Amino Acid Mutation N348I in the Connection Subdomain of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Multiclass Resistance to Nucleoside and Nonnucleoside Reverse Transcriptase Inhibitors[▽][†]

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We identified clinical isolates with phenotypic resistance to nevirapine (NVP) in the absence of known nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations. This resistance is caused by N348I, a mutation at the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Virologic analysis showed that N348I conferred multiclass resistance to NNRTIs (NVP and delavirdine) and to nucleoside reverse transcriptase inhibitors (zidovudine [AZT] and didanosine [ddI]). N348I impaired HIV-1 replication in a cell-type-dependent manner. Acquisition of N348I was frequently observed in AZT- and/or ddI-containing therapy (12.5%; $n = 48$; $P < 0.0001$) and was accompanied with thymidine analogue-associated mutations, e.g., T215Y ($n = 5/6$) and the lamivudine resistance mutation M184V ($n = 1/6$) in a Japanese cohort. Molecular modeling analysis shows that residue 348 is proximal to the NNRTI-binding pocket and to a flexible hinge region at the base of the p66 thumb that may be affected by the N348I mutation. Our results further highlight the role of connection subdomain residues in drug resistance.

Combinations of multiple drugs used for clinical treatment of human immunodeficiency virus type 1 (HIV-1) infections in highly active antiretroviral therapies (HAART) can dramatically reduce viral load, increase levels of CD4positive cells, improve survival rates, and delay the onset of AIDS. HAART typically includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (17). After prolonged therapy, however, an increasing number of treatment failures are caused by the emergence of multidrug-resistant (MDR) variants. For example, treatment with zidovudine (AZT) and dideoxynuleoside RT inhibitors such as didanosine (ddI) may result in the "Q151 complex" of clinical mutations in RT (A62V/V751/F77L/F116Y/Q151M) which causes high-level resistance to multiple NRTIs, AZT, ddI, zalcitabine (ddC), and stavudine (d4T) (21, 38). Another MDR complex of RT mutations is the "fingers insertion" complex that includes an insertion of two residues at the fingers subdomain of the p66 subunit of RT in the presence of AZT resistance mutations, e.g., M41L and T215Y (M41L/T69SSG/T215Y). This complex can emerge during combination treatment that includes NRTIs (10, 41) and confers resistance to multiple drugs by en-

hancing the excision reaction that causes resistance by unblocking NRTI-terminated primers (40). G333E or G333D polymorphisms with thymidine analogue-associated mutations (TAMs) and M184V have also been reported to facilitate moderate resistance to at least two NRTIs, AZT and lamivudine (3TC) (7, 22). RT mutations K103N, V106M, and Y188L are associated with resistance to multiple NNRTIs (1, 5). Since all NNRTIs bind at the same hydrophobic binding pocket, mutations in the binding pocket may result in broad cross-resistance between members of this family of drugs.

The presence of variants that are resistant to multiple drugs limits significantly the available therapeutic strategies and, even more profoundly, therapeutic options. However, so far all reports of viruses that acquire resistance to members of both families of RT inhibitors describe variants with multiple mutations at several residues that confer either NRTI or NNRTI resistance. Recently, Paolucci et al. reported that Q145M/L mutations confer cross-resistance to some NRTIs and NNRTIs (31, 32). Similarly, an NNRTI resistance mutation, Y181I, also confers resistance to d4T at the enzyme level (2). The frequency of these mutations in clinical isolates does not appear to be significant, according to the Stanford HIV resistance database (http://hivdb.stanford.edu/index.html); there is no deposition for Q145M/L, and Y181I has a prevalence of 0.02% in drug-naïve or NRTI-treated patients and 0.9% in NNRTI-treated patients.

We report here that N348I is a multiclass resistance mutation involved in resistance to both NRTIs and NNRTIs and present in a significant number of clinical isolates. Residue 348 is at the RT connection subdomain outside the region usually

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sequenced as the drug resistance assay in clinical settings. The role of connection subdomain mutations in AZT resistance has been highlighted recently by Pathak and colleagues (28). The present work shows that N348I confers resistance not only to the NRTI AZT but also to another NRTI, ddI, and two NNRTIs, nevirapine (NVP) and delavirdine (DLV). Importantly, we show that the N348I variant emerges frequently during chemotherapy containing AZT and/or ddI. To our knowledge, this is the first example of a clinically significant and high-prevalence multiclass RTI resistance mutation that highlights the need for extensive phenotypic and genotypic assays to detect novel mutations with important implications on future therapeutic strategies.

MATERIALS AND METHODS

Reagents and cells. AZT, ddI, ddC, and d4T were purchased from Sigma (St. Louis, MO). 3TC, DLV, and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). NVP, abacavir (ABC), and efavirenz (EFV) were generously provided by Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), GlaxoSmithKline (Philadelphia, PA), and Merck Co. Inc. (Rahway, NJ), respectively. Loviride was kindly provided by S. Shigeta, Fukushima Medical University (Fukushima, Japan). MT-2, SupT1, PM1, H9, Cos-7, and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously (14). Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated with phytohemagglutinin (PHA) for 3 days and grown in RPMI 1640 medium with 10% fetal calf serum and 10 U of interleukin-2 as described previously (15, 23).

Clinical isolates. Clinical isolates were obtained from fresh plasma of an HIV-1-infected patient attending the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, using MAGIC-5 cells. The isolates were stored at −80°C until use, and infectivity was measured as blue cell-forming units (BFU) of MAGIC-5 cells. The Institutional Review Board approved this study (IMCJ-H13-80), and written informed consent was obtained from the patient.

Viruses and construction of recombinant HIV-1 clones. An HIV-1 infectious clone, pNL101, was kindly provided by K.-T. Jeang (NIH, Bethesda, MD) and used for generating recombinant HIV-1 clones (15). A wild-type (WT) HIV-1, designated HIV-1$_{WT}$, was constructed by replacing the *pol*-coding region (nucleotides [nt] 2006 of ApaI site to 5785 of SalI site of pNL101) with the HIV-1 BH10 strain. The *pol*-coding region contains a silent mutation at nt 4232 (TTTAGA to TCTAGA; mutation is italicized) for generation of an XbaI unique site. The DNA fragments amplified by reverse transcription-PCR from the primary isolates were digested with appropriate restriction enzymes and cloned into pNL-RT$_{WT}$. The nucleoside sequences of the PCR-amplified fragments were verified with a model 3730 automated DNA Sequencer (Applied Biosystems, Foster, CA). Viral stocks were obtained by transfection of each molecular clone into Cos-7 cells, harvested, and stored at −80°C until use.

