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Drug-associated changes in amino acid residues in Gag p2, p7^{NC}, and p6^{Gag}/p6^{Pol} in human immunodeficiency virus type 1 (HIV-1) display a dominant effect on replicative fitness and drug response

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ABSTRACT

Regions of HIV-1 *gag* between p2 and p6^{Gag}/p6^{Pol}, in addition to protease (PR), develop genetic diversity in HIV-1 infected individuals who fail to suppress virus replication by combination protease inhibitor (PI) therapy. To elucidate functional consequences for viral replication and PI susceptibility by changes in Gag that evolve *in vivo* during PI therapy, a panel of recombinant viruses was constructed. Residues in Gag p2/p7^{NC} cleavage site and p7^{NC}, combined with residues in the flap of PR, defined novel fitness determinants that restored replicative capacity to the posttherapy virus. Multiple determinants in Gag have a dominant effect on PR phenotype and increase susceptibility to inhibitors of drug-resistant or drug-sensitive PR genes. Gag determinants of drug sensitivity and replication alter the fitness landscape of the virus, and viral replicative capacity can be independent of drug sensitivity. The functional linkage between Gag and PR provides targets for novel therapeutics to inhibit drug-resistant viruses.

Introduction

Introduction in the mid 1990s of combination antiretroviral therapy (ART) resulted in a dramatic decrease in the proportion of HIV-infected individuals who progress to AIDS. However, resistant viruses emerge as a result of several factors including the error-prone nature of reverse transcriptase (Battula and Loeb, 1976) and suboptimal levels of drugs due to poor adherence to therapeutic regimens, differing bioavailablities of drugs, and anatomical or cellular reservoirs inaccessible to drugs. Resistance, defined as diminished susceptibility to a protease inhibitor (PI), involves accumulation and persistence of multiple amino acid changes in the HIV-1 protease (PR) gene (Erickson et al., 1999). Resistance to PI can be attributed to multiple mechanisms. For example, polymorphisms in PR known to reduce or increase sensitivity to PI can occur in therapy-naive patients (Brown et al., 1999; Lech et al., 1996; Leigh Brown et al., 2004; Martinez-Picado et al., 2005; Perez et al., 2001; Rose et al., 1996). In addition, as a result of suboptimal drug therapy, viruses begin to accumulate mutations in PR in a stepwise fashion (Condra et al., 1996; Erickson et al., 1999; Molla et al., 1996). The first PR mutations selected in the presence of PI are primary, or major, mutations, which cause resistance by lowering inhibitor affinity (Cote et al., 2001; Muzammil et al., 2003), accompanied by a decrease in the replicative capacity of the virus, due in part to inefficient processing by PR (Borman et al., 1996; Croteau et al., 1997; Kaplan et al., 1994; Martinez-Picado et al., 1999; Resch et al., 2005; Rose et al., 1996; Watkins et al., 2003; Zennou et al., 1998). Secondary, or compensatory, mutations can be present before therapy or appear in response to inhibitors and help to restore the replicative capacity of the virus (Barrie et al., 1996; Doyon et al., 1996; Kaplan et al., 1994; Nijhuis et al., 1999; Perez et al., 2001; Rose et al., 1996; Shao et al., 1997; Zhang et al., 1997).

PR genotype is used to predict phenotypic drug resistance, and various algorithms have been developed to identify reduced susceptibility to inhibitors, particularly to determine transmission of drug resistant viruses and to identify treatments that might suppress viruses rebounding from an initial therapy (Johnson et al., 2007; Perez et al., 2001; Shafer et al., 2007). Predictions of PR susceptibility or resistance to inhibitors rely primarily on PR genotype, but are imperfect, in part

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because of the multiple mutations in PR that accumulate to increase resistance, new inhibitors that select for novel combinations of mutations, and cross resistance to different inhibitors that combinations of mutations in PR can produce.

Novel amino acid substitutions accumulate in Gag and Gag-Pol concomitant with PR mutations during suboptimal therapy and, similar to PR mutations, persist over time. Changes develop in a subset of the cleavage sites that serve as substrates for PR, as well as non-cleavage site residues in p2, nucleocapsid ($p7^{NC}$), and $p6^{Gag}/p6^{Pol}$ (Borman et al., 1996; Condra et al., 1995; Doyon et al., 1998; Erickson et al., 1999; Gatanaga et al., 2002; Kaplan et al., 1994; Maguire et al., 2002; Mammano et al., 1998; Markowitz et al., 1995; Martinez-Picado et al., 1999; Molla et al., 1996; Rose et al., 1996; Zennou et al., 1998; Zhang et al., 1997). Considerable evidence supports the functional relationship between Gag and PR and the role that Gag sequences can play in modulating pretherapy, wild-type PR activity (Bloom et al, 1998; Goodenow et al., 2002; Pettit et al., 2003; Pettit et al., 2005; Verheyen et al., 2006). Drug-associated changes in Gag amino acid residues may contribute to PR drug response and to viral fitness. Although PR determinants of *ex vivo* viral fitness and drug response are well characterized, contributions by mutations in Gag and Gag-Pol to replicative capacity and PI susceptibility are less well defined. We designed a study to assess the contribution of amino acid changes in Gag, which developed in viruses *in vivo* during continued replication in the presence of PI combination antiretroviral therapy, on viral replicative capacity and drug sensitivity *ex vivo* in primary CD4 T lymphocytes.

Results

Pre-and posttherapy gag-pol alleles

The pretherapy *gag-pol* region that predominated in the peripheral blood 7 years prior to the initiation of ART differed by 23 amino acids from the posttherapy region (Fig. 1). Nine of the 23 amino acid changes evolved over 7 years in the absence of therapy, appeared as the dominant population at baseline immediately prior to initiation of ART, and persisted throughout the period of treatment. Amino acid polymorphisms that accumulated before therapy were distributed throughout the Gag-PR region and involved I376V within the $p2/p7^{NC}$ cleavage site; K452R within the $p1/p6^{Gag}$ cleavage site; E464A and P482T in $p6^{Gag}$; R464S, G465R, and A482D in the overlapping $p6^{Pol}$ reading frame; and M36I and T37N in PR (Fig. 1).

