Cytidine deamination induced HIV-1 drug resistance

Lubbertus C. F. Mulder*, Ariana Harari*, and Viviana Simon*†‡

*Department of Medicine, Division of Infectious Diseases, and †Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1090, New York, NY 10029

Edited by Malcolm A. Martin, National Institutes of Health, Bethesda, MD, and approved February 15, 2008 (received for review October 25, 2007)

The HIV-1 Vif protein is essential for overcoming the antiviral activity of DNA-editing apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3) cytidine deaminases. We show that naturally occurring HIV-1 Vif point mutants with suboptimal anti-APOBEC3G activity induce the appearance of proviruses with lamivudine (3TC) drug resistance-associated mutations before any drug exposure. These mutations, ensuing from cytidine deamination events, were detected in >40% of proviruses with partially defective Vif mutants. Transfer of drug resistance from hypermutated proviruses via recombination allowed for 3TC escape under culture conditions prohibitive for any WT viral growth. These results demonstrate that defective hypermutated genomes can shape the phenotype of the circulating viral population. Partially active Vif alleles resulting in incomplete neutralization of cytoplasmic APOBEC3 molecules are directly responsible for the generation of a highly diverse, yet G-to-A biased, proviral reservoir, which can be exploited by HIV-1 to generate viable and drug-resistant progenies.

hypermutation | reservoir | diversity | reverse transcription

Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3) cytidine deaminases restrict endogenous and exogenous retroviruses (1–3). Four of the seven APOBEC3 molecules identified in humans (APOBEC3B, APOBEC3DE, APOBEC3F, and APOBEC3G) inhibit HIV replication to a varying degree (1, 4). HIV-1, by means of its Vif protein, counteracts the antiviral activity of APOBEC3G, APOBEC3F, and APOBEC3DE (5–9) by promoting proteasome-dependent degradation (6) and inhibiting packaging (10) in the producer cell.

The mechanisms underlying APOBEC3-mediated HIV-1 restriction are a combination of editing (11) and nonediting activities (4). In the absence of a functional HIV-1 Vif, the budding virion, incorporates different APOBEC3 family members (e.g., APOBEC3G or APOBEC3F), which then deaminate cytosines of the newly synthesized cDNA minus strand during reverse transcription (RT) in the next cycle of infection (3, 12, 13). As a result, numerous guanosine (G) to adenosine (A) changes in the plus strand of the provirus occur [also referred to as "hypermutated" proviruses (3, 12, 13)]. In addition, APOBEC3 may exert antiviral activity in the target cell in an editing-independent manner, by interfering with tRNA primer binding (14), provirus formation, and/or viral cDNA integration (15–17).

Protection of HIV-1 from cytidine deamination *in vivo*, however, is not absolute, because full-length Vif alleles that fail to neutralize one or several APOBEC3 enzymes have been identified (18, 19). Indeed, HIV-1 sequences carrying the genetic footprints of past deamination have been detected in treated and untreated chronically infected patients (20–22), vertically infected infants (23), and long-term nonprogressors (18, 24) suggesting that variations in HIV-1 Vif's anti-APOBEC3 activity occur in many different clinical settings.

Here, we investigate the consequences of partially defective Vif alleles on HIV-1 replication, sequence variability, and functional diversification. We show that Vif-mediated defects in APOBEC3 neutralization modulate HIV-1 fitness and increase viral diversity at endogenous levels of APOBEC3 protein expression as found in human peripheral blood mononuclear cells (PBMCs). Moreover, viral genomes rendered defective on the protein level by cytidine deamination are of critical importance for viral escape from the antiretroviral drug lamivudine (3TC). These results indicate that HIV-1 strains encoding partially active Vif alleles generate a highly diverse pool of viral quasispecies which, in turn, provides the genetic reservoir necessary for rapid viral escape from particular antiretroviral inhibitors.

Results

Description of Partially Active HIV-1 Vif Mutants. The two Vif mutants studied are located within the putative APOBEC3 interacting regions of Vif (25). In overexpression experiments these mutants display activity against APOBEC3F but no (K22E) or little (E45G) activity against APOBEC3G (18). They were initially identified in primary viral isolates (18) and queries into the Los Alamos HIV sequence database (www.hiv.lanl.gov) revealed that these specific mutations (K22E, E45G) have also been detected in patients infected with subtype B, D, and AE viruses (Fig. 1). Vif mutant (144AAA) carrying inactivating alanine substitutions in the functional BC-box motif SLQ 144–146 of Vif [144AAA (26)] served as a negative control in all experiments.

Partially Defective Vif Mutants Display Reduced Fitness in PBMC Cultures. We analyzed the fitness of viruses carrying Vif mutations K22E and E45G relative to WT HIV-1 by monitoring their replication in mitogen-activated PBMCs from three different HIV-1 negative donors.

Fitness differences between WT and Vif-defective HIV-1 (144AAA) ranged between 37-fold (donor 1; Fig. 2) and 7-fold (donor 3, Fig. 2). The replication characteristics of Vif mutant E45G were comparable to WT in donor 3 but attenuated in donors 1 and 2 (Fig. 2*A*), whereas Vif mutant K22E resembled negative control 144AAA in PBMCs of all three donors (Fig. 2*B*). Sequencing of the proviral Vif region at the end of each infection revealed maintenance of the specific Vif mutations.

Of note, viral stocks were produced in permissive HEK 293T cells, resulting in viruses that are fully infectious in the initial round of PBMC infection. The low-level p24 production observed in K22E and 144AAA infections likely reflects noninfectious particle release from cell populations infected during the first round of replication.

