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Persistence of mutations during replication of an HIV library containing combinations of selected protease mutations

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Abstract

It has been known that, in some cases, accumulation of specific mutations in HIV-1 protease leads to multi-protease inhibitor (PI) resistance. We examined the persistence of mutations detected in HIV-1 clinical isolates cross-resistant to the current PIs using an HIV-1 protease restricted library (HXB2 protease in an HIV-1_{NL4-3} background) in the absence of protease inhibitors. The virus library contained combinations of 0–11 amino acid substitutions (4,096 possible combinations) in the protease-encoding region. We examined the frequency of each amino acid substitution in the library using a T cell line, MT-2. The frequency of the amino acid substitutions V82T/I and L90M decreased rapidly with a short half life ($t_{1/2} < 10$ days). However, the mutations M36I, M46I and I84V were relatively persistent: $t_{1/2} = 34.2$, 28.1 and 30.6 days, respectively. Other amino acid substitutions, i.e., L10I, I54V, L63P, A71V and V82A, were well retained ($t_{1/2} > 36$ days). By contrast, the half lives ($t_{1/2}$) of the D30N and N88D mutations associated with nelfinavir (NFV) resistance were only 7.2 and 1.8 days, respectively. These results indicate that this type of the HIV-1 protease restricted library is useful to evaluate the persistence of PI resistance-associated mutations in the absence of drug selective pressure.
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Keywords: HIV; Resistance; Protease inhibitors; Mutations; Library

1. Introduction

HIV-1 protease functions during viral maturation by processing the Gag and Gag-Pol polyproteins at the late stage of the HIV life cycle (Debouck, 1992; Kaplan et al., 1993; Kohl et al., 1988; Oroszlan and Luftig, 1990). The HIV-1 protease is one of the major molecular targets for antiviral agents in highly active antiretroviral therapy (HAART) (Gulick et al., 1997; Hammer et al., 1997; Palella et al., 1998). However, the emergence and persistence of mutant viruses with cross-resistance to unused protease inhibitors (PIs) is a serious obstacle for efficient PI salvage therapy with alternative PIs (Kantor et al., 2002; Yoshimura et al., 1999). The emergence of such viral variants has been extensively described and is found even under combination retroviral therapy (Martinez-Picado et al., 2000; Miller, 2001). The objective of this work is to determine persistence of mutations detected in multi-PI-resistant virus using an HIV-1 protease restricted library in the absence of PIs. Using the virus library, we could deal with large number (>4000) of possible combinations of mutations. We chose eight positions of amino acid substitutions found in multidrug-resistant HIV-1 isolates from seven patients who had received various protease inhibitors, including indinavir (IDV), ritonavir (RTV), saquinavir (SQV), and/or amprenavir (APV) (Yoshimura et al., 1999). In addition to these mutations, we introduced the nelfinavir (NFV) resistance-associated amino acid substitutions D30N and N88D (Patick et al., 1996), and the I84V mutation which is associated with RTV, IDV and SQV resistance (Kuiken et al., 2000; Shafer et al., 1999).

2. Materials and methods

2.1. Construction of HIV-1 library containing 0–11 amino acid substitutions in viral protease

The HIV-1 library carrying a set of random amino acid substitutions in the viral protease (Fig. 1A) was constructed as described previously (Yusa et al., 2002), except for the 286-bp DNA fragment encoding the part of protease with the six degenerated mutations. The 587 bp *Apa* I–*Sma* I fragment including protease from HIV-1 HXB2D (Davis et al., 1999; Ratner et al., 1985) was subcloned into pCR7ΔZ (Yusa et al.,