During 93 weeks of PI-containing ART, changes accumulated at fourteen additional amino acid residues in Gag-PR. Although most (10 of 14) developed during the first 6 months of ART, fewer than half (6 of 14) of the amino acid changes occurred in PR (Fig. 1B). Posttherapy PR differed from pretherapy PR in (V82A) or near the active site (L10I and I15V), in the hinge (E34Q, M36I, and T37N), and in the flap (I54A and Q58E). Similar to PR mutations, eight novel amino acid substitutions in Gag-Pol also accumulated during the course of treatment. The Gag-Pol substitutions included E398V within the first zinc finger motif of $p7^{NC}$; E458A, D484G, L487P (in the AIP-1 binding site) (Popov et al., 2008; Strack et al., 2003; von Schwedler et al., 2003), and K494R (in the VPR binding site) (Andersen et al., 2006; Elder et al., 2000; He et al., 1995; Jowett et al., 1995; Poon et al., 1997; Re et al., 1995) in $p6^{Gag}$ (Fig. 1A); and D437N (in $p6^{Pol}$ cleavage site) (Chen et al., 2001; Lindhofer et al., 1995; Louis et al., 1998; Pettit et al., 2005; Pettit et al., 2004; Pettit et al, 2003; Phylip et al., 1995), R458S, and G471R in $p6^{Pol}$ (Fig. 1B). Specific mutations in the $p7^{NC}/p1$ and p1/p6 cleavage sites occur in some treated individuals and can act to increase the fitness of a resistant virus (Maguire et al., 2002; Mammano et al., 1998; Zhang et al., 1997). In our cohort of more than 50 treated individuals, the $p7^{NC}/p1$ and p1/p6 cleavage sites, as well as the $p24^{CA}/p2$, $p6^{Pol}$/PR, and PR/RT cleavage sites were highly conserved prior to and following therapy (Barrie et al., 1996 and data not shown). In contrast, the $p2/p7^{NC}$ cleavage site was polymorphic in nearly all subjects within our cohort.

Replicative fitness and drug sensitivity of pre-and posttherapy viruses in PBMC

Prior to construction of recombinant viruses, the PR genes from pre-and posttherapy were shown to encode functional protease enzymes that processed the Gag polyproteins *in vitro* (Goodenow et al., 2002; data not shown). When replicative capacity of the recombinant

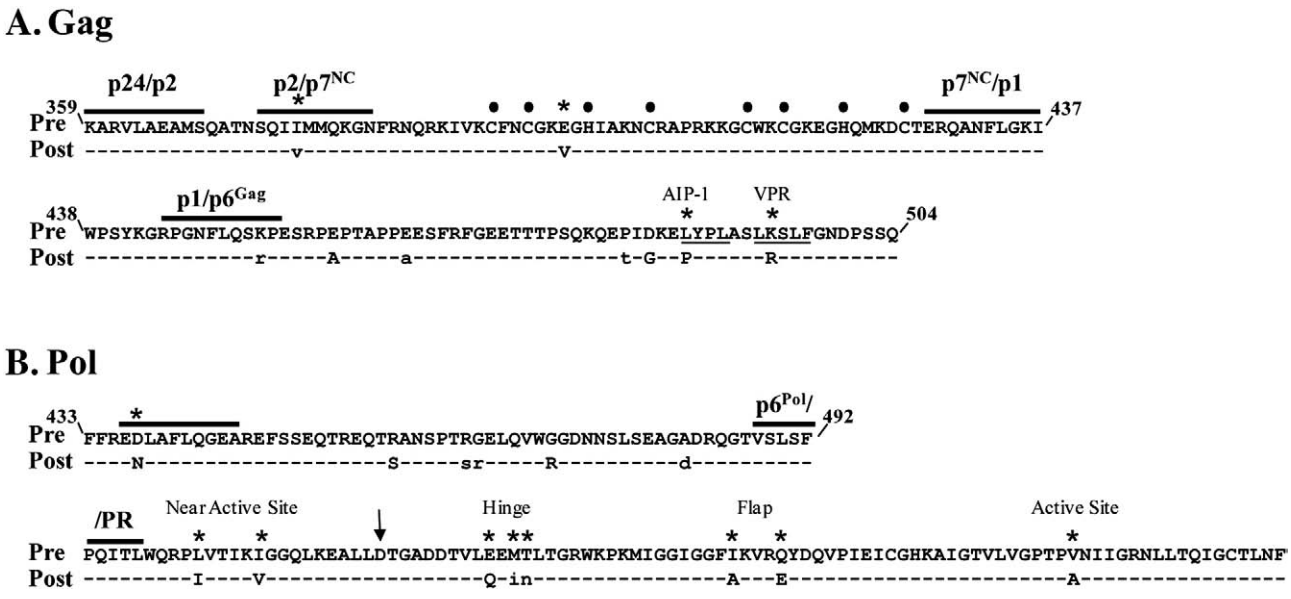

Fig. 1. Sequence alignment of pre- and posttherapy Gag-PR variants. (A) Gag p2, $p7^{NC}$, and $p6^{Gag}$ sequences. (B) $p6^{Pol}$ and PR sequences. Gag and Gag-Pol cleavage sites are marked with lines above the sequence. Polymorphisms that developed over 7 years prior to ART are shown in lower case; amino acid changes that appeared during the 93 weeks of PI-containing ART are shown in upper case. Filled circles designate cysteine and histidine residues comprising the Zn finger motifs in $p7^{NC}$. Asterisks denote amino acids targeted for mutagenesis. Functional regions in $p6^{Gag}$ and PR are noted above the asterisks. Arrow denotes the catalytic aspartate residue at position 25 in PR.

viruses with pre-or posttherapy alleles was assessed in parallel infections of PBMC, the pretherapy recombinant virus replicated to high levels compared to the posttherapy recombinant virus (Fig. 2A). In general, over the course of 6 to 10 days in culture, replication by the posttherapy virus achieved levels that were only about 10% to 20% of replication levels by the pretherapy virus. To confirm relative fitness, the viruses were tested in direct competition with each other. When the pretherapy or posttherapy virus was cultured alone for 5 weeks as a control, 100% of the viruses sequenced at the end of 1 and 5 weeks in culture were pretherapy or posttherapy virus, respectively (Fig. 2B, left and right bars). When PBMC were coinfected by equal $TCID_{50}$ of the pre-and posttherapy viruses, the high fit pretherapy virus outgrew completely the low fit posttherapy virus after 1 week (data not shown) and after 5 weeks (Fig. 2B, center striped bar).

Mutations at positions 10, 36, 54, and 82 in PR confer resistance to RTV therapy, as well as cross resistance to IDV (Johnson et al., 2007), demonstrating that the posttherapy PR developed genotypic resistance *in vivo* consistent with the PIs administered. To directly evaluate sensitivity for RTV or IDV, IC_{50} values for the pretherapy and posttherapy recombinant viruses in PBMC from a series of independent donors were determined. The posttherapy virus had a mean IC_{50} value of 5150 nM for RTV, 6-fold higher than the mean IC_{50} of 870 nM for the pretherapy virus, and a mean posttherapy IC_{50} value of 200 nM for IDV compared with a mean IC_{50} value of 30 nM for the pretherapy virus. Overall, mean IC_{50} values for the pretherapy virus were about 15% to 18% of values for the posttherapy virus. Pre-and posttherapy viruses displayed a direct relationship between PR genotypic and phenotypic resistance to PIs, as well as an inverse relationship between PI resistance and fitness in PBMC in the absence of PI.