These data suggest that single-nucleotide mutations in Vif associated with suboptimal APOBEC3G activity affect viral fitness at endogenous APOBEC3G expression levels, with individual host characteristics further influencing the degree of restriction on WT and Vif mutant HIV-1.

Author contributions: L.C.F.M. and V.S. designed research; L.C.F.M., A.H., and V.S. performed research; L.C.F.M., A.H., and V.S. analyzed data; and L.C.F.M. and V.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

‡To whom correspondence should be addressed. E-mail: viviana.simon@mssm.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0710190105/DCSupplemental.

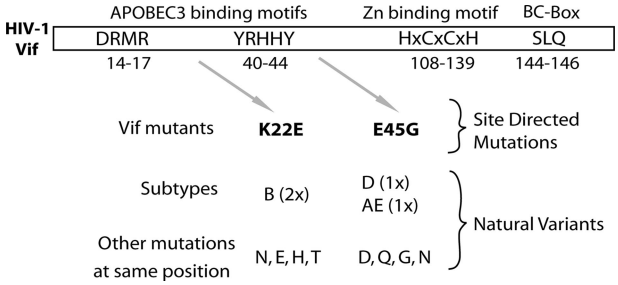

Fig. 1. Schematic representation of HIV-1 Vif. The identity and position of the Vif mutations chosen for this study are depicted in the context of functional domains (25, 37, 38, 40). Each Vif mutation was introduced into the molecular clone HIV-1 NL4-3 and subsequently characterized. Number and subtypes of patient-derived viral isolates encoding these specific Vif mutations are listed under residue and subtypes (based on HIV Vif sequence DNA aligment 2006 from the Los Alamos HIV database; www.hiv.lanl.gov).

HIV-1 Vif Mutants Induce High Sequence Diversity. PBMC infections were initiated with homogeneous viral stocks differing only by one single nucleotide in Vif and, after 2 weeks of propagation, we determined the sequence diversity associated with each virus. The complete dataset included 10–13 cloned *pol* fragments for each virus resulting in a total of 13,750 to 16,250 nucleotides sequenced per infection (three independent infections for each of the viruses, total of 132 independent clones; Fig. 3*A*).

WT viruses were found to have the lowest mutation frequency (mean 0.083 ± 0.02%; Fig. 3*A*), whereas the Vif-defective control 144AAA displayed 20 times more mutations (mean 1.767 ± 0.60%; Fig. 3*A*), with >90% of the mutations being G-to-A substitutions (Fig. 3 *A* and *B*). Vif mutants K22E and E45G, which fail to efficiently neutralize APOBEC3G (18), displayed high to intermediate mutation frequencies (K22E: 2.603 ± 0.74%; E45G: 0.803% ± 0.45) and were characterized by a strong bias toward G-to-A mutations in an APOBEC3G-favored nucleotide context (e.g., GG; Fig. 3*B*). Mutations in a GA context occurred at least five times less frequently than mutations in GG contexts (Fig. 3*B*) with no differences in the relative GG contribution among Vif mutants (% GG: K22E 86 ± 3.06%; E45G 93 ± 1.6%; 144AAA: 88 ± 4.2%). The rate of G-to-A mutations in GC or GT contexts was comparable to the substitutions rates of adenosine, thymidine or cytidine.

Deamination-driven mutagenesis resulting from incomplete APOBEC3 neutralization by Vif mutants exceeded the frequency of RT-generated errors, on average, by an order of magnitude (Fig. 3*A*) and the mutation frequency inversely correlated with the fitness of HIV-1 Vif mutants in PBMC (Fig. 3*C*). The increased genetic diversity in conjunction with attenuation observed with these two partially defective Vif mutants is reminiscent of the lower plasma viremia observed in HIV-1-infected patients harboring hypermutated proviruses (20).

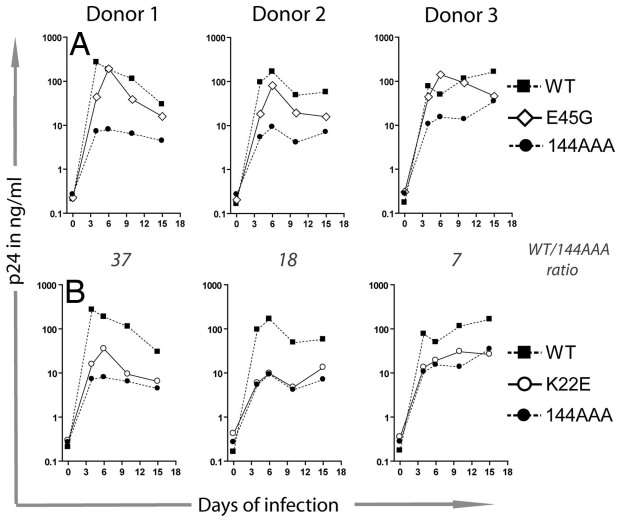

Fig. 2. Single Vif mutations impact on viral fitness. Replication characteristics of WT and Vif mutant HIV-1 molecular clones in PBMCs derived from three different healthy donors. PHA-stimulated PBMCs were infected in parallel with each viral stock, and HIV-1 p24 production in the culture supernatants was measured. Results from a representative complete infection experiment are shown. WT and Vif defective HIV-1 (144AAA) are shown with closed symbols to serve as reference for Vif mutant viruses E45G (*A*) and K22E (*B*). WT/144AAA ratio represents the fold difference between WT and Vif-defective 144AAA p24 values at day 4 of culture.

