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Impaired Fitness of Human Immunodeficiency Virus Type 1 Variants with High-Level Resistance to Protease Inhibitors

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One hope to maintain the benefits of antiviral therapy against the human immunodeficiency virus type 1 (HIV-1), despite the development of resistance, is the possibility that resistant variants will show decreased viral fitness. To study this possibility, HIV-1 variants showing high-level resistance (up to 1,500-fold) to the substrate analog protease inhibitors BILA 1906 BS and BILA 2185 BS have been characterized. Active-site mutations V32I and I84V/A were consistently observed in the protease of highly resistant viruses, along with up to six other mutations. In vitro studies with recombinant mutant proteases demonstrated that these mutations resulted in up to 10^4-fold increases in the K_i values toward BILA 1906 BS and BILA 2185 BS and a concomitant 2,200-fold decrease in catalytic efficiency of the enzymes toward a synthetic substrate. When introduced into viral molecular clones, the protease mutations impaired polyprotein processing, consistent with a decrease in enzyme activity in virions. Despite these observations, however, most mutations had little effect on viral replication except when the active-site mutations V32I and I84V/A were coexpressed in the protease. The latter combinations not only conferred a significant growth reduction of viral clones on peripheral blood mononuclear cells but also caused the complete disappearance of mutated clones when cocultured with wild-type virus on T-cell lines. Furthermore, the double nucleotide mutation I84A rapidly reverted to I84V upon drug removal, confirming its impact on viral fitness. Therefore, high-level resistance to protease inhibitors can be associated with impaired viral fitness, suggesting that antiviral therapies with such inhibitors may maintain some clinical benefits.

Initial antiviral strategies against human immunodeficiency virus type 1 (HIV-1) concentrated mostly on inhibiting the reverse transcriptase (RT) enzyme. Both nucleoside and nonnucleoside analog inhibitors of this enzyme have shown good antiviral activity (6). However, due to its high replication rate and the low fidelity of the RT enzyme, which has no proofreading activity (11, 32, 41), HIV-1 rapidly develops resistance to both classes of compounds (8, 21). Indeed, variants showing up to 1,000-fold-decreased susceptibility to RT inhibitors have been reported in vitro and in vivo (21). The molecular basis for this resistance involves mutations in the enzyme active site (nucleoside analogs) or in binding pockets (nonnucleoside analogs), both of which decrease inhibitor binding (reviewed in reference 37). Despite these mutations, however, no change in activity has been detected with most drug-resistant RT enzymes compared to the wild type (14, 17).

HIV-1 also encodes a small homodimeric aspartic protease, which mediates the cleavage of polyprotein precursors Gag and Gag-Pol during virion maturation (5, 40, 43) (reviewed in reference 29). Following processing, six structural proteins (p17, p24, p2, p7, p1, and p6) and four enzymes (protease, RT, RNase H, and integrase) are released. Protease function is essential for HIV-1 replication, as deletions or point mutations that abolish protease activity result in the production of uninfectious viral particles (15, 28). Protease inhibitors have therefore been developed and have also proven to be effective antiviral agents (4, 42). Since the protease is a small protein with only 99 amino acids per monomer, it was thought that it may tolerate only limited mutational events and therefore be refractory to the development of resistance involving multiple mutations. In a number of instances, however, HIV-1 variants showing up to 100-fold-decreased susceptibility to protease inhibitors have been obtained both in vivo and in vitro (2, 21, 22). Not surprisingly, resistance has been attributed to active-site mutations in the protease including R8Q/K, V32I, I47V, I50V, P81T, V82I,A,T,F, and I84V (9, 21). Most of these mutations were shown to decrease inhibitor binding (13, 18, 25, 36), but, in contrast to RT mutations, protease mutations also seem to decrease enzyme function toward peptide-based substrates, the extent of the decrease being directly correlated with the number of active-site mutations in the enzyme (10, 18, 26, 36). In variants resistant to protease inhibitors, this decrease in enzyme function can become so important that compensatory cleavage site mutations, improving the functional nature of these sites as substrates for the protease, become essential for viral replication (7). This latter observation clearly demonstrates the negative effects of protease mutations on viral fitness and therefore raises the possibility that protease inhibitor treatment will still have a therapeutic advantage despite the development of resistance.

To better characterize the appearance and the extent of impaired viral fitness of protease inhibitor-resistant viruses, HIV-1 was maintained for up to 58 passages in culture under increasing selective pressure by the substrate analog protease inhibitors BILA 1906 BS and BILA 2185 BS. Variants obtained at various passages were characterized in terms of viral resistance and viral fitness. Results show that during 58 passages in culture, variants showing from 2- to 1,500-fold resistance can be selected. These variants contain one to eight mutations in the protease gene along with one or two cleavage site mutations, have up to 2,200-fold reduction in protease activity, and are still viable. Viruses containing only one active-site mutation are relatively unaffected in fitness, whereas viral

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replication of HIV containing eight mutations in the protease, four being in the active site of the enzyme, is considerably reduced despite the presence of p7/p1 and p1/p6 cleavage sites mutations. These results demonstrate that treatment with protease inhibitors can indeed lead to the selection of unfit HIV if an aggressive selection protocol, allowing the survival only of viruses containing multiple mutations in the protease, is applied.