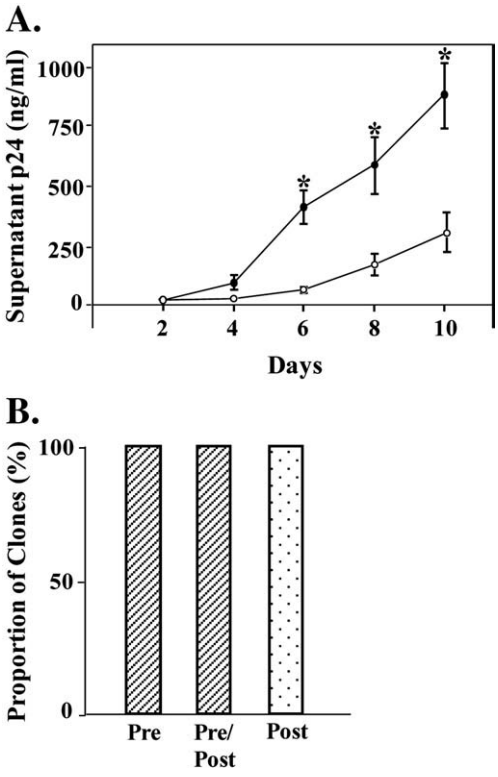

Fig. 2. Replicative capacity of pre-and posttherapy Gag-PR recombinant viruses in PBMC cultures. (A) Parallel infections. Filled circles represent the pretherapy virus and open circles represent the posttherapy virus. Data represent mean and SEM of 7 independent infections (*$P< 0.05$). (B) Competitive infections. Proportion of virus clones (expressed as percent of total clones) present after 5 passages in culture. Approximately 25–30 clones were sequenced for each infection. Striped bar, pretherapy virus; dotted bar, posttherapy virus.

Differences in replicative capacity and drug sensitivity could reflect in part an intrinsic difference between the drug-sensitive or drug-resistant PR to process the Gag-Pol polyprotein. To examine this possibility, Gag-Pol polyprotein processing by the PI^{sen} and PI^{res} PR region was assessed in the presence or absence of PI in a cell-free transcription/translation system. In the absence of PI, processing between the PI^{sen} and PI^{res} PR variants was indistinguishable, while including RTV or IDV in the reaction inhibited production of Gag p24/p25 by the PI^{sen}, but not the PI^{res} PR (data not shown).

Strategy to restore fitness

To test a contribution by the amino acids in Gag to viral fitness, a stepwise mutagenesis strategy that reverted, either alone or in combination, the amino acids found in the posttherapy Gag-PR to the residues present in the pretherapy Gag-PR was developed to measure gain-of replicative capacity (Fig. 3). Because a strict timeline for sequential accumulation of the mutations in Gag-PR residues could not be determined, selection of amino acid residues was based on functional domains in Gag or PR. The first set of studies addressed the potential role of the pretherapy residues in the $p2/p7^{NC}$ cleavage site and $p7^{NC}$ in replication of a virus with posttherapy PR; the second set of studies combined pretherapy $p2/p7^{NC}$ cleavage site and $p7^{NC}$ residues with changes in posttherapy PR; and the final study introduced changes at selected residues in $p6^{Gag}/p6^{Pol}$ into the virus with pretherapy $p2/p7^{NC}$ and PR sequences. A panel of 14 recombinant viruses, including the pre-and posttherapy viruses, was evaluated for replicative capacity in PBMC and for changes in sensitivity to RTV or IDV.

Residues in $p2/p7^{NC}$ cleavage site and $p7^{NC}$ contribute to replication and modulate PI sensitivity of a drug-resistant virus

To determine if $p2/p7^{NC}$ residues could modulate replicative capacity, Viruses 1–3 combined pretherapy amino acid residues at positions 376 and/or 398 in the $p2/p7^{NC}$ region with drug-resistant PR alleles (Fig. 4). A change from valine to pretherapy isoleucine at position 376 in $p2/p7^{NC}$ cleavage site (Virus 1) or valine to glutamic acid at residue 398 in $p7^{NC}$ (Virus 2) produced little if any improved replicative capacity compared to the posttherapy virus. In contrast, combination of both pretherapy residues I376 and E398 in the same viral genome (Virus 3) improved replication to levels that were about four-fold above the posttherapy virus and about 50% of pretherapy virus, indicating that residues in Gag p2 and $p7^{NC}$ combined to contribute to replicative fitness.

To assess if residues 376 and/or 398 might also contribute to PI response, IC_{50} values for RTV and IDV were determined (Fig. 4). In the presence of RTV, Virus 1 and Virus 2 each displayed hypersensitivity with IC_{50} levels that were almost a log-fold reduced relative to pretherapy virus. Combining $p2/p7^{NC}$ cleavage site and $p7^{NC}$ pretherapy residues in Virus 3 partially restored drug response by either of the single variants to levels similar to the pretherapy virus. In the presence of IDV, Virus 1 was as sensitive as the pretherapy virus, Virus 2 displayed a level of sensitivity that was intermediate between the pre-and posttherapy viruses, and Virus 3 maintained a fully resistant phenotype to IDV, similar to the posttherapy virus. Results from this series of experiments indicated that determinants localized outside PR in $p2/p7^{NC}$ cleavage site and $p7^{NC}$ modulated response to PI by viruses with genotypic and phenotypic drug resistant PR.

Residues in PR alter replicative fitness independent of PI sensitivity

Since pretherapy residues in $p2/p7^{NC}$ cleavage site and $p7^{NC}$ failed to reconstitute full replicative capacity to the posttherapy virus, a role for changes in PR to enhance replication by Virus 3 was examined (Fig. 5). Changing the active site residue at position 82 from the

