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Role of Baseline *pol* Genotype in HIV-1 Fitness Evolution

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Summary: Viral fitness can be modified upon development of antiretroviral drug resistance, usually by selection of compensatory mutations. In this study, we have used HIV-1 isolates from individuals receiving a protease inhibitor (PI)-based regimen to analyze the impact of basal genetic background on viral fitness evolution. Paired plasma samples and HIV-1 isolates were obtained from 10 PI-naive HIV-infected individuals enrolled in 2 different studies of combination antiretroviral therapy. Genomic regions from *pol* and *env* were sequenced. Viral fitness was measured using growth competition experiments followed by heteroduplex tracking analysis. Baseline genotypic analyses of *pol* showed that 9 of 10 viruses had a different degree of secondary mutations in the protease gene at codons associated with PI resistance (i.e., 10I, 36I, 63P, 71T, and 77I). After 48 weeks of PI-based therapy, a strong correlation was observed between protease genetic divergence and viral fitness difference ($r = 0.78$, $P = 0.03$), but not with reverse transcription or Env divergence, suggesting that genotypic changes in the protease gene were driving HIV-1 evolution in these patients. As expected, an inverse correlation was observed between the number of protease and reverse transcription primary mutations and viral fitness ($r = -0.65$, $P < 0.0001$). However, our results suggest that the preexistence of secondary mutations in protease genetic background may have implications in HIV-1 fitness evolution and virologic response to antiretroviral therapy. **Key Words:** viral fitness, secondary mutations, protease

Treatments with combinations of antiretrovirals do not completely inhibit HIV-1 replication, and this often leads to treatment failure.[1] The effectiveness of all antiretroviral drugs is limited by the emergence and selection of drug-resistant variants, frequently preexisting at low levels in the HIV-1 quasispecies of patients undergoing no therapy with the relevant inhibitors.[2,3] In the absence of antiretroviral therapy, viruses containing primary drug resistance mutations have a reduced fitness (often referred to as viral replication capacity) compared with wild-type viruses, and as a result, wild-type variants are prevalent within the population in the absence of therapy.[4]

During the past few years, multiple studies have described a significant reduction in HIV-1 fitness as a consequence of resistance to protease inhibitors (PIs).[5,6] Most of these mutations reduce replication capacity but several amino acid substitutions have been shown to re-

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store or even increase fitness over that of wild-type virus.[6] Therefore, 2 types of mutations associated with HIV-1 drug resistance to PIs have been described: primary mutations, which allow the wild-type virus to escape drug inhibition, and secondary or compensatory mutations, which may increase the fitness of the drug-resistant virus.[6,7] Accordingly, 2 phases in the evolution of viral fitness during antiretroviral therapy have been proposed: selection of drug-resistant variants, usually accompanied by a decrease in viral fitness, and selection of compensatory mutations, which may not increase drug resistance but give rise to variants with increased replication capacity.[8,9] However, appearance of specific mutations is often highly dependent on the baseline sequence and the sequential selection of compensatory mutations that contribute to viral fitness.[10–12]

Although previous studies have described the role of HIV-1 genetic background in development of resistance to PIs,[10,13] the mechanism through which secondary protease mutations improve fitness is still unclear. It is possible that mutations in these regions provide better peptide substrates for the mutated protease, which partially compensate for the resistance-associated loss of viral fitness.[14] Furthermore, secondary mutations or natural polymorphisms at numerous protease codons (e.g., 10, 20, 36, 63, 71, and 77) have been identified in PI-naive HIV-infected individuals,[3,15,16] which may have implications for viral fitness evolution and clinical relevance for antiretroviral therapy.

Recent studies have focused on discordant responses in HIV-infected patients treated with PIs (i.e., high viral loads and sustained CD4 T-cell counts), and a potential correlation with the viral fitness of PI-resistant variants.[17–19] In addition, it has been suggested that continuation of antiretroviral therapy in patients with persistently detectable viral load and presence of multidrug-resistant variants still provides benefit.[20–22] A previous study using viral isolates from the AIDS Clinical Trials Group (ACTG) 315 study monitored the fitness of 5 paired viral isolates (pre- and posttreatment) in human peripheral blood leukocyte–reconstituted severe combined immunodeficient (hu-PBL-SCID) mice.[18] As expected, 3 of 5 posttreatment PI-resistant HIV-1 isolates exhibited decreased replication in hu-PBL-SCID compared with the paired pretreatment virus.[18] Thus, subtle losses in the replication capacity of PI-resistant viruses may contribute to relative preservation of CD4 cell counts in persons who experience virologic failure.[17,18]

In the present study, we have analyzed the basal genetic background in *pol* (protease and reverse transcription) and *env* (C2V3 region) genes of 10 PI-naive HIV-infected individuals enrolled in 2 different PI-based antiretroviral regimens (ACTG 315 and IRIS studies) and their potential role in viral fitness evolution. As expected, a higher divergence in protease sequences seems to have driven HIV genetic evolution in these patients during combined antiretroviral treatment. In addition, growth competition experiments showed a correlation between HIV-1 fitness and protease genetic divergence. Interestingly, the presence of secondary PI resistance mutations at baseline seems to accelerate the recovery of viral fitness in PI-resistant HIV-1 isolates, which correlated with a poor response to combined therapy.

MATERIALS AND METHODS

Patients and Samples From the ACTG 315 and IRIS Studies

The ACTG protocol 315 enrolled HIV-infected individuals with CD4 T-lymphocyte count of 100–300 cells/μL and no history of treatment with lamivudine (3TC) or any PI. Previous antiretroviral treatment with zidovudine (AZT) was interrupted 5 weeks prior to study entry, and on day 0 all patients initiated treatment with ritonavir (RTV). AZT and 3TC were added to the combination regimen at day 10.[23,24] Virologic and immunologic responses in the first year of treatment have been described elsewhere.[23–25] For this study, 4 patients from the ACTG 315 trial were selected. Plasma and peripheral blood mononuclear cells (PBMCs) were collected at baseline and 12, 24, and 48 weeks after treatment.

To analyze the role of basal secondary PI-associated mutations in viral fitness evolution, a second set of plasma and HIV-1 isolates was obtained from 6 PI-naive HIV-1-infected individuals enrolled in an open-label randomized clinical trial comparing 2 different types of combination therapy: ritonavir/saquinavir plus 1 nucleoside reverse transcription inhibitor (NRTI) versus indinavir plus 2 NRTIs (IRIS study), performed in Antwerp, Belgium.[26] Paired, pre-, and posttreatment, samples were obtained from 6 patients based on clinical evidence of virologic failure (i.e., 1 or 2 logs of viral load decrease from baseline with further rise to similar pretherapy plasma HIV-1 RNA levels) and viral genotypic analyses (i.e., absence of primary mutations associated with PI resistance and presence of different patterns of secondary mutations in the protease gene). However, HIV-1 isolates were obtained from 9 of 12 blood samples. Clinical and virologic indices for both sets of samples are described in Table 1. Finally, HIV-1 *env* genetic subtype and biophenotype (i.e., nonsyncytium- or syncytium-inducing and coreceptor usage) were estimated by nucleotide sequencing using a fragment of the gp120-coding region of the *env* gene.

