Human Immunodeficiency Virus Type 1 Protease Inhibitor Drug-Resistant Mutants Give Discordant Results When Compared in Single-Cycle and Multiple-Cycle Fitness Assays[▽]

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The replication fitness of HIV-1 drug-resistant mutants has been measured using either multiple-cycle or single-cycle assays (MCAs or SCAs); these assays have not been systematically compared. We developed an MCA and an SCA that utilized either intact or *env*-deleted recombinant viral vectors, respectively, in which virus-infected cells were detected by flow cytometry of a reporter gene product. Fitness was measured using each assay for 11 protease mutants, 9 reverse transcriptase mutants, and two mutants with mutations in *gag* p6, which is important for the release of virus particles from the cell membrane. In the SCA, fitness (replication capacity [RC]) was defined as the proportion of cells infected by the mutant compared to the wild type 40 h after infection. MCA fitness (1+s) was determined by comparing the changes in the relative proportions of cells infected by the mutant and the wild type between 3 and 5 days after infection. Five protease mutants showed statistically different fitness values by the MCA versus the SCA: the D30N, G48V, I50V, I54L, and I54M mutants. When all the mutants were ranked in order from most to least fit for both assays, 4 protease mutants moved more than 5 positions in rank: the D30N, I54L, I54M, and V82A mutants. There were no significant differences in fitness for the *gag* p6 or reverse transcriptase mutants. We propose that discordant results in the MCA and SCA are due to alterations in late events in the virus life cycle that are not captured in an SCA, such as burst size, cell-to-cell transmission, or infected-cell life span.

Fitness, as defined in population biology, is the ability of a variant to contribute to successive generations (reviewed in reference 16). The more fit a variant, the more likely it is to contribute its genotype to its offspring. Human immunodeficiency virus type 1 (HIV-1) occurs as a quasispecies in patients, in which many genetically related but distinctly different variants exist together as an evolving group (17). It has been hypothesized that the most dominant variant in a patient is that which is most fit under current selective pressures (11). When selective pressures change, for example, by the introduction of drug therapy or through action by the immune system, the most dominant variant will change. There are phenomena in patients in which fitness might play a role, such as the likelihood that a variant will be transmitted and how quickly the disease progresses.

The role of fitness in the development of drug resistance, transmission, and disease progression has been studied using many types of fitness assays. These studies have found that drug-resistant variants replicate less efficiently than their drug-sensitive counterparts (4, 13, 15, 24, 32, 36, 39, 48) and that fitness is one factor that determines the relative prevalence of drug-resistant variants in a patient failing drug therapy. Studies

of the role of fitness in disease progression and transmission have been performed using both single-cycle and multiple-cycle assays (SCAs and MCAs) and have yielded conflicting results (42). Therefore, it is still unclear what role fitness may play in pathogenesis and what impact the assay itself has on the result.

In order to understand the role of fitness in HIV-1 pathogenesis, more information is needed on how well different cell culture fitness assays correlate. Five different characteristics have been used to describe such fitness assays: whole virus versus recombinant virus, growth competitions versus parallel infections, direct measurement of a viral gene product versus a reporter gene, primary cells versus a cell line, and a single cycle versus multiple cycles (19). MCAs are performed with whole virus or recombinant vectors that have all the genes in the genome that are essential for growth in culture and can undergo multiple rounds of replication over the course of the experiment (5, 21, 27, 31). SCAs are performed with a recombinant vector in which in an essential gene, usually envelope, is deleted and which can undergo only a single round of replication (51). SCAs have the advantage of being performed in a shorter time frame, usually 48 h, and therefore have a higher throughput, than MCAs, which take days to weeks to complete. However, it is not known whether mutants of HIV-1 have the same relative fitness when compared against the wild type (WT) in a multiple-cycle assay and in a single-cycle assay. We hypothesized that the SCA would detect abnormalities in virus entry, reverse transcription, integration, and protein expression but would not reflect abnormalities in late stages of

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FIG. 1. HIV-1 vectors pDAT1, used for the SCA (A), and pAT1, used for the MCA (B). The open reading frames for *gag*, *pol*, the accessory genes, *Thy1* markers, and long terminal repeats are shown, as are the bacterial pUC19 sequences (pUC) and the 5' cellular flanking sequence. Vertical lines indicate the locations of restriction enzymes. A black triangle indicates the location of the deletion in the envelope in the pDAT vector used for the SCA.

the virus replication cycle, such as burst size, cell-to-cell transmission, or infected-cell life span. In contrast, an MCA should reflect all the steps in the virus life cycle. In addition, small differences in fitness may be amplified over several rounds of replication, resulting in a larger fitness deficit in the MCA than in the SCA.

In order to determine whether the use of an MCA versus an SCA results in different relative fitness values, we developed an SCA analogous to a flow cytometry-based MCA that has been described previously (21) and compared the two assays using a panel of HIV-1 mutants resistant to protease and reverse transcriptase (RT) inhibitors.