HIV-1 Vif Mutants Lead to the Accumulation of 3TC-Resistant Proviruses Before Drug Exposure. High-level resistance to the nucleoside RT inhibitor 3TC is associated with mutations M184I, M184V, or M184T in the active site of RT (27). A third of all clones sequenced encoded HIV-1 drug-resistance mutation M184I (33%, 44/132 clones; Fig. 4*A*), which results from a G-to-A substitution in an APOBEC3G-favored dinucleotide context. On the other hand, M184V or M184T, which are caused by A-to-G or T-to-C substitutions (27), were never observed. Proviruses with M184I were detected in every single infection with Vif mutants (K22E, 24/34, 70%; E45G, 5/33, 15%; 144AAA, 15/32, 47%) but were notably absent in all WT infections (Fig. 4*A*).

The codon composition of certain amino acids makes them potential target sites: deamination of the third position of tryptophan codons (TGG), for example, results in a stop codon (TGA). Sixty-nine of all 132 (52%) clones encoded stop codons with all but one of the 44 M184I clones also including stop codons (mean 8; range 0–14) in the 1,250-nt-long sequenced RT region. Fig. 4 shows that the numbers of stop codons correlated well with the overall level of deamination.

Therefore, partially active Vif mutants generate a pool of replication-defective proviruses that are likely to encode 3TC resistance (Fig. 4*A*). Because the underlying mechanism is cytidine deamination in the target cell, the extensive accumulation of resistant genomes is rapid, but independent, of drug exposure. Lastly, our findings recapitulated very well the spectrum, the positions of G-to-A mutations, and the high frequency of M184I detected in RT sequences derived from resting $CD4^+$ T lymphocytes of highly active antiretroviral therapy (HAART)-treated patients (21).

Genotypic and Phenotypic Characterization of Proviruses Derived from Vif Mutant E45G Infection. We created a quasi-species representative of viral populations generated by replication of partially defective Vif mutants to mimic a putative skewed proviral reservoir. For this purpose we chose Vif mutant E45G from donor 1 PBMC infection because of its intermediate level of attenuation (Fig. 2*A*) and sequence diversity (Fig. 4*A*). A fragment spanning the C terminus of *gag* to the middle of *vpr* was amplified and cloned in the corresponding region of WT NL4-3, and 32 individual clones were randomly selected for further analysis (referred to as the E45population).

Sequencing of the p6, protease, and the RT region of the 32 clones (residues 1–250) revealed that seven of these clones (7/32, 22%) [supporting information (SI) Fig. S1] were hypermutated with four of them encoding M184I (3TC resistance; Fig. 5*A*) in addition to premature stop codons. The overall mutation frequency (0.58) and the frequency of M184I (4/32) pertinent to the E45population are comparable to the data independently derived from Vif mutant E45G donor 1 infection (mutation frequency: 0.39, Fig. 3*A*; frequency of M184I: 1/11, Fig. 4*A*).

A

	Donor 1				Donor 2				Donor 3			
	G-to-A	Other	Total	Freq.	G-to-A	Other	Total	Freq.	G-to-A	Other	Total	Freq.
WT	3	12	13750	0.11	5	6	13750	0.08	1	7	13750	0.06
K22E	258	27	16250	1.75	333	12	12500	3.12	391	13	13750	2.94
E45G	44	10	13750	0.39	165	11	13750	1.28	96	6	13750	0.74
144AAA	317	21	13750	2.46	158	12	13750	1.36	190	13	13750	1.48

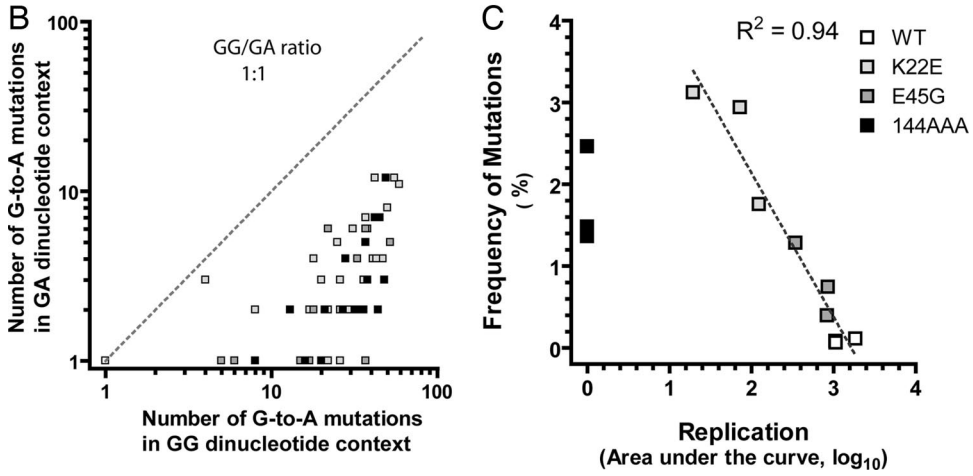

Fig. 3. Determinants of APOBEC3-driven HIV-1 sequence variation. (*A*) The viral diversity resulting from 2 weeks of propagation of homogenous viral stocks in PBMCs was assessed by analyzing a total of 165,000 nt (132 clones; 1,250 nt each) derived from parallel infections with WT and Vif mutant viruses. The number of G-to-A mutations (G-to-A), the number of any other mutations (Other), the total number of sequenced nucleotides (Total), and the frequency of mutations (Freq, in percent) are listed. (*B*) Comparison of G-to-A mutations in GG versus GA dinucleotide contexts in all clones derived from WT, K22E, E45G, and 144AAA. The data from the three different PBMC donors were combined. The dotted line symbolizes a 1:1 GG-to-GA ratio. (*C*) Inverse correlation between frequency of mutations and viral replication in PBMCs. Mutation rates were derived from the total numbers of mutations per nucleotides sequenced, and replication was expressed as area under the curve (AUC) for the entire time of infection (Fig. 2). The data from the three different PBMC donors were combined but 144AAA was excluded from the nonlinear regression. R^2 denotes the goodness of fit.