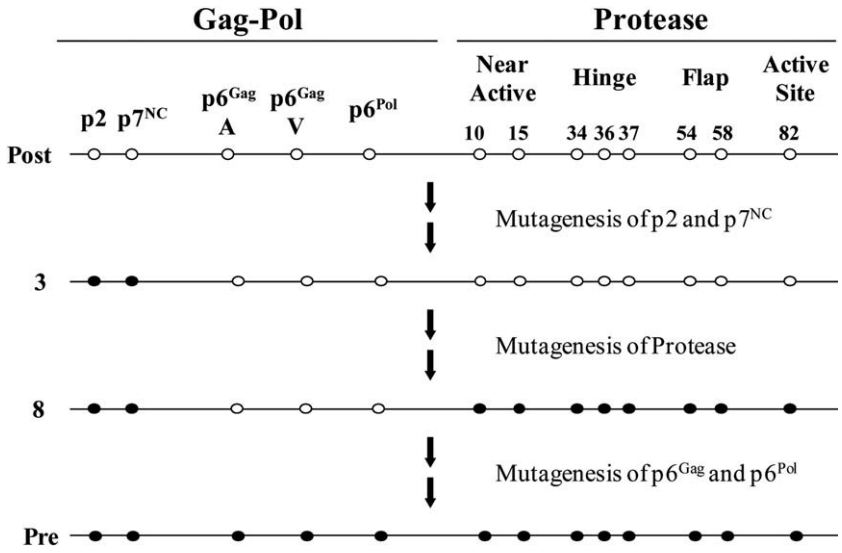

Fig. 3. Mutagenesis strategy to restore replicative capacity. The posttherapy Gag-PR recombinant virus was used as the backbone to introduce pretherapy residues into p2 and $p7^{NC}$ (Virus 3). Virus 3 was then used as the backbone to introduce a series of pretherapy residues into PR (Virus 8). Virus 8 provided the backbone for the final mutations in the $p6^{Gag}$ AIP-1 binding site ($p6^{Gag}$ A), the $p6^{Gag}$ VPR binding site ($p6^{Gag}$ V), and $p6^{Pol}$. Positions and their locations are noted above the constructs. The same designations appear in Figs. 4–6. Open circles represent amino acids present in the posttherapy Gag-PR region and filled circles represent residues in the pretherapy Gag-PR region.

posttherapy alanine to pretherapy valine produced Virus 5 which, similar to Virus 3, replicated at about 50% of the wild-type pretherapy virus, indicating no increased fitness advantage by V82. As expected, V82 in PR reduced resistance to either RTV or IDV by Virus 5 to about 50% of posttherapy levels.

Pretherapy amino acid residues in the near active site (positions 10 and 15) combined with the active site (residue 82) in Virus 6 failed to improve replication compared with posttherapy virus and actually suppressed the positive replicative effect by pretherapy $p2/p7^{NC}$ alone (Fig. 5). Combining pretherapy amino acid residues in the PR hinge (residues 34, 36, and 37) and flap (residues 54 and 58) regions in Virus 7 also failed to restore any replicative capacity to the posttherapy virus. While pretherapy residues in the active site or the hinge/flap regions provided no improvement in replication, Viruses 6 and 7 each showed reduced resistance to PI with IC_{50} values similar to the pretherapy virus.

Introducing pretherapy amino acids into the PR flap residues of Virus 3 produced Virus 4, which displayed enhanced replicative capacity that was almost two-fold greater than Virus 3 and 85% of the level of pretherapy virus (Fig. 5). Results identify positions in the flap of PR, in combination with $p2/p7^{NC}$, as fitness determinants. In contrast to increased replication, changes in the flap of PR in Virus 4 produced dichotomous responses to PIs. Compared with Virus 3 and the posttherapy virus, Virus 4 was more sensitive to RTV, but as resistant to IDV. Results indicate that replication fitness does not necessarily correlate with susceptibility to PI.

Residues in $p6^{Gag}$ and $p6^{Pol}$ contribute to PI hypersensitivity

A role for the multiple amino acid changes in $p6^{Gag}/p6^{Pol}$ that accumulated prior to and during PI treatment in further enhancement of replicative capacity was evaluated. The posttherapy $p6^{Gag}/p6^{Pol}$ region was combined with pretherapy $p2/p7^{NC}$ and PR in Virus 8, which displayed a level of replication equivalent to the pretherapy virus, indicating little, if any contribution by the combination of residues in $p6^{Gag}/p6^{Pol}$ to replicative capacity (Fig. 6). To determine if individual positions in $p6^{Gag}/p6^{Pol}$ could modulate replication, selected residues (487P and 494R in Gag and 437N in Pol) were changed, alone or in combination, to the pretherapy amino acids L, K, or D, respectively (refer to Fig. 1). Single substitutions in Viruses 9, 10

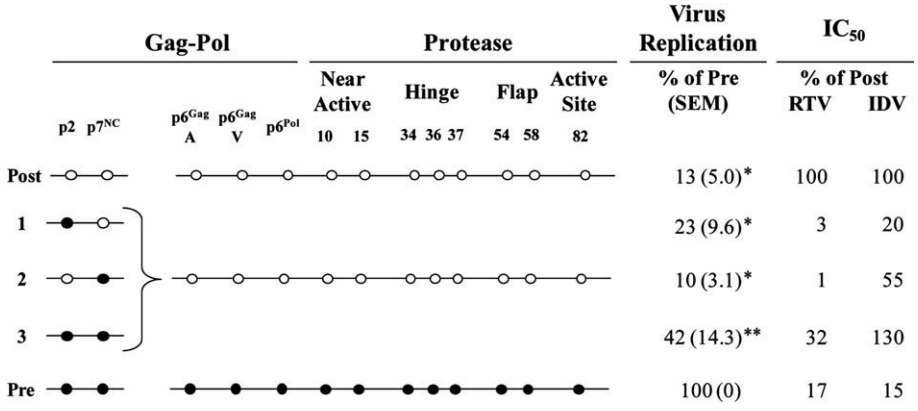

Fig. 4. Replicative capacity and PI sensitivity of p2 and $p7^{NC}$ revertants. Representations of viral constructs are shown on the left. Open circles indicate resides that are identical to the posttherapy virus and filled circles represent amino acids identical to the pretherapy virus. Viral replication on day 8 is expressed as a percent of the pretherapy virus. Data represent mean and SEM of 6 independent experiments (*$P<0.001$, **$P=0.006$). IC_{50} values for ritonavir (RTV) and indinavir (IDV) are expressed as a percent of the posttherapy virus, and are shown in the last two columns on the right. Actual IC_{50} values for RTV are as follows: Pretherapy, 870 nM; Posttherapy, 5150 nM; Virus 1, 130 nM; Virus 2, 60 nM; Virus 3, 1650 nM. IC_{50} values for IDV are: Pretherapy, 30 nM; Posttherapy, 200 nM; Virus 1, 40 nM; Virus 2, 110 nM; Virus 3, 260 nM.