Sequencing analysis of HIV-1 RT region. Viral RNA was extracted from plasma and/or culture supernatant of clinical isolates and subjected to reverse transcription-PCR using a OneStep RNA PCR Kit (Takara Bio, Otsu, Japan). Nested PCR was subsequently conducted for direct sequencing. Primer pairs used for amplification of the DNA fragment from nt 2574 to 3333 of pNL101 were T1 (5′-AGGGGGAATTGGAGGTTT; nt 2393 to 2410) and T4 (5′-TTCT GTTAGTGCTTTGGTT; nt 3422 to 3404) for the first PCR and T12 (5′-CCAG TAAAATTAAAGCCAG; nt 2574 to 2592) and T15 (5′-TCCCACTAACTTCT GTATGTC; nt 3335 to 3315) for the second PCR (15). Primer pairs used for amplification of DNA fragment from nt 3288 to 4316 were 3244F (5′-AT GAACTCCATCCTGACAAATG; nt 3244 to 3265) and 4428R (5′-TGTA CAATCTAATTGCCATAT; nt 4428 to 4407) for the first PCR and 3288F (5′-CCAGAAAAAGACAGCTGGACT; nt 3288 to 3308) and 4316R (5′-TG GCAGATTAAAATCACTAGCC; nt 4316 to 4295) for the second PCR (13). The nested PCR products were then subjected to the direct sequencing of the entire RT coding region, and some PCR products were further analyzed with clonal sequence determination as described previously (13, 15).

Drug susceptibility assay. HIV-1 sensitivity to various RTIs was determined in triplicate using MAGIC-5 cells as described previously (14). MAGIC-5 cells were infected with diluted virus stock (100 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed, and stained with X-Gal (5-bromo-4-chloro-3-indolyl-βD-galactopyranoside). The stained cells were counted under

a light microscope. Drug concentrations reducing the cell number to 50% of that of the drug-free control (EC$_{50}$) were determined by referring to the dose-response curve.

Competition assay of HIV-1 replication. MT-2, SupT1, PM1, and H9 cells (2.5×10^5 cells/5 ml) and PHA-stimulated PBMCs (2.5×10^6 cells/5 ml) were infected with each virus preparation (500 BFU) for 4 h. The infected cells were then washed and cultured in a final volume of 5 ml. Culture supernatants (100 μl) were harvested from days 1 to 7 after infection, and the p24 antigen amounts were quantified (27).

Freshly prepared H9 cells (3×10^5 cells/well) were exposed to the mixture of viral preparations (300 BFU) and cultured to compare their replicative capacities, as previously described (15). On day 1 in culture, one-third of the infected H9 cells were harvested and washed twice with phosphate-buffered saline, followed by DNA extraction. Purified DNA was subjected to nested PCR to sequence the HIV-1 RT genes. The supernatant of the viral culture was transferred to uninfected H9 cells at 7-day intervals, and the cells harvested at each passage were subjected to direct DNA sequencing of the HIV-1 RT gene. Population change of the viral mixture was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed in all infectious clones used in this assay.

Molecular modeling studies. The SYBYL and O programs were used to prepare molecular models of the complexes of WT and N348I HIV-1 RT with DNA, NVP, and the triphosphates of AZT and ddI. Starting atomic coordinates of HIV-1 RT in complex with DNA were obtained from the structures described by Tuske et al. (40), Sarafianos et al. (36), and Huang et al. (20) (Protein Data Bank [PDB] code numbers 1T05, 1N6Q, and 1RTD, respectively). Because there is no available structure of RT in complex with both NNRTI and DNA, we used structures of RT in complex with NNRTI to obtain initial coordinates of the NNRTI-binding pocket (9, 12). Specifically, we used the coordinates of the two β-sheets of the polymerase active site (β6-β9-β10 that contains the three catalytic aspartates and the YMDD motif as well as β12-β13 of the primer grip) to replace the corresponding regions in the RT-DNA complex. The N348I side chain mutation was manually modeled in the p66 subunit, and all structures were optimized using energy minimization protocols in SYBYL. The triphosphates of AZT and ddI were built based on the structures of AZT monophosphate and dTTP in PDB 1N6Q (36) and 1RTD (20) or of TDF diphosphate in the ternary complex of HIV-1 RT/DNA/TFV-DP, PDB 1T03 (40). The coordinate vector of the resulting structures was varied using a minimization procedure to minimize the potential energy by relieving short interatomic distances while maintaining structural integrity.

RESULTS

Resistance to NNRTIs observed in HIV-1 isolates. The clinical history of the patient is summarized in Fig. 1 and includes the variation of genotypic and phenotypic drug resistance profiles of sequential isolates with time (see also Table S1 and Fig. S1 in the supplemental material). In spite of the combination therapy, little immunologic and virologic response was observed; at time point 2, the CD4 count was 25/μl, and the plasma HIV-1 RNA levels were 2.1×10^6 copies/ml. However, no known drug resistance mutations associated to both NRTIs and NNRTIs were detected in the RT region at this point (Fig. 1B). Due to poor adherence, upon changing the regimen we observed only partial suppression of viral replication and limited increase in the CD4 count. TAMs with N348I accumulated during time points 3 to 6 (Fig. 1). In February 2000, the treatment was interrupted due to severe adverse effects, resulting in a rebound of viral load. In July 2000, the same therapy was resumed for approximately 1 year. No drug resistance-associated mutations were detected upon initiation of this therapy (time point 7). At time point 8, mixtures of two amino acid insertions at codon 69 with TAMs and N348I were detected, although these mutations disappeared after the treatment interruption at time point 10.

