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Nelfinavir-Resistant, Amprenavir-Hypersusceptible Strains of Human Immunodeficiency Virus Type 1 Carrying an N88S Mutation in Protease Have Reduced Infectivity, Reduced Replication Capacity, and Reduced Fitness and Process the Gag Polyprotein Precursor Aberrantly

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The evolution of human immunodeficiency virus type 1 (HIV-1) strains with reduced susceptibility to protease inhibitors (PIs) is a major cause of PI treatment failure. A subset of subjects failing a therapy regimen containing the PI nelfinavir developed mutations at position 88 in the protease region. The N88S mutation occurring in some of these subjects induces amprenavir hypersusceptibility and a reduction of fitness and replication capacity. Here we demonstrate that substitutions L63P and V77I in protease, in combination, partially compensate for the loss of fitness, loss of replication capacity, loss of specific infectivity, and aberrant Gag processing induced by the N88S mutation. In addition, these mutations partially ablate amprenavir hypersusceptibility. Addition of mutation M46L to a strain harboring mutations L63P, V77I, and N88S resulted in a reduction of fitness and infectivity without changing Gag-processing efficiency, while amprenavir hypersusceptibility was further diminished. The ratio of reverse transcriptase activity to p24 protein was reduced in this strain compared to that in the other variants, suggesting that the M46L effect on fitness occurred through a mechanism different from a Gag-processing defect. We utilized these mutant strains to undertake a systematic comparison of indirect, single, cycle-based measures of fitness with direct, replication-based fitness assays and demonstrated that both yield consistent results. However, we observed that the magnitude of the fitness loss for one of the mutants varied depending on the assay used.

The rapid, error-prone replication of human immunodeficiency virus (HIV) allows viral populations in the host to adapt quickly to changing selective pressures. One example of this adaptability is the failure of antiretroviral therapy due to the emergence of virus populations with reduced drug susceptibility caused by well-defined mutations in the protease region. The majority of such resistance-associated mutations are rarely observed in the absence of drug selection and have therefore been hypothesized to be of lesser fitness than the predominant, drug-susceptible virus strains (15, 16, 35). Indeed, it has been possible to demonstrate an impairment of the catalytic efficiency and polyprotein precursor processing of protease (PR)-bearing resistance-associated mutations, as well as reduced replicative capacity, fitness, and infectivity of resistant virus strains (1, 3, 5, 11, 13, 18, 20–22, 26, 32, 34, 39).

Mutations at position 88 of PR cause a reduction of susceptibility to the PR inhibitors nelfinavir, BMS-232632, and SC-55389A (9, 23, 36). N88S/D mutations emerged in protease sequences derived from 20% of individuals who had failed a therapy regimen containing nelfinavir, though the two mutations occured in different sequence contexts (23). A subset of virus strains with reduced susceptibility to nelfinavir was subsequently shown to have a 3- to 12-fold increase in susceptibility to amprenavir, another PR inhibitor (40). The amprenavir hypersusceptibility was most strongly linked to the N88S mutation, which by itself resulted in a 25-fold increase in amprenavir susceptibility. L63P led to a decrease of amprenavir hypersusceptibility to 10-fold, and mutations M46L, L63P, and V77I together led to an augmentation of the nelfinavir resistance associated with N88S (Table 1) (40). The fitness of variants carrying the N88S mutation has been reported to be reduced compared to that of the wild type, as measured by growth kinetics and replication capacity in a single cycle (36, 37, 40).

In an extension of previous observations, we employed four experimental approaches to quantify the reduction of fitness (parallel growth kinetics and virus cocultures) or components of fitness (single-cycle replication capacity and specific infectivity) associated with the mutation N88S in *pro* and the ability of mutations M46L, L63P, and V77I, which can be found in association with N88S in vivo, to compensate for the fitness loss induced by N88S. This work not only extends previous observations but also provides a systematic comparison of results obtained from commonly used assays of relative fitness with assays that measure components of relative fitness, such as infectivity and replication capacity. Finally, we demonstrate that Gag processing is aberrant in strains carrying the N88S

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TABLE 1. Relative susceptibility of HIV-1 protease mutants to nelfinavir (NFV) and amprenavir (AMP)[a]

Virus strain	Relative susceptibility to:	
	NFV	AMP
NL4-3 (W)	1.00	1.00
N88S (S)	2.40	0.04
L63P/V77I/N88S (T)	12.90	0.08
M46L/L63P/V77I/N88S (Q)	12.20	0.14

[a] Data taken from reference 40.

mutation and is partially restored with additional mutations, although not with M46L.

MATERIALS AND METHODS

Construction of recombinant viral genomes. The construction of full-length recombinant genomes and retroviral vectors was described previously (40).

