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Mutagenesis of the Glu-89 Residue in Human Immunodeficiency Virus Type 1 (HIV-1) and HIV-2 Reverse Transcriptases: Effects on Nucleoside Analog Resistance

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A Glu-89 → Gly alteration in the human immunodeficiency virus type 1 reverse transcriptase (RT) was previously shown to result in resistance to several dideoxynucleoside analogs and to phosphonoformic acid (PFA; foscarnet). This residue was altered to Ala, Val, Ser, Thr, Gln, Asp, Asn, or Lys, and the ddGTP and PFA sensitivities of the mutant RTs were measured. Replacements with Ala, Gly, Val, and Thr led to resistance to inhibition by ddGTP, while mutants with amino acid Ser, Gln, Asn, Asp, or Lys displayed only moderate or no resistance. A similar result was obtained with inhibition by PFA, except that the Asp-89 mutant also displayed resistance. Furthermore, the introduction of Glu-89 → Gly alteration into the RT of human immunodeficiency virus type 2 likewise rendered it resistant to both ddGTP and PFA.

The emergence of drug-resistant viruses in patients receiving long-term therapy is a major problem in the treatment of AIDS. Variants of human immunodeficiency virus type 1 (HIV-1) resistant to 3′-azido-3′-deoxythymidine (zidovudine; AZT), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxycytidine (ddC) have been isolated from patients receiving therapy (1, 7, 8, 14), while those resistant to AZT (2, 6) and pyridinone drugs (11) have also been isolated by selecting for growth in the presence of the drug in cell culture. It is important to know whether resistance will arise against new drugs and to know the frequency and types of mutations that are needed to render HIV resistant to a given drug. Furthermore, the identification of residues involved in drug resistance may throw light on the architecture of the catalytic site to help design better, efficacious drugs. Previously, we described an in vitro screen employing a bacterially expressed HIV reverse transcriptase (RT) to expedite the identification of mutations that render RT resistant to substrate analog drugs (12, 13, 15). The procedure yielded a variant RT, ddGTP^r-1, in which the alteration of a glutamic acid at the 89th position to glycine rendered the enzyme resistant to phosphonoformic acid (PFA; foscarnet) and several deoxynucleoside triphosphate (dNTP) analog inhibitors including ddGTP, ddATP, ddCTP, ddTTP, and 2′,3′-dideoxy, 3′-azidothymidine triphosphate (AZTTP). The mutant RT, unlike the wild-type enzyme, displayed a lack of preference for magnesium. On the basis of these results, we speculated that the 89th residue in the wild-type RT may be involved in dNTP binding (13). Although the sequence around the 89th residue is in a region of HIV RT that is not highly conserved among retroviral RTs, it is well-conserved among lentiviruses. In this communication, we describe the effects on nucleoside analog sensitivity of amino acid residues other than glycine at the 89th residue in HIV-1 RT and show that an identical mutation introduced into HIV-2 RT leads to a very similar phenotype.

Mutagenesis at the 89th codon. In trying to understand the basis for resistance to ddGTP and to PFA induced by the Glu-89 → Gly alteration in HIV-1 RT, we wished to determine the effect of various amino acid residues in the place of Glu-89. In order to perform site-directed mutagenesis, a 1.7-kb *Bam*HI-*Xba*I fragment containing HIV RT sequence was excised from the expression plasmid pHRTRX2 and cloned into M13. Mutagenesis was carried out by using Kunkel's procedure (5) with a mutant oligonucleotide that is fully degenerate with respect to the nucleotides encoding the 89th residue. The resulting clones were subjected to sequence analysis to identify the existence and type of mutations. Eight variant RTs were obtained, with replacements of Glu-89 by Ala, Val, Gln, Asp, Asn, Lys, Ser, or Thr. All the mutant RTs were recloned into the expression plasmid. *Escherichia coli* HB101 cells carrying each of the mutant plasmids, including the Glu-89 → Gly mutant, or the wild type were induced as described previously (16), and the extracts were assayed for RNA-dependent DNA polymerase activity. One microliter of the soluble extract (containing 2 μg of protein) was added to reaction mixtures containing 50 mM Tris · Cl (pH 8.0), 20 mM dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 10 μg of poly(rC) per ml, 5 μg of oligo(dG) per ml, 10 mM $MgCl_2$, and 10 μM dGTP ([α-^{32}P]dGTP;1 Ci/mmol), and the mixtures were incubated at 37°C for 60 min. The incorporation of radioactivity was measured after aliquots were spotted onto DE-81 paper, washed, and counted. Table 1 shows a comparison of these mutants with the wild type and the previously described E89G mutant enzyme. Gly and Lys resulted in highest reduction in activity (35 to 40% of wild-type activity), Ser, Thr, and Asn showed approximately 50% reduction, and the rest showed marginal to no decrease in polymerase activity (Table 1). The mutants showed no alteration in RNase H activity, and the stability and the levels of expression of the proteins were very similar to those of the wild type (data not shown).

