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Effect of Raltegravir Resistance Mutations in HIV-1 Integrase on Viral Fitness

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Abstract

Raltegravir resistance is conferred by mutations at integrase codons 143, 148 and 155 together with associated secondary mutations. The N155H mutants emerge first, and are eventually replaced by Q148H mutants, usually in combination with G140S. These mutations have different effects on susceptibility and replication capacity, but data on the relative fitness of RAL-resistant viruses are limited. To understand the impact of the different RAL resistance pathways on viral fitness, mutations at IN codons 74, 92, 138, 140, 148, 155 and/or 163 were introduced into HIV-1_{NL4-3} by site-directed mutagenesis and expressed in recombinant viruses. Relative fitness and drug susceptibility were determined in the absence or presence of RAL. In the absence of drug, RAL-resistant mutants were less fit than wild-type, and the Q148H mutant was significantly less fit than the N155H mutant. Fitness was partially restored by the presence of additional RAL resistance mutations at positions G140S and E92Q or E138K, respectively. In the presence of RAL, the N155H mutant remained fitter than the Q148H mutant, but the G140S/Q148H double mutant was fitter than single mutants or the E92Q/N155H double mutant. These findings correspond well with the clinical trials data and help explain the temporal pattern of RAL resistance evolution.

INTRODUCTION

Integration into the host chromosome of the double-stranded DNA product of reverse transcription is an essential step in the life cycle of human immunodeficiency virus type 1 (HIV-1) 1–3. This process is facilitated by integrase (IN), a 32-kDa virally encoded enzyme. Following reverse transcription, HIV-1 IN, along with other viral and cellular proteins, binds to specific sequences in the long terminal repeats (LTRs) of the viral cDNA to form the pre-integration complex. IN cleaves two conserved nucleotides from the 3'-ends of both strands of the viral cDNA and, after nuclear importation, ligates the 5'-ends of the viral cDNA to the host chromosomal DNA by a process called strand transfer. Integrase strand-transfer inhibitors (INSTIs) block infection by preventing integration of viral double-stranded DNA into the host cell DNA 1–4–7.

Raltegravir is the first integrase inhibitor approved for the treatment of HIV-1 infection⁷. As with other antiretroviral drugs, resistance to RAL can emerge in the setting of incomplete viral

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suppression. Data from clinical trials show that RAL resistance involves IN mutations Y143C, Q148H or R or K or N155H^{8, 9}, together with associated secondary mutations that result in higher levels of resistance^{10–12}. These mutations are located within the catalytic core domain of IN, and have been shown to reduce viral replication capacity¹³. The N155H mutant generally emerges first, and is eventually replaced by Q148H mutants, usually in combination with G140S^{14, 15}. To date, only limited information is available on the effect of RAL resistance mutations on relative viral fitness.

To explore virologic consequences of INSTI resistance we constructed a series of recombinant viruses carrying various INSTI resistance mutations and compared their effect on viral fitness as determined by growth competitions assays in the absence and presence of RAL.

MATERIALS AND METHODS

Reagents and cells

Raltegravir tablets (Issentress, 400 mg; Merck) were purchased from the Massachusetts General Hospital pharmacy. MT-2 cells were grown in R-10 medium (RPMI 1640 [Cellgro, Herndon, Virginia] supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin [100 U/ml], and streptomycin [100 µg/ml]). The cell lines 293T, U87-CD4-X4 and TZM-HeLa CD4-LTR/β-gal, obtained from the AIDS Research and Reference Reagent Program, were propagated in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, hygromycin B (0.2 mg/ml), and geneticin (0.2 mg/ml).

