

Functional Correlation between a Novel Amino Acid Insertion at Codon 19 in the Protease of Human Immunodeficiency Virus Type 1 and Polymorphism in the p1/p6 Gag Cleavage Site in Drug Resistance and Replication Fitness

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Population-based sequence analysis revealed the presence of a variant of human immunodeficiency virus type 1 (HIV-1) containing an insertion of amino acid Ile in the protease gene at codon 19 (19I) and amino acid substitutions in the protease at codons 21 (E21D) and 22 (A22V) along with multiple mutations associated with drug resistance, M46I/P63L/A71V/I84V/I93L, in a patient who had failed protease inhibitor (PI) therapy. Longitudinal analysis revealed that the P63L/A71V/I93L changes were present prior to PI therapy. Polymorphisms in the Gag sequence were only seen in the p1/p6 cleavage site at the P1' position (Leu to Pro) and the P5' position (Pro to Leu). To characterize the role of these mutations in drug susceptibility and replication capacity, a chimeric HIV-1 strain containing the 19I/E21D/A22V mutations with the M46I/P63L/A71V/I84V/I93L and p1/p6 mutations was constructed. The chimera displayed high-level resistance to multiple PIs, but not to lopinavir, and grew to 30% of that of the wild type. To determine the relative contribution of each mutation to the phenotypic characteristic of the virus, a series of mutants was constructed using site-directed mutagenesis. A high level of resistance was only seen in mutants containing the 19I/A22V and p1/p6 mutations. The E21D mutation enhanced viral replication. These results suggest that the combination of the 19I/E21D/A22V mutations may emerge and lead to high-level resistance to multiple PIs. The combination of the 19I/A22V mutations may be associated with PI resistance; however, the drug resistance may be caused by the presence of a unique set of mutations in the p1/p6 mutations. The E21D mutation contributes to replication fitness rather than drug resistance.

Treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a combination of anti-human immunodeficiency virus (HIV) drugs is highly effective in controlling HIV-1 replication and decreases AIDS-related morbidity and mortality (29, 43). Treatment reduces viral load in plasma below 50 copies per ml and increases total CD4⁺ T-cell counts. Treatment failure, however, does occur and is typically defined as an increase in viral load, a decrease in CD4⁺ T-cell counts, and the emergence of drug-resistant variants.

Drug-resistant variants in the genetic pool of HIV-1 in patients emerge as the predominant variants under the selective pressure of anti-HIV drug. The character of the highly error-prone HIV-1 reverse transcriptase (RT) results in the emergence of a high level of mutations and increasing genetic diversity (32). This characteristic of RT makes it possible for a number of amino acid substitutions to occur in the protease (PR), RT, and envelope of HIV-1. A number of amino acid substitutions in the PR and RT have been identified as mutations conferring drug resistance (34). Recently, the emergence

of variants containing amino acid insertions in RT or PR has also been reported. The insertions in RT emerge between codons 69 and 70 or near codon 103 (7, 21, 22, 27, 37, 38, 39). The insertion of 2, 5, 8, 11, or 15 amino acids at these positions has been reported. Nearly all variants containing insertions also possess well-described mutations associated with nucleoside RT inhibitors. The T215Y/F change is associated with insertions between codons 69 and 70, while the Q151M or M184V mutation is seen with insertions near codon 103.

The insertions in PR appear between codons 17 and 18, 21 and 25, 31 and 38, 70 and 71, and 95 and 96 of the PR (18, 35, 36, 40). Approximately two-thirds of the PR insertions are also contained with well-described mutations associated with PR resistance, e.g., I84V and L90M. The PR insertions by themselves have not shown to be associated with drug resistance. Mutants containing the insertions in association with known protease inhibitor (PI)-resistant mutations show reduced susceptibility to PIs comparable to that of drug-resistant variants without the insertions; however, the range of the reduction is not significant. Thus, it appears that the PR insertions do not contribute directly to drug resistance (18).

We have identified the presence of a variant containing a novel insertion of the amino acid Ile in the PR between codon 19 and 20 (19I), amino acid substitutions Glu to Asp at codon

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21 (E21D), and Ala to Val at 22 (A22V) with multiple well-described drug-resistant mutations in a patient who had been treated with a combination of PIs. We have also determined that the variant contained mutations in the p1/p6 cleavage site at the P1' and P5' positions. In this study, we demonstrated the impact of the mutations in the PR and the p1/p6 cleavage sites on drug susceptibility and replication fitness and showed that the insert could contribute to inducing the high level of multiple PI resistance in the presence of a unique set of circumstances.

MATERIALS AND METHODS

Cells and reagents. MT-2 cells (12, 13) and the HIV-1 proviral DNA clone pNL4.3 (1) were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. MT-2 was obtained from Douglas Richman, and pNL4.3 was obtained from Malcolm Martin. RD cells (a human embryonal rhabdomyosarcoma cell line) were provided by the American Type Culture Collection (Rockville, MD). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood from healthy donors using lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH) (15) and then stimulated with 5 μ g/ml of phytohemagglutinin (Sigma, St. Louis, MO). MT-2 and PBMCs were cultured in RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 10 mM L-glutamine, 100 U of penicillin/ml (Invitrogen, Carlsbad, CA), and 100 μ g of streptomycin/ml (Invitrogen) (RPMI-10). RD cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 10 mM L-glutamine, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml as previously described (15).

DNA sequencing of HIV protease and gag. HIV RNA was isolated from 130 μ l of plasma by using the QIAamp viral RNA mini kit (QIAGEN, Valencia, CA) as previously described (15). HIV-1 RNA corresponding to full-length gag or part of gag, full-length PR, and part of RT (p7-PR-RT) was amplified by PCR as described before. DNA sequencing was performed using an ABI Prism genetic analyzer 3130x (Applied Biosystems, Foster City, CA). The nucleotide sequences of gag, protease, and the reverse transcriptase gene were translated and aligned with Sequence Navigator (Applied Biosystems). Changes in the gag, protease, and reverse transcriptase region were compared with HIV-1 consensus B sequence as a reference sequence. Population-based sequence analysis was performed using the TruGene system by following the manufacturer's protocol (Bayer Diagnostic, Berkeley, CA).

