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Interplay of Reverse Transcriptase Inhibitor Therapy and Gag p6 Diversity in HIV Type 1 Subtype G and CRF02_AG

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Abstract

The gag p6 region of HIV-1 has various nonsubstitutionary mutations, including insertions, duplications, deletions, and premature stop codons. Studies have linked gag p6 mutations to reduced susceptibility to antiretroviral therapy in HIV-1 subtype B. This study examined the relationship between antiretroviral therapy and gag p6 diversity in HIV-1 CRF02_AG and subtype G. p6 data were generated for secondary analyses following Viroseq genotyping of *pol* gene sequences in plasma samples from HIV-1-infected Nigerians on reverse transcriptase inhibitor therapy, with virologic failure (repeat VL $>$ 2000 copies/ml). p6 sequence chromatograms were available for 40 CRF02_AG and 43 subtype G-infected individuals. Subjects who had not received their supply of antiretroviral drugs for at least 2 months prior to the plasma sampling were classified as nonadherent. p6 sequences from therapy-adherent individuals had more nonsubstitutionary mutations than sequences from drug-naive individuals ($p = 0.0005$). The P5L/T mutation was inversely correlated with the presence of K27Q/N in p6, with each mutation being more prominent in subtype G and CRF02_AG, respectively. The data also suggested that P5L/T may be a compensatory mutation for the loss of an essential phosphorylation site in p6. In addition, there was an inverse association between P5L/T mutations in p6 and thymidine analog mutations in reverse transcriptase ($p = 0.0001$), and drug nonadherence was associated with an 8-fold lower risk of having a nonsubstitutionary mutation in p6 (95% CI = 1.27–52.57). Our data suggest that antiretroviral therapy influences gag p6 diversity, but further studies are needed to clarify these observations.

Introduction

The human immunodeficiency virus (HIV-1) *gag* gene produces a 55-kDa precursor polyprotein, which is cleaved during viral maturation into the matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p9), p1, p7, and p6 proteins. Retroviral Gag polyproteins have specific regions, commonly referred to as late assembly (L) domains, which are required for the efficient budding of assembled virions from the host cell. This function has been mapped to the C-terminal p6 domain of HIV-1 gag and contains an essential P(T/S)AP core motif that is widely conserved among lentiviruses.[1–4] The p6 protein is unique to lentiviruses within the retrovirus family and overlaps part of the *pol* gene in the region encoding for the protease enzyme.[5] By contrast, the L domains of oncoretroviruses such as Rous sarcoma virus (RSV), murine leukemia virus, and Mason Pfizer monkey virus have a PPxY core motif and are located 150–200 amino acid residues from the Gag N terminus.[6–9] The L domain in p6 mediates the detachment of the virion by protein–protein interactions with two host proteins, Tsg101 and AIP1/ALIX, which are human homologs of the yeast class E vacuolar protein sorting (Vps) machinery.[10]

HIV-1 p6 phosphorylation is important for viral budding and p6 has previously been shown to be the major phosphoprotein of the HIV-1 virion.[11] Phosphorylation is catalyzed by a mitogen-activated protein kinase (MAPK) and occurs, in subtype B, on a threonine at position 23 (T23),[11,12]

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which is part of the signature S/T-P phosphorylation motif for MAPK.[13] p6 has also been found to be vital for the incorporation of Vpr into the virion via the interaction of Vpr with two short regions within the p6 domain. These motifs, PTAP and LXXLF, are highly conserved among primate immunodeficiency viruses and are located near the N-terminus and C-terminus, respectively, of the p6 domain.[14–17]

HIV-1 p6 protein exhibits great sequence variability, with two broad categories of mutations: substitutionary amino acid point mutations, in which p6 retains its original amino acid length, and nonsubstitutionary (NS) mutations, in which insertions, duplications, deletions, and premature or absent stop codons lead to p6 sequences with variable lengths. There may be subtype-related differences, as subtype C viruses appear to have a higher frequency of PTAP duplications than other subtypes.[18,19]

