ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2005, p. 2015–2025 Vol. 49, No. 5
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.5.2015–2025.2005

Novel Human Immunodeficiency Virus Type 1 Protease Mutations Potentially Involved in Resistance to Protease Inhibitors

Valentina Svicher,[1]† Francesca Ceccherini-Silberstein,[1]†* Fulvio Erba,[1] Maria Santoro,[1] Caterina Gori,[2] Maria Concetta Bellocchi,[2] Sara Giannella,[2] Maria Paola Trotta,[2] Antonella d'Arminio Monforte,[3] Andrea Antinori,[2] and Carlo Federico Perno[2]

Department of Experimental Medicine, University of Rome "Tor Vergata," Via Montpellier 1, 00133 Rome,[1] National Institute for Infectious Diseases "L. Spallanzani," Via Portuense 292, 00149 Rome,[2] and Institute of Infectious and Tropical Diseases, University of Milan, Hospital Sacco, Via G. B. Grassi 74, 20157 Milan,[3] Italy

Received 6 August 2004/Returned for modification 12 October 2004/Accepted 3 January 2005

Plasma-derived sequences of human immunodeficiency virus type 1 (HIV-1) protease from 1,162 patients (457 drug-naïve patients and 705 patients receiving protease inhibitor [PI]-containing antiretroviral regimens) led to the identification and characterization of 17 novel protease mutations potentially associated with resistance to PIs. Fourteen mutations were positively associated with PIs and significantly correlated in pairs and/or clusters with known PI resistance mutations, suggesting their contribution to PI resistance. In particular, E34Q, K43T, and K55R, which were associated with lopinavir treatment, correlated with mutations associated with lopinavir resistance (E34Q with either L33F or F53L, or K43T with I54A) or clustered with multi-PI resistance mutations (K43T with V82A and I54V or V82A, V32I, and I47V, or K55R with V82A, I54V, and M46I). On the other hand, C95F, which was associated with treatment with saquinavir and indinavir, was highly expressed in clusters with either L90M and I93L or V82A and G48V. K45R and K20T, which were associated with nelfinavir treatment, were specifically associated with D30N and N88D and with L90M, respectively. Structural analysis showed that several correlated positions were within 8 Å of each other, confirming the role of the local environment for interactions among mutations. We also identified three protease mutations (T12A, L63Q, and H69N) whose frequencies significantly decreased in PI-treated patients compared with that in drug-naïve patients. They never showed positive correlations with PI resistance mutations; if anything, H69N showed a negative correlation with the compensatory mutations M36I and L10I. These mutations may prevent the appearance of PI resistance mutations, thus increasing the genetic barrier to PI resistance. Overall, our study contributes to a better definition of protease mutational patterns that regulate PI resistance and strongly suggests that other (novel) mutations beyond those currently known to confer resistance should be taken into account to better predict resistance to antiretroviral drugs.

Human immunodeficiency virus type 1 (HIV-1) is characterized by a high degree of genetic variability, mainly due to the intrinsic inability of HIV-1 reverse transcriptase to carry out proofreading of DNA during replication (20), and is exacerbated by the high rate of HIV-1 replication in vivo ($\approx 10^9$ viral particles are produced daily), the accumulation of proviral variants, and genetic recombination (2). Among the different areas of the viral genome, the *pol* gene, which encodes enzymes such as reverse transcriptase and protease, is subjected not only to natural variation pressure but also to mutations imposed by the pharmacological treatment (6, 31).

The HIV-1 protease enzyme is responsible for the posttranslational processing of the viral polyproteins encoded by *gag* (p55) and *gag-pol* (p160), thus yielding the mature structural core proteins (the matrix, capsid, nucleocapsid, and p6 proteins) and the essential enzymes (protease, reverse transcriptase, and integrase) required to produce mature infectious particles (32).

For its crucial role in the HIV-1 life cycle, protease represents an important target for the antiretroviral therapy. To date seven protease inhibitors (PIs; indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, lopinavir, and atazanavir) have been approved by the Food and Drug Administration (FDA) and are clinically available. Unfortunately, when antiretroviral therapy fails to be fully suppressive, viral variants with reduced susceptibilities to PIs can emerge (8, 10, 21). Resistance to PIs is mediated by the appearance of protease amino acid substitutions (at positions either in direct contact with the inhibitor or at distant sites) that reduce the binding affinity between the inhibitor and the mutant protease enzyme (6). These amino acid substitutions, defined in the literature as major mutations, may deeply impair the protease catalytic activity and, consequently, the replication capacity of the virus (5, 19). Restoration of the replication capacity, which allows the viability of virus particles, is due to the presence of mutations defined in the literature as compensatory mutations (5, 9). Several studies have contributed to our current knowledge of the drug-related variants of HIV-1 protease. To date, mutations at 50 of 99 residues of protease have been related to one or more experimentally tested PIs, thus attesting to the high degree of flexibility of the protease enzyme (11, 13, 23, 29, 37); 22 of these residues are involved in resistance to the PIs used

* Corresponding author. Mailing address: Department of Experimental Medicine, University of Rome "Tor Vergata," Via Montpellier 1, 00133 Rome, Italy. Phone: 39-06-72596553. Fax: 39-06-72596039. E-mail: ceccherini@med.uniroma2.it.

† V.S and F.C.-S. contributed equally to this work.

in clinical practice (13; Stanford HIV Drug Resistance Database [http://hivdb.Stanford.edu]). Generally, single mutations confer only a modest reduction in drug susceptibility (with some exceptions, such as D30N, I47A, G48V, and I50L/V), while for many PIs, particularly if they are boosted with ritonavir, a stepwise accumulation of several protease mutations is required for the development of high-level drug resistance. This requirement of multiple mutations to overcome the activities of PIs has been referred to as a high genetic barrier to drug resistance (7, 16, 22). Furthermore, most drug resistance mutations in the protease confer cross-resistance to many PIs, so this resistance should be considered class specific rather than drug specific (10, 15, 17, 27).

Since protease is characterized by a high degree of structural flexibility and resistance to PIs is a such complex phenomenon, it is conceivable that the mutational pathways of HIV protease are far more complex than is currently known and that some mutations (and associations of mutations) have been not yet precisely defined. For instance, previous work recently identified some positions or mutations positively associated with treatment with PIs; however, their exact role in PI resistance has not yet been precisely clarified (3, 23, 37). Thus, we decided to focus our attention on these uncharacterized (novel) mutations to better understand their association with specific PIs and PI resistance-associated mutations and thus contribute to obtaining a better definition of the protease mutational patterns that regulate PI resistance.

The identification of new mutational clusters also suggests that these additional mutations may interact as part of higher-order networks and strongly suggests that other mutations beyond those currently known to be associated with PI resistance should be considered to define precise algorithms able to predict resistance to antiretroviral drugs.

MATERIALS AND METHODS

Patients. The study included 1,162 patients enrolled either in the Italian Cohort of Antiretroviral Naïve patients (ICONA) or in different clinical centers in central Italy; 457 patients were naïve to treatment with antiretroviral drugs, and 705 were failing an antiretroviral regimen containing at least one PI. Drug-naïve patients underwent genotypic tests between 1 January 1997 and 31 December 2003, while PI-treated patients underwent genotypic tests between 1 January 1998 and 31 December 2003. Overall, drug-treated patients were exposed to an average of two PIs, 680 (96.5%) were receiving a highly active antiretroviral therapy, and 701 (99.4%) PI-treated patients had a well-characterized antiretroviral treatment history. In detail, 61.1% of patients had been treated with indinavir, 56.4% had been treated with nelfinavir, 44.3% had been treated with saquinavir, 29.8% had been treated with ritonavir, 6.8% had been treated with lopinavir/ritonavir, 1.1% had been treated with amprenavir, and 0.1% had been treated with tipranavir. At the time of genotypic analysis, 47.5% of patients were receiving treatment with nelfinavir (median time, 426 days), 29.6% were receiving treatment with indinavir (median time, 615 days), 18.6% were receiving treatment with ritonavir (median time, 239 days), 16% were receiving treatment with saquinavir (median time, 302 days), 6% were receiving treatment with lopinavir/ritonavir (median time, 223 days), 1.1% were receiving treatment with amprenavir (median time, 271 days), and 0.1% were receiving treatment with tipranavir (median time, 14 days). Patients carrying non-B clades of HIV *pol* were excluded from this analysis. Data for all patients were stored in a specifically designed anonymous database that included mutational, demographic, immunologic, virologic, and therapeutic parameters.