Generation of viral stocks. A series of chimeric HIV clones was constructed by replacing the region from p7 to PR in a modified cloned proviral DNA, pNL4.3PFB (wild type [WT]) (15) with the equivalent region derived from a clinical isolate. To generate recombinant infectious HIV-1_{NL4.3} virus stocks, pNL4.3 was transfected into RD cells using TransIT LT-1 (PanVera, Madison, WI) as previously described (15). After 24 h, the transfected RD cells were cocultured with fresh MT-2 cells for an additional 24 h. The MT-2 cells thus infected were collected and cultured for 3 days. Cell-free culture supernatants were then obtained and stored at -80°C . The 50% tissue culture infectious dose of each stock was determined as previously described (15).

Drug resistance assay. Drug resistance assays were performed using MT-2 or PBMCs as previously described (15). HIV replication on day 7 was monitored by the p24 amount in culture supernatants using a p24 antigen capture assay kit (Beckman-Coulter, Miami, FL). Each assay was performed in quadruplicate. 3'-Azido-3'-deoxythymidine (AZT) was purchased from Sigma; ritonavir (RTV), saquinavir (SQV), nelfinavir (NFV), lopinavir (LPV), atazanavir (AZV), and amprenavir (APV) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Indinavir (IDV) was provided by Merck Research Laboratory (West Point, PA). Sensitivities were reported as the concentrations of the drugs that inhibited p24 production by 50% (IC_{50} s) (15).

Comparative replication assay and competitive replication assay. To compare the level of HIV-1 replication capacity, a comparative replication assay was performed as previously described (15). Briefly, HIV-infected MT-2 cells were cultured in 2 ml of RPMI-10 in the absence of PIs at a density of $0.1 \times 10^6/\text{ml}$ in 24-well plates. Each assay was performed in triplicate. The p24 levels in day 7 culture supernatants were measured by p24 antigen assays; results were expressed as a percentage of growth of the WT, NL4.3. To determine the growth advantage between two strains, a competitive assay was performed as described previously (15). To assess the viral population changes in each passage, HIV-1 RNA was isolated from culture supernatants, reverse transcribed to cDNA, and

amplified by PCR, followed by direct DNA sequencing. The relative peak heights of each nucleotide at codon 19 were compared by using the 4Peaks software package (version 1.5; The Netherlands Cancer Institute) (14).

Western blotting. A series of mutant plasmids of HIV-1_{NL4.3} was transfected into RD cells (2.5×10^6 cells) in a T75 flask as described above and then cultured for 3 days. Culture supernatants were clarified by low-speed centrifugation ($500 \times g$ for 5 min) and filtered through a Millex-GV, 0.45- μm polyvinylidene difluoride membrane (Millipore, Billerica, MA) to remove cellular debris. To pellet HIV-1 virions in the supernatants, ultracentrifugation ($10,000 \times g$, 2 h at 4°C) was performed using 20% sucrose (wt/vol) in 150 mM sodium chloride (NaCl) and 50 mM HEPES, pH 7.4. The pelleted virions were resuspended in radioimmuno-precipitation assay lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 [Roche Molecular Biochemical], 0.5% sodium deoxycholate [Sigma], 0.1% sodium dodecyl sulfate [Sigma], $1 \times$ protease inhibitor cocktail [Sigma]). The total p24 amount in the viral lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL). The lysate (1 μ g of total protein) was loaded onto a 4 to 12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). Blots were then probed with diluted mouse anti-p24 or RT monoclonal antibodies (Advanced Biotechnologies, Inc., Columbia, MD): goat anti-p17, rabbit anti-p7, or rabbit anti-p6 sera (kindly provided by R. J. Gorelick, SAIC-Frederick, Inc.). The primary antibody was detected with a horseradish peroxidase-conjugated anti-mouse immunoglobulin (Ig) or anti-rabbit IgG (Amersham Life Science, Piscataway, NJ) or anti-goat Ig (Santa Cruz Biotechnology, Santa Cruz, CA). A positive signal was detected with the ECL Plus Western blotting detection system (Amersham Life Science). The membrane was stripped with the Restore Western blot stripping buffer (Pierce) and then reprobed with other antibodies.

Transmission EM. RD cells were transfected with plasmids as described above. The transfected cells were cultured for 2 days at 37°C and then washed with warmed phosphate-buffered saline. The transfectants were fixed using 2% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate trihydrate [Sigma], pH 7.0) and processed for electron microscopy (EM) analysis, as previously described (11).

Statistical analysis. Differences between HIV variants in comparative replicative ability were calculated by using the unpaired *t* test using the StarView program (Abacus Concepts, Berkeley, CA).

Nucleotide sequence accession number. The GenBank accession numbers of the protease sequences containing the 19L/A22V/L63P/A71V/I93L and 19I/E21D/A22V/M46I/L63P/A71V/I84V/I93L mutations are DQ431463 and DQ431464, respectively.

RESULTS

Response of a patient to therapy. Figure 1 shows changes in HIV-1 viral load and CD4^+ T-cell number in the blood of a patient who transiently has taken combination therapy using RT inhibitors and PIs with one cycle of intermittent interleukin 2 administration (20). The patient was initially treated with AZT plus stavudine (D4T) plus didanosine (ddI) therapy prior to a PI-containing therapy. PI therapy was started on the patient from April 1995, with IDV monotherapy for 2 weeks. The therapy was subsequently switched to a combination of D4T plus ddI plus AZT plus lamivudine (3TC) followed by combination of SQV plus RTV plus D4T. In October 1998, the regimen was switched to a combination of APV plus NFV plus efavirenz plus 3TC, used until October 2000. After a 1-month cessation of drug therapy, the regimen was resumed in December 2000 with a combination of ddI plus D4T plus LPV plus RTV. During the course of therapy, particle-associated HIV-1 RNA levels in plasma were determined by version 3.0 of the branched-DNA signal amplification assay (detection limit, 50 copies per ml; Bayer Health Care, Tarrytown, NY) (9). The HIV copy numbers in blood had consistently been higher than 1,000 copies/ml. The regimen containing LPV plus RTV plus D4T plus ddI led the viral load to be below the sensitivity of the assay. Thus, it was speculated that clinical isolates derived from

