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Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors

(combination therapy/BMS-186318/saquinavir/A-77003)

RONALD E. ROSE, YI-FEI GONG, JILL A. GREYTOK, CLIFFORD M. BECHTOLD, BRIAN J. TERRY, BRETT S. ROBINSON, MASUD ALAM, RICHARD J. COLONNO, AND PIN-FANG LIN*

Department of Virology, Bristol-Myers Squibb Company, 5 Research Parkway, Wallingford, CT 06492

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ABSTRACT **The observed *in vitro* and *in vivo* benefit of combination treatment with anti-human immunodeficiency virus (HIV) agents prompted us to examine the potential of resistance development when two protease inhibitors are used concurrently. Recombinant HIV-1 (NL4-3) proteases containing combined resistance mutations associated with BMS-186318 and A-77003 (or saquinavir) were either inactive or had impaired enzyme activity. Subsequent construction of HIV-1 (NL4-3) proviral clones containing the same mutations yielded viruses that were severely impaired in growth or nonviable, confirming that combination therapy may be advantageous. However, passage of BMS-186318-resistant HIV-1 (RF) in the presence of either saquinavir or SC52151, which represented sequential drug treatment, produced viable viruses resistant to both BMS-186318 and the second compound. The predominant breakthrough virus contained the G48V/A71T/V82A protease mutations. The clone-purified RF (G48V/A71T/V82A) virus, unlike the corresponding defective NL4-3 triple mutant, grew well and displayed cross-resistance to four distinct protease inhibitors. Chimeric virus and *in vitro* mutagenesis studies indicated that the RF-specific protease sequence, specifically the Ile at residue 10, enabled the NL4-3 strain with the triple mutant to grow. Our results clearly indicate that viral genetic background will play a key role in determining whether cross-resistance variants will arise.**

Current therapy for human immunodeficiency virus type 1 (HIV-1) is limited to four reverse transcriptase inhibitors (zidovudine, didanosine, zalcitabine, and stavudine). The clinical efficacy of these inhibitors has been limited by adverse side effects, inadequate viral suppression, and/or the emergence of resistant viral variants (1–3). HIV protease is a virus-encoded enzyme that is essential for processing of Gag and Gag–Pol into structural proteins and replication enzymes required for virion production (4). A number of potent HIV-1 protease inhibitors including A-77003, ABT-538, saquinavir, MK-639, SC52151, and BMS-186318 have been described (5–13). Several of these have been recently tested in human clinical trials and demonstrated significant reductions in viral load (14, 15). However, similar to results obtained with reverse transcriptase inhibitors (1), HIV variants with reduced sensitivity to these inhibitors have emerged in cell culture and in treated patients (14, 16–24). Resistance mutations have been mapped to HIV-1 protease substitutions R8Q/M46I, M46F, and V32I/V82I for A-77003, I84V/V82F for AB-538, and G48V/L90M for saquinavir (16, 19, 21–23).

We have previously isolated an HIV-1 RF variant that was 15-fold resistant to the aminodiol protease inhibitor BMS-186318, slightly cross-resistant (3-fold) to A-77003, and sensitive to saquinavir and SC52151 (25). The resistant virus contained the substitutions A71T and V82A in the protease, with the V82A mutation being the key change responsible for viral resistance to BMS-186318 (25). Combination antiviral therapies have been shown to be more effective in reducing viral replication and delaying resistance development than monotherapy (26, 27, †). Since HIV-1 protease is a relatively small protein (99 amino acids), it may have limited tolerance for multiple-resistance mutations (28) and be a more effective target for combination therapy. Studies were therefore undertaken to investigate the potential for resistance development when combination treatment involved the use of two protease inhibitors. We evaluated the compatibility of multiple protease mutations by constructing HIV-1 (NL4-3) recombinant proteases and viruses containing various combinations of BMS-186318/saquinavir- and BMS-186318/A-77003-resistance mutations. We also studied the possibility for the development of HIV-1 variants cross-resistant to multiple protease inhibitors after sequential drug treatment by passage of the BMS-186318-resistant HIV-1 RF virus in the presence of either saquinavir or SC52151.