HIV sequencing. HIV genotype analysis was performed with plasma samples by means of a commercially available kit (The ViroSeq HIV-1 Genotyping System; Applied Biosystems) (3, 26). Briefly, RNA was extracted, retrotranscribed with murine leukemia virus reverse transcriptase, and amplified with the AmpliTaq Gold polymerase enzyme by using two different sequence-specific primers for 40 cycles. The sense and antisense orientations of the full lengths of the *pol*-amplified products (containing the entire protease gene and the first 320 amino acids of the reverse transcriptase open reading frame) were sequenced by using seven different overlapping sequence-specific primers and an automated sequencer (ABI 3100) (3, 26). Sequences with a mixture of wild-type and mutant residues at single positions were considered to have the mutation(s) at that position. The isolates were subtyped by comparing their sequences to reference sequences of known subtype (http://hivdb.Stanford.edu).

Statistical analysis. To assess the association of protease mutations with PI treatment, we calculated their frequencies in isolates both from untreated patients and from PI-treated patients and performed chi-square tests of independence (based on a two-by-two contingency table) to verify whether the differences in frequency between the two groups of patients were statistically significant ($P < 0.05$). We considered novel mutations (T12A, A22V, E34Q, E35G, K43T, K45R, K55R, Q58E, I62V, L63Q, H69N, T74S, I85V, Q92K, and C95F) to be those that have not yet been reported to be associated with PI resistance by the Stanford HIV Drug Resistance Database (http://hivdb.Stanford.edu) and by the International AIDS Society (13). We also included in our analysis the novel mutations K20I and K20T, reported by the International AIDS Society to be specifically associated with resistance to atazanavir (approved by FDA in June 2003) and tipranavir (not yet approved by FDA), respectively, and also recently reported in the Stanford HIV Drug Resistance Database.

To assess the association of each novel mutation with treatment with a specific PI, we compared the rate of occurrence of each novel mutation in the subpopulation that experienced treatment with a specific PI with that in the subpopulation that did not experience treatment with that PI; we then performed chi-square tests of independence to verify statistically significant differences ($P < 0.05$).

We used the Benjamini-Hochberg method (1, 37) to identify results that were statistically significant in the presence of multiple-hypothesis testing. A false discovery rate of 0.05 was used to determine statistical significance.

Mutation covariation. We calculated the binomial correlation coefficient (phi) for all the possible pairwise combinations between novel mutations and the other protease mutations in the set of PI-treated patients. Statistically significant pairwise correlations were those with a P value of <0.05. We used the Benjamini-Hochberg method (1) to identify pairwise combinations that were significant in the presence of multiple-hypothesis testing. A false discovery rate of 0.05 was used to determine statistical significance.

For each pair, two positions each with a mixture of two or more mutations were excluded from the covariation analysis, since it is impossible to discriminate whether these mutations fall in the same viral genome.

Mutational clusters were defined as clusters of three or more mutated positions in which each position was significantly correlated with each other. Mutational clusters were identified by a computational technique that evaluated all possible clusters that can be formed from the significant correlated pairwise combinations of mutated positions. The computational technique was performed with the S-Plus program by implementation of hierarchical clustering algorithms. For the box-plot analysis, we considered D30N, V32I, M46I/L, I47A, G48V, I50L/V, I54A/L/M/S/T/V, V82A/F/S/T, I84V, N88D/S, and L90M to be major mutations (13; Stanford HIV Drug Resistance Database [http://hivdb.Stanford.edu]).

Structural analysis. The X-ray crystallographic coordinates with the code 1HIH.pdb deposited in the Protein Data Bank (PDB; http://www.rcsb.org/PDB/) were used as the template for structural analysis. Deep View (version 3.7) software was used to map amino acids onto the three-dimensional representation of the protease enzyme and to inspect the structures for hydrogen bond formation between residues. To calculate the interatomic distances between statistically significantly correlated residues, a script was developed to measure the distance between each pair of atoms within two different amino acids. The interresidue distances are referred to as the shortest interatomic distance between any atoms in two residues. Residues within 8 Å in the protease structure were considered near enough to interact (37).

Nucleotide sequence accession numbers. The majority of nucleotide sequences from drug-naïve patients have been submitted previously to GenBank (26). The 485 new sequences of isolates from patients treated with at least a PI within the HAART regimens (3) were submitted to GenBank and assigned the following accession numbers: AY855351 to AY855439, AY855441 to AY855458, AY855460 to AY855502, AY855504 to AY855556, AY855558 to AY855773, AY855775 to AY855795, AY855797 to AY855818, AY855820 to AY855836, and AY995408 to AY995555.

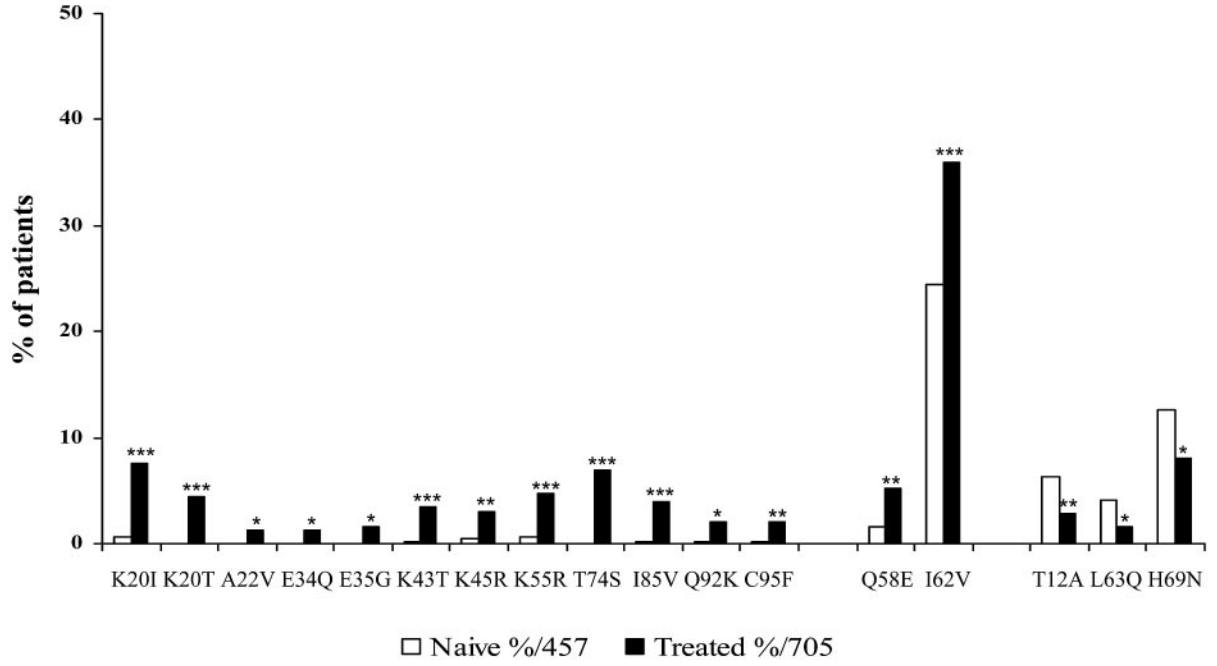

FIG. 1. Frequency of novel protease mutations in isolates from drug-naïve and PI-treated HIV-1-infected patients. Statistically significant differences were assessed by chi-square tests of independence. *P* values were significant at a false discovery rate of 0.05 following correction for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

Novel protease mutations and their association with treatment. By evaluating the entire protease sequences derived from 457 drug-naïve patients and 705 PI-treated patients, we identified 17 novel protease mutations significantly associated with treatment with PIs. These mutations were grouped in three classes (Fig. 1).