Single-cycle replication capacity assay. Single-cycle replication capacity was measured using a replication-incompetent HIV type 1 (HIV-1) vector in which a luciferase gene was inserted into a deleted portion of the *env* gene as described previously (25). Briefly, human embryonic kidney cell 293 cultures were cotransfected by calcium phosphate precipitation with the HIV-1 luciferase vector encoding the PR sequences of interest and either an amphotropic murine leukemia virus (MLV) *env* gene or an HIV-1 *env*-expressing DNA construct. Transfection efficiency, determined by luciferase expression in the transfected cells, displayed less than 10% variability between samples transfected on the same day. Virus particles were harvested 48 h after transfection and were used to infect target 293 cells. Luciferase activity in target cells infected with mutant strains was measured 48 to 72 h after infection and was normalized to luciferase activity expressed by cells infected with the parental strain to give relative light units or single-cycle replication capacity.

Virus stocks. For transfection, 293 cells were seeded at 4×10^5 cells/well in six-well plates. One day after seeding, the cultures were transfected with 0.8 μg of plasmid DNA using Effectene reagents (Qiagen) according to the manufacturer's instructions. Virus supernatants were collected 48 h after transfection, clarified by centrifugation for 1 min at $3{,}000 \times g$, and stored in aliquots at −80°C. To generate virus stocks for coculture experiments, virus from the transfection supernatants was passaged once through CEMx174 cells (AIDS Research and Reference Reagent Program, National Institutes of Health) to remove residual plasmid DNA. Sequences of the PR-coding domain and the CA/p2, p2/NC, NC/p1, and p1/p6 processing sites were verified after this passage by sequencing reverse transcriptase (RT)-PCR products.

RT activity assay. To quantitate virus growth, the activity of RT in the culture supernatant was measured as described earlier (2), with the following modifications. The radionucleotide incorporated into the nascent DNA strand was ^{33}P, a vacuum manifold (Bio-Rad) was used to capture the newly synthesized DNA on anion-exchange paper, and storage phosphor autoradiography (Storm 840 PhosphorImager; Molecular Devices) was used to quantitate incorporated radioactivity.

Quantitative determination of capsid protein. The amount of capsid protein (p24) in transfection supernatants was measured with an enzyme-linked immunosorbent assay using a commercially available set of reagents (New England Nuclear) according to the manufacturer's instructions.

Specific infectivity assay. Virus stocks were generated by transfection of plasmid DNA harboring full-length HIV proviral genomes in triplicate as described above. For each transfection supernatant, RT activity and the amount of capsid protein were measured as described above. Virus titers in each transfection supernatant were determined with the multinuclear activation of a galactosidase indicator (MAGI) assay (14). Specific infectivity was calculated as the number of infectious units per nanogram of capsid protein and was normalized to the parental strain. Three to six transfection supernatants were used to calculate the mean and standard deviation of specific infectivity.

Virus growth kinetics in parallel cultures. Aliquots of 6.3×10^4 infectious units (IU) of virus, as determined by the MAGI assay, were allowed to adsorb to 3×10^6 CEMx174 cells for 2 h at 37°C in a volume of 0.5 ml. Unbound virus was removed by two washes with phosphate-buffered saline, and cultures were maintained in 10 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Cells were transferred to fresh medium daily, and virus growth was measured by determining the amount of RT activity in the culture supernatants as described above and by calculating a dimensionless ratio of the amount of radioactivity incorporated into DNA by RT activity present in the culture supernatants at time t divided by the amount of radioactivity incorporated by the RT activity present in the supernatant of the same culture at the peak of virus production (RT/RT_{max}).

The exponential growth model

$$\frac{RT(t)}{RT_{\max}} = \frac{RT(0)}{RT_{\max}} \cdot e^{(r-\delta)t} = \frac{RT(0)}{RT_{\max}} \cdot e^{qt} \quad (1)$$

was fit to the data to obtain an estimate of the net exponential growth constant q (1/day). Note that q is a net rate constant that includes the rate of new infections (r) and the rate of death of productively infected cells (δ).