MATERIALS AND METHODS

Cells and viruses. The cell lines C8166 (obtained from J. Sullivan), 293 (American Type Culture Collection), and H-9 chronically infected with HIV-1 (IIIB) (30, 31, 33) (obtained from Robert Gallo, AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) were used. Peripheral blood mononuclear cells (PBMC) from healthy donors (negative for Epstein-Barr virus, cytomegalovirus, and HIV) were kindly provided by Eric Cohen (University of Montréal, Montréal, Canada). All cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 μg of gentamicin per ml, and 10^{-5} M β-mercaptoethanol. For PBMCs, 10 U of interleukin-2 (Gibco/BRL) per ml and 1% phytohemagglutinin A (Gibco/BRL) were added to the cultures every 2 to 3 days. Viral stocks were prepared from chronically infected H-9 cells. Proviral DNA pNL4.3 (1) was obtained from Malcolm Martin, AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases.

Drugs. BILA 1906 BS and BILA 2185 BS are pipecolinic acid derivatives that correspond to inhibitors 1 and 4, respectively (nomenclature as in reference 38). The RT inhibitors azidothymidine (AZT) and nevirapine were generous gifts from Glaxo/Wellcome Inc. and Boehringer Ingelheim Pharmaceutical Inc. (Ridgefield, Conn.), respectively.

Generation of variants resistant to drugs. Protease inhibitor-resistant HIV-1 variants were obtained by infecting C8166 cells with an initial multiplicity of infection (MOI) of 1 HIV-1 (IIIB) infectious particle per cell. The cells were then cultured in the presence of 50% effective concentrations (EC_{50}) of drug. Viral replication was maintained by serial passages on C8166 cells in the presence of the drug. At each passage (3 to 4 days), 10^6 fresh C8166 cells were infected with viral supernatant from the previous passage. The drug concentration was increased if the cytopathic effect increased in the previous passage. Aliquots of supernatant from each passage were frozen for subsequent viral titer determination, EC_{50} determination, and cross-resistance studies.

Viral titer and p24 Ag determination. For viral titer determination, limiting dilutions of viral supernatants were used to infect 5×10^4 C8166 cells adhered on poly-D-lysine (Sigma)-coated 96-well flat-bottom plates. Cytopathic effect was monitored 3 days postinfection. The 50% tissue culture infectious dose was determined by the Karber method through a computerized program (19). For quantification of p24 antigen (Ag) in culture supernatants, the Coulter HIV-1 p24 Ag enzyme immunoassay was used.

EC_{50} determination. The drug EC_{50} was determined on acutely infected C8166 cells. The cells were infected at 0.001 MOI and plated in 96-well plates at 5×10^4 cells/well. Serial twofold dilutions of inhibitors were added to wells (eight replicates per dilution) and incubated for 3 days. Extracellular p24 Ag levels of pooled replicates were then determined and used to generate the EC_{50}s with the SAS statistical software system (SAS Institute Inc., Cary, N.C.).

PCR amplification of HIV-1 variant protease genes. The protease gene from resistant variants was PCR amplified from genomic DNA of HIV-1-infected C8166 cells with either of two sets of primers as described previously (7).

Enzyme preparation. PCR-amplified mutant or wild-type proteases were cloned in the pET11a expression vector (Novagen) and expressed in *Escherichia coli* BL21(DE3)pLysS. Protease expression was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 2 h at 37°C. Enzyme preparations consisted either of pure preparation (HIV-2 [27]), partially pure preparations (HIV-1 [24]), refolded enzymes isolated from inclusion bodies, or cell lysates. Inclusion bodies were prepared by lysing cells in buffer A (10 mM Tris [pH 8.0], 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 0.1% Nonidet P-40, 10 mM $MgCl_2$, and 4 U of DNase I per ml. Lysates were sonicated and centrifuged (10,000 × *g* for 20 min) to pellet the insoluble fraction containing inclusion bodies. The inclusion bodies were then washed three times in buffer A containing 2 M urea and 1% Triton X-100, solubilized in buffer A containing 8 M urea, diluted to 200 μg/ml, and dialyzed at 4°C against three changes of refolding buffer (25 mM sodium acetate [pH 4.5], 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol). Kinetic measurements on refolded enzymes were performed as described below.

***K_i*, *K_m*, and *k_{cat}* determinations.** To assess the sensitivity of proteases to the inhibitors BILA 1906 BS and BILA 2185 BS, the inhibition constant K_i, was determined by the method of Morrison and Walsh (23) for tight-binding inhibitors. For classical competitive inhibitors, the velocity of the inhibited reaction was measured at several fixed inhibitor concentrations while varying the substrate concentration. Enzyme activity was monitored by measuring the change in fluorescence associated with the cleavage of the fluorogenic substrate 2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2 (39) as described previously (16), using the appropriate enzyme and inhibitor concentrations. The kinetic parameters K_m and k_{cat} were obtained under similar assay conditions, in the presence of 0.25 to 10 μM of substrate, and were calculated by nonlinear regression of the velocity data with the GraFit software (version 3.0; Erithacus Software Ltd., Staines, United Kingdom).

TABLE 1. In vitro selection of HIV-1 variants resistant to BILA 1906 BS or BILA 2185 BS

Compound	Passage	Mutations[a]	Fold increase in EC_{50}
BILA 1906 BS	12	V32I A71V	3
	22	(V32I) M46I,L A71V (I84V)	5
	33	(V32I) M46I,L A71V I84A*	520
BILA 2185 BS	14	I84V	5
	37	(L10I) (L23I) (V32I) M46I (A71V) I84V,A*	350
	58	(L10F) L23I V32I M46I I47V I54M A71V I84V**	1,500

[a] Mutations in parentheses do not occur in all clones. A L→F mutation in the P1′ position of the Gag p1/p6 cleavage site was observed in variant populations marked *, whereas the same mutation along with QA→RV mutations in the P3 and P2 positions of the Gag p7/p1 cleavage site were observed in variant populations marked **.