Interestingly, HIV-1 isolates at time points 5 and 6 showed resistance to NVP (44- and 25-fold, respectively) and to DLV
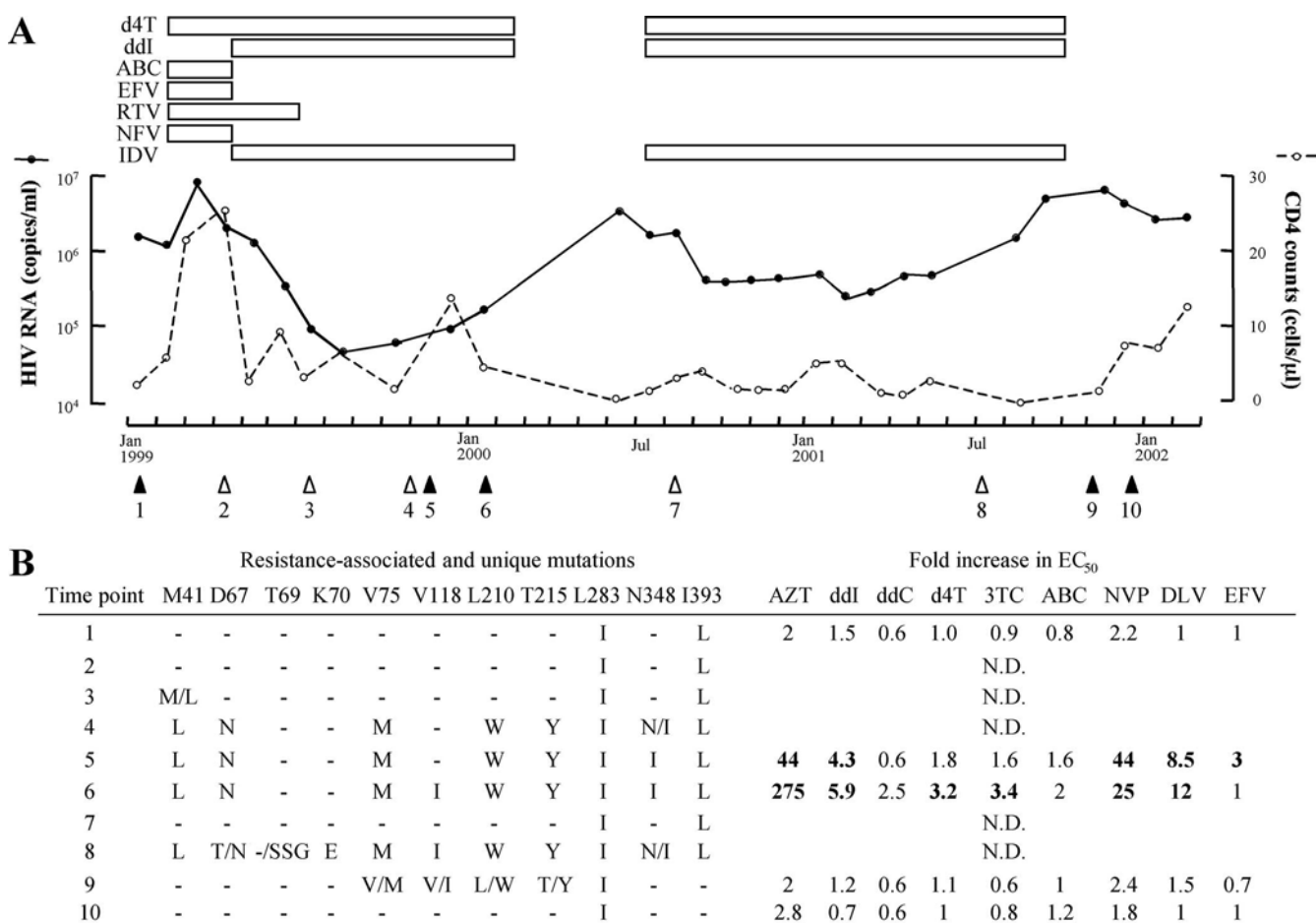
FIG. 1. The course of patient and drug resistance profiles of clinical isolates obtained from the patient. (A) The drug treatment history is indicated at the top of the graph. The virologic responses represented by plasma viral load and CD4 counts of peripheral blood are shown. Open triangles indicate the time points of genotypic assays. Closed triangles indicate the time points of isolation of clinical isolates for genotypic assays (also see Fig. S1 in the supplemental material) and phenotypic assays (also see Table S1 in the supplemental material showing actual EC_{50} values as mean values and standard deviations from three independent experiments). From February to June 2000 and after October 2001, the chemotherapy was interrupted due to severe adverse effects. (B) The viruses acquired NRTI resistance mutations sequentially as shown. Susceptibility to compounds tested in at least three independent experiments is shown as the relative increase in the EC_{50} compared to HIV-1$_{WT}$ obtained from a pNL4-3-based plasmid. An increase larger than 3.0-fold is indicated in bold. NRTI or NNRTI resistance mutations were reported in the HIV drug resistance database maintained by International AIDS Society 2006, the Stanford University (Stanford, CA) and Los Alamos National Laboratory (Los Alamos, NM), http://hivdb.stanford.edu/and http://resdb.lanl.gov/Resist_DB/, respectively. RTV, ritonavir; NFV, nelfinavir; IDV, indinavir.

(8.5- and 12-fold, respectively) but lacked any known NNRTI resistance-associated mutations except for L283I, which influences susceptibility of NNRTIs when combined with I135L/M/T (6) (Fig. 1B). However, L283I was detected at all points without I135L/M/T even in phenotypically sensitive viruses; therefore, it is unlikely that this single mutation is involved in the resistance. After the interruption at time points 9 and 10, the majority of HIV-1 detected in the plasma reverted to WT and was susceptible to all RTIs tested. The patient was previously treated with a regimen containing EFV, not NVP, for several months prior to the appearance of the N348I mutation. Importantly, this mutation was not detected in genotypic assays during treatment with EFV, but it was first detected 6 months after removal of EFV and use of ddI in the following regimen. Phenotypic and genotypic information at time point 5 shows that resistance to NVP and DLV was present while the patient

was on a regimen that did not include any NNRTIs and in the absence of any known NNRTI resistance-related mutations. Thus, it is unlikely that the phenotypically identified NNRTI resistance in the patient was induced by the previous EFV-containing therapy.

RT C-terminal region confers NVP resistance. To identify the mutation(s) responsible for the resistance to NVP and DLV, we constructed chimeric clones with cDNA fragments of the RT region derived from the clinical isolates. Briefly, the N-terminal (amino acids 15 to 267) and C-terminal (amino acids 268 to 560) RT coding regions of clinical isolates were PCR amplified separately and used for replacement of the corresponding regions in the WT sequence of pNL-RT$_{WT}$. These chimeric clones were then examined for their susceptibility to RTIs (Table 1). Only the clones containing the C-terminal region derived from CL-6 isolated at time point 6 and

TABLE 1. Susceptibility of chimeric HIV-1 clones with N- and/or C-terminal RT region substitutions

RT-replaced region		EC$_{50}$ (fold increase)[a]				
N terminus[b]	C terminus[c]	AZT	ddI	NVP	DLV	EFV
WT[d]	WT	0.038 ± 0.012	2.6 ± 1.04	0.05 ± 0.01	0.03 ± 0.01	0.003 ± 0.001
CL-6[e]	CL-6[e]	3.37 ± 0.97 (**89**)	14.3 ± 0.58 (**5.5**)	1.2 ± 0.21 (**24**)	0.16 ± 0.02 (**5.3**)	0.007 ± 0.004 (2.3)
CL-9[f]	CL-9[f]	0.04 ± 0.01 (1.1)	2.3 ± 1.21 (0.9)	0.13 ± 0.07 (2.6)	0.06 ± 0.02 (2)	0.004 ± 0.002 (1.3)
CL-6	WT	1.24 ± 0.34 (**33**)	4.6 ± 1.50 (1.8)	0.12 ± 0.06 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)
WT	CL-6	0.19 ± 0.04 (**5**)	13.7 ± 2.31 (**5.3**)	1.67 ± 0.23 (**33**)	0.39 ± 0.06 (**13**)	0.006 ± 0.002 (2)
CL-6	CL-9	1.50 ± 0.95 (**39**)	5.9 ± 1.21 (2.3)	0.10 ± 0.05 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)