MATERIALS AND METHODS

Cells, Viruses, and Compounds. The CEM-SS T-cell line, HeLa CD4$^+$ clone 1022, MT-2 cells, RF strain of HIV-1, and HIV-1 pNL4-3 proviral clone were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, and were contributed by P. Nara, B. Chesebro, D. Richman, R. Gallo, and M. Martin, respectively. The protease inhibitors BMS-186318, A-77003, saquinavir, SC52151, and MK-639 were synthesized at Bristol-Myers Squibb.

Purification of Mutant Proteases and Protease Assay. HIV-1 NL4-3 protease genes containing the amino acid substitutions were generated as described (25). The mutant protease genes were cloned into the pMAL-P2 expression vector and expressed in *Escherichia coli*, and the fusion protein was purified as reported (29), with the exception of allowing the combination mutant proteases to autoprocess for longer times (16–48 h) before FPLC Mono S chromatography.

Proteases were assayed as described (30). Inhibition constants were determined from reaction velocities measured at six inhibitor concentrations by using an equation for tight binding inhibitors from at least two enzyme preparations (31).

Construction of Recombinant HIV-1 Variants and Chimeric NL4-3 Proviral Clones Containing the RF Protease Genes. HIV-1 strains with defined mutations in the protease

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Abbreviation: HIV, human immunodeficiency virus.

*To whom reprint requests should be addressed.

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gene were constructed as described (25). NL4-3 proviral clones containing the wild-type or mutant RF protease genes were constructed by replacing the *Cla* I–*Xba* I fragment of pNL4-3-PXC with the appropriate *Cla* I–*Xba* I protease fragments that were PCR-amplified and cloned from the RF viruses (25).

Analysis of Gag Processing on Western Blots. To study the ability of virion mutant proteases to process the Gag polyprotein, HeLa CD4$^+$ cells were transfected with 30 μg of proviral DNA. Four days after transfection, the filtered supernatant was layered onto 20% (wt/vol) sucrose and centrifuged for 2 h at 20,000 × *g* in a Beckman SW 41 rotor. The resuspended viral pellets were then examined by SDS/PAGE and Western blot analysis using a p24 monoclonal antibody (NEA-9304; DuPont/NEN) and the ECL Western blotting detection system (Amersham). The relative amount of p24 compared to the other total processing intermediates was determined by scanning densitometry.

Growth Properties of Mutant Viruses. To study the replication kinetics and viability of mutant viruses, virus stocks were first propagated in HeLa CD4$^+$ cells by transfection with 3 μg of proviral DNA using the calcium phosphate precipitation method. Five days after transfection, growth properties of mutant viruses were evaluated by the infection of MT-2 cells with equal amounts of mutant viruses normalized by p24 antigen (DuPont/NEN) to the parental NL4-3 virus titered by a quantitative syncytia assay (32). After infection, MT-2 cells were washed and virus replication was determined by measuring the levels of p24 in culture supernatants.

Due to instability of the NL4-3 (G48V/A71T/V82A) virus, the subsequent growth kinetics studies involving this virus were determined in HeLa CD4$^+$ cells after transfection of 3 μg of proviral DNA. Virus replication was monitored by levels of p24.

Rescue of HIV-1 Protease Mutants. To ensure that noninfectivity of certain viruses was due to a defective protease gene, the mutant gene was rescued with a wild-type protease sequence by cotransfection experiments. The wild-type protease gene pRR127-1 was generated by cloning the 833-bp *Apa* I–*Pst* I fragment of pNL4-3 into pBluescript II SK+. Prior to transfection, the mutant HIV-1 pNL4-3 proviral clones were digested with *Cla* I and *Xba* I to release the protease gene (25) and pRR127-1 was digested with *Apa* I and *Pst* I to liberate the protease fragment. HeLa CD4$^+$ cells were cotransfected with 10 μg of each plasmid and supernatants from the 7-day transfected culture were then used to infect MT-2 cells. Viability of the rescued virus was monitored over 14 days after infection by determining the p24 levels and cytopathic effect.

Sequential Selection of HIV-1 Variants and DNA Sequencing. HIV-1 variants resistant to protease inhibitors were selected as described (25). Briefly, HIV-1 (RF) containing the mutations A71T/V82A was passed in the presence of increasing concentrations of saquinavir (final 1.5 μM) or SC52151 (final 2 μM). Viral stocks from the supernatants were used in drug susceptibility assays. Cell pellets were also collected at each passage for DNA sequence analysis (25).