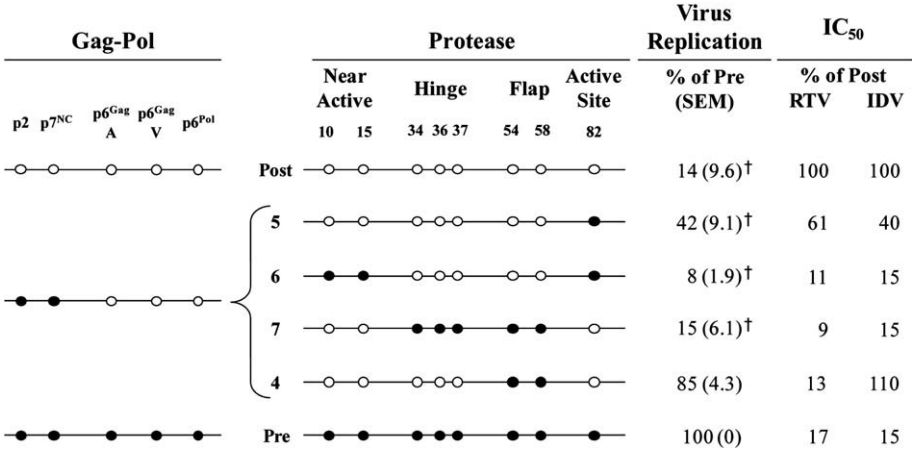

Fig. 5. Replicative capacity and PI sensitivity of PR revertants. Designations are the same as described for Fig. 4. Data represent mean and SEM of 3 independent experiments (†$P<0.05$). Actual IC_{50} values for RTV are as follows: Pretherapy, 870 nM; Posttherapy, 5150 nM; Virus 4, 690 nM; Virus 5, 3150 nM; Virus 6, 590 nM; Virus 7, 450 nM. IC_{50} values for IDV are: Pretherapy, 30 nM; Posttherapy, 200 nM; Virus 4, 220 nM; Virus 5, 80 nM; Virus 6, 30 nM; Virus 7, 30 nM.

and 11 failed to alter replicative capacity relative to pretherapy virus, while combining the three pretherapy amino acid residues in the background of posttherapy $p6^{Gag}/p6^{Pol}$ diminished replication by Virus 12 to levels that were about 70% of the pretherapy virus.

When responses to PI were evaluated, this series of recombinant viruses displayed significant hypersensitivity to both IDV and RTV compared with the pretherapy virus (Fig. 6). Virus 8 was reduced by about eight-fold in sensitivity to RTV or about two-fold to IDV, relative to pretherapy virus. More striking results were the IC_{50} values displayed by Viruses 9, 10, 11, or 12, which were almost two logs lower than pretherapy virus for RTV, or decreased by three-to five-fold for IDV relative to the pretherapy virus. Although combinations of residues in $p6^{Gag}/p6^{Pol}$ had limited effect on replicative capacity relative to the pretherapy virus, residues in this region enhanced pretherapy PR sensitivity to inhibitors.

Phenotypic drug response can be independent of PR genotype or replicative capacity

The relationship between replicative capacity and drug susceptibility characteristics among the viruses was displayed in a fitness landscape (Fig. 7). Pretherapy and posttherapy recombinant viruses defined the upper and lower boundaries, respectively, of replication capacity in the fitness landscape. In contrast, while the posttherapy virus defined the upper limit of drug resistance to RTV, Viruses 3 and 4 were more resistant to IDV than the posttherapy virus. Furthermore, the pretherapy virus failed to define the lower limit of sensitivity to either RTV or IDV. With few exceptions, any change in Gag increased sensitivity to PI to levels that were well-below the levels displayed by the pretherapy virus. Viruses 8–12 with $p2/p7^{NC}$ and PR regions identical to the pretherapy virus moved in the fitness landscape toward increased sensitivity to inhibitors by virtue of single amino acid changes in $p6^{Gag}/p6^{Pol}$. A striking result was that Viruses 1, 2, and 3, with $p6^{Gag}/p6^{Pol}$ and drug-resistant PR genes identical to the posttherapy virus, moved in the fitness landscape based solely on limited changes in Gag. Overall, changes in phenotypic drug response could be disconnected from either replicative capacity or PR genotype.

Discussion

HIV-1 PR activity is targeted to and intimately linked with substrates in the Gag and Gag-Pol polyprotein. Determinants within PR that alter enzymatic activity and contribute to replicative fitness of the virus in the presence and absence of a variety of protease inhibitors are well defined (Clemente et al., 2004; Croteau et al., 1997; Liu et al., 2006; Mahalingam et al., 1999; Mammano et al., 2000;

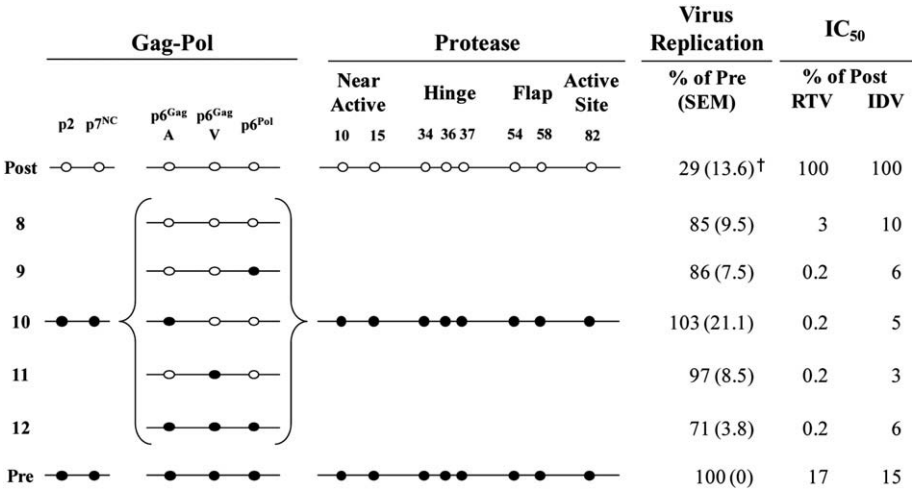

Fig. 6. Replicative capacity of $p6^{Gag}$ and $p6^{Pol}$ revertants. Designations are the same as described for Fig. 4. Data represent mean and SEM of two independent experiments performed in triplicate (†$P<0.05$). Actual IC_{50} values for RTV are as follows: Pretherapy, 870 nM; Posttherapy, 5150 nM; Virus 8, 170 nM; Virus 9, 10 nM; Virus 10, 9 nM; Virus 11, 9 nM; Virus 12, 10 nM. IC_{50} values for IDV are: Pretherapy, 30 nM; Posttherapy, 200 nM; Virus 8, 20 nM; Virus 9, 11 nM; Virus 10, 10 nM; Virus 11, 5 nM; Virus 12, 12 nM.