Sensitivity of the E89 mutants of HIV-1 RT to ddGTP and PFA. We first tested the effect of mutagenesis at the 89th residue on resistance to ddGTP. The inhibition was measured by including increasing concentrations of ddGTP in

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TABLE 1. Levels of RNA-dependent DNA polymerase activity of mutant HIV-1 RTs

Amino acid alteration at position 89[a]	RNA-dependent DNA polymerase activity (% of wild type [Glu])	IC_{50} of:			
		ddGTP		PFA	
		μM	Fold increase	μM	Fold increase
Vector	1	ND	ND	ND	ND
Glu	100	0.20	1.0	10	1
Asp	71	0.70	3.5	340	34
Lys	35	1.00	5.0	80	8
Gly	39	3.10	15.5	20,000	2,000
Ala	105	2.05	10.2	800	80
Val	77	2.05	10.2	305	30
Ser	55	0.50	2.5	40	4
Thr	56	3.00	15.0	900	90
Gln	196	0.20	1.0	20	2
Asn	65	0.55	2.7	36	3

[a] Glu is the wild-type residue, and Gly is the residue found in the ddGTP^r-1 mutant.

the standard RT cocktail described above, and incubations were done for 10 min. The levels of inhibition by ddGTP were measured and 50% inhibitory concentrations (IC_{50}s) were estimated. As shown in Table 1, with the exception of glutamic acid and glutamine, any alteration at the 89th residue appears to result in some degree of resistance to ddGTP. However, interestingly, both hydrophobic amino acid residues tested (Ala and Val) resulted in a pronounced (10- to 17-fold) increase in the IC_{50} just as the original Gly mutation did. Charged amino acids (Lys, Asp, and Glu) displayed minimal or no increase in the IC_{50}s. Replacements with neutral, polar amino acids (Ser, Gln, and Asn) also seemed to have a minimal effect, with the exception of Thr, which showed a higher fold increase in the IC_{50}. Therefore, it appears that replacements of Glu-89 with hydrophilic or polar amino acids have minimal or no effect on the sensitivity to inhibition by ddGTP.

When tested with several inhibitors, the original mutant E89G was most resistant to ddGTP and PFA. Therefore, the panel of mutants were also tested for inhibition by PFA in the standard assay as described for ddGTP. The IC_{50}s were determined as described above, and the fold increases are shown in Table 1. The general pattern is the same: mutants with hydrophobic residues at the 89th position showing the least sensitivities and charged or polar amino acid residues showing wild-type-like sensitivity (with two exceptions: Asp [34-fold increase in IC_{50}] and Thr [90-fold increase in IC_{50}] showed a greater decrease in sensitivity).

Mutagenesis of HIV-2 RT. To compare results obtained here for HIV-1 RT with those for a related RT, we chose to introduce the same mutation into HIV-2 RT. First, an expression plasmid encoding a TrpE-HIV-2 RT fusion protein was created by cloning a 1.82-kb *Bsu*36I (flush-ended with Klenow enzyme)-*Hin*cII fragment from the pHIV-2 ROD clone (provided by Keith Peden, National Institutes of Health, Bethesda, Md.) into the *Sma*I site of the pATH2 vector. In the fusion gene thus created, the *trpE* sequences are followed by the HIV-2 sequence in which RT is flanked by seven residues of protease and 43 residues of integrase. The bacteria carrying this construct were induced for expression by tryptophan starvation, and the bacterial lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Figure 1 shows the accumulation of an approximately 112-kDa fusion protein in bacteria carrying the HIV-2 RT expression plasmid. Enzymatic assays of lysates showed that both RNA-dependent DNA polymerase and the RNase H activities (data not shown) were present.