Interestingly, we detected two more drug resistance-associated mutations resulting from G-to-A mutations in an APOBEC3-favored context in the E45population: G73S in protease (clones E45pop-1 and 17, both in combination with M184I) and D67N in RT (clone E45pop-46) (Fig. S1). Interestingly, D67N results from a G-to-A mutation in an APOBEC3F-favoured dinucleotide context, whereas G73S, like M184I, is a mutation in a dinucleotide context preferred by APOBEC3G (Table 1).

The majority of the viral stocks produced by the selected E45Gpop clones displayed <10% of the infectivity measured for the WT control (28/32; Fig. 5*A*), suggesting that mutagenesis rendered these clones replication defective. In addition to the seven hypermutated clones, it is reasonable to assume that the remaining ones that appear to be WT in the genotypic analysis are likely to encode a lethal amino acid substitution outside of the sequenced area. Four clones produced infectious particles (E45pop clones 2, 6, 7, and 38). These clones encoded mutation E45G in Vif but no other changes in the 1,150-nt-long sequenced *pol* region (Fig. S1).

Taken together, 15% (5/32) of the proviruses derived from Vif

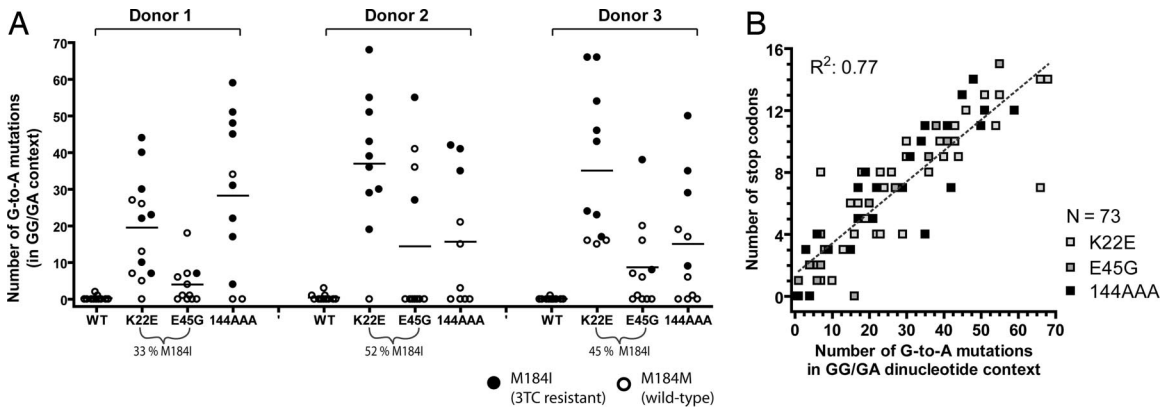

Fig. 4. HIV-1 Vif mutants induce accumulation of 3TC-resistant proviruses. Forty-three percent (29/67) of the clones derived from PBMC infections with partially defective Vif mutants (K22E, E45G), but none of the clones derived from the Vif WT infections carry resistance-associated mutations in RT. (*A*) Proviruses carrying M184M (WT RT, open symbols, $n = 88$) and M184I (3TC-resistant, closed symbols, $n = 44$) display a range of deamination events. M184V was never detected. The mean number of G-to-A mutations in GG/GA dinucleotide context per infection is depicted by a horizontal line. (*B*) Correlation between the degree of deamination of Vif mutants (G-to-A mutations in GG/GA dinucleotide context) and stop codons in all clones ($n = 73$). Forty-three of these clones encoded M184I. The data from the three different PBMC donors were combined. R^2 denotes the goodness of fit.