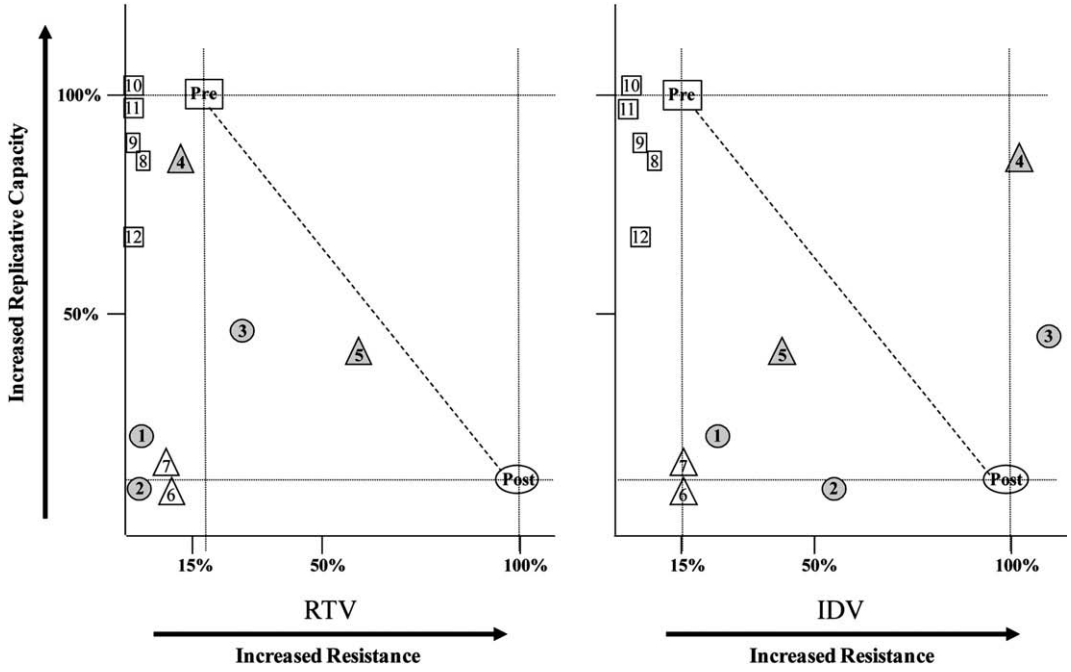

Fig. 7. Viral fitness and inhibitor sensitivity can be modulated independently by sequences in Gag. Viral replication is expressed as a percent of the pretherapy virus and PI resistance is expressed as a percent of the posttherapy virus. Circles (Viruses 1–3) indicate viruses with PR sequences identical to posttherapy (Post) virus. Squares (Viruses 8–12) indicate viruses with PR sequences identical to pretherapy genotypic sensitive PR allele (Pre). Triangles (Viruses 4–7) indicate viruses with partial genotypic resistant PR alleles. Shaded symbols indicate differences based on inhibitor.

Martinez-Picado et al., 1999; Molla et al., 1996; Muzammil et al., 2003; Myint et al., 2004; Nijhuis et al., 1999; Resch et al., 2005; Watkins et al., 2003; Zhang et al., 1997). In our study, PR developed *in vivo* genotypic changes that conferred phenotypic resistance to RTV and IDV, PIs administered sequentially as part of combination therapies. An unexpected finding from our studies is that Gag can be a dominant modulator of PR drug resistant phenotype and increase drug susceptibility of a genotypic and phenotypic drug-resistant virus.

Residues in Gag cleavage sites can evolve *in vivo* during incomplete suppression of virus replication by PI-containing antiretroviral therapies (Feher et al., 2002; Kolli et al., 2006; Malet et al., 2007; Mammano et al., 1998; Verheyen et al., 2006) and contribute to phenotypic drug resistance and replication *in vitro* (Maguire et al., 2002; Mammano et al., 1998; Yates et al., 2006; Zhang et al., 1997). However, highly resistant viruses without cleavage site mutations occur (Gatanaga et al., 2002), and in our cohort, cleavage sites, other than the p2/p7^{NC} cleavage site, were not variable. This study shows that mutations in the p2/p7^{NC} cleavage site can act in a compensatory manner in the absence of changes at other cleavage sites, such as p7^{NC}/p1 and p1/p6, demonstrating that there are multiple ways to achieve the same phenotype with different cleavage site mutations.

PI treatment-related changes did accumulate outside the cleavage sites in Gag, similar to other studies, although a genotypic profile for Gag determinants that modulate replicative capacity or drug response is not well defined (Doyon et al., 1998; Gatanaga et al., 2002; Mammano et al., 1998; Zhang et al., 1997). The number of unique residues that developed in Gag and Pol outside the cleavage sites during antiretroviral therapy in our study led us initially to consider that the changes could contribute to enhanced fitness of the virus. Even though every possible combination of amino acid differences between pre-and posttherapy Gag-PR regions in our study was not examined, a key to defining novel fitness determinants that could fully restore the reduced replicative capacity of the posttherapy virus to levels displayed by the pretherapy virus, was assessment of combinations of residues, rather than single amino acids. Fitness determinants are complex, discontinuous, and context dependent, raising the possibility that additional determinants might be identified by assessing alternative combinations of residues. In our previous studies, polymorphisms in Gag p7^{NC} and PR were found to modulate wild-type PR processing activity and diminish replicative fitness of therapy naive viruses (Bloom et al., 1998; Goodenow et al., 2002). The current studies implicate the same regions of p7^{NC} as fitness determinants in the context of drug-resistant PR.

Replicative fitness alone accounts for the function of only a subset of the drug-associated changes in Gag and Pol, although fitness can be associated with drug responsiveness. For example, drug resistance by a wild type PR is increased by substitutions that map exclusively in the p7^{NC}/p1 cleavage site (Nijhuis et al., 2007), while wild type PR among viruses from therapy naive individuals display hypersusceptibility to PI (Leigh Brown et al., 2004; Martinez-Picado et al., 2005) The Gag mutations identified in our study accumulated concomitantly with PR mutations in the presence of PI and persisted over time, similar to characteristics of amino acid residues in PR that confer drug resistance. Indeed, the predominant effect for most of the drug-associated amino acid substitutions in Gag-Pol in our study was to enhance resistance to inhibitors, as any reversions to pretherapy residues increased drug sensitivity. Protease inhibitor drug resistant viruses are less fit than wild-type viruses in the absence of antiretrovirals, with an inverse relationship between level of response to drug and replicative capacity in the absence of inhibitor (Coffin, 1995; Croteau et al., 1997; Harrigan et al., 1998; Martinez-Picado et al., 1999; Rose, 2002; Stoddart et al., 2001). Our study shows that viral replicative capacity can be independent of drug sensitivity.

If PR genotype alone were sufficient to predict the drug response phenotype of virus, then viruses with identical PR sequences should have identical resistance to PI. Yet, PR genotype was a poor predictor of drug resistance for the variants that were tested in this study. Viruses with identical PR genes failed to exhibit similar phenotypes because of complex determinants that required combinations of amino acid residues in regions of Gag or Gag-Pol. PR with resistance mutations can exhibit differential susceptibility to inhibitors (Gonzalez et al., 2003; Resch et al., 2005), but differential sensitivity to inhibitor