Construction of molecular clones. Molecular clones were constructed in the pNL4.3 background and transfected by the calcium phosphate method in 293 cells, as described previously (7). For clarity purposes, clones are identified by the positions of the protease mutations they contain, followed by one or two asterisks. One asterisk represents the p1/p6 cleavage site mutation, while two asterisks represents the presence of both the p1/p6 and the p7/p1 cleavage site mutations. Since two different mutations were observed at position 84 of the protease (I84V and I84A), clones containing either of these mutations are identified as 84A or 84V.

Western blot analysis. Viral supernatants of transfected 293 cells were collected 3 days after transfection, centrifuged at low speed, and filtered on 0.22-μm-pore-size Millex-GV Millipore membranes to get rid of remaining cells and cellular debris. Supernatants were then centrifuged at 20,000 × *g* for 2 h to pellet virus particles. The pellets were resuspended in 1 × Laemmli sample buffer (0.06 M Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% sucrose, 5% β-mercaptoethanol) and heated at 100°C for 5 min. Sample aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide), transferred onto nitrocellulose membranes, and hybridized with a monoclonal antibody directed against the HIV-1 p24 Ag (Cellular Products Inc., no. 0801080) followed by ^{125}I-labeled sheep anti-mouse antibody (Amersham; no. IM131). Blots were exposed on a PhosphorImager screen (Molecular Dynamics Inc.).

Viral growth kinetic studies. C8166 and PBMCs were infected with viral molecular clones at a MOI of 0.001 and 0.01, respectively, for 1 h at 37°C. The cells were then washed twice and maintained in either 10-ml (C8166) or 2-ml (PBMCs) cultures. For PBMC cultures, freshly stimulated cells were added on day 3 whereas medium containing interleukin-2 and phytohemagglutinin A was added every 2 to 3 days. For all cultures, aliquots of supernatant were harvested every day and kept at −80°C until p24 Ag determination.

Coculture experiments. For coculture experiments, C8166 cells were separately infected with different molecular clones (MO1, 0.001) on day 1 and then mixed at a 1:1 ratio for subsequent culture. Viral cultures were maintained by serial passages of cell-free supernatant on fresh C8166 cells every 3 to 4 days. DNA of infected cells was extracted at some passages, and the viral protease gene was PCR amplified and sequenced as described above.

RESULTS

HIV-1 variants resistant to BILA 1906 BS and BILA 2185 BS generated in vitro. BILA 1906 BS and BILA 2185 BS are substrate analog protease inhibitors that prevent HIV-1 replication in vitro (EC_{50}s of 1 and 2 nM, respectively). Serial passages of HIV-1 in the presence of increasing concentrations of BILA 1906 BS and BILA 2185 BS, however, led to the selection of variants with decreased susceptibility to these compounds. PCR amplification followed by sequencing of the protease gene of outgrowing variants showed that two major breakthroughs of resistant viruses occurred with both BILA 1906 BS and BILA 2185 BS (Table 1). For BILA 1906 BS, a

first resistant variant population containing the active-site mutation V32I as well as a mutation outside of the active site (A71V) emerged after 12 passages in culture and was associated with a threefold increase in the EC_{50} of the drug. Selection with a closely related compound has indicated that the single mutation V32I occurs prior to A71V in resistant variants (data not shown). Following the outgrowth of this population, variants with very heterogeneous genotypes were obtained (Table 1, passage 22). Several combinations of the mutations V32I, M46I,L, A71V, and I84V were indeed observed in the protease of variants cultured for 22 passages in the presence of BILA 1906 BS, although no significant change in drug susceptibility could be detected. After 33 passages in culture, a homogeneous population outgrew, showing either of two dominant genotypes: V32I M46I A71V I84A or M46L A71V I84A. In these populations, two active-site mutations (V32I and I84A) were observed, with I84A involving a double nucleotide change. A mutation outside of the protease gene locus, located in the P1′ position (L→F) of the p1/p6 cleavage site, was also present in all variants from passage 33. These variants were associated with a dramatic (520-fold) increase in the EC_{50} of BILA 1906 BS and were considered highly resistant to the drug.

Passage of HIV-1 in the presence of BILA 2185 BS gave rise to resistant variant populations with different genotypes and resistance phenotypes (Table 1). A population containing the active-site mutation I84V and showing fivefold resistance to BILA 2185 BS was the first to appear after 14 passages in the presence of the drug. Populations of variants showing mixed genotypes were then obtained after 37 passages in culture, with several combinations of the mutations L10I, L23I, V32I, M46I, A71V, and I84V,A present in the protease. Interestingly, three of five variants sequenced from this passage also had the L→F mutation in the p1/p6 cleavage site previously observed in variants highly resistant to BILA 1906 BS. BILA 2185 BS-resistant variants from passage 37 were associated with a 350-fold increase in the EC_{50} of the drug. Further passages with BILA 2185 BS led to the selection of a quite homogeneous population bearing the protease mutations L23I, V32I, M46I, I47V, I54M, A71V, and I84V, sometimes in the presence of L10F. These genotypes included the active-site mutations at positions 32 and 84 previously observed in BILA 1906 BS-resistant variants and also included active-site mutations L23I and I47V. In addition, BILA 2185 BS-resistant variants from passage 58 had mutations in both the p1/p6 and the p7/p1 cleavage sites, the latter involving changes in both the P2 and P3 positions (QA→RV) of the cleavage site. These variants were associated with a 1,500-fold increase in the EC_{50} of BILA 2185 BS. Therefore, HIV-1 variants with 500- to 1,500-fold increases in resistance to protease inhibitors can be selected in vitro, and distinct but structurally related compounds give rise to variants bearing similar active-site mutations.

