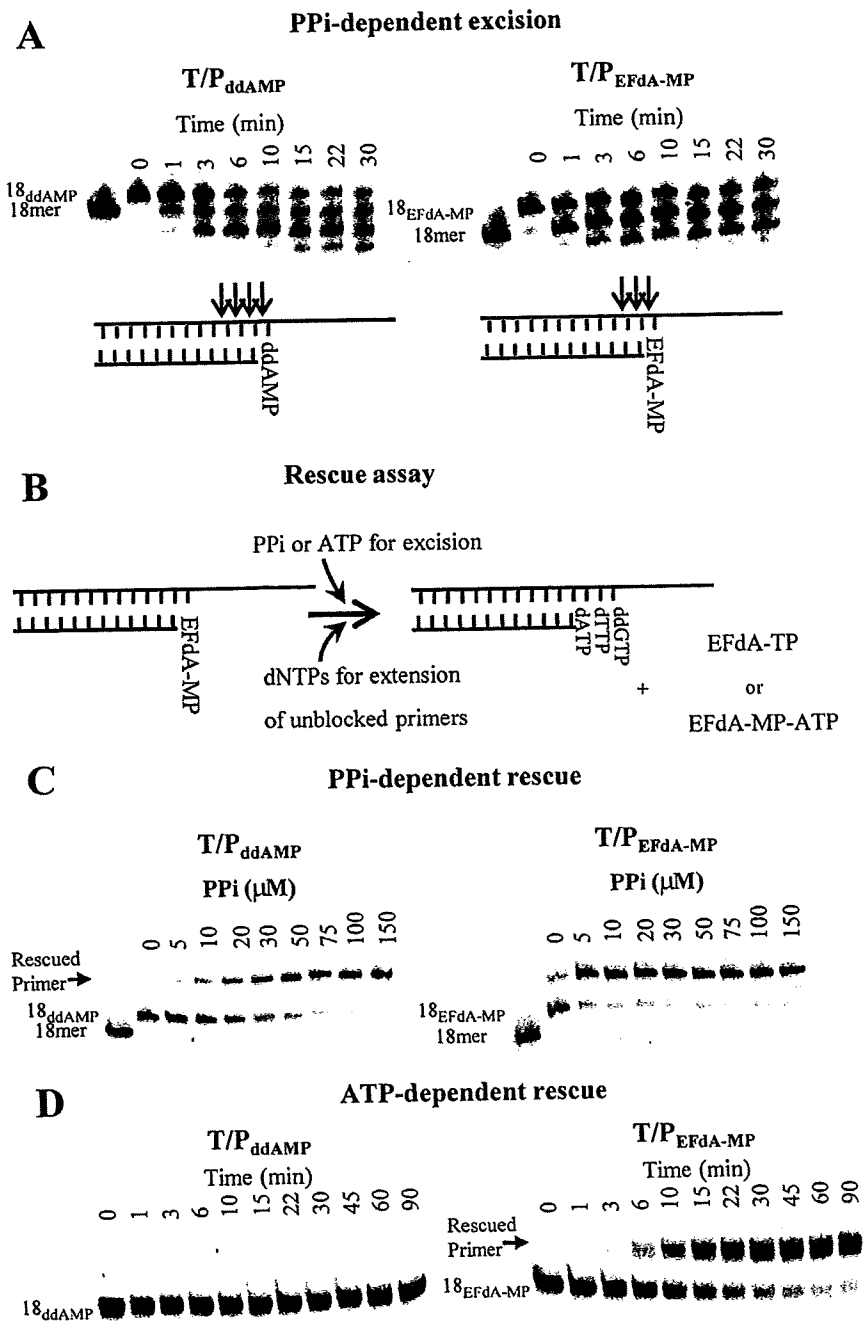

FIGURE 6. **PP_i and ATP-dependent unblocking of ddAMP and EFdA-MP terminated primers.** *A*, PP_i-dependent unblocking of T/P_{ddAMP} and $T/P_{EFdA\text{-}MP}$. Purified T/P_{ddAMP} or $T/P_{EFdA\text{-}MP}$ was incubated with HIV-1 RT in the presence of 6 mM $MgCl_2$ and 150 μM PP_i at 37 °C. Aliquots were removed and reactions stopped at the indicated time points (0–30 min). Cleavage sites are indicated with *arrows* in the schemes below the gels. *B*, schematic representation of PP_i- and ATP-dependent rescue assay. The excision products of PP_i- and ATP-dependent excision of EFdA-MP are EFdA-TP and the EFdA-MP-ATP dinucleoside tetraphosphate, respectively. *C*, PP_i-dependent rescue of T/P_{ddAMP} and $T/P_{EFdA\text{-}MP}$. Purified $T/P_{EFdA\text{-}MP}$ or T/P_{ddAMP} was incubated with HIV-1 RT in the presence of various amounts of PP_i (0–150 μM), dATP (100 μM), dTTP (0.5 μM), or ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped after 10 min. *D*, ATP-dependent rescue of T/P_{ddAMP} or $T/P_{EFdA\text{-}MP}$. Purified $T/P_{EFdA\text{-}MP}$ or T/P_{ddAMP} was incubated with HIV-1 RT in the presence of ATP (3.5 mM), dATP (100 μM), dTTP (0.5 μM), or ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped at the indicated time points (0–90 min).

has to date been unclear. The detailed *in vitro* biochemical studies presented in this article show that EFdA inhibits HIV-1 reverse transcriptase mainly by blocking translocation after incorporation at the 3′-primer end and functioning as a TDRTI. The specificity of inhibition can vary depending on the type and sequence of the template (Fig. 2). Our studies also suggest that both the 3′-OH and the 4′-ethynyl groups of EFdA play important roles in the high antiviral potency exerted by this nucleoside analog.

The 4′-ethynyl group is essential for the mechanism of EFdA inhibition of RT-catalyzed DNA synthesis. The present work shows that EFdA-TP acts mainly as a potent terminator of RT-catalyzed DNA synthesis, despite having a 3′-OH group. It is possible that the presence of a 4′-ethynyl substitution on the ribose ring somehow affects the geometry and reactivity of its 3′-OH. However, in the presence of physiological concentrations of dNTPs (<50 μM) the chain terminating activity of EFdA appears to arise mainly from the difficulty of RT to translocate the 3′-EFdA-MP-terminated primer ($T/P_{EFdA\text{-}MP}$) following incorporation of the inhibitor. Under these circumstances the dNTP-binding site is not accessible, and incorporation of the next complementary nucleotide is prevented (Fig. 7*C*). Hence, EFdA appears to act as a translocation-defective RT inhibitor. The 4′-ethynyl moiety appears to be the critical factor in the difficulty presented by translocation of DNA primers with a 3′-terminal EFdA-MP residue. Our molecular model of the RT·DNA·EFdA-TP ternary complex suggests that the 4′-ethynyl moiety fits nicely into a hydrophobic pocket in the RT active site defined by residues Ala-114, Tyr-115, Phe-160, and Met-184 and the aliphatic portion of Asp-185 (Fig. 7*A*), similar to the proposed interactions of 4′-Ed4T at the same site (32). Once EFdA-MP has been added to the primer end to form the pre-translocation (or N-site) reaction product, these same RT residues serve to stabilize the terminal EFdA-MP in

clinically used NRTIs (Table 2 and Ref. 23), consistent with the inhibitory data obtained in transformed T-cell lines (13). The molecular basis for this exceptional antiviral activity of EFdA

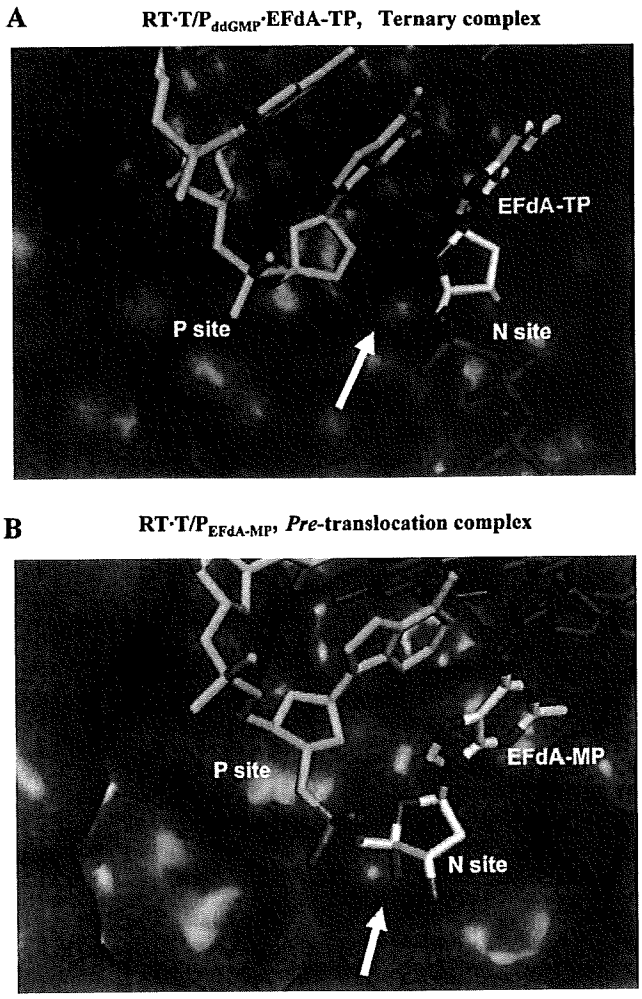

FIGURE 7. **Molecular models representing intermediates of the DNA polymerization reaction.** *A*, molecular model of a ternary complex among RT, DNA, and EFdA-TP. The primer is bound at the P-site, and the incoming EFdA-TP is bound at the N-site. The 4′-ethynyl group of EFdA-TP is bound at a hydrophobic pocket (shown by a *yellow arrow*) defined by residues Met-184, Ala-114, Tyr-115, and Phe-160 and the aliphatic chain of Asp-185. For purposes of clarity the p66 fingers subdomain is not shown. *B*, molecular model of RT bound to EFdA-MP-terminated T/P immediately after incorporation of the inhibitor at the primer terminus and before translocation. The EFdA-MP of the 3′-primer terminus is positioned at the N-site. *C*, schematic representation of RT inhibition by EFdA. After incorporation of EFdA-MP at the 3′-primer terminus RT remains bound to T/P$_{EFdA}$ mostly in a pre-translocation binding mode (*top*). In that binding mode the EFdA-MP at the 3′-primer terminus blocks binding of the incoming dNTP, thus inhibiting DNA polymerization.

the pre-translocation state (Fig. 7*B*). Hence, the stabilization of the primer 3′-terminal EFdA-MP in the pre-translocation state helps it to remain in a position antagonistic to further nucleotide addition and to inhibit DNA polymerization (Fig. 7*C*).

We have observed that in one instance RT stopped not only at the point of EFdA incorporation but also at the position following (Fig. 2*A*, positions *6* and *7*, respectively). Interestingly, we found that RT has enhanced translocation efficiency at this site, both on T/P$_{EFdA-MP}$ and on T/P$_{ddAMP}$ (data not shown). It appears that some translocated T/P$_{EFdA-MP}$ is also elongated by an additional nucleotide. Further polymerization may be inhibited by unfavorable interactions between the 4′-ethynyl group in the elongated template/primer (T/P$_{EFdA-MP-dNMP}$) with protein residues upstream in the DNA-binding cleft. The effect of template on the inhibition mechanism by EFdA is the subject of an ongoing investigation.

RT-catalyzed phosphorolytic excision of chain-terminating NRTIs is a major mechanism of HIV-1 resistance to the nucleoside analog class of therapeutics (26–28, 33). Our previous studies have shown that the NRTI phosphorolytic excision reaction is favored when the primer 3′-terminal nucleotide is in the pre-translocation or N-site (19, 34). The preference of the primer 3′-terminal EFdA-MP to remain in this site suggests that terminal EFdA-MP should undergo facile phosphorolytic removal. EFdA-MP was subject to excision by pyrophosphorolysis somehow faster than ddAMP, which tends to localize in the post-translocation site when at the 3′ terminus of the primer (see Fig. 5*A*). Although EFdA-MP can undergo excision, this process is not overly efficient, apparently because once the nucleotide is excised through pyrophosphorolysis to form EFdA-TP, the latter is rapidly reincorporated. These findings suggest that phosphorolysis may not play a significant role in HIV-1 resistance to EFdA, consistent with the relatively small loss of antiviral potency of EFdA against excision-enhanced HIV-1-containing mutations associated with resistance to AZT (13).

The importance of the 3′-OH in antiviral activity of EFdA is perhaps best highlighted by the observation that EFdA is 10,000-fold more potent at inhibiting HIV-1 replication in PBMCs than is the identical nucleoside lacking a 3′-OH, namely EFddA (Table 2). The 3′-OH on EFdA appears to play a number of roles in contributing to the exceptional antiviral potency of the compound. The 3′-OH on natural dNTP substrates contributes to the efficiency with which RT uses these substrates, and in general NRTIs that lack a 3′-OH are used less efficiently by RT than the base-analogous dNTP (35) (Table 3). Our *in vitro* biochemical data demonstrate that EFdA-TP is approximately 1 and 2 orders of magnitude more potent an inhibitor of RT-catalyzed DNA synthesis *in vitro* than are the

adenine-based NRTIs ddATP and TFV-DP, respectively (Fig. 1*D*), neither of which has a 3′-OH. Indeed, it appears that under identical conditions, RT is at least twice as likely to use EFdA-TP as a substrate over the natural nucleotide dATP (Table 3). This suggests that during HIV-1 reverse transcription, EFdA-TP might be preferentially incorporated, thereby leading to early and profound chain termination and contributing to the observed potent antiviral activity of this nucleoside analog.