modulated by Gag is a novel finding. In our study, differential sensitivity to RTV or IDV was conferred to a virus with a genotypic and phenotypic resistant PR gene by a single amino acid residue in Gag p2 or $p7^{NC}$. One potential explanation for the discordance in drug sensitivities could be related to the PIs affinity for P-glycoprotein (P-gp). PIs are known substrates of P-gp (Kim et al., 1998; Lee et al., 1998), and recent studies in our lab indicate that different PIs have different affinities for P-gp (Ho et al., unpublished). P-gp acts to pump PIs out of the cell, so a PI with a higher affinity for P-gp will be pumped out at a greater rate, resulting in a lower intracellular concentration of that PI. Although RTV and IDV have the same mechanism of action and bind in the active site of PR, changes in Gag-PR can alter sensitivity to one PI, but not the other, suggesting that Gag-PR interactions outside the active site may represent novel drug targets. Directing a drug to a region outside the active site that interferes with protein-protein interactions may produce an alternative inhibitory effect. Susceptibility of pretherapy PR to inhibitor was enhanced significantly by single amino acids in $p6^{Gag}$ or $p6^{Pol}$. Furthermore, differences between the pretherapy virus and Virus 12 in IC_{50} values particularly for RTV indicate that additional residues in the $p6^{Gag}/p6^{Pol}$ region contribute to PI sensitivity. The $p6^{Gag}/p6^{Pol}$ sites examined in our study were chosen based on known functional activity and were sufficient to restore replicative capacity to the posttherapy virus. Yet, ten additional amino acid residues in Gag or Gag-Pol differed between the pretherapy virus and Virus 12, which enhanced susceptibility to PI by wild type PR. These data underscore the necessity to examine regions upstream of PR for predictive sequences that, in combination with PR, will enhance genotype predictions and modulate phenotypic assays of drug susceptibility.

Gag proteins, particularly in $p7^{NC}$ and $p6^{Gag}$, are multifunctional proteins that modulate multiple facets of the virus life-cycle and interactions with host cell proteins (Andersen et al., 2006; Berthoux et al., 1997; Buckman et al., 2003; Burnett and Spearman, 2007; Dorfman et al., 1993; Elder et al., 2000; Goodenow et al., 2002; He et al., 1995; Jowett et al., 1995; Poon et al., 1997; Poon et al., 1996; Popov et al., 2008; Re et al., 1995; Strack et al., 2003; Thomas et al., 2006; von Schwedler et al., 2003). While mechanisms for increased sensitivity to inhibitors by drug-associated changes in Gag could be mediated through changes in diverse Gag functions, the predicted consequences would be diminished fitness, rather than hypersensitivity to drugs (Gatanaga et al., 2002). We favor a model that more directly explains the results: Gag-PR is a functional unit, and interactions between Gag and PR are required for PR processing of Gag in virion maturation. The functional interactions between Gag and PR predict that mutations in Gag would cause conformational changes that modulate interactions between active site and substrate. Taken together, our results define a dominant effect by Gag on fitness and drug resistance, as well as a functional interrelationship among the regions of Gag-PR, and suggest that novel therapeutics could be designed to disrupt these interactions.

Methods

Construction of replication competent gag-pol recombinant viruses

Replication competent recombinant viruses containing selected *gag-pol* alleles were constructed as described previously (Rose, 2002). Briefly, the molecular clone pLAI.2 (Peden et al., 1991) was modified by site-directed mutagenesis to remove the SpeI restriction site at nucleotide position 7 to produce the molecular clone pLAI.4. pLAI.4 was then digested with the restriction enzymes SpeI (HIV_{LAI} position 1553, New England Biolabs, Inc, Beverly, MA, NEB) and Bstz17I (HIV_{LAI} position 3011, NEB) to remove the LAI *gag-pol* region, and a 1.5 kb fragment amplified from patient samples was inserted into the pLAI.4 backbone. Recombinant viruses were made with *gag-pol* alleles obtained from an HIV-infected pediatric subject (D1) enrolled in a protocol to examine the impact of HIV-1 genotype on therapy outcome. The *gag-pol* alleles were isolated from serial blood samples obtained over 7 years before therapy initiation (pretherapy) and after the development of multiple drug resistance following 77 weeks of initial combination therapy including ritonavir (RTV) and an additional 16 weeks of treatment with indinavir (IDV)-containing antiretroviral therapy (posttherapy). The pretherapy allele was referred to previously as D1.10 (Barrie et al., 1996; Goodenow et al., 2002). Subject D1 is currently alive after 18 years of infection.

Production of virus stocks and PBMC infections

Virus stocks were generated by transfection of HEK293 cells (Graham et al., 1977) as described previously (Goodenow et al., 2002), and $TCID_{50}$ values were calculated using the Spearman–Karber method (Division of AIDS, 1997). For parallel infections, phytohemaglutinin (PHA)-stimulated PBMC were infected with 1500 $TCID_{50}$ of virus stock per 1.2×10^6 cells (Goodenow et al., 2002). Supernatants (20 µL aliquots) were harvested every 2 days for 10 days, concurrent with a 10% media change. Supernatant p24 antigen levels were determined by the HIV-1 p24 Antigen Assay ELISA (Beckman Coulter, Fullerton, CA) following the manufacturer's protocol. For competition experiments that consisted of 5 one-week passages, 5×10^6 PBMC were infected with two viruses, each at 2000 $TCID_{50}$. At the end of each passage, supernatant p24 antigen levels were determined, DNA was extracted from the cells for sequence analysis, and 1 ml of syringe filtered (0.45 µm Acrodisc) supernatant was used to infect a new batch of PBMC from the same donor.

Sequence analysis

Cells were lysed with Proteinase K (Fisher Scientific, Pittsburgh, PA), and the DNA was isolated using the QiaAmp® DNA Blood Mini Kit (Qiagen) following manufacturer's protocol. The *gag-PR* region was amplified using two rounds of PCR. First round amplification was performed with forward primer Gag 7 (5'-GTTAAAAGAGACCATCAAT-3', nucleotides 1389–1407) and reverse primer Pol 4 (5'-TCCTACATA-CAAATCATCC-3', nucleotides 3101–3119), followed by second round with nested primers G100 (forward, 5'-TAGAAGAAATGATGACAG-3', nucleotides 1817–1834) and Pol I (reverse, 5'-ACTTTTGGGCCATC-CATTCCTGGC-3', nucleotides 2588–2611). Amplifications were performed in a 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) and consisted of an initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, with a final elongation for 10 min at 72 °C. Amplified products were cloned using the TOPO TA Cloning® Kit (Invitrogen) and transformed into TOP10F' competent cells (Invitrogen). Recombinant plasmids were purified using the QIAprep DNA Miniprep Kit (Qiagen), and the presence of an insert was confirmed by restriction digest. Sequences were prepared with DYEnamic ET dye terminator cycle sequencing kit for MegaBACE DNA Analysis Systems (GE Healthcare, Chalfont St. Giles, United Kingdom) and run on a MegaBACE 1000 (GE Healthcare) in the Genome Sequencing Service Laboratory at the University of Florida.