The susceptibility of resistant variants to the protease inhibitors BILA 1906 BS and BILA 2185 BS, as well as to the clinically approved RT inhibitors AZT and nevirapine, was assessed. Table 2 shows that BILA 1906 BS-resistant variants (BILA 1906^r) from passages 12 or 22 have three- to fivefold increases in resistance to the protease inhibitors BILA 1906 BS and BILA 2185 BS. The switch from I84V to I84A observed in variants from passage 33 confers cross-resistance (200- to 500-fold) to both protease inhibitors. BILA 2185 BS-resistant variants (BILA 2185^r) containing the single mutation I84V (P14) are only moderately resistant to both protease inhibitors. Variants from passages 37 or 58, on the other hand, whatever their genotypes, seem to be cross-resistant to all protease inhibitors tested (Table 2 and data not shown). In no case was a significant shift of susceptibility toward the RT inhibitors AZT and nevirapine observed with any of these variants.

TABLE 2. Cross-resistance of BILA 1906 BS- and BILA 2185 BS-resistant variants

Population	EC_{50} (nM) of:			
	BILA 1906 BS	BILA 2185 BS	AZT	nevirapine
Wild type	1	2	5	12
BILA 1906^r				
P12	3	8	6	
P22	5	8	4	
P33	520	400	5	15
BILA 2185^r				
P14	2	11	12	16
P37	122	700	8	
P58	360	2,713	4	5

Recombinant mutant proteases are associated with an increased inhibition constant (K_i) of BILA 1906 BS and BILA 2185 BS. To characterize the protease of the drug-resistant variants, wild-type and mutant protease genes were expressed in *E. coli* and the protease was isolated as described in Materials and Methods. The K_i of BILA 1906 BS and BILA 2185 BS for these proteases was then determined (Table 3). The results showed that with mutant protease V32I, BILA 1906 BS had a K_i of 39 pM, a 22-fold increase compared to that for the wild-type HIV-1 enzyme. The mutation A71V did not significantly affect drug binding, as the double mutant V32I-A71V was associated with a BILA 1906 BS K_i of 45 pM, representing a 26-fold increase compared to that for the wild type. The additional mutations M46I and I84A, however, drastically increased the K_i of BILA 1906 BS, which shifted to 20,710 pM, a 12,000-fold increase. Mutations present in variants selected with BILA 1906 BS thus decrease protease susceptibility toward this drug. Proteases cloned from BILA 2185 BS-resistant variants were also analyzed in similar experiments. Compared to the wild-type HIV-1 enzyme, the single mutation I84V caused a 14-fold increase in the K_i of BILA 2185 BS (K_i = 5.5 and 77 pM respectively). The mutant enzyme L10I L23I M46I I84V, containing three additional mutations, caused a K_i of 197 pM, a 36-fold increase, while a protease containing seven mutations (L23I, V32I, M46I, I47V, I54M, A71V, and I84V) caused a K_i of 68,100 pM, a 12,000-fold increase compared to the wild-type enzyme. Interestingly, in these studies, all mutant proteases were associated with concomitant increases in the K_i

TABLE 3. K_i s of BILA 1906 BS and BILA 2185 BS

Protease	K_i (pM) of:	
	BILA 1906 BS	BILA 2185 BS
HIV-1	1.7	5.5
HIV-2	49	24
V32I	39	83
V32I A71V	45	60
V32I M46I A71V I84A	20,710	24,770
I84V	27	77
L10I L23I M46I I84V	71	197
L23I V32I M46I I47V I54M A71V I84V	10,300	68,100

TABLE 4. In vitro catalytic efficiencies of mutant proteases

Protease	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
HIV-1	0.58	8.95	15.5
HIV-2	15.6	3.02	0.19
V32I	7.37	2.48	0.34
V32I A71V	7.04	2.59	0.37
V32I M46I A71V I84A	15.5	0.11	0.007
I84V	6.48	3.43	0.53
L10I L23I M46I I84V	6.79	2.63	0.39
L23I V32I M46I I47V I54M A71V I84V	27.3	0.56	0.02

of both BILA 1906 BS and BILA 2185 BS (Table 3), in agreement with cross-resistance results obtained with variant populations (Table 2). Altogether, these results confirm that mutant proteases are less susceptible to inhibition by BILA compounds. However, the shifts in K_i observed for mutant proteases were consistently more significant than the increases in EC_{50} observed for variant populations containing the corresponding enzymes (Tables 3 and 2, respectively).

Mutant proteases have reduced catalytic efficiencies toward a fluorogenic substrate. As an assessment of mutant protease function, the proteolytic cleavage of the fluorogenic substrate 2-aminobenzoyl-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2 (40) was compared between wild-type and mutant enzymes. Results from Table 4 show that the mutant enzyme V32I causes a 45-fold decrease in k_{cat}/K_m of the fluorogenic substrate compared to the effect of the wild-type HIV-1 enzyme (0.34 versus 15.5 $\mu M^{-1} s^{-1}$, respectively), mostly due to an increase in the K_m of the substrate. As for the K_i, the addition of mutation A71V does not significantly affect the catalytic efficiency of the V32I mutant protease, giving a 42-fold decrease in efficiency. The V32I M46I A71V I84A protease, on the other hand, showed poor activity toward the fluorogenic substrate (k_{cat}/K_m = 0.007 $\mu M^{-1} s^{-1}$, a 2,200-fold decrease) due to both an increase in K_m and a decrease in k_{cat}. Proteases resistant to BILA 2185 BS showed similar reductions in activity, as I84V alone caused a 29-fold reduction in k_{cat}/K_m (0.53 $\mu M^{-1} s^{-1}$) which was again mostly attributable to an increase in K_m. Additional mutations L10I, L23I, and M46I further reduced activity to 0.39 $\mu M^{-1} s^{-1}$, a 40-fold decrease, while seven mutations in the protease gave a reduction in catalytic efficiency of 775-fold, to 0.02 $\mu M^{-1} s^{-1}$, attributable to both an increase in K_m and a decrease in k_{cat}. Therefore, although mutant proteases are less susceptible to inhibition by BILA compounds, they show decreased catalytic efficiencies toward a synthetic substrate.