Drug Susceptibility Assay. For drug susceptibility assays, viral stocks were titered by an infectivity assay (33). After infection, MT-2 cells were incubated with various concentrations of inhibitors for 4 days. Viral yields were quantitated by a reverse transcriptase assay (34) and the results from four experiments were used to calculate ED_{50} values.

RESULTS AND DISCUSSION

Compatibility and Resistance Phenotype of Multiple Protease Mutations. To study the interactions of multiple-resistance mutations, recombinant HIV-1 (strain NL4-3) proteases possessing A71T/V82A, R8Q/M46I, G48V/L90M, and combinations of these resistance mutations were tested in drug susceptibility assays against five distinct protease inhibitors (Table 1). The mutations responsible for resistance to a single drug were examined first for cross-resistance. The recombinant protease containing the A71T/V82A mutations was resistant to BMS-186318 (33-fold) as previously observed, but showed only 3- to 4-fold reduced sensitivity to the other inhibitors tested. In contrast, recombinant proteases containing either the R8Q/M46I or G48V/L90M mutations showed significant cross-resistance to all inhibitors except BMS-186318. Thus, the resistance profile of BMS-186318 appeared to be different from that of A-77003 and saquinavir. Addition of the A71T mutation to the R8Q/M46I or G48V/L90M double mutations had no significant effect as expected from previous studies (25). Enzymes incorporating the V82A mutation along with either the R8Q/M46I or G48V/A71T mutations displayed cross-resistance to all five inhibitors, indicating that proteases can be generated that are cross-resistant to a wide variety of inhibitors. However, the enzymes containing these particular combinations of amino acid substitutions were significantly defective in protease activity as measured by relative enzyme velocity determinations at 4 mM substrate (4-fold K_m of wild-type enzyme) (Table 1). Of particular importance was the finding that proteases containing one of the triple (G48V/L90M/V82A) and both quadruple mutations R8Q/M46I/A71T/V82A and G48V/L90M/A71T/V82A were inactive despite several purification attempts. Since the level of protein expression was very similar for each construct, the lack of enzyme activity was due to either the inability to refold and autoprocess or an intrinsic lack of

Table 1. Effect of combination resistance mutations on HIV-1 protease

Protease mutation(s)	Enzyme K_i, nM (fold increase compared to wild type)					Protease activity, % wild-type
	BMS-186318	A-77003 (Abbott)	Saquinavir (Roche)	SC52151 (Searle)	MK-639 (Merck)	
Wild type	34	1.2	0.98	2.3	0.95	100
V82A	1080 (32)	6.1 (5)	2.8 (3)	2.2 (1)	2.6 (3)	25
A71T/V82A	1140 (33)	5.0 (4)	1.5 (1)	3.4 (1)	3.3 (3)	13
R8Q/M46I	47 (1)	42 (35)	11 (11)	21 (9)	7.6 (8)	15
R8Q/M46I/A71T	43 (1)	36 (30)	13 (13)	13 (6)	5.7 (6)	12
R8Q/M46I/V82A	380 (11)	67 (55)	37 (38)	36 (16)	10 (11)	7
R8Q/M46I/A71T/V82A*						
G48V/L90M	70 (2)	25 (21)	26 (27)	73 (32)	7.0 (7)	1
G48V/L90M/A71T	64 (2)	35 (29)	21 (21)	69 (30)	4.5 (5)	16
G48V/A71T/V82A	810 (24)	65 (54)	36 (37)	65 (28)	12 (13)	6
G48V/L90M/V82A*						
G48V/L90M/A71T/V82A*						

Protease activity was measured as the relative velocity of peptide cleavage with 4 mM H_2N-VSQN-(β-napthylalanine)-PIV-COOH.
*Inactive enzyme.

protease activity. These results therefore suggested that the HIV-1 NL4-3 protease had a limited tolerance for cumulative-resistance mutations.