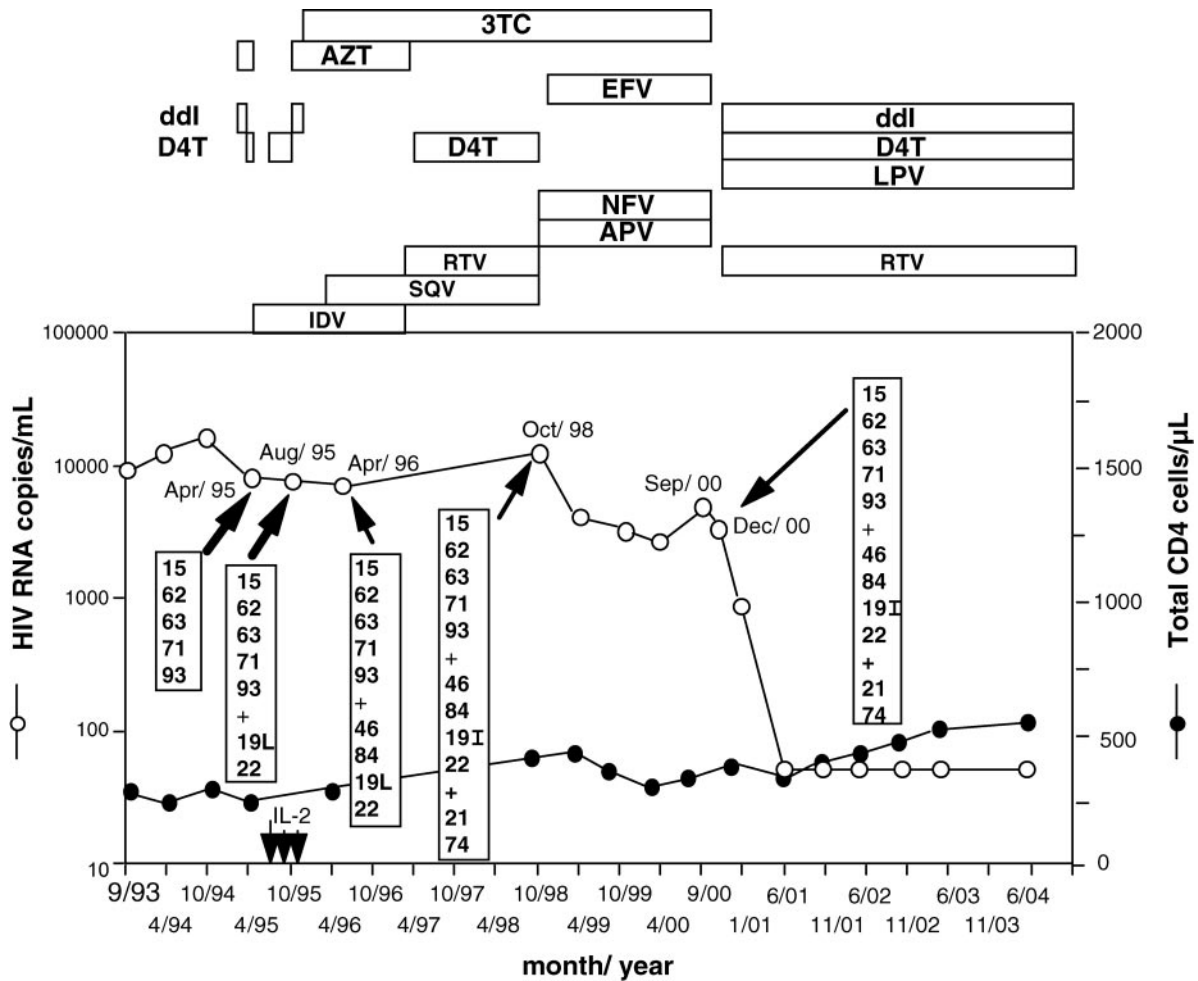


FIG. 1. Virological and immunological responses of the patient to antiretroviral therapy and HIV PR genotype. Particle-associated HIV RNA levels in plasma were determined by the branched-DNA signal amplification assay; the detection limit was 50 copies per ml. The drug treatment history is shown above the graph. Sequence data were obtained from RT-PCR-amplified HIV RNA. Boxed numbers 15, 21, 22, 46, 62, 63, 71, 74, 84, and 93 refer to amino acid changes at the indicated codons, as follows: 15, Ile to Val; 21, Glu to Asp; 22, Ala to Val; 46, Met to Ile; 62, Ile to Val; 63, Leu to Pro; 71, Ala to Val; 74, Thr to Pro; 84, Ile to Val; 93, Ile to Lys. 19L and 19I refer to Leu and Ile insertion at codon 19, respectively. Short arrows indicate time points when interleukin-2 was administered. The drug treatment history is shown in boxes above the graph. EFV, efavirenz.

the patient might have acquired resistance to some PIs but remained sensitive to LPV.

To elucidate the nucleotide substitutions in the HIV sequence, a population-based sequence analysis was first performed using a plasma sample from December 2000. The genotypic analysis showed that the PR contained resistance mutations at codons 46 (M46I), 63 (L63P), 71 (A71V), 84 (I84V), and 93 (I93L) with several polymorphisms in the amino acid sequence at codons 15 (I15V), 62 (I62V), and 74 (T74P) (42). Of note, the PR contained a novel insertion of Ile at codon 19 (19I) and a novel amino acid change at codon 21 (E21D) with a recently reported amino acid change at 22 (A22V) (42). Results from genotyping of RT showed that the virus contained drug resistance mutations at codons 67 (D67N), 69 (T69D), 70 (K70R), 103 (K103N), 215 (T215V/F), 219 (K219Q), and 225 (P225H). Polymorphisms were noted at codons 122 (E122K), 123 (E123E), 135 (I135L), 207 (Q207E), 214 (L214F), and 228 (L228H). No novel amino acid substitu-

tions were seen in the RT sequence. The HIV db:drug resistance algorithm (beta test) (<http://hivdb.stanford.edu/index.html>) predicted that the variant might show high-level resistance to APV, NFV, 3TC, abacavir, AZT, D4T, dideoxycytosine, ddI, and nonnucleoside RT inhibitors; intermediate resistance to AZV, IDV, RTV, and SQV; and low-level resistance to LPV and tenofovir.