RT-PCR and PCR of the HIV-1 *pol* and *env* Genes

Plasma viral RNA was purified from pelleted virus particles (cell-free plasma centrifuged at 32,000*g* for 40 minutes) using QIAamp Viral RNA mini kit (Qiagen). Viral RNA was reverse transcribed using the GeneAmp Gold RNA polymerase chain reaction (PCR) kit (PE Biosystems) for the *pol* fragment and the OneStep RT-PCR kit (Qiagen) for the *env* fragment, and the corresponding antisense external primer. Viral cDNA was then PCR amplified using a set of external and nested primers. HIV-1 genomic regions encoding the protease and reverse transcription (*pol* gene) were amplified using the following primer pairs: external PCR, PRTO5 (5′-GCCCCTAGGAAAAAGGGCTGT-

TABLE 1. *Clinical and virologic parameters of HIV-infected individuals from the ACTG 315 and IRIS studies*

Patient	Sample*	Date†	CD4 count (cells/μL)	Viral load (log_{10} copies/mL)	Antiviral treatment	PR mutations‡	RT mutations‡	Viral fitness§ (% relative to WT)
ACTG315 study								
610471	K0	5/1996	156	4.85	NRTI experienced	63P	67N, **70R, 215F,** 219Q	48
(71K)	K12	7/1966	331	2.65	AZT + 3TC + RTV	63P	67N, **70R, 215F,** 219Q	74
	K24	10/1996	411	2.65	AZT + 3TC + RTV	63P	67N, **70R, 215Y,** 219Q	43
	K48	4/1997	443	4.30	AZT + 3TC + RTV	63P, **82A**	67N, **70R, 184V, 215F,** 219Q	35
610363J	J0	6/1966	205	4.08	NRTI experienced	63P	None	41
(63J)	J12	9/1996	395	2.00	AZT + 3TC + RTV	63P	None	58
	J24	12/1996	322	2.00	AZT + 3TC + RTV	63P	None	74
	J48	5/1997	302	2.00	AZT + 3TC + RTV	63P	None	72
610185H	J0	6/1996	149	4.40	NRTI experienced	36I, 77I	41L, 210W, **215Y**	41
(85H)	H12	9/1996	276	2.00	AZT + 3TC + RTV	10I, 36I, 77I	41L, 67N, 210W, **215Y**	87
	H24	12/1966	309	2.00	AZT + 3TC + RTV	36I, 77I	41I, 67N, 210W, **215Y**	62
	H48	5/1997	267	3.93	AZT + 3TC + RTV	10I, 36I, 54V, 77I, **82A**	41L, 67N, **184V,** 210W, **215Y**	62
610500F	F0	10/1996	260	5.04	NRTI experienced	None	44D	102
(00F)	F12	1/1997	276	5.04	AZT + 3TC + RTV	None	44D	92
	F24	4/1997	147	4.92	AZT + 3TC + RTV	None	44D	95
	F48	9/1997	128	5.45	AZT + 3TC + RTV	None	44D	96
IRIS study								
1C	C0	9/1998	622	4.48	Untreated	63P, 71T, 77I	None	92
	C48	9/1999	580	4.06	d4T + 3TC + IDV	63P, 71T, 77I	None	89
2S	S0	7/1997	19	5.50	Untreated	10I, 63P, 77I	None	79
	S48	7/1998	236	3.71	3TC + RTV + SQV	20R, 63P	None	51
3M	M0	11/1997	199	5.14	Untreated	36I	None	65
	M24	4/1998	249	4.44	d4T + RTV + SQV	36I	None	67
4L	L0	4/1998	12	4.71	Untreated	63P	None	ND
	L36	12/1998	156	4.21	d4T + 3TC + IDV	63P, 77I	None	84
5W	W0	1/1998	252	3.02	AZT + 3TC	36I	**184V**	ND
	W48	12/1998	205	3.72	AZT + 3TC + IDV	36I	**184V**	45
6I	I0	9/1997	315	3.42	AZT + ddC	36I, 77I	None	ND
	I48	9/1998	505	3.18	3TC + RTV + SQV	36I, 77I	**184V**	42

* The letter indicates samples in each patient taken at specific weeks (number) after PI regimen initiation (e.g., K12, sample obtained from patient 610471K at 12 weeks posttherapy).

† Date at which blood samples were obtained (month/year).

‡ Drug resistance–associated mutations as previously described (http://www.iasusa.org). None, wild-type sequence as compared to HIV-1_{HXB2} (http://hiv-web.lanl.gov). Primary mutations are indicated in boldface letters.

§ Viral fitness is the average of 2 relative fitness values, corresponding to the competitions of each HIV-1 isolate with two HIV-1 control strains (see text for details) and then calculated relative to the wild-type HIV-$1_{92US076}$ virus control (100%).

ACTG, AIDS Clinical Trials Group; IRIS, indinavir ritonavir inhibitors study; PI, protease inhibitor; PR, protease; RT, reverse transcription; WT, wild type; NRTI, nucleoside reverse transcription inhibitor; AZT, zidovudine; 3TC, lamivudine; RTV, ritonavir; IDV, indinavir; SQV, saquinavir; ddC, zalcitabine; d4T, stavudine; ND, not determined.

TGG-3′, nt position 2008 corresponding to the HIV-1_{HXB2} isolate, http://hiv-web.lanl.gov) and IN3 (5′-CATTGCTCTCCAATTACTGTGATATTTCTCATG-3′, nt position 4263); and nested PCR, PRTI5 (5′-TGAAAGATTGTACTGAGAGACAGG-3′, nt position 2057), and OUT3 (5′-TCTATTCCATCTAAAAATAGTACTTTCCTGATTCC-3′, nt position 4212). The C2V3 fragment of the gp120-coding region (*env* gene) was PCR amplified using a set of external primers, E80-ED33,[27] followed by nested amplification using the ES7-E125 primer pair[27] as previously described.[28] Both external and nested PCR reactions were carried out in a 100-μL reaction mixture with defined cycling conditions.[28] Finally, for all growth competition experiments (see below), proviral DNA was extracted from lysed PBMCs using the QIAamp DNA Blood Kit (Qiagen), then PCR amplified using a set of external and nested primers, as previously described.[28,29] PCR-amplified products were isolated in agarose gels then purified using the QIAquick PCR Purification Kit (Qiagen).

