6) | >20 (≥35) | 4.93 (3.9) |

Thr at position 215 were somewhat more sensitive to inhibition than those with Tyr at this position. The reason for the discrepancy between determinations based on excision followed by extension reactions and those obtained by monitoring ternary complex formation relates to the presence of the PPI donor. Using an excess of foscarnet (a PPI analogue), all of the RTs displayed similar susceptibility to inhibition by the next complementary dNTP. The apparent Kd[dNTP] values were similar to the IC50 values reported for the excision reactions. These results suggest that despite being outside the dNTP-binding site and away from the catalytic pocket, Tyr-215 exerts a significant influence on the conformation of the dNTP-binding pocket. This effect can be counteracted in the presence of a PPI analogue.

Modeling studies have led authors to propose that Tyr-215 (and other TAMs) could participate in ATP binding to facilitate nucleotide analogue excision of chain-terminated primers when the primer terminus sits at the N site (52, 53). Tyr-215 is exposed to the solvent in the crystal structures of RTs containing the mutation T215Y (and other TAMs) (54, 55), and a direct participation of this residue in binding the adenine base of ATP through van der Waals’ interactions could occur. However, ATP cannot contact simultaneously the mutated side chains of residues 41 and 215. Available biochemical data are also not consistent with the hypothesis that ATP is bound more tightly by AZT-resistant RTs (15). The available crystal structures of AZT-resistant RTs are complexes of RT bound to a non-nucleoside RT inhibitor and show a conformation where the distance between fingers and thumb subdomains is even larger than in the “open” conformation of the binary complex. In this scenario, the possibility of Tyr-215 (and other TAMs) going through large conformational changes during polymerization that could explain their effects on phosphorolysis cannot be excluded. Another factor that adds further complexity to the study of the mechanism of ATP-based excision is that the removal reaction requires millimolar concentrations of ATP, which are high enough to compete with dNTPs for binding at the N site (56). Available crystal structures of binary and ternary complexes containing the wild-type RT show significant conformational changes at the fingers and thumb subdomains (Fig. 7) that result in a large movement of the β3-β4 hairpin loop that contains the insertion in the SS RT. In the closed configuration of the ternary complex, Lys-65 interacts directly with the γ phosphate of the incoming dNTP (50), whereas in the binary complex, this residue is at the tip of the fingers domain, far from the dNTP-binding site (51). In contrast, conformational changes affecting β strand 11a, which contains Thr-215, are less important. Our data suggest that under physiological conditions (i.e., high concentrations of ATP), the resistance mechanism is dependent on the orientation of the PPI moiety of the
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donor, which appears to be largely affected by the side chain of Tyr-215 and to a lesser extent by the conformation of the β3-β4 hairpin loop.

Previous studies have shown that interrupting nucleoside analogue therapy leads to replacement of the multidrug-resistant virus by wild-type strains (42, 57). Replacing Tyr with Thr involves two nucleotide substitutions. However, one mutation (i.e., T215N or Y181C) would be sufficient to abrogate thymidine analogue resistance, thereby facilitating an alternative resistance pathway. This reversion pathway has not been described in patients infected with strains having insertion-containing RTs, but it is relatively common in patients having the classical AZT resistance mutations (58, 59), where mutations in T215D, T215C, and T215S have a relatively low fitness cost in the absence of drugs and are often observed in transmitted virus (60, 61). Mutation D67N is very rare in insertion-containing RTs. Virus population dynamics studies have shown that in some isolates this mutation is detected very early together with T215Y and the insertion but is quickly replaced by D67S, suggesting that D67N confers a fitness loss to the multidrug-resistant virus. Our biochemical studies did not reveal any significant differences between the SS RT and mutant SS_D67N, whereas recombinant virus containing those enzymes were viable and replicated efficiently in SupT1 cells. Detailed studies are currently in progress to measure any possible fitness differences between both enzymes.

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Tania Matamoros, Sandra Franco, Blanca M. Vázquez-Alvarez, Antonio Mas, Miguel Ángel Martínez and Luis Menéndez-Arias

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