Virus cocultures. H9 cells (AIDS Research and Reference Reagent Program, National Institutes of Health) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Cultures were started with 3×10^6 H9 cells and 1,000 to 5,000 total IU as determined by the MAGI cell assay in a total volume of 0.5 ml. After 2 h, excess virus was removed by two washes with phosphate-buffered saline and the cultures were maintained as described in the previous section. The less fit virus strain was introduced into the culture at a 2- to 10-fold excess of IU over the fitter strain. RT activity in the culture supernatant was measured to monitor the extent of virus replication, and the ratio of the two strains was determined daily from viral RNA in the culture supernatant using RT-PCR and a heteroduplex tracking assay (HTA) (31). Briefly, viral RNA was extracted from culture supernatants using the QIAmp viral RNA kit (Qiagen), RT-PCR products spanning codons 18 to 99 of *pro* were generated and annealed to a radioactively labeled DNA probe, and the resulting DNA heteroduplexes were resolved by native polyacrylamide gel electrophoresis. The heteroduplex bands corresponding to the two virus strains in the culture were quantitated by storage phosphor autoradiography to determine the relative abundance of the two virus strains. Two different probes were used to resolve the different virus strain combinations. To resolve the mixture of parental strain with the strain containing only the N88S substitution, probe 16.12EB was used. This is a site-specific probe derived from probe 6.1 described previously (31). All other combinations of virus strains were resolved using probe 5.5/1 derived from an HIV-1 clade A virus isolate. This isolate differs from clade B consensus in 6% of the nucleotide positions of the probe, which allows the nonspecific detection of multiple mutations. These procedures are similar to previously published applications of cocultures (12, 21, 28, 30).

Computation of relative fitness from cocultures and parallel cultures. The fitness difference of two continuously replicating organisms is defined as the difference of their replication rate constants and is dependent on the absolute replication rates realized in the experimental setup. However, the fractional difference s (selection coefficient) between the replication rates is not dependent on the absolute rates. It is defined as

$$r_M = (1 + s)r_W \quad (2)$$

$$s = \frac{r_M - r_W}{r_W}$$

where r_M and r_W are replication rates of a mutant and wild-type strain, respectively, and $1 + s$ is the relative fitness or difference (n-fold) between the growth constants. One can calculate an apparent selection coefficient, s_{app}, from the net replication rates obtained from single virus cultures using equation 2

$$s_{\text{app}} = \frac{q_M - q_W}{q_W} \quad (3)$$

For the relationship between s_{app} and s, we find

$$s_{\text{app}} = s \cdot \frac{r_W}{r_W - \delta} \quad (4)$$

In the text we estimate the magnitude of the error associated with using the net replication rates as opposed to the real replication rates from equation 4. The use of net replication rates results in an overestimate of the absolute value of s.

The most general model for the determination of the selection coefficient from coculture experiments has been proposed by Marée and coworkers (19). In this model, without requiring that growth rates be constant over the course of the experiment, the selection coefficient s can be computed as

$$s = \frac{\ln \frac{H(t)}{H(0)}}{\ln \frac{W(t)}{W(0)} + \delta t} \quad (5)$$

where $W(t)/W(0)$ is the ratio of wild-type virus at time t and time 0 or the expansion (n-fold) of the wild-type virus, $H(t)/H(0)$ is the change (n-fold) of the mutant-to-wild- type ratio $H = M/W$, and δ is the rate of decay of productively infected cells ($1/\delta$ is the average life span of an infected cell). This equation was rewritten as

$$\ln \frac{H(t)}{H(0)} = s\left[\ln \frac{W(t)}{W(0)} + \delta t\right] \quad (6)$$

so that s could be determined as the slope of the best-fit line obtained by linear regression of $\ln\frac{H(t)}{H(0)}$ versus $\ln\frac{W(t)}{W(0)} + \delta t$. The expansion ($n$-fold) of the wild-type strain in coculture experiments was calculated from the increase (n-fold) of RT activity in the culture supernatant, and the abundance of the wild-type strain was determined using HTA. The ratio of the two viruses was determined by HTA.

The average life span of infected cells is unknown for the in vitro culture conditions employed here. This problem can be approached in two ways. First, δ can be ignored ($\delta = 0$), and s_{app} can be calculated. Again, this leads to an overestimate of the absolute value of s. The relationship between s_{app} and s was derived from equation 5

$$s_{app} = s\left[1 + \delta \frac{t}{\ln\left[\frac{W(t)}{W(0)}\right]}\right] \quad (7)$$

An estimate of the difference (n-fold) between s_{app} and s is given in the text. The mean of the constant $t/\ln[W(t)/W(0)]$, which was determined experimentally and used in the estimate, was 0.7 day (99% confidence interval, 0.6 to 0.8).

The model proposed by Marée et al. can be rewritten into the model proposed by Holland and coworkers (12) if it is assumed that the replication rate does not change over the course of the experiment. The assumption of a constant replication rate is valid in the exponential phase of virus growth. This model predicts that the logarithm of the mutant-to-wild-type ratio changes linearly with time t (equation 8, which follows below) and that the slope p of that line, where $p = r_W s$, represents the difference of the replication rate constants of the two viruses.

$$\ln \frac{M(t)}{W(t)} = pt + \ln \frac{M(0)}{W(0)} \quad (8)$$

Equation 8 can be used to calculate the change (n-fold) of the mutant-to-wild-type ratio per time period d (ω_d) in the coculture as

$$\omega_d = e^{rw\ sd} \quad (9)$$

The terminology of wild-type and mutant strains is arbitrary and is used here for simplicity only. The same models apply to any combination of strains.