Nucleotide Sequence Analysis

The full protease-coding region, the first 296 amino acids from the reverse transcription polymerase domain, and 105 amino acids spanning the C2V3 gp120-coding region of all 25 HIV-1 isolates from both patient groups were sequenced using AP Biotech DYEnamic ET Terminator cycle with Thermosequenase II (Davis Sequencing LCC, Davis, CA). Primers used in the sequencing reactions [*pol*, 3′T7PROT, 5′SP6P66/OUT, 3′HALFPOL,[30] and *env*, ES7[27]] have been described. Nucleotide sequences were edited and translated by BioEdit Sequence Alignment Editor v.5.0.7.[31] Multiple alignment and phylogenetic analyses were performed using the CLUSTAL X v.1.8 program.[32] Nucleotide sequences described in this study have been submitted to GenBank under the following accession numbers: IRIS study (*pol*, AY140807–AY140815) and ACTG 315 study (*pol*, AY140816–AY140831; *env*, AY140832–AY140847).

Cells and Viruses

PBMCs from HIV-seronegative blood donors were obtained by Ficoll-Hypaque density centrifugation of heparin-treated venous blood. Prior to HIV-1 infection, the cells were stimulated with 2 μg of phytohemagglutinin (PHA; Gibco BRL) per milliliter for 3–4 days and maintained in RPMI 1640/2 mM L-glutamine media (Cellgro; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Cellgro), 10 mM HEPES buffer (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Cellgro), 1 ng/mL of interleukin-2 (IL-2) (Gibco, BRL), 100 U of penicillin/mL, and 100 μg of streptomycin/mL (both from Cellgro). HIV-1 isolates were purified from PBMCs as previously described.[28] All viral stocks were propagated and expanded in PHA-stimulated, IL-2–treated PBMCs. Three syncytium-inducing HIV-1 primary isolates (A-92UG029, B-92US076, and AE-CMU06) were obtained from the AIDS Research and Reference Reagent Program. Tissue culture dose for 50% infectivity ($TCID_{50}$) was determined for each isolate in triplicate with serially diluted supernatants of each viral propagation. Reverse transcription[33] activity in culture supernatants on day 8 of culture was used to calculate $TCID_{50}$ values using the Reed and Muench[34] method. Titers were expressed as infectious units per milliliter (IU/mL). Finally, to verify that the viruses used to estimate viral fitness (obtained from PBMCs) matched the viruses circulating in plasma, the protease gene from each HIV-1 isolate was sequenced and compared with the sequence obtained from plasma HIV-1 RNA.

Growth Competition Experiments to Estimate HIV-1 Fitness

All dual infection/competition experiments were carried out as previously described,[28] with minor modifications. Briefly, each HIV-1 isolate was competed against 2 different HIV-1 control strains (A-92UG029 and AE-CMU06) in a 1:1 initial proportion using a multiplicity of infection of 0.01 IU/cell. One milliliter of these virus mixtures was incubated with 1×10^6 PBMCs for 2 hours at 37°C, 5% CO_2. Subsequently, the cells were washed 3 times with 1× phosphate-buffered saline and then resuspended in culture medium (1×10^6/mL). Cells were washed and fed with medium after 4 days. Supernatants and cells were harvested at day 8, resuspended in dimethyl sulfoxide/fetal bovine serum, and stored at −80°C for subsequent analysis. Nested PCR products, C2V3 of the *env* gene, were analyzed using the heteroduplex tracking analysis as previously described.[28] It is important to note that this viral fitness assay has been established and validated in multiple studies.[28,29,35] Finally, increases in PI resistance are often associated with substitutions in the protease cleavage sites (*gag* and *pol* genes).[6,14,36] By using HIV-1 isolates, our ex vivo fitness assay accounts for changes in these genomic regions. However, potential mutations in these regions and their contribution to overall viral fitness were not analyzed in this study.

To estimate the fitness of the HIV-1 isolates from the ACTG 315 and IRIS studies, the final ratio of the 2 viruses produced from each dual infection was determined by heteroduplex tracking analysis and compared with production in the monoinfections, as previously described.[28,29] Briefly, a *relative fitness* value for each virus in the competition was estimated by the production of each individual HIV-1 strain in the dual infection. A *total relative fitness* was calculated as the average of the 2 relative fitness values, corresponding to competition between each HIV-1 isolate from the patients and both HIV-1 control strains. The total relative fitness was then compared and expressed as a percentage of a wild-type subtype B HIV-1 primary isolate (B-92US076, taken as 100%).[29]

Statistical Methods

Differences between 2 different groups (e.g., CD4 cell counts or viral loads vs. relative fitness HIV-1 values) were determined by the Mann-Whitney rank sum test. Spearman rank order correlation coefficient was used to determine the strength of association or correlation between different parameters. All statistical tests were performed using SigmaStat v.2.03 program (SPSS, Chicago, IL).

RESULTS

Clinical and Virologic Data

The 2 clinical trials were designed to study the efficacy of PI-based regimens in PI-naive HIV-1–infected individuals. In the present study, we have selected 10 subjects from these 2 cohorts to evaluate HIV-1 fitness evolution. Sixteen viral isolates from 4 patients participating in ACTG 315 were analyzed. Of the 4 subjects, only 1 responded to the treatment (63J, Table 1) with plasma HIV-1 RNA levels decreasing after 12 weeks of treatment to values below the limit of sensitivity of the assay (≤ 2 $\log_{10}$ copies/mL). No primary mutations associated with PI or reverse transcription inhibitor resistance were detected in any plasma viral RNA sample from this patient (Table 1). However, 3 subjects clearly failed the treatment (measured as a rebound on plasma viral load). Plasma HIV-1 RNA levels of patient 85H rose to 3.93 $\log_{10}$ copies/mL at 48 weeks together with a decrease in CD4 cell count (Table 1). The presence of the primary mutations V82A (RTV resistance) and M184V (3TC resistance) in viral RNA sequences at 48 weeks is likely responsible for this increase in viral load. Interestingly, a discordant response was observed in patient 71K: plasma HIV-1 RNA level increased to 4.3 $\log_{10}$ copies/mL at 48 weeks (perhaps as a consequence of the selection of RTV-, AZT-, and 3TC-resistant viruses), but CD4 cell counts were 3-fold higher than the baseline value (Table 1, see below). Finally, patient 00F apparently was not adherent to the antiretroviral treatment. Plasma HIV-1 RNA levels remained elevated during the course of the treatment and CD4 cell counts dropped to 128 cells/μL at week 48 (Table 1). In addition, no PI resistance mutations were detected, although all viral isolates carried the E44D substitution in the reverse transcription gene associated with 3TC resistance when accompanied by several of the multi-NRTI-associated mutations (Table 1) (see also http://www.iasusa.org).[37]

Additional plasma and HIV-1 isolates were obtained from 6 PI-naive HIV-1-infected adults enrolled in the IRIS study. These patients were treated with 1 of 2 combination antiretroviral therapies: RTV + SQV + 1 NRTI (2S, 3M, and 6I subjects) or IDV + 2 NRTI (1C, 4L, and