[a] The data shown are mean values ± standard deviations obtained from the results of at least three independent experiments, and the relative increase in the EC$_{50}$ values for recombinant viruses compared with WT is shown in parentheses. Bold indicates an increase in EC$_{50}$ value greater than threefold relative to the WT.
[b] RT N-terminal region contains mainly the domains of finger and palm and partially thumb (amino acid positions 15 to 267).
[c] RT C-terminal region contains domains of thumb, connection, and RNase H (amino acid positions 268 to 560).
[d] DNA fragment is identical to pNL-RT$_{WT}$.
[e] N- and C-terminal regions of CL-6 contained T39A/M41L/K43E/D67N/V75M/V118I/I132V/L210W/T215Y and N348I/I393L in their coding regions, respectively (see also Fig. S1 in the supplemental material).
[f] No resistance-associated mutations were observed in either the N- or C-terminal region of CL-9 (also see Fig. S1 in the supplemental material).

showed resistance (Fig. 1; see also Fig. S1 in the supplemental material) to NVP and DLV. Interestingly, the C-terminal region also conferred resistance to AZT and ddI even in the absence of AZT resistance mutations that normally reside at the N-terminal region within amino acids 41 to 219. Recently, mutations in the connection subdomain, including G335D, N348I, and A360T, have been shown to confer AZT resistance (28). In these clinical isolates the C-terminal region contained four unique mutations in the connection subdomain: G335D, N348I, A360T, and I393L (see Fig. S1 in the supplemental material). G335D and A360T were continuously observed at every time point and are polymorphisms related to subtype D. Since these isolates showed no phenotypic resistance (Table 1 and Fig. 1B), it is unlikely that G335D and A360T are involved in the resistance, at least in subtype D. I393L was also continuously detected from time point 1 but disappeared after the treatment interruption at time point 9 (Fig. 1) while N348I appeared only from time points 4 to 6 and at point 8 under treatment.

To further clarify the effect of mutations at residues 348 and 393 on drug resistance, we generated the N348I and/or I393L mutations in the C-terminal region by site-directed mutagenesis on a pNL-RT$_{WT}$ background. Consistent with the phenotypic experiments and the experiments with chimeric viruses, we found that the N348I substitution conferred resistance to AZT, ddI, NVP, and DLV. In contrast, we found that the I393L mutation caused no significant resistance by itself (Table 2). Furthermore, the combination of I393L with N348I did not show any significant increase in NVP resistance compared to N348I alone.

To address whether N348I further increases the level of AZT resistance in the presence of TAMs, we examined the effect of N348I on AZT susceptibility in the presence or absence of the classical AZT resistance mutations M41L/T215Y. M41L/T215Y or N348I showed only moderate resistance to AZT whereas a combination of M41L/T215Y and N348I further enhanced AZT resistance (Table 2). These data demonstrate that the N348I mutation is responsible for this cross-resistance to multiple members of the NRTI and NNRTI families and enhances AZT resistance induced by TAMs.

Viral replication kinetics. Since N348I and I393L immediately disappeared after cessation of HAART, we examined

whether these mutations have an effect on viral replication kinetics using the p24 antigen production assay and a competitive HIV-1 replication assay (CHRA). In the p24 antigen production assay, acquisition of N348I drastically impaired replication in MT-2 and SupT1 cells (Fig. 2A and B). However, a moderately low reduction of replication kinetics was observed in PM1, H9 cells, and PHA-stimulated PBMCs (Fig. 2C, D, and E). HIV-1 carrying the mutation I393L (HIV-1$_{I393L}$) showed comparable replication kinetics in all cells tested. A combination of I393L with N348I showed no apparent change of replication kinetics in MT-2, SupT1 cells, and PHA-stimulated PBMCs (Fig. 2A, B, and E) and reduction in PM1 cells (Fig. 2C) compared to N348I alone. CHRA was performed for further comparison of replication kinetics in H9 cells. During 6 weeks in culture, we observed little difference in viral replication in H9 cells (Fig. 2F). A lack of an effect of I393L on the replication of N348I was confirmed by CHRA (Fig. 2G). These results indicate that N348I impairs viral replication in a cell-type-dependent manner and that I393L exerts little effect on viral replication of either the WT or N348I clones. Thus, I393L appears to be one of the specific polymorphisms for this isolate.

Insertion at 69 and N348I. At time point 8 we detected the transient presence of the fingers insertion mutation, a 2-amino-acid insertion at codon 69 in the presence of TAMs known to confer resistance to NRTIs by enhancing the excision reaction (3) (Fig. 1). Interestingly, at time point 8 WT N348 coexisted with resistant I348. To address whether these two MDR mutations were introduced onto the same RNA genome, we carried out clonal sequence analysis of PCR products. The results show that the fingers insertion and the N348I mutations were randomly introduced; seven, three, one, and six clones (n = 17) contained both mutations, the fingers insertion only, N348I only, and no mutation or insertion, respectively, in the background of TAMs (Table 3). In previous studies the fingers insertion complex emerged with the K70E mutation that was selected in vitro with adefovir (8) and β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (18), and it conferred low level resistance to TDF, ABC, and 3TC (39). The effect of K70E on resistance or enzymatic activity influenced by the fingers insertion remains to be elucidated. These results suggest that there is no correlation between the N348I and the

TABLE 2. Drug susceptibilities of HIV-1 variants constructed by site-directed mutagenesis