NRTIs are administered therapeutically as prodrugs; these must undergo phosphorylation in target cells in order to exert their antiviral activity. The lack of a 3′-OH on current clinically used NRTIs can reduce recognition by cellular nucleoside/nucleotide kinases that have evolved to interact with the 3′-OH present in their natural nucleoside substrates (9). Preliminary studies suggest that EFdA appears to undergo rapid and facile intracellular conversion to the active antiviral EFdA-TP (36), showing that the 4′-ethynyl group does not interfere with recognition by cellular nucleoside/nucleotide kinases. Furthermore, the presence of fluorine at position 2 of the adenine base of EFdA helps to stabilize intracellular levels of EFdA and its phosphorylated products by hindering adenosine deaminase-catalyzed degradation of the molecule (13). Thus, both the 2-fluoro and the 3′-OH of EFdA may contribute to the intracellular accumulation of the antiviral EFdA-TP, thereby leading to its pronounced antiviral activity. We are presently carrying out detailed studies of the intracellular pharmacokinetics of EFdA in comparison with other NRTIs to better understand the dynamics of EFdA phosphorylation and turnover as contributors to the exceptional potency and persistence of its antiviral activity.

Other nucleoside analog inhibitors of HIV-1 RT that possess a 3′-OH have been described (21, 37–42), although these have mechanisms of action quite distinct from that of EFdA. North-methanocarba-2′-deoxyadenosine triphosphate and North-methanocarba-2′-thymidine triphosphate inhibit HIV-1 RT *in vitro* by a mechanism of delayed chain termination, where RT-catalyzed DNA synthesis pauses after the addition of several nucleotides following incorporation of the inhibitor (38, 39). Neither of these compounds has antiviral activity, presumably because of poor intracellular phosphorylation. Entecavir is a nucleoside analog with a 3′-OH that is approved for treatment of hepatitis B infection. Entecavir-TP also has been shown to inhibit HIV-1 RT-catalyzed DNA synthesis by a mechanism of delayed chain termination (40). Entecavir has only weak antiviral activity against HIV-1.

Additionally, nucleoside analogs substituted at the 4′-position have also been described previously (43–46). For example, 4′-azidothymidine and 4′-azidoadenosine both inhibit HIV-1 replication (46) although with potencies 200–2000-fold less than that of EFdA. Both 4′-azido nucleosides also have poor *in vitro* selectivity indices because of significant cytotoxicity. Azidothymidine-TP was shown to inhibit RT-catalyzed DNA synthesis by a type of delayed chain termination; incorporation of two sequential azidothymidine-MP molecules blocked DNA synthesis (44, 45). 4′-Methyl thymidine and 4′-ethyl thymidine both seem to cause pauses and stops in DNA synthesis at the point of incorporation (39). However, neither of these compounds has antiviral activity, because they cannot be phosphorylated by cellular nucleoside kinases. An analog of d4T that has a 4′-ethynyl substitution (Ed4T) is ~10 times more active than the parent compound (47, 48). Because Ed4T lacks a 3′-OH, it inhibits RT as a conventional chain terminator. Interestingly, Ed4T is a better substrate than d4T for phosphorylation by human thymidine kinase 1 (47–50), a property that leads to its increased antiviral potency compared with d4T. The antiviral activity of Ed4T is ~50-fold lower than that of EFdA.

In summary, EFdA is a TDRTI with two functionalities lacking in current therapeutic NRTIs, namely a 4′-ethynyl group and a 3′-OH. These additional properties impart superior antiviral activity to the compound and contribute to its mechanism of action, namely inhibition of primer translocation following EFdA-MP incorporation. This mechanism allows EFdA to act mainly as a *de facto* chain terminator of RT-catalyzed DNA synthesis, despite the presence of a 3′-OH. The present study validates RT nucleic acid translocation as a potential therapeutic target.

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Non-Cleavage Site Gag Mutations in Amprenavir-Resistant Human Immunodeficiency Virus Type 1 (HIV-1) Predispose HIV-1 to Rapid Acquisition of Amprenavir Resistance but Delay Development of Resistance to Other Protease Inhibitors▽

Manabu Aoki,[1,2] David J. Venzon,[3] Yasuhiro Koh,[1] Hiromi Aoki-Ogata,[1] Toshikazu Miyakawa,[1] Kazuhisa Yoshimura,[1] Kenji Maeda,[1,4] and Hiroaki Mitsuya[1,4]*

Departments of Hematology and Infectious Diseases, Kumamoto University Graduate School of Medical and Pharmaceutical Sciences, Kumamoto 860-8556, Japan[1]; Institute of Health Sciences, Kumamoto Health Science University, Kumamoto 861-5598, Japan[2]; and Biostatistics and Data Management Section[3] and Experimental Retrovirology Section, HIV and AIDS Malignancy Branch,[4] Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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In an attempt to determine whether mutations in Gag in human immunodeficiency virus type 1 (HIV-1) variants selected with a protease inhibitor (PI) affect the development of resistance to the same or a different PI(s), we generated multiple infectious HIV-1 clones carrying mutated Gag and/or mutated protease proteins that were identified in amprenavir (APV)-selected HIV-1 variants and examined their virological characteristics. In an HIV-1 preparation selected with APV (33 passages, yielding HIV_{APVp33}), we identified six mutations in protease and six apparently critical mutations at cleavage and non-cleavage sites in Gag. An infectious recombinant clone carrying the six protease mutations but no Gag mutations failed to replicate, indicating that the Gag mutations were required for the replication of HIV_{APVp33}. An infectious recombinant clone that carried wild-type protease and a set of five Gag mutations ($rHIV_{WTpro}^{12/75/219/390/409gag}$) replicated comparably to wild-type HIV-1; however, when exposed to APV, $rHIV_{WTpro}^{12/75/219/390/409gag}$ rapidly acquired APV resistance. In contrast, the five Gag mutations significantly delayed the acquisition of HIV-1 resistance to ritonavir and nelfinavir (NFV). Recombinant HIV-1 clones containing NFV resistance-associated mutations, such as D30N and N88S, had increased susceptibilities to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy. The present data suggest that the preexistence of certain Gag mutations related to PI resistance can accelerate the emergence of resistance to the PI and delay the acquisition of HIV resistance to other PIs, and these findings should have clinical relevance in the therapy of HIV-1 infection with PI-including regimens.

Combination antiretroviral therapy using reverse transcriptase inhibitors and protease inhibitors (PIs) produces substantial suppression of viral replication in human immunodeficiency virus type 1 (HIV-1)-infected patients (3, 27, 28, 42). However, the emergence of drug-resistant HIV-1 variants in such patients has limited the efficacy of combination chemotherapy. HIV-1 variants resistant to all of the currently available antiretroviral therapeutics have emerged both in vitro and in vivo (6, 16, 27, 30). Of note, a number of PI resistance-associated amino acid substitutions in the active site of protease have been identified, and such substitutions have considerable impact on the catalytic activity of protease. This impact is reflected by impaired processing of Gag precursors in mutated-protease-carrying virions and by decreased catalytic efficiency of the protease toward peptides with natural cleavage sites (7, 29, 31, 43).

However, the highly PI-resistant viruses frequently have amino acid substitutions at the p7-p1 and p1-p6 cleavage sites in Gag. These mutations have been identified in PI-resistant HIV-1 variants selected in vitro (2, 5, 8, 29) and in HIV-1 isolated from patients with AIDS for whom chemotherapy including PIs was failing (26, 40, 47, 48). These mutations are known to compensate for the enzymatic impairment of protease, per se, resulting from the acquisition of PI resistance-conferring mutations within the protease-encoding region. Moreover, certain mutations at non-cleavage sites in Gag have been shown previously to be essential for the replication of HIV-1 variants in the presence of PIs (14, 15). Although a few amino acid substitutions at cleavage and non-cleavage sites in Gag have been shown to be associated with resistance to PIs, the roles and impact of amino acid substitutions in Gag for the HIV-1 acquisition of PI resistance remain to be elucidated.

In the present study, we identified novel Gag non-cleavage site mutations in addition to multiple mutations in the protease gene during in vitro selection of HIV-1 variants highly resistant to amprenavir (APV). We show that the non-cleavage site mutations were important for not only the replication of the mutated-protease-carrying HIV-1 but also the accelerated acquisition of HIV-1 resistance to APV and an unrelated PI, nelfinavir (NFV). We also show that recombinant HIV-1 clones containing NFV resistance-associated mutations, such

* Corresponding author. Mailing address: Department of Hematology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Phone: (81) 96-373-5156. Fax: (81) 96-363-5265. E-mail: hm21q@nih.gov.

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as D30N and N88S, had increased susceptibility to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy.

MATERIALS AND METHODS

Cells and antiviral agents. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium, and 293T cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (HyClone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. APV was kindly provided by GlaxoSmithKline, Research Triangle Park, NC. Saquinavir (SQV) and ritonavir (RTV) were provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. NFV and indinavir (IDV) were kindly provided by Japan Energy Inc., Tokyo.

Generation of PI-resistant HIV-1 in vitro. For the generation of PI-resistant HIV-1, various PI-resistant HIV-1 strains were propagated in the presence of increasing concentrations of a drug in a cell-free fashion as described previously (44, 45). In brief, on the first passage, MT-2 or MT-4 cells (5×10^5) were exposed to 500 50% tissue culture infective doses ($TCID_{50}$) of each infectious molecular HIV-1 clone and cultured in the presence of various PIs at initial concentrations of 0.01 to 0.06 μM. On the last day of each passage (approximately day 7), 1 ml of the cell-free supernatant was harvested and transferred to a culture of fresh uninfected cells in the presence of increased concentrations of the drug for the following round of culture. In this round of culture, three drug concentrations (increased by one-, two-, and threefold compared to the previous concentration) were employed. When the replication of HIV-1 in the culture was confirmed by substantial Gag protein production (greater than 200 ng/ml), the highest drug concentration among the three concentrations was used to continue the selection (for the next round of culture). This protocol was repetitively used until the drug concentration reached the targeted concentration. Proviral DNA from the lysates of infected cells at various passages was subjected to nucleotide sequencing.

Determination of nucleotide sequences. Molecular cloning and the determination of nucleotide sequences of HIV-1 passaged in the presence of each PI were performed as described previously (44, 45). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-2 and MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first-round PCR amplification of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5'-GAT GCT ACA TAT AAG CAG CTG C-3') and PR12 (5'-CTC GTG ACA AAT TTC TAC TAA TGC-3'). The first-round PCR mixture consisted of 5 μl of proviral DNA solution, 2.0 U of premix *Taq* (Ex *Taq* version; Takara Bio Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions used were an initial 2-min step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8 min of extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR F2 (5'-GAG ACT CTG GTA ACT AGA GAT C-3') and Ksma2.1 (5'-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3') under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Generation of recombinant HIV-1 clones. The PCR products obtained as described above were digested with two of the three enzymes BssHII, ApaI, and SmaI, and the obtained fragments were introduced into $pHIV\text{-}1_{NLSmu}$, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of $pHIV\text{-}1_{NL4\text{-}3}$ (15, 19). To generate HIV-1 clones carrying the mutations, site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed, and the mutation-containing genomic fragments were introduced into $pHIV\text{-}1_{NLSma}$. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. 293T cells were transfected with each recombinant plasmid by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the thus-obtained infectious virions were harvested 48 h after transfection and stored at −80°C until use.

Drug sensitivity assays. Assays for HIV-1 p24 Gag protein production were performed with MT-4 cells as described previously (1, 20, 24). In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$ of infectious molecular HIV-1 clones in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amounts of p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentrations [IC_{50}]) were determined by comparing the levels of p24 production with that in a drug-free control cell culture. All assays were performed in triplicate.

Replication kinetic assay. MT-2 or MT-4 cells (10^5) were exposed to each infectious HIV-1 clone (5 ng of p24 Gag protein/ml) for 3 h, washed twice with phosphate-buffered saline, and cultured in 10 ml of complete medium as described previously (1, 14). Culture supernatants (50 μl) were harvested every other day, and the p24 Gag amounts were determined as described above.

CHRA. Two titrated infectious clones to be compared for their replicative capabilities or fitness in the competitive HIV-1 replication assay (CHRA) were combined and added to freshly prepared MT-4 cells (2×10^5) in the presence or absence of various concentrations of PIs as described previously (21, 36). Briefly, a fixed amount (200 $TCID_{50}$) of one infectious clone was combined with three different amounts (100, 200, and 300 $TCID_{50}$) of the other infectious clone, and the mixture was added to the culture of MT-4 cells. On the following day, one-third of infected MT-4 cells were harvested and washed twice with phosphate-buffered saline, and cellular DNA was extracted and subjected to nested PCR and sequencing as described above. The HIV-1 coculture that best approximated a 50:50 mixture on day 1 was further propagated, and the remaining cultures were discarded. Every 7 days, the cell-free supernatant of the virus coculture was transmitted to fresh uninfected MT-4 cells. The cells harvested at the end of each passage were subjected to direct DNA sequencing, and viral population changes were determined. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay.