5W subjects) (Table 1). A diverse virologic and immunologic response to the antiretroviral therapy was observed among these patients (i.e., decay of plasma HIV-1 RNA levels, with stable or increased CD4 cell counts) (Table 1).[26] Forty-eight weeks after therapy, only 2 subjects had viruses that carried the 3TC resistance M184V substitution in the RT. Finally, although all HIV-1 isolates from these patients carried wild-type protease genes, these protease sequences harbored different patterns of secondary mutations associated with PI resistance (Table 1).

pol and *env* Genetic Evolution During Combined Therapy

Three different regions of HIV-1 (*pol*, PR and RT; *env*, C2V3) were reverse transcribed, PCR amplified, and sequenced from 28 plasma samples obtained from 10 HIV-infected individuals from the ACTG 315 and IRIS studies. Intra- and interpatient nucleotide sequence diversities were calculated from a distance matrix based on the Kimura[38] 2-parameter model. Neighbor-joining trees were then constructed to compare the phylogenetic relationships of all *pol* and *env* sequences and confirm the presence of intrapatient clusters. As expected, HIV-1 sequences from each subject (intrapatient) formed a monophyletic group in each genomic region analyzed (Fig. 1). However, interpatient branches in the phylogenetic trees (i.e., genetic distances) were longer among sequences from the IRIS study due to the natural genetic diversity of nonsubtype B sequences (Fig. 1B, see below).

pol and *env* sequences were used to compare the genetic divergence (i.e., genetic distance between pre- and posttherapy sequences) from each patient with ≥2

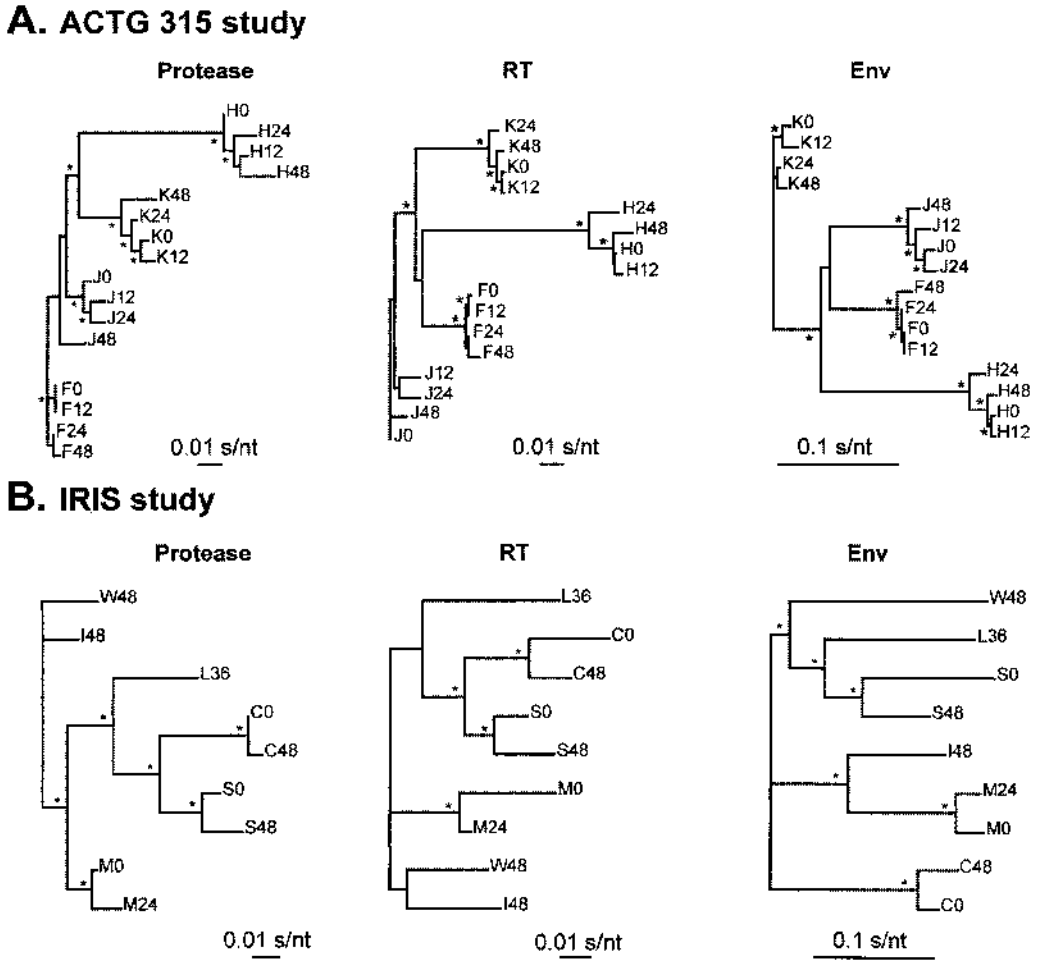

FIGURE 1. Phylogenetic tree analysis of the *pol* (protease and RT) and *env* (C2V3 region) genes from 25 HIV-1 isolates. HIV-1 sequences from the ACTG 315 (A) and IRIS (B) studies were used to construct neighbor-joining trees as described in "Materials and Methods." Bootstrap resampling values (1000 sets) >70% are indicated by an asterisk. The distance between two sequences in each tree is obtained by summing the length of the connecting branches, using the corresponding scale (in nucleotides substitutions per nucleotide, s/nt).

HIV-1 isolates. This genetic divergence was used to estimate the evolution of HIV-1 protease, reverse transcription, and Env sequences in PI-naive patients during combined antiretroviral therapy. Consequently, with a PI-based regimen, 48-week protease gene sequences from 3 ACTG 315 subjects (71K, 63J, and 85H) showed a 2.7 ± 0.3% (average ± SD) divergence from the baseline sequences (Fig. 2). A lower evolution (0.7%) was observed in protease sequences from the nonadherent 00F patient (Fig. 2). However, the average genetic divergence in reverse transcription was similar among all 4 ACTG 315 subjects (0.7 ± 0.3%) as was the average of *env* divergence (2.0 ± 0.7%) (Fig. 2). HIV-1 sequences from the 3 IRIS subjects with 2 longitudinal samples (i.e., 1C, 2S, and 3M) showed a similar evolution pattern for the protease gene (divergence, 2.2 ± 1.0%), with slightly higher divergence values for the reverse transcription (2.7 ± 0.3%) and Env (7.5 ± 3.4%) sequences (data not shown).