Class I included mutations (K20I, K20T, A22V, E34Q, E35G, K43T, K45R, K55R, T74S, I85V, Q92K, and C95F) that occurred at a very low frequency (<1%) in isolates from drug-naïve patients and showed a significant increase ($P = 0.05$ to 0.001) in isolates from PI-treated patients. K20T, A22V, E34Q, E35G, and T74S never occurred in isolates from drug-naïve patients, while K20T and T74S showed a remarkable increase in frequency in isolates from PI-treated patients (up to 4.4% and 7%, respectively; $P < 0.0001$). The frequencies of the K43T, K45R, I85V, Q92K, and C95F mutations never exceeded 4% in isolates from PI-treated patients, while the frequencies of the K55R and K20I mutations reached 4.7% and 7.2%, respectively ($P < 0.001$).

Class II included mutations (Q58E and I62V) that were already occurring in isolates from drug-naïve patients at a frequency >1% but that showed a significant increase ($P < 0.01$) in isolates from PI-treated patients (Q58E from 1.5% to 5.2% and I62V from 24.5% to 35.9%).

Interestingly, and different from the other classes, class III included mutations (T12A, L63Q, and H69N) that showed a significant decrease in isolates from PI-treated patients compared with that in isolates from drug-naïve patients. In a comparison of drug-naïve and PI-treated patients, the frequency of T12A decreased from 6.3% to 2.8%, that of L63Q increased from 4.2% to 1.6%, and that of H69N increased from 12.7% to 8.1% ($P < 0.05$ for all comparisons).

Many class I mutations and both class II mutations showed remarkable increases in frequency in isolates from patients who had experienced more than three PIs (Table 1). In particular, for E34Q, Q58E, I62V, and C95F, the increase in frequency observed in isolates from patients who experienced more than three PIs was statistically significant ($P < 0.05$) compared with that observed not only in isolates from drug-naïve patients but also in isolates from patients who experienced one or two PIs. On the other hand, class III mutations showed a progressive decrease in prevalence, despite experience with increased numbers of PIs (Table 1).

Most class I mutations were significantly associated ($P < 0.05$) with the use of specific PIs (Table 1). In detail, K55R, I85V, and C95F were specifically associated with indinavir treatment; K20T, K45R, and T74S were specifically associated with nelfinavir treatment; and E34Q and K43T were specifically associated with lopinavir/ritonavir treatment. K55R was also associated with lopinavir/ritonavir and nelfinavir treatment, while C95F was associated with saquinavir treatment. None of the other mutations was significantly associated with treatment with any specific PI, at least in this analysis.

Associations between protease mutations. A further step in our study was to assess the association of novel protease mutations with other mutations observed in PI-treated patients, with the focus of our attention on those involved in PI resistance.

Many mutations of class I or class II usually occurred in isolates with one to four major PI resistance mutations. More than three major mutations were found in >50% of the iso-

TABLE 1. Association of novel protease mutations with PI treatment

Novel mutation	Frequency (%) of mutations			Association with PIs[a]
	Naïve patients (n = 457)	PI-experienced patients[a]		
		Two or fewer (n = 508)	Three or more (n = 193)	
K20I	0.7	**6.9**	**9.3**	
K20T	0	**4.3**	**4.1**	**NFV (P = 0.0001)**
A22V	0	1.0	**2.1**	
E34Q	0	0.4	**3.6***	**LPV/r (P = 0.0001)**
E35G	0	**1.6**	1.6	
K43T	0.2	**2.6**	**6.2**	**LPV/r (P = 0.002)**
K45R	0.4	**3.9**	0.5	NFV (P = 0.035)
K55R	0.7	**3.7**	**7.3**	**IDV, LPV/r (P = 0.0001); NFV (P = 0.004)**
T74S	0	**6.3**	**8.8**	**NFV (P = 0.003)**
I85V	0.2	**4.5**	**2.6**	IDV (P = 0.01)
Q92K	0.2	**2.0**	**2.6**	
C95F	0.2	1.0	**4.7***	**IDV (P = 0.006)**; SQV (P = 0.02)
Q58E	1.5	3.7	**9.3***	
I62V	24.5	**33.1**	**43***	
T12A	6.3	3.3	**1.6**	
L63Q	4.2	1.8	1.0	
H69N	12.7	9.1	**5.7**	

[a] Values in boldface indicate statistically significant differences, corrected for multiple-hypothesis testing by the Benjamini-Hochberg method, with a false discovery rate of 0.05 with respect to the results for isolates from drug-naïve patients. Asterisks indicate statistically significant differences between isolates from patients experienced with PIs less than or equal to two and isolates from patients experienced with three or more PIs.

[b] Statistically significant association ($P < 0.05$) with PIs are reported. P values in boldface were significant at a false discovery rate of 0.05 following correction for multiple-hypothesis testing. Abbreviations: NFV, nelfinavir; LPV/r, lopinavir/ritonavir; IDV, indinavir; SQV, saquinavir.

lates from patients harboring one of the following mutations: E34Q, K43T, K55R, I85V, Q92K, C95F, or Q58E (Fig. 2). Among these, E34Q, K55R, and C95F were never found in the absence of major mutations, whereas isolates with class III mutations had a median number of major mutations ranging from 0 to 1 (Fig. 2).

To identify the patterns of pairwise correlations between novel mutations and specific protease mutations observed in isolates from PI-treated patients, we calculated the pairwise binomial correlation coefficient (phi) and its probability for each pair of mutations (Table 2).

Class I and class II mutations were positively correlated as pairs with many different PI resistance mutations. The most frequently selected multidrug resistance mutation, L90M (prevalence, 34.6%), was correlated with the novel mutation K20I (phi = 0.26), I62V (phi = 0.20), or C95F (phi = 0.17).