MATERIALS AND METHODS

Reagents. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: SV-A-MLV-env, from Nathaniel Landau and Dan Littman (29); the PM1 cell line, a clonal derivative of HUT 78 that is permissive for both macrophage-tropic and lymphocyte-tropic strains of HIV-1, from Marvin Reitz (33); and the MT-4 T cell line, from Douglas Richman (26, 30). The 293 and COS-1 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Plasmid pHCMV-g expresses the vesicular stomatitis virus g protein (VSV-g) under the control of the human cytomegalovirus promoter (18, 50). Fetal bovine serum (FBS) was obtained from Valley Biomedical (Winchester, VA). Restriction enzymes were obtained from either New England Biolabs (Beverly, MA) or MBI Fermentas (Hanover, MD). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat Thy1.1 (HIS51) and R-phycoerythrin (PE)-conjugated rat anti-mouse Thy1.2 (30-H12) monoclonal antibodies were obtained from BD Biosciences (San Jose, CA).

Cell culture. PM1 and MT-4 cells were cultured in the presence of RPMI medium (Cellgro, Herndon, VA) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 U/ml). 293 and COS-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). Primary human peripheral blood mononuclear cells (PBMCs) isolated from HIV-negative donors were purified by Ficoll-Hypaque density centrifugation, stimulated for 2 days with 5 μ g/ml phytohemagglutinin P (PHA-P; Sigma-Aldrich) and 5% interleukin-2 (IL-2), and cultured in RPMI 1640 supplemented with 20% (vol/vol) FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and 5% IL-2. All cells were propagated under 5% CO₂ at 37°C.

Construction of the pDAT1 and pDAT2 HIV-1 vectors. The construction of the pAT1 and pAT2 vectors has been described previously (21). Briefly, the pAT1 and pAT2 constructs are identical to NL4-3 except that they contain the mouse *Thy1.1* or *Thy1.2* gene, respectively, in place of *nef*, have silent *Xma*I and *Xba*I restriction sites that flank RT to facilitate the cloning of patient RT sequences, and have the *Xba*I site removed from between the 3' long terminal repeat (LTR) and the pUC19 plasmid sequence. pDHIV3.Thy1.1 is derived from pNL4-3 and

has a 700-bp deletion in *env* and the mouse *Thy1.1* gene cloned in place of *nef* (18). *env*-deleted versions of pAT1 and pAT2 were made by replacing the *Sal*I/*Bst*EII fragment spanning *env* with the same fragment from pDHIV3.Thy1.1 to produce pDAT1 and pDAT2, respectively (Fig. 1).

Introduction of drug resistance mutations. Mutations in protease and RT were introduced into pRHAXX (20) using the QuikChange II mutagenesis kit (Stratagene). pRHAXX was made previously by introducing silent *Xma*I and *Xba*I restriction sites into pRHA, which was created by subcloning the *pol* gene of pNL4-3 into the phagemid vector pTZ18U (Bio-Rad, Hercules, CA) by using *Sph*I and *Eco*RI restriction endonuclease sites (20, 24). The generation of pAT2 containing either the L90M, D30N, K103N, V106A, G190S, or P236L mutant has been described previously (21). Other mutants used in this study were generated using pRHAXX and the primers given in parentheses (the sense version of the primer set is shown) as follows: the protease L33F (5'-GGAGCAGATGATAGTATTCGAAGAAATGAATTTGCCA), G48V (5'-AAACCAAAAAATGATAGTGGGAATTGGAGGTTT), I50L (5'-AAAAATGATAGGAGGAGTGGAGGAGGTTTATCAAAA), I50V (5'-CCAAAAATGATAGGAGGAGTGGAGGTTTATCAAAAGTA), I54L (5'-GGGGGAATTGGAGGTTTCTCAAAGTAAAGACAGTATGAT), I54M (5'-GGGGGAATTGGAGGTTTATGAAAGTAAGACAGTATGAT), V82A (5'-GTAGGACCTACACCTGCCAACATAATGGGAAGAAAT), V82T (5'-TTAGTAGGACCTACACCTACCAACATAATGGGAAGAAATCTG), and I84V (5'-CTACACCTGTCAACATAATTGGAAAGAAATCTG) mutants; the RT K65R (5'-GTATTTGCCATAAAGAGAAAAAGACAGTAAATGG), K70R (5'-GAAAAAAGACAGTGGGATGGGAAGAAATAGTAG), Q151L (5'-CCACTGGGATGGAAAGGATCACCAGCAATATTC) (used to generate the template for Q151M), Q151M (5'-CCAAATGGGATGGAAAGGATCACCAGCAATATTC), M184V (5'-CATCTATCAATACGTGGATGATTTGTATGTAGG), and T215Y (5'-TGAGGTGGGGATTTTACACACAGACAAAAAAC) mutants; and the *Gag* p6 PTAPAPP (5'-CCAGAGCCAACAGCCCCACCAGCCCCACCAGAAGAGAGC) and PTAPDUP (5'-CCAGAGCCAACAGCCCCACCAGGCCAACAGCCCCACCAGAAGAGAGC) constructs.