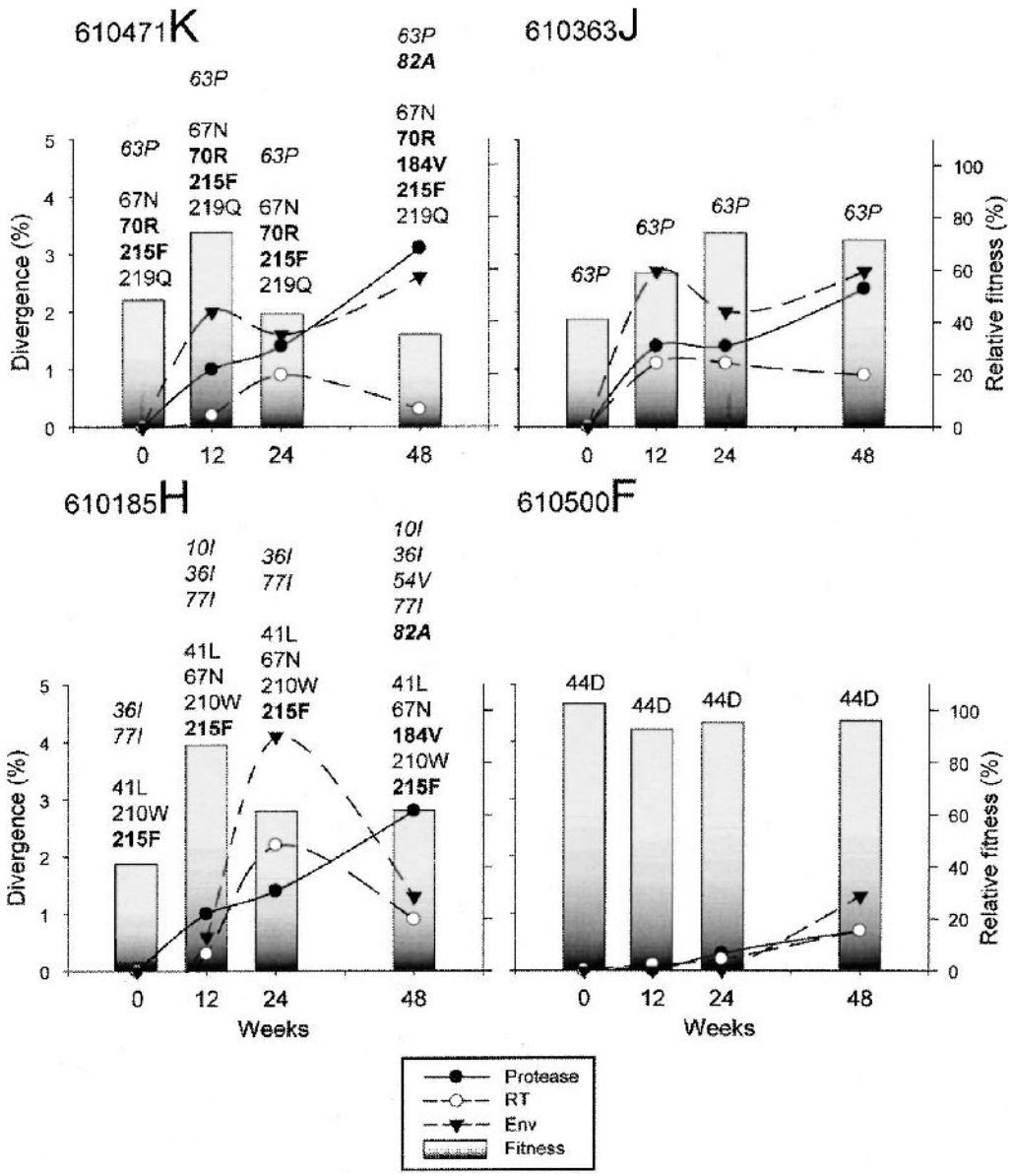

FIGURE 2. Analysis of HIV-1 *pol* and *env* genetic divergence in 4 HIV-1–infected patients from the ACTG 315 cohort and its relationship with viral fitness. Nucleotide genetic distances between sequences at baseline and weeks 12, 24, and 48 posttherapy were determined using the MEGA v.1.02 program,[59] and plotted as genetic divergence (%) from the baseline sample. A relative fitness was calculated for each HIV-1 isolate as described in "Materials and Methods" (see Table 1 for details). Drug resistance–associated mutations in each particular time point are indicated. Protease inhibitor (PI) mutations are depicted in italics, while primary mutations are indicated in boldface letters.

Taken together, these results suggest that in most cases both protease and Env HIV-1 genes were driving the viral genetic evolution in these HIV-infected individuals, perhaps due to the PI-based antiretroviral treatment and host immune selection, respectively.

Correlating Genetic Divergence With Viral Fitness in the Presence of Antiretroviral Therapy

Many studies have suggested that constant intrapatient HIV-1 evolution, and subsequent changes in viral fitness, may play a role in viral tropism, disease progression, and response to antiretroviral treatment.[5,6] In this study, we have used growth competition experiments to examine the relationship between HIV-1 *pol* and *env* divergence and viral fitness. As described earlier, 22 HIV-1 isolates corresponding to longitudinal samples from 2 different studies (ACTG 315 and IRIS) were competed against 2 different control HIV-1 primary isolates to get relative fitness values. This total relative fitness was then expressed as a percentage of a wild-type subtype B HIV-1 primary isolate (Table 1).

Evolution of wild-type HIV-1 protease and Env sequences from the responder 63J ACTG 315 subject appears to correlate with an increase in viral fitness over the 48-week period (a statistically significant correlation was not obtained due to the small number of points, data not shown) (Fig. 2). On the other hand, a reduced genetic evolution was observed in all 3 HIV-1 genomic regions analyzed from the nonadherent 00F patient, corresponding to relatively small changes in viral fitness (Fig. 2). However, a very different scenario was observed when HIV-1 sequences from the subjects with virologic failure (71K and 85H) were analyzed. Multiple mutations associated with PI and reverse transcription inhibitor resistance were detected in sequences from both patients (Table 1 and Fig. 2). As expected, viral isolates from both individuals showed a high divergence in the protease gene (Fig. 2). In addition to the L63P polymorphism in the PR gene, baseline HIV-1 quasispecies from patient 71K evolved to select only the V82A (RTV resistance) and M184V (3TC resistance) primary mutations (protease and reverse transcription genes, respectively) after 48 weeks of antiretroviral therapy (Table 1 and Fig. 2). These mutations led to a 27% reduction in viral fitness at 48 weeks posttherapy relative to the baseline HIV-1 isolate (Table 1 and Fig. 2). On the other hand, baseline HIV-1 isolates from the 85H subject carried 2 secondary mutations (i.e., M36I and V77I) in the protease gene in addition to several mutations related to AZT resistance (Table 1). During the 48 weeks of treatment, this virus accumulated additional secondary mutations in the protease gene (i.e., L10I and I54V), until a virus resistant to RTV, 3TC, and AZT emerged. Interestingly, this HIV-1 isolate was 50% more fit than its own baseline virus, perhaps as a consequence of the basal secondary mutations in the protease gene (Table 1 and Fig. 2).