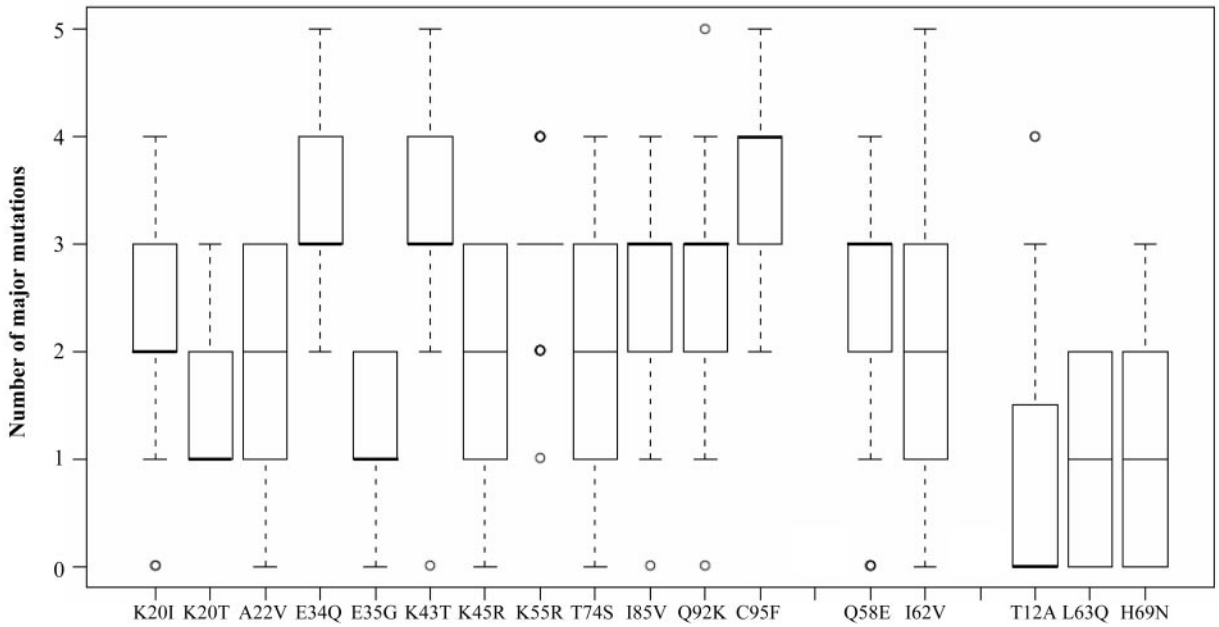

FIG. 2. Box plot representing the number of major HIV-1 drug resistance mutations (13; http://hivdb.Stanford.edu) associated with each novel protease mutation. The medians, interquartile ranges, upper and lower whiskers, and outlier values (○) are shown.

TABLE 2. Significantly correlated pairs of mutations

Novel mutation	Frequency (no)[a]	Correlated mutation[b]	Frequency (no)[a]	Covariation frequency (%[c])	Interresidue distance (Å)[d]	Phi value	*P* value[e]
K20I	53	L90M	244	40 (75.5)	12.7	0.26	6.3e−12
		G73S	88	20 (37.7)	14.8	0.22	3.9e−9
		M46I	158	24 (45.3)	8.9	0.17	5.0e−6
		T74S	49	10 (18.9)	12.5	0.13	3.9e−4
		A71V	277	27 (51)	13.2	0.13	7.9e−4
K20T	31	E35G	11	4 (13)	4.6	0.20	2.0e−7
		I15V	140	16 (51.6)	4.7	0.17	6.3e−6
		M36I	218	20 (64.5)	3.4	0.16	3.2e−5
		L90M	244	20 (64.5)	12.7	0.13	3.2e−4
		I13V	208	18 (58.1)	2.8	0.13	4.0e−4
E34Q	9	L33F	26	4 (44.4)	1.3	0.25	6.3e−11
		F53L	23	3 (33.3)	8.4	0.19	3.2e−7
E35G	11	K20T	31	4 (33.7)	4.6	0.20	2.0e−7
		M36I	218	11 (100)	1.3	0.19	5.0e−7
K43T	25	V32I	34	10 (40)	11.1	0.31	6.3e−17
		I47V	14	6 (24)	9.4	0.30	1.0e−15
		V82A	145	19 (76)	16.8	0.26	3.2e−12
		G48V	40	8 (32)	13.1	0.22	6.3e−9
		I54A	12	4 (16)	9.5	0.21	2.0e−8
		M46I	158	13 (52)	7.0	0.14	3.2e−4
		L10I	245	17 (68)	24.1	0.13	4.0e−4
		I54V	110	10 (40)	9.5	0.13	6.3e−4
K45R	20	N88D	66	8 (40)	9.5	0.18	2.0e−6
		D30N	98	8 (40)	4.5	0.13	6.3e−4
K55R	33	M46I	158	24 (72.7)	3.5	0.27	1.3e−12
		I54V	110	18 (55.5)	1.3	0.24	2.5e−10
		L24I	37	9 (27.3)	19.6	0.22	6.33e−9
		V82A	145	19 (57.6)	11.7	0.21	2.5e−8
		L10I	245	24 (72.7)	19.7	0.19	3.2e−7
		R57K	73	10 (30.3)	4.7	0.15	1.3e−4
T74S	49	G48V	40	11 (22.4)	13.1	0.21	5.0e−8
		I64V	137	24 (48.9)	7.0	0.20	6.3e−8
		K20I	53	10 (20)	13.9	0.13	4.0e−4
		V82A	145	19 (38.7)	12.7	0.12	1.0e−3
I85V	28	Q92R	9	4 (14.3)	8.4	0.24	4.0e−10
		I84V	72	11 (39.2)	1.3	0.20	2.0e−7
		V82T	16	4 (14.3)	6.8	0.16	1.3e−5

Continued on following page

I84V, another mutation conferring cross-resistance to all PIs, had as novel covariates either I85V (phi = 0.20) or Q92K (phi = 0.15). Different mutations at positions 82 and 54 were correlated as pairs with various novel mutations. V82A showed pairwise correlations with either K43T (phi = 0.26), K55R (phi = 0.21), or Q58E (phi = 0.18), while V82T recognized either C95F (phi = 0.18) or I85V (phi = 0.16) as a covariate. None of the other variations at position 82 (F, L, and S) was significantly associated with any novel mutations, probably because of their low frequency (<1.1%). I54V, the most common amino acid substitution at position 54 (15.6% of prevalence), showed a very strong pairwise correlation with Q58E (phi = 0.27), but it was also correlated with K55R (phi = 0.24). On the other hand, mutation I54A, which occurred at a very low frequency (1.7%), was correlated with K43T (phi = 0.21). The D30N and N88D mutations, specifically associated with resistance to nelfinavir, were correlated only with K45R, thus confirming the observed association of this mutation with treatment with this drug. Mutation G48V, which conferred high-level resistance to saquinavir (31), was associated with either C95F (phi = 0.27), K43T (phi = 0.22), or T74S (phi = 0.21). Among these mutations, only C95F was associated with saquinavir treatment.

Class III mutations did not show any positive correlations with PI resistance-associated mutations; on the contrary, H69N was negatively associated with the compensatory mutations M36I (phi = −0.14) and L10I (phi = −0.13). In addition, H69N showed a weak negative correlation with the major mutation M46I (phi = −0.05), although it was not statistically significant.

TABLE 2—*Continued*

Novel mutation	Frequency (no)[a]	Correlated mutation[b]	Frequency (no)[a]	Covariation frequency (%[c])	Interresidue distance (Å)[d]	Phi value	*P* value[e]
		I15V	140	14 (50)	7.7	0.15	5.0e−5
		L10I	245	19 (67.8)	8.2	0.15	6.3e−5
		M46I	158	13 (46.4)	15.5	0.12	1.0e−3
		I62V	253	17 (60.7)	9.9	0.11	2.5e−3
Q92K	15	I84V	72	6 (40)	12.5	0.15	1.3e−4
C95F	14	G48V	40	7 (50)	19.9	0.27	4.0e−13
		V82T	16	3 (21.4)	15.9	0.18	1.3e−6
		L90M	244	13 (92.8)	3.0	0.17	4.0e−6
		L10I	245	13 (92.8)	11.2	0.17	4.0e−6
		I54V	110	8 (57.1)	22.0	0.16	1.6e−5
		I93L	241	10 (71.4)	3.4	0.11	3.2e−3
		V82A	145	7 (50)	15.9	0.10	6.3e−3*
Q58E	37	I54V	110	21 (56.7)	9.0	0.27	1.3e−12
		V82A	145	19 (51.3)	14.5	0.18	2.0e−6
		L10I	245	24 (64.8)	21.4	0.15	8.0e−5
		K20M	29	6 (16.2)	15.1	0.15	1.0e−4
I62V	253	L90M	244	118 (46.6)	11.1	0.20	1.0e−7
		L10I	245	119 (47)	18.1	0.20	1.3e−7
		M36I	218	105 (41.5)	7.8	0.18	2.5e−6
		G73S	88	46 (18.2)	2.7	0.13	6.3e−4
		M46I	158	74 (29.2)	16.0	0.13	8.0e−4
		185V	28	17 (6.7)	9.9	0.11	2.5e−3
		I93L	241	99 (39.1)	10.4	0.10	1.0e−2*
L63Q	11	I64V	137	8 (72.7)	1.3	0.17	6.3e−6
H69N	57	I72V	83	15 (26.3)	7.2	0.14	3.2e−4
		M36I	218	5 (8.7)	15.1	−0.14	1.6e−4
		L10I	245	7 (12.3)	13.1	−0.13	4.0e−4