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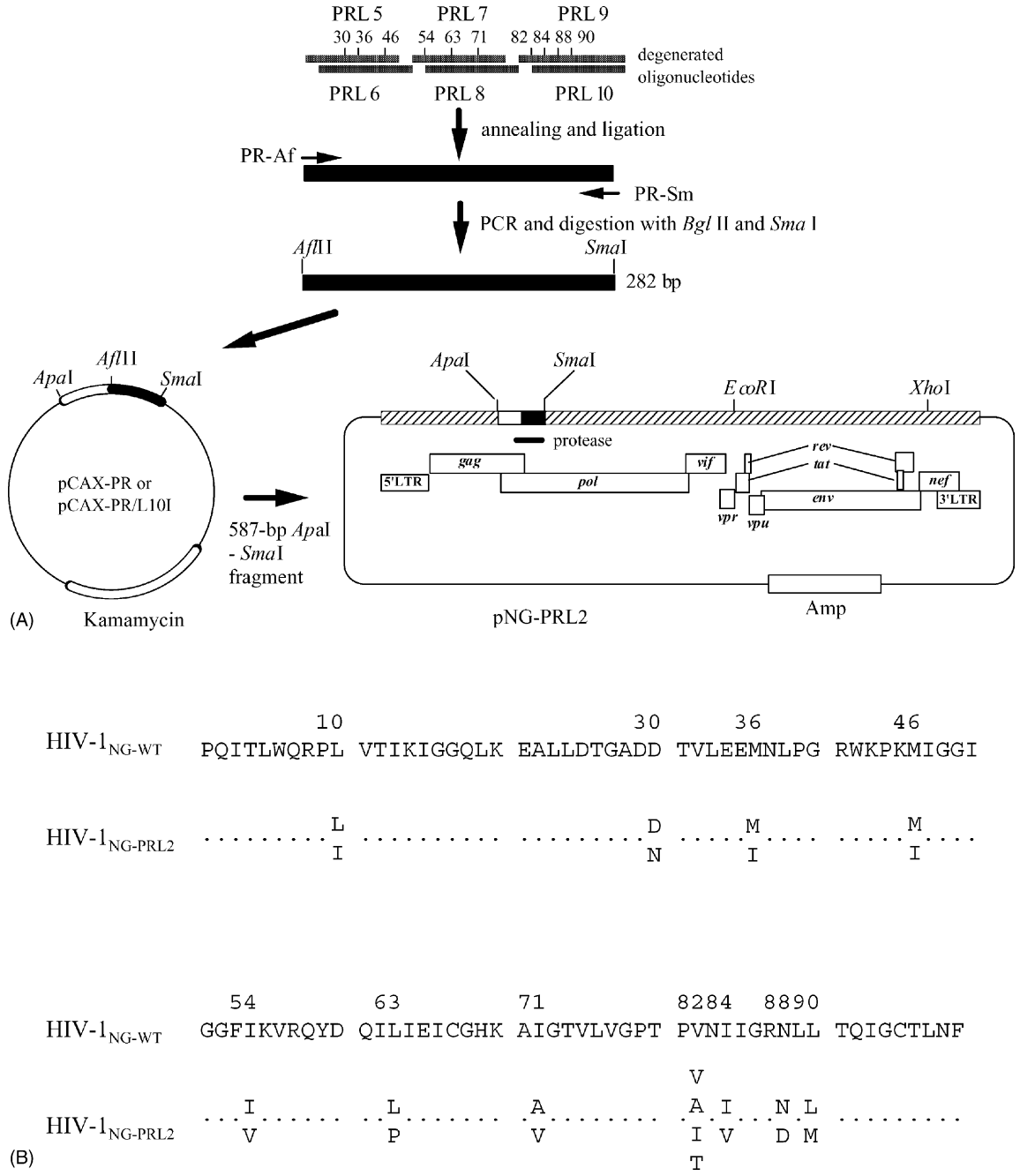


Fig. 1. (A) HIV-1 library pNG-PRL2 containing 0–11 amino acid substitutions in protease. Degenerated oligonucleotides were used for the insertion of 12 nucleotide substitutions in protease (the residue at 82 contains two sequential nucleotide substitutions). The number of combinations of 0–11 amino acid substitutions was 4096, because amino acid residue 82 contained four possibilities (Val, Ala, Ile or Thr) and 10 other amino acid residues contained two possibilities. (B) Combinations of 0–11 amino acid substitutions in HIV-1 library pNG-PRL2.