Gag Processing and Infectivity of Viruses Carrying Multiple Protease Mutations. To preclude that the observed reduction in enzyme activity was due to our inability to efficiently renature these mutant enzymes, we subsequently constructed HIV-1 NL4-3 proviral clones containing the same set of multiple protease changes and used them to transfect HeLa $CD4^+$ cells. Gag processing in virions released from the transfected cells was monitored by Western blot analysis and the relative amounts of p24 and processing intermediates (p25, p41, p47, and p55) were determined (Fig. 1 and Table 2). The mutants A71T, V82A, and A71T/V82A appeared to process Gag similarly to wild-type virus. All of the remaining combination mutants showed the presence of processing intermediates with an overall decrease in the proportion of p24 produced relative to total protein. These results demonstrate that recombinant HIV-1 NL4-3 viruses encoding combination protease mutations were all somewhat defective in protease activity.

The effect of these multiple mutations on the viability of virus was also monitored after infection of MT-2 cells with viral particles from transfected HeLa $CD4^+$ cells. Quantitation of p24 levels over a 14-day period after infection showed increase in p24 levels of three orders of magnitude when wild-type HIV-1 NL4-3 was utilized. In contrast, the triple mutants R8Q/M46I/A71T and G48V/V82A/L90M, and the quadruple mutant G48V/A71T/V82A/L90M failed to yield viable virus (Fig. 2 and Table 2). The same results were obtained when the R8Q/M46I/V82A and R8Q/M46I/A71T/V82A mutant proviral clones were used in a separate experiment (Table 2). The G48V/A71T/V82A mutant appeared to produce a low level (less than one order of magnitude increase in p24 levels) of virus after prolonged culture. However, genotypic analysis indicated that virus recovered at week 6 was a mixed population with reversion observed at residue G48V. To demonstrate that the impaired growth or lack of viability of HIV-1 mutant viruses was due to the specific mutations in the protease gene and not to unintended mutations elsewhere in the genome, proviral DNA representing the multiple mutants was cotransfected with a wild-type protease DNA fragment to show that infectivity could be restored (Table 2). Infectious virus as measured by p24, virus-induced cytopathic effect, and infectivity in MT-2 cultures was obtained after cotransfection, with the rescued viruses appearing to grow normally. These results confirmed that the recombinant NL4-3 viruses encoding multiple protease mutations were defective in replication solely due to their lack of proteolytic activity. These

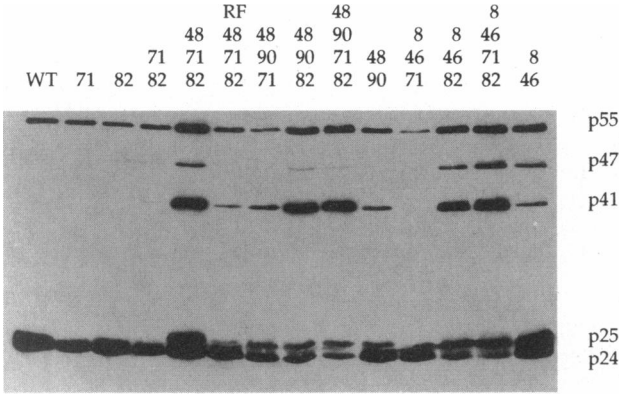

FIG. 1. Western blot analysis of Gag processing by NL4-3 viruses containing mutant proteases. Numbers above the lanes indicate specific mutations within the viral protease: WT, wild type; 71, A71T; 82, V82A; 48, G48V; 90, L90M; 8, R8Q; 46, M46I; RF, HIV-1 RF.

Table 2. Summary of Gag processing and infectivity of HIV-1 NL-4-3 mutant viruses

Genotype	% viral Gag processing	p24, ng/ml: Viral viability	p24, ng/ml: Growth rescued by wild-type protease DNA
Wild type	83	393	ND
A71T	77	ND	ND
V82A	89	361	ND
A71T/V82A	74	382	ND
R8Q/M46I	42	ND	ND
R8Q/M46I/A71T	73	1	>20
R8Q/M46I/V82A	7	2	>40
R8Q/M46I/A71T/V82A	3	1	>8
G48V/L90M	63	350	ND
G48V/L90M/A71T	55	84	ND
G48V/A71T/V82A	30	6	>40
G48V/L90M/V82A	21	1	>20
G48V/L90M/A71T/V82A	12	<1	>8

Data for Gag processing were derived from Western blot analysis followed by densitometer tracing (Fig. 1). Gag processing is expressed as the percent of p24 relative to the processing intermediates (p25, p41, p47, and p55). The data for viral viability and growth rescue were determined from culture supernatants 14 days after infection. ND, not determined.

results were quite encouraging and suggested that the proper combination of protease inhibitors might be an effective approach to treatment.