Construction of HIV-1 vectors with an integrase deletion

A 3,868-bp fragment encompassing the entire coding sequence of the HIV-1 *pol* gene (corresponding to nucleotides 1970 to 5838 of the HIV-1_{NL4-3} sequence [http://hiv-web.lanl.gov]) was amplified by PCR from proviral clone pNL4-3 and cloned into pGEM-T-Easy vector to generate plasmid pGEM-Pol. Subsequently, 864 nucleotides of the IN coding sequence (corresponding to nucleotides 4230 to 5094 in HIV-1_{NL4-3}) were deleted, and a unique PmeI restriction enzyme site introduced at the deletion junction by PCR-based cloning. The *pol* gene carrying this deletion was then cloned into pNL4-3 to yield pHIVΔIN.PmeI. A segment of the *Salmonella enterica* serovar Typhimurium histidinol dehydrogenase gene¹⁶ or the green fluorescent protein gene (GFP) were then introduced into the XhoI site in *nef* to serve as sequence tags, yielding plasmids pHIVΔin.PmeInef-hisD or pHIVΔin.PmeInef-GFP, respectively.

Site-directed mutagenesis

The wild-type IN-coding region of HIV-1_{NL4-3} *pol*, along with approximately 3868 bp of flanking sequence (corresponding to nucleotides 1970 to 2253 and 5095 to 5838 of the HIV-1_{NL4-3} sequence [http://hiv-web.lanl.gov]) was cloned into the pGEM®-T-easy-vector (Promega, Madison, WI). Mutations at IN codons 74, 92, 138, 140, 148, 155 and/or 163 were introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The presence of mutant sequences was confirmed by automated sequencing of the final plasmid clones on an ABI 377 automated sequencer.

Generation of recombinant marker viruses

Infectious recombinant marker viruses carrying the desired IN mutations were generated by co-transfecting linearized pHIVΔin.PmeInef-hisD or pHIVΔin.PmeInef-GFP along with the wild-type or mutant IN gene of interest into 293T cells. The IN-coding sequence was amplified from proviral DNA of infected cells at the end of virus culture and analyzed by automated DNA sequencing to verify presence of the correct alleles at codons 74, 92, 138, 140, 148, 155

and/or 163. Homologous recombination between flanking sequences 5'- and 3'- to IN and the IN-deleted HIV plasmid results in generation of intact, fully infectious viruses.

Raltegravir susceptibility testing

The susceptibility of HIV-1 recombinants to RAL was determined by a single-cycle HIV-1 infectivity assay using TZM-bl cells. Raltegravir stock solutions were prepared by dissolving crushed tablets containing 400 mg of drug in 90 ml of water to yield a nominal concentration of 0.01 mM. The solution was passed through a 0.22 micron filter to remove insoluble excipients and to sterilize the solution. Two-fold serial dilutions of raltegravir (range, 5000 to 5 nM) in a total of 100 μ l DMEM were added to the wells of a 96-well microtiter plate. An amount of virus sufficient to produce β -galactosidase activity equivalent to 1×10^5 counts per second (CPS) was added to each well except for uninfected control wells, after which the cell suspension was added into each well (1×10^4 TZM-bl cells in 50 μ l/well); the final volume in each well was 200 μ l. After incubation at 37°C for 48 hours, β -galactosidase activity was quantified using Galacto-Light Plus (Applied Biosystems, Foster City, California) and expressed as chemiluminescence units (CPS). All infections were performed in triplicate and experiments repeated at least twice. Percent inhibition was expressed as CPS generated at various inhibitor concentrations relative to the no-drug control. Drug susceptibility was calculated by plotting the percent inhibition of virus replication (β -galactosidase activity) versus the $\log_{10}$ drug concentration to derive the IC_{50} . The drug susceptibility curves were fitted by non-linear regression using GraphPad Prism 5 (GraphPad Software, Ann Arbor, MI) ¹⁷.