Mutation[a]	EC$_{50}$ (fold increase)[b]										
	NRTI							NNRTI			
	AZT	ddI	ddC	d4T	3TC	ABC	TDF	NVP	DLV	Lovride	EFV
WT	0.035 ± 0.01	2.3 ± 0.14	0.7 ± 0.13	3.6 ± 1.36	2.1 ± 0.2	3.4 ± 0.14	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	1.4 ± 0.38	0.003 ± 0.0008
N348I	0.24 ± 0.04 **(6.9)**	12 ± 1.0 **(5.2)**	0.74 ± 0.58 (1.1)	2.9 ± 0.21 (0.8)	1.7 ± 0.36 (0.8)	3.4 ± 1.11 (1)	0.02 ± 0.01 (0.7)	1.07 ± 0.06 **(27)**	0.22 ± 0.04 **(5.5)**	2.4 ± 0.35 (1.7)	0.005 ± 0.0005 (1.7)
I393L	0.06 ± 0.01 (1.7)	2 ± 1.37 (0.9)	0.42 ± 0.23 (0.6)	1.8 ± 1.21 (0.5)	1.5 ± 0.74 (0.7)	2.4 ± 0.95 (0.7)	0.02 ± 0.01 (0.7)	0.05 ± 0.01 (1.3)	0.04 ± 0.01 (1.0)	2.2 ± 0.4 (1.6)	0.003 ± 0.001 (1)
N348I/I393L	0.23 ± 0.03 **(6.6)**	11.3 ± 1.53 **(4.9)**	0.49 ± 0.01 (0.7)	4.2 ± 1.12 (1.2)	1.7 ± 0.40 (0.8)	2.7 ± 0.26 (0.8)	0.02 ± 0.01 (0.7)	1.02 ± 0.51 **(26)**	0.28 ± 0.06 **(7)**	2.6 ± 0.42 (1.8)	0.005 ± 0.001 (1.7)
M41L/I393L	0.28 ± 0.06 **(8)**	4.5 ± 1.55 (2)	ND	ND	1.3 ± 0.25 (0.6)	ND	ND	0.05 ± 0.01 (1.3)	0.04 ± 0.02 (1)	ND	0.002 ± 0.0004 (0.7)
M41L/T215Y/ N348I	1.37 ± 0.21 **(39)**	9.9 ± 0.99 **(4.3)**	ND	ND	1.4 ± 0.20 (0.7)	ND	ND	1.11 ± 0.69 **(28)**	0.15 ± 0.06 **(3.8)**	ND	0.002 ± 0.0004 (0.7)

[a] See Materials and Methods for the construction of clones.
[b] Data are means ± standard deviations from at least three independent experiments. The relative increase in the EC$_{50}$ value compared with that in HIV-1$_{WT}$ is given in parentheses. Boldface indicates an increase greater than threefold. ND, not determined.
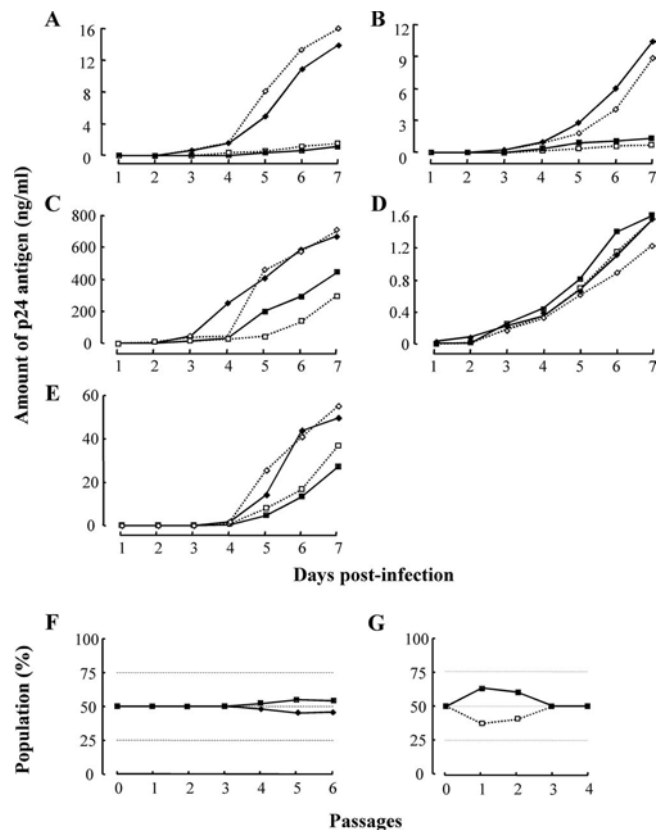

FIG. 2. Viral replication kinetics. Production of p24 antigen in culture supernatant was determined with a commercially available p24 antigen kit. Profiles of replication kinetics (p24 production) of HIV-1$_{WT}$ (closed diamonds), HIV-1$_{N348I}$ (closed squares), HIV-1$_{I393L}$ (open diamonds) and HIV-1$_{N348I/I393L}$ (open squares) were determined with MT-2 (A), SupT1 (B), PM1 (C) and H9 cells (D) and PHA-stimulated PBMCs (E). Representative results from at least two (or three) independent single determinations of p24 production with newly titrated viruses are shown. A competitive HIV-1 replication assay was performed in H9 cells to compare the replication kinetics of HIV-1$_{WT}$ (closed diamond) and HIV-1$_{N348I}$ (closed squares) (F) and of HIV-1 N348I (closed squares) and HIV-1$_{N348I/I393L}$ (open square) (G).

finger insertion mutations. Because our studies show that N348I does not confer d4T resistance, we speculate that the fingers insertion mutation was introduced to overcome the drug pressure by d4T.

TABLE 3. Sequences of HIV-1 RT-coding region of clinical samples

No. of clones[a]	Resistance-associated and unique mutation at the indicated position									
	M41	D67	T69	K70	V75	V118	L210	T215	N348	I393
5	L	N			M	I	W	Y		L
3	L	T	SSG	E	M		W	Y	I	L
3	L	T	SSG	E	M	I	W	Y	I	L
2	L	T	SSG	E			W	Y		L
1	L	T	SSG	E			W	Y	I	L
1	L	T	SSG	E	M	I	W	Y		L
1	L				M					
1	L				M	I	W	Y	I	L

[a] The PCR product at time point 8 was subcloned and sequenced (n = 17).

TABLE 4. Frequency of N348I acquisition in clinical isolates

| Treatment group | No. of isolates (%) | | P value[a] |
	Total in group	With N348I	
AZT and/or ddI	48	6 (12.5)	<0.0001
AZT	22	2 (9.1)	0.011
ddI	16	2 (12.5)	0.006
AZT/ddI	10	2 (20)	0.002
Control	183	0	
Antiretrovirals with neither AZT nor ddI	55	0	
No antiretrovirals	128	0	
Deposited in Los Alamos database	328	3 (0.9)	0.0002

[a] The P value was determined by the Fisher's exact test. For the AZT and/or ddI treatment groups, values were compared with the control group. The P value for isolates deposited in the Los Alamos database was determined based on a comparison with the AZT and/or ddI treatment group.