HIV-1 isolates from 2 longitudinal time points were available from 3 IRIS subjects (i.e., 1C, 2S, and 3M) (Table 1). As described, these patients had detectable plasma viral loads but carried wild-type HIV-1 isolates after 48 weeks posttreatment. However, all these viruses harbored different secondary mutations in the protease gene (Table 1). In general, these mutations did not affect the fitness of the virus (e.g., L63P, A71T, and V77I in viruses from the 1C subject) (Table 1). However, in the 2S patient, selection of the K20R mutation in the protease gene at week 48 may have had an impact on the 35% decrease in viral fitness relative to the baseline virus (79–51%) (Table 1). Interestingly, a relatively low viral fitness was calculated for both HIV-1 isolates from the 3M subject (65 and 67% relative to the wild-type control, respectively), which carried viruses with the secondary M36I mutation in the protease gene (Table 1). It is important to note that a considerable decrease in HIV-1 fitness due to selection and accumulation of drug-resistant mutations in the *pol* gene may overcome the effect of other viral genomic regions, allowing the use of *pol* recombinant viruses or HIV-1 isolates to estimate replication capacity in viruses from patients treated with PIs or reverse transcription inhibitors. However, a definitive demonstration of the potential effects of these mutations in viral fitness would require the analyses of protease-recombinant viruses.

Finally, a fitness difference was calculated between the baseline and final HIV-1 isolates from each patient and plotted against protease, reverse transcription, and Env sequence divergences from the paired virus. As genetic divergence is an absolute number (i.e., how many mutations have been selected, fixed, and accumulated in relation to the baseline sequence), we compared this parameter with equal absolute fitness values (i.e., changes in viral fitness between baseline and 48-week samples). There was no correlation between reverse transcription and Env genetic divergence and changes in viral fitness ($r = 0.01$, $P > 0.9$ and $r = 0.13$, $P > 0.7$, respectively; Spearman rank order correlation). However, a positive correlation was observed between sequence divergence in the protease gene of each patient HIV-1 isolate and the respective HIV-1 fitness difference ($r = 0.78$, $P < 0.001$, Spearman rank order correlation) (Fig. 3A).

Because HIV-1 isolates from PI-naive individuals are subject to high selection pressure under a PI-based regimen,[8] perhaps even higher than the pressure of the host immune response over the *env* gene, PI selective pressure

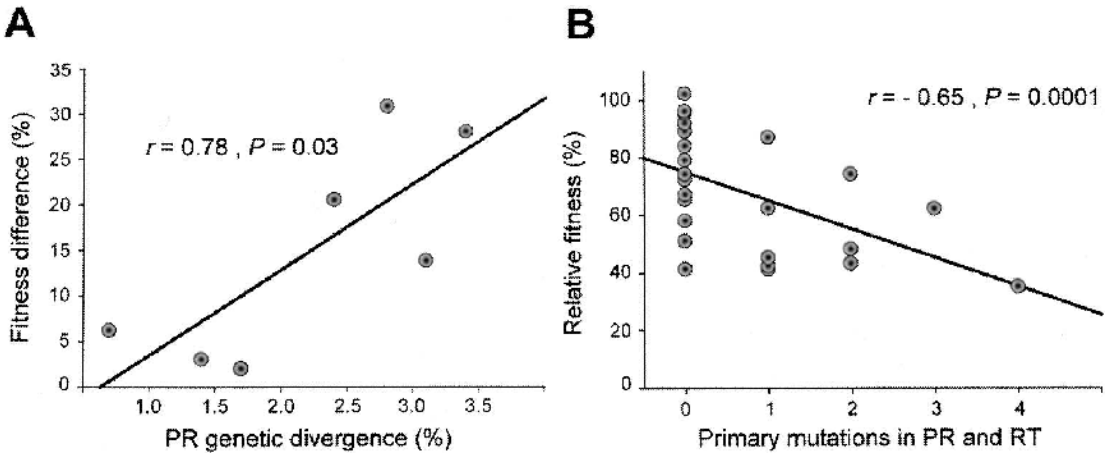

FIGURE 3. (A) Correlation between viral fitness difference and protease (PR) genetic divergence in 7 HIV-1–infected individuals from the ACTG 315 and IRIS cohorts. A difference in relative fitness between HIV-1 isolates at baseline and the last sample available for each patient (Table 1) was calculated and then plotted against paired PR genetic divergences. (B) Total number of primary drug resistance mutations in the PR and reverse transcriptase (RT) genes were plotted against the relative fitness of all 25 HIV-1 isolates from both cohorts.

seems to be driving both genetic and viral fitness evolution in viruses from these patients. A similar effect has been observed during the natural course of viral infection[39] and selective pressure on reverse transcription.[40]

Role of HIV-1 Fitness in Virologic/Immunologic Response to Antiretroviral Therapy

Several studies have associated HIV-1 fitness with changes in plasma HIV-1 RNA levels or CD4 T-cell counts.[17,28] However, the clinical significance of these relationships is not fully understood. As described above, we used growth competition experiments to estimate the relative fitness of 25 HIV-1 isolates from 2 different studies, ACTG 315 and IRIS. This relative fitness was then compared with several virologic and immunologic parameters (e.g., plasma HIV-1 RNA levels, CD4 T-cell counts, and number of primary and secondary mutations associated with resistance to PI and reverse transcription inhibitors). Based on *env* sequences, all HIV-1 isolates from the ACTG 315 study were classified as subtype B, whereas most of the C2V3 sequences from the European IRIS cohort clustered with nonsubtype B strains (i.e., subtype A, 3M and 6I; subtype B, 1C; subtype C, 5W; and subtype D, 2S and 4L) (data not shown). In addition, C2V3 sequences suggested that all HIV-1 isolates used in this study were nonsyncytium-inducing/CCR5-tropic (NSI/R5) viruses (data not shown). The absence of syncytium-inducing/CXCR4-tropic (SI/X4) viruses among these isolates circumvented the controversy of comparing fitness of viruses with 2 different biophenotypes.[28,41]

We analyzed viral fitness evolution over a 48-week trial period for each infected individual in this study. Patient 63J responded to the PI-based treatment with a steady low viral load ($\leq$2 $\log_{10}$ copies/mL) and CD4 cell counts >300 cell/μL, whereas the relative fitness of the wild-type HIV-1 isolates increased over time (Table 1 and Fig. 4). Conversely, all 4 HIV-1 isolates from the nonadherent 00F patient had a similar viral fitness, with a reduced genetic evolution, high viral loads, and depletion of CD4 cell counts (Table 1 and Fig. 4). Interestingly, the virologic and immunologic response to treatment was different between the subjects failing the ACTG 315 protocol (71K and 85H). Both patients entered the study with similar CD4 cell counts and plasma HIV-1 RNA levels (156 vs. 149 cells/μL and 4.8 vs. 4.3 $\log_{10}$ copies/mL, respectively; Table 1). At baseline, both subjects carried AZT-resistant HIV-1 isolates, and at 48 weeks, these viruses had selected the same primary mutations (V82A and M184V) responsible for reverse transcription and lamivudine resistance (Table 1). In addition, the fitness of the baseline HIV-1 isolates was similar between both individuals (48 and 41% relative to the wild-type control, respectively). However, at 48 weeks' posttherapy, a more marked increase in CD4 cell counts was observed in the 71K subject than in the 85H patient (Table 1 and Fig. 4). Our relative fitness assay showed that, whereas the fitness of the 71K HIV-1 isolate at 48 weeks had decreased to 35% of the wild-type control, the 85H HIV-1 isolate increased its fitness to 62% (i.e., a decrease of 27% and an increase of 50%, relative to their respective baseline relative fitness values, respectively) (Table 1 and Fig. 4).