Sequential Drug Selection and Characterization of Cross-Resistant Viruses. Since the replicative dynamics of HIV-1 in patients results in a vast sequence heterogenity (15, 35) and demonstrates ability to circumvent a variety of antivirals (36), we investigated whether the virus could devise an alternative pathway to cross-resistance. A series of selection experiments were conducted by challenging BMS-186318-resistant HIV-1 RF virus containing the A71T/V82A mutations with increasing concentrations of either saquinavir or SC52151 in culture. No BMS-186318 was present during these passages and thus these experiments represented sequential treatment with two protease inhibitors. Viruses escaping the inhibitory effect of the newly added compound slowly appeared over 4 months. Drug susceptibility testing of the emerging viruses showed that HIV-1 RF viruses resistant to the second inhibitor were present after such a sequential drug treatment. Passage of HIV-1 RF (A71T/V82A) in the presence of SC52151 yielded

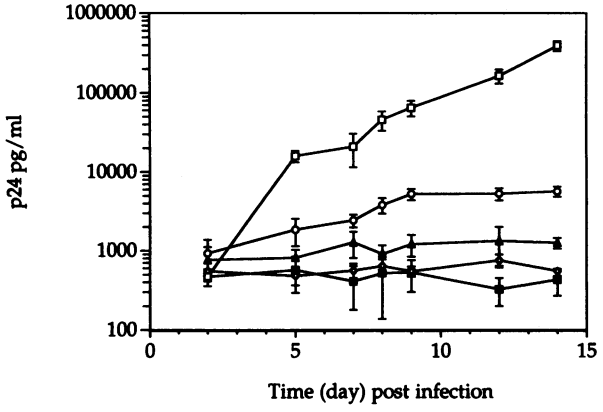

FIG. 2. Replication of protease mutant viruses after infection of MT-2 cells. The replication of NL4-3 wild type (□), NL4-3 G48V/A71T/V82A (○), NL4-3 R8Q/M46I/A71T (▲), NL4-3 G48V/V82A/L90M (◇), and G48V/A71T/V82A/L90M (■) viruses was determined by the levels of p24 in the supernatants. Error bars indicate the range of values obtained from triplicate data points.

Table 3. Cross-resistance phenotypes and protease genotypes of HIV-1 RF after sequential drug treatment

Selection		Drug sensitivity ED_{50}, μM (fold resistance)				Protease mutations	
Virus	Drug	BMS-186318	Saquinavir	SC52151	MK-639	Preexisting	Induced
WT	NA	0.097 ± 0.018	0.0064 ± 0.0008	0.077 ± 0.007	0.008 ± 0.002	NA	NA
BMS-186318 resistant	None	3.11 ± 0.09 (32)	0.013 ± 0.002 (2)	0.16 ± 0.06 (2)	0.09 ± 0.01 (11)	A71T/V82A	NA
BMS-186318 resistant	SC52151	0.53 ± 0.05 (5)	ND	2.0 ± 0.9 (26)	ND	A71T/V82A	G48V/V13I (6/12)* G48V/G16E (5/12) V13I/L23R/Q58H (1/12)
BMS-186318 resistant	Saquinavir	1.9 ± 0.08 (20)	0.11 ± 0.005 (17)	2.56 ± 0.55 (33)	0.26 ± 0.04 (33)	A71T/V82A	G48V/I54V (9/12) G48V/I54N (1/12) G48V/I54V/F53S (1/12) G48V/I54V/W42Stop G78R/G86R/G96S (1/12)

WT, wild-type HIV-1 RF; NA, not applicable; ND, not determined.
*Number of clones with this genotype out of the 12 sequenced is in parentheses.