[a] The frequency was determined for 705 isolates from PI-treated patients.
[b] Positive and negative correlations with phi ≥ 0.10 or phi ≤ -0.10, with $P < 0.05$ are shown.
[c] The percentages were calculated for patients containing each specific novel mutation.
[d] The distance between wild-type residues is referred to as the shortest interatomic distance between any atoms in two residues.
[e] *P* values were corrected for multiple comparison (by the Benjamini-Hochberg method), with an false discovery rate of 0.05. Asterisks indicate *P* values not significant at a false discovery rate of 0.05.

Cluster of correlated positions. The mathematical approach used in our study let us define some clusters of mutated positions in which all possible pairs were mutually correlated. Among the class I mutations, K20I, K43T, K45R, K55R, I85V, and C95F were involved in one or more clusters that ranged in size from three to seven mutated positions (Table 3). K45R specifically clustered only with D30N and N88D. K43T was involved in two clusters, both of which included V82A; one was in the presence of I54V, while the other was in the presence of both I47V and V32I. Similarly, C95F was involved in two different and separated clusters. The most frequent cluster (71.4% of isolates with C95F) included L90M with the compensatory mutation I93L, while the other cluster (35.7% of isolates with C95F) included V82A with other major mutations (G48V and I54V). The presence of V82A was also observed in a large cluster involving K55R together with the major mutations M46I and I54V and the compensatory mutations L10I and L24I. Clusters involving I85V contained only I84V as the major mutation. K20I frequently clustered with the major mutation I54V and the compensatory mutation M36I. The other novel mutations of class I (A22V, K20T, E34Q, E35G, T74S, and Q92K) were not found to cluster at a significant level with PI resistance mutations.

Among the class II mutations, Q58E was involved in one cluster (present in 32.4% of the isolates with Q58E) with three major mutations (M46L, I54V, and V82A) and the compensatory mutation L10I. This cluster was the only one in which M46L was predominant instead of M46I (which was present in all other clusters involving mutations at this position). On the other hand, I62V was involved in four types of clusters, with prevalences ranging from 3.9% to 31.6%. Two of them involved L90M with or without M46I. It is noteworthy that among all clusters analyzed, the compensatory mutation L63P, which was present at a very high frequency in isolates from PI-treated patients (72%), was observed to be specifically involved in only one cluster with I62V and L90M.

Locations of novel mutations in the three-dimensional structure of protease. The distribution of the novel mutations was widespread in the HIV-1 protease structure (Fig. 3A), and the mutations were not directly involved in protease binding sites and did not directly affect the dimerization interface (36). However, several novel mutation residues were located at min-

TABLE 3. Clusters involving novel mutations and PI resistance-associated mutations

Novel mutation[a]	PI resistance-associated mutation(s)[b]	Cluster	Cluster prevalence	
			No. (%) of all PI-treated patients	No./frequency no. of novel mutation (%)
K20I	**M36I + I54V**	**20 + 36 + 54**	**12 (1.7)**	**12/53 (22.6)**
	L10I + I72L + G73S	10 + 20 + 72 + 73	7 (1)	7/53 (13.2)
	L10I + I54V + A71V	10 + 20 + 54 + 71	6 (0.8)	6/53 (11.3)
	L10I + M46I + A71V + G73S + 184V + L90M	10 + 46 + 71 + 73 + 84 + 90	3 (0.4)	3/53 (5.6)
K43T	**I54V + V82A**	**43 + 54 + 82**	**12 (1.7)**	**12/25 (48)**
	V32I + 147V + V82A	32 + 43 + 47 + 82	6 (0.8)	6/25 (24)
K45R	D30N + N88D	30 + 45 + 88	6 (0.8)	6/21 (28.6)
K55R	**L10I + M46I + I93L**	**10 + 46 + 55 + 93**	**10 (1.4)**	**10/33 (30.3)**
	L10I + L24I + M46I + I54V + V82A	**10 + 24 + 46 + 54 + 55 + 82**	**8 (1.1)**	**8/33 (24.2)**
I85V	**L10I + I62V + I93L**	**10 + 62 + 85 + 93**	**11 (1.6)**	**11/28 (39.3)**
	L10I + I84V	**10 + 84 + 85**	**11 (1.6)**	**11/28 (39.3)**
	I84V + Q92R	84 + 85 + 92	4 (0.6)	4/28 (14.3)
C95F	**L10I + L90M + I93L**	**10 + 90 + 93 + 95**	**10 (1.4)**	**10/14 (71.4)**
	L10I + G48V + I54V + V82A	10 + 48 + 54 + 82 + 95	5 (0.7)	5/14 (35.7)
Q58E	**L10I + M46L + I54V + V82A**	**10 + 46 + 54 + 58 + 82**	**12 (1.7)**	**12/37 (32.4)**
I62V	**L10I + L63P + L90M**	**10 + 62 + 63 + 90**	**80 (11.3)**	**80/253 (31.6)**
	L10I + I72L/M/T/V + G73S	10 + 62 + 72 + 73	22 (3.1)	22/253 (8.7)
	L10I + I85V + I93L	10 + 62 + 85 + 93	11 (1.6)	11/253 (4.3)
	L10I + M46I + G73S + L90M + I93L	10 + 46 + 62 + 73 + 90 + 93	10 (1.4)	10/253 (3.9)

[a] Boldface indicates clusters that occurred at a frequency >1% in which a novel mutation occurred at a frequency of >20%.
[b] PI resistance-associated mutations occurring in the cluster with a frequency >50%.

imal interatomic distances from PI resistance-associated residues. In particular, residues 43, 45, and 55, which are located in the flap, showed interatomic distances <8 Å with several PI resistance-associated residues (residues 43 to 46, 45 to 30, 55 to 46, and 55 and 54), and residue 85 showed an interatomic distance <8 Å with substrate binding sites (V82 and I84). Overall, among the 61 statistically significant correlated pairs of residues, 23 (37.7%) were within 8 Å of each other; and in particular, 4 of the 23 correlated pairs of residues were contiguous (residues 34 and 33, 35 and 36, 55 and 54, and 85 and 84) (Table 2).

Interestingly, the residue at position 45, which clustered with residues 30 and 88, showed interatomic distances of 4.5 Å with residue 30 and 9.5 Å with residue 88. Furthermore, the electrostatic representation of the molecular surface of the protease with the mutations D30N, K45R, and N88D showed that the D30N mutation determined a loss of a negative charge on the protease surface that was restored by the mutation N88D, while the mutation K45R, which resulted in an increase in the size of the side chain, contributed to the enlargement of the positively charged area near position 30 (Fig. 3B).

Finally, among the highly correlated residues, the novel residue 95 showed interatomic distances <4 Å with residues 90 and 93. In particular, the three residues are involved in a hydrogen bond network, and the carbonylic oxygen in the backbone of residue 90 interacts with the nitrogens of residues 93, 94, and 95 (Fig. 3C).