2002) digested with *Apa* I and *Sma* I, resulting in pCAX9. pCAX9^{Afl} was created by introduction of Afl II site into pCAX9 without amino acid substitution by Quikchange^{TM} site-directed Mutagenesis Kit (Stratagene Cloning System, La Jolla, CA) with primers 5′-ATA GGG GGG CAA CTT AAG GAA GCT CTA TTA-3′and 5′-TAA TAG AGC TTC CTT AAG TTG CCC CCC TAT-3′. pCAX9^{Afl}-L10I containing an amino acid substitution Leu-to-Ile at position 10 was also created by employing the same procedure with primers 5′-TTT GGC AAC GAC CCC TCG TCA CAA TAA AGA-3′ and 5′-TCT TTA TTG TGA CGA GGG GTC GTT GCC AAA-3′. Six oligonucleotides PRL5, PRL6, PRL7, PRL8, PRL9 and PRL10 (Yusa et al., 2002) were commercially synthesized to generate three short DNA fragments with sticky ends. The respective pair of complementary oligonucleotides at 0.20 μg/μl was denatured for 2 min at 90 °C in a heat block, and then left in the heat block off for 30 min in 10 mM $MgCl_2$ and 10 mM Tris–HCl (pH

8.0) at room temperature for annealing. The resultant short DNA fragments were designated as PRL5/6 (95 bp), PRL7/8 (83 bp) and PRL9/10 (108 bp), respectively. PRL5/6 contained three degenerated codons (GAT (Asp 30) or AAT (Asn 30); ATG (Met 36) or ATA (Ile 36); ATG (Met 46) or ATA (Ile 46)), PRL7/8 contained three degenerated codons (ATC (Ile 54) or GTC (Val 54); CTC (Leu 63) or CCC (Pro 63); GCT (Ala 71) or GTT (Val 71)) and PRL9/10 contained four degenerated codons (GTC (Val 82), ACC (Thr 82), GCC (Ala 82) or ATC (Ile 82); ATA (Ile 84) or GTA (Val 84); AAT (Asn 88) or GAT (Asp 88); TTG (Leu 90) or ATG (Met 90)). These three DNA fragments (0.12 μg each) were ligated by T4 DNA ligase (New England Biolabs, Inc., Beverly, MA) and the resultant 286-bp DNA fragment was purified via 1.5% agarose electrophoresis. One hundred nanograms of the purified DNA fragment was used for polymerase chain reaction (PCR) as the DNA template with Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The upstream primer was PRL-Af (5′-GGG GCA ACT TA A GCT GCT ATT AGA TAC-3′) and the downstream primer was PRL-Sm (5′-CAT CCC GGG CTT TAA TTT TAC TGG TAC AGT CTC AAT AG-3′). PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystems Inc., Foster City, CA). The PCR product (5 μg) was purified by Concert Rapid PCR Clean-up System (Invitrogen) and digested with *Afl* II and *Sma* I, and ligated to the *Afl* II and *Sma* I sites of pCAX9^{Afl} (5 μg) and pCAX9^{Afl}-L10I (5 μg) mixture. The ligation products was purified by Microcon-30 (Millipore Corporate, Bedford, MA) and used to transform *Escherichia coli* strain DH5alpha by electroporation using ECM 600 (BTX, San Diego, CA) at 2.5 kV, as described previously (Yusa et al., 2002). Then, pCAX^{Afl}-PRL containing 2.5×10^6 independent clones with a *Afl* II–*Sma* I fragment was generated. After purification of pCAX^{Afl}-PRL DNA, the *Apa* I–*Sma* I fragment from 15 μg of the plasmid was cloned into the *Apa* I and *Sma* I sites of pNL4-3Sma. pNL4-3Sma contains full-length NL4-3 (Adachi et al., 1986) in which the *Sma* I site has been introduced in pol (nt 2589). pNL4-3Sma was a gift from Dr. Takamasa Ueno (Kumamoto University). The ligation products (0.2 μg) were transformed into *E. coli* strain JM109 by electroporation, as described above. Finally, the HIV-1 library pNG-PRL2 contained 1.5×10^5 independent clones containing a 496-bp pol DNA fragment (ligation efficiency: 75%). The HIV-1 library pNG-PRL2 used in this report contained 1.5×10^5 independent clones and this complexity was large enough to provide 4096 possibilities of 0–11 combinations of amino acid substitutions in the viral protease. Incorporation of a random combination of the mutations in the protease-encoding sequence was confirmed with 42 randomly selected clones, and at an average 0.09% of unintended nucleotide changes were detected.