The antisense primers used for mutagenesis were the reverse and complement of each sense primer. The resultant clones were sequenced to confirm the presence of the mutation and the absence of spurious mutations. Protease inhibitor resistance mutations and p6 proline-threonine-alanine-proline (PTAP) insertion mutations were introduced into pAT2 and pDAT2 by subcloning the *Apal*-*Xma*I fragment from the mutant pRHAXX constructs, and RT mutations were introduced by subcloning the *Xma*I-*Age*I fragment from the mutant pRHAXX constructs. All mutant pDAT2 and pAT2 clones were sequenced to verify the presence of the mutation and the integrity of the cloning sites.

Generation of virus stocks. pAT1WT and pAT2 mutant virus stocks were made by transfecting 293 cells as described previously (21). Five micrograms of pDAT1WT or pDAT2 mutant constructs was used to transiently transfect 1×10^6 COS-1 or 4×10^6 293 cells seeded in 10-cm-diameter plates the day before, along with 5 μ g of pHCMV-g (expressing the VSV-g envelope) or pSV-A-MLV-ENV (expressing the murine leukemia virus [MLV] envelope), by using 25 μ l of Lipofectamine LTX (Invitrogen). DNA and Lipofectamine LTX were incubated for 30 min in 2 ml of Opti-MEM (Invitrogen). Eight milliliters of DMEM containing 10% FBS and Pen/Strep were added to each transfection mixture

before it was added to the COS-1 cells. Supernatants were harvested 72 h later and were clarified by centrifugation at $400 \times g$. HIV-1 capsid protein (p24) quantitation was performed on virus stocks using an HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) (Perkin-Elmer, Norwalk, CT), and 25 to 100 ng of each virus stock was used per infection. Virus stocks were tested in triplicate on 96-well plates, and competing stocks were tested in the same assay.

VSV-g protein quantitation. VSV-g protein quantitation was performed by ELISA using a horseradish peroxidase-conjugated anti-VSV-g antibody (Abcam, Cambridge, MA). DAT virus stocks were tested in triplicate on 96-well plates, and competing stocks were tested on the same assay. Virus stocks were diluted 1:2 with phosphate-buffered saline (PBS), and 200 μ l was incubated with 20 μ l of 5% Triton X-100 and 0.02% sodium azide in PBS. Stocks were incubated for 2 h at 37°C and were then washed twice with wash solution (0.2% Tween 20 in PBS). Blocking solution (2% bovine serum albumin [BSA] and 0.2% Tween 20 in PBS) was then added, and the mixture was incubated for 90 min at room temperature. The plates were washed twice, and 100 μ l of anti-VSV-g antibody diluted 1:500 in blocking solution was added for 2 h at room temperature. The plates were washed 4 times, and 100 μ l of tetramethylbenzidine (TMB) substrate (Pierce) was incubated for 1 h at room temperature in the dark. The reaction was stopped by the addition of 100 μ l of 2 M sulfuric acid, and absorbance was measured at 450 nm. A single wild-type virus stock was used as a control for each assay. The ratio of the optical density (OD) of the test stock to that of the control stock was used to compare the input inoculum for the SCA to the inoculum calculated using the p24 content and was used to determine the input inoculum relative to wild-type virus in some SCAs.

Multiple-cycle and single-cycle growth competition assays. The MCAs in PM1 cells were performed as previously described (21). Briefly, 7 million PM1 cells were coinfecting with equal amounts of wild-type and mutant viruses as determined by p24 capsid protein quantitation. At days 3, 4, 5, and 6, half the culture was removed and replaced with fresh medium. The number of viable cells was determined by trypan blue staining. One-half million cells were stained with the anti-Thy1.1 or anti-Thy1.2 antibody alone, or with the anti-Thy1.1 and anti-Thy1.2 antibodies together, diluted 1:100 and 1:200, respectively, in fluorescence-activated cell sorter (FACS) buffer (PBS with 0.02% FBS, 0.02% sodium azide, and 0.5 mM EDTA). The MCA performed in other cell types was the same as that in PM1 cells except for the virus inoculum and cell number (5 ng p24 of each virus infected 14 million MT4 cells subcultured at 200,000 cells/ml, and 70 ng p24 of each virus infected 10 million human PBMCs, which were subcultured at 2 million cells/ml). These inocula were determined empirically so as to give approximately 0.5% infected cells 3 days after infection.