Evolutionary path of the emergence of 19I, D21E, and A22V changes. To define when and how the 19I, E21D, and A22V mutations were selected under therapy, we performed DNA sequencing using longitudinally stored plasma samples (Table 1). Amino acid substitutions of I15V/I62V/L63P/A71V/I93L in PR existed prior to the initiation of PI therapy in April 1995. The amino acid insertion at codon 19 and A22V changes emerged under the selective pressure of IDV, D4T, and ddI in July 1995. The inserted amino acid was Leu (19L). The inserted DNA sequence was a duplicate of the nucleotide sequence of codon 19, CTA. Subsequently, M46I/I84V mutations and

TABLE 1. Longitudinal analysis of p1/p6 cleavage site and HIV PR^a

Time point	p1/p6 sequence		Mutation(s) at ^b :												
	RPGNF	LQSRP	I15	L19	Insertion	K20	E21	A22	M46	I62	L63	A71	T74	I84	I93
April 1995	-----	P---L	V	—	X	—	—	—	—	V	P	V	—	—	L
June 1995	-----	P---L	V	—	X	—	—	— (6)/V (4)	—	V	P	V	—	—	L
July 1995	-----	P---L	V	—	L (6)/X (2)	—	—	V	—	V	P	V	—	— (6)/V (2)	L
August 1995	-----	P---L	V	—	L	—	D	V	I	V	P	V	—	V	L
October 1995	-----	P---L	V	—	I	—	D	V	I	V	P	V	—	V	L
April 1996	-----	P---L	V	—	I	—	D	V	I	V	P	V	—	V	L
October 1998	-----	P---L	V	—	I	—	D	V	I	V	P	V	P	V	L
December 2000	-----	P---L	V	—	I	—	D	V	I	V	P	V	P	V	L

^a HIV RNA was isolated from plasma, and then RT-PCR was performed to amplify the full-length gag region. All data were obtained from 8 to 10 clones at each time point.

^b Major mutations in HIV PR are listed first, followed by slashes (/) and minor mutations. Numbers in parentheses indicate the number of clones that contained the specific polymorphism. Dashes (—) denote identity with the HIV-1 consensus B sequence; X denotes absence of insertion.

E21D emerged under drug pressure of IDV plus 3TC plus AZT in August 1995. The nucleotide sequence, CTA changed to ATA; the resulting change 19L to 19I was seen under a treatment using SQV plus RTV plus D4T plus 3TC in October 1995.

As we first reported, PI resistance in clinical isolates can be caused by accumulation of mutations in PR and amino acid changes in the Gag cleavage sites (45). To define the correlation between emergence of the mutations in PR and changes in gag sequence, a full-length gag was amplified from clinical isolates from the longitudinal plasma samples. Genotypic analyses revealed the presence of polymorphisms in the p1/p6 cleavage site prior to PI therapy. The amino acid changes of Leu to Pro at the P1' position (LP1'P) and Pro to Leu at the P5' position (PP5'L) in p1/p6 were observed. These changes have been reported as polymorphisms (2, 5, 8). The polymorphisms were preserved during the course of therapy without changing. No amino acid substitutions were seen in other Gag cleavage sites (data not shown).

Impact of 19I/E21D/A22V and polymorphisms in p1/p6 on drug susceptibility. Since a variant containing an insertion at codon 19 and the A22V changes emerged prior to the selection of drug-resistant mutations and persisted during therapy, it was speculated that a combination of these mutations might be associated with drug resistance. To define the impact of the combination of 19I/E21D/A22V to drug susceptibility and replication capacity, a recombinant chimeric HIV-1 isolate was constructed by replacing the region from p7 to PR in a mod-

ified cloned proviral DNA, pNL4.3: pNL4.3PFB (WT) (15), with the equivalent region derived from the clinical isolate from December 2000. The resulting chimeric HIV, HIV3116 contained 19I/E21D/A22V with the M46I/L63P/A71V/I84V/I93L and p1/p6 mutations. The chimera also contained natural polymorphisms of I15V/I62V/T74P in PR. In addition, to assess the impact of the 19I/E21D/A22V mutations in the phenotypic assay, a variant lacking the motif but otherwise identical to HIV3116 was constructed (resulting clone, HIV3121). 19I was deleted, and the consensus B sequence was induced at codons 21 and 22 in HIV3116 using site-directed mutagenesis.

Results of phenotypic analyses of these constructs expressed as mean IC₅₀s from at least three independent assays are shown in Table 2 and Fig. 2. In these assays, a nucleoside RT inhibitor, AZT, was used as an internal control. The presence of the 19I/E21D/A22V motif (HIV3116) led to 2- to >10-fold-higher IC₅₀s to PIs than those lacking the motif (HIV3121). These substitutions had no impact on susceptibility to AZT. A similar drug resistance profile for HIV3116 was observed in a culture system using phytohemagglutinin-stimulated PBMC (data not shown); thus, the resistant profile for HIV3116 is not associated with host cell factors. HIV3121 showed susceptibility to nearly all tested PIs but resistance to RTV and NFV; therefore, the increased IC₅₀ in the variants containing the 19I/E21D/A22V motif is associated with drug resistance rather than with either genetic effect.

A viral replication assay was performed in the absence of drugs to determine the replication competence of each vari-

TABLE 2. Drug resistance profile of HIV mutants

HIV clone ^b	IC ₅₀ (nM) (fold increase) of ^a :							
	AZT ^c	PI						
		IDV	RTV	SQV	NFV	AZV	LPV	APV
217	11 ± 2.1 (1.0)	19 ± 4.5 (1.0)	18 ± 4.5 (1.0)	4.6 ± 1.8 (1.0)	3.4 ± 0.9 (1.0)	2.8 ± 0.6 (1.0)	7.8 ± 1.7 (1.0)	12 ± 2.3 (1.0)
3116	26 ± 3.2 (2.4)	310 ± 56 (16)	1,700 ± 290 (94)	210 ± 50 (46)	>1,000 (>290)	25 ± 7.8 (8.9)	30 ± 5.8 (3.8)	203 ± 61 (17)
3121	14 (1.3)	24 ± 4.3 (1.3)	153 ± 13 (8.5)	15 ± 1.5 (3.3)	35 ± 4.8 (10)	7.6 ± 3.0 (2.6)	14 ± 4.7 (1.8)	36 ± 11 (3.0)
3138	13 (1.2)	11 ± 4.4 (0.6)	43 ± 7.8 (2.4)	5.0 ± 1.4 (1.1)	15 ± 3.2 (4.4)	1.5 ± 0.3 (0.5)	2.4 ± 0.5 (0.3)	4.5 ± 0.8 (0.4)

^a Data show means ± standard errors of IC₅₀s. Numbers in parentheses show relative increases compared with wild-type HIV217. The values were derived from at least three independent assays.