viruses resistant to both BMS-186318 (5-fold) and SC52151 (26-fold) (Table 3). Similarly, passage of HIV-1 RF (A71T/V82A) in the presence of saquinavir produced viruses cross-resistant to both BMS-186318 (20-fold) and saquinavir (17-fold) (Table 3). Unexpectedly, this pool of virus also displayed reduced sensitivity to SC52151 (33-fold) and MK-639 (33-fold) despite the absence of these inhibitors during the selection process. Therefore, sequential treatment with BMS-186318 and saquinavir engendered virus cross-resistant to at least four protease inhibitors. A similar result was obtained in the clinic after prolonged monotherapy with MK-639 (14), in which extended treatment with MK-639 resulted in the appearance of variants cross-resistant to several protease inhibitors. Genotypic analysis of the protease genes from selected viruses (Table 3) showed that the predominant mutations induced by saquinavir and SC52151 were G48V/I54V and G48V, respectively. It was also noted that the A71T/V82A substitutions were still present despite the absence of BMS-186318 in culture for 4 months. The appearance of these cross-resistant viruses was unexpected since the NL4-3 recombinant virus containing the G48V/A71T/V82A mutations was growth-defective (Fig. 2 and Table 2).

To investigate this observation further, virus pools displaying the cross-resistant phenotype were cloned by two cycles of limiting dilution (an HIV-1 RF proviral clone is currently unavailable for *in vitro* mutagenesis) and their protease sequences were confirmed. The cloned HIV-1 RF strain containing the G48V/A71T/V82A protease displayed cross-resistance to BMS-186318 (9-fold), saquinavir (9-fold), SC52151 (25-fold), and MK-639 (12-fold) (Table 4). Similarly, the variant containing the G48V/I54V/A71T/V82A mutations was also 13-, 18-, 34-, and 13-fold cross-resistant to each of the aforementioned compounds, respectively (Table 4). These data clearly suggested that the RF, and not the NL4-3 viral background, can accommodate the G48V/A71T/V82A triple mutation and enable the resistant virus to grow. To confirm this observation, we have subsequently passaged the HIV-1 RF (A71T/V82A) and NL4-3 (A71T/V82) viruses in parallel against saquinavir and found that breakthrough virus appeared from the RF but not the NL4-3 virus (data not shown). Thus, these results imply that viral backbone plays an important role in whether a viable cross-resistant variant can arise.

Effect of Viral Genetic Background on Resistance Development. To definitely prove that viral background plays an important role in viral resistance, a chimeric proviral DNA clone containing the RF (G48V/A71T/V82A) protease was constructed within the NL4-3 backbone. Proviral DNA was then transfected into HeLa $CD4^+$ cells and the growth of the chimeric virus was compared to that of NL4-3 carrying the same triple mutation. The chimeric variant showed significant growth capability compared to the equivalent NL4-3 triple mutant (Fig. 3). Western blot analysis of Gag processing using both strains of virus showed that the NL4-3 triple mutant was more defective in Gag processing than the chimeric RF variant (Fig. 1). These results confirmed that the HIV-1 RF protease background can accommodate the presence of the G48V/A71T/V82A mutations to a greater extent than the HIV-1 NL4-3 background.

HIV-1 RF and NL4-3 proteases differ at only three amino acid residues: I10L, V13I, and K41R. We next determined whether all three substitutions were required to allow the growth of the NL4-3 triple mutant. NL4-3 (G48V/A71T/V82A) proviral clones containing each of the possible mutation subsets were constructed and transfected into HeLa $CD4^+$ cells and viral growth monitored over time using a p24 assay. Despite the inherent variation in these assays, results from three experiments consistently showed that only the proviral

Table 4. Drug sensitivity of the cloned HIV-1 RF mutant viruses

	Drug susceptibility ED_{50}, μM (fold resistance)			
Protease genotype	BMS-186318	Saquinavir	SC52151	MK-639
Wild type	0.40 ± 0.21	0.019 ± 0.007	0.044 ± 0.028	0.018 ± 0.010
G48V/A71T/V82A	3.72 ± 1.64 (9)	0.17 ± 0.09 (9)	1.11 ± 0.29 (25)	0.22 ± 0.14 (12)
Wild type	0.24 ± 0.06	0.0091 ± 0.0035	0.053 ± 0.032	0.031 ± 0.006
G48V/I54V/A71T/V82A	3.08 ± 1.32 (13)	0.16 ± 0.09 (18)	1.79 ± 0.03 (34)	0.40 ± 0.07 (13)