Cleavage of polyprotein precursors is less efficient in viruses with protease mutations. To determine if the decreased in vitro catalytic efficiency of mutant proteases resulted in impaired polyprotein processing in virions, viral molecular clones containing mutant proteases were constructed. To accurately represent the resistant variants selected in culture, molecular clones were designed to contain both protease and cleavage site mutations if these were present in corresponding variants. Therefore, the clones containing mutations V32I (clone 32), V32I and A71V (32/71), and V32I, M46I, A71V, and I84A with a mutation in the p1/p6 cleavage site (32/46/71/84A*) were constructed to represent variants resistant to BILA 1906 BS and clones containing mutations I84V (84V), L10I, L23I, M46I, and I84V with the p1/p6 mutation (10/23/46/84V*), and L23I, V32I, M46I, I47V, I54M, A71V, and I84V with both p1/p6 and p7/p1 mutations (23/32/46/47/54/71/84V**) were constructed to represent variants resistant to BILA 2185 BS. In culture assays, the drug susceptibility of these clones was comparable to that of corresponding variant populations (reference 7 and data not shown). Viruses produced upon transfection of DNA from these clones were therefore harvested and lysed, and polyprotein processing was analyzed by anti-p24 Western blot assay. By using this technique, wild-type viral particles were shown to contain mature p24 proteins as well as $Pr55^{Gag}$ precursors, but no intermediate cleavage products were detected (Fig. 1). A single mutation (clone 32 or 84V) in the protease active site, however, gave rise to a 41-kDa intermediate containing both p17 and p24 proteins, in addition to the 55-kDa precursor and the mature p24 protein. This intermediate became more obvious in viral particles containing multiple mutations in the protease (clones 32/46/71/84A* and 23/32/46/47/54/71/84V**). In clone 32/46/71/84A*, an additional p25 band was detected, representing an uncleaved p24-p2 polyprotein. Although some clones contained cleavage site mutations, the latter were previously shown to improve polyprotein processing in virions (7) and most probably minimize the real impact of protease mutations on p24 maturation in this experiment. Therefore, in agreement with the catalytic efficiency data, inefficient processing of the Gag precursors did occur in viral particles containing altered proteases. Interestingly, despite the unusual presence of uncleaved precursors, a similar amount of mature p24 protein was detected in all viral particles.

Although $Pr55^{Gag}$ processing is altered in drug-resistant molecular clones, it is relatively unaffected by the presence of protease inhibitors, consistent with the increased K_i s obtained with these proteases. Indeed, Fig. 1 B and C show that although BILA 1906 BS and BILA 2185 BS efficiently blocked the maturation of p24 proteins in a dose-dependent manner in wild-type virus, it only slightly affected processing in clones containing multiple mutations in the protease. Indeed, in wild-type virus, p24 production was completely blocked in the presence of 100 nM BILA 1906 BS while $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ processing was blocked at 1 μM drug. Similarly, BILA 2185 BS blocked polyprotein processing and p24 production in wild-type virus at 1 μM (Fig. 1B). In the highly resistant clones 32/46/71/84A* and 23/32/46/47/54/71/84V**, however, almost no difference in Gag processing was observed even in the presence of 1 μM protease inhibitors (Fig. 1C). The only slight effect of inhibitors was the appearance of a 49-kDa intermediate in clones 32/46/71/84A* grown in the presence of 100 nM or 1 μM BILA 1906 BS. Interestingly, this intermediate was also present in wild-type virus grown in the presence of high concentrations of BILA compounds. Therefore, it seems that when expressed in viral molecular clones, mutations in the protease impair normal polyprotein precursor processing but considerably reduce the susceptibility of proteolytic processing to inhibition by protease inhibitors.

Molecular clones with mutations in the protease gene show impaired growth kinetics. Since protease mutations impair polyprotein processing and lead to the accumulation of polyprotein precursors in virions, these mutations could also affect viral replication. This can be assessed by comparing the growth kinetics of wild-type and mutant viruses on T cells in culture. PBMCs were therefore infected at a low MOI (0.01) with viral molecular clones, and viral growth was monitored for 2 to 8 days postinfection. Figure 2A shows that BILA 1906 BS-resistant clones bearing the V32I or V32I A71V mutations in the protease grow only slightly more slowly than wild-type virus on PBMCs, giving total p24 values less than 1 order of