^b 217, wild type; 3116, chimeric HIV containing 19I/E21D/A22V plus M46I/L63P/A71V/I84V/I93L; 3121, chimeric HIV containing M46I/L63P/A71V/I84V/I93L; 3138, recombinant HIV containing 19I/E21D/A22V.

^c AZT is an RT inhibitor.

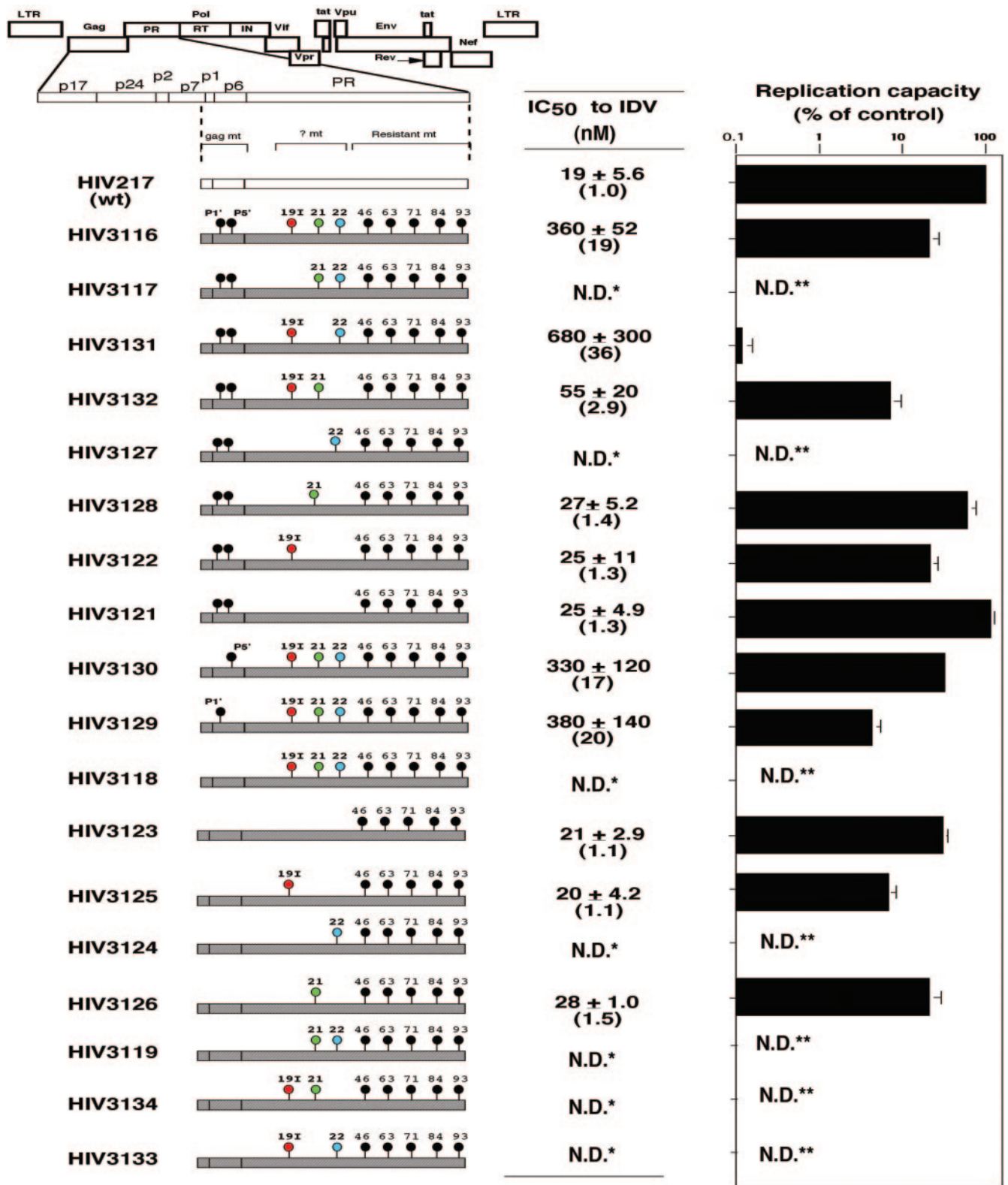


FIG. 2. Drug susceptibility to IDV and replication properties of HIV-1 constructs. Recombinant HIV-1 mutants were constructed using site-directed mutagenesis. MT-2 cells were infected with 2,500 50% tissue culture infectious dose per 4×10^6 cells. In the drug resistance assay, the infected cells were cultured for 7 days in the presence of different concentrations of IDV. IC₅₀s were determined by at least three independent assays. Data are means ± standard errors (SE). Numbers in parentheses show relative increases compared with those for WT HIV217. In the replication capacity assay, the infected cells were cultured for 7 days in the absence of drug. Results are expressed as mean percentages of growth ± SE compared to WT growth. In this assay, the p24 concentration of the WT was 1,566 ± 281 ng/ml. N.D.*, not done; N.D.**, not detected.

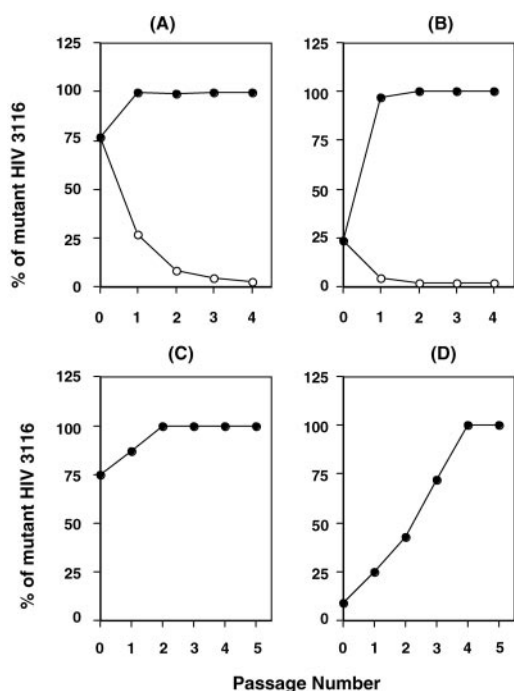


FIG. 3. Competitive replication assay of WT and mutant virus. WT and HIV3116 virus were mixed at 1:1 (A) or 10:1 (B). Infected cells were cultured in the presence (closed circles) or absence (open circles) of 1 μ M IDV. To assess the fitness impact of the amino acid change in the insert, HIV3116 and a newly described mutant, HIV3173, in which the 19I was changed to 19L, were mixed at a ratio of 1:1 (C) or 1:10 (D) and then used for infection. The infected cells were cultured in the absence of drug. Data were generated based on relative peak heights of electropherogram produced from direct DNA sequencing of viral RNA from tissue culture supernatant at the end of each passage.