Site directed mutagenesis

To prepare for mutagenesis, a 1.7 kb *gag-pol* fragment was digested from the pLAI.4-posttherapy *gag-pol* recombinant virus using the restriction enzymes SphI (NEB) and EcoRV (NEB) and ligated into the $pGEM5Zf^+$ cloning vector (Promega, Madison, WI) that was previously digested with SphI and EcoRV and treated with calf intestinal alkaline phosphatase (CIAP, Promega). Following transformation of DH5α cells (Invitrogen, Carlsbad, CA), ten clones were picked, grown overnight at 37 °C, and plasmid DNA was extracted using a QIAprep™ Miniprep Kit

Table 1
Mutagenesis primers

Primer name	HXB2 positions	Primer sequence (5'–3')
CSITE1	1979–2013	GCACCAATTCACAGATCATAATGATGCAGAAAGGC
NC	2052–2076	TTGTGGCAAAGAGGGGCACATAGCC
A	2355–2388	CGACCCCTCGTCACAATAAAGATAGGGGGACAGC
B	2421–2464	GATACAGTATTAGAAGAAATGACTTTGACAGGAAGATGGAAACC
C	2475–2517	GGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGG
D	2562–2593	GTAGGACCTACACCTGTCAACATAATTGGAAG
E	2161–2199	GCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGG
F	2304–2335	GATAGGCAAGGAACTGTATCCCTTAGCTTCCC
G	2323–2352	CCCTTAGCTTCCCTCAAATCACTCTTTGGC

(Qiagen, Valencia, CA) following the manufacturer's protocol. Insertion of the *gag-pol* fragment was confirmed by restriction digest using SpeI and BstZ17I.

The QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and primers in Table 1 were used to introduce specific mutations into the *gag-pol* region. Primer CSITE1 was used to convert a valine to an isoleucine in p2 (within the p2/p7^{NC} cleavage site) (Virus 1), and primer NC1 was used to mutate valine to glutamic acid in p7^{NC} (Virus 2). To produce Virus 3, primer NC1 was used on Virus 1.

Additional mutations were introduced into the protease region of Virus 3. Primer A was used to mutate position 10 from isoleucine to leucine and position 15 from valine to isoleucine. Primer B was designed to mutate position 34 from glutamine to glutamic acid, position 36 from isoleucine to methionine, and position 37 from asparagine to threonine. Primer C mutated position 54 from alanine to isoleucine and position 58 from glutamic acid to glutamine (Virus 4), and Primer D changed position 82 from alanine to valine (Virus 5). Primers A and D were combined in the same reaction to produce Virus 6, primers B and C together produced Virus 7, while Virus 8 was produced by using primers A and D on Virus 7.

The construct which contained both the p2 and p7^{NC} mutations and all 8 protease mutations (Virus 8) was then used as the backbone for mutagenesis of p6^{Gag} and p6^{Pol} using primers E, F, and G. Primer E was used to mutate the fifth amino acid of p6^{Pol} from asparagine to aspartic acid (Virus 9). Primer F mutated a proline to a leucine in the AIP-1 binding site in p6^{Gag} (Virus 10) and Primer G changed an arginine to a lysine in the VPR binding site in p6^{Gag} (Virus 11). All three mutations were combined in the genome of Virus 12. The mutations in the codons were engineered to change selectively the amino acids in either the Gag or Pol reading frame without changing the alternative reading frame.

The mutagenic primers, reaction buffer, and QuikChange® Multi enzyme blend were added to the pGEM5Zf^{+} template DNA with appropriate target sequences following manufacturer's protocol. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 65 °C for 9 min. Mutagenesis was confirmed by sequencing using ABI Prism BigDye terminator cycle sequencing and ABI Prism 377XL automated sequence instrumentation (Perkin-Elmer, Wellesley, MA). *Gag-pol* regions were digested from pGEM5Zf^{+} with SpeI and Bstz17I and ligated into the pLAI.4 backbone for construction of replication competent virus stocks, as described above. Sequences have been submitted to GenBank with accession numbers pending.

Determination of IC$_{50}$

Indinavir and ritonavir were obtained from the NIH AIDS Research and Reference Reagent Program (McKesson BioServices Corporation, Germantown, MD). Following PBMC stimulation with PHA and virus infection, IDV or RTV was added to the culture to achieve final concentrations ranging between 10^1 and 10^5 nM. IDV or RTV was added during media changes to maintain PI concentration for the entire length of the infection. Virus replication was determined by measuring supernatant p24 antigen and IC$_{50}$ values were calculated with a nonlinear regression analysis using the GraphPad Prism 4.03 software package (GraphPad Software, Inc., San Diego, CA).

Statistical analysis

Statistical analysis was performed using SigmaStat 3.0 software (Jandel Scientific Corp, San Rafael, CA). Differences between the replication of pre-and posttherapy viruses were tested with t-tests. A P value of <0.05 was considered significant.

In vitro transcription/translation for Gag-Pol processing

The LAI *gag-pol* sequence was amplified using forward primer 5'-GGTTTCTCGAGCGGAGGCTAGAAGGAGAGAGA-3', and reverse primer 5'-TTCCCTCTAGACCCCTAGCTTTCCCTGAAACA-3' engineered to insert an XhoI restriction site at the 5' end and an XbaI at the 3' end of the sequence for directional cloning into the expression vector TNT. A region in LAI *gag-pol* extending from SpeI in p24^{CA} to BstZ17I in reverse transcriptase (nucleotides 1507 to 2924) was exchanged with the SpeI to BstZ17I *gag-pol* fragments. To obtain efficient production of the Gag-Pol polyprotein, a continuous *gag-pol* open reading frame was created by site-directed mutagenesis (Stratagene) to reproduce exactly the amino acid sequences of the Gag-Pol proteins found in virions. The primers used were: forward 5'-GAGAGACAGGC-TAACTTCCTCCGCGAAGACTTGGCCTTCCTACAAGGG-3' and reverse 5'-CCCTTGTAGGAAGGCCAAGTCTTCGCGGAGGAAGTTAGCCTGTCTCTC-5' and the products were verified by sequencing.

For *in vitro* transcription/translation reactions, purified plasmid DNA template (600 ng/μl) was mixed with a solution containing 20 μl TNT T7 Quick Master Mix (Promega) that contained rabbit reticulocte lysate and a mixture of all the amino acids except methionine, 1 μl ^{35}S-Met (1000 Ci/mmol at 10 mCi/ml) and nuclease-free water up to 25 μl final volume, and incubated at 30 °C. PIs were added at a final concentration of 10 μM. Samples were taken at 0 and 4 h, quenched with 2× Laemmli Sample Buffer (BioRad), heated for 3 min at 85 °C and run on a precast 10–20% Tris-HCl SDS-PAGE Criterion gel (BioRad). Gels were fixed, soaked in 10% glycerol solution for 5 min, dried, and exposed to either a XAR-5 film (Kodak) or a phosphor-screen at room temperature for 12 to 24 h. Amount of labeled protein was quantified by scanning the screens using a Storm 860 Molecular Dynamics PhosphorImager.

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