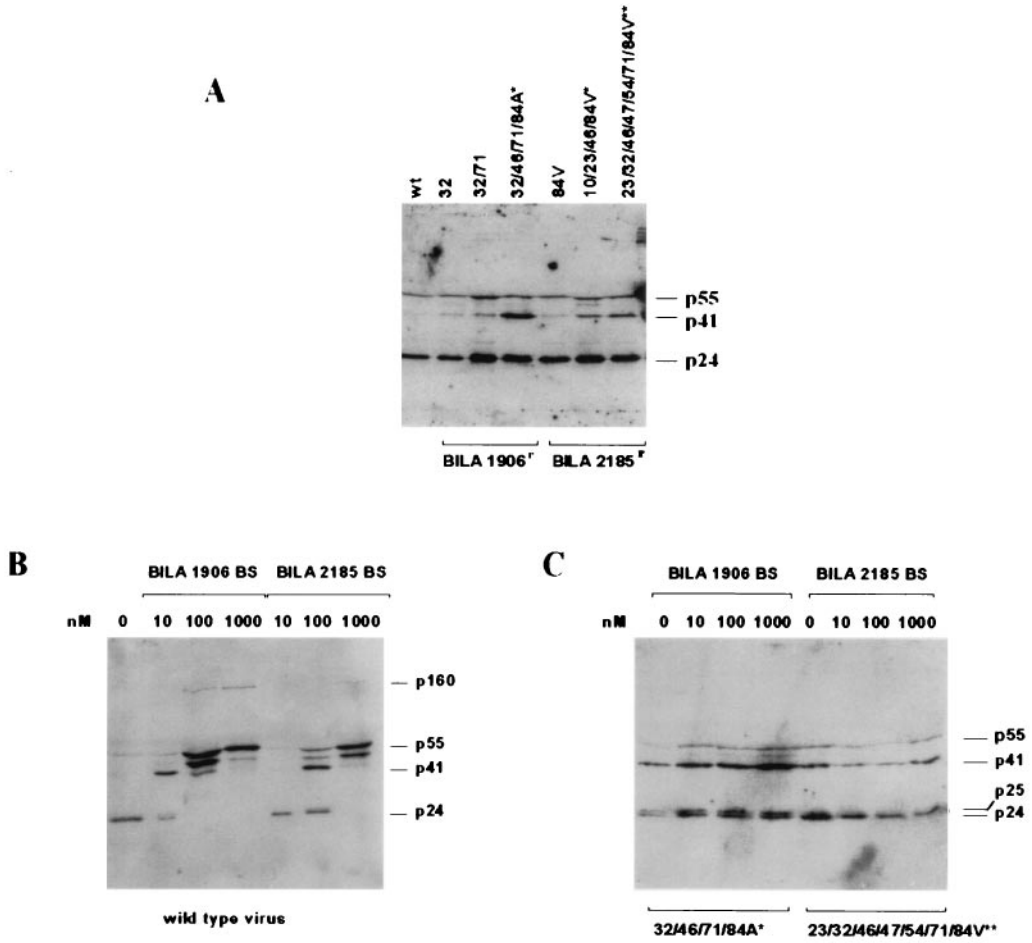

FIG. 1. Anti-p24 Western blot analysis of viral molecular clones. (A) Mutations in the protease lead to the accumulation of polypeptide precursors in viral molecular clones. Molecular clones were transfected in 293 cells in the absence of protease inhibitors, and virus was recovered by centrifugation after 3 days. Equivalent amounts of virus particles were loaded in each lane and analyzed by anti-p24 Western blotting as described in Materials and Methods. BILA 1906^r indicates clones representing BILA 1906 BS-resistant variants from different passages, while BILA 2185^r indicates BILA 2185 BS-resistant variants from different passages. (B) BILA 1906 BS and BILA 2185 BS efficiently block p24 protein maturation in wild-type molecular clones. A molecular clone containing a wild-type protease was transfected in 293 cells in the presence of 0, 10, 100, or 1,000 nM BILA 1906 BS or BILA 2185 BS. Virus was recovered after 3 days and analyzed for the presence of p24 Ag by Western blotting. (C) Molecular clones 32/46/71/84A* and 23/32/46/47/54/71/84V** were transfected in the presence of protease inhibitors and analyzed for the presence of p24 Ag as described for panel B.

magnitude lower than wild-type virus on most days. The determination of progression rates on semilogarithmic plots indeed could not distinguish the weak differences in kinetics among these three viruses (wild type, 32, and 32/71). In contrast, molecular clone 32/46/71/84A* grew significantly more slowly than the wild-type virus on PBMCs. This is represented by a significant decrease in the slope of the semilogarithmic growth curve. Again, cleavage site mutations were previously shown to improve the growth of viruses containing multiple mutations in the protease (7), therefore probably underestimating the growth deficiencies observed in this experiment. The growth of clones representing BILA 2185 BS-resistant variants was also analyzed. Mutations I84V and L10I L23I M46I I84V did not affect viral replication, as clones bearing these mutations grew as well as the wild-type virus (Fig. 2B). Clone 23/32/46/47/54/71/84V**, on the other hand, showed significant growth impairment as indicated by the considerable decrease in the slope of the semilogarithmic curve. Therefore, the HIV-1 protease can tolerate single protease active-site mutations without impairing viral growth properties, whereas multiple mutations in the active site of the enzyme considerably decrease viral growth.

In another set of experiments, resistant viruses were serially passaged on a T-cell line, C8166, to monitor for genotype stability and address the possible reversion of protease mutations in the absence of drugs. No amino acid change was observed in the protease of clones containing mutations V32I, I84V, or L23I V32I M46I I47V I54M A71V I84V** even after 7 to 10 passages (40 days) on C8166 cells. However, 50% of clones containing the mutations V32I M46I A71V I84A* showed reversion of mutation I84A to I84V after seven passages (25 days), indicating that the I84A mutation was unstable in the absence of drugs.