ant. The results are expressed as mean percentages of growth of variants compared with that of WT (HIV217) (Fig. 2). In the absence of 19I/E21D/A22V, a variant containing only the drug-resistant mutations (HIV3121) showed growth activity similar to that of WT; however, the presence of 19I/E21D/A22V with the drug resistance mutations (HIV3116) showed 30% of the replication activity of the WT. These results suggest that the presence of the combination of 19I/E21D/A22V leads to higher-level drug resistance at the expense of replication capacity.

To further compare the replication fitness of the mutant to that of the WT, a competitive fitness assay was performed. WT and HIV3116 were mixed at different ratios and cultured in the presence or absence of 1 μ M IDV. In the absence of IDV, the WT became the dominant species by the second tissue culture passage. In the presence of 1 μ M IDV, however, the mutant virus rapidly became the predominant species within the first passage (Fig. 3A and B). These results suggested that HIV3116 has a disadvantage in viral growth in the absence of drug.

As described above, longitudinal analysis showed that the 19L insertion was selected by a duplication of DNA sequence of codon 19, and then the amino acid was changed to 19I. This evolutionary change implicated that 19I may have advantages in replication and/or PI susceptibility. To address the impact of the change, a site-directed mutagenesis was performed on

HIV3116 to induce a 19L change from 19I. The resulting clone, HIV3173, showed drug susceptibility similar to that of HIV3116 (data not shown) but less replication capacity (11% of WT, one-third of the growth of HIV3116). To demonstrate that HIV3116 possesses a relatively better replication capacity than HIV3173, a competitive replication assay was performed with HIV3116 and HIV3173 at the ratios 1:1 or 1:10 and cultured in the absence of drug. The results from the assays showed that the 19I mutant (HIV3116) outgrew the 19L mutant (Fig. 3C and D). These results suggest that the evolution from 19L to 19I leads to enhanced replication fitness rather than an increase in drug resistance.

Characterization of mutations at codons 19, 21, and 22 and gag cleavage sites. To further characterize the impact of each mutation of 19I, E21D, and A22V on drug susceptibility and replication activity, a series of mutants were constructed by site-directed mutagenesis on HIV3116. Drug susceptibility was analyzed using IDV (Fig. 2). Of note, variants containing known PI resistance mutations and A22V but not 19I (HIV3117 and HIV3127) were defective. Variants containing 19I with the context of PI-resistant mutations but without A22V (HIV3121, HIV3122, and HIV3132) had modest changes in drug susceptibility and showed a reduced replication activity; however, the presence of both 19I and A22V in the context of PI-resistant mutations (HIV3131) caused a high level of drug resistance with a low level of replication activity. The presence of E21D by itself in the context of the PI-resistant mutations (HIV3128) had no effect on either drug susceptibility or replication activity. These results suggest that 19I or A22V alone in the context of PI-resistant mutations is not associated with drug resistance; however, a combination of the two mutations is associated with a high level of multiple-PI resistance but induces impaired replication. It appears that the addition of E21D to the impaired variant compensates replication. Since a variant containing A22V alone with PI-resistant mutations (HIV3127) was noninfectious, it was postulated that the A22V mutation by itself induced immature, nonviable particles. To address the impact of A22V on replication activity, A22V was induced in NL4.3. The resulting strain was defective (data not shown). Thus, the A22V change by itself leads to a defective protease.

Mutations at the P1' position in p1/p6 were reported as compensational mutations for viral fitness in the activity of PI-resistant mutations in PR. It is reported that a polymorphism of PP5'L in p1/p6 may be associated with drug resistance through the I84V mutation (2). To address the roles of each mutation in drug susceptibility and replication fitness, an additional series of recombinant variants was constructed using HIV3116. Variants lacking both mutations at the P1' and P5' positions (HIV3118, HIV3119, HIV3124, and HIV3133) were nonviable (Fig. 2). The presence of a single mutation at the P1' (HIV3129) or P5' (HIV3130) position restored replication activity to 9% and 30%, respectively. These results suggest that the presence of the p1/p6 mutations restores replication activity in the context of 19I/E21D/A22V with other PI resistance mutations. Thus, the p1/p6 mutations are required to allow emergence of the 19I/E21D/A22V mutations.

To define whether the complex of 19I/E21D/A22V mutations alone induces PI resistance, the mutations were induced in NL4.3 using site-directed mutagenesis. The resulting variant