For the SCA, 1 million PM1 cells were coinfecting with 100 ng p24 (or, in some cases, 1.0 OD unit of VSV-g) each of WT and mutant virus. Cells were incubated with virus at 37°C in a total volume of 0.8 ml for 1 h; then they were washed with PBS, centrifuged at $500 \times g$, and seeded at 200,000 cells/ml in the appropriate medium. At 40 h, trypan blue staining was used to determine the viable cell count. At this time point, 0.5 million cells were also stained with the anti-Thy1.1 and anti-Thy1.2 antibodies and were analyzed by FACS using the same methods as those for the MCA (21).

Fitness calculation and statistical analysis. For the MCA, the 1+s value was determined using the method of Wu et al. (49). A fitness calculator, available at <http://bis.urmc.rochester.edu/vFitness/>, was used to obtain the average 1+s value by using the numbers of infected cells from days 3 to 5. For the SCA, fitness was calculated using the following equation: replication capacity (RC) = (% mutant-infected cells)/(% WT-infected cells). Cells that were infected with both mutant and WT viruses were included in both the numerator and the denominator. For assay and stock variability studies, the absolute deviation of each observation from the sample median (robust measure of variability) was constructed. Two-way analysis of variance (ANOVA) was performed to test for variability differences in mean deviations between stock preparations. Two-way ANOVA tests were also performed to account for any differences between mutants in testing for relative fitness value differences by assay type or cell line. Significant effects were followed with Wilcoxon two-sample exact tests due to the small sample sizes. All reported *P* values are Bonferroni adjusted for multiple comparisons within each experiment, maintaining an overall experiment-wide alpha value of 0.05. Log transformations were taken to normalize relative fitness value (RFV) measurements for ANOVA tests. Analyses were performed using SAS, version 9.2 (SAS Institute, Cary, NC).

RESULTS

Assay design. We designed a single-cycle fitness assay that can differentiate wild-type from mutant virus in the same cul-

FIG. 2. Experimental design of the SCA. Circular versions of the constructs from Fig. 1 are shown with the wild type in black and the mutant in gray. COS-1 or 293 cells were cotransfected with pCMV-g or pSV-A-MLV-ENV and either wild-type pDAT1 (pDAT1WT) or mutant pDAT2 (pDAT2MUT). Wild-type (black) and mutant (gray) virus stocks are represented by hexagons. The titers of virus stocks were determined by measuring the p24 or VSV-g protein content. PM1 cells were coinfecting with equal amounts of wild-type and mutant viruses. After 40 h, cells were harvested, and a subset of cells was stained with either an anti-Thy1.1 antibody, an anti-Thy1.2 antibody, or both. Four different types of cells can be observed during analysis: uninfected cells, cells infected with wild-type virus only, cells infected with mutant virus only, and cells infected with both viruses (dually infected).

ture and that is otherwise the same as a previously described MCA (21). Both the wild-type MCA and SCA vectors have the mouse Thy1.1 gene cloned in place of *nef* and are identical to each other except that the SCA vector pDAT has a 570-bp deletion in the *env* gene (Fig. 1). Virus derived from the pDAT vectors was pseudotyped with either VSV-g or MLV *env*. Site-directed mutants were cloned into the Thy1.2 versions of the pAT and pDAT constructs, and independent virus stocks were produced by transfection. Cells were coinfecting with wild-type and mutant viruses, and the proportion of cells infected with wild-type or mutant virus was determined by flow cytometry (Fig. 2). Each culture was stained with each Thy antibody individually; one aliquot was also stained with both antibodies. Thresholds for Thy1.1 and Thy1.2 positivity were determined by using the heterologous anti-Thy antibody as a control on the singly stained samples; one blinded observer set all gates. We compared the replication of mutant and wild-type reference viruses using the dually stained sample and compared the RC value for each mutant as determined by the SCA with the 1+s value determined by the MCA.

Assay variability. We have previously observed that for the MCA there is more variability between cultures than there is between virus stocks (data not shown). Since SCA stocks are made by cotransfection of an envelope vector and the parental

FIG. 3. Assay and stock variability. For assay variability, single VSV-g/DAT2G190S and VSV-g/DAT2K103N stocks were tested against a single WT stock in 6 independent assays set up on different days. For stock variability, nine independently transfected G190S or K103N stocks were tested against a single WT stock by the SCA. Four independently transfected G190S or K103N stocks were tested on different days, each in triplicate, against the same WT stock for the multiple-cycle assays. The relative input used for each stock was determined by the p24 protein content. The relative fitness value for either the SCA (RC) or the MCA (1+s) is shown along the y axis. Each box indicates the upper and lower quartiles; the solid line within the box represents the mean; and the whiskers show the range.