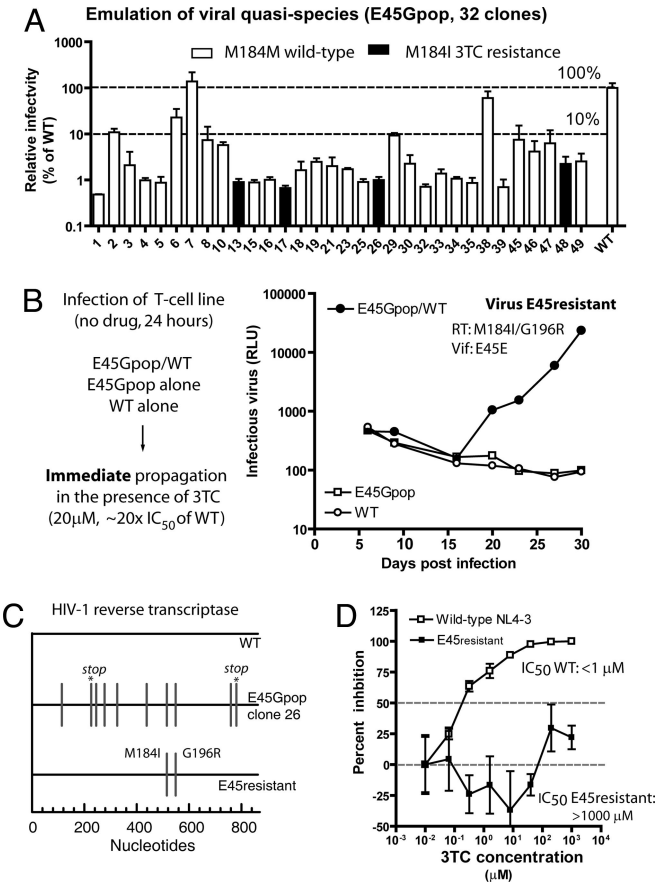

Fig. 5. Complementation of WT HIV-1 with Vif mutant, drug-resistant viral population. (*A*) The viral quasi-species resulting from replication of partially defective Vif mutant was emulated by generating a viral stock comprising the combination of 32 individual HIV-1 clones derived from donor 1 PBMC infection with Vif mutant E45G. Infectivity of the individual clones was assessed by using TZM-bl reporter cells and expressed as percent of WT virus production. Presence of M184M (WT, empty bars) or M184I (3TC resistance, filled bars) is indicated for each clone. (*B*) Virus stocks (viral quasi-species population E45Gpop alone, WT alone, or a combination of both) were used to infect MT2 T cells. (*Left*) Twenty-four hours after infection, cells were put under selection with 20 μM 3TC. (*Right*) The presence of replication-competent viruses under 3TC drug selection was assessed by measuring infectious virus release on TZM-bl cells starting at day 6 after infection. Drug-resistant virus appeared in the E45Gpop/WT infection at day 20 (virus $E45_{resistant}$), and the mutations present in RT and Vif are listed. C) Genotypic characterization of virus $E45_{resistant}$. The RT regions from WT NL4-3, E45Gpop clone 26, and virus $E45_{resistant}$ are shown (nucleotides correspond to RT residues 48–267). All 10 mutations differing from NL4-3 are G-to-A mutations. Stop codons are identified by *. Results represent findings from two independent experiments. (*D*) Phenotypic characterization of virus $E45_{resistant}$. Viral susceptibility to the nucleoside analogue 3TC was determined by infecting TZM-bl cells in the presence of increasing drug concentrations. Infectivity was determined by quantification of β-galactosidase activity. IC_{50} is depicted by a horizontal line.

mutant E45G PBMCs encode drug resistance-associated mutations, but all of these viruses are replication-defective.

Complementation of WT HIV-1 with a Deaminated Drug-Resistant Provirus Population. Hypermutated proviruses are regarded as an evolutionary dead-end given the occurrence of multiple premature stop codons that cause them to become replication-defective. Hypermutated, integrated proviruses, however, remain transcriptionally competent. Therefore, we tested whether fragments of hypermutated genomes (e.g., the region comprising the RT drug resistance M184I) could be transferred through *in vivo* recombination to a replication-competent WT virus. Indeed, recombination that occurs during RT by template switching of the RT enzyme between two heterogeneous RNA genomes is an important mechanism of sequence variation and HIV-1 evolution (28–30).

Table 1. Summary of HIV-1 drug resistance mutations that result from APOBEC3-driven mutagenesis (GG/GA nucleotide context)

Protease	RT	Envelope
G16E	**D67N**	G36S/D
D30N	**M184I**	
M36I	G190S/E	
M46I		
G73S		

The mutations in bold were detected in E45 population clones. The analyzed drug-resistance mutations are based on the 2007 update from the International AIDS Society (41). Amino acid abbreviations used: G, glycine; E, glutamate; D, aspartate; N, asparagine; M, methionine; I, isoleucine; S, serine.

Protocols for selection of drug-resistant viruses generally call for serial passages of drug-sensitive HIV-1 in the presence of increasing concentrations of antiretroviral drug, a procedure that results in stepwise viral adaptation. We developed a different experimental approach, in which infection was allowed to proceed in the absence of 3TC for about one cycle of infection followed by immediate propagation in high 3TC concentration (Fig. 5*B Left*). The MT2 T cells were inoculated in the absence of drugs for 24 h with viral stocks produced by transfection of WT alone or a combination of the 32 E45G mutant clones either with or without WT (E45pop/WT and E45Gpop alone, respectively). Subsequent propagation of E45pop/WT in the presence of 20 μM 3TC, the equivalent of 20 times the IC_{50}, resulted reproducibly in the rapid appearance of infectious, drug-resistant viruses (e.g., $E45_{resistant}$ at day 20 postinfection; Fig. 5*B Right*), whereas WT alone always failed to replicate in the presence of 20 μM 3TC. In one experiment, viruses derived from E45Gpop alone showed replication in the presence of 3TC although this event occurred ≈3 weeks after the corresponding E45Gpop/WT breakthrough.

Sequence comparison between WT NL4-3, $E45_{resistant}$, and the RT regions of the 32 clones comprised in E45Gpop identified a specific clone, E45pop clone 26, as a potential resistance donor, because a 300-nt-long region surrounding M184I and G196R matched the corresponding region of $E45_{resistant}$ (Fig. 5*C*). Phenotypic characterization showed that virus $E45_{resistant}$ was 1,000-fold less susceptible to 3TC than WT ($IC_{50} > 1{,}000$ μM 3TC compared with <1 μM for NL4-3; Fig. 5*D*). Genotypic analysis revealed that all clones derived from $E45_{resistant}$ carried M184I and G196R in RT combined with WT Vif (E45E; Fig. 5*B*), supporting recombination as the underlying rescue mechanism.

These results indicate that hypermutated proviruses can shape the circulating viral quasi-species by recombination during coinfection/superinfection. Furthermore, our data indicate the importance of a reservoir of hypermutated, replication-defective proviruses for viral drug escape, suggesting that sporadic and partial Vif inactivation can be beneficial for HIV-1 evolution in certain biological environments.