Highly active antiretroviral therapy (HAART) has become the mainstay of treatment for HIV infection, and involves combinations of inhibitors of the reverse transcriptase (RT) and protease (PR) enzymes.[20] Therapy failure, however, can occur either due to problems with adherence or the development of drug resistance mutations (DRMs). Studies have shown that mutations at the gag cleavage sites may modulate viral replication and drug susceptibility.[21] NS mutations in p6 have recently been found to be involved with resistance to nucleoside reverse transcriptase inhibitors (NRTIs). The NS mutations include insertions/duplications of the PTAP and KQE motifs, various deletions of amino acids within the central region of the protein, and the absence of stop codons.[21–23] Most of these studies have mostly been carried out in Europe where subtype B is the predominant viral subtype. However, it is not known if antiretroviral therapy for infection with HIV-1 subtype G and CRF02, the predominant viruses in Nigeria, will be associated with mutations in gag p6. We therefore sought to characterize p6 diversity and determine associations that may exist between p6 and these subtypes in patients on HAART.

Materials and Methods

Study design

Plasma samples were obtained from patients enrolled in the APIN Plus/ President's Emergency Plan for AIDS Relief (PEPFAR) program in Nigeria who were receiving a treatment regimen of stavudine + lamivudine + nevirapine. These patients were enrolled and followed in clinics where they receive the drugs on a monthly basis and had blood samples drawn periodically for monitoring of virological, immunological, and biochemical parameters. The study received ethical approval from the Institutional Review Boards of the Harvard School of Public Health and all local implementing institutions in Nigeria. Informed consent was also obtained from the subjects.

The RT and PR genes were sequenced from plasma RNA of HIV-1-infected patients with evidence of virologic failure (repeat VL > 2000 copies/ml). HIV-1 viral RNA load was determined using the Roche Amplicor viral load assay kit according to the manufacturer's specifications. The Celera Diagnostics' ViroSeq HIV-1 Genotyping System v2.0 was used with Stanford and ViroSeq algorithms to predict drug resistance.

Viroseq genotyping assay

We performed HIV-1 genotyping using the ViroSeq assay according to the manufacturer's instructions. Blood was collected in EDTA tubes and separated. The plasma was subjected to high-speed centrifugation at 4°C to concentrate the viral particles, followed by treatment with viral lysis buffer and isopropanol-ethanol precipitation for RNA purification. The purified RNA was resuspended in RNA diluent and 10 μl of resuspended RNA was used for the RT (65°C for 30 s, 42°C for 65 min, and 99°C for 5 min) with Moloney murine leukemia virus reverse transcriptase enzyme. Following reverse transcription, a 1.8-kb region of the HIV-1 *pol* gene encoding the 99 amino acids of protease and the first 335 amino acids of RT was amplified by a 40-cycle polymerase chain reaction (PCR) using a 30-μl reaction mix containing AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), dNTPs, forward and reverse primers, and AmpErase uracil *N*-glycosylase (temperatures: 50°C for 10 min, 93°C for 12 min, 93°C for 20 s, 64°C for 45 s, 66°C for 3 min, and 72°C for 10 min). PCR products were purified with spin columns and quantified. DNA sequencing was then carried out using BigDye terminator chemistry (Applied Biosystems) with seven different primers. HIV-1 pol sequence data were analyzed by adapting the Celera Diagnostics ViroSeq HIV-1 Genotyping algorithm and data from the Stanford HIV Drug Resistance Database.

p6 sequence analyses

Since the 5′ end of the raw nucleotide sequences obtained corresponds to the p6 region, secondary analyses of this region was carried out manually using the Lasegene's DNASTAR SeqMan II program to extract p6 sequence information. p6 data were obtained for three sets of subjects.

1. p6 sequence chromatograms were obtained for 34 CRF02_AG and 39 subtype G-infected individuals adherent to HAART for at least 24 months. Each individual had repeat viral load levels >2000 copies/ml and harbored at least one drug resistance mutation. Of these 73 subjects, 72 had nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations (in varying combinations of K103N, Y181C, and V108I), while 66 subjects had NRTI mutations [including 21 subjects with thymidine analog mutations (TAMs)].