Statistical analysis of selection profiles of infectious HIV-1 clones. The selection profiles of various infectious HIV-1 clones were compared as follows. The logarithms of the concentrations were modeled as normally distributed variables with possible left censoring. The mean was assumed to be a quadratic function of the passage number. The difference between two curves was assessed by combining the estimated covariance-weighted differences of the linear and quadratic coefficients and comparing the result to computer simulations for the same quantity generated under the specific null hypothesis for that difference. SAS 9.1.3 (SAS Institute, Cary, NC) was used for all the computations. All *P* values are two tailed, and for figures with more than two curves, the values were corrected by the Hochberg method for multiple pairwise comparisons.

RESULTS

Amino acid sequences of Gag and protease of HIV-1 passaged in the presence of APV. A wild-type HIV-1 strain (HIV_{WT}) was propagated in MT-2 cells in the presence of increasing concentrations of APV, and the proviral DNA sequences in those MT-2 cells were determined at passages 3, 12, and 33 (Fig. 1). By passage 3, when HIV-1 was propagating in the presence of 0.04 μM APV (yielding HIV_{APVp3}), no amino acid substitutions in protease were identified but 5 of 10 clones had acquired the substitution of arginine for leucine at position 75 (L75R) in Gag. By passage 12 (at 0.18 μM APV), two APV-related resistance mutations (L10F and M46L) in protease had emerged and one mutation (H219Q) in Gag had been added. By passage 33 (at 10 μM; yielding HIV_{APVp33}), six APV-related amino acid substitutions, one primary mutation (I84V) and five secondary mutations (L10F, V32I, M46I, I54M, and A71V), in protease had emerged (Fig. 1A). In addition, a p1-p6 cleavage site mutation in Gag (L449F) was identified in all 10 HIV-1 clones of HIV_{APVp33} examined, and five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) were seen in Gag of HIV_{APVp33} (Fig. 1B). Cleavage site mutations have been known to emerge when amino acid substitutions in protease are accumulated and HIV-1 develops resistance to PIs both in vitro and in vivo (5, 8). Intriguingly, the present data suggest that certain amino acid substitutions in non-cleavage sites of Gag (i.e., L75R and

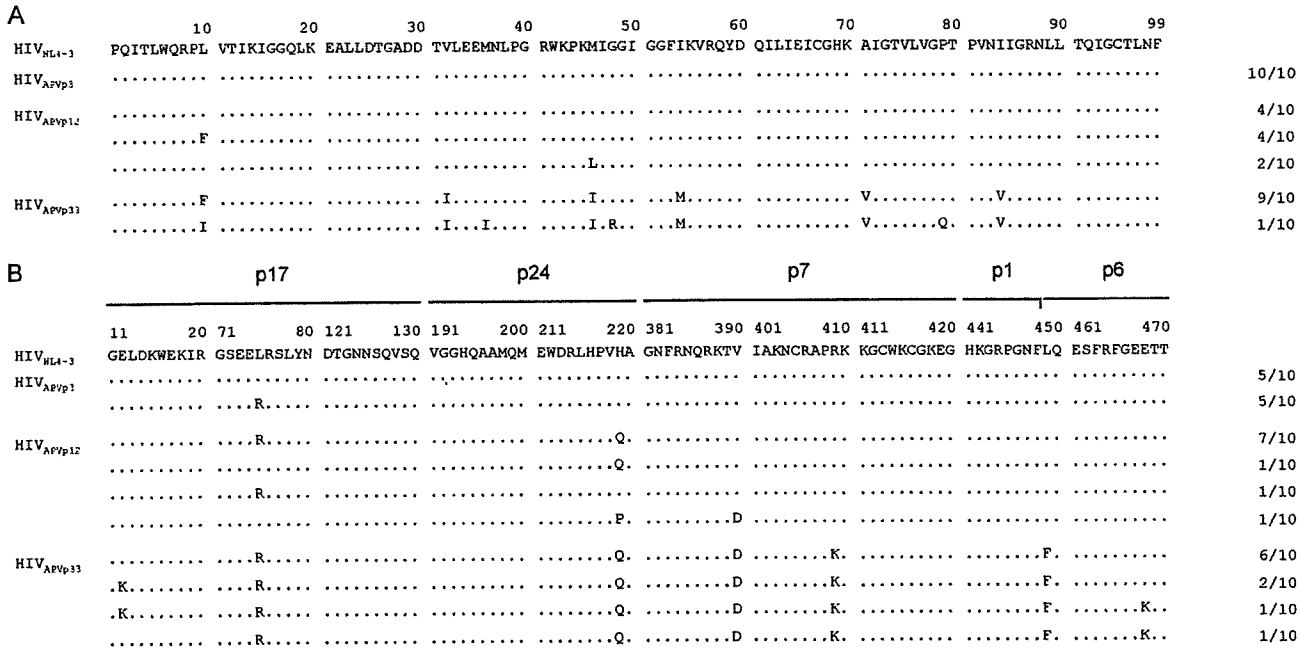

FIG. 1. Amino acid sequences deduced from the nucleotide sequences of protease (A)- and Gag (B)-encoding regions of proviral DNA isolated at the indicated passages (p3, p12, and p33) from HIV-1_{NL4-3} variants selected in the presence of APV. The amino acid sequences of the protease and Gag proteins of wild-type HIV-1_{NL4-3} are shown at the top as a reference. Identity to the sequence at individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 10 clones are listed.

H219Q) may emerge earlier and in greater numbers than amino acid substitutions in protease, at least in the case of HIV-1 selection with APV. The amino acid substitutions that emerged in the virus and the pattern and order of such substitutions were largely in agreement with the data in the previous report by Gatanaga et al. (15). The present results suggested that the non-cleavage site mutations observed may play a key role in the development of HIV-1 resistance against PIs and that especially the two Gag mutations H219Q and R409K may be required for the development of PI resistance.

Mutations in Gag are required for the replication of HIV_{APVp33}. In order to examine the effects of the mutations identified in Gag as described above on the replication profile of HIV-1, we generated infectious recombinant HIV-1 clones containing the six mutations (L10F, V32I, M46I, I54V, A71V, and I84V) in protease seen in HIV_{APVp33}. A recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate in MT-2 cells over the 7-day period of culture (Fig. 2A), indicating that these Gag species do not support the growth of HIV_{APVp33}. Therefore, we next generated a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the Gag protein with the five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K; $rHIV_{APVp33pro}^{12/75/219/390/409gag}$), which replicated moderately under the same conditions (Fig. 2A). The addition of the cleavage site mutation L449F, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, further improved the replication of the virus. In MT-4 cells, in which HIV-1 generally replicates more quickly and efficiently than in MT-2 cells, $rHIV_{APVp33pro}^{WTgag}$ and $rHIV_{APVp33pro}^{449gag}$ replicated moderately; however, both $rHIV_{APVp33pro}^{12/75/219/390/409gag}$ and $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$ replicated comparably to HIV_{WT} (Fig. 2B), due presumably to the greater replication of HIV-1 in MT-4 cells, making the difference relatively indistinct. These data clearly indicate that both non-cleavage site and cleavage site mutations in Gag contribute to the robust fitness of HIV_{APVp33}. We also attempted to examine the effects of combined Gag mutations on the replication of HIV-1 containing wild-type protease and generated three recombinant HIV clones, $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and $rHIV_{WTpro}^{12/75/219/390/409gag}$. The replication rates of these three recombinant clones turned out to be comparable to that of HIV_{WT} when examined in MT-2 and MT-4 cells (Fig. 2C and D), unlike the finding by Doyon and his colleagues that the cleavage site mutation L449F compromised the replication of HIV-1 containing wild-type protease (8).

Gag mutations predispose HIV-1 to rapidly acquire APV resistance. The appearance of two non-cleavage site mutations (L75R and H219Q) in Gag prior to the emergence of mutations in protease (Fig. 1) prompted us to examine whether these two Gag mutations predisposed the virus to the acquisition of APV resistance-associated mutations in protease. We thus attempted to select APV-resistant HIV-1 by propagating HIV_{NL4-3} (HIV_{WT}) and $rHIV_{WTpro}^{75/219gag}$ in the presence of increasing concentrations of APV (Fig. 3). When we compared the selection curves of these two viruses, there was no significant difference (P, 0.53 and 0.65 for propagation in MT-2 and MT-4 cells, respectively). We then examined the effects of two mutated Gag species containing two and five mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and

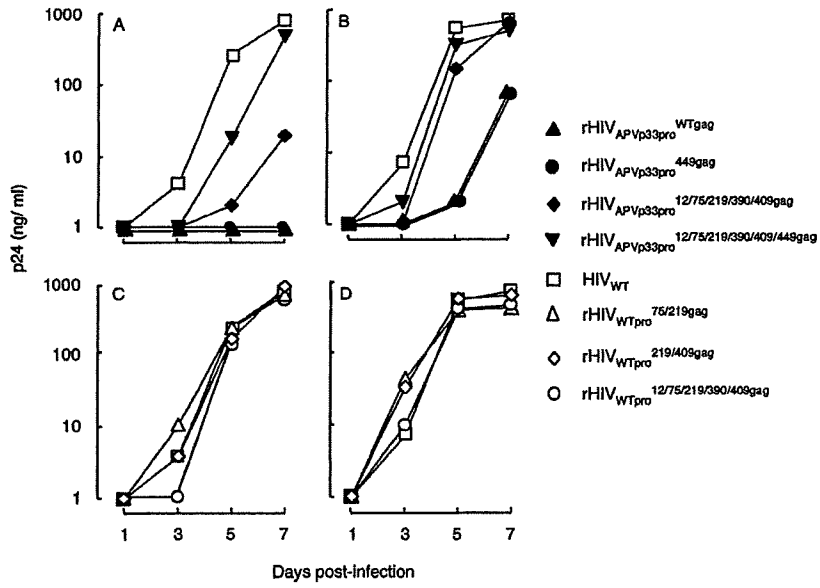

FIG. 2. Replication kinetics of Gag mutant clones with or without protease mutations. MT-2 cells (A and C) and MT-4 cells (B and D) were exposed to Gag mutant clones with (A and B) or without (C and D) protease mutations. Virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. The results shown are representative of results from three independent experiments. HIV$_{APVp33}$ variants had six mutations (L10F, V32I, M46I, I54M, A71V, and I84V) in the viral protease.

R409K [yielding mGag$^{12/75/219/390/409gag}$], respectively) on the selection curves. The selection profile of a newly generated recombinant HIV clone (rHIV$_{WTpro}^{219/409gag}$) was not different from that of HIV$_{WT}$ in MT-2 cells ($P = 0.22$); however, rHIV$_{WTpro}^{219/409gag}$ acquired resistance to APV much earlier than HIV$_{WT}$ when propagated in MT-4 cells ($P < 0.0001$). The recombinant clone with five non-cleavage site mutations (rHIV$_{WTpro}^{12/75/219/390/409gag}$) started to propagate in both cell lines in the presence of APV significantly earlier than HIV$_{WT}$, with P values of 0.0080 and <0.0001 for MT-2 and MT-4 cells, respectively (Fig. 3).

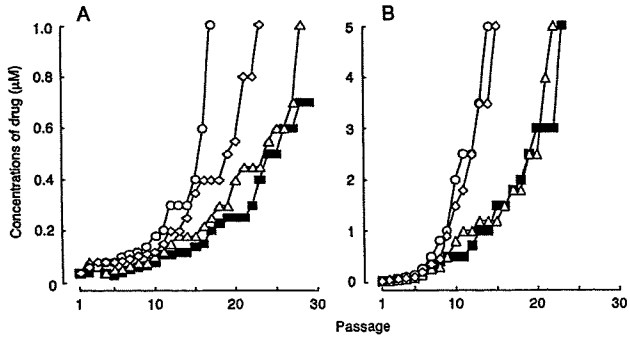

FIG. 3. In vitro selection of APV-resistant variants using HIV-1 carrying Gag mutations. HIV$_{WT}$ (■) and three infectious HIV clones, rHIV$_{WTpro}^{75/219gag}$ (△), rHIV$_{WTpro}^{219/409gag}$ (◇), and rHIV$_{WTpro}^{12/75/219/390/409gag}$ (○), were propagated in the presence of increasing concentrations of APV (starting at 0.03 μM) in MT-2 cells (A) or MT-4 cells (B). The selection was carried out in a cell-free manner for a total of 14 to 29 passages. The results of statistical evaluation of the selection profiles are as follows: panel A, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.53$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0080$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.22$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0065$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.15$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.0018$, and panel B, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.65$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.088$.