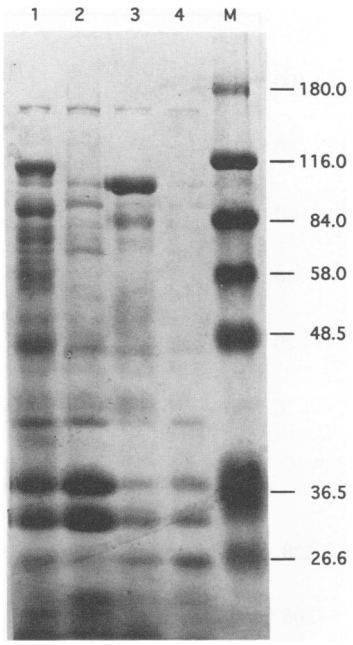

FIG. 1. Analysis of total proteins from *E. coli* bearing HIV-2 RT expression plasmid on SDS-PAGE. Lanes indicate lysates from *E. coli* induced by tryptophan starvation prepared from hosts carrying no plasmid (HB101) (lane 4); pATH 2 vector without any insert (lane 2); pRx3B2, the HIV-1 RT expression plasmid (lane 3); and pHIV-2 RT plasmid (lane 1). Lane M is molecular weight markers (in thousands) for proteins, as indicated on the right.

We first examined whether HIV-1 and HIV-2 RTs differed in their sensitivities to ddGTP and PFA. Lysates of bacteria carrying each of the expression plasmids were used in standard reactions to measure the extent of inhibition. The patterns of inhibition, shown in Fig. 2a, indicate that the two RTs are equally sensitive to inhibition by ddGTP (IC_{50}s of 0.2 μM for HIV-1 and 0.3 μM for HIV-2), while HIV-2 RT is more sensitive to inhibition by PFA than HIV-1 RT (IC_{50}s of 4.8 μM for HIV-1 and 0.7 μM for HIV-2).

A comparison of the amino acid sequence around the Glu-89 residue of HIV-1 and HIV-2 RTs showed that the E89 residue and the region around it are >90% conserved. In fact, the conservation extends to other lentiviruses as well. To examine whether the Glu-89 → Gly mutation introduced into HIV-2 will result in a similar drug resistance phenotype, we introduced the Glu → Gly alteration at the 89th residue of

TABLE 2. Sensitivities of wild-type and Glu-89 → Gly versions of HIV-2 RT to inhibition by ddGTP and PFA

Expression vector	IC_{50} of:			
	ddGTP		PFA	
	μM	Fold increase	μM	Fold increase
HIV-2 RT	0.50	1.00	1.8	1.0
HIV-2 RT E89G	10.80	21.60	90.00	50.00