Analysis of Gag processing. A 500-μl aliquot of transfection supernatant containing HIV-1 virions was centrifuged at 21,000 × *g* for 1.5 h in a microfuge. Pellets containing virus particles were resuspended and lysed in 50 μl of sample buffer (0.45 M Tris HCl, pH 8.45, 12% [vol/vol] glycerol, 4% [wt/vol] sodium dodecyl sulfate [SDS], 50 mM dithiothreitol, 0.0075% Coomassie blue G, and 0.0025% phenol red). Approximately equal amounts of lysed virus particles as determined by the amount of RT activity in the transfection supernatant were subjected to denaturing tricine-SDS-polyacrylamide gel electrophoresis as described previously (33). Proteins were transferred to a polyvinylidene difluoride membrane, and viral proteins were detected with either monoclonal mouse anti-p24 (Cellular Products) or polyclonal rabbit anti-p15 (provided by S. Erickson-Viitanen) antibodies using enhanced chemiluminescence (ECLplus; Amersham). ECLplus allows the detection of luminescence as well as blue-excited fluorescence (Storm 840 PhosphorImager; Molecular Devices) for quantitation. This anti-p15 antibody appears to be largely specific for NC and NC-p1, as its reactivity in Western analysis is identical to that of an anti-NC monoclonal antibody (data not shown).

RESULTS

The N88S mutation in PR reduces specific infectivity and single-cycle replication capacity. The N88S mutation in PR has been observed during failure of antiretroviral treatments containing nelfinavir (23, 40). In some viruses, N88S was associated with mutations at PR positions 46, 63, and 77, and these mutations were found to augment the reduction of nelfinavir sensitivity associated with N88S (Table 1) and partly relieve defects in single-cycle replication capacity induced by the N88S substitution (40). We examined specific infectivity and single-cycle replication capacity of four virus strains—N88S (single mutant, S), L63P/V77I/N88S (triple mutant, T), and M46L/L63P/V77I/N88S (quadruple mutant, Q)—compared to the parental NL4-3 strain (wild type, W) and compared the observed impairment of these measures with the reduction of fitness determined in virus cocultures and parallel growth kinetics experiments (see subsequent sections).

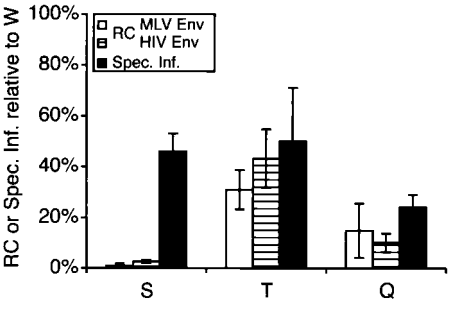

FIG. 1. Single-cycle replication capacity (RC) and specific infectivity (Spec. Inf.) of HIV-1 strains carrying N88S mutations in PR relative to the parental strain (W). Single-cycle replication capacity was measured using a retrovirus vector pseudotyped with MLV amphotropic Env protein or HIV Env protein. Specific infectivity was measured as the number of IU per nanogram of p24 capsid protein relative to the parental strain using infectious molecular clones. Note that all single-cycle-based assays showed similar results for two strains (T and Q) and that strain S showed a marked difference between the single-cycle replication capacity and specific infectivity assays. Error bars represent standard deviations. S, N88S; T, L63P/V77I/N88S; and Q, M46L/L63P/V77I/N88S.

Specific infectivity or average per-particle infectivity was measured as the number of IU (MAGI assay) per nanogram of capsid protein of a mutant relative to the parental strain in transfection supernatants generated from plasmid DNA (Fig. 1). The specific infectivity of the three mutant strains was reduced to 46% ± 7% (S; mean and standard deviation), 50% ± 21% (T), and 24% ± 5% (Q) relative to W. Strain Q appeared to be less infectious than S, while T was indistinguishable from S. We also noted that, in strain Q (adding a M46L mutation), the ratio of RT activity to capsid protein in the transfection supernatants was reduced to 68% of that found in the wild type (data not shown).