Discussion

Cytoplasmic APOBEC3 cytidine deaminases act as potent mutagens of retroviral genomes (1, 2, 31, 32), but these antiviral effects are generally inactivated by HIV-1 Vif. Because of their failure to efficiently counteract APOBEC3, naturally occurring APOBEC3G/3F neutralization-defective Vif proteins have the potential to determine HIV/AIDS disease outcome by reducing viral infectivity and increasing viral diversification.

Most of our understanding about APOBEC3 activity and corresponding Vif defects has been gained by using overexpression systems (18, 19, 25). In this study, we demonstrate that HIV-1 Vif mutants with suboptimal anti-APOBEC3G activity display reduced fitness in a multiple round replication assay with human PBMCs. Importantly, the attenuation of Vif mutant K22E and E45G was observed at endogenous APOBEC3 expression levels (Fig. 2), and reduced fitness of Vif mutant viruses correlated with increase viral sequence diversity (Fig. 3*A* and *C*). Thus, variation in Vif activity may lead to reduced viremia, as reported for some untreated chronically infected patients harboring hypermutated proviruses (20).

After only 2 weeks of propagation, the proviral reservoir associated with Vif mutants K22E and E45G was shaped by G-to-A substitutions at levels that were far superior to RT-introduced errors (Fig. 3*A*). The predominant effect of APOBEC3G-driven rather than APOBEC3F-driven cytidine deamination (Fig. 3*B*) reflects the impairment of these Vif mutants to efficiently neutralize APOBEC3G but not APOBEC3F (18). Individual donor-specific factors may also play a role as suggested by the variable degree of deamination observed in Vif mutant E45G (Fig. 4*A*). APOBEC3G/3F expression and packaging efficiency (33) and single-nucleotide polymorphisms in the APOBEC3 genes could contribute to such an individual deamination threshold.

As a consequence of G-to-A substitutions, we found an abundance of proviruses with the 3TC resistance-associated mutation M184I and stop codons in all Vif mutant infections. Comparable high numbers of M184I RT mutants were detected in HIV-1 genomes recovered from resting $CD4^+$ cells of HAART-treated patients (21). HIV-1 sequence diversification is thought to originate from random errors introduced during RT and caused by either the low fidelity of retroviral RT or recombination events (34). Using an amended drug-resistance selection protocol, we demonstrate that viral stocks produced by a mixture of hypermutated and WT genomes have a clear growth advantage over pure WT HIV-1 controls when propagated in the presence of high concentrations of 3TC. As a general underlying mechanism, we propose that transcriptionally active deaminated proviruses, upon coinfection/ superinfection with replication-competent viruses, form heterogeneous genome-containing virions. In the next cycle of infection, selected fragments of the hypermutated genomes (e.g., drug resistance-containing regions) recombine with WT ones during RT. Incomplete neutralization of host cytidine deamination in conjunction with recombination could also explain why M184I (G-to-A mutation in an APOBEC3G-favored dinucleotide context) as opposed to M184V variants (a mutation introduced by RT errors) appears first in patients failing 3TC treatment (35). A number of drug-resistance mutations result from G-to-A mutations in an APOBEC3-favored context (Table 1), and certain clinical settings (e.g., advanced disease or suboptimal treatment efficacy) could predispose emergence of these variants. Coinfection or superinfection are prerequirements for recombination, and the frequency of coinfection has been associated with emergence of less fit, but more pathogenic, viruses (36).

Although this study harbors the limitations of *in vitro* experimental settings (e.g., high multiplicities of infections, production of viruses by transfection), it highlights that partial neutralization of APOBEC3G represents an important additional mechanism for viral sequence diversification. Cytidine deamination, in combination with viral recombination, allows for the selection of replication-competent viruses with beneficial mutations (as exemplified in this study by the appearance of drug resistance). We report that hypermutated genomes affect circulating virus population, which implies that HIV-1 can exploit a restriction mechanism as a means of escape. The clinical relevance of deaminated proviral reservoirs will need to be investigated in light of these findings (e.g., avoidance of 3TC in certain patients, HIV-1 subtypes specific pathways to drug resistance).

Several potential APOBEC3-Vif interaction motifs have recently been described (37, 38). Our results support the validity of this putative drug target but also inform about the unintended consequences of therapies targeting this protein–protein interaction with less than absolute efficacy. This limitation would be especially relevant in sanctuaries inaccessible to antiretroviral treatments, such as the brain, that could become not only centers for viral replication but also for recombination.

Materials and Methods

Plasmids. NL4-3 Vif mutants pCRV1-vif22E, 45G, and SLQ144AAA have been described (18). Replication competent molecular clones encoding the above-mentioned HIV-1 Vif mutants were generated by overlapping PCR and inserted into the HIV-1 NL4-3 by using standard molecular biology techniques. The sequences of inserts from all constructs were verified by sequencing.

Culture of Cell Lines and PBMCs. HEK 293T and reporter cells TZM-bl cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin/ streptomycin, and 2 mM L-glutamine.

Human PBMCs were obtained by Ficoll density centrifugation from three HIV-1-negative blood donors (Mount Sinai School of Medicine Blood Bank). Cells were cyropreserved in liquid nitrogen. PBMCs were stimulated with phytohemagglutinin (PHA, 1 μg/ml) for 48 h and cultured in RPMI medium 1640 supplemented with 10% FBS, penicillin/streptomycin, 2.3 mg/ml Hepes, glutamine, and 20 units/ml IL-2.