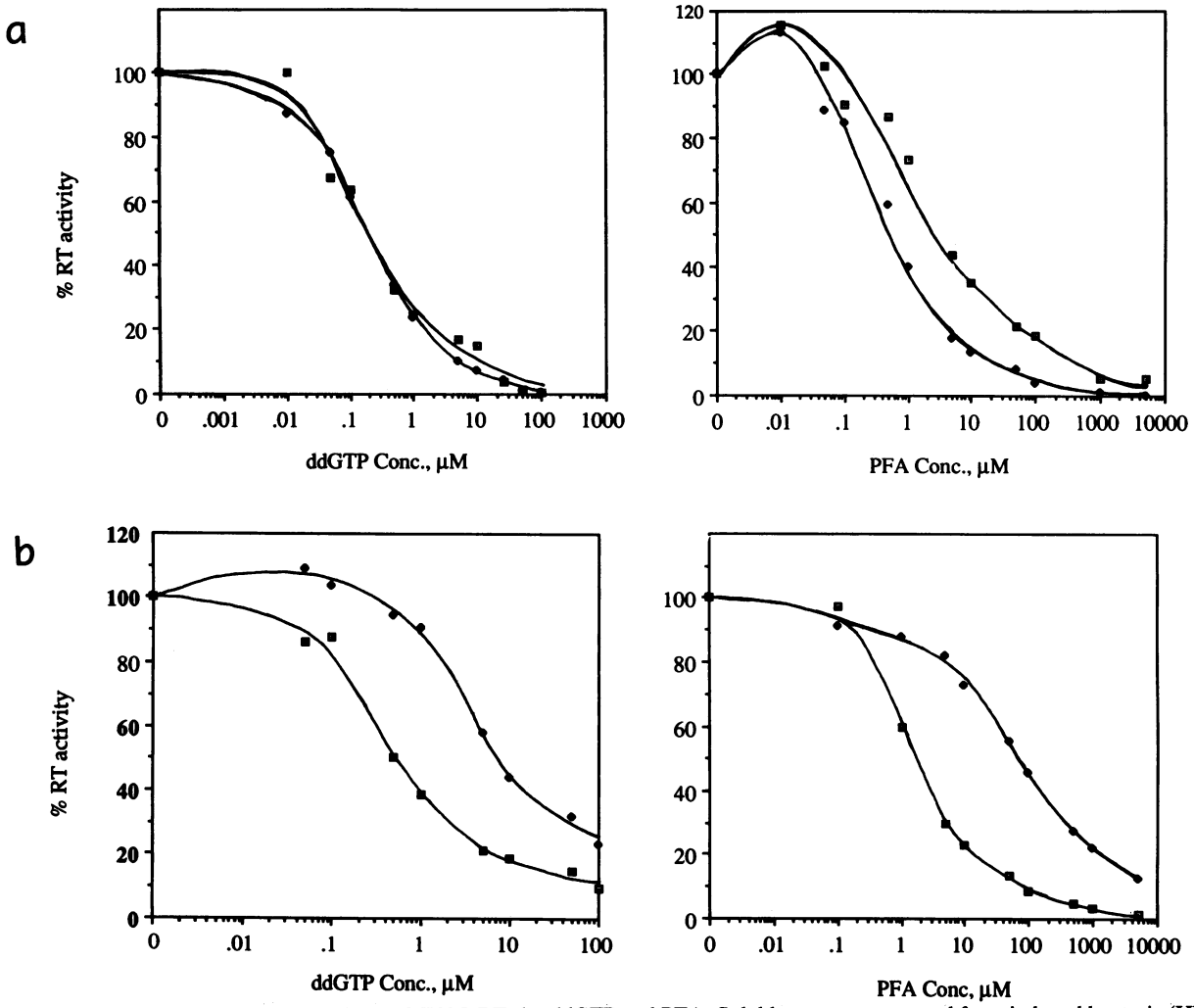

FIG. 2. Inhibition of HIV-1, HIV-2, and HIV-2 E89G RTs by ddGTP and PFA. Soluble extracts prepared from induced bacteria (HB101) carrying each of the above expression plasmids were tested for inhibition at various concentrations of the drug in a standard cocktail. Comparisons of HIV-1 (Rx3B2) (□) and HIV-2 (◆) (a) and HIV-2 (□) and HIV-2 E89G (◆) (b) RTs for sensitivity to inhibition by ddGTP and PFA are shown.

HIV-2 RT by oligonucleotide-directed mutagenesis of an M13 version of the HIV-2 RT clone. The mutation was reintroduced into the expression plasmid (HIV-2 RT E89G), the mutant protein was induced, and lysates were prepared and tested for sensitivity to inhibition by both ddGTP and PFA alongside the wild-type HIV-2 enzyme. Figure 2b shows the patterns of inhibition by ddGTP and PFA for both the wild-type and mutant HIV-2 RTs. Interestingly, Glu-89 → Gly alteration in HIV-2 RT caused a decrease in sensitivity to both drugs as did the Glu-89 → Gly version of HIV-1 RT (fold increases of ~22 for ddGTP and ~50 for PFA; Table 2).