We then asked whether additional amino acid substitutions occurred and accelerated the acquisition of APV resistance by the virus when the Gag mutations were present. To investigate this issue, we determined the nucleotide sequence of the protease-encoding gene of each virus. Only one protease mutation (L10F) was seen by passage 20 when HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ were propagated in MT-2 cells in the presence of APV (Fig. 4A and B). In contrast, two mutations (M46L and I84V) had been acquired by rHIV$_{WTpro}^{219/409gag}$ by passage 20. Of note, when rHIV$_{WTpro}^{12/75/219/390/409gag}$ was propagated in MT-2 cells in the presence of APV, a mutation (L10F) had occurred by an early passage (passage 5) and four mutations (L10F, V32I, M46I, and I84V) had emerged by passage 17 (Fig. 4D). When examined in MT-4 cells, HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ had acquired two mutations (L10F and I84V and M46L and I84V, respectively) by passage 10, although rHIV$_{WTpro}^{219/409gag}$ and rHIV$_{WTpro}^{12/75/219/390/409gag}$ had acquired three and four mutations (L10F, M46I, and I84V and L10F, V32I, M46I, and I84V, respectively) by the same passage (Fig. 4E to H). These data, taken together, indicate that the two sets of Gag mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) clearly predisposed the virus to rapidly acquire APV resistance-associated mutations in protease and begin to propagate in the presence of APV.

Gag mutations in HIV$_{APVp33}$ delay viral acquisition of resistance to other PIs. We next asked whether the presence of

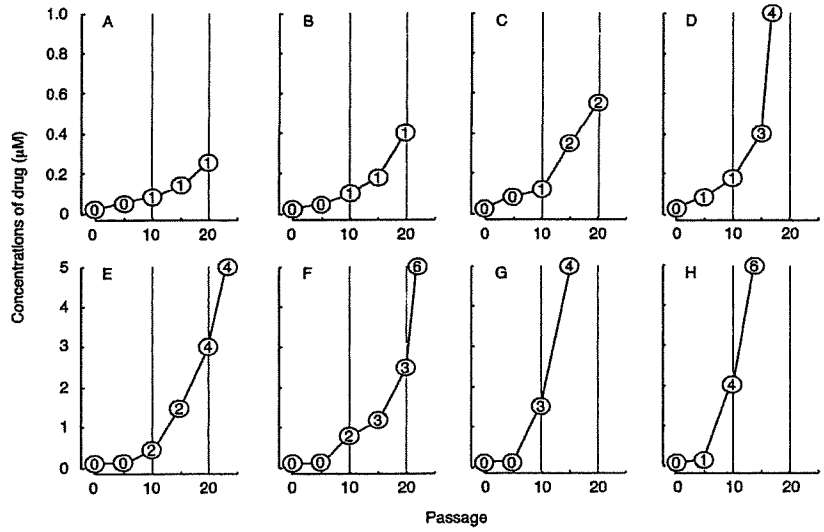

FIG. 4. Number of amino acid substitutions corresponding to the protease-encoding region of each infectious HIV-1 clone selected in the presence of APV. Nucleotide sequences of proviral DNA of HIV_{WT} (A and E) and three infectious HIV-1 clones, $rHIV_{WTpro}^{75/219gag}$ (B and F), $rHIV_{WTpro}^{219/409gag}$ (C and G), and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (D and H), were determined using lysates of HIV-1-infected MT-2 cells (*A* to D) and MT-4 cells (E to H) at the termination of each passage and compared to the nucleotide sequence of $HIV\text{-}1_{NL4\text{-}3}$. The number within each symbol represents the number of mutations identified in the protease when each infectious HIV-1 clone was selected in the presence of APV.

the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) accelerated the viral acquisition of resistance to other currently available PIs (SQV, IDV, RTV, and NFV) (Fig. 5). To this end, we propagated two HIV-1 strains (HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$) in MT-4 cells in the presence of increasing concentrations of each PI and compared the replication profiles. The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by a concentration of up to 5 μM. The selection was carried out in a cell-free manner for a total of 13 to 32 passages as described previously (44, 45). There was no significant difference in the selection profiles of the two strains when they were passaged in the presence of SQV ($P = 0.8$) or IDV ($P = 0.22$) (Fig. 5A and B). However, $rHIV_{WTpro}^{12/75/219/390/409gag}$ started to replicate significantly later in the presence of RTV ($P = 0.0001$ (Fig. 5C). The selection profiles of HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the presence of NFV were examined in two independent experiments. Both curves in the first and second sets depicted in Fig. 5D showed statistically significant difference, with *P* values of <0.0001 and 0.0016, respectively. These data strongly suggest that the Gag mutations seen in HIV_{APVp33} predispose HIV-1 to the rapid acquisition of APV resistance; however, such Gag mutations delay the viral acquisition of resistance to other PIs.

Gag mutations seen in HIV_{APVp33} do not affect viral susceptibilities to PIs. Since the Gag mutations seen in HIV_{APVp33} were found to contribute to the rapid acquisition of viral resistance to APV but they delayed the emergence of viral resistance to other PIs, we examined whether such Gag mutations affected the susceptibilities of HIV-1 to various PIs in the HIV-1 drug susceptibility assay. As shown in Table 1, none of three sets of Gag mutations, as examined in the context of $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and

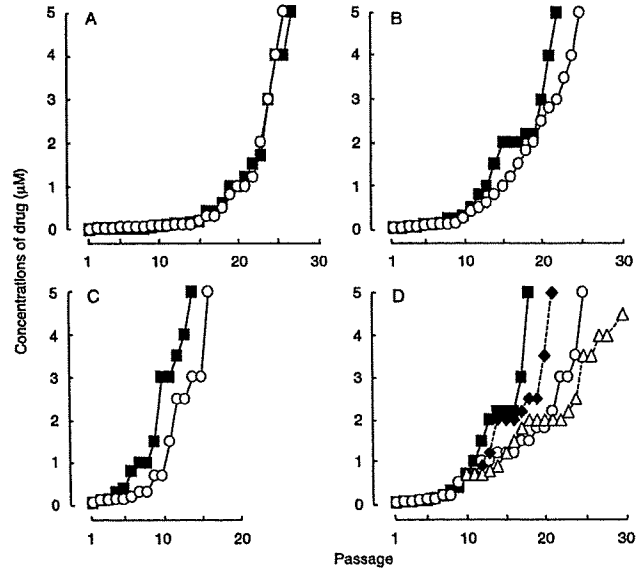

FIG. 5. In vitro selection of PI-resistant variants using HIV-1 carrying Gag mutations. HIV_{WT} (■ and ◆) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○ and △) were propagated in MT-4 cells in the presence of increasing concentrations of SQV (A), IDV (B), RTV (C), or NFV (D). The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by up to a 5 μM concentration of each PI. The selection was carried out in a cell-free manner for a total of 13 to 32 passages. NFV selection was performed twice. Data from the first selection are shown with a solid line; the second selection was started using the HIV-1 from passage 10 of the first selection (with NFV at 0.7 μM), and the data are shown with a dashed line. The results of statistical evaluation of the selection profiles are as follows: panel A, $P = 0.80$; panel B, $P = 0.22$; panel C, $P = 0.0001$; and panel D, first selection, $P < 0.0001$, and second selection, $P = 0.0016$.

TABLE 1. Sensitivities of infectious HIV-1 clones with Gag mutations to various PIs

Infectious HIV-1 clone	IC_{50}[a] (μM) of:				
	APV	SQV	IDV	RTV	NFV
HIV_{WT}	0.031 ± 0.0008	0.021 ± 0.002	0.032 ± 0.002	0.032 ± 0.0005	0.028 ± 0.002
$rHIV_{WTpro}^{75/219gag}$	0.031 ± 0.003	0.017 ± 0.003	0.032 ± 0.003	0.031 ± 0.0007	0.029 ± 0.003
$rHIV_{WTpro}^{219/409gag}$	0.029 ± 0.003	0.020 ± 0.01	0.032 ± 0.001	0.031 ± 0.004	0.028 ± 0.002
$rHIV_{WTpro}^{12/75/219/390/409gag}$	0.032 ± 0.0001	0.023 ± 0.005	0.032 ± 0.003	0.032 ± 0.0001	0.028 ± 0.002

[a] Data shown are mean values (with 1 standard deviation) derived from the results of three independent experiments conducted in triplicate. The IC_{50}s were determined by employing MT-4 cells exposed to each infectious HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point.

$rHIV_{WTpro}^{12/75/219/390/409gag}$, affected the susceptibility of HIV-1 to any of five PIs (APV, SQV, IDV, RTV, and NFV). Indeed, the IC_{50}s for HIV_{WT} were highly comparable to those for any of the three recombinant clones carrying combined Gag mutations.

Replication rate difference is not the cause of the contrasting resistance acquisition patterns. Our observations of the contrasting resistance acquisition patterns, in which $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} when selected with APV (Fig. 3) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs compared to HIV_{WT} (Fig. 5), prompted us to ask whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} were differentially affected by the presence of PIs. We therefore compared the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the presence or absence of APV, SQV, IDV, RTV, or NFV by using the CHRA (21). As shown in Fig. 6, $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the absence or presence of PIs. Comparing the divergence patterns of the curves for $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the absence and presence of APV (Fig. 6A and B) revealed that those for growth in the presence of APV diverged more quickly than those for growth in the absence of APV (Fig. 6B). However, similar divergence patterns were seen with SQV, IDV, RTV, and NFV (Fig. 6C, D, E, and F), suggesting that the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$ seen in the CHRA was not the cause for the observed contrasting resistance acquisition patterns.

NFV resistance-conferring protease mutations increase HIV-1 susceptibility to APV. There have been reports that an

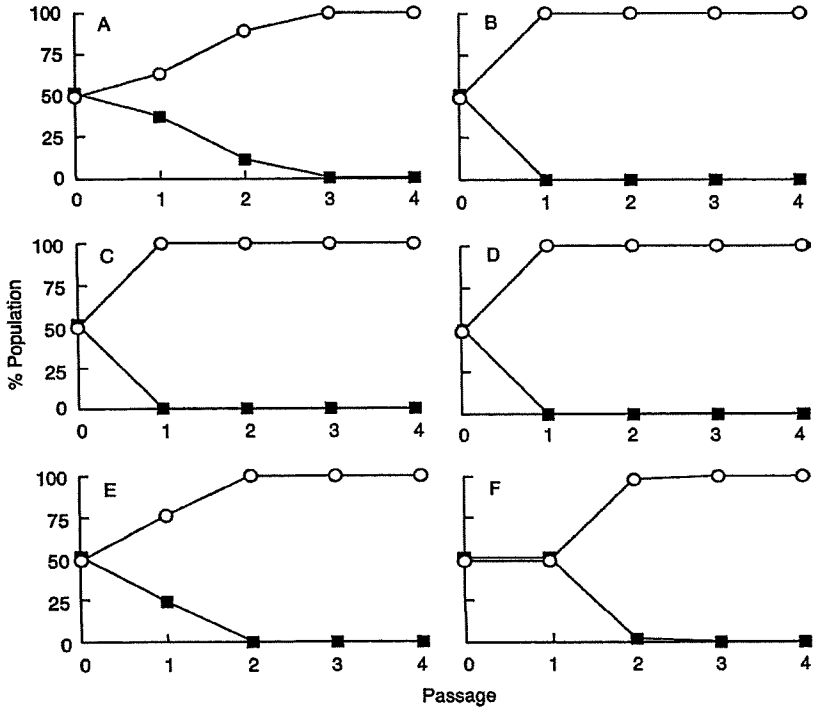

FIG. 6. Results from the CHRA for HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the absence or presence of each drug. Replication profiles of HIV_{WT} (■) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○) in the absence (A) or presence of 0.03 μM APV (B), 0.02 μM SQV (C), 0.03 μM IDV (D), 0.03 μM RTV (E), or 0.03 μM NFV (F) were examined by the CHRA. The cell-free supernatant was transferred to fresh MT-4 cells every 7 days. High-molecular-weight DNA extracted from infected cells at the end of each passage was subjected to nucleotide sequencing, and the proportions of Arg and Lys at position 409 in Gag were determined.

TABLE 2. Phenotypic sensitivities of recombinant HIV-1 clones passaged with NFV[a]

Infectious HIV-1 clone	IC_{50} (μM) ± SD (change, *n*-fold) of:	
	APV	NFV
HIV_{WT}	0.031 ± 0.0008 (1)	0.028 ± 0.002 (1)
$rHIV_{N88Spro}{}^{WTgag}$	0.0015 ± 0.0007 (0.05)	0.028 ± 0.001 (1)
$rHIV_{D30Npro}{}^{WTgag}$	0.0031 ± 0.0001 (0.1)	0.045 ± 0.001 (1.6)
$rHIV_{10/30/45/71pro}{}^{WTgag}$	0.014 ± 0.0021 (0.45)	0.26 ± 0.03 (9)
$rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$	0.020 ± 0.002 (0.64)	0.32 ± 0.03 (11)
$rHIV_{30/46/77pro}{}^{WTgag}$	0.0069 ± 0.0024 (0.22)	0.25 ± 0.04 (9)
$rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$	0.0046 ± 0.0019 (0.15)	0.21 ± 0.06 (8)

[a] Recombinant HIV clones $rHIV_{10/30/45/71pro}{}^{WTgag}$ and $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$ were generated to have a set of four protease mutations (L10F, D30N, K45I, and A71V) and wild-type Gag or Gag with five mutations, while other clones, $rHIV_{30/46/77pro}{}^{WTgag}$ and $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, were generated with three protease mutations (D30N, M46I, and V77I) and wild-type Gag or Gag with five mutations. Both sets of protease mutations were seen when HIV-1 was propagated in the presence of NFV. The IC_{50}s were determined by employing MT-4 cells exposed to each recombinant HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point. All values were determined in triplicate, and the data are shown as mean values ±1 standard deviation of results from two or three independent experiments. The numbers in parentheses are changes (*n*-fold) compared to the IC_{50} of each PI for HIV_{WT}.

NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49). Since the acquisition of viral resistance to PIs such as NFV was significantly delayed when HIV-1 had the Gag mutations seen in HIV_{APVp33}, we asked if another NFV-related resistance mutation (D30N) would render HIV-1 more susceptible to APV. We also asked whether the presence of multiple NFV resistance-associated mutations (D30N, M46I, and V77I) would make HIV-1 susceptible to APV. Moreover, we examined the effects of the Gag mutations seen in HIV_{APVp33} on HIV-1 susceptibilities to APV and NFV.

As shown in Table 2, the N88S mutant clone $rHIV_{N88Spro}{}^{WTgag}$ was more susceptible to APV than HIV_{WT} by a factor of 20, in agreement with the reports by Ziermann et al. and Resch et al. (33, 49). We found that the D30N mutation in $rHIV_{D30Npro}{}^{WTgag}$ also made HIV-1 more susceptible to APV, by a factor of 10. Interestingly, $rHIV_{10/30/45/71pro}{}^{WTgag}$, with the four mutations L10F, D30N, K45I, and A71V, was more resistant to NFV than HIV_{WT} by a factor of 9; however, the recombinant virus remained more susceptible to APV than HIV_{WT} (Table 2). The introduction of the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) into $rHIV_{10/30/45/71pro}{}^{WTgag}$, generating $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$, did not change the susceptibility profile (Table 2). Another recombinant HIV-1 clone with three protease mutations (D30N, M46I, and V77I), $rHIV_{30/46/77pro}{}^{WTgag}$, was also more resistant to NFV (by a factor of 9) and more susceptible to APV than HIV_{WT}. The introduction of the five Gag mutations, generating $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, did not affect the susceptibility of $rHIV_{30/46/77pro}{}^{WTgag}$ to APV or NFV (Table 2).

Taken together, the data suggest that, as seen in the case of the lamivudine (3TC) resistance-associated mutation M184V that restores zidovudine (ZDV) sensitivity (37), NFV resistance-associated mutations paradoxically render HIV-1 more susceptible to APV.

DISCUSSION

Certain amino acid substitutions in Gag are known to occur in common with resistance to PIs (11, 15, 32, 36); however, no salient features such as patterns and orders of the occurrence have been identified for a number of amino acid substitutions seen in Gag in PI-resistant HIV-1 variants. The roles and impact of such amino acid substitutions in Gag for the replication of HIV-1 have not been delineated, either. These limitations have been worsened since the functions and tertiary structures of entire HIV-1 Gag proteins remain to be determined, although some structures of certain parts of Gag proteins have been lately elucidated (13, 34, 41).

In the present study, we attempted to determine the effects of non-cleavage site mutations in Gag which emerged during the in vitro selection of HIV-1 in the presence of APV on the viral acquisition of resistance to APV and other currently existing PIs. When we selected HIV-1 in vitro in the presence of increasing concentrations of APV, six amino acid substitutions apparently critical for the development of APV resistance emerged. Such substitutions included five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) and one cleavage site mutation, L449F (Fig. 1B).

HIV-1 variants containing PI resistance-conferring amino acid substitutions in protease plus wild-type Gag often have highly limited replicative abilities (7, 31). Indeed, in the present study, the recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}{}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}{}^{449gag}$) failed to replicate in MT-2 cells (Fig. 2A), indicating that neither of the two Gag species supported the growth of HIV_{APVp33}. However, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the five Gag non-cleavage site mutations, $rHIV_{APVp33pro}{}^{12/75/219/390/409gag}$, replicated moderately under the same conditions (Fig. 2A), an observation in agreement with reports by others that some PI resistance-associated mutations compromise the catalytic activity of protease and/or alter polyprotein processing, often leading to slower viral replication (29, 36, 43). Since some of the five non-cleavage site mutations emerged before mutations in protease developed, we examined the effects of three sets of non-cleavage site amino acid mutations upon the emergence of APV resistance. Interestingly, HIV-1 with either of two sets of Gag mutations ($rHIV_{WTpro}{}^{219/409gag}$ and $rHIV_{WTpro}{}^{12/75/219/390/409gag}$) acquired APV resistance significantly faster than HIV_{WT} (Fig. 3), while such mutations alone did not alter the susceptibilities of HIV to the PIs examined (Table 1), a finding providing the first report that Gag mutations expedite the emergence of PI-resistant HIV-1 variants. At this time, it is apparently unknown whether certain Gag mutations associated with viral resistance to PIs persist when highly active antiretroviral therapy (HAART) regimens including a PI(s) are interrupted or changed to regimens containing no PIs. However, the non-cleavage site mutations in Gag examined in this study did not reduce the viral fitness (Fig. 2 and 6), suggesting that Gag mutations may persist longer in circulation and/or in the HIV-1 reservoir in the body than mutations in protease when antiretroviral therapy including a PI(s) is interrupted. Such persisting Gag mutations may enable HIV-1 to rapidly acquire resistance

to that very PI when treatment with the PI is resumed. It is of note that on the other hand, two sets of Gag non-cleavage site mutations seen in HIV_{APVp33} (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) significantly delayed the emergence of resistance to other PIs such as RTV and NFV (Fig. 5). These data suggest that if a HAART regimen including APV is changed to an alternative regimen, the inclusion of a different PI in the alternative regimen is likely to delay the emergence of resistance to the different PI.

It is known that the L449F cleavage site mutation renders recombinant HIV-1 carrying a protease mutation (I50V) more resistant to APV (25). In the present study, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate (Fig. 2A). These data strongly suggest that the L449F mutation alone prevents HIV_{APVp33} from replicating, although HIV_{APVp33} did not contain the I50V mutation. The observation in the present study that the addition of five non-cleavage site mutations to $rHIV_{APVp33pro}^{449gag}$, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, restored the replicative ability of the virus indicates that the presence of non-cleavage site Gag mutations plays an important role in the replication of APV-resistant HIV-1 variants.

Since $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} (Fig. 3), while $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs (Fig. 5), we examined whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} were associated with the observed contrasting resistance acquisition patterns by using the CHRA (21). We found that $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the presence or absence of PIs (Fig. 6), suggesting that the difference in the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} was not the cause for the contrasting resistance acquisition patterns. As for the reason why $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew HIV_{WT}, it is well explained by the presence of the H219Q mutation. His-219 is located within the cyclophilin A (CypA) binding loop of p24 Gag protein. It is thought that CypA plays an essential role in the HIV-1 replication cycle (4, 35) by destabilizing the capsid (p24 Gag protein) shell during viral entry and uncoating (12) and/or by performing an additional chaperone function, thus facilitating correct capsid condensation during viral maturation (17, 39). CypA is also known to support the replication of HIV-1 by binding to the Ref-1 restriction factor and/or TRIM5α, the human cellular inhibitors that impart resistance to retroviral infection (18, 38). It has also been demonstrated previously that the effect of CypA on HIV-1 replicative ability is bimodal: both high and low CypA contents limit HIV-1 replication (14). We have demonstrated previously that certain human cells, such as MT-2 and H9 cells, contain large amounts of CypA (14). We have determined more recently that MT-2 cells contain more CypA by about fivefold and that MT-4 cells contain about three times more than peripheral blood mononuclear cells (PBMCs) (unpublished data). In fact, HIV-1 produced in MT-4 cells contains large amounts of CypA, presumably resulting in compromised replication of the HIV-1. However, the H219Q mutation apparently reduces the incorporation of CypA into the virions through significantly distorting the CypA binding loop and restores the replicative ability of virions produced in MT-4 cells (14). Therefore, H219Q should contribute at least in part to the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$. It is noteworthy that of 156 different HIV-1 strains whose sequences were compiled in the *HIV Sequence Compendium 2008* (22), 95 and 45 strains had histidine and glutamine, respectively, at position 219. Hence, position 219 is a polymorphic amino acid site, and it is thought that this polymorphic position is associated with the acquisition of resistance to certain PIs. Indeed, we have observed that $rHIV_{WTpro}^{219gag}$ overgrew $rHIV_{WTpro}^{WTgag}$ in the CHRA using fresh phytohemagglutinin-stimulated PBMCs (14). Since H219Q confers a replication advantage on HIV-1 in PBMCs, it is likely that HIV-1 with H219Q may acquire resistance more rapidly than HIV-1 without H219Q.

Two groups, Ziermann et al. and Resch et al., have reported that an NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49), and indeed, Zachary et al. have reported an anecdotal finding that the infection of an individual with HIV-1 containing N88S was successfully managed with an ensuing APV-based regimen (46). Therefore, we examined the effect of another NFV resistance-associated mutation, D30N, in addition to that of the N88S mutation on HIV-1 susceptibility to APV. It was found that the mutations (D30N and N88S) clearly increased the susceptibility of HIV-1 to APV by 10- and 20-fold, respectively. These data are reminiscent of the observation that the 3TC resistance-associated mutation M184V in a background of mutations conferring resistance to ZDV restores ZDV sensitivity (37) and that ZDV-3TC combination therapy has proven to be more beneficial than ZDV monotherapy in patients harboring HIV-1 with the M184V mutation (9, 23), although the structural mechanism of the restoration of ZDV sensitivity by M184V is not clear. When a set of four protease mutations (L10F, D30N, K45I, and A71V), which had emerged by passage 10 when HIV_{WT} was selected with NFV, were introduced into HIV_{WT}, generating $rHIV_{10/30/45/71pro}^{WTgag}$, the recombinant HIV-1 clone was more resistant to NFV than HIV_{WT} by a factor of 9 while the clone was slightly more sensitive to APV (Table 2). When we introduced $mGag^{12/75/219/390/409gag}$ into HIV-1 containing a set of three NFV resistance-associated protease mutations (D30N, M46I, and V77I), generating $rHIV_{30/46/77pro}^{12/75/219/390/409gag}$, the recombinant clone was more resistant to NFV by a factor of 8 but more sensitive to APV by a factor of 6.7 (Table 2).

There has been a report that dual PI therapy with APV plus NFV is generally safe and well tolerated and that the combination of APV with NFV may have the most beneficial pharmacokinetic interactions, based on the results of a phase II clinical trial of dual PI therapies, APV in combination with IDV, NFV, or SQV, although this phase II trial was handicapped by the presence of substantial PI resistance at the baseline and the small number of patients in the study, precluding conclusions about the relative activities or toxicities of the dual PI combinations (10). The hypothesis that a HAART regimen combining APV with NFV may bring about more

favorable antiviral efficacy for HIV-1-infected individuals should merit further study.

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GRL-02031, a Novel Nonpeptidic Protease Inhibitor (PI) Containing a Stereochemically Defined Fused Cyclopentanyltetrahydrofuran Potent against Multi-PI-Resistant Human Immunodeficiency Virus Type 1 In Vitro[▽]

Yasuhiro Koh,[1] Debananda Das,[2] Sofiya Leschenko,[3] Hirotomo Nakata,[1,2] Hiromi Ogata-Aoki,[1] Masayuki Amano,[1] Maki Nakayama,[1] Arun K. Ghosh,[3] and Hiroaki Mitsuya[1,2]*

Departments of Hematology and Infectious Diseases, Kumamoto University Graduate School of Medical and Pharmaceutical Sciences, Kumamoto 860-8556, Japan[1]; Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892[2]; and Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907[3]

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We generated a novel nonpeptidic protease inhibitor (PI), GRL-02031, by incorporating a stereochemically defined fused cyclopentanyltetrahydrofuran (Cp-THF) which exerted potent activity against a wide spectrum of human immunodeficiency virus type 1 (HIV-1) isolates, including multidrug-resistant HIV-1 variants. GRL-02031 was highly potent against laboratory HIV-1 strains and primary clinical isolates, including subtypes A, B, C, and E (50% effective concentration [EC_{50}] range, 0.015 to 0.038 μM), with minimal cytotoxicity (50% cytotoxic concentration, >100 μM in $CD4^+$ MT-2 cells), although it was less active against two HIV-2 strains ($HIV\text{-}2_{EHO}$ and $HIV\text{-}2_{ROD}$) (EC_{50}, ~0.60 μM) than against HIV-1 strains. GRL-02031 at relatively low concentrations blocked the infection and replication of each of the $HIV\text{-}1_{NL4\text{-}3}$ variants exposed to and selected by up to 5 μM of saquinavir, amprenavir, indinavir, nelfinavir, or ritonavir and 1 μM of lopinavir or atazanavir (EC_{50} range, 0.036 to 0.14 μM). GRL-02031 was also potent against multi-PI-resistant clinical HIV-1 variants isolated from patients who had no response to the conventional antiretroviral regimens that then existed, with EC_{50}s ranging from 0.014 to 0.042 μM (changes in the EC_{50}s were less than twofold the EC_{50} for wild-type HIV-1). Upon selection of $HIV\text{-}1_{NL4\text{-}3}$ in the presence of GRL-02031, mutants carrying L10F, L33F, M46I, I47V, Q58E, V82I, I84V, and I85V in the protease-encoding region and G62R (within p17), L363M (p24-p2 cleavage site), R409K (within p7), and I437T (p7-p1 cleavage site) in the *gag*-encoding region emerged. GRL-02031 was potent against a variety of $HIV\text{-}1_{NL4\text{-}3}$-based molecular infectious clones containing a single primary mutation reported previously or a combination of such mutations, although it was slightly less active against HIV-1 variants containing consecutive amino acid substitutions: M46I and I47V or I84V and I85V. Structural modeling analysis demonstrated a distinct bimodal binding of GRL-02031 to protease, which may provide advantages to GRL-02031 in blocking the replication of a wide spectrum of HIV-1 variants resistant to PIs and in delaying the development of resistance of HIV-1 to GRL-02031. The present data warrant the further development of GRL-02031 as a potential therapeutic agent for the treatment of infections with primary and multidrug-resistant HIV-1 variants.