The effect of the mutations in PR on single-cycle replication capacity was determined using an HIV-1-based resistance-testing vector encoding a luciferase gene (25). Pseudotyped virus particles were generated with two different Env proteins to exclude the possibility of entry phenomena confounding measurements of PR mutants. Single-cycle replication capacity of virus strain S was reduced to 1% ± 1% ($n = 12$) and 3% ± 1% ($n = 3$) compared to that of the wild type, using MLV and HIV *env*, respectively (Fig. 1). This phenotype was partially rescued by the addition of mutations L63P and V77I in strain T, raising single-cycle replication capacity to 31% ± 8% ($n = 12$; MLV *env*) and 43% ± 12% ($n = 3$; HIV *env*). The addition of M46L

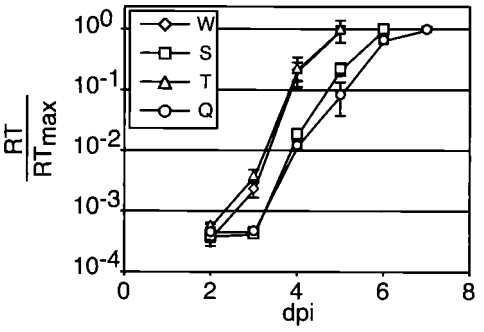

FIG. 2. Replication kinetics of HIV-1 strains carrying N88S mutations in PR. The three mutant strains and the parental strain were inoculated into three (W and T) or four (S and Q) independent cultures, and the rate of virus spread through the culture was followed by measuring the amount of RT activity in the culture supernatant. RT activities were normalized to the peak RT level (RT_{max})and averaged. Error bars represent standard deviations. W, parental strain (NL4-3); S, N88S; T, L63P/V77I/N88S; and Q, M46L/L63P/V77I/N88S. dpi, days postinfection.

resulted in an intermediate phenotype between the S and T mutants (15% ± 10%, MLV *env*, $n = 6$; 10% ± 4%, HIV *env*, $n = 3$), indicating that M46L does not act as a compensatory mutation in this assay. These results are consistent with the specific infectivity for strains T and Q, but the impairment of strain S was significantly greater in the replication capacity assay than in the specific infectivity assay. There was no statistically significant difference between measurements carried out with the different Env proteins.

Strains S and Q have reduced replication kinetics compared to the parental strain in parallel cultures. The kinetics of the accumulation of RT activity in CEMx174 cells was measured to estimate the replicative disadvantage associated with the N88S mutation alone or in combination with substitutions at positions 46, 63, and 77 during multiple rounds of replication (Fig. 2). Qualitatively, it appeared that strains W and T grew at comparable rates, whereas strains S and Q spread at lower rates. An exponential growth model was fit to the data from each culture to estimate the net growth rate constant q. This suggested that the four virus strains in order of fitness were W ($q = 2.9 \pm 0.02$ day^{-1}), T ($q = 2.7 \pm 0.1$ day^{-1}), S ($q = 2.2 \pm 0.1$ day^{-1}), and Q ($q = 2.0 \pm 0.1$ day^{-1}), though the differences between W and T and those between S and Q were small. Growth constants are given as a mean and 1 standard deviation.

The fractional difference of mutant and wild-type net growth constants was used to estimate the fitness of the mutant strains relative to that of the wild type (see equations 2 and 3) as 76 ± 3% (S), 92 ± 5% (T), and 68 ± 2% (Q) (summarized in Fig. 4). Equation 4 (Materials and Methods) was used to estimate the error associated with using net growth constants in the calculation of relative fitness. For this estimate we used previously published values for the decay rate constant of infected cells in vivo of approximately 0.5 day^{-1} (10, 24, 29, 38). Under these conditions, the absolute value of the apparent selection coefficient is a 1.2- to 1.3-fold overestimate of the true value of s, i.e., the relative fitness would be underestimated. The decay rate constant may be lower in vitro than in vivo, due to the absence of cytotoxic T cells, consistent with a report of a somewhat longer life span of infected cells observed in transformed T cells (8). A lower decay rate (longer life span) would reduce the magnitude of the error.

Strains S, T, and Q display reduced fitness relative to that of the parental strain in virus cocultures. To confirm the validity of the growth kinetics results, internally controlled virus cocultures were carried out in H9 cells. Virus coculture experiments, also termed growth competition cultures, were started at a summed multiplicity of infection of between 0.0003 and 0.002 for pairs of viruses. The cocultures were carried out with mixtures of two strains at different initial ratios with the less fit virus usually being in excess. The total amount of virus over the course of the experiment was measured using an RT assay for supernatant virus, and the ratio of the two strains in each culture was determined daily from supernatant viral RNA using RT-PCR and an HTA. The abundance of the two bands detected by HTA reflects the relative abundance of the two virus strains in the culture. Cultures representative of the three to six cultures carried out for each combination of two strains are shown in Fig. 3A to E. These results suggested that all three mutant strains had impaired replication capacity compared to that of the parental strain, that S and T were comparable in replication capacity, and that Q was marginally less fit than S.

To determine a selection coefficient analogous to the previous section, we used a modification of a model proposed by Marée and coworkers (19) (see equation 6 in Materials and Methods). Using this model, s can be determined by linear regression. Since the decay rate of productively infected cells, $\Delta\delta$, is not known precisely for our experimental conditions, we decided to calculate an apparent selection coefficient by ignoring the δt term. As in the previous section, we caution that s_{app} represents a 1.3- to 1.4-fold overestimate of s (underestimate of relative fitness) as computed from equation 7 (Materials and Methods), assuming that the average life span of infected cells is similar to that published previously for cells in vivo.