WT and Vif Mutant Viral Stocks. HIV-1 viral stocks were obtained by transfection of HEK 293T cells using 4 μg/ml polyethylenimine (Polysciences). Two days after transfection supernatants were collected, filtered (0.45 μM), aliquoted, and stored at -80°C. Viral concentrations were obtained by measurement of p24-capsid by using commercial p24 ELISA (Coulter). $TCID_{50}$ values were determined on TZM-bl reporter cells by infection of triplicate serial dilutions of each stock.

PBMC Infections with NL4-3 Vif Mutants. A total of 0.7–1 $\times$ 10^6 PHA-stimulated PBMCs were infected overnight with 20 ng of p24 equivalent of viral stocks in the presence of 2 μg/ml polybrene. Infectious dose input per infection varied from 1,500 (WT, K22E, E45G) to 700 (144AAA), resulting in multiplicities of infection from 0.003 to 0.007. After infection, cells were washed three times and maintained in complete RPMI medium 1640/ IL-2 (20 units/ml) over 15–18 days. Culture supernatants for p24 antigen quantification were collected every 2–4 days. Experiments were performed twice with three different HIV-1-negative donors.

Assessment of Viral Diversity. After 15 days of infection, PBMCs were collected and DNA was extracted by a using DNeasy DNA isolation kit (Qiagen). To confirm the presence of the single-nucleotide mutation in Vif at the end of the infection, Vif regions were amplified by using Platinium *Taq* HiFi and primer Vif1S (HXB2: residues 4923–4951, gatccactttggaaaggaccagcaaagct) and Vif2as (HXB2: residues 5737–5712, atggcttccactcctgcccaagtatc). PCR products were purified (Qiagen PCR purification kit) and directly sequenced. To assess the identity and frequency of proviral mutations, a region spanning from RT to the central polypurine tract (cPPT) in integrase was selected for amplification with primers NL4-3-S (HXB2: residues 2928–2947, tatactrcatttaccataccc) and cPPT-AS (HXB2: residues 4814–4833, ctactattctttcccctgca) by using PicoMax (Stratagene) followed by cloning with a StrataClone Kit (Stratagene). We pooled two PCRs for each cloning to limit amplification bias, especially in low replication settings (such as Vif mutants K22E and 144AAA). The PCRs and the cloning were repeated at least twice. Each clone from the Vif mutant infections represents a unique sequence. Vif clones identical in sequences are only listed if they were generated in distinct experiments. DNA sequencing reactions were performed with BigDye Terminator v3.1 reagents and analyzed on an ABI PRISM 3730xl (Agencourt Bioscience). The 1,250-nt-long sequences (HXB2: residues 2931–4180) were manually edited and aligned by using DNASTAR and Bioedit software packages. Analysis of genetic distances and mutation frequency was performed with PAUP (version 4.0 beta), Bioedit, and Hypermut (original, version 2.0) programs (39).

Selection of 3TC Drug Resistance. At the completion of the infection experiment (day 15; Fig. 2), total DNA was extracted with the DNeasy DNA isolation kit (Qiagen) from donor 1's PBMCs infected with the E45G mutant (Fig. 4). A 4,250-nt-long fragment including C terminus of *gag*, full-length *pol*, *vif*, and parts of *vpr* was amplified from the integrated provirus population and cloned

into NL4-3 WT. Mixed viral populations were obtained by transfecting a DNA preparation consisting of 32 individual clones with or without NL4-3 (WT/E45Gpop and E45Gpop, respectively) into HEK 293T cells. These viral populations were used to infect the T cell line MT2, which was treated with 20 μM 3TC 24 h postinfection. Culture supernatants were collected at regular intervals, and 3TC was replenished at least once a week.

Phenotypic resistance testing was performed by infecting TZM-bl reporter cells, in triplicate with WT HIV-1 or virus $E45_{resistant}$ in the presence of increasing concentrations of 3TC (5-fold dilutions, maximum 3TC concentration 1,000 μM). β-Galactosidase activity was quantified 48 h after infection by using chemiluminescent substrate, as described (18). RT and Vif regions of virus $E45_{resistant}$ were sequenced and compared with corresponding regions of the 32 individual clones comprised in the viral population E45Gpopulation. Two independent experiments were performed.

Statistical Analysis. Prism software (version 4.0, GraphPad) was used to perform all statistical tests. *P* values are two-sided, and values <0.05 are considered to be significant.

ACKNOWLEDGMENTS. We thank P. Bieniasz, L. Chakrabarti, I. Chen, C. Cheng-Mayer, and P. Palese for helpful discussions and Y. Safavi and F. Yu for excellent technical assistance provided by in the early phase of the project. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: 3TC, TZM-bl cells (provided by J. C. Kappes, X. Wu, and Transzyme Inc.) and IL-2 (provided by Roche). This work was supported by National Institutes of Health, National Institute of Allergy and Infectious Diseases, Grant R01 AI064001 (to V.S.).