vector, we wanted to determine if the assay-to-assay variability was higher than the stock-to-stock variability. The assay-to-assay variability of the SCA was determined by measuring the fitness of a VSV-g-pseudotyped stock derived from a single transfection in six independent cultures set up on six different days. Each culture contained a wild-type reference derived from a single transfection. The stock-to-stock variability using VSV-g- or MLV-pseudotyped virus was determined by measuring the RCs of nine pseudotyped stocks (three independent stocks made on each of three different days). Each of these infections contained a wild-type reference derived from a single transfection. The G190S mutant, a reverse transcriptase mutant known to have reduced fitness in this MCA, and the K103N mutant, a reverse transcriptase mutant with preserved fitness in this MCA (21), were used for this comparison. We compared the mean absolute deviations (measure of variability) of the RCs generated from the VSV-g assay-to-assay variability tests with those of the RCs generated from the VSV-g stock-to-stock variability tests. Overall, there was significantly less assay variability (P , 0.0437; two-way ANOVA main effect) than stock variability. This comparison was the same for the G190S and K103N mutants (P , 0.1038).

Because MLV and VSV-g envelope proteins are commonly used to pseudotype HIV-1 (2, 3, 42, 43, 45), we compared the variability in RC values generated by viruses pseudotyped with these two envelopes and also compared the RC values to the 1+s values derived from the MCA. The stock-to-stock variability of the MCA was evaluated with four stocks, each made on a different day (Fig. 3). The mean RC for the K103N mutant was not significantly different when either MLV or

VSV-g was used as the envelope (P , 0.4961). The mean RC for the G190S mutant was somewhat higher when MLV was used as an envelope (0.63 versus 0.38; P , 0.01); however, this did not hold true for the other mutants tested (the D30N, L90M, and V106A mutants [data not shown]). In order to determine if there was more variability between the SCA and the MCA, we compared the mean absolute deviations of the SCA MLV and SCA VSV-g data for the K103N or G190S mutant with the MCA data for the K103N or G190S mutant. Overall, there was a marginally significant (P , 0.0501) difference in the variability (mean deviations) between stock preparations. Further posthoc comparison tests showed no significant differences in variability between any of the stock preparations (P , 0.9363) for the G190S mutant. For the K103N mutant, there was a significant difference in mean variability (P , 0.0405) when all 3 stock preparations were compared; however, any pairwise comparison of stock preparations was statistically nonsignificant (P , >0.05). These results suggest, particularly for the K103N mutant, more variability in the VSV-g stocks than in the VSV-g assays and more variability in the VSV-g SCA than in the MLV SCA. Therefore, we tested 6 independent stocks in single competitions for the remaining SCA studies and chose the MLV envelope for the remaining assays.

Effect of the virus input measurement method on RC. One potential explanation for the greater variability in the SCA than in the MCA is that the SCA only measures the replication of the mutant versus the wild-type reference strain at a single time point. Variation in the measurement of the relative inocula of wild-type and mutant viruses would therefore have a greater impact on the RC value than on the (1+s) value derived from the MCA, which is based on the relative prevalence of mutant versus wild-type virus using three time points. Since virus was pseudotyped by cotransfecting the DAT construct with the MLV envelope or VSV-g expression construct, we hypothesized that there could be different efficiencies of envelope incorporation into the different stocks, which could impact relative infectivity, and therefore RC. To test this, we measured the VSV-g protein content of each K103N and G190S stock by ELISA, measured the RC by coinfecting cells with equal amounts of VSV-g protein, and compared that to the RC obtained when the p24 protein content was used. Since the assay variability for the SCA was shown to be low, we tested each stock only once. There were no significant differences in the mean RC values for the K103N mutant (RC, 1.47 for p24-defined input versus 1.64 for VSV-g; P , 0.7422). There were also no differences in RC for the G190S mutant depending on whether p24 or VSV-g was used to determine input (0.38 for p24 versus 0.39 for VSV-g; P , 0.8125). These findings indicate that similar ratios of VSV-g to p24 antigen exist in the different stocks and that this explanation does not account for the greater variability in RC observed with VSV-g-pseudotyped stocks.

Comparing the SCA to the MCA. We next determined if the relative fitness values for different drug-resistant mutants would be similar by the SCA and the MCA. We chose to test the mutants in the SCA using MLV envelope, since this assay appeared to be less variable than the assay that utilized VSV-g-pseudotyped stocks. The mean fitness values of the mutants tested in the MCA ranged widely, from 1.36 to 0.18 (Fig. 4A). When the same mutants were tested in the MLV-SCA, the

FIG. 4. Comparison of the SCA with the MCA. (a) MCA. The site-directed mutants tested against the WT are shown along the x axis. The 1+s value, as calculated from the fitness website, is shown along the y axis. Each bar represents the average and standard deviation for at least 6 replicate cultures. (b) SCA. The relative input used for each stock was determined by the p24 protein content. The site-directed mutants tested against the WT are shown along the x axis. The RC value is shown along the y axis. Each bar represents the average and standard deviation for at least 6 independent virus stocks tested in single cultures and against the same WT stock. Circles highlight the positions of the D30N and I54L mutants, and squares highlight those of the I54M and V82A mutants, on each x axis.

mean RC values had a range of 4.26 to 0.046 (Fig. 4B). When we controlled for the effect of the mutant ($P, <0.0001$), there was no significant difference in mean RFV between the MCA and the SCA ($P, 0.10$), although the effect of the assay is modified by the significant interaction of mutant and assay ($P, <0.0001$).