The currently available combination therapy or highly active antiretroviral therapy (HAART) with two or more reverse transcriptase inhibitors and protease inhibitors (PIs) for human immunodeficiency virus (HIV) type 1 (HIV-1) infection and AIDS has been shown to suppress the replication of HIV-1 and extend the life expectancy of HIV-1-infected individuals (35, 38). However, the ability to provide effective long-term antiretroviral therapy for HIV-1 infection has become a complex issue, since those who initially achieved favorable viral suppression to undetectable levels have experienced treatment failure (11, 18, 28). In addition, it is evident that with these anti-HIV drugs, only partial immunologic reconstitution is attained in patients with advanced HIV-1 infection.

Nevertheless, recent analyses have revealed that the life expectancy of HIV-infected patients treated with HAART increased between 1996 and 2005, that the mortality rates for HIV-infected persons have become much closer to general mortality rates since the introduction of HAART, and that first-line HAART with boosted PI-based regimens results in less resistance within and across drug classes (2, 3, 18, 46).

In the development of new anti-HIV-1 therapeutics, we have faced a variety of challenges different from those faced during the design of the first-line drugs (7, 10, 39). The issue of the emergence of drug-resistant HIV-1 variants is one of the most formidable challenges in the era of HAART. Indeed, it is of note that the very features that contribute to the specificities and the efficacies of reverse transcriptase inhibitors and PIs provide the virus with a strategy to develop resistance (15, 19, 35), and it seems inevitable that this resistance issue will remain problematic for many years to come, although a few recently developed drugs, such as darunavir (DRV) and tipranavir, have been relatively successful as treatments for individuals carrying multidrug-resistant HIV-1 variants (5, 20).

In particular, a number of studies indicate that cross-resis-

* Corresponding author. Mailing address: Department of Hematology, Kumamoto University Graduate School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Phone: (81) 96-373-5156. Fax: (81) 96-363-5265. E-mail: hmitsuya@helix.nih.gov.

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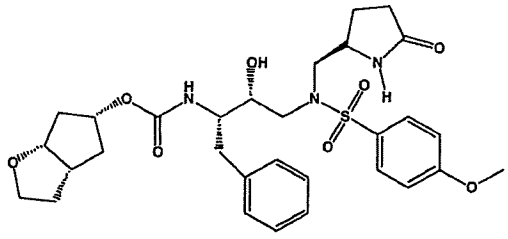

FIG. 1. Structure of GRL-02031.

tance is a major obstacle to antiviral therapy with PIs (19, 24). Obviously, the emergence of viral resistance, difficulties with compliance with the complicated treatment protocols, and adverse side effects urge the development of new classes of PIs (i) that have potent activities against existing resistant HIV-1 variants and that do not allow or delay the emergence of resistance, (ii) that have improved pharmacokinetics parameters in humans, and (iii) that have less severe side effects (43).

The present paper represents the first demonstration of the results of antiviral analyses of a novel PI which contains cyclopentanyltetrahydrofuran (Cp-THF) and which is highly potent against a wide spectrum of HIV isolates, including a variety of multi-PI-resistant clinical strains, in vitro. In addition, we selected GRL-02031-resistant HIV-1 variants in vitro and characterized their virological properties and susceptibilities to other PIs. We also demonstrated that the emergence of HIV-1 variants resistant to GRL-02031 requires multiply accumulated amino acid substitutions in the protease-encoding region. Moreover, in an attempt to explain why GRL-02031 can exert potent activity against a wide spectrum of HIV-1 variants resistant to multiple PIs, we performed structural modeling and molecular docking and examined the interactions of GRL-02031 with HIV-1 protease.

MATERIALS AND METHODS

Cells and viruses. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, MD), 50 U/ml penicillin, and 50 μg/ml of streptomycin. The following HIV-1 strains were employed for the drug susceptibility assay (see below): HIV-1_{LAI}, HIV-$1_{Ba\text{-}L}$, HIV-1_{JRFL}, HIV-$1_{NL4\text{-}3}$, HIV-2_{EHO}, and HIV-2_{ROD}; two clinical HIV-1 strains isolated from drug-naive patients with AIDS, HIV-$1_{ERS104pre}$ and HIV-1_{MOKW} (30, 45); and seven HIV-1 clinical isolates which were originally isolated from patients with AIDS who had received 9 to 11 anti-HIV-1 drugs over the past 32 to 83 months and which were genotypically and phenotypically characterized as multi-PI-resistant HIV-1 variants (47, 48). HIV-$1_{92UG037}$, HIV-$1_{97ZA003}$, and HIV-$1_{92TH019}$ were obtained from the NIH AIDS Reagent Program. All primary HIV-1 strains were passaged once or twice in 3-day-old phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMs), and the culture supernatants were stored at −80°C until use.

Antiviral agents. GRL-02031 (Fig. 1), a novel nonpeptidic PI containing Cp-THF, was designed and synthesized. Detailed methods for the synthesis of GRL-02031 will be described elsewhere by A. K. Ghosh et al. 3′-Azido-2′,3′-dideoxythymidine (AZT; zidovudine) was purchased from Sigma (St. Louis, MO). Saquinavir (SQV) and ritonavir (RTV) were kindly provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. Amprenavir (APV) was a kind gift from Glaxo-Wellcome, Research Triangle Park, NC. Nelfinavir (NFV) and indinavir (IDV) were kindly provided by Japan Energy Inc, Tokyo, Japan. Lopinavir (LPV) was synthesized by previously published methods (48). Atazanavir (ATV) was a kind gift from Bristol-Myers Squibb (New York, NY).

Drug susceptibility assay. The susceptibilities of HIV-1_{LAI}, HIV-$1_{Ba\text{-}L}$, HIV-2_{EHO}, HIV-2_{ROD}, and the primary HIV-1 isolates to various drugs were determined as described previously (26), with minor modifications. Briefly, MT-2 cells (2×10^4/ml) were exposed to 100 50% tissue culture infectious dose ($TCID_{50}$s) of HIV-1_{LAI}, HIV-$1_{Ba\text{-}L}$, HIV-2_{EHO}, or HIV-2_{ROD} in the presence or the absence of various concentrations of drugs in 96-well microculture plates; and the plates were incubated at 37°C for 7 days. After 100 μl of the medium was removed from each well, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μl, 7.5 mg/ml in phosphate-buffered saline) was added to each well in the plate, followed by incubation at 37°C for 2 h. After incubation, to dissolve the formazan crystals, 100 μl of acidified isopropanol containing 4% (vol/vol) Triton X-100 was added to each well and the optical density was measured in a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate.

To determine the sensitivities of the primary HIV-1 isolates to drugs, PHA-PBMs (10^6/ml) were exposed to 50 $TCID_{50}$s of each primary HIV-1 isolate and cultured in the presence or the absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates. To determine the drug susceptibilities of certain laboratory HIV-1 strains, MT-4 cells were employed as target cells, as described previously (26), with minor modifications. In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$s of drug-resistant HIV-1 strains in the presence or the absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatants were harvested and the amounts of the p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo, Japan) (29). The drug concentrations that suppressed the production of p24 Gag protein by 50% (EC_{50}) were determined by comparison of the amount of p24 Gag protein produced in drug-treated cell cultures with the level of p24 Gag protein produced in a drug-free control cell culture. All assays were performed in triplicate.

Generation of PI-resistant HIV-1 variants in vitro. MT-4 cells (10^5/ml) were exposed to HIV-$1_{NL4\text{-}3}$ (500 $TCID_{50}$s) and cultured in the presence of various PIs at an initial concentration of 0.01 to 0.03 μM. Viral replication was monitored by determination of the amount of p24 Gag produced by MT-4 cells. The culture supernatants were harvested on day 7 and were used to infect fresh MT-4 cells for the next round of culture in the presence of increasing concentrations of each drug. When the virus began to propagate in the presence of the drug, the drug concentration was generally increased two- to threefold. Proviral DNA samples obtained from the lysates of infected cells were subjected to nucleotide sequencing. This drug selection procedure was carried out until the drug concentration reached 5 μM.

Determination of nucleotide sequences. Molecular cloning and determination of the nucleotide sequences of HIV-1 isolates passaged in the presence of anti-HIV-1 agents were performed as described previously (26, 47). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first round of PCR of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR-F1 (5′-GAT GCT ACA TAT AAG CAG CTG C-3′) and PR12 (5′-CTC GTG ACA AAT TTC TAC TAA TGC-3′). The first-round PCR mixture consisted of 5 μl of proviral DNA solution, 2.0 U of Premix *Taq* (Ex *Taq* version; Takara Bio Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions employed were as follows: an initial 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8-min extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR-F2 (5′-GAG ACT CTG GTA ACT AGA GAT C-3′) and Ksma2.1 (5′-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3′) under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR columns; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with an ABI model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The viral RNA in the selection culture should contain a number of noninfectious (or dead) virions due to randomly occurring amino acid substitutions, which could provide misleading results if the sequences of such noninfectious or dead virions were erroneously taken into account. The viral DNA extracted from the newly infected cells in the present cell-free transmission system represents the infectious virions in the previous culture.

Generation of recombinant HIV-1 clones. The PCR products obtained as described above were digested with two enzymes, ApaI and SmaI; and the fragments obtained were introduced into pHIV-1_{NLSma}, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of pHIV-$1_{NL4\text{-}3}$, as described previously (14, 25). To generate HIV-1 clones carrying the desired mutations, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the mutation-containing genomic fragments were introduced into pHIV-1_{NLSma}. Determination of the nucleotide

TABLE 1. Antiviral activity of GRL-02031 against HIV-1_{LAI}[a]

Compound	EC_{50} (μM)	CC_{50}(μM)	Selectivity index
GRL-02031	0.028 ± 0.003	>100	>3,600
SQV	0.014 ± 0.005	9.9 ± 3.6	710
APV	0.033 ± 0.012	>100	>3,000
IDV	0.044 ± 0.007	69.8 ± 3.1	1,600
RTV	0.038 ± 0.004	21.3 ± 0.9	560
NFV	0.023 ± 0.006	ND	ND
LPV	0.032 ± 0.007	ND	ND

[a] MT-2 cells (2×10^4/ml) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI} and were cultured in the presence of various concentrations of PIs, and the EC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate. The data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments. ND, not determined. Selectivity index, CC_{50}/EC_{50}.

sequences of the plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each recombinant plasmid was transfected into 293T cells with Lipofectoamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), and the infectious virions thus generated were harvested for 48 h after transfection and stored at −80°C until use.

Structural analysis of GRL-02031 interactions with wild-type HIV-1 protease. The interactions of GRL-02031 with wild-type HIV-1 protease were examined by computational structural modeling and molecular docking on the basis of the published crystallographic data for protease complexed with PIs. Besides accounting for the conformational flexibility of the inhibitor, the polarization induced in the inhibitor by the protease was taken into consideration by employing polarizable quantum charges in the docking computations. The use of polarizable quantum charges has recently been shown to substantially improve the prediction of protein-ligand complex structures (4). The quantum mechanical polarized ligand docking protocol provided with the Glide (version 4.5), QSite (version 4.5), Jaguar (version 7.0), and Maestro (version 8.5) software (Schrödinger, LLC, New York, NY) was used as described below. The crystal structures 2FDE (protease-brecanavir complex) and 2IEN (protease-DRV complex) were used as templates in separate docking calculations to determine the binding mode of GRL-02031 with wild-type protease. The crystal coordinates were obtained from the Protein Data Bank (http://www.rcsb.org/). Hydrogens were optimized by placing constraints on the heavy atoms. The crystal water that mediates the interaction between PIs and the protease flap was retained, and all other crystal waters were deleted. Close interaction in the protease was annealed, and the docking grid was set up. Polarizable ligand charges were determined at the B3LYP/6-31G* level. The extraprecision mode of the Glide program (12, 13), which has a higher penalty for unphysical interactions, was used.

RESULTS

In vitro activity of GRL-02031 against laboratory and primary HIV strains and cytotoxicity of GRL-02031. We designed and synthesized ~80 different novel nonpeptidyl PIs containing a Cp-THF moiety and examined them for their anti-HIV activities and cytotoxicities in vitro. Among them, we found that GRL-02031 (Fig. 1) was the most potent against a laboratory HIV-1 strain, HIV-1_{LAI}, and had a favorable cytotoxicity profile, as examined with target MT-2 cells. As shown in Table 1, GRL-02031 showed an anti-HIV-1 activity profile comparable to that of most of the Food and Drug Administration (FDA)-approved PIs examined in the present study, although its toxicity profile was apparently more favorable, with a 50% cytotoxic concentration (CC_{50}) of >100 μM and a selectivity index (CC_{50}/EC_{50}) of >3,600.