2.2. Cell culture

MT-2 cells were grown in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). 293T and MAGI/CCR5 cells (Chackerian et al., 1997) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum.

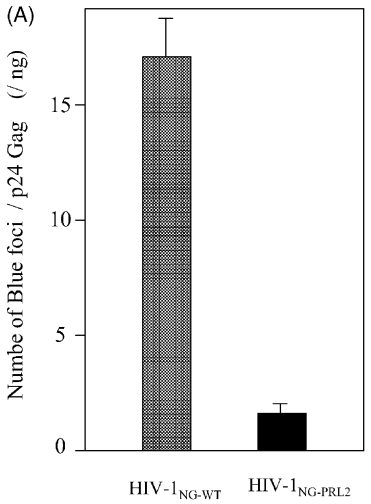
2.3. Passage of the library virus in the absence of PI(s)

On day 0, HIV-1 library DNA pNG-PRL2 was transfected into 293T cells. Transfections were performed with the calcium phosphate-mediated method (Chen and Okayama, 1987). Briefly, 0.5 ml of 0.25 M $CaCl_2$ containing 20 μg of pNG-PRL2 was mixed with 0.5 ml of 2× BBS (50 mM *N*,*N*-bis[2-hydroxyethyl]-2-aminoethaesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95), and incubated for 15 min at room temperature. Then the DNA–calcium phosphate mixture was added to 293T cells (1×10^6) in a 100-mm Collagen Type I coated dish (Iwaki Glass Co. Ltd., Chiba, Japan) with 10 ml of growth medium. The cells were incubated for 14 h at 37 °C in 5% CO_2–95% humidified air, washed twice, and then replaced with 10 ml of growth medium. After another 10 h incubation, the supernatants of five dishes were collected and used for infection of MT-2 cells (day 1). p24 Gag antigen was determined by p24 Gag antigen ELISA system using RETRO-TEK (ZeptoMetrix, Co., Buffalo, NY). MT-2 cells (2×10^5) were infected with HIV-$1_{NG\text{-}PRL2}$ (250 ng of p24 Gag antigen) and maintained in the absence of PI in a 25-cm^2 flask. The viruses were transferred to uninfected MT-2 cells every 3 days. On days 4, 13, 25 and 37, DNA from the infected cells was extracted for sequencing. DNA was extracted using urea lysis buffer (7.0 M urea, 2% sodium dodecyl sulfate, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris–HCl buffer) (Lee et al., 1989). A 706-bp fragment containing a viral protease-encoding sequence was amplified by PCR in a 50-μl reaction volume comprising 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, and 2 U ampliTaq (Applied Biosystems Inc.) with primers PR5 (5′-GTT AAG TGT TTC AAT TGT GGC AAA GAA GGG C-3′) and PR12 (5′-CTC TGT ACA AAT TTC TAC TAA TGC-3′). PCR products were purified via 1% agarose electrophoresis and cloned into *Stu* I digested pCRΔZ vector created from pCR-blunt (Invitrogen) by deletion of a *Pml* I fragment containing Zeocin cassette. Cloned sequences were sequenced using the ABI Prism 377 (Applied Biosystems Inc.). The half life for mutations required to decrease its frequency to levels 50% of those at day 0 was calculated.