In a final set of experiments and to unambiguously demonstrate the growth deficiency of protease-mutated viruses, molecular clones were cocultured on C8166 cells and the genotype of outgrowing viruses was determined. C8166 cells were infected with either wild-type or mutant molecular clones, and infected cells were subsequently mixed at a 1:1 ratio (1 wild-type-infected cell to 1-mutant-infected cell). The cells were then maintained in culture for 7 to 10 passages (40 days), infecting fresh cells with supernatant at each passage (3 to 4 days per passage). Protease gene sequencing at the last passage indicated which virus was dominant and had outgrown the

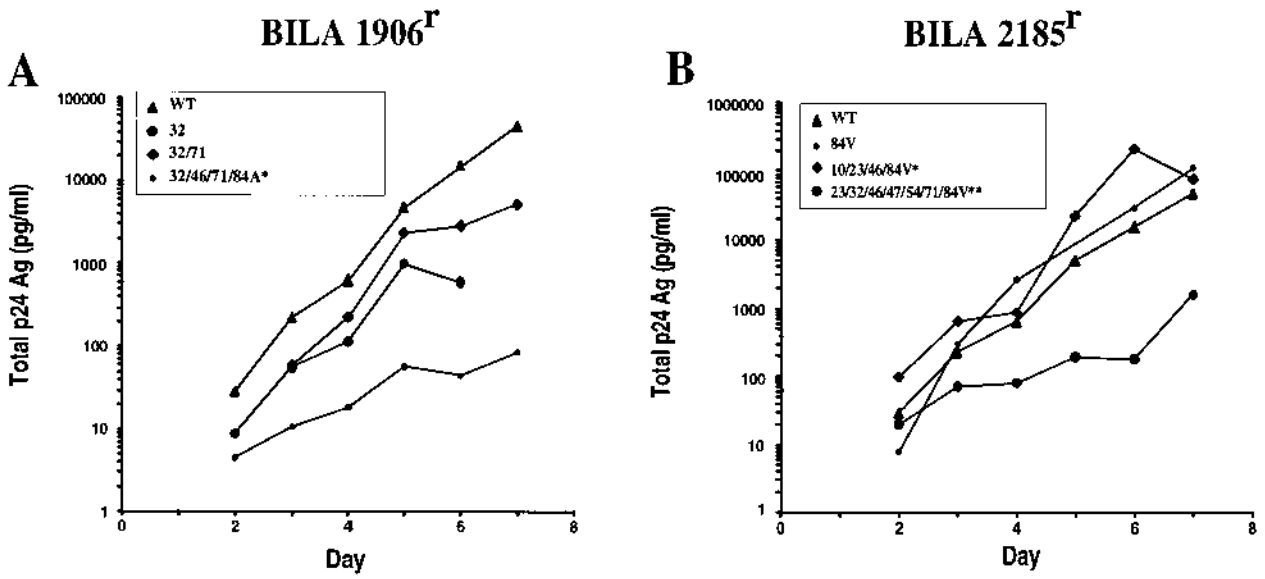

FIG. 2. Growth kinetics of protease-mutated viral molecular clones on PBMCs. Human PBMCs were infected on day 0 with 0.01 MOI of viral clones and maintained in culture for 8 days. Total p24 Ag levels were determined by quantification of p24 Ag in aliquots of supernatants on days 2 to 8. (A) Clones representing BILA 1906 BS-resistant variants. (B) Clones representing BILA 2185 BS-resistant variants. Symbols: *, clone 32/46/71/84A* also contains a L→F mutation in the P1′ position of the Gag p1/p6 cleavage site; **, clone 23/32/46/47/54/71/84V** also contains a L→F mutation in the P1′ position of the Gag p1/p6 cleavage site and QA→RV mutations in the P3 and P2 positions of the p7/p1 Gag cleavage site.

culture at this time. Table 5 shows that in WT:32 and WT:84V mixed cultures, both clones were still detected after 10 passages, confirming that clones 32 and 84V grew fairly well compared to the wild-type virus. In contrast, in WT:32/46/71/84A* and WT:23/32/46/47/54/71/84V** mixed cultures, only wild-type virus was detected after seven passages in culture. The complete disappearance of mutant clones in these cultures clearly indicates that these clones grow more slowly than wild-type virus on T cells.

DISCUSSION

By using the potent protease inhibitors BILA 1906 BS and BILA 2185 BS, HIV-1 variants showing up to 1,500-fold-increased resistance were selected. These levels of resistance come close to the levels obtained with RT inhibitors and thus compromise the widespread thinking that the protease is much less prone to resistance than the RT. BILA 1906 BS- and BILA 2185 BS-resistant variants contain up to eight mutations in the protease gene, including four in the active site of the enzyme (L23I, V32I, I47V, and I84V,A). The mutations V32I and I84V,A are active-site mutations consistently observed during selection with BILA compounds. These have been observed during in vitro selection with various other protease inhibitors (9, 21), but the combination of V32I and I84V,A seems unique to BILA compounds, as does the double-nucleotide change involved in I84A. This latter mutation is critical for high-level resistance to BILA 1906 BS and for cross-resistance to all protease inhibitors tested (3). Variants highly resistant to BILA 1906 BS and BILA 2185 BS also contain one or two mutations in the Gag precursor cleavage sites. These second-locus cleavage site mutations, however, have been shown not to contribute directly to drug resistance but, rather, to improve polyprotein processing and outgrowth of protease mutated viruses (7). Clearly, BILA 1906 BS and BILA 2185 BS select variants containing unique mutational patterns.