Prevalence of N348I. We obtained viral specimens from 231 infected patients who visited our clinical center from May 1997 to July 2003 and analyzed HIV-1 sequences by direct sequencing (Table 4). The viral specimens were classified in two groups: (i) those from patients treated with AZT and/or ddI ($n = 48$) and (ii) those from patients treated by regimens with neither AZT nor ddI (control group, $n = 183$). The group treated with AZT and/or ddI was further divided into three subgroups based on the treatment received: with AZT, with ddI, and with the AZT/ddI combination (Table 4). During chemotherapy containing AZT ($n = 22$), ddI ($n = 16$), or the combination of AZT and ddI ($n = 10$), two patients each harbored HIV-1 with the N348I mutation. Acquisitions of N348I in all of the subgroups was statistically significant ($P = 0.011$, 0.006, and 0.002, respectively). In contrast, none of the patients in the control group ($n = 183$) harbored N348I variants. Only three variants with N348I are deposited in the Los Alamos HIV sequence database that includes subtypes B, D, and CRF14 (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html). Thus, prevalence of N348I was statistically significant in the group treated that received chemotherapy containing AZT and/or ddI ($P < 0.0001$).

Because at present the numbers of NVP- or DLV-containing regimens without AZT and/or ddI are limited in our cohort ($n = 6$ or $n = 0$, respectively), we were not able to detect acquisition of N348I in these groups. Acquisition of N348I was observed in two patients treated with EFV (Table 5). Notably, these two patients were simultaneously treated with AZT and ddI, suggesting that the significance of EFV treatment for the emergence of N348I remains unknown.

Profiles of patients infected with HIV-1 containing the N348I mutation. We further analyzed the profiles of HIV-1

TABLE 5. Profiles of patients infected with HIV-1 containing the N348I mutation

| Patient | Subtype of RT region[a] | Antiretroviral treatment | Duration (mo) | HIV RNA (copies/ml) | N348I | RT mutation(s) by region | |
						Polymerase subdomain	Connection subdomain[d]
Case 1[b]	D	d4T, ddI, IDV	6	6.1×10^4	+/−	M41L, D67N, V75M, L210W, T215Y	G335D, A360T
		d4T, ddI, IDV	7	ND[c]	+	M41L, D67N, V75M, L210W, T215Y	G335D, A360T
Case 2	B	AZT, ddC, NFV	1	7.9×10^3	−		A360T
		AZT, ddC, NFV	4	9×10^3	−		A360T
		AZT, ddC, NFV	6	1.2×10^4	+/−	T215N/S/Y	A360T
		AZT, ddC, NFV	10	3.5×10^4	+	D67N, K70R, T215Y[e]	A360T
		d4T, 3TC, RTV, SQV	8	<50	ND	ND	ND
Case 3	B	AZT, 3TC, RTV, SQV	7	3.5×10^5	−		A360T, A376T
		AZT, 3TC, RTV, SQV	8	1.9×10^5	+	M41L, D67N, T69D, M184V, L210L/W, T215Y	A360T, A376T
		None (interruption)	7	1.2×10^5	+	M41L, D67N, T69D, M184M/V, L210L/W, T215Y	A360T, A376T
		ABC, EFV, RTV	3	60	ND	ND	ND
Case 4	B	AZT, 3TC, ddI, EFV	3	1.7×10^3	+	M184V	
Case 5	B	d4T, ddI, RTV, SQV	23	9.9×10^3	+	M41L, L210W, T215Y	
		AZT, ddI, RTV, SQV	3	6.3×10^4	+	M41L, T69D, L210W, T215Y, K219R	
		None (interruption)	7	1.8×10^5	−		
		ABC, TDF, LPV, EFV	7	<50	ND	ND	ND
Case 6	B	AZT, ddI, EFV	3	180	−		ND
		AZT, ddI, EFV	5	540	+/−	T215T/Y	ND
		AZT, ddI, EFV	6	1.1×10^4	+	T215Y	
		None (interruption)	2	2.4×10^5	−		
		d4T, 3TC, LPV	8	<50	ND	ND	ND

[a] The RT regions were sequenced and subjected to subtype analysis (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).
[b] This patient is described in this study.
[c] ND, not detectable.
[d] G335D is an observed polymorphism in subtype D. A360I/V and A376S were reported to be AZT-resistant mutations (24).
[e] Phenotype assays were performed at 10 months for a regimen combining ddC, AZT, and NFV; resistance to AZT, ddI, and NVP was induced 52-, 6.8-, and 8.3-fold, respectively.
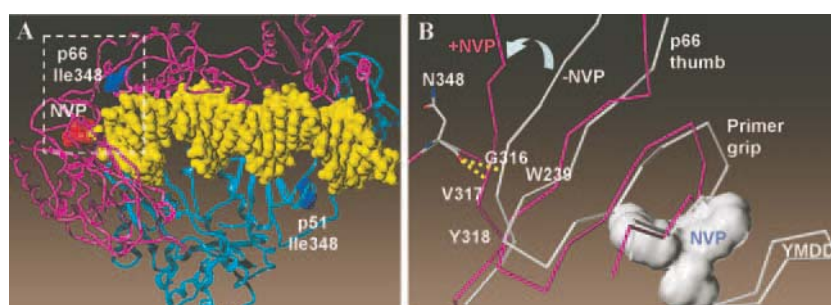
FIG. 3. Location of N348I in the modeled HIV-1 RT with NVP. (A) The N348I mutation (blue Van Der Waals volume) is shown in the connection subdomains of both p66 (purple) and p51 (cyan) subunits. The 348 residue of the p51 subunit is distant from the nucleic acid, shown as yellow Van Der Waals surfaces. In the p66 subunit (purple) the 348 residue is in a position to affect the flexibility of the p66 thumb, which in turn might affect binding of the nucleic acid. NVP is shown bound at the NNRTI binding pocket (red Van Der Waals volume). Magnification of the frame area of the enzyme is shown in panel B. (B) The main chain C=O of N348 is shown to interact with the N-H of 317 (yellow broken line) through a hydrogen bond interaction. Binding of NVP (white ball) repositions the p66 thumb subdomain with respect to (i) the polymerase active site (β6-β9-β10) that contains the three catalytic aspartates and the YMDD motif and (ii) the primer grip (β12-β13) of p66. The movement of the thumb subdomain is in a hinge-like motion that is based at the position where residue 348 interacts with residue 317.

with N348I from the six infected patients described in Table 4. The results of this analysis are shown in Table 5. The RT regions were sequenced and subjected to analysis with the software Genotyping, which uses the BLAST algorithm to determine homologies with known subtypes (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). HIV-1 variants in case 1 belonged to subtype D, and the others belonged to subtype B. All six patients received therapy containing AZT and/or ddI. Among them, two patients (cases 4 and 6) were under therapy with EFV. However, none of them was treated with NVP or DLV. The five N348I-containing variants were in the presence of TAMs that emerged during the therapies. TAMs in case 1 and some TAMs (M41L, L210W, and T215Y) in case 5 seemed to be induced by d4T, not by AZT. In case 3, the 3TC resistance mutation M184V that attenuates TAM-induced AZT resistance (24) was present together with N348I. Similarly, in case 4, M184V may confer AZT hypersusceptibility. In case 6, N348I was present together with a classical AZT resistance mutation, T215Y. Thus, except for case 5, even under AZT-containing therapy, the HIV-1 resistance level to AZT and ddI seemed to be intermediate and weak, respectively. Additionally, viral load in cases 2, 3, 5, and 6 dramatically decreased after introduction of a new regimen without AZT and/or ddI. These results indicated that N348I may enhance AZT resistance and at least act as a primary mutation for ddI.