3. Results

3.1. Passage of the HIV-1 library in T cell line in the absence of PIs

On day 0, we transfected the library DNA (Fig. 1A and B) into 293T cells and then maintained the virus mixture in MT-2 cells every 3 days from day 1. The frequency of the intended 11 mutations was examined on days 1, 4, 13, 25



(B)

	10 30 36 46 54 63 71 8284 8890	
HIV-1_{NG-WT}	PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF	n
#01	.....D.....	1
#02	.....N.....V.....P.....A.....I.V...D.M.....	8
#03	.....I.....V.....P.....	3
#04	.....I.....I.....V.....M.....	4
#05	.....I.....N.....I.....V.....A.V.....	6
#06	.....I.....I.....I.....V...D.....	5
#07	.....N.....I.....V.....I....D.....	5
#08	.....I.....N.....I.....V.....V.....	5
#09	.....I.....G.....V.....A.....M.....	4
#10	.....N.....I.....I.....V.....A.....V.....	6
#11	.....I.....N.....V...D.....	4
#12	.....I.....G.I.....V.....T.V.....	5
#13	.....A.....I.....	2
#14	.....N.....V.....A.....	3
#15	.....T.V.....	2
#16	.....I.....I.....I.....V.....V.....	5
#17	.....L.....P.....A.....T.V.....M.....	6
#18	.....N.....V.....V.....M.....	4
#19	.....N.....I.....V.....A.V.....	5
#20	.....I.....V.....V...D.....	4
#21	.....V.....A.V.....	3
#22	.....V.....	1
#23	.....I.....I.....V.....P.....	4
#24	.....I.....V.....P.....I.V.....	5
#25	.....I.....I.....P.....A.....A.V.....	6
#26	.....I.....P.....A.....D.....	4
#27	.....I.....I.....P.....T.....D.....	5
#28	.....V.....A.....M.....	3
#29	.....I.....N.....I.....I.....V.....P.....T.V.....M.....	9
#30	.....N.....T.....D.....	3
#31	.....N.....I.....V.....A.....I.....D.....	6
#32	.....I.....N.....P.....T.....M.....	5
#33	.....S.....N.....P.....A.....V.....M.....	4
#34	.....I.....A.....I.V...D.....	5
#35	.....N.....I.....V.....M.....	4
#36	.....I.....A....S.....I.V.....M.....	5

Fig. 2. (A) Infectious virions in of HIV-1_{NG-WT} and HIV-$1_{NG-PRL2}$ used for the experiments were evaluated by using a single viral infectivity assay (Kimpton and Emerman, 1992). Error bars indicate the standard deviation of three experiments. (B) Amino acid sequences of 36 protease recombinants of HIV-$1_{NG-PRL2}$ randomly sequenced on day 1. The virus mixture generated from the 293T cells transfected with pNG-PRL2 was precipitated and subjected to RT–PCR using ImProm-II reverse transcription system (Promega Co., Madison, WI). The PCR products were subcloned into pCRΔZ and sequenced. *n*, number of intended amino acid substitutions.