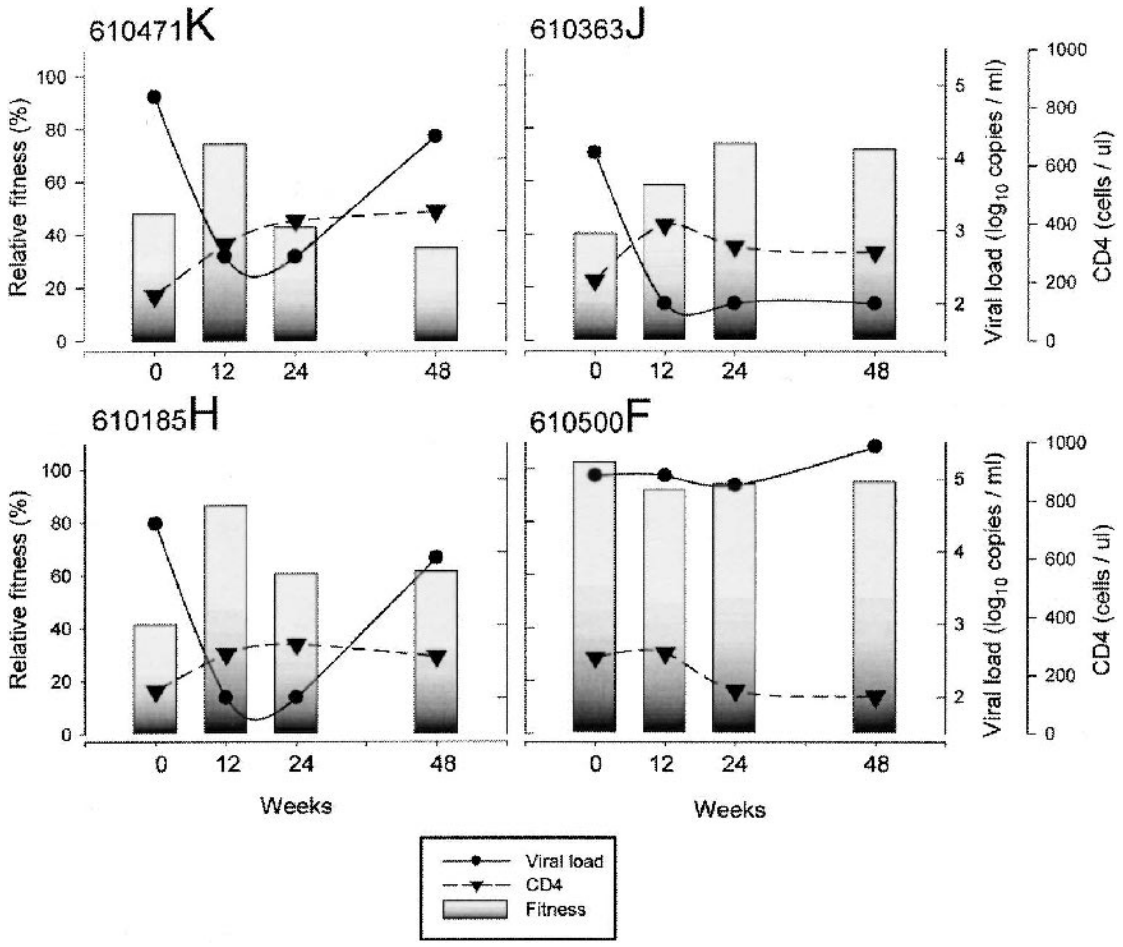

FIGURE 4. Relationship between viral fitness and virologic (plasma HIV-1 RNA levels) or immunologic ($CD4^+$ T-cell counts) response to a protease inhibitor (PI)-based regimen in 4 HIV-1 infected subjects from the ACTG 315 study. See Table 1 and Figure 2 for details.

Finally, each relative fitness value of all ACTG 315 and IRIS HIV-1 isolates was plotted against HIV-1 RNA loads, CD4 cell counts, and primary or secondary mutations associated with PI and reverse transcription inhibitor resistance. There was no correlation between relative viral fitness and CD4 cell counts ($r = 0.12$, $P > 0.56$, Spearman rank order correlation) or between relative fitness and plasma HIV-1 RNA levels ($r = -0.22$, $P > 0.30$, Spearman rank order correlation) in these treated patients. However, a negative correlation was observed between the relative fitness of each HIV-1 isolate and the number of primary mutations associated with PI and reverse transcription resistance ($r = -0.65$, $P = 0.0001$, Spearman rank order correlation) (Fig. 3B).

DISCUSSION

An initial decrease in viral fitness usually coincides with the appearance of primary substitutions conferring direct drug resistance during antiretroviral therapy. Continued drug pressure allows the selection of secondary or compensatory mutations to restore the enzymatic activity of the drug-targeted resistant enzyme (protease or reverse transcription) leading to a rebound in fitness.[5,6,8,9] However, preexistence of resistance-associated polymorphisms has the potential to contribute to a poor virologic response to antiretroviral drug treatment, perhaps by accelerating the recovery of viral fitness after selection of primary mutations, which may lead to treatment failure in HIV-infected drug-naive individuals. In this study, we have analyzed genetic and fitness evolution of HIV-1 isolates from 10 PI-naive HIV-1-infected individuals under 2 different PI-based antiretroviral protocols (ACTG 315 and IRIS) and its relationship with basal genetic background.

Phylogenetic analyses of *pol* and *env* sequences from these 10 HIV-1 isolates showed clear monophyletic intrapatient clusters (Fig. 1). HIV-1 evolution was esti-

mated by calculating the genetic divergence in 3 different regions (i.e., protease, reverse transcription, and envelope) from each patient with ≥2 longitudinal samples. HIV-1 protease genetic divergence was similar in 6 of 7 subjects (mean of 2.5%), whereas a 4-fold lower evolution was observed in protease sequences from the nonadherent 00F subject (0.7%). It is evident that the selection pressure of the PI regimen was driving the evolution of HIV-1 isolates in those patients under antiretroviral treatment. A lower genetic divergence was estimated for reverse transcription sequences from all HIV-1-infected individuals. Six of 10 subjects were treated previously with different reverse transcription inhibitors (e.g., AZT in all 4 ACTG 315 patients and a combination of 2 NRTIs in 2 subjects in the IRIS study) before being randomized to a PI-based regimen.[25,26] It is possible that pretreatment with reverse transcription inhibitor had diminished the selection effect of these drugs in comparison with the newly added PIs. Interestingly, changes in protease sequences due to PI regimen selective pressure had an effect over *env* gene evolution (Fig. 2). We observed a relatively stable genetic variation in the ACTG 315 subject nonadherent to the antiretroviral therapy (00F), with an expected higher genetic divergence in the *env* gene at 48 weeks. However, development of genotypic resistance to a PI seems to have affected *env* genetic variation in the other 3 patients, perhaps by reducing the size of the replicating viral population.[42] In general, population bottlenecks produced during antiviral therapy may randomly select changes in other genomic regions not directly implicated in drug resistance. Although contradictory results have been described relating the impact of AZT treatment on the evolution of the *env* gene,[43,44] changes in *env* during the emergence of resistance to a PI have been previously reported,[42,45,46] suggesting that viral bottlenecks during a strong PI selective pressure might be important in intrapatient viral evolution and HIV-1 pathogenesis. For example, this reduction in viral population, together with the enzymatic impairment of the mutated protease, may have a significant effect on viral fitness of drug-resistant variants as predicted by the "Muller ratchet" hypotheses.[47]