In these six patients, HIV-1 with the G335D mutation was observed only in case 1. In the Los Alamos HIV sequence database, G335D has been observed in 77% of subtype D HIV-1 isolates ($n = 35$). A360T was detected in two isolates of subtype B and one isolate of subtype D and was observed in 13 and 51% of drug-naïve isolates of subtypes B and D, respectively. This suggests that A360T is also one of the polymorphisms. The A360V or A360I mutation has been reported to have a modest effect on AZT resistance (28). Meanwhile, none of N348I-containing subtype B variants ($n = 5$) had mutations associated with AZT resistance in the connection subdomain (28) (Table 5).

Molecular modeling. Residue 348 is located close to the hinge site of the thumb subdomain. Mutations at the virus level affect both subunits of RT. Figure 3 shows that residue 348 of the p51 subunit is located remotely from the polymerase active site (~60 Å) and from the NNRTI binding pocket (~55 Å). Furthermore, it is not in close proximity to the interface of the two subunits (~20 Å) or the DNA in the nucleic acid binding cleft (~15 Å). On the other hand, residue 348 of the p66 subunit is proximal to the NNRTI-binding site and the nucleic acid binding cleft. These relative distances suggest that it is more likely that the interactions involve mainly residue 348 of the p66 subunit. Subunit-specific biochemical analysis would determine the precise contribution of the N348I mutation in each subunit to the drug resistance phenotype. In the p66 subunit, the main chain of the 348 residue interacts through a hydrogen bond with the main chain of V317 of the p66 thumb subdomain (Fig. 3). To determine the degree of flexibility of this part of the structure of RT, we superposed 23 structures of RT complexes. The comparison revealed measurable differences. The length of the amide bond between the main chain C=O of residue 348 and N-H of V317 varies considerably (from 2.5 to 3.6 Å), suggesting a flexibility at the junction of the connection, thumb, and palm subdomains. It is likely that the N348I mutation affects the interactions of this residue with a number of neighboring residues. In the RT/DNA/deoxynucleoside triphosphate or RT/DNA/TDF structures of ternary catalytic complexes (PDB code 1RTD or 1T05, respectively), the change of N348 to a more hydrophobic Ile would improve the hydrophobic interactions with T351 of the p66 connection subdomain and with G316 and I270 of the p66 thumb subdomain. In other structures of complexes of RT with various NNRTIs (PDB codes 1S1X, 1S6P, 1S1U, 1S1T, 1S1W, 1TKZ, 1TKX, 1TL1, 1SUQ, 1SV5, 1HNI, 1HQU, and 1HNV), residue W239 appears to be in the vicinity of these residues and likely to be affected directly or indirectly by the N348I mutation. Notably, residue W239 interacts through P-P interactions with Y318, which has been involved in resistance to NNRTIs (NVP and DLV) (19, 33).

DISCUSSION

Two previous reports have shown that two rare mutations, Q145M/L and Y181I, can confer cross-resistance to some NRTIs and NNRTIs (31, 32). N348I appears to be the first reported high-prevalence amino acid mutation to confer resistance to multiple members of the NRTI and NNRTI families. N348 is highly conserved in HIV-1 strains, including subtype O. Interestingly, the equivalent residue in HIV-2 and other retroviruses is an isoleucine (Los Alamos Sequence Data Base, http://hiv-web.lanl.gov/content/hiv-db/). Similarly, WT HIV-2 RT resembles NNRTI-resistant HIV-1 RTs at the NNRTI binding pocket region, e.g., V/I at 181 and L at 188 (34). Any of these differences from the HIV-1 enzyme, including N348I, may contribute to the observed NNRTI resistance of the HIV-2 RT. The significance and role of I348 in the natural resistance of HIV-2 to NNRTIs and susceptibility to NRTIs remain to be elucidated by further experiments.

Recently, Shafer et al. proposed criteria for evaluating the relevance of mutations to drug resistance based on extensive resistance surveillance data (37). In this review the mutations related to drug resistance were assessed by the following: (i) correlations between a mutation and treatment (whether the drug therapy selects for the mutation), (ii) correlations between a mutation and decreased in vitro drug susceptibility, and (iii) correlations between a mutation and a diminished in vivo virologic response to a new antiretroviral regimen.

Regarding the first criterion, we showed that the N348I mutation was induced by AZT and/or ddI treatment (Table 4). For the second criterion, we showed that N348I decreases susceptibility to AZT, ddI, NVP, and DLV (Table 2). The AZT and ddI resistance of the N348I clone was comparable to that of M41L/T215Y and L74V, respectively. Additionally, N348I showed 27-fold increased resistance to NVP. Regarding the third criterion, our data on patient viral load levels shown in Table 5 indicate that N348I affected the clinical outcome. Specifically, in case 6, the viral load clearly increased upon acquisition of N348I. Moreover, dramatic decreases in viral load were observed after introduction of a new regimen without AZT and/or ddI, especially in cases 2, 3, 5, and 6. Hence, the N348I mutation meets the accepted criteria for being a drug resistance mutation.

At present, it is not possible to accurately compare the incidence of N348I with that of other resistance mutations. Genotypic analysis of the largest and most recent drug resistance surveillance examined 6,247 patients treated with well-characterized RTIs, mainly performed within amino acids 1 to 240 of the RT region (35). In this surveillance, the incidences of the Q151M complex and fingers insertion were 2.6 and 0.5%, respectively. Because the connection subdomain is located outside the region sequenced in the majority of genotypic assays, only limited data are available for connection subdomain mutations such as G333E/D and N348I. Nonetheless, the incidence of N348I in our cohort is higher than other MDR mutations such as that of the Q151M complex and the insertion mutations. Furthermore, prevalence of N348I in a Canadian cohort (11.3%) (42) is comparable to that in our Japanese cohort.