2. As a comparison group, p6 sequence data were retrospectively obtained from the drug-naive subjects from Ibadan and Saki whose samples were previously described.[24] Of these drug-naive individuals, chromatograms were available for 13 CRF02_AG and 11 subtype G-infected individuals. None of these individuals had drug resistance mutations.

3. We also had p6 data for 10 treated individuals who had viral load levels similar to the first group, but who had been delinquent in reporting to the clinics to receive their supply of antiretroviral drugs for at least 2 months prior to the plasma sampling. Viroseq sequencing revealed they had no drug resistance mutations. The data obtained from these individuals provided an opportunity to test for associations between drug adherence and gag p6 diversity.

For all generated p6 sequences, multiple alignments were performed using Clustal X[25] and Se-Al v2.0 (http://tree.bio.ed.ac.uk/software/seal/), while PAUP phylogenetic analyses were performed on the 1.8-kb fragment obtained from the Viroseq assay to determine subtypes. Nucleotide se-

quences were translated to amino acid sequences using the MacClade 3.08a software.[26]

Statistical analyses

Comparisons between drug-naive and treated individuals were tested with the Fisher's exact test. Based on the length of the p6 protein, statistical significance for amino acid alignment comparisons was set at $p = 0.001$ after correction for 50 multiple comparisons using the Bonferroni method. Odds ratio with 95% confidence intervals (and $p = 0.05$) was used to describe associations between gag p6 diversity and drug adherence.

Results

Subtype-specific polymorphisms in gag p6

The 13 CRF02 and 11 subtype G sequences from drug-naive subjects were aligned and the wild-type consensus sequences of these variants were determined. Baseline polymorphisms with respect to consensus subtype B are shown in Fig. 1a. Both CRF02 and subtype G sequences have a premature stop codon at position 50. Thus, the CRF02_AG gag p6 is a 50-amino acid protein, while subtype G p6 is 49 amino acids long, in contrast to the 52-amino acid p6 in subtype B. Amino acid T23 was deleted in both CRF02 and subtype G (Fig. 1a). However, these subtypes have P25S and Q26P polymorphisms that restore the phosphorylation motif. Interestingly, 4 of the 11 subtype G sequences had a P26Q mutation, without restoration of the MAPK phosphorylation site, but each of these sequences had a P5L polymorphism (Fig. 1b).

We also retrieved all 29 subtype G p6 sequences known to be from drug-naive individuals in the Los Alamos National Laboratory (LANL) database. Comparison of positions 5 and 26 confirmed the relationship that we found between P5L/T and P26Q/L, although there was more variation in the LANL data. Fourteen of 15 (93%) sequences with the P5L/T mutation also had the P26Q/L mutation whereas 6 of 7 (84%) sequences with wild-type p26 also had wild-type P5 (two-tailed, $p = 0.035$).

The influence of RTI therapy on gag p6 diversity

Table 1 shows the frequencies of nonsubstitutionary and point mutations by subtype and compares these frequencies between drug-naive and HAART-adherent individuals. Due