Growth competition assays

The fitness of various raltegravir-resistant mutants was compared to wild-type virus and to each other in pair-wise growth competition assays as described ^{16, 18}. Briefly, recombinant marker viruses of interest carrying the hisD or GFP sequence tags were mixed together at ratios of 50:50; 80:20; or 20:80, respectively, and inoculated onto 1.5×10^6 MT-2 cells suspended in 300 μ l of R-10 medium to yield an m.o.i. of 0.001. After incubation at 37 °C for 2 hours cells were washed twice with phosphate-buffered saline (PBS), re-suspended in 10 mL of R-10 medium at a concentration of 0.15×10^6 cells/mL in 25-cm² tissue culture flasks and re-incubated ¹⁹. Cultures were passaged by inoculating 200 μ l of supernatant onto 10×10^6 fresh MT-2 cells every 3 or 4 days. The proportion of the two competing variants was estimated by quantifying GFP and hisD sequences present in culture supernatants on days 1, 4, 7, 11, and 14 using real time reverse transcriptase-coupled PCR (RT-PCR) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems; see Supplemental Information for primer and probe sequences). Viral RNA was extracted from culture supernatants using the Qiagen kit and treated with RNase-free DNase (Qiagen, Valencia, California). Parameters for the real-time PCR were as described (23), except that the initial reverse transcription reaction was performed at 50°C for 30 minutes. Quantitative real time RT-PCR was performed in triplicate for each sample. Control experiments in which two wild-type recombinants tagged with the hisD and GFP markers, respectively, were competed against each other showed no change in relative proportions of the two viruses over time, indicating that the sequence tags had no significant effect on relative fitness of the recombinants (data not shown). For each pair of viruses tested, reciprocal growth competition assays were conducted in which the IN genes of interest were linked to hisD or GFP and vice versa; in each case, similar results were obtained. Therefore, data shown represent the means $\pm$ S.D. of reciprocal experiments ¹⁸.

Estimation of viral fitness

Quantitative estimates of relative viral fitness were calculated as described ¹⁸. For each pair of recombinant viruses tested, the final ratio of the two viruses was determined by quantitative

real-time PCR at day 11 as described above. The relative fitness (w) of each virus was obtained from the average of the results of three independent growth competition assays (inoculated at ratios of 80:20, 50:50, and 20:80). The fitness difference (WD) was estimated by the ratio of the relative fitness values ($WD = WM/WL$), where WM is the more fit and WL the less fit virus in the growth competition assay. Growth competition assays that compared two wild-type recombinants carrying the *hisD* and GFP markers, respectively, gave a mean fitness difference ($\pm$ S.D.) of 1.0 ± 0.02 -fold. Therefore, we considered fitness differences greater than 1.1 to be significant.

RESULTS

Raltegravir susceptibility of IN mutants

Table 1 shows the raltegravir susceptibility of recombinant viruses carrying various mutations in HIV-1_{NL4-3} integrase. Compared to WT, the N155H mutation conferred 19-fold resistance, and the Q148H(R) (K) mutations conferred 7- to 22-fold resistance to raltegravir. The addition of secondary mutations L74M or E92Q to N155H resulted in 28- and 55-fold resistance, respectively, but addition of G163R did not result in any substantial change in raltegravir resistance. The addition of secondary mutations E138K or G140S to Q148H resulted in 36- and 245-fold raltegravir resistance, respectively.

Fitness of N155H mutants

The relative replicative fitness of recombinant viruses carrying the N155H mutation was compared to WT in growth competitions assays. In these experiments, the N155H recombinants were substantially less fit than WT in the absence of raltegravir and more fit than WT in presence of 5.0 μ M RAL (Fig. 1). In the absence of drug, the relative fitness difference for WT versus N155H was 3.4-fold, whereas in the presence of drug the N155H mutant had a relative fitness that was 2.0-fold greater than wild-type (Table 2).