1. Cullen BR (2006) Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J Virol* 80:1067–1076.
2. Bieniasz PD (2004) Intrinsic immunity: A front-line defense against viral attack. *Nat Immunol* 5:1109–1115.
3. KewalRamani VN, Coffin JM (2003) Virology: Weapons of mutational destruction. *Science* 301:923–925.
4. Holmes RK, Malim MH, Bishop KN (2007) APOBEC-mediated viral restriction: Not simply editing? *Trends Biochem Sci* 32:118–128.
5. Dang Y, Wang X, Esselman WJ, Zheng YH (2006) Identification of APOBEC3DE as another antiretroviral factor from the human APOBEC family. *J Virol* 80:10522–10533.
6. Mehle A, *et al.* (2004) Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J Biol Chem* 279:7792–7798.
7. Zheng YH, *et al.* (2004) Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J Virol* 78:6073–6076.
8. Liddament MT, Brown WL, Schumacher AJ, Harris RS (2004) APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 *in vivo*. *Curr Biol* 14:1385–1391.
9. Wiegand HL, Doehle BP, Bogerd HP, Cullen BR (2004) A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J* 23:2451–2458.
10. Kao S, *et al.* (2003) The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *J Virol* 77:11398–11407.
11. Miyagi E, *et al.* (2007) Enzymatically active APOBEC3G is required for efficient inhibition of human immunodeficiency virus type 1. *J Virol* 81:13346–13353.
12. Yu Q, *et al.* (2004) Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat Struct Mol Biol* 11:435–442.
13. Lecossier D, Bouchonnet F, Clavel F, Hance AJ (2003) Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* 300:1112.
14. Guo F, *et al.* (2007) The interaction of APOBEC3G with human immunodeficiency virus type 1 nucleocapsid inhibits tRNA3Lys annealing to viral RNA. *J Virol* 81:11322–11331.
15. Iwatani Y, *et al.* (2007) Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G. *Nucleic Acids Res* 35:7096–7108.
16. Mbisa JL, *et al.* (2007) Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration. *J Virol* 81:7099–7110.
17. Luo K, *et al.* (2007) Cytidine deaminases APOBEC3G and APOBEC3F interact with human immunodeficiency virus type 1 integrase and inhibit proviral DNA formation. *J Virol* 81:7238–7248.
18. Simon V, *et al.* (2005) Natural variation in Vif: differential impact on APOBEC3G/3F and a potential role in HIV-1 diversification. *PLoS Pathog* 1:e6.
19. Tian C, *et al.* (2006) Differential requirement for conserved tryptophans in human immunodeficiency virus type 1 Vif for the selective suppression of APOBEC3G and APOBEC3F. *J Virol* 80:3112–3115.
20. Pace C, *et al.* (2006) Population level analysis of human immunodeficiency virus type 1 hypermutation and its relationship with APOBEC3G and vif genetic variation. *J Virol* 80:9259–9269 and erratum (2007) 81:8843–8845.
21. Kieffer TL, *et al.* (2005) G → A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting $CD4^+$ T cells *in vivo*. *J Virol* 79:1975–1980.
22. Janini M, Rogers M, Birx DR, McCutchan FE (2001) Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of $CD4^+$ T cells. *J Virol* 75:7973–7986.
23. Koulinska IN, Chaplin B, Mwakagile D, Essex M, Renjifo B (2003) Hypermutation of HIV type 1 genomes isolated from infants soon after vertical infection. *AIDS Res Hum Retroviruses* 19:1115–1123.
24. Wei M, *et al.* (2004) Biased G-to-A hypermutation in HIV-1 proviral DNA from a long-term nonprogressor. *AIDS* 18:1863–1865.
25. Russell RA, Pathak VK (2007) Identification of two distinct HIV-1 Vif determinants critical for interactions with human APOBEC3G and APOBEC3F. *J Virol* 81: 8201–8210.
26. Marin M, Rose KM, Kozak SL, Kabat D (2003) HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* 9:1398–1403.
27. Diallo K, Gotte M, Wainberg MA (2003) Molecular impact of the M184V mutation in human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* 47:3377–3383.
28. Jung A, *et al.* (2002) Multiply infected spleen cells in HIV patients. *Nature* 418:144.
29. Nikolenko GN, Svarovskaia ES, Delviks KA, Pathak VK (2004) Antiretroviral drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase increase template-switching frequency. *J Virol* 78:8761–8770.
30. Malim M, Emerman M (2001) HIV-1 sequence variation: Drift, shift, and attenuation. *Cell* 104:469–472.
31. Trono D (2004) Retroviruses under editing crossfire: A second member of the human APOBEC3 family is a Vif-blockable innate antiretroviral factor. *EMBO Rep* 5:679–680.
32. Harris RS (2003) DNA deamination: Not just a trigger for antibody diversification but also a mechanism for defense against retroviruses. *Nat Immunol* 4:641–643.
33. Wang T, *et al.* (2007) 7SL RNA mediates virion packaging of the antiviral cytidine deaminase APOBEC3G. *J Virol* 81:13112–13124.
34. Frost SD, Dumaurier MJ, Wain-Hobson S, Brown AJ (2001) Genetic drift and within-host metapopulation dynamics of HIV-1 infection. *Proc Natl Acad Sci USA* 98:6975–6980.
35. Keulen W, Back NK, van Wijk A, Boucher CA, Berkhout B (1997) Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. *J Virol* 71:3346–3350.
36. Wodarz D, Levy DN (2007) Human immunodeficiency virus evolution toward reduced replicative fitness *in vivo* and the development of AIDS. *Proc Biol Sci* 274:2481–2490
37. Mehle A, *et al.* (2007) Identification of an APOBEC3G binding site in HIV-1 Vif and inhibitors of Vif-APOBEC3G binding. *J Virol* 81:13235–13241.
38. Xiao Z, *et al.* (2007) Characterization of a novel Cullin5 binding domain in HIV-1 Vif. *JMol Biol* 373:541–550.
39. Rose PP, Korber BT (2000) Detecting hypermutations in viral sequences with an emphasis on G → A hypermutation. *Bioinformatics* 16:400–401.
40. Mehle A, Thomas ER, Rajendran KS, Gabuzda D (2006) A zinc-binding region in Vif binds Cul5 and determines cullin selection. *J Biol Chem* 281:17259–17265.
41. Johnson VA, *et al.* (2007) *Top HIV Med* 15:119–125.