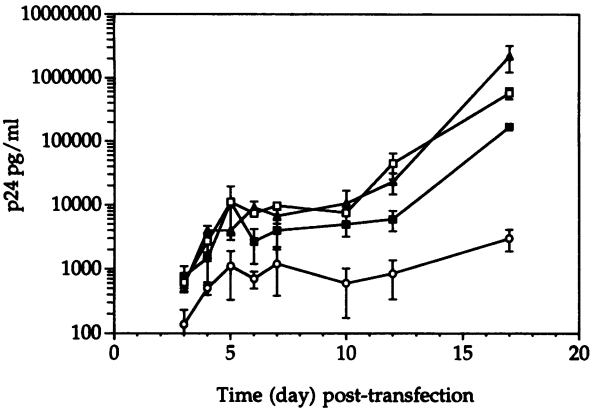

FIG. 3. Growth kinetics of protease mutant viruses after transfection of HeLa CD4$^+$ cells. The replication of NL4-3 wild type (□), NL4-3 G48V/A71T/V82A (○), NL4-3 containing the wild type HIV-1 RF protease gene (▲), and NL4-3 containing the RF G48V/A71T/V82A protease gene (■) was determined by the levels of p24 in the supernatant. Error bars indicate the range of values obtained from triplicate data points.

clones containing the L10I substitution could rescue the impaired growth of the NL4-3 (G48V/A71T/V82A) mutant and yield a virus with growth properties equivalent or superior to that of the RF (G48V/A71T/V82A) chimeric virus (Table 5). Thus the L10I is a silent mutation necessary to accommodate the triple mutation responsible for cross-resistance. Improving the growth of HIV-1 protease mutants by other silent mutations has also been noted with A-77003- and ABT-538-resistant mutants (21, 23).

Subsequent drug sensitivity studies in MT-2 cells revealed that the chimeric NL4-3 virus carrying the RF (G48V/A71T/V82A) protease gene displayed minimal levels of cross-resistance to BMS-186318 (3-fold), saquinavir (2-fold), SC52151 (4-fold), and MK-639 (3-fold); these resistance levels were noticeably less than that of the RF triple mutant in the full HIV-1 RF viral background (Table 4). These results agree with our previous observation (25) that the HIV-1 RF (A71T/V82A) virus conferred 15-fold resistance to BMS-186318, but the HIV-1 NL4-3 strain of the double-mutant was consistently less resistant (8-fold). The strain-dependent resistance expression was also demonstrated by another example in this report; the MK-639-resistant phenotype was only observed in the RF (A71T/V82A) virus (Table 3) but not in the equivalent NL4-3 virus (data not shown). It appears that other factors or HIV-1 sequences outside of the protease gene may also contribute to the resistance phenotype.

In conclusion, HIV-1 proteases containing multiple resistance mutations may exhibit a greatly reduced enzyme activity and result in either growth impaired or nonviable virus.

Table 5. Identification of amino acid residues responsible for growth-positive phenotype of NL4-3 virus containing the G48V/A71T/V82A (48/71/82) triple mutation

Virus	Additional mutation(s)	p24, pg/ml		Fold enhancement
		Day 1	Day 14	
NL4-3 (48/71/82)	None	62	15,750	—
NL4-3/RF (48/71/82)	None	49	296,150	18.8
NL4-3 (48/71/82)	10/13/41	43	108,000	6.9
NL4-3 (48/71/82)	10/13	31	105,300	6.7
NL4-3 (48/71/82)	10/41	1	674,100	42.8
NL4-3 (48/71/82)	13/41	36	6,500	0
NL4-3 (48/71/82)	10	169	397,100	25.2
NL4-3 (48/71/82)	13	92	29,350	1.9
NL4-3 (48/71/82)	41	18	4,300	0

However, other silent mutations within and outside the protease gene, which emerge frequently during HIV-1 replication, can compensate for impaired viral growth and confer viral resistance. Therefore, HIV genetic background plays an important role in determining whether HIV-1 can become resistant and able to overcome combination treatment.

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