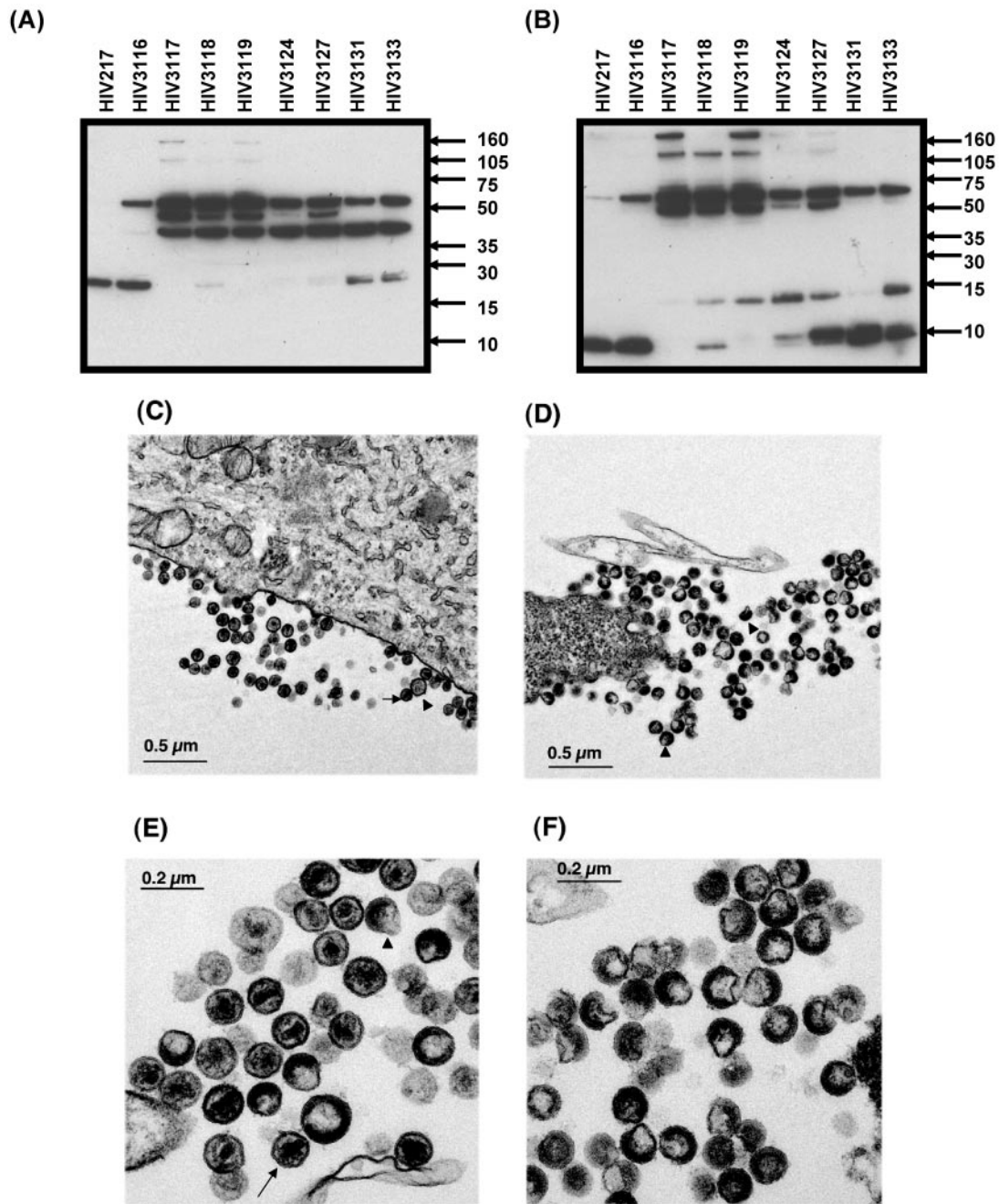


FIG. 4. Western blot and EM pictures of a series of defective HIV mutants. RD cells were transfected with a series of plasmids encoding nonviable viruses (Fig. 2) and cultured for 3 days. HIV-1 virions were isolated from culture supernatants of RD cells using ultracentrifugation as described in Materials and Methods. For each blot, 1 μ g of total protein was loaded on 4 to 12% bis-Tris sodium dodecyl sulfate gels, and Western blotting was performed using an anti-p24 antibody (A) and antisera against p7 (B). The tested variants were HIV217 (WT), HIV3116 (containing the p1/p6 mutations and 19I/E21D/A22V), HIV3117 (lacking 19I), HIV3118 (lacking the p1/p6 mutations), HIV3119 (lacking 19I and the p1/p6 mutations), HIV3124 (lacking 19I, E21D, and the p1/p6 mutations), HIV3127 (lacking 19I and E21D), HIV3131 (lacking E21D), and HIV3133 (lacking E21D and the p1/p6 mutations). Molecular size markers (in kilodaltons) are indicated to the right of the panels. RD cells were transfected with plasmid encoding HIV3116 (C and E) or HIV3117 (D and F) and cultured for 2 days, followed by fixation. Arrows indicate mature virions, and arrowheads indicate immature forms of virions. The electron microscopic pictures were taken as described in Materials and Methods (11).

was nonviable (data not shown); however, addition of the LP1'P and PP5'L mutations to the variant restored replication activity. A variant containing 19I/E21D/A22V with PP5'L (resulting strain, HIV3138) replicated comparably to the WT, was

susceptible to all drugs tested (Table 2), and showed hypersensitivity to AZV, LPV, and APV.

Impact of mutations on cleavage of Gag precursor and structure of HIV-1 virion. The HIV-1 PR cleaves the Gag and

the Gag-Pol precursor in the virion to compose the mature form of the infectious virions (19). As described above, absent the 19I insertion or the p1/p6 mutations in the chimeric HIV, the virus demonstrates a defect in replication. The absence of E21D in the chimeric HIV also led to a decrease in replication activity of the chimeric HIV. Thus, it was speculated that the nonviable mutants might be due to the presence of nonfunctional PR. To address the correlation between the PR mutations and the p1/p6 mutations in the cleavage site, Western blotting was performed using antibodies against p24 or RT and antisera against p6, p7, or p17 with virus lysate of isolated HIV virions, as described in Materials and Methods.

Figure 4A and B show results from the Western blot using anti-p24 antibody and anti-p7 serum, respectively. All variants tested showed uncleaved Gag precursors (57 kDa) in virions. Since the partially cleaved products were also seen, the mutated PR in these nonviable viruses retained some degree of enzymatic function.

All mutants possessed a 38-kDa fragment, which is equivalent to the molecular size of uncleaved p17/p24/p2. HIV3117, lacking the 19I insertion, possessed fragments associated with full-length Gag (57 kDa) and Gag-Pol (110 kDa) but not the p24 and p7 fragments, and an unexpected 50-kDa fragment was seen. The 50-kDa fragment also cross-reacted with anti-p6 and anti-p17 antisera but not to the anti-RT antibody (data not shown). HIV3118 lacking the p1/p6 mutations possessed fragments associated with p24, p7, and p7/p1/p6 and the 50-kDa fragment. HIV3131 lacking E21D was infectious (Fig. 2) and contained fragments associated with p24, p24/p2, p7, and p7/p1 and a low amount of p7/p1/p6 but not a significant amount of the 50-kDa fragment. HIV3133, lacking both E21D and the p1/p6 mutations, was defective and contained fragments of p24, p24/p2, p7, p7/p1, and p7/p1/p6 but not the 50-kDa fragment. Overall, the defective viruses contain the 50-kDa fragment or the p7/p1/p6 fragment.