GRL-02031 was further tested against two R5 laboratory HIV-1 strains (HIV-$1_{Ba\text{-}L}$ and HIV-1_{JRFL}), three different subtypes of primary HIV-1 strains (HIV-$1_{92UG037}$ [subtype A], HIV-$1_{97ZA003}$ [subtype C], and HIV-$1_{92TH019}$ [subtype E]), and two HIV-2 strains (HIV-2_{ROD} and HIV-2_{EHO}). GRL-02031 was found to be potent against all these HIV-1 strains and had EC_{50}s that ranged from 0.015 to 0.038 μM, as tested by the use of target PHA-PBMs, while GRL-02031 was moderately active against two HIV-2 strains (EC_{50}, ~0.60 μM), as tested by the use of MT-2 cells (data not shown).

GRL-02031 exerts potent activity against a wide spectrum of primary HIV-1 variants resistant to multiple PIs. We next examined the activity of GRL-02031 against a variety of primary HIV-1 strains which were isolated from those with AIDS who had failed a number of anti-HIV therapeutic regimens after they had received 9 to 11 anti-HIV-1 drugs over the previous 32 to 83 months and who proved to be highly resistant to multiple PIs (47, 48). These primary strains contained 9 to 14 amino acid substitutions in the protease-encoding region of the HIV-1 genome which have been reported to be associated with HIV-1 resistance to various PIs (RTV, IDV, NFV, SQV, APV, and LPV) (8). The substitutions identified included Leu-10 → Ile (L10I; seven of seven isolates), M46I/L (six of seven isolates), I54V (five of seven isolates), L63P (seven of seven isolates), A71V/T (six of seven isolates), V82A or V82T (seven of seven isolates), and L90M (five of seven isolates) (see footnote *a* of Table 2).

All drugs examined showed potent activity against two reference wild-type primary strains (X4 HIV-$1_{ERS104pre}$ [45] and R5 HIV-1_{MOKW} [30]), with the EC_{50}s ranging 0.004 to 0.036 μM (Table 2). However, all the primary strains examined were highly resistant to AZT, with the EC_{50}s being from 24- to >200-fold greater than the EC_{50} against HIV-$1_{ERS104pre}$. It was noted that SQV and LPV were still active against one or two of the seven strains and had EC_{50}s that differed 3- to 4-fold from those for HIV-$1_{ERS104pre}$; however, all the other FDA-approved PIs examined in this study except DRV failed to exert activity and had EC_{50}s 6- to >63-fold greater than the EC_{50} for HIV-$1_{ERS104pre}$. In contrast, GRL-02031, like DRV, potently blocked all seven primary strains and had EC_{50}s that ranged from 0.014 to 0.043 μM. It should be noted that the change in the EC_{50} of GRL-02031 for all seven multi-PI-resistant isolates tested was less than twofold compared with the EC_{50} for a wild-type primary strain, HIV-$1_{ERS104pre}$.

Selection of HIV-$1_{NL4\text{-}3}$ with GRL-02031. We then attempted to select a laboratory X4 HIV-1 strain (HIV-$1_{NL4\text{-}3}$) by propagating it in MT-4 cells in the presence of increasing concentrations of APV, IDV, or GRL-02031, as described previously (47). The virus was initially exposed to 0.03 μM APV, 0.02 μM IDV, or 0.02 μM GRL-02031. At passages 21 and 27, HIV-$1_{NL4\text{-}3}$ was capable of propagating in the presence of 167- and 250-fold greater concentrations of APV and IDV, respectively. At passage 26, HIV-$1_{NL4\text{-}3}$ was capable of propagating in the presence of a 250-fold greater concentration of IDV; however, 37 passages were required until the virus became similarly resistant to GRL-02031 and capable of propagating in the presence of 5 μM (Fig. 2).

We also determined the nucleic acid sequences of the protease-encoding region of the proviral DNA isolated from the cells exposed to GRL-02031 at passages 5, 15, 22, 30, and 37 (Fig. 3). At passage 5, no significant amino acid substitutions were identified; however, by passage 15, the virus had acquired the L10F substitution, which has been reported to be associated with PI resistance (6, 32). By passage 22, all eight clones of the virus examined had additionally acquired a flap muta-

TABLE 2. Antiviral activity of GRL-02031 against clinical HIV-1 isolates in PHA-PBMs[a]

Virus	Phenotype	EC_{50} (μM)								
		AZT	SQV	APV	IDV	NFV	RTV	LPV	DRV	GRL-02031
HIV-$1_{ERS104pre}$ (wild type)	X4	0.005 ± 0.002	0.009 ± 0.005	0.025 ± 0.007	0.021 ± 0.008	0.016 ± 0.004	0.036 ± 0.008	0.032 ± 0.002	0.0035 ± 0.0003	0.027 ± 0.002
HIV-1_{MOKW} (wild type)	R5	0.016 ± 0.011	0.004 ± 0.002	0.015 ± 0.004	0.019 ± 0.005	0.023 ± 0.008	0.036 ± 0.008	0.029 ± 0.0005	0.003 ± 0.0005	0.026 ± 0.007
HIV-1_{TM}(MDR)	X4	0.89 ± 0.11 (178)	0.27 ± 0.08 (30)	0.35 ± 0.11 (14)	>1 (>48)	>1 (>63)	>1 (>30)	0.23 ± 0.18 (7)	0.004 ± 0.001 (1)	0.026 ± 0.004 (1)
HIV-1_{MM}(MDR)	R5	0.45 ± 0.08 (90)	0.27 ± 0.06 (30)	0.40 ± 0.10 (16)	>1 (>48)	>1 (>63)	>1 (>30)	0.62 ± 0.28 (19)	0.017 ± 0.008 (5)	0.041 ± 0.004 (2)
HIV-1_{ISL} (MDR)	R5	0.18 ± 0.11 (36)	0.32 ± 0.13 (36)	0.66 ± 0.17 (26)	>1 (>48)	>1 (>63)	>1 (>30)	0.74 ± 0.32 (23)	0.026 ± 0.005 (7)	0.043 ± 0.003 (2)
HIV-1_A(MDR)	X4	0.12 ± 0.11 (24)	0.10 ± 0.07 (11)	0.16 ± 0.08 (6)	>1 (>48)	0.33 ± 0.07 (21)	>1 (>30)	0.32 ± 0.003 (10)	0.003 ± 0.0003 (1)	0.014 ± 0.007 (1)
HIV-1_B(MDR)	X4	>1 (>200)	0.30 ± 0.02 (33)	0.31 ± 0.04 (12)	>1 (>48)	>1 (>63)	>1 (>30)	0.25 ± 0.007 (8)	0.026 ± 0.017 (7)	0.029 ± 0.007 (1)
HIV-1_C(MDR)	X4	0.28 ± 0.20 (56)	0.033 ± 0.010 (4)	0.22 ± 0.13 (9)	>1 (>48)	0.36 ± 0.12 (23)	>1 (>30)	0.46 ± 0.24 (14)	0.007 ± 0.005 (2)	0.027 ± 0.009 (1)
HIV-1_G(MDR)	X4	0.60 ± 0.56 (120)	0.027 ± 0.006 (3)	0.23 ± 0.13 (9)	0.34 ± 0.07 (16)	0.23 ± 0.11 (14)	>1 (>30)	0.13 ± 0.04 (4)	0.007 ± 0.004 (2)	0.028 ± 0.001 (1)

[a] The amino acid substitutions identified in the protease-encoding region of HIV-$1_{ERS104pre}$, HIV-1_{MM}, HIV-1_{ISL}, HIV-1_A, HIV-1_B, HIV-1_C, and HIV-1_G compared to the consensus B sequence cited from the Los Alamos National Laboratory database include L63P, L10I/K14R/R41K/M46L/I54V/L63P/A71V/V82A/L90M/I93L, L10I/K43T/M46L/I54V/L63P/A71V/V82A/L90M/Q92K, L10I/L24I/L33F/E35D/M36I/N37S/M46L/I54V/R57K/I62V/L63P/A71V/G73S/V82A, L10I/I15V/E35D/N37E/K45R/I54V/L63P/A71V/V82T/L90M/I93L/C95F, L10I/K14R/L33I/M36I/M46I/F53L/K55R/I62V/L63P/A71V/G73S/V82A/L90M/I93L, L10I/I15V/K20R/L24I/M36I/M46L/I54V/I62V/L63P/K70Q/V82A/L89M, and L10I/V11I/T12E/I15V/L19I/R41K/M46L/L63P/A71T/V82A/L90M, respectively. HIV-1_{MOKW} was confirmed to lack known drug resistance-associated amino acid substitutions. The EC_{50}s were determined by employing PHA-PBMs as target cells and the inhibition of p24 Gag protein production as the endpoint. All values were determined in triplicate, and those shown were derived from the results of three independent experiments. Numbers in parentheses represent the fold changes in the EC_{50} for each isolate compared to the EC_{50} for HIV-$1_{ERS104pre}$. MDR, multidrug resistant.

FIG. 2. In vitro selection of HIV-1 variants resistant to GRL-02031. HIV-1_{NL4-3} was passaged in the presence of increasing concentrations of APV (open circles), IDV (solid circles), or GRL-02031 (open triangles) in MT-4 cells. The selection was carried out in a cell-free manner for a total of 21 to 37 passages with drug concentrations escalating from 0.02 to 5 μM. The nucleotide sequences of the proviral DNAs were determined by using cell lysates of HIV-1-infected MT-4 cells at the termination of each indicated passage.

tion (I47V) and the primary substitution (V82I). By passage 30, in addition to L10F, I47V, and V82I, the virus had acquired L33F, M46I, and I85V. By passage 37, the virus had gained I84V and another Q58E substitution, an unusual mutation not associated with resistance to other HIV PIs. Genetic characterization of the protease-encoding gene of the HIV-1 variants selected with APV and IDV revealed that those variants had acquired previously reported mutations, such as L10F, V32I, M46I, and I84V in APV-selected HIV-1 (HIV-1_{APV^r}) and L10F, V32I, K43T, M46I, L63P, G73C, and V82A in HIV-1_{IDV^r}.

Amino acid substitutions in Gag protein of HIV-1 exposed to GRL-02031. We also determined the amino acid sequences of the Gag proteins of HIV-1_{NL4-3} exposed to GRL-02031. By passage 15, an unusual amino acid substitution, L363M, located at the p24-p2 cleavage site, had emerged. By passage 22, the virus had acquired another amino acid substitution, I437T, located close to the p7-p1 cleavage site. By passage 30 and beyond, G62R (within p17) and R409K (within p7) were seen in addition to L363M and I437T (data not shown).

Anti-HIV-1 profile of a GRL-02031-resistant HIV-1 variant. We then examined the anti-HIV-1 profile of a GRL-02031-resistant HIV-1 variant. In this experiment, we employed eight HIV-1 variants which were selected in the presence of up to 1 or 5 μM of each of seven FDA-approved PIs and GRL-02031. Each of these eight variants contained a variety of previously known PI resistance-associated amino acid substitutions (see footnote *a* of Table 3). As shown in Table 3, when each HIV-1 variant was examined in target MT-4 cells, each HIV-1 variant except the variant selected with 1 μM LPV in vitro (HIV-$1_{LPV\text{-}1\ \mu M}$) and the variant selected with 1 μM ATV in vitro (HIV-$1_{ATV\text{-}1\ \mu M}$), proved to be highly resistant to the PI with which the virus was selected, with EC_{50}s exceeding 1 μM. However, GRL-02031 was potent against all the PI-resistant variants, although the compound was relatively less potent against the variant selected with 5 μM SQV in vitro (HIV-