To determine how the secondary RAL resistance mutations L74M, E92Q and G163R affect the fitness of N155H mutants, we compared the relative replicative fitness of the N155H mutants to that of the L74M/N155H, E92Q/N155H or N155H/G163R double mutants. Introduction of E92Q or G163R into an N155H backbone resulted in a virus with greater fitness than N155H mutant both in the absence and presence of RAL. The relative fitness difference for E92Q/N155H versus N155H was 2.8-fold in absence of drug, and 6.7-fold in the presence of RAL (Fig. 1 and Table 2). The relative fitness difference for N155H/G163R versus N155H was 3.4-fold in absence of drug, and 2.1-fold in the presence of RAL. By contrast, introduction of L74M into a N155H backbone further reduced viral fitness in the absence of drug, but resulted in increased fitness in the presence of drug. The N155H mutant had a 5.7-fold relative fitness advantage over the L74M/N155H mutant in the absence of raltegravir, whereas in the presence of RAL the L74M/N155H mutant had a 2.1-fold relative fitness advantage (Table 2). When competed against each other in the presence of raltegravir, the N155H/G163R mutant showed 2.1-fold greater fitness than the L74M/N155H mutant and the E92Q/N155H mutant in turn had a 3.7-fold relative fitness advantage over the N155H/G163R mutant (Table 2).

Fitness of the Q148H(R)(K) mutants

The relative replicative fitness of Q148H(R)(K) mutants was compared to WT in growth competitions assays. Recombinants carrying each of these mutations were substantially less fit than WT in the absence of raltegravir and more fit than WT in presence of 5.0 μ M drug (Fig. 1). The relative fitness difference for WT versus Q148H was 13.7-fold in absence of raltegravir, whereas the mutant had a 1.8-fold relative fitness advantage over WT in the presence of drug (Table 2). Similarly the Q148K and R mutants were less fit than WT in the absence of RAL and more fit than WT in the presence of drug (Table 2). To determine the

effect of the secondary raltegravir resistance mutations E138K and G140S on the fitness of the Q148H mutant, we compared the relative replicative fitness of the Q148H mutant to that of double mutants E138K/Q148H and G140S/Q148H. Introduction of either E138K or G140S into a Q148H backbone increased viral fitness both in the absence and presence of raltegravir (Fig. 1 and Table 2). In the presence of raltegravir, the E138K/Q148H had a 3.0-fold relative fitness advantage over the Q148H mutant. Similarly, the G140S/Q148H mutant had a relative fitness advantage of over Q148H. However, the E138K/Q148H mutant was less fit than G140S/Q148H in the absence and presence of drug. The relative fitness difference for G140S/Q148H versus E138K/Q148H was 3.7-fold in absence of drug, and 3.1-fold in the presence of RAL (Table 2).

Relative fitness of Q148H versus N155H mutants

To help understand why the N155H mutant usually emerges before the Q148H mutant, we compared the relative replicative fitness of recombinant viruses carrying the Q148H and N155H mutations in growth competitions assays. In these experiments, the Q148H recombinant was substantially less fit than N155H both in the absence and presence of 5.0 μ M RAL (Fig. 2). The N155H mutant had a relative fitness advantage over the Q148H mutant of 3.4-fold in absence of raltegravir, and a 2.4-fold advantage in the presence of drug (Table 2). The Q148R and Q148K mutants were fitter than the N155H mutant in absence of drug, but less fit in the presence of 5.0 μ M RAL (data not shown).

We then compared the fitness of various Q148H and N155H double-mutants to each other in pair-wise growth competition assays. In these experiments, the Q148H/G140S recombinant was substantially fitter than E92Q/N155H both in the absence and presence of raltegravir (Fig. 2). The Q148H/G140S mutant had a relative fitness advantage of 2.9-fold over the E92Q/N155H mutant in absence of drug, and 3.9-fold in the presence of 5.0 μ M raltegravir (Table 2). Similarly, the E138K/Q148H mutant was fitter than the L74M/N155H mutant both in the absence and presence of 5.0 μ M RAL (Table 2). By contrast, the E92Q/N155H mutant was fitter than the E138K/Q148H mutant both in the absence and presence of 5.0 μ M RAL (Table 2).