Based on the model of all 22 mutants, further multiple comparison testing of each mutant showed that the relative fitness value for each mutant did not differ by assay, with the

exception of 3 mutants in the MCA and 2 mutants in the SCA replicating more poorly than in the alternative assay (Table 1). The D30N mutant, whose relative fitness was only slightly reduced in the SCA compared to that of the wild type, significantly replicated more poorly in the MCA. Similarly, the G48V and I54L mutants had reduced relative fitness in the MCA compared to the relative fitness in the SCA. In contrast, the I50V and I54M mutants had reduced relative fitness in the SCA compared to the relative fitness in the MCA.

TABLE 1. Comparison of the relative fitnesses of protease mutants with different relative fitnesses in the single-cycle assay versus the multiple-cycle assay^a

Mutant	Result (95% CI) by:		<i>P</i>
	SCA (RC)	MCA (1+s)	
D30N	0.80 ± 0.16 (0.68–0.92)	0.32 ± 0.10 (0.21–0.42)	0.0088
G48V	0.34 ± 0.13 (0.21–0.47)	0.18 ± 0.02 (0.16–0.20)	0.0484
I54L	0.89 ± 0.16 (0.72–1.06)	0.49 ± 0.11 (0.38–0.60)	0.0484
I50V	0.036 ± 0.006 (0.030–0.043)	0.29 ± 0.045 (0.24–0.33)	0.0484
I54M	0.34 ± 0.09 (0.25–0.43)	0.79 ± 0.04 (0.75–0.83)	0.0484

^a SCA, single-cycle assay; RC, replication capacity; CI, confidence interval; MCA, multiple-cycle assay.

We hypothesize that the MCA and SCA fitness values take into account different steps of the virus life cycle. If this is true, statistical comparison of the absolute fitness values of the mutant tested in each assay may not be valid. We think it more biologically relevant to compare the rank order among mutants between the two assays. This analysis would help determine how much a mutant's fitness changes relative to that of the wild type. Therefore, we ranked the mutants from most fit to least fit in the MCA and determined if any mutants changed more than 5 rank positions in the SCA. When this analysis was performed, the D30N and I54L mutants (Fig. 4; marked with circles) moved 8 and 6 spots, respectively, and the I54M and V82A mutants (Fig. 4; marked with boxes) both moved down 11 spots. The D30N and I54L mutants were more fit in the SCA, and the I54M and V82A mutants were less fit in the SCA.

Effect of cell line on MCA. Cong et al. have also tested the relative fitness of several mutants resistant to nucleoside RT inhibitors and nonnucleoside RT inhibitors (NNRTIs) by using an MCA (13). Of note is that some nucleoside-resistant mu-

tants that showed reduced fitness in their MCA did not have a discernible replication deficit in our MCA. Two important differences between their assay and ours were the cells used (PM1 cells in our assay versus MT-4 cells and PBMCs in theirs) and the viral sequence backbone (NL4-3 versus HXB-2). We therefore evaluated whether the cell line used in the assay could affect the relative fitness of a subset of mutants, using MT-4 cells or primary human PBMCs (Fig. 5). The overall test of the main effect of cell line was not significant (*P*, 0.8055), although there was a significant interaction between the mutant and the cell line (*P*, <0.0001). However, further comparison testing showed that the relative fitness of each of these mutants in the PM1 cell line did not differ significantly (*P*, >0.05) from the relative fitness based on either the MT4 or the PBMC line.

DISCUSSION

We have developed two HIV-1 replication fitness assays that use flow cytometry to detect the number of cells infected by a drug-resistant mutant, compared to the number infected by a WT reference strain in the same culture. The SCA utilizes a vector in which *env* is deleted and viruses are pseudotyped with MLV envelope. Therefore, viruses undergo a single round of infection with no subsequent rounds. Mutant virus produced after transfection is normalized for p24 capsid protein against WT, and cells are subsequently infected with equal amounts of WT and mutant viruses. The SCA vector has the mouse *Thy* gene cloned in place of *nef*, resulting in the expression of Thy protein on the surfaces of infected cells. In contrast, the MCA, which we have described previously (21), utilizes Thy-expressing virus with an intact envelope; viruses therefore undergo

FIG. 5. Measurement of fitness by the MCA using different cell lines. The relative input used for each stock was determined by the p24 protein content. The site-directed mutants tested against the WT are shown along the x axis. The 1+s value, as calculated from the fitness website, is shown along the y axis. Each bar represents the average and standard deviation for at least 6 replicate cultures. Mutants were tested against the WT in PM1 cells (filled bars), MT4 cells (shaded bars), and human PBMCs (open bars).

several rounds of replication during the course of the experiment.