Evolution of phenotypic resistance to HIV-1 PIs is associated with the accumulation of amino acid substitutions in the protease.[48–50] Many of these substitutions, especially secondary mutations, have also been shown to exist in untreated, PI-naive populations.[3,15,16] In this study, natural PI-associated polymorphisms at baseline protease sequences were observed in 9 of 10 HIV-1 isolates, independently of the *env* subtype classification (Table 1), corroborating previous reports that showed no differences in frequency and distribution of intrinsic PI resistance–associated mutations and drug susceptibility between subtype B and nonsubtype B HIV-1.[51–54] Although the effect of these naturally occurring secondary mutations is usually manifested only in the presence of primary mutations, preexisting secondary mutations (or viral genetic background) may reduce the effectiveness of PI treatment through a more rapid progression to a resistant phenotype.[1,10,13,50] Thus, the number of secondary mutations needed to achieve a "fit" viral population during a PI-based therapy may determine the duration of the relative suppression of viral replication as well as affect the immunologic benefit. A previous description of the ACTG 315 study showed that polymorphisms at protease codons 10, 36, and 71 were associated with significantly faster second-phase decay rates.[25] In addition, a more recent study identified mutations at codons 10 and 36 of protease as stronger predictors of virologic failure.[55] We observed different patterns of secondary mutations or polymorphisms in 9 baseline protease sequences analyzed, including 10I, 36I, 63P, 71T, and 77I (Table 1), which may have influenced the rate at which PI-resistant viruses were selected during therapy and contributed to viral fitness evolution.

A statistically significant correlation was obtained between the number of primary mutations associated with drug resistance and viral fitness, but not for the number of secondary mutations at baseline. However, the best-fit HIV sequence may differ strongly between patients due to variation in multiple host (e.g., immune response) and viral (e.g., genetic background and replication capacity) factors.[5,6,9] Wild-type viruses from treatment-naive patients have a broad range of replicative capacities (47–89%, median 73%) compared with the HIV-1_{NL4-3} virus used as control.[56] Our results showed wild-type viruses with different polymorphisms in protease sequences and diverse viral fitness values (e.g., virus 1C-0 harbored substitutions at codons 63, 71, and 77 with a 92% relative fitness, whereas 3M-0 carried only the protease mutation 36I and had a 65% relative fitness). Thus, in wild-type HIV-1 strains, the real contribution of secondary PI-associated mutations to viral fitness, in the absence of antiretroviral drug selection, is still unclear. Finally, although viruses with the 3TC resistance mutation M184V in the reverse transcription showed a low viral fitness (i.e., 5W-48 45% and 6I-48 42%) as previously described,[57] all 4 HIV-1 isolates from patient 00F harboring the 3TC mutation E44D had a high viral fitness relative to the wild-type control (Table 1), corroborating the effect of this mutation only when accompanied by several of the multi-NRTI-associated mutations (www.iasusa.org).

As described earlier, a relatively poor response to therapy has been associated with a high number of baseline polymorphisms in the protease gene.[13] This was evident in the contrasting HIV-1 fitness evolution and virologic/immunologic response to the PI-based regimen of 3 subjects from the ACTG 315 study. These HIV-infected individuals had similar CD4 cell counts and plasma HIV RNA levels before initiation of the combined antiretroviral therapy (Table 1). However, their HIV-1 genotypic background was different at baseline: 63J (protease, 63P; reverse transcription, wild type); 71K (protease, 63P; reverse transcription AZT resistant), and 85H (protease, 36I and 77I; reverse transcription AZT resistant). Thus, these differences in pretherapy *pol* sequences may have been partially responsible for the success or failure of the antiretroviral treatment. The patient with wild-type genotype (63J) responded to the treatment, despite an increase in viral fitness over the 48-week treatment period. This is consistent with recent results describing an augmentation in wild-type HIV-1 fitness during the course of the viral infection (Collins and Arts, personal communication). However, we observed a greater increase in CD4 cell counts in the 71K patient with no secondary PI-associated mutations at baseline compared with the patient 85H with the polymorphisms 36I and 77I in pretreatment protease sequences. Interestingly, these differences in CD4 cell counts and basal genetic background correlated with changes in viral fitness at 48 weeks' posttherapy (i.e., −27% and +50%, respectively). Recent studies have correlated the impaired viral fitness of PI-resistant variants with an atypical response to antiretroviral therapy,[17,19] i.e., continued immunologic benefit (improvement in CD4 T-cell counts) despite virologic failure of the antiretroviral regimen (increase of plasma HIV RNA levels). Thus, a diminished replicative fitness due to the absence of protease secondary mutations at baseline may be sufficient to explain the relative preservation of immune cells observed in patient 71K. Furthermore, it seems plausible that preexistence of secondary mutations in protease genetic background allowed a rapid recovery of viral fitness in HIV-1 isolates from patient 85H and the consequent virologic and immunologic therapy failure. A previous study using HIV-1 isolates from one of these patients (71K) in the hu-PBL-SCID animal model showed diminished viral replication and preservation of CD4 T cells.[18] Unfortunately, the authors were not able to analyze viral fitness of HIV-1 isolates from the second patient (85H) to corroborate our results. At this point it is important to remember that viral fitness is intimately ligated to environmental factors, and that ex vivo or in vitro viral fitness determinations may or may not correlate to in vivo viral fitness in an HIV-infected human host.[6,58]

In conclusion, we have described viral genetic and fitness evolution in 10 PI-naive HIV-infected individuals receiving a PI-based antiretroviral treatment. Altogether, our results suggest that baseline genotypic-associated resistance in drug-naive HIV-infected individuals may have implications in viral fitness evolution and a further impact in resistance testing in patient management. Because identification of polymorphisms in any of these residues could be relevant in interpreting genotypic resistance data, drug therapies for HIV-1–infected individuals may need to be adjusted to preexisting viral protease gene profiles. Finally, further phenotypic and clinical evaluations are needed to verify whether PI resistance, viral fitness increase, and virologic/immunologic failure occurs more rapidly in individuals infected with HIV-1 strains harboring secondary PI resistance mutations.

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