DISCUSSION

In this study we determined the relative fitness of INSTI-resistant mutants of HIV-1 in comparison to wild-type virus and to each other, in the presence and absence of raltegravir. The results of growth competition assays showed that in the absence of drug, the Q148H and N155H mutations, which confer raltegravir resistance, are associated with a substantial reduction in viral fitness as compared to wild-type. As expected, the mutant viruses were fitter than wild-type in the presence of drug, with the N155H mutant being more fit than the Q148H mutant. However, the G140S/Q148H double-mutant was fitter than the E92Q/N155H double mutant. These results are consistent with the patterns of raltegravir resistance observed in samples from patients with virologic failure on a raltegravir-containing regimen, and suggest that N155H mutants emerge first because they are fitter than the N148H mutants. With the addition of the G140S mutation the G140S/N148H mutants enjoy a substantial fitness advantage over other raltegravir-resistant mutants and therefore become the dominant species in the virus population^{10, 13, 20}. Our findings compliment results of another study in which the relative infectivity of various raltegravir-resistant mutants was compared to that of wild-type virus at different drug concentrations²¹. That study did not perform direct growth competition assays; however, so relative fitness differences could not be calculated.

A previous study compared the integrase-mediated replication capacity of plasma viruses from two raltegravir-treated patients using the PhenoSense IN assay¹⁰. In that study, the appearance of viruses with the Q148R or N155H mutations initially was associated with reduced

replication capacity, but replication capacity returned towards wild-type levels as Q148H and G140S mutations emerged. Clones with G140S/Q148H showed higher integrase-mediated replication capacity as compared to clones with Q148R or N155H alone; the G140S/Q148H clones also exhibited higher levels of raltegravir resistance.

Another study performed clonal analysis of raltegravir-resistant viruses obtained from patients after failure of a raltegravir-containing regimen in the phase 3 clinical trials of that drug¹³. That study showed that the N155H and Q148H(R)(K) mutations appear to be mutually exclusive. The secondary mutation E92Q occurred only in combination with N155H, whereas the G140S mutation occurred only with Q148H(R)(K). Site-directed mutants carrying N155H showed lower replication capacity than wild-type, but higher replication capacity than Q148H(R)(K). The E92Q/N155H double-mutant had a lower replication capacity than the N155H mutant, whereas the G140S/Q148H mutant showed a replication capacity that was close to that of the wild-type control. These results are generally similar to the results of our study using growth competition experiments in the absence of drug, except that the E92Q/N155H mutant showed greater fitness than the N155H single mutant. One possible explanation for these conflicting results is the difference in viral backbones used in the two sets of experiments (HIV_{IIIB} versus HIV-1_{NL4-3}, respectively). A third study found the G140A mutation in place of G140S in viruses that carried G148R substitution¹¹. It would be interesting to compare the fitness of the G140A and S mutants in combination with the different codon 148 mutants.

Reductions in relative fitness and replication capacity associated with INSTI resistance mutations are most likely due to impaired enzyme function^{2, 19, 22–28}. The N155H mutation results in reduced strand-transfer activity, whereas the E92Q mutation is associated with modest reductions in both 3'-processing and strand transfer activities of the enzyme; both activities of the enzyme are substantially reduced in the G140S/Q148H mutant^{27, 29–31}. Whether these alterations in integrase function and the associated reductions in viral fitness and replication capacity are manifested clinically by lower level viremia at the time of raltegravir failure remains uncertain, as no consistent pattern has emerged in the examples reported to date^{10, 29, 32}. Partial treatments interruption studies in which raltegravir are discontinued in patients with raltegravir-resistant virus while maintaining the background antiretroviral regimen are needed to resolve this question.

One limitation of our study is that the mutants we analyzed were constructed by site-directed mutagenesis of integrase in an HIV-1_{NL4-3} backbone. Different results might have been obtained using other viral backbones, including those of non-B subtypes, or using raltegravir-resistant clinical isolates. The close parallels between the results of the growth competition studies performed in this study and previously reported replication capacity data support the generalizability of our results. As with other in vitro studies, our study also suffers from the limitations inherent in comparing relative fitness in a system in which target cells are not limiting and in the absence of an antiviral immune response. The fitness effect of raltegravir resistance mutations could be measured in vivo by determining the rate at which these mutations disappear following discontinuation of the drug^{33, 34}.