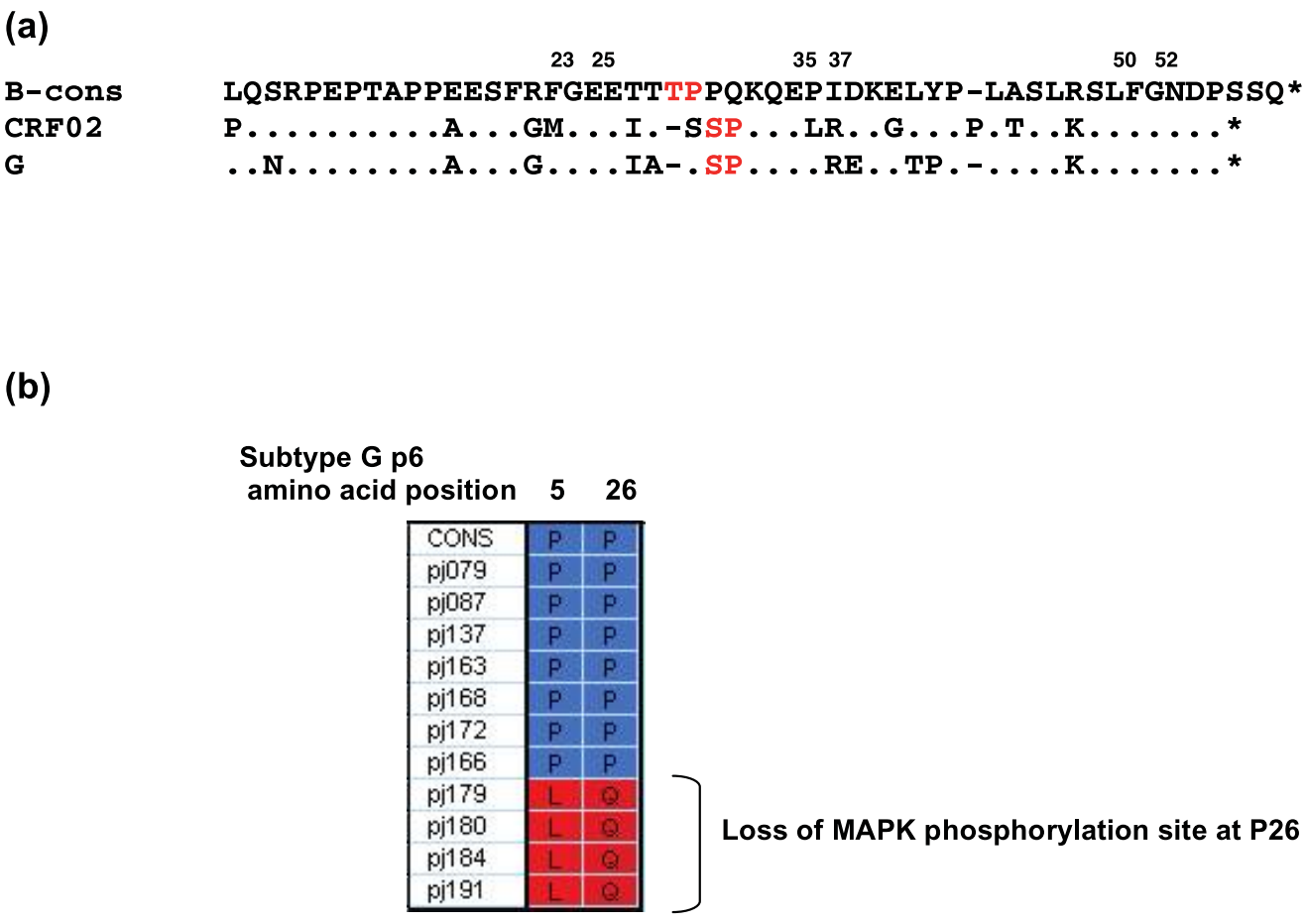

FIG. 1. Subtype-specific polymorphisms in gag p6. Consensus sequences generated from multiple alignments of HIV-1 p6 sequences obtained from 13 CRF02 and 11 subtype G-infected drug-naive Nigerians are depicted (**a**). The consensus subtype B p6 sequence is based on the 709 p6 sequences available on the Los Alamos National Laboratory (LANL) database. The asterisk (*) denotes a stop codon. The S/T-P motifs for MAPK phosphorylation are shown in red. (**b**) A color-coded alignment truncated to show p6 mutations at positions 5 and 26 in drug-naive subtype G-infected subjects. The P5L/T mutation is found only in sequences lacking the MAPK phosphorylation SP motif due to a P26Q substitution.