We used these flow cytometry-based MCAs and SCAs to test a panel of 22 drug-resistant mutants. The most striking finding of this study was that four protease inhibitor-resistant mutants had marked differences in relative fitness by the two assays (Fig. 4). The replication deficit of the D30N mutant was moderate (20% reduction relative to WT) in the SCA and more pronounced (68% reduction) in the MCA (Table 1). Similarly, the I54L mutant had a fitness similar to that of the wild type in the SCA but a significant reduction in the MCA (Table 1). In contrast, the I54M and V82A mutants had greater decreases in fitness in the SCA, 66% and 56%, respectively, and were similar to the WT in the MCA (Fig. 4). The G48V and I50V mutants also had significant differences in their fitness values between the two assays; however, their relative fitness compared to that of the wild type in both assays was very poor (Table 1).

We hypothesize that the SCA, in which the lack of an envelope gene results in noninfectious progeny, should detect abnormalities in virus entry, reverse transcription, integration, and protein expression but would be insensitive to changes in the virus life cycle after early gene expression, such as the production of infectious virions from infected cells (i.e., burst size), the efficiency of cell-to-cell transmission, or the life span of infected cells. The MCA should reflect all these steps in the virus life cycle and may also reflect compensatory interactions between events that require multiple rounds of infection and those that can be measured by both assays (e.g., increases in cell-to-cell transmission could completely or partially compensate for reductions in protease activity). We believe that discordant results for the protease mutants provide important mechanistic clues as to which specific steps in the HIV replication cycle are affected by the mutation. The protease activity of the D30N, I54L, I54M, and V82A mutants correlates with the relative fitness measured in our SCA, indicating that infectivity is one step of the viral life cycle measured by the SCA (1, 10, 34). The fitness defects of the D30N and I54L mutants were even more pronounced in the MCA, which could be due to defects in both maturation and steps in virus spread that occur only in the MCA. One possible explanation as to why the I54M and V82A mutants are more fit in the MCA is that defects in particle production and maturation, which would be detected in both assays, are compensated for by improvements in steps of viral replication that are measured only in the MCA. This would result in an overall improvement in fitness in the MCA for the V82A mutant.

To determine whether burst size might explain the fitness differences seen for the protease mutants, two duplication mutants of the PTAP motif of p6 were made. This motif has been shown to help the release of particles from the cell by sequestering TSG101 (22, 25). The duplications used in this study have been observed in drug-experienced patients and in cytotoxic T-lymphocyte (CTL) escape variants (9, 41). It is hypothesized that these duplications increase the virus's ability to release particles from the cell surface. In our assays, the fitness of these mutants was not significantly different between the two assays. Therefore, either these mutants do not increase particle production, or burst size is measured in both assays and does not influence only the MCA fitness value.

The SCA has two technical differences from the MCA that could cause the potential variability that was observed in this study. The first is the need to cotransfect producer cell lines with the vector and envelope. Differences in the abilities of the two clones to be comparatively expressed from one transfection to another can introduce variability in the amount of envelope protein that is incorporated into the virion. Even in the face of equal expression, there is also the potential for variability in the amount of envelope protein that is functionally incorporated into virions. A second difference is that the RC is determined by a measurement at a single time point. Since relative fitness is not measured as a change over time, as in the MCA, the assay is much more sensitive to the relative amounts of WT and mutant viruses that are added. These two differences potentially create more variability in the RC values of the SCA than in the MCA values and result in the need to average the results for several different stocks.

For the panel of mutants we tested, some previously published studies gave results similar to ours and some gave dissimilar results. There is no previously published information comparing the fitness of HIV-1 drug-resistant mutants in multiple- versus single-cycle assays. In one previous report, the fitness deficit of the D30N mutant was 40% (40) compared to the WT in an SCA and was reduced 60% in an independent MCA (36). Like our studies, this MCA study also showed that the fitness of the L90M mutant was similar to that of the WT and greater than that of the D30N mutant. Our results for the I50V mutant are also consistent with another report in which the fitness of this mutant was reduced 90% relative to that of the wild type as measured in an SCA in combination with the L10F and M46I mutants (44). Our results are also consistent with our previously published results for NNRTI drug-resistant mutants. The fitness of the K103N mutant is similar to that of the WT, whereas the fitness levels of the V106A, G190S, and P226L mutants are reduced (4, 24, 28, 48).