We have shown here that the nature of the amino acid side chain at the 89th position of the HIV-1 RT sequence has a minimal effect on the enzymatic properties of this enzyme but plays a significant role in rendering it ddGTP or PFA resistant. It is interesting that no alteration of the 89th residue abolished the enzymatic activity of HIV-1 RT. In fact, when mutations A, E, S, T, and K were reconstructed into infectious molecular clones of HIV-1 and introduced into $CD4^+$ T lymphocytes, no deleterious effect on viral replication was seen (10a). A lack of conservation for a residue leading to profound effects upon genetic alteration is not entirely unprecedented, as mutations in RT of ddC-, ddI-, and AZT-resistant variants of HIV are also located in poorly conserved regions (1, 8, 14). The lack of conservation and the fact that alteration does not affect enzymatic activity may suggest that the 89th residue is not critical for catalytic function and that it probably serves some secondary function. On the other hand, although not necessarily conserved, the role of this residue on nucleoside analog sensitivity suggests a crucial position for this residue in the dNTP-binding pocket of this enzyme (see below).

Many of the replacements tested here lead to resistance to both ddGTP and PFA. The fact that the presence of hydrophobic residues at the 89th position leads to a greater resistance is intriguing. It is tempting to speculate that the presence of a hydrophobic or a hydrophilic residue determines the strength of interaction between the dNTP-binding pocket of RT and 2′-deoxy- or 2′,3′-dideoxynucleoside triphosphates. However, a possible role of the size of the side chain cannot be ruled out. Im et al. have recently shown that mutagenesis of Val-90 to Ala confers multidrug resis-

tance to HIV-1 RT (3). They found that there was an inverse correlation between the length of the hydrophobic residue and the level of resistance of the enzyme to AZTTP, PFA, and d4TTP (2',3'-dideoxy, 2',3'-didehydrothymidine triphosphate). They also found that the alteration led to a loss of magnesium preference (3).

These results strengthen our findings and suggest that the 89th residue, along with the 90th, may form a portion of the dNTP-binding pocket. Indeed, the recently published 3.5-Å (0.35-nm) resolution of the HIV-1 RT crystal structure allows one to approximately determine the location of the 89th residue with respect to the catalytic site. The HIV-1 RT structure has been likened to a right hand consisting of finger, palm, and thumb subdomains with the thumb linked to the RNase H domain via a connection subdomain (4). The template-primer DNA complex is thought to bind to a DNA-binding cleft formed by portions of the finger, palm, and thumb subdomains of p66 and the thumb subdomain of p51. While the clear identification of 89th residue is still not possible, according to the data of Kohlstaedt et al. (4), the 89th residue is located between the end of the B helix in the finger subdomain and the beginning of β strand 5 in the palm subdomain. Residues 66 through 75, which include most of the mutations identified in AZT- and ddI-resistant viruses, are located close to the template-primer site in the DNA-binding cleft. The physical nearness in the three-dimensional structure of Glu-89, residues 66 to 75 (1, 8, 14), and residues 185 and 186 (which are actually at the catalytic site) and the fact that the Glu-89 is at the juncture of the finger and palm subdomains suggest its likely participation in forming the template-binding site. Until proven, this remains speculative. Other interpretations of our results, such as a distance effect on another residue directly involved in binding to ddGTP, cannot be completely ruled out at present. Several residues involved in sensitivity of HIV-1 RT to AZTTP have been previously identified (9, 10). These include amino acid residues Asp-113, Ala-114, Tyr-115, Lys-55, and Gln-151. Furthermore, a separate set of residues, Asp-67, Asp-69, Lys-70, Leu-74, and Thr-215, lead to AZT and ddI resistance in HIV (1, 8, 14). It is interesting that most of these mutations along with Glu-89 are clustered in a 100-amino-acid region of HIV-1 RT. All or some of these residues together may form the nucleotide-binding pocket of the HIV RT.

Furthermore, our results show that the mutation that rendered HIV-1 RT resistant to ddGTP, when introduced into the RT of a related virus, brings about a very similar phenotype. This suggests that the Glu-89 residue plays similar roles in the RT of both these viruses, although the two differ significantly in their overall amino acid sequences.

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