and 37. The library DNA theoretically contained combinations of 0–11 amino acid substitutions (4096 possible combinations). The infectivity of the virus library HIV-$1_{NG\text{-}PRL2}$ used for this experiment was 9.4% of that of wild type HIV-$1_{NG\text{-}WT}$ evaluated using a single-round viral infectivity assay (Kimpton and Emerman, 1992; Yusa et al., 2002) (Fig. 2A). There were no clones containing identical combinations of mutations amongst 36 clones randomly sequenced on day 1 (Fig. 2B). The wild type (no mutation detected in protease) was contained in the virus mixture shown on day 25, though there was no wild type detected in the clones randomly sequenced on days 1, 4, 13 and 37 (Fig. 3). The number of intended mutations in the isolated clones on day 1 ranged from 1 to 9, whereas the number of intended mutations on day 37 ranged from 1 to 6. The peak frequency of mutation number in the virus shifted from 5 to 3 mutations between days 1 and 37. Most of the higher number of random combinations of the 11 amino acid residues in the protease lead to a replicative disadvantage compared to the clones carrying lower numbers of mutations. However, replacement of the diverse mutants with wild type or viruses with a single amino acid substitution in protease did not occur within 37 days. The content of the wild type or viruses with a secondary or polymorphic amino acid residue was low in the library DNA (0.024% each). The competitive replication for 37 days was not sufficient to crowd out the clones with multiple mutations.

In addition to the intended 11 mutations, the virus library on day 0 contained 0.16% (6/3564 amino acid residues) of other amino acid substitutions in the protease, and 14% (5/36) of the clones contained unintended amino acid substitutions (Fig. 2B). It is conceivable that they were incorporated during PCR for construction of the library since the viruses did not contain any additional mutations in other regions including cleavage sites NC/p1, p1/$p6^{gag}$, TFP/pol or protease/reverse transcriptase on days 0, 1, 4, 13, 25 and 37 (data not shown).

Table 1
Persistence of amino acid substitutions in HIV-1 protease library

Amino acid substitution	$t_{1/2}$ (days)
L10I	>36
D30N	7.2
M36I	34.2
M46I	28.1
I54V	>36
L63P	>36
A71V	>36
V82A	>36
V82T	2.1
V82I	7.2
N88D	1.8
I84V	30.6
L90M	5.1

3.2. *Half lives ($t_{1/2}$) of the amino acid substitutions in the absence of PI*

Using the time course data measuring the frequency of each intended mutation for 37 days, we determined the half life ($t_{1/2}$) to compare the persistence of each amino acid substitution in the library (Fig. 4, Table 1). D30N has been reported solely in patients receiving nelfinavir and does not confer cross-resistance to the other PIs (Markowitz et al., 1998; Patick et al., 1998; Winters et al., 1998). N88D occurs in patients receiving nelfinavir and occasionally in patients receiving indinavir (Patick et al., 1998). In mixed