In the patient case presented in Fig. 1, there is strong evidence that N348I was not present during and at least 6 months after cessation of NNRTI-based therapy. Still, because of the limited number of such cases in our cohort, it remains unclear if N348I can be induced by NNRTI-containing regimens. According to the Stanford HIV drug resistance database, the incidence of N348I in patients treated with NNRTIs is 5.8% ($n = 13/224$), significantly higher than in the untreated group (0.1%; $n = 2/1095$, $P < 0.0001$). We report here that N348I confers significant and moderate resistance to NVP and DLV, respectively. Most recently, Yap et al. also reported that combined treatment with AZT and NVP was associated with increased risk in the emergence of N348I (42). They mention that other mutations, e.g., K103N, may further enhance N348I-induced resistance to EFV. Thus, it is possible that HIV-1 also acquires N348I under NNRTI-containing therapy. Further experiments and surveillance are needed in patients treated with NNRTI(s) as well as NRTIs.

Mutations at multiple residues are present in the MDR variants of the Q151M and the fingers insertion complexes. Q151M complexes typically contain at least four mutations, including V75I, F77L, and F116Y in addition to Q151M (21). Insertion complexes generally contain an insertion of six bases that code for two amino acids in the background of the classical AZT resistance backbone such as T215Y (41). These results suggest that genetic barriers to developing these MDR mutations appear to be high, consistent with their low incidence (35). Genetic barriers to the G333D/E complex also seem to be high, since G333D/E requires other TAMs to develop this certain resistance phenotype (7). In contrast, a single nucleotide substitution (AAT to ATT) is sufficient to develop the N348I mutation, indicating that the genetic barrier to N348I is low. This may contribute to an increased prevalence of N348I during prolonged chemotherapy with AZT and/or ddI.

The disappearance of N348I was relatively rapid following interruption of treatment (Fig. 1 and Table 5). This was consistent with the observed replication kinetics of N348I HIV-1 where strong impairment was observed in MT-2 and SupT1 cells (Fig. 2). However, in PM1 cells and PHA-stimulated PBMCs, this reduction was moderate, and in H9 cells little reduction was observed. Since both PM1 and H9 cells were originally derived from the same T-cell line, Hut78 (25, 26), some properties for HIV replication may be identical. Availability of deoxynucleoside triphosphates or some cellular factors may compensate the effect of N348I on RT activity, suggesting that some cell populations in patients might harbor HIV-1 with N348I due to its comparable replication kinetics with the WT.

How might the N348I mutation affect resistance to NRTI and NNRTI inhibitors that act with entirely different mechanisms and target different binding sites? Theoretically, it is possible that the N348I mutation at either p66 or p51 or both subunits is responsible for the resistance phenotype. It is also possible that NRTI and NNRTI resistance do not involve the same subunit. However, the N348I mutation in p51 is 50 to 60 Å away from the polymerase active site and the NNRTI binding pocket where the affected inhibitors are expected to bind. Similarly, the mutation site in p51 is 15 to 20 Å away from the interface of the two subunits or the DNA binding cleft. Meanwhile, the mutation site in the p66 subunit is close to the NNRTI-binding pocket and the nucleic acid binding cleft. Hence, it is more likely that the effects of the N348I mutation

are mediated through the p66 subunit mutation, although an involvement of the mutation at the p51 subunit currently cannot be ruled out and should be addressed by biochemical experiments.

In terms of NNRTI resistance, our molecular modeling analysis is consistent with a hypothesis that the mutation is likely to affect the flexibility and mobility of the p66 thumb subdomain. Extensive crystallographic work with HIV-1 RT in several forms, including an unliganded form, in complex with DNA substrates or NNRTIs has revealed that during the course of DNA polymerization, the p66 thumb subdomain undergoes major conformational motions that are critical for efficient catalysis. Alignment of multiple structures of HIV RT suggests that the p66 thumb moves as a rigid body with its base hinged to the palm subdomain exactly near residue 348 (Fig. 3). Residue 348 is proximal to, and likely to affect, the relative interactions between residues of the p66 connection (T351) and p66 thumb subdomains (V317, I270, P272, W239, and eventually Y318). The proximity of residue 348 to this hinge region leads us to believe that changes imparted by the N348I mutation alter the mobility and flexibility of the thumb subdomain. Subtle changes in the interactions between V317 and N348 may also reposition W239 and its neighboring Y318 in the NNRTI-binding pocket. Interestingly, the Y318F mutation affects NNRTI resistance in a similar way as N348I: it decreases susceptibility to NVP and DLV but not to EFV (19, 33). Biochemical binding experiments of RTs with NNRTIs would directly evaluate this hypothesis.

The effect of the N348I mutation on NRTI resistance cannot be rationalized by direct interactions of the mutated residue with the NRTI binding site. It is tempting to speculate that minor changes in the p66 thumb subdomain hinge motions also have minor effects on the positioning of the nucleic acid, which in turn affects the ability to discriminate between NRTI and the normal substrate by an as yet undefined mechanism. However, direct biochemical experimental evidence will be needed to determine the precise molecular details of the specific mechanisms of NRTI resistance.

It has been proposed previously that an imbalance between reverse transcription and RNA degradation plays an important role in NRTI resistance (25). Pathak and colleagues proposed that connection subdomain mutations may result in a slower RNase H reaction, and this in turn may provide an increased time period available for AZT excision, especially with TAMs (28–30). In the case of N348I, Yap et al. recently reported that N348I decreases RNase H enzymatic activity (42). At present, available evidence is consistent with a model in which these connection subdomain mutations alter the affinity of the RT for template/primer, enhance nucleoside excision, and reduce template switching.

Several studies, including recent work by Delviks-Frankenberry et al. and Brehm et al. (4, 11), highlighted the necessity to expand sequencing analysis to include the connection and RNase H subdomains. This contention is further supported by results in this work and by others (16, 28, 42, 43) showing that mutations at the connection subdomain influence susceptibility to some antiretroviral drugs. Hence, there is a growing interest in obtaining genotypic information from expanded areas of RT that would be useful for a more complete analysis of HIV drug resistance. Interestingly, already two out of four commercially

available genotypic and phenotypic assay kits are designed to include in their analysis at least part of the connection subdomain (Antivirogram by Virco up to RT residue 400 and ViroSeq by Abbott/Celera Diagnostics up to RT residue 335).

The present study identifies N348I as a MDR mutation in HIV-1 RT. This knowledge provides information that may be useful in designing more efficient therapeutic strategies that can improve clinical outcome and help prevent the emergence of MDR variants, especially in salvage therapy. This work further highlights the functional role of the HIV-1 RT connection subdomain in drug resistance. Future studies that focus on the structural and biochemical properties of connection subdomain RT mutants should reveal the molecular details of NRTI and NNRTI drug resistance caused by connection subdomain residues.

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