Table 1. Effect of HAART on p6 Diversity

	HAART naive			*HAART adherent*			
	AG	*G*	*Total*	*AG*	*G*	*Total*	
	n = *13*	n = *11*	n = *24*	n = *34*	n = *39*	n = *73*	p *value*[a]
PTAP duplications	0	0	0	4	5	9	
KQE insertions	0	1	1	0	8	8	
Other insertions	0	1	1	2	4	6	
Deletions	0	0	0	6	8	14	
No stop codon	0	0	0	0	7	7	
Subjects with nonsubstitutionary mutations[b]	0	2	2 (8%)	11	24	35 (48%)	0.00050
Point mutations							
P5L/T	0	4	4 (16%)	12	26	38 (52%)	0.0038
K27Q/N	1	0	1 (4%)	12	1	13 (18%)	0.177
				12	1		(*0.00035*)[c]

[a]$p < 0.001$ is considered as statistically significant, reflecting Bonferroni correction for multiple comparisons.
[b]Some subjects had multiple mutations and these numbers represent number of subjects, not number of mutations.
[c]The *p* value in parentheses refers to comparison between the subjects within the HAART-adherent group.

to the small number of samples, the NS mutations were grouped together rather than analyzed individually, and both subtypes were assessed as a group. The *p* values were corrected for multiple comparisons across 50 amino acid positions.

There was a low frequency of NS mutations in drug-naive subjects. Only 2 of 24 (8%) drug-naive individuals compared with 35 of 73 (48%) HAART-adherent individuals had NS mutations ($p = 0.0005$). With respect to substitutionary mutations, there was a trend for P5L/T occurrence at higher frequencies in the drug-treated individuals ($p = 0.0038$), but this was not statistically significant after correction for multiple comparisons. Interestingly, we noticed that P5L/T and K27Q/N occurred almost mutually exclusively from each other (Fig. 2a). Forty of 42 (95%) individuals with P5L/T did not have K27Q/N, while 14 of 16 (88%) of individuals with K27Q/N did not bear the P5L/T mutation ($p < 0.00001$). Furthermore, there was differential subtype preference under treatment, with K27Q/N occurring almost exclusively in CRF02_AG infection ($p = 0.00035$) (Table 1).

Notably, the presence of P5L/T was found to be significantly associated with the absence of an MAPK phosphorylation site in the 23–26 amino acid p6 region in treated individuals in both subtypes (Fig. 2b). Twenty of 25 (80%) sequences without the MAPK site had P5L/T while 36 of 58 (62%) sequences that retained the phosphorylation site did not have the P5L/T mutation ($p = 0.00065$). Interestingly, the relationship between loss of the MAPK site and treatment is subtype dependent. Thirty-five of 40 (88%) sequences from treated CRF02_AG-infected subjects retained their p6 MAPK sites but 20 of 43 (47%) sequences from treated subtype G-infected subjects lost the MAPK site ($p = 0.00082$).

We were also intrigued to find an inverse relationship between the presence of P5L/T in gag p6 and the presence of TAMs in the RT of subtype G-infected individuals. Thirteen subjects had K70R and/or K219Q/E, two major mutations in the TAM2 pathway. Twenty-six of 30 (87%) of individuals with P5L/T in p6 did not have these TAM2 mutations while 10 of 13 (77%) individuals who did not have P5L/T had at least one of the TAM2 mutation in RT ($p = 0.0001$).

Gag p6 diversity is associated with drug adherence

We tested for associations between drug adherence and gag p6 diversity using samples from 10 subjects who were not adherent to therapy for at least 2 months prior to plasma sample collection. Only 1 of the 10 (10%) sequences from nonadherent individuals had an NS mutation, while 35 of 73 (48%) sequences from adherent subjects had at least one NS mutation. The odds ratio for the likelihood of occurrence of NS mutations in gag p6 associated with adherence to the HAART regimen was 8.29 (95% CI = 1.27–52.57; Chi-square $p = 0.023$). This suggests an association exists between drug adherence and the presence of an NS mutation in gag p6, although other variables like subtype and viral load might also play a role.

Discussion

Resistance mutations usually occur within specific drug targets, although changes in the diversity of other proteins have been previously described. For example, Nijhuis *et al.* found that development of resistance to ritonavir, a protease inhibitor, induced bottleneck effects in the *env* gene, with the genetic variation of *env* decreasing significantly during treatment.[27] Mutations in gag have been previously described in connection with protease inhibitor therapy. These mutations occur in the nucleocapsid/p1 and p1/p6 cleavage sites of Gag, both *in vitro* and *in vivo*, and influence polyprotein processing and viral maturation.[28] Peters *et al.* also demonstrated that PTAP duplications were associated with stavudine and didanosine therapy.[29] This study therefore examined the relationship between reverse transcriptase inhibitor therapy and gag p6 diversity in the predominant HIV-1 subtypes in Nigeria, CRF02_AG and subtype G.