DISCUSSION

Our study suggests that other HIV-1 protease mutations, beyond the currently reported PI resistance-associated mutations, may regulate the development or maintenance of resistance to PIs. In fact, 17 novel mutations have been identified, characterized, and grouped into three distinct classes. Class I mutations were rare or completely absent in isolates from drug-naïve patients. In contrast, their frequency significantly increased in isolates from patients treated with at least one PI, thus suggesting the requirement of selective pressure with a PI for their emergence at virological failure. In confirmation of this, some mutations (K43T, T74S, I85V, Q92K, and C95F) rapidly disappeared during therapy interruption, with the dynamics of disappearance being similar to that observed for major PI resistance-associated mutations (data not shown). The frequency of class II mutations, which were already present in isolates from drug-naïve patients, significantly increased in isolates from PI-treated patients, thus suggesting the positive association of these mutations with PI treatment. In particular, I62V, observed in 24.5% of isolates from drug-naïve patients, may be considered a common protease polymorphism.

These results are consistent with recent analyses conducted with a large number of protease sequences by two independent groups. In fact, both class I and class II mutations were included in a list of 22 newly described treatment-associated mutated positions (37). The fact that Wu et al. (37) observed a

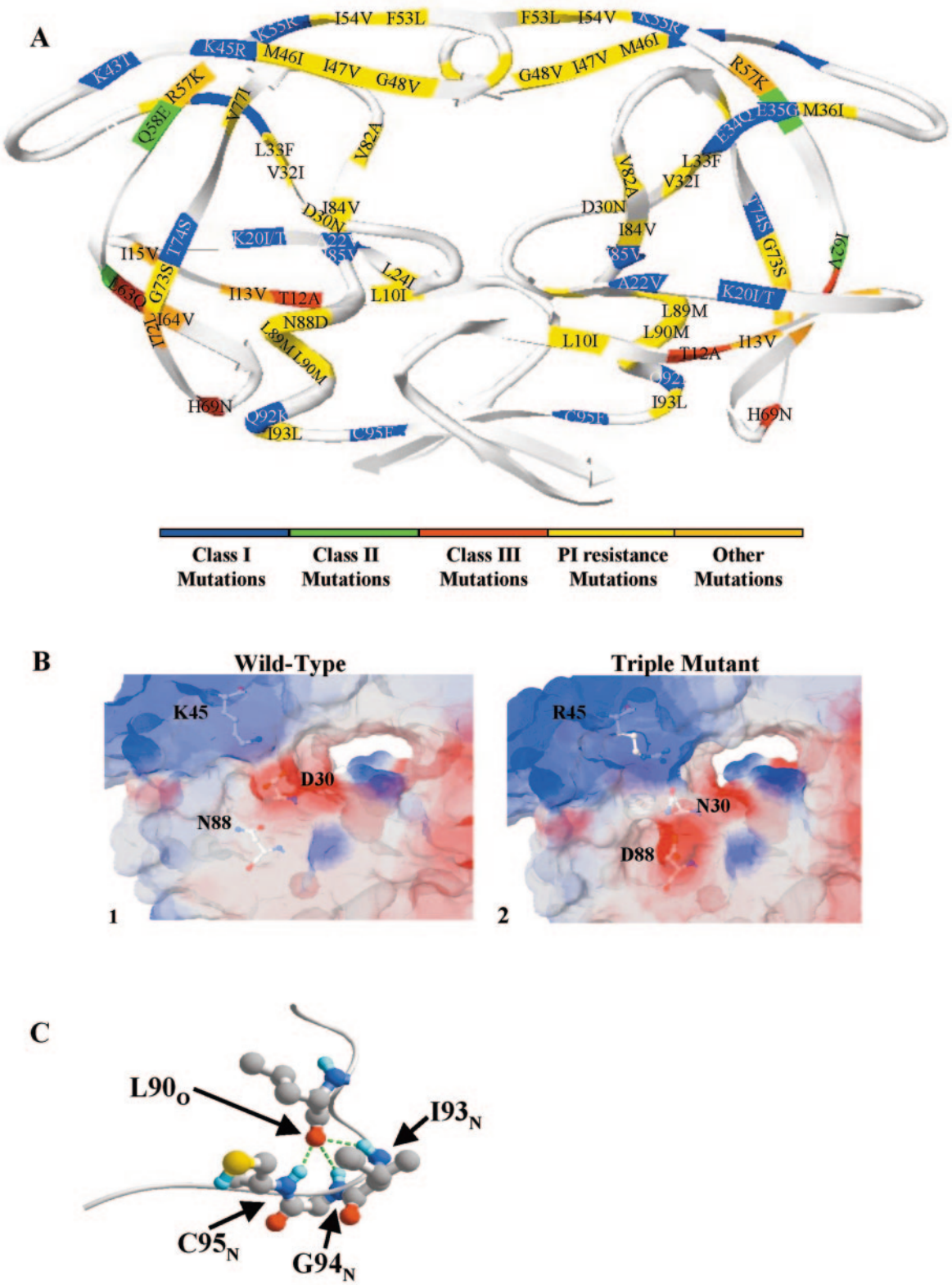

FIG. 3. Localization of novel mutations in the structure of HIV-1 protease. (A) A ribbon model of HIV-1 protease showing the three different classes of novel mutations and all mutations correlated. (B) Insights into the protease molecular surface showing changes in charge distribution due to mutations D30N, K45R, and N88D. Red and blue, negatively and positively charged regions, respectively. In panel 2 side chains of mutated residues are rotated to minimize strong van der Waals clashes. (C) Hydrogen bond networks among residues at positions 90, 93, 94, and 95. Grey, carbonium atoms; red, oxygen atoms; blue, nitrogen atoms; yellow, the sulfur atom of Cys.

higher number of new treatment-associated mutated positions may probably be related to the different number of patients analyzed, the patients' characteristics, and the therapeutic regimens used. In addition, most class I and II mutations (K20T, E34Q, K43T, K45R, K55R, T74S, I85V, Q92K, Q58E, and I62V) were included as candidate mutations for drug resistance or positive reproductive fitness by having a ratio of the number of nonsynonymous mutations to the number of synonymous mutations >1, with values similar to those observed for the majority of the already-known PI resistance-associated mutations (4).

Many class I and class II mutations were strongly correlated (both in pairs and in clusters) with PI resistance-associated mutations and rarely occurred in the absence of major mutations, suggesting that they may require the accumulation of PI resistance-associated mutations in the protease enzyme for their emergence. In particular, correlations frequently involved one or more multi-PI resistance-associated mutations at positions 82, 84, and 90, thus confirming the wide cross-resistance that characterizes PIs. We have no evidence how each mutation could influence (enhance) drug resistance or increase replication capacity; it is conceivable that class I and class II mutations may act as minor mutations able to rescue the loss of fitness or to increase the level of resistance. As confirmation of these hypotheses, K20I and K20T were recently included in the set of mutations associated with reduced susceptibility to atazanavir and tipranavir, respectively, even if their exact role has not been determined (13). Parkin et al. (24) included E34Q, K43T, Q58E, and T74S in the list of lopinavir mutational scores used as a predictor of reduced lopinavir susceptibility; in particular, E34Q and K43T are associated with lopinavir resistance in the rules-based CREST algorithm (http://assign.dci.uwa.edu.au/crest/crest5_table.asp). Furthermore, it was observed to be a better predictor of lopinavir resistance by use of an artificial neural network when a set of mutations, including K43T, K55R, Q58E, T74S, I85V, and C95F, was used (34). Consistent with this report, our analysis shows that E34Q, K43T, and K55R occurred in isolates from patients who failed an antiretroviral therapy containing lopinavir/ritonavir and correlated with mutations associated with lopinavir resistance (13, 31). In fact, E34Q was specifically associated with either L33F or F53L, and K43T was specifically associated with I54A and clustered with multi-PI resistance mutations (V82A and I54V or V82A, V32I, and I47V), while K55R clustered with multi-PI resistance mutations (V82A, I54V, and M46I). Therefore, these data strongly suggest that E34Q, K43T, and K55R will need consideration in more than one resistance algorithm to correctly predict lopinavir resistance. In addition, K55R significantly occurred also in patients failing a nelfinavir-containing regimen, thus supporting the previously reported association of K55R with reduced susceptibility to nelfinavir (18).