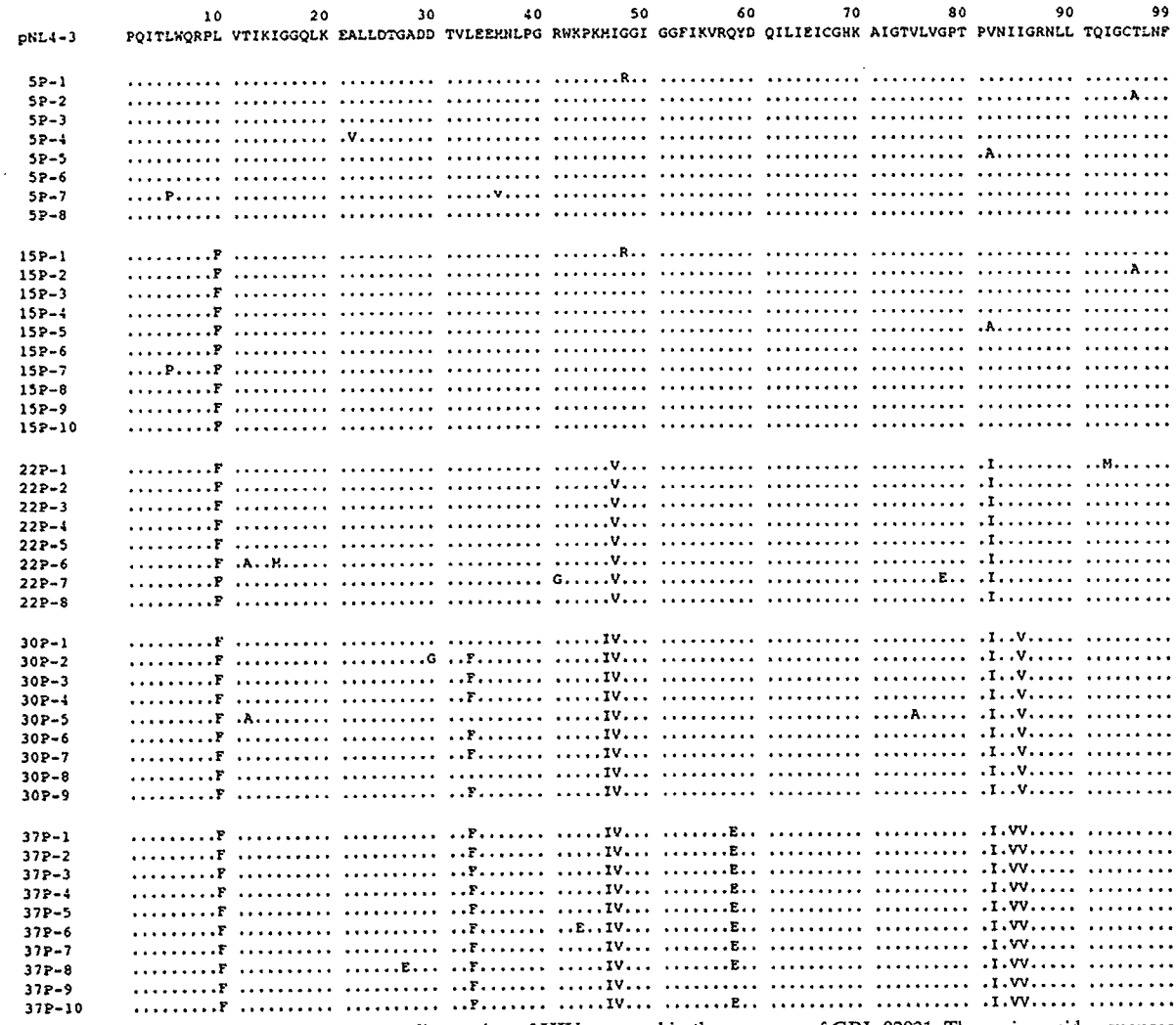

FIG. 3. Sequence analysis of the protease-encoding region of HIV passaged in the presence of GRL-02031. The amino acid sequences of the proteases deduced from the nucleotide sequences of the protease-encoding region of HIV clones determined at five different passages are illustrated. The identity of each amino acid with that from pNL4-3 (top row) at each individual amino acid position is indicated by a dot.

$1_{SQV\text{-}5\ \mu M}$) (a sixfold increase in the EC_{50} of SQV compared with that of GRL-02031).

When the virus was selected with up to 5 μM GRL-02031 (HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$) was examined in MT-4 cells, the EC_{50} of GRL-02031 turned out to be >1 μM although HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$ remained susceptible to other PIs, in particular, SQV, IDV, and NFV. The HIV-$1_{LPV\text{-}1\ \mu M}$ variant was substantially resistant to APV, IDV, NFV, RTV, LPV, and ATV; however, this variant was highly susceptible to GRL-02031 and had an EC_{50} of 0.038 μM (Table 3). HIV-$1_{ATV\text{-}1\ \mu M}$ variant was also substantially resistant to IDV, NFV, and ATV; however, this variant was susceptible to LPV and GRL-02031. Of note, LPV, which has currently been widely used as a first-line therapeutic among HAART regimens, was not active against three HIV-1 variants (HIV-$1_{SQV\text{-}5\ \mu M}$, a variant selected with 5 μM IDV [HIV-$1_{IDV\text{-}5\ \mu M}$], and a variant selected with 5 μM NFV [HIV-$1_{NFV\text{-}5\ \mu M}$]), with the differences in the EC_{50}s being more than 16-fold compared to the value for wild-type strain HIV-$1_{NL4\text{-}3}$. This anti-HIV-1 profile of LPV greatly contrasted with that of GRL-02031. GRL-02031 was highly potent against all the variants examined except HIV-$1_{SQV\text{-}5\ \mu M}$ (sixfold change in the EC_{50} compared to that for HIV-$1_{NL4\text{-}3}$). It is also noteworthy that SQV, IDV, and NFV remained potent against HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$, suggesting that the combination of GRL-02031 and SQV, IDV, or NFV could exert complementarily augmented activity against multi-PI-resistant HIV-1 variants.

Sensitivities of infectious molecular HIV-1 clones carrying various amino acid substitutions to GRL-02031. Finally, we attempted to determine the profile of the activity of GRL-02031 against a variety of HIV-$1_{NL4\text{-}3}$-based molecular infectious clones containing a single primary mutation previously reported or a combination of such mutations (Table 4) (21, 31, 40–42). Interestingly, no significant changes in EC_{50}s were observed when HIV-1 clones containing only one of the amino acid substitutions (L10F, L33F, M46I, I47V, Q58E, V82I,

TABLE 3. Antiviral activities of GRL-02031 against laboratory PI-resistant HIV-1 variants[a]

Virus	EC_{50} (μM)							
	SQV	APV	IDV	NFV	RTV	LPV	ATV	GRL-02031
$HIV\text{-}1_{NL4\text{-}3}$	0.008 ± 0.004	0.028 ± 0.009	0.014 ± 0.004	0.018 ± 0.009	0.021 ± 0.009	0.018 ± 0.001	0.0043 ± 0.0004	0.023 ± 0.008
$HIV\text{-}1_{SQV\text{-}5\ \mu M}$	>1 (>125)	0.21 ± 0.09 (8)	>1 (>71)	0.32 ± 0.04 (18)	>1 (>48)	0.70 ± 0.22 (39)	0.32 ± 0.02 (74)	0.14 ± 0.06 (6)
$HIV\text{-}1_{APV\text{-}5\ \mu M}$	0.016 ± 0.010 (2)	>1 (>36)	0.22 ± 0.15 (16)	0.17 ± 0.10 (10)	>1 (>48)	0.14 ± 0.05 (8)	0.0032 ± 0.012 (1)	0.037 ± 0.004 (2)
$HIV\text{-}1_{IDV\text{-}5\ \mu M}$	0.022 ± 0.009 (3)	0.26 ± 0.14 (9)	>1 (>71)	0.65 ± 0.16 (36)	>1 (>48)	>1 (>56)	0.063 ± 0.022 (15)	0.047 ± 0.002 (2)
$HIV\text{-}1_{NFV\text{-}5\ \mu M}$	0.028 ± 0.009 (4)	0.078 ± 0.026 (3)	0.27 ± 0.06 (19)	>1 (>56)	0.08 ± 0.05 (4)	0.29 ± 0.03 (16)	0.024 ± 0.005 (6)	0.036 ± 0.002 (2)
$HIV\text{-}1_{RTV\text{-}5\ \mu M}$	0.010 ± 0.008 (1)	0.43 ± 0.27 (15)	0.30 ± 0.07 (21)	0.24 ± 0.10 (13)	>1 (>48)	0.11 ± 0.08 (6)	0.021 ± 0.007 (5)	0.077 ± 0.031 (3)
$HIV\text{-}1_{LPV\text{-}1\ \mu M}$	0.033 ± 0.003 (4)	0.32 ± 0.02 (11)	>1 (>71)	0.51 ± 0.06 (28)	>1 (>48)	0.30 ± 0.04 (17)	0.041 ± 0.002 (10)	0.038 ± 0.004 (2)
$HIV\text{-}1_{ATV\text{-}1\ \mu M}$	0.034 ± 0.006 (4)	0.18 ± 0.06 (6)	0.33 ± 0.10 (24)	0.21 ± 0.03 (12)	0.14 ± 0.02 (7)	0.025 ± 0.011 (1)	0.31 ± 0.05 (72)	0.033 ± 0.002 (1)
$HIV\text{-}1_{GRL\text{-}02031\text{-}5\ \mu M}$	0.008 ± 0.001 (1)	0.21 ± 0.02 (8)	0.044 ± 0.015 (3)	0.011 ± 0.004 (1)	0.26 ± 0.10 (12)	0.14 ± 0.09 (8)	0.036 ± 0.005 (8)	>1 (>43)

[a] The amino acid substitutions identified in the protease-encoding region of $HIV\text{-}1_{SQV\text{-}5\ \mu M}$, $HIV\text{-}1_{APV\text{-}5\ \mu M}$, $HIV\text{-}1_{IDV\text{-}5\ \mu M}$, $HIV\text{-}1_{NFV\text{-}5\ \mu M}$, $HIV\text{-}1_{RTV\text{-}5\ \mu M}$, $HIV\text{-}1_{LPV\text{-}1\ \mu M}$, $HIV\text{-}1_{ATV\text{-}1\ \mu M}$, and $HIV\text{-}1_{GRL\text{-}02031\text{-}5\ \mu M}$ compared to the consensus B sequence cited from the Los Alamos National Laboratory database include L10I/G48V/I54V/L90M, L10F/V32I/M46I/I54M/A71V/I84V, L10F/L24I/M46I/L63P/A71V/G73S/V82T, L10F/D30N/K45I/A71V/T74S, M46I/V82F/I84V, L10F/M46I/I54V/V82A, L23I/K43T/M46I/I50L/G51A/A71V, and L10F/L33F/M46I/I47V/Q58E/V82I/I84V/I85V, respectively. MT-4 cells (1×10^4) were exposed to each HIV-1 isolate (100 $TCID_{50}$s), and the inhibition of p24 Gag protein production by the drug was used as the endpoint. The numbers in parentheses represent the fold changes in the EC_{50}s for each isolate compared to the EC_{50} for $HIV\text{-}1_{NL4\text{-}3}$. The data shown are mean values (±1 standard deviation) derived from the results of three independent experiments conducted in triplicate.

I84V, or I85V) which emerged in the selection process in the present work (Fig. 3) were tested with GRL-02031. We tested each of these one-mutation-containing infectious clones against a selected PI, and again, no significant changes in EC_{50}s were seen (Table 4).

It was noted that increases in the EC_{50}s of GRL-02031 were seen only when more than two amino acid substitutions were introduced into $HIV\text{-}1_{NL4\text{-}3}$. A substantial reduction in susceptibility (differences in EC_{50}s of more than threefold) was seen when the virus had two mutations (M46I/I47V or I84V/I85V). Further increases in the EC_{50}s were seen when four substitutions (L10F/I47V/V82I/I85V) or five substitutions (L10F/M46I/I47V/V82I/I85V) were introduced. Moreover, we generated molecular clones containing a primary mutation with which HIV-1 is known to acquire substantial resistance to a PI(s) (such as D30N, G48V, I50V, and L90M) and determined the EC_{50}s of GRL-02031 (Table 4). We found that GRL-02031 was potent against all such molecular clones with a primary mutation, with the differences in the EC_{50}s being 0.7- to 1.7-fold in comparison with the EC_{50} for $HIV\text{-}1_{NL4\text{-}3}$, although $HIV\text{-}1_{D30N}$ and $HIV\text{-}1_{G48V}$ showed moderate to substantial levels of resistance to NFV (5.6-fold change) and SQV (5.1-fold change) (Table 4). These data suggest that HIV-1 can acquire substantial resistance to GRL-02031 only when it gains multiple mutations in the protease, a potentially advantageous property of GRL-02031.

Structural analysis of GRL-02031 interactions with wild-type protease. Finally, we conducted molecular and structural analyses of the interactions of GRL-02031 with protease (Fig. 4). By refined structural modeling based on the previously published crystal structures 2FDE (protease-brecanavir complex) and 2IEN (protease-DRV complex), we found that the oxygen atom of Cp-THF has a hydrogen bond interaction with Asp29 in the S-2 pocket of the protease. The hydrogen bond interactions of GRL-02031 with Asp25 and Gly27, which have been observed for various PIs, were also predicted to be present. In addition, GRL-02031 has hydrogen bond interactions mediated through a water molecule with flap residues Ile50 and Ile50′. Of note, GRL-02031 has an *R* configuration at the pyrrolidone stereocenter. Interestingly, the structural models demonstrated that for the *R*-stereochemical configuration, two distinct binding modes of GRL-02031 were found in the S-2′ pocket. The 2-pyrrolidone group and the methoxybenzene moiety can orient toward Asp29′ and Asp30′ for configuration 1 and configuration 2, respectively (Fig. 4A and B). In configuration 1, the 2-pyrrolidone oxygen has hydrogen bond interactions with Asp29′ in the S-2′ pocket. In configuration 2, the methoxybenzene orients toward the S-2′ pocket and forms tight hydrogen bonds with Asp30′. The interactions of PIs (48) with Asp29′ and/or Asp30′ have been reported to be mediated by water molecules. It is likely that the presence of water molecules may influence the relative abundance of configurations 1 and 2. The alternate bimodal binding feature observed in this molecular analysis should provide advantages to the PI in maintaining its antiviral potency when the HIV-1 protease either has a polymorphism or develops amino acid substitutions under drug pressure.

We also examined the lipophilic potential of the computationally defined cavity for the binding of GRL-02031 within the HIV protease (Fig. 4C). It was revealed that GRL-02031 fits