Figure 3F shows an example of the analysis of the coculture data using this model. The slope of the regression line equals s_{app}. Model fit was similar to that for the example shown in Fig. 3F for all competitions. When the results from multiple independent coculture experiments were analyzed, no significant differences of relative fitness were found between the three mutants in competition with the wild type, although the trend of W(100%) > T(83% ± 8%) > Q(79% ± 6%) > S(72% ± 8%) is suggested (Fig. 4). In direct competition, however, Q was consistently less fit than S, whereas S and T had similar fitness.

Polyprotein precursor processing is aberrant in viral strains carrying the N88S mutation. Processing of the polyprotein precursor Gag by the virally encoded PR is a required step in the formation of infectious virus particles, and processing defects are associated with reduced infectivity (39). To test if aberrant processing of Gag correlated with the observed deficiencies in infectivity, Gag processing was examined in virus particles generated after transfection of the infectious molecular clones of NL4-3 carrying the four different *pro* genes (Fig. 5). Compared to what was found for the wild type, a higher proportion of partially processed Gag intermediates was detected with both antibodies in the S strain. While the p24 capsid processing appeared to be wild-type-like in the T and Q

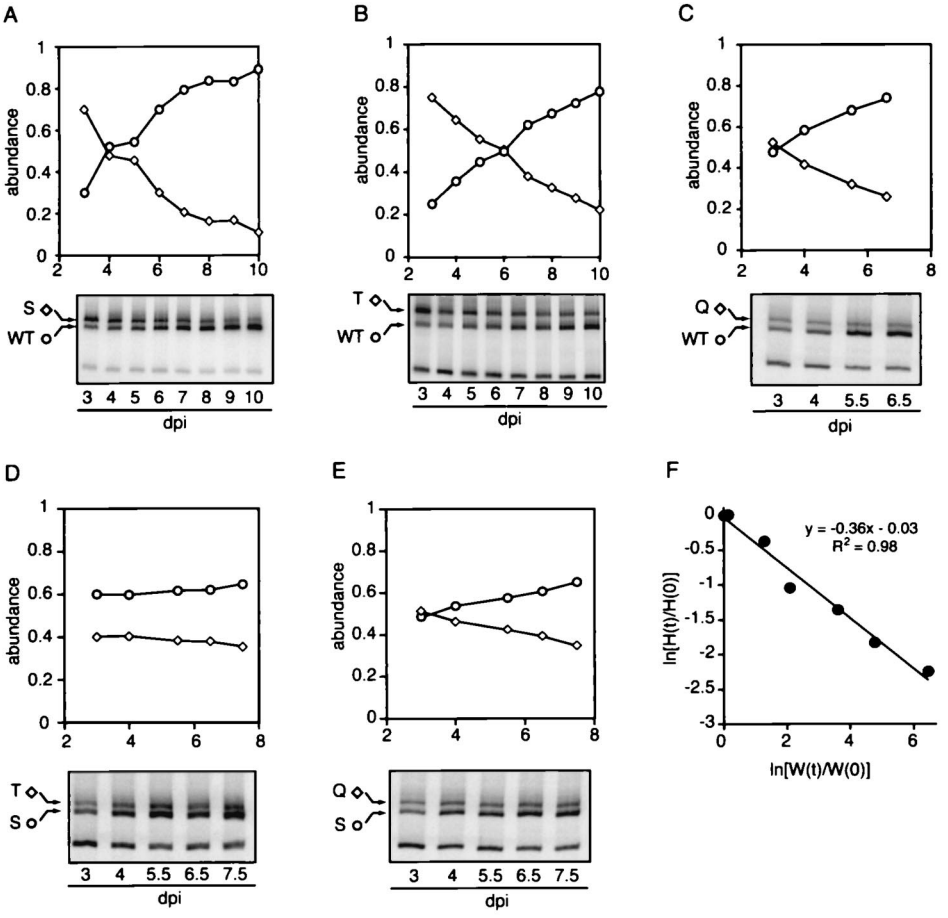

FIG. 3. Representative virus coculture experiments. Cultures were inoculated with mixtures of two virus strains at a low multiplicity of infection, and the rate of change of the ratio of the two strains was measured over time. For each combination of strains tested, the abundance of either strain over time as well as the HTA gel used to measure the abundances is shown for one representative experiment. (A) S versus W; (B) T versus W; (C) Q versus W; (D) T versus S; and (E) Q versus S. (F) Analysis of the results of an S-versus-W competition culture using a model proposed by Mareé and coworkers (19). The slope of the line represents *s*, the fractional difference of the fitness of strain S relative to strain W. W, parental strain (NL4-3); S, N88S; T, L63P/V77I/N88S; Q, M46L/L63P/V77I/N88S; dsP, double-stranded probe; dpi, days postinfection; H, M/W; WT, wild type.

strains, NC processing was only partially restored in these strains. Particularly pronounced in all three mutant strains is an apparent reduction in the efficiency of the NC-p1 site cleavage, as indicated by the detection of a protein with an apparent molecular mass of 8 kDa. The presence of the putative NC-p1 protein correlated better with reduced infectivity and fitness than did the presence of partially processed, capsid-containing Gag fragments.