In contrast to our study, the replication deficit of the V82A mutant in an NL4-3 background has been reported to be similar to the WT in an SCA, only 20% reduced, as measured by a chlorophenol red- β -D-galactopyranoside (CPRG)/ β -galactoside assay (35). However, this study used a different method to measure the infectivity of the mutants and did not compare the WT and mutant in the same culture. Competitions where WT and mutant viruses are in the same culture have been shown to be more sensitive to differences (12). Therefore, differences in sensitivity may explain why our SCA may have detected a greater reduction in fitness for the V82A mutant. In support of our results, the V82A mutant had reduced fitness in the context of a clinical isolate sequence in the single-cycle Monogram RC assay, again indicating that the sequence backbone may influence the results (7). The results of the current study and previous work further support the idea that the method used to measure fitness may influence the relative fitness of some mutants. We suspect that these methodological differences can account for at least some of the contradictory fitness values for drug-resistant mutants that have been reported in some previously published studies.

Cong et al. have tested the relative fitness, which was different from our results, of several RT mutants using an MCA (13). There were three main differences between the two assays used: the vector backbone (NL4-3 versus HXB2), the cell type

(PM1 versus MT4 cells), and the method used to detect the proportion of viruses (flow cytometry versus sequencing). We have shown previously that the flow cytometry used in our study and the sequencing used in the Cong study give similar relative ratios of mutant to WT virus (21), and the studies described here show that the relative fitness in MT4 and PM1 cells is the same. The only explanation we could find for the discordant results is differences in sequence backbones. We and others have previously shown that the background sequence in which a drug-resistant mutant develops can influence its relative fitness, prevalence in patients, and level of drug resistance (20, 23, 46, 47).

There are three differences between the SCA and MCA that may or may not explain the differences in fitness seen for the protease mutants. These include the use of MLV envelope to pseudotype the SCA virus stocks, a different transfection reagent, and differences in the length of time the virus particles are in the culture supernatant before they find a target cell. However, we believe these differences are unlikely explanations for the differences seen between the SCA and MCA, since they cannot explain the fact that some mutants had higher relative fitness in the SCA than in the MCA while other mutants had higher relative fitness in the MCA. Therefore, the difference would have to be dependent on the specific mutant being tested. In addition, even though differences in the envelope could impact binding and entry, there is no evidence that protease activity is needed for binding and entry, and the transfecting reagents are present during transfection only and are not present during infection.

The fitness difference between the multiple-cycle and single-cycle assays for the V82A, I54M, I54L, and D30N mutants ranges from 39% to 48%. Is this magnitude of difference biologically relevant? Several studies have looked at the correlation of fitness with clinical measures such as viral load and CD4 count. A study of chronically infected subjects using a whole-virus MCA showed that a 20% decrease in fitness correlates with at least a 0.5 log₁₀ decrease in viral load (8). Therefore, the mutants identified here, which have about a 40% different in fitness, could impact the viral load by 1 log₁₀. Another study, with acutely infected subjects, showed that subjects with an RC value of <0.42 measured using an SCA had an average CD4 cell count that was >100 cells/μl higher than that of subjects with an RC value of >0.42 (6). Therefore, mutants such as the D30N and I54M mutants, which cross the 0.42 threshold, could have an impact on CD4 cell counts. Another study showed that a cutoff of 65% for RC predicted better response, such that subjects with an RC of <65% had a better outcome than subjects with an RC of >65% (14). Again, the difference in fitness for our mutants crosses this threshold. Other studies, with elite controllers, whose viral load is never >2,000 copies/ml without therapy, show that their viruses have 20% lower fitness than those of progressors (37, 38). Our group has also shown that NNRTI-resistant mutants, which have a 30 to 70% decrease in fitness compared to highly prevalent mutants, are less prevalent *in vivo* (24, 28). These studies serve as examples that the magnitude of the difference we see between the SCA and the MCA for protease mutants could be clinically important.

More studies are warranted to determine if fitness as measured by an SCA or an MCA correlates better with clinical

outcome. Until that is determined, studies of fitness, particularly for protease mutants, warrant the use of both types of assays. Currently, the Phenosense assay from Monogram Biosciences, a drug resistance assay, is the only assay that also provides clinicians with an RC value measured using an SCA. Our work indicates that patient samples containing protease mutants that are tested by this assay may not have an accurate fitness measurement. Therefore, clinicians should use this value cautiously. The magnitude of the difference measured between the SCA and the MCA is large enough to have a biological impact *in vivo*. Since this work is the most comprehensive comparison of drug-resistant mutants in both assays to date, more studies in different clinical settings are needed to determine its clinical importance.

Our results show that the type of fitness assay that is chosen to analyze the relative fitness of drug-resistant mutants may influence the result. Direct comparison of relative fitness using a multiple-cycle and a single-cycle assay may be a way to determine whether a particular mutant is likely to affect early or late steps in the life cycle. Studies are under way to determine if the early steps of the virus life cycle affect fitness using both the SCA and MCA and if late steps in the life cycle only affect fitness using the MCA.

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