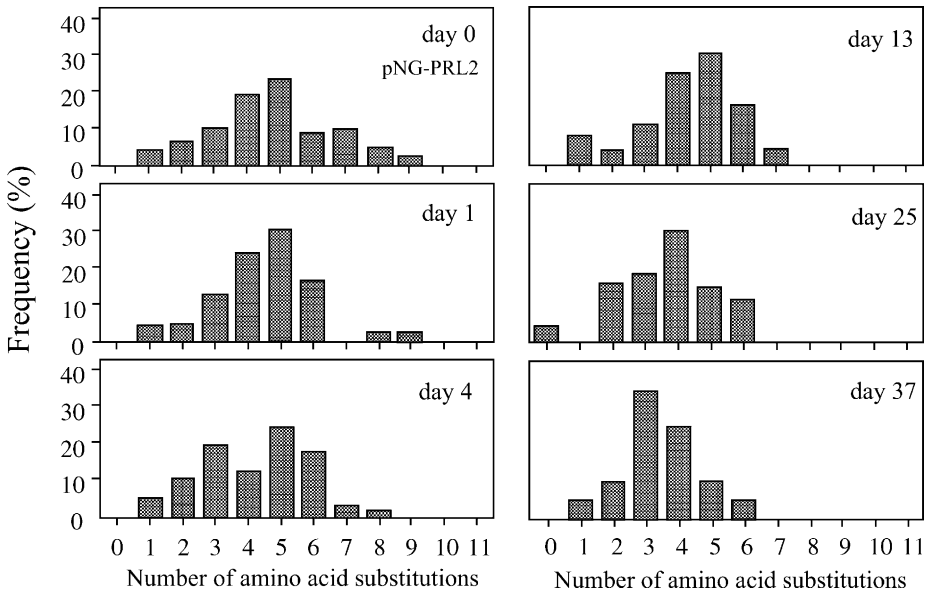


Fig. 3. Number of intended amino acid substitutions in protease of HIV-$1_{NG\text{-}PRL2}$ randomly sequenced on days 0, 1, 4, 13, 25 and 37. Forty-two clones for day 0, 27 clones for day 1, 25 clones for day 13, 25 clones for day 25 and 25 clones for day 37 were sequenced.

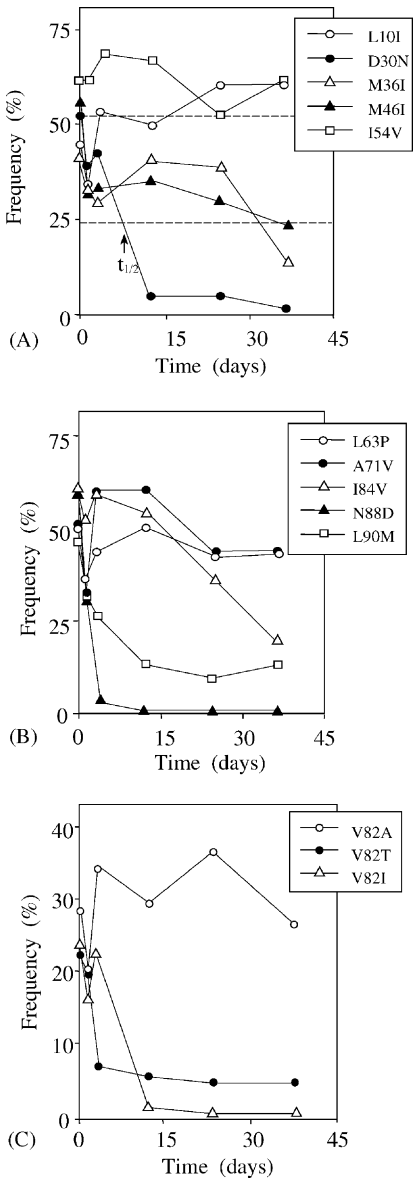


Fig. 4. Frequency of viral clones containing each amino acid substitution on days 0, 1, 4, 13, 25 and 37 was plotted. (A) L10I, D30N, M36I, M46I and I54V. (B) L63P, A71V, I84V, D88N and L90M. (C) V82A, V82I and V82T. The frequency of the mutations on day 0 was obtained from 42 randomly selected clones of the HIV-1 library pNG-PRL2.

infections starting with 53% D30N in the library, a sharp decrease shortly after infection was found. This decrease was sustained throughout the experiment (Fig. 3). The half life ($t_{1/2}$) of D30N was 7.2 days, indicating that the incorporation of D30N with various combinations of other mutations caused a marked decrease of infectivity. This might be partially because combination of D30N with L90M causes severe impairment of processing of viral polyproteins (Sugiura et al., 2002). Similarly, viruses containing the N88D mutation rapidly decreased in the absence of NFV.

V82A/T occurs predominantly in HIV-1 isolates from patients receiving treatment with IDV and RTV (Condra et al., 1996; Molla et al., 1996). V82A persisted in the viral mixture and the half life ($t_{1/2}$) was >36 days, indicating that, in most cases, incorporation of V82A did not lead to a serious replicative disadvantage when combined with other PI resistance-associated mutations. In contrast, V82T and V82I declined quickly shortly after infection; the half lives ($t_{1/2}$) were 2.1 days for V82T and 7.2 days for V82I (Table 1). M46I and I84V were relatively persistent; the half lives ($t_{1/2}$) were 28.1 and 30.6 days, respectively.