DISCUSSION

The goals of this study were threefold. First, we attempted to extend previous observations of reduced replication capacity of nelfinavir-resistant and amprenavir-hypersusceptible HIV-1 strains harboring an N88S mutation and to test the ability of second-site mutations in PR to rescue the reduction of fitness. Second, we carried out various well-established measurements used to determine relative fitness of virus strains in a effort to compare qualitative and quantitative outcomes of these assays. Third, we investigated the correlation of measures of fitness with aberrant processing of the Gag polyprotein precursor.

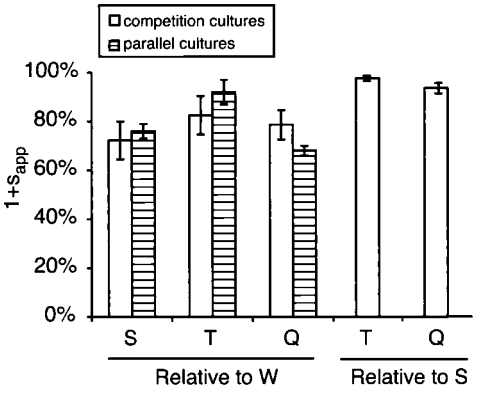

FIG. 4. Relative fitness ($1 + s$) measured by cocultures and parallel cultures. Relative fitness measured by cocultures (white bars; S versus W, $n = 3$; T versus W, $n = 6$; Q versus W, $n = 5$; T versus S, $n = 3$; Q versus S, $n = 3$) and parallel cultures is shown. Error bars represent standard deviations. Note that the results are comparable except for the smaller fitness reduction of strain T suggested by the parallel cultures. s, selection coefficient or fractional fitness difference; $1+ s_{app}$, relative fitness; W, parental strain (NL4-3); S, N88S; T, L63P/V77I/N88S; and Q, M46L/L63P/V77I/N88S.

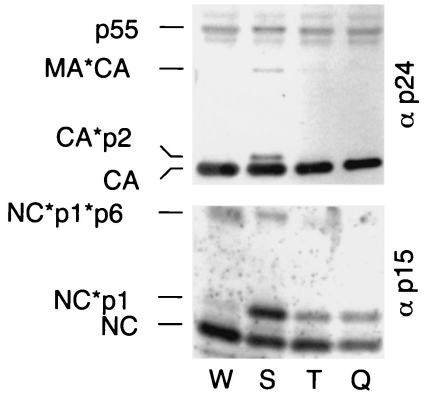

FIG. 5. Processing of Gag polyprotein precursor. Virus particles of the HIV-1 strains W, S, T, and Q were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-p15 and anti-p24 antibodies. Only the relevant parts of the gels are shown here. Incomplete processing of nucleocapsid (NC)- and capsid (C)-containing fragments was apparent in strain S. The addition of the L63P and V77I mutations in strains T and Q resulted in a compensation of the capsid-processing defect, whereas there was only a partial recovery of the nucleocapsid-processing defect in strains T and Q. W, parental strain (NL4-3); S, N88S; T, L63P/V77I/N88S; Q, M46L/L63P/V77I/N88S; and MA, matrix. All lanes contained viral proteins from the comparable numbers of virions.

In all four assays (specific infectivity, single-cycle replication capacity, parallel cultures, and virus cocultures), strain T (L63P/V77I/N88S) performed consistently better than strains S (N88S) and Q (M46L/L63P/V77I/N88S) but did worse than the parental wild-type strain, though the differences were not significant in all assays. This suggests that the mutations L63P and V77I, in combination, partially compensate for the loss of fitness caused by N88S, while at the same time increasing nelfinavir resistance from 2- to 13-fold and decreasing amprenavir hypersusceptibility from 25- to 13-fold. We hypothesize that the hypersusceptibility of strain S to amprenavir is at least partially a result of reduced fitness due to a decrease of total PR activity in the virion. This is consistent with improved processing of the NC-p1 PR cleavage site in strain T compared to strain S and with the previous observation of a general increase in catalytic efficiency of PR mediated by the L63P mutation (34). In addition, L63P has been reported to be a compensatory mutation for L90M (21). The correlation of the incomplete cleavage of the NC-p1 site with reduction of fitness is in agreement with the high frequency of substitutions at this site in PR inhibitor-resistant strains (4). However, increased amprenavir binding affinity of the mutant PRs cannot be excluded as a possible explanation for hypersusceptibility (40).