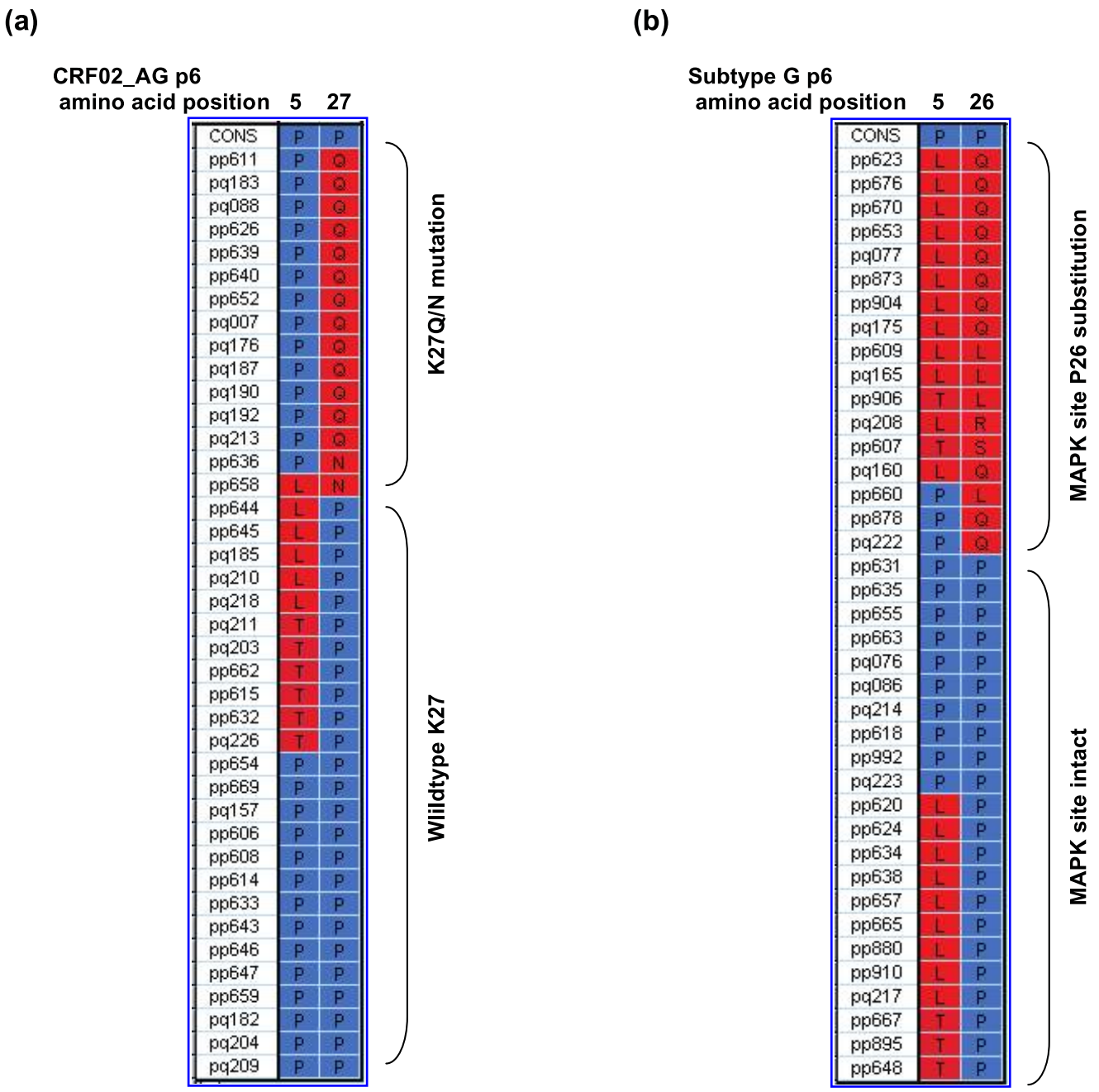

FIG. 2. Relationship of the P5L/T mutation with the K27Q/N mutation and loss of the MAPK phosphorylation site in HIV-1 p6. Nucleotide alignments of HIV-1 p6 and reverse transcriptase (RT) sequences obtained from HAART-treated subjects were generated and edited to highlight the coexpression of amino acids at key positions and are presented in three panels. Each panel compares a pair of alignments and the first row of each alignment represents the consensus (CONS) amino acid at each position. The consensus (wild-type) amino acid at each position has a blue color code, while mutant amino acids are coded in red. The consensus for CRF02_AG and subtype G did not differ from subtype B at each position. (**a**) The relationship between K27Q/N and P5L/T mutations in treated CRF02_AG-infected subjects; (**b**) the association between the loss of the MAPK phosphorylation site at position 26 and the P5L/T mutation in treated subtype G-infected subjects. Relationships were statistically significant, with $p < 0.001$, after Bonferroni correction for multiple comparisons.

We found, as previously described,[18,19,30] that the p6 proteins of CRF02_AG and subtype G viruses have shorter lengths than subtype B p6. This was due to a T23 deletion and premature stop codons in both variants. CRF02_AG viruses also have a P37 duplication. Unlike subtype C gag p6, there was a low incidence of PTAP duplications in drug-naive individuals in CRF02_AG and subtype G.

There was a higher frequency of NS mutations in treated individuals in our cohort, as a group, but the increased frequencies of individual mutations such as PTAP duplications

and deletions did not attain statistical significance. The thymidine analog, stavudine, is a component of the first-line antiretroviral regimen in Nigeria,[31] and it has been associated with increased frequencies of PTAP duplications.[29] Therefore, the increased gag p6 diversity observed in our subjects may be explained in part by the stavudine-based therapy regimen of our subjects.