In summary, mutations that confer resistance to the integrase strand-transfer inhibitor raltegravir are associated with reduced viral fitness when compared to wild-type virus in the absence of drug. The greater fitness of the N155H mutant as compared to Q148H in the presence of drug likely contributes to the earlier emergence of N155H mutants in the setting of raltegravir failure. The eventual predominance of the G140S/Q148H double mutant is explained in part by its greater fitness in the presence of drug as compared to other raltegravir-resistant mutants. Additional in vivo studies are needed to establish the clinical significance of these findings.

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Fig. 1.

Growth competition assays between recombinant virus carrying WT (open triangle) or N155H (open circles) (A, B), double mutations N155H/E92Q (closed triangle) (C, D), Q148H (cross) (E, F) or double-mutant G140S/Q148H (closed circles) (G,H) in the absence (A, C, E, G) and presence (B, D, F, H) of 5.0 μ M RAL. The recombinant viruses were inoculated at a ratio of 50:50. The data shown are the means $\pm$ S.D. of two independent experiments.

Fig. 2.
Growth competition assays between recombinant virus carrying Q148H (cross) versus N155H (open circles) (A, B) or G140S/Q148H (closed circles) versus E92Q/N155H (closed triangle) (C, D) in the absence (A, C) and presence (B, D) of 5.0 μ M RAL. The recombinants were inoculated at a ratio of 50:50. The data shown are the means $\pm$ S.D. of two independent experiments.

Table 1

Raltegravir susceptibility of HIV-1 recombinants carrying resistance mutations in integrase.

Mutants	IC ₅₀ ^a (μM)	Fold Change ^b
Wild Type	0.008	---
Q148H	0.093	12
Q148R	0.059	7
Q148K	0.177	22
E138K/Q148H	0.287	36
G140S/Q148H	1.96	245
N155H	0.151	19
L74M/N155H	0.222	28
E92Q/N155H	0.436	55
N155H/G163R	0.126	16

^aData shown are the means of two independent determinations.

^bFold change in raltegravir IC₅₀ compared to wild-type.

Table 2

Relative fitness of raltegravir-resistant HIV-1 recombinants

IN mutants tested in growth competition assays ^a	RAL ^b	Production of individual recombinant relative to initial inoculum (f_{t/t_0}) ^c			Relative fitness ^d	Fitness difference (fold) ^e	
		80:20	50:50	20:80			
155H mutants	WT	–	2.96	1.39	1.16	1.84	3.4
	N155H		0.57	0.63	0.40	0.54	
	WT	+	0.78	0.61	0.74	0.71	
	N155H		1.82	1.37	1.06	1.42	2.0
	E92Q/N155H	–	1.09	1.27	3.13	1.83	2.8
	N155H		0.66	0.75	0.53	0.65	
	E92Q/N155H	+	1.21	1.68	3.54	2.15	6.7
	N155H		0.19	0.36	0.42	0.32	
	N155H/G163R	–	1.15	1.40	3.08	1.88	3.4
	N155H		0.47	0.63	0.54	0.55	
	N155H/G163R	+	1.10	1.30	2.11	1.50	2.1
	N155H		0.66	0.72	0.74	0.71	
148H/R/K vs WT	L74M/N155H	–	0.45	0.21	0.46	0.37	
	N155H		1.14	1.86	3.31	2.11	5.7
	L74M/N155H	+	1.76	1.32	1.15	1.41	2.1
	N155H		0.82	0.69	0.47	0.66	
	N155H/G163R	+	2.34	1.30	1.19	1.61	2.1
	L74M/N155H		0.69	0.72	0.32	0.58	
	E92Q/N155H	+	1.74	1.83	1.17	1.58	3.7
	N155H/G163R		0.82	0.23	0.31	0.45	
	WT	–	4.10	1.87	1.19	2.39	13.7
	Q148H		0.32	0.20	0.30	0.27	
	WT	+	0.80	0.69	0.76	0.75	
	Q148H		1.76	1.29	1.06	1.37	1.8
WT	–	2.19	1.32	1.13	1.55	2.4	
Q148K		0.71	0.71	0.55	0.65		