Similar to K55R, Q58E clustered with positions 10, 46, 54, and 82; in this context, the most common amino acid substitution at position 46 was M46L and not M46I. We then suppose that M46I and M46L may have different compensatory pathways mediated by K55R and Q58E, respectively, thus supporting the hypothesis formulated by Hoffman et al. (12), who suggested that as a consequence of different codon usage at position 46, M46I and M46L lie along divergent evolutionary pathways.

On the other hand, C95F clustered with G48V and V82A, whose copresence reflects prolonged saquinavir exposure (30, 35). As C95F was associated with saquinavir use, we may suppose that this mutation may contribute to saquinavir resistance. However, C95F was also strongly associated with indinavir use and was strongly associated in a cluster with L90M and I93L. Our structural analysis showed that residues at positions 90, 93, and 95 directly interact through a hydrogen bond network that stabilizes the ending loop from positions 92 to 95 of the protease enzyme.

Two mutations (K45R and K20T) were specifically associated with nelfinavir. K45R occurred in isolates from patients who failed a first-line regimen containing nelfinavir and showed a highly specific association with nelfinavir-associated mutations D30N and N88D (25, 28). Kaldor et al. (14) demonstrated that the D30N mutation reduces a hydrogen bond with bound nelfinavir. However, D30N also determines a loss of a negative charge on the molecular surface of the protease enzyme. The restoration of this negative charge is somewhat related to the emergence of N88D, thus helping the overall increase in protease efficiency. K45R, which results in an increased size of the side chain, may contribute to an enlargement of the positively charged area around position 30. K20T significantly occurred in patients who failed nelfinavir as the second PI and was significantly correlated with the multi-PI resistance-associated mutation L90M, suggesting that K20T may contribute to L90M-mediated resistance to nelfinavir. Also, K20I and I62V were strongly correlated with L90M, but they were not associated with any specific PI, thus suggesting that they may play their compensatory roles in the context of cross-resistance.

Although biochemical experiments are required to demonstrate the mechanism of correlation between pairs of residues, our study provides preliminary hypotheses on the importance of these novel mutations. In addition, the fact that many class I and II residues are located at minimal interatomic distances with their positively correlated PI resistance-associated residues in the three-dimensional protease structure confirms a role of the local environment in the interactions among mutations.

Class III mutations are common polymorphisms in isolates from drug-naïve patients, and their frequency decreased in isolates from patients who failed an antiretroviral regimen containing at least one PI, thus suggesting the negative association of these mutations with PI treatment and failure. Class III mutations were rarely found in the presence of major mutations and were never positively correlated with any PI resistance-associated mutations. If anything, H69N showed negative correlations with the minor mutations M36I and L10I. These data suggest a protective role for class III mutations that may prevent the appearance of PI resistance-associated mutations under PI pressure, thus contributing to increases in the level of the genetic barrier to PI resistance. However, this hypothesis needs confirmation in in vitro studies and clinical practice.

It should be noted that all patients analyzed in this study carried the HIV-1 clade B subtype. Further studies should also investigate the prevalence and the role of the novel mutations in non-B subtype clades. In fact, it is known that pathways of viral evolution toward drug resistance may proceed through

distinct steps and at different rates among different HIV-1 subtypes (33). Also, we cannot exclude the possibility that novel mutations, and in particular, the observed patterns of correlated mutations, may be the result of pharmacological pressure imposed by the drug regimens that were used by our cohort, while other treatment regimens may lead to the development of pathways in part different from those that we observed. Further studies and extended databases (with genotypic and/or phenotypic data) may provide insights regarding this important point.

In conclusion, our study strongly suggests that PI resistance is a very complex phenomenon and is regulated both positively and negatively by a highly ordered network of mutations, which also reflects the high genetic barrier to PI resistance. On this basis, novel mutations must be taken into account to define more precise algorithms able to correctly predict resistance to antiretroviral drugs.

ACKNOWLEDGMENTS

This work was financially supported by grants from the Italian National Institute of Health, the Ministry of University and Scientific Research, Current and Finalized Research of the Italian Ministry of Health, and the European Community (grant QLKT-CT-2000-00291 and the Descartes Award). The ICONA network is supported by unrestricted educational grants from Glaxo-Smith-Kline, Italy.

We thank Ada Bertoli, Alessandra Cenci, Federica Forbici, Roberta D'Arrigo, Fabio Continenza, Andrea Bidditu, and Sandro Bonfigli for sequencing and data management and all the ICONA study group participants and members.