The P5L/T mutation has been previously demonstrated to be associated with antiretroviral therapy.[23] We found this mutation in higher frequencies in treated individuals infected with subtype G viruses compared with CRF02_AG. Other p6 mutations have been shown to enhance virus escape from NRTI pressure through increased packaging of RT molecules per virion.[29] The incorporation and retention of key viral proteins are essential for successful assembly of retroviruses. The incorporation of Gag-Pol polyproteins during virus assembly requires the Gag domains that are shared by the Gag and Gag-Pol precursors. Yu *et al.* demonstrated that the amount of Pol proteins in virions was drastically reduced in p6 truncation mutants as well as in virions with P(T/S)APP mutations.[32] Dettenhofer and Yu further narrowed this effect down to amino acid substitutions at proline residues 5 and 7 of p6. This was found to be cell-type specific as there was a 16-fold reduction in Pol protein levels in mutant virions produced from H9 cells, but not mutant virions produced from Jurkat cells.[33]

Interestingly, the P5R and P7Q mutants used in the Dettenhofer and Yu study were constructed with single-point nucleotide mutations, which resulted in synonymous mutations in the *pol* reading frame. There was a high frequency of P5L in the treated subjects in our study. P5L also involves a single nucleotide change from CCX to CTX (where X is any nucleotide), without amino acid mutations in *pol*. Arginine (R) is a basic (polar) amino acid with a long side chain while leucine (L) is nonpolar and closer in size to proline. It is thus possible that the deleterious effect of the P5R mutation in the Dettenhofer study may be mediated in part by changes in the hydrophobicity and structural geometry of the protein that may inhibit interactions essential to p6 function. It is possible that the virus naturally selects P5L for some yet undescribed beneficial effects.