IN mutants tested in growth competition assays ^a	RAL ^b	Production of individual recombinant relative to initial inoculum (ρ/ρ_0) ^c			Relative fitness ^d	Fitness difference (fold) ^e
		80:20	50:50	20:80		
148H mutants	WT	+	0.84	0.80	0.67	0.77
	Q148K		1.66	1.19	1.08	1.31
	WT	-	2.79	1.29	1.09	1.72
	Q148R		0.57	0.74	0.62	0.64
	WT	+	0.85	0.82	0.72	0.80
	Q148R		1.62	1.18	1.07	1.29
	E138K/Q148H	-	1.24	1.85	2.68	1.92
	Q148H		0.25	0.22	0.64	0.37
	E138K/Q148H	+	1.75	1.68	1.18	1.54
	Q148H		0.81	0.33	0.37	0.51
148H vs. 155H	G140S/Q148H	-	1.24	1.89	3.59	2.24
	Q148H		0.12	0.06	0.29	0.16
	G140S/Q148H	+	1.21	1.81	4.17	2.39
	Q148H	-	0.24	0.22	0.29	0.25
	G140S/Q148H		3.03	1.41	1.17	1.87
	E138K/Q148H		0.53	0.61	0.38	0.51
	G140S/Q148H	+	2.19	1.52	1.16	1.63
	E138K/Q148H		0.72	0.51	0.34	0.52
	N155H	-	2.82	1.45	1.15	1.81
	Q148H		0.59	0.60	0.45	0.54
148H vs. 155H mutants	N155H	+	1.97	1.46	1.11	1.52
	Q148H		0.76	0.57	0.62	0.62
	G140S/Q148H	-	2.88	1.31	1.11	1.77
	E92Q/N155H		0.58	0.70	0.58	0.62
	G140S/Q148H	+	3.08	1.30	1.22	1.87
	E92Q/N155H		0.51	0.71	0.21	0.48
	E138K/Q148H	-	3.35	1.24	1.13	1.91
	L74M/N155H		0.48	0.78	0.51	0.59

IN mutants tested in growth competition assays ^a	RAL ^b	Production of individual recombinant relative to initial inoculum (f_0/f_0) ^c			Relative fitness ^d	Fitness difference (fold) ^e
		80:20	50:50	20:80		
E138K/Q148H	+	1.98	1.34	1.11	1.48	2.3
L74M/N155H		0.74	0.66	0.53	0.64	
E92Q/N155H	-	3.13	1.43	1.15	1.90	3.7
E138K/Q148H		0.53	0.60	0.44	0.52	
E92Q/N155H	+	1.12	1.46	2.14	1.57	2.6
E138K/Q148H		0.56	0.54	0.74	0.61	

^aGrowth competition assays to compare relative fitness of the indicated pairs of HIV-1 recombinants were performed with three dual infections. Viruses were inoculated at three different ratios (80:20, 50:50, 20:80) as described Materials and Methods.

^bAssays were performed in absence (-) or presence (+) of raltegravir.

^cSingle-virus production (w_s) was determined as the amount of each virus produced from the dual infection at day 11 (f_0) divided by the proportion of that virus in the initial inoculum (i_0). Values shown represent means of at least two independent experiments.

^dRelative fitness (w) is the average of the three single virus production values (w_s) determined at each inoculum.

^eFitness difference (W_D) between HIV-1 recombinants in the growth competition assays was estimated as the ratio of the relative fitness values ($W_D = W_M/W_L$), where W_M and W_L represent the fitness of the most and least fit recombinants, respectively, in each set of pairwise growth competition assays. Growth competition assays between isogenic recombinants differing only in their sequence tags gave mean fitness differences of 1.0 ± 0.02 . Therefore, fitness differences greater than 1.1-fold were considered significant.