REFERENCES

1. **Benjamini, Y., and Y. Hochberg.** 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B **57:**289–300.
2. **Burke, D. S.** 1997. Recombination in HIV: an important viral evolutionary strategy. Emerg. Infect. Dis. **3:**253–259.
3. **Ceccherini-Silberstein, F., F. Erba, F. Gago, A. Bertoli, F. Forbici, M. C. Bellocchi, C. Gori, R. D'Arrigo, L. Marcon, C. Balotta, A. Antinori, A. d'Arminio Monforte, and C. F. Perno.** 2004. Identification of the minimal conserved structure of HIV-1 protease in the presence or absence of drug pressure. AIDS **18:**11–19.
4. **Chen, L., A. Perlina, and C. J. Lee.** 2004. Positive selection detection in 40,000 human immunodeficiency virus (HIV) type 1 sequences automatically identifies drug resistance and positive fitness mutations in HIV protease and reverse transcriptase. J. Virol. **78:**3722–3732.
5. **Chen, Z., Y. Li, H. B. Schock, D. Hall, E. Chen, and L. C. Kuo.** 1995. Three-dimensional structure of a mutant HIV-1 protease displaying cross-resistance to all protease inhibitors in clinical trials. J. Biol. Chem. **270:** 21433–21436.
6. **Clavel, F., and A. J. Hance.** 2004. HIV drug resistance. N. Engl. J. Med. **350:**1023–1035.
7. **Condra, J. H., D. J. Holder, W. A. Schleif, O. M. Blahy, R. M. Danovich, L. J. Gabryelski, D. J. Graham, D. Laird, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, T. Yang, J. A. Chodakewitz, P. J. Deutsch, R. Y. Leavitt, F. E. Massari, J. W. Mellors, K. E. Squires, R. T. Steigbigel, H. Teppler, and E. A. Emini.** 1996. Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J. Virol. **70:**8270–8276.
8. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
9. **Hertogs, K., M. Van Houtte, and B. A. Larder.** 1999. Testing for HIV-1 drug resistance: new development and clinical implications. Rec. Res. Dev. Antimicrob. Agents Chemother. **3:**83–104.
10. **Hertogs, K., S. Bloor, S. D. Kemp, C. Van den Eynde, T. M. Alcorn, R. Pauwels, M. Van Houtte, S. Staszewski, V. Miller, and B. A. Larder.** 2000. Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples. AIDS **14:**1203–1210.
11. **Hirsch, M. S., F. Brun-Vezinet, R. T. D'Aquila, S. M. Hammer, V. A. Johnson, D. R. Kuritzkes, C. Loveday, J. W. Mellors, B. Clotet, B. Conway, L. M. Demeter, S. Vella, D. M. Jacobsen, and D. D. Richman.** 2000. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society—USA Panel. JAMA **283:**2417–2426.
12. **Hoffman, N. G., C. A. Schiffer, and R. Swanstrom.** 2003. Covariation of amino acid positions in HIV-1 protease. Virology **314:**536–548.
13. **Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, R. T. D'Aquila, L. M. Demeter, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman.** 2004. Update of the drug resistance mutations in HIV-1: 2004. Top. HIV Med. **12:**119–124.
14. **Kaldor, S. W., V. J. Kalish, J. F. Davies, B. V. Shetty, J. E. Fritz, K. Appelt, J. A. Burgess, K. M. Campanale, N. Y. Chirgadze, D. K. Clawson, B. A. Dressman, S. D. Hatch, D. A. Khalil, M. B. Kosa, P. P. Lubbehusen, M. A. Muesing, A. K. Patick, S. H. Reich, K. S. Su, and J. H. Tatlock.** 1997. Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. J. Med. Chem. **40:**3979–3985.
15. **Kemper, C. A., M. D. Witt, P. H. Keiser, M. P. Dube, D. N. Forthal, M. Leibowitz, D. S. Smith, A. Rigby, N. S. Hellmann, Y. S. Lie, J. Leedom, D. Richman, J. A. McCutchan, and R. Haubrich.** 2001. Sequencing of protease inhibitor therapy: insights from an analysis of HIV phenotypic resistance in patients failing protease inhibitors. AIDS **15:**609–615.
16. **Kempf, D. J., J. D. Isaacson, M. S. King, S. C. Brun, Y. Xu, K. Real, B. M. Bernstein, A. J. Japour, E. Sun, and R. A. Rode.** 2001. Identification of genotypic changes in human immunodeficiency virus protease that correlate with reduced susceptibility to the protease inhibitor lopinavir among viral isolates from protease inhibitor-experienced patients. J. Virol. **75:**7462–7469.
17. **Kozal, M.** 2004. Cross-resistance patterns among HIV protease inhibitors. AIDS Patient Care STDs **18:**199–208.
18. **Lawrence, J., J. Schapiro, M. Winters, J. Montoya, A. Zolopa, R. Pesano, B. Efron, D. Winslow, and T. C. Merigan.** 1999. Clinical resistance patterns and responses to two sequential protease inhibitor regimens in saquinavir and reverse transcriptase inhibitor-experienced persons. J. Infect. Dis. **179:**1356–1364.
19. **Lin, Y., X. Lin, L. Hong, S. Foundling, R. L. Heinrikson, S. Thaisrivongs, W. Leelamanit, D. Raterman, M. Shah, B. M. Dunn, and J. Tang.** 1995. Effect of point mutations on the kinetics and the inhibition of human immunodeficiency virus type 1 protease: relationship to drug resistance. Biochemistry **34:**1143–1152.
20. **Mansky, L. M.** 1998. Retrovirus mutation rates and their role in genetic variation. J. Gen. Virol. **79:**1337–1345.
21. **Miller, V.** 2001. Resistance to protease inhibitors. J. Acquir. Immune Defic. Syndr. **26:**34–50.
22. **Molla, A., M. Korneyeva, Q. Gao, S. Vasavanonda, P. J. Schipper, H. M. Mo, M. Markowitz, T. Chernyavskiy, P. Niu, N. Lyons, A. Hsu, G. R. Granneman, D. D. Ho, C. A. Boucher, J. M. Leonard, D. W. Norbeck, and D. J. Kempf.** 1996. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat. Med. **2:**760–766.
23. **Parikh, U., C. Calef, B. A Larder, R. Schinazi, and J. W. Mellors.** 2002. Mutations in retroviral genes associated with drug resistance, p. 95–183. *In* C. Kuiken, B. Foley, E. Freed, B. Hahn, B. Korber, P. Marx, F. McCutchan, and J. W. Mellors (ed.), HIV sequence compendium 2002. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.M.
24. **Parkin, N. T., C. Chappey, and C. J. Petropoulos.** 2003. Improving lopinavir genotype algorithm through phenotype correlations: "novel" mutation patterns and amprenavir cross-resistance. AIDS **17:**955–961.
25. **Patick, A. K., M. Duran, Y. Cao, D. Shugarts, M. R. Keller, E. Mazabel, M. Knowles, S. Chapman, D. R. Kuritzkes, and M. Markowitz.** 1998. Genotypic and phenotypic characterization of human immunodeficiency virus type 1 variants isolated from patients treated with the protease inhibitor nelfinavir. Antimicrob. Agents Chemother. **42:**2637–2644.
26. **Perno, C. F., A. Cozzi-Lepri, C. Balotta, F. Forbici, M. Violin, A. Bertoli, G. Facchi, P. Pezzoti, G. Cadeo, G. Tositti, S. Pasquinucci, S. Pauluzzi, A. Scalzini, B. Salassa, A. Vincenti, A. N. Phillips, F. Dianzani, A. Apice, G. Angarano, L. Monno, G. Ippolito, M. Moroni, and A. d'Arminio Monforte.** 2001. Secondary mutations in the protease region of human immunodeficiency virus and virological failure in drug-naïve patients treated with protease inhibitor based therapy. J. Infect. Dis. **184:**983–991.
27. **Race, E., E. Dam, V. Obry, S. Paulous, and F. Clavel.** 1999. Analysis of HIV cross-resistance to protease inhibitors using a rapid single-cycle recombinant virus assay for patients failing on combination therapies. AIDS **13:**2061–2068.
28. **Roge, B. T., T. L. Katzenstein, H. L. Nielsen, and J. Gerstoft.** 2003. Drug resistance mutations and outcome of second-line treatment in patients with first-line protease inhibitor failure on nelfinavir-containing HAART. HIV Med. **4:**38–47.
29. **Schinazi, R. F., B. A. Larder, and J. Mellors.** 2000. Mutations in retroviral genes associated to drug resistance: update 2000–2001. Int. Antivir. News **8:**65–91.
30. **Sevin, A. D., V. De Gruttola, M. Nijhuis, J. M. Schapiro, A. S. Foulkes, M. F. Para, and C. A. Boucher.** 2000. Methods for investigation of the relationship between drug-susceptibility phenotype and human immunodeficiency virus 1 genotype with application to AIDS clinical trial group 333. J. Infect. Dis. **182:**59–67.

31. **Shafer, R. W.** 2002. Genotypic testing for human immunodeficiency virus type 1 drug resistance. Clin. Microbiol. Rev. **15:**247–277.
32. **Turner, B., and M. Summers.** 1999. Structural biology of HIV. J. Mol. Biol. **285:**1–32.
33. **Wainberg, M. A.** 2004. HIV-1 subtype distribution and the problem of drug resistance. AIDS **18**(Suppl. 3)**:**63–68.
34. **Wang, D., and B. A. Larder.** 2003. Enhanced prediction of lopinavir resistance from genotype by use of artificial neural networks. J. Infect. Dis. **188:**653–660.
35. **Winters, M. A., J. M. Schapiro, J. Lawrence, and T. C. Merigan.** 1998. Human immunodeficiency virus type 1 protease genotypes and in vitro protease inhibitor susceptibility of isolates from individuals who were switched to other protease inhibitors after long term saquinavir treatment. J. Virol. **72:**5303–5306.
36. **Wlodawer, A., and A. Gustchina.** 2000. Structural and biochemical studies of retroviral proteases. Biochim. Biophys. Acta **1477:**16–34.
37. **Wu, T. D., C. A. Schiffer, M. J. Gonzales, J. Taylor, R. Kantor, S. Chou, D. Israelski, A. R. Zolopa, W. J. Fessel, and R. W. Shafer.** 2003. Mutation patterns and structural correlates in human immunodeficiency virus type 1 protease following different protease inhibitor treatments. J. Virol. **77:**4836–4847.