When present, the K27Q/N mutation was found almost exclusively in the absence of P5L/T, especially in treatment-exposed CRF02_AG-infected individuals. The two mutations may therefore play similar roles in influencing p6 structure/function but in a nonredundant fashion. In addition, p6 interacts covalently with small ubiquitin-like modifier 1 (SUMO-1) at position K27 and this interaction is associated with the production of defective viruses.[34] Therefore, the loss of K27 may be beneficial to the virus by limiting SUMO-1-related production of defective viruses. Conversely, the role of Tsg101 in viral budding is strengthened by covalent attachment of monovalent ubiquitin to HIV-1 p6 at K27. Therefore, the role of K27 mutations in p6 biology requires further investigation.

p6 has previously been shown to be the major phosphoprotein of the HIV-1 virion.[11] This MAPK-mediated phosphorylation is essential for viral budding and occurs at position T23 in subtype B.[11,12] Although T23 was deleted in subtype G and CRF02, a P25S polymorphism restored the S/T-P motif in most of the p6 sequences from drug-naive subjects. One of the intriguing observations we made was that the loss of this site was associated with the presence of the P5L/T mutation. Due to the important role of this phosphorylation site for p6 function and viral budding, P5L/T is probably a compensatory mutation mediating a function that mimics phosphorylation. This phenomenon was observed in both drug-naive and treated subtype G-infected subjects. Among the drug-naive individuals, P5L/T was found only in those whose p6 proteins lacked the phosphorylation site, suggesting it is not a natural polymorphism, and that viruses with P5L/T in gag p6 may be less fit than wild-type viruses in the absence of therapy.

The negative correlation observed between the occurrence of TAM2 mutations in RT and P5L/T in p6 in subtype G viruses is of interest. Considering that some P5L/T mutations occur prior to treatment and the TAMs most likely resulted from stavudine therapy in these patients, it is tempting to speculate that perhaps viruses with P5L/T prior to treatment are less likely to develop TAMs. It could be expected that if the virus already harbors a mutation thought to be compensatory for fitness, the potential benefit of that mutation is lost upon subsequent development of the DRM for which it is compensatory. Thus, following treatment, such viruses may be less fit than viruses without the compensatory mutation, and double mutants may be observed at a lower degree in the quasispecies. This does not imply that the DRM does not occur; the virus harboring the DRM in RT alongside the compensatory mutation in p6 is simply less fit. It is also possible that P5L/T fades upon interruption of therapy, whereas TAMs persist.

We also found a strong positive correlation between the frequency of NS mutations and drug adherence, although this relationship is confounded by the absence of DRMs in all nonadherent subjects. Nonetheless, this finding is still consistent with the hypothesis that increased p6 diversity is secondary to therapy (since DRM appearance is dependent on therapy).

P5L/T in gag p6 appears to play a central role in maintaining viral fitness both before and after reverse transcriptase inhibitor treatment through several mechanisms, especially in HIV-1 subtype G. We speculate that the P5L/T mutation compensates for MAPK phosphorylation and for viral fitness following drug resistance to thymidine analog therapy. This may be an example of positive epistasis in which a deleterious mutation in one gene is compensated for by another deleterious mutation in another gene.[35,36]

Resistance to antiviral drugs is usually due to mutations in the target genes, but this observed phenomenon of mutations in p6 following reverse transcriptase inhibitor therapy is more analogous to the gene dosing or inoculum effect seen in bacteria. For example, mutations in the *inhA* gene in *Mycobacterium tuberculosis* mediate resistance to isoniazid by increasing the levels of the drug target, enoyl reductase.[37] P5L/T may have a similar effect, compensating for p6 MAPK phosphorylation and/or RT drug resistance by increasing or stabilizing the amount of Pol proteins incorporated into the virions.

It is possible that the diversity and relationships observed in this study are purely epiphenomenal and without clinical significance. We recently commenced a study to follow p6 and RT diversity changes in subjects with available paired pretreatment and posttreatment samples. Our preliminary findings indicate that there are no NS p6 mutations in a

group of drug-adherent patients lacking drug resistance mutations after at least 18 months of treatment (data not shown). This supports the hypothesis that the increased p6 diversity observed in this study is most likely secondary to the development of drug resistance mutations in RT, but further studies are needed to clarify these observations.

Sequence Data

The sequences have been submitted to GenBank and have the following accession numbers: EU268910–EU269016.

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Disclosure Statement

No competing financial interests exist.

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