To clarify the morphology of the defective HIV mutants, electron micrographic analyses were performed using RD cells transfected with a series of cloned proviral DNAs, as described in Materials and Methods. The infectious chimeric virus, HIV3116, demonstrated many normal budding, immature, and mature viral particles. Mature particles contained a condensed core of electron-dense material in the center of the virions (Fig. 4C and E). The mutant lacking 19I (HIV3117) demonstrated normal budding and only immature teardrop viral particles lacking the core material (Fig. 4D and F). High-power magnifications revealed that HIV3117 only demonstrated a double layer membrane, likely due to lack of complete Gag processing (Fig. 4F). The same results were seen with other defective viruses, HIV3118, HIV3119, HIV3124, HIV3127, and HIV3133 (data not shown).

DISCUSSION

The emergence of 1 to 6 amino acid insertions in PR at codons near 18, 25, 36, 70, and 95 in clinical isolates has been reported previously (18, 35, 36, 40). The reports indicate that (i) most variants containing amino acid insertions also have one or more well-described PI resistance mutations, (ii) variants containing the insertions in the absence of drug resistance

mutations have been shown to be fully susceptible to all approved PIs, (iii) the presence of the insertions in the context of drug resistance leads to a modest decrease in PI susceptibility, and (iv) the presence of the insertions in the context of PI resistance confers a replication advantage rather than an increase in drug resistance (18, 35). In our study, we demonstrated that an amino acid insertion may be selected at codon 19, but the insertion by itself may not contribute to drug susceptibility. We, however, showed that a combination of the insertion and the A22V mutation in the context of drug resistance synergistically induced a high level of multiple-PI resistance. To our knowledge, this is the first report that the amino acid insertion may contribute to high-level PI resistance, although only in the presence of a set of unique circumstances between PR and Gag.

Longitudinal analysis using stored samples showed that the mutations at the p1/p6 cleavage sites existed prior to PI therapy, and the 19L insertion initially emerged followed by the 19I change. It is reported that PR-containing insertions appear in nearly 0.1% of patients (16, 18, 35), and the prevalence of the mutation at the P1' position with the P5' position at the p1/p6 cleavage site is nearly 5% (2). We demonstrated that the complex of 19I/E21D/A22V lacking the p1/p6 mutations was nonviable; therefore, it appears that the emergence of the complex in PR may be regulated by the Gag sequence prior to PI therapy, and thus, the prevalence of the complex of 19I/A22V mutations is most likely only lower than suggested by previous reports regarding insertions in PR. Since it has been reported that the emergence of mutations in Gag is concomitant with the development of PI resistance (3, 4, 6, 10, 17, 24, 26, 28, 31, 33, 44, 45), further studies of the correlation between Gag sequences and insertions may provide more insights regarding the possible mechanism of an evolutionary pathway in the emergence of insertions in PR.

Longitudinal analysis showed that the nucleotide sequence of the insertion was initially a duplicate of the sequence of codon 19, CTA, which encodes Leu; subsequently, a C-to-A change led to a Leu-to-Ile change in the insertion. The comparative replication assay showed that replication capacity in the 19I variant was higher than that of the 19L. Since the drug susceptibility was not seen as significantly different, it appears most likely that the Leu-to-Ile change was selected because of an advantage in growth rather than because of drug susceptibility.

It has been reported that, *in vitro*, PR cleaves the Gag precursor (p17/p24/p2/p7/p1/p6) in the order of p2/p7, p17/p24, and p1/p6; p7/p1; and p24/p2. The cleavages of the p7/p1 and p24/p2 fragments are the two slowest steps (30). Therefore, after initial cleavage of the Gag precursor, the resulting fragments are p17/p24/p2 (43 kDa) and p7/p1/p6 (14 kDa). A chimeric infectious clone, HIV3116, contained lower amounts of noncleaved Gag precursor and 43-kDa fragments than defective clones. Defective viruses accumulated the 43-kDa fragment with low amounts of the p24 fragment. Thus, the mutated PR studied here may inefficiently cleave p17/p24 and subsequently lead to an accumulation of the 43-kDa fragment.

Virions lacking 19I or the Gag mutations contained an unexpected 50-kDa fragment as well as partially cleaved Gag-Pol fragments. The 50-kDa fragment cross-reacted with anti-p6, -p7, -p17, and -p24 antibodies but not with anti-RT antibody.

Even though further study is needed to identify the components of the fragment, the mutated PR may have a different substrate specificity than WT PR and produce the unexpected fragment from the Gag or Gag-Pol precursors.

The variants lacking the p1/p6 mutations contained the non-cleaved p7/p1/p6 fragment. The p7/p1/p6 fragments were also observed in virions derived from a variant lacking 19I and E21D (HIV3127 retaining A22V). Thus, the p1/p6 mutations and/or A22V may affect the cleavage of p7/p1/p6 fragment.

The crystal structure of multiple-drug-resistant PR has been compared with that of WT PR (23, 25). These studies demonstrated that the presence of drug-resistant mutations in PR led to an expansion of the active site. The same principle may be applied here. Since the codons 19, 21, and 22 are located near the active site in the PR (41), the insertion at codon 19 and the A22V mutation add additional pressure outside the active site, presumably compressing it such that there is an inability of drug to bind (high level of drug resistance), and decrease the replication capacity due to this compromise. The subsequent E21D change helps to relieve this compromise and increases replication competency. Further molecular study may need to address the role of each mutation in the context of a unique set of mutations.

In this study, we have shown that emergence of a novel insertion (19I), a novel amino acid change (E21D), and the A22V mutation may be selected under PI pressure in a clinical isolate. A combination of the 19I and A22V changes are associated with drug resistance, and the E21D mutation plays a role in compensation of replication fitness. The complex of 19I/E21D/A22V mutations may emerge in the presence of mutations in the p1/p6 cleavage site at P1' and P5' positions. Thus, a low overall prevalence of this variant is predicted; however, viruses with this genotype demonstrate a high level of multiple drug resistance to PIs but remain sensitive to LPV.

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