TABLE 5. Coculture of wild-type and mutant viruses on C8166 cells

Clone	No. of clones at passage 7 or 10[a]
WT:32	8/10 WT, 2/10 32
WT:84V	2/9 WT, 7/9 84V
WT:32/46/71/84A*	6/6 WT
WT:23/32/46/47/54/71/84V**	7/7 WT

[a] C8166 cells were separately infected with wild-type (WT) or mutant molecular clones at an MOI of 0.001 and cocultured for 7 (mixed cultures WT:32/46/71/84A* and WT:23/32/46/47/54/71/84V**) or 10 (mixed cultures WT:32 and WT:84V) passages. Results indicate the number of clones with the desired genotype over the total number of clones sequenced at that passage for that particular mixed culture.

The dramatic increase in the K_i of BILA 1906 BS and BILA 2185 BS, which shifts from 2–5 pM for wild-type protease to more than 68 nM for highly mutated enzymes, confirms that mutations in the protease confer resistance to protease inhibitors. However, protease mutations, especially those in the active site of the enzyme such as V32I and I84V,A, considerably affect normal protease function. Indeed, results from enzymatic studies suggest that the single-active-site mutations V32I and I84V reduce catalytic efficiency toward a synthetic substrate by up to 45-fold, a result already suggested by other studies (10). In virions, this is reflected by the accumulation of an uncleaved p41 precursor, which is almost undetectable in wild-type virus. In contrast, viral replication seems to tolerate single mutations, since only limited reduction, if any, in growth kinetics is observed with clone 32 or 84V (Fig. 2). The presence of four to seven mutations in the protease, however, as in clones 32/46/71/84A* and 23/46/47/54/71/84V**, has a dramatic effect on all parameters studied including growth. This is reflected by a 12,000-fold increase in K_i, by an 800- to 2,200-fold decrease in the activity of the protease towards a synthetic substrate, by the accumulation of precursors in virions, and by a 2 to 3 log unit reduction in viral growth. High-level resistance

to protease inhibitors can therefore develop in culture but resistant variants show considerable decreases in viral fitness.

In this study, proteases showing up to 2,200-fold reduction in activity still give rise to infectious particles. These results are in contrast to those from previous studies, which suggested that a 50-fold reduction in protease activity was sufficient to prevent the formation of infectious particles in vitro (34). Clones highly resistant to BILA 1906 BS and BILA 2185 BS, however, also contain cleavage site mutations, which were shown to be better substrates for the protease (7) and which may bypass a limiting step in poorly active protease function. The minimal level of protease activity required for viral replication could therefore be underestimated in the presence of such improved cleavage site substrates. By constructing chimeric viruses in which cleavage site mutations have been replaced with wild-type sequences, previous results have shown that the mutations V32I, M46I, A71V, and I84A in the protease still give rise to infectious particles (7). Therefore, in this system, the protease can tolerate a 2,200-fold reduction in catalytic efficiency and still generate infectious virus in the presence of wild-type cleavage sites. Therefore, a threshold of protease activity, in the range of 0.01 $\mu M^{-1}\ s^{-1}$, seems to be required to support viral replication, and below this point compensatory cleavage site mutations may be necessary. The contrasting results obtained in other studies (34) could be due either to the nature of the mutations in the protease or to the nature of the substrates used for the determination of enzyme activity. Taken together, results in this study suggest that HIV-1 has evolved to tolerate significant decreases in protease activity while maintaining infectivity but that, if needed, HIV-1 can acquire compensatory cleavage site mutations. These results highlight the considerable pressure that must exist to maintain viral fitness.

The fitness of inhibitor-resistant variants is an issue with obvious clinical relevance. Indeed, viral replication of reduced-fit variants may decrease the viral load and generate a lower virologic set point in patients, an outcome associated with a decreased risk of disease progression. Few studies, however, have described variants with altered growth kinetics (12, 20, 35). In this study, clones 32/46/71/84A* and 23/32/46/47/54/71/84V** have significant reduction in viral growth as determined by semilogarithmic progression analysis. Moreover, these clones are rapidly outgrown by wild-type virus in coculture experiments, confirming their growth disadvantage. The major mutational contributors to this phenotype seem to be the combination of active-site mutations V32I and I84V,A. Indeed, this combination seems necessary, as clones harboring only mutation V32I or I84V alone grow as well as the wild-type virus does. The double-nucleotide change I84A, on the other hand, must add considerable pressure on its own, as clones harboring the single mutation I84A do not give rise to infectious particles (3). The impact of this mutation on viral growth is also obvious by the rapid reversal of I84A to I84V upon removal of drugs for seven passages. The fact that the growth of viral clones is impaired compared to that of wild-type virus even in the presence of compensatory cleavage site mutations confirms that protease mutations put considerable pressure on the viral life cycle, an impact that the virus obviously has great difficulty overcoming.

Since growth is significantly affected in viruses containing multiple active-site protease mutations and second-locus cleavage site mutations, one can only wonder whether these highly resistant variants are likely to be selected in vivo. Indeed, the limited data on in vivo resistance to protease inhibitors only report on the selection of variants showing 2- to 100-fold resistance (2, 22). The requirement of a stepwise accumulation of mutations in HIV for high-level resistance, however, highlights the potential of BILA compounds to delay the emergence of resistance in vivo by suppressing the replication of early variants in the resistance pathway. As reported recently by Molla et al. (22), a higher level of ritonavir in plasma was associated with a more durable antiviral response and with a delay in the appearance of resistance mutations. If highly resistant variants do emerge in the face of high suppressive concentrations in plasma, clinical benefits may still be observed with a significant reduction in viral load.

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