Other amino acid substitutions classified as secondary mutation or polymorphism were more persistent; the half life ($t_{1/2}$) of M36I was 34.2 days and the half lives of L10I, I54V, L63P and A71V were >36 days, indicating that most of the recombinant viruses containing these mutations had less of an effect on viral replication.

4. Discussion

It is generally assumed that HIV-1 variants with different mutations are generated because of the high replication rate of HIV-1 (Perelson et al., 1996), low fidelity of HIV-1 reverse transcriptase (Mansky and Temin, 1995) and high frequency of recombination among heterogeneous viral genomes (Burke, 1997; Moutouh et al., 1996). Compared to the diverse and large population of HIV-1 in vivo, the incorporation rate of mutations and size of a virus population in vitro is highly limited for the viral protease. To overcome this issue, we prepared a virus mixture containing 0–11 mutations associated with resistance to PIs (Yusa et al., 2002). Therefore, recipient cells with a high susceptibility for virus infection was crucial to retain diversity of the library virus. We maintained the library virus using the human T cell line MT-2. The library could not be maintained in activated peripheral blood mononuclear cells (PBMCs) because of their low susceptibility towards virus infection. The maximum level of infectious virions released from the infected PBMCs reached only 3% of that of MT-2 cells and the obtained library virus, after only four passages in the infected PBMC, showed a highly restricted number of mutants (data not shown).

The library containing L10I, M36I, M46I, I54V, L63P, A71V, V82A/T and L90M amino acid substitutions were chosen from the seven clinical isolates and showed ≥31-, ≥17-, ≥3-, ≥18-fold resistance to RTV, IDV, SQV and NFV, respectively (Yoshimura et al., 1999). These mutations are supposed to be key signature substitutions for multi-PI-resistance. D30N or N88D rarely co-exist at positions 82 and/or 84 (Ziermann et al., 2000). In the library, half lives ($t_{1/2}$) of D30N and N88D were 7.2 and 1.8 days, respectively. These results indicated that any combination of these mutations with the other mutations were a replicative disadvantage in most cases. Even in the presence of NFV alone, mutant viruses containing D30N and N88D were not dominantly isolated (data not shown). These results are consistent with previous data that the D30N mutation occurs

in patients receiving NFV and confers no in vitro or clinical cross-resistance to the other PIs (Markowitz et al., 1998; Patick et al., 1996; Winters et al., 1998; Zolopa et al., 1999).

V82A was the most persistent mutation at position 82. V82A was retained in the absence of drug pressure throughout the experiment until 36 days, whereas V82I and V82T showed a sharp decrease shortly after infection. This indicates that V82A can be maintained in the virus population for a relatively longer duration and is retained in a minor population as a reservoir of mutations even without drug pressure. Amino acid substitution at position 82 occurs predominantly in HIV-1 isolates from patients receiving treatment with IDV and RTV, and V82A has been the most commonly found mutation (Condra et al., 1996; Molla et al., 1996). The result obtained here also supports that the persistence contributed to the highly frequent emergence in vivo of PI-resistant viruses carrying the V82A mutation. These results indicate that this type of HIV-1 library is useful to evaluate the persistence of the diverse mutations in the viral protease.

In this report, we measured persistence of the mutations of HIV-1_{HXB2} protease in an HIV-1_{NL4-3} background. The persistence of these mutations may be influenced by the viral background. Also combination with other PI-associated mutations such as K20R, L24I, L33F, M46L, A71V and/or G73S (detected in the primary HIV-1 isoltes from seven heavily drug-experienced patients (Yoshimura et al., 1999)) and other types of mutations such as I50V/L (Markland et al., 2000) and/or N88S (Ziermann et al., 2000) may have different effects on persistence of the mutations concerned. Further analysis of the persistence of mutations associated with multi-PI resistance, additional mutations in protease, protease cleavage sites (Cote et al., 2001; Doyon et al., 1996) or Gag encoding sites (Gatanaga et al., 2002) may influence the persistence of each mutation.

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