The addition of the M46L mutation to strain T to yield strain Q resulted in a decrease of fitness and infectivity in all assays without changing the pattern of processing at the NC-p1 site or further increasing nelfinavir resistance. However, the hypersusceptibility to amprenavir was reduced to sevenfold, while the ratio of RT activity to capsid protein fell to 68% of the ratio observed in the parental strain. The reduction of the RT-activity-to-p24-protein ratio is consistent with the previously reported reduction of virion-associated RT activity for other PR inhibitor-resistant strains (6). These observations suggest that the M46L mutation had a modest effect on amprenavir sensitivity and may have resulted in reduced virion incorporation of the RT-containing Gag-Pro-Pol precursor protein.

Strain Q, in direct competition, was less fit than strain S and had a lower specific infectivity and slower replication kinetics in parallel cultures and thus is the most impaired mutant in this study. The reduction in fitness associated with the inclusion of the M46L mutation did not correlate with a reduction of the NC-p1 cleavage efficiency (Fig. 3), suggesting a distinct mechanism of fitness loss for this mutation. Surprisingly, the single-cycle replication capacity measured for strain S was considerably lower (<5% of wild type) than expected, given the results of all other assays. One possible explanation for this difference may have been a reduced stability of strain S that could have led to different estimates of fitness reduction depending on the storage and treatment conditions during the experiment. However, in a preliminary analysis, we found no evidence for reduced stability of strain S compared to that of the other strains (data not shown). Alternatively, technical differences between the experiments, such as the use of different cell lines for infection, could account for the observed differences in infectivity. A difference between replication capacity and specific infectivity has been observed with several other mutant PRs (unpublished observation), indicating that this phenomenon is not restricted to the N88S class mutants. The magnitude of reduction of specific infectivity reported here for the N88S-carrying strain was similar to the reduction of PR catalytic efficiency to 20 to 40% of that found in the wild type, observed for a different mutation at position 88 linked to nelfinavir resistance (N88D) (17), and the relative fitness measurements were comparable to the 80% relative fitness observed in vivo for virus strains harboring the N88D substitution (7).

In order to compare the reduction of specific infectivity and single-cycle replication capacity to the reduction of fitness measured in cocultures during multiple rounds of infection, we reasoned that the fold change of the mutant-to-wild-type ratio in the virus cocultures per generation should be comparable to the single-cycle replication capacity and specific infectivity, if the reduction of infectivity alone is sufficient to explain the loss of fitness. The fold change of the mutant-to-wild-type ratio per generation can be calculated as $\omega = e^{r_W s\tau}$, where τ is the generation time in days and r_W the replication rate of the wild type. From our virus coculture experiments, we estimated r_W to be 2 days^{-1} (95% confidence interval, 1.8 to 2.2), assuming that the turnover rate of infected cells was approximately 0.5 day^{-1} and that the generation time τ was approximately 2 days, as has been determined previously by in vivo and in vitro experiments (8, 10, 24, 29, 38). Table 2 compares the results from single-cycle assays with relative fitness and ω. We found that the change of specific infectivity is sufficiently close to the change (*n*-fold) of the mutant-to-wild-type ratio in the competition cultures to conclude that the reduction of specific infectivity is the main cause for the reduction of viral fitness. In the set of virus strains discussed here, the correlation between the two approaches was sufficient to suggest the use of the significantly faster and more reproducible single-cycle-based assays as a surrogate for fitness during multiple cycles of infection for mutants in which the reduction of fitness is largely caused by a

TABLE 2. Comparison of different measures of fitness[a]

Strain	Single-cycle replication capacity mean % (SD)	Specific infectivity mean % (SD)	% Relative fitness: $1 + s_{app}$ [$(1 + s_{app})$ (100%)]		$w(100\%)^b$ range
			Parallel culture mean (SD)	coculture mean (SD)	
S	3 (1)	46 (7)	76 (3)	72 (8)	29–37
T	43 (12)	50 (20)	92 (5)	83 (8)	46–53
Q	10 (4)	24 (5)	68 (2)	79 (6)	39–53

[a] All values given relative to wild type.
[b] Expected change of mutant-to-wild-type ratio per generation in cocultures.

reduction of specific infectivity or replication capacity. Similar concordance of single-cycle assays and virus culture assays has been observed by others (27). However, this correlation will need to be extended to other classes of PR mutants to understand more fully the differences between the single-cycle replication capacity assay and the specific infectivity assay and the impact of different mechanism of fitness loss and compensation on these assays. This is especially important in the light of the discordance between one of the single-cycle assays and all other assays for one of the mutant strains.

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