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Isolation and Molecular Characterization of a Nelfinavir (NFV)-Resistant Human Immunodeficiency Virus Type 1 That Exhibits NFV-Dependent Enhancement of Replication

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During the use of a phenotypic anti-human immunodeficiency virus type 1 (HIV-1) drug resistance assay in a large set of clinical virus isolates, we found a unique variant (CL-4) that exhibited a high level of nelfinavir (NFV) resistance and rather enhanced replication under subinhibitory concentrations of NFV (0.001 to 0.1 μM). Comparison of *gag-pol* sequences of the CL-4 variant and its predecessor virus isolates showed a stepwise accumulation of a total of 19 amino acid substitutions in protease (PR) and Gag p17 during 32-month NFV-containing antiretroviral therapy, while other Gag regions including the cleavage sites of the p55 precursor remained highly conserved. To understand the relationship between the genetic and phenotypic changes in CL-4, we constructed chimeric viruses using pNL4-3, replacing the PR, p24PR, or p17PR gene segment of CL-4 or its predecessor. A series of tissue culture infections with the chimeras in the absence or presence of increasing concentrations of NFV demonstrated that only the p17PR segment of CL-4 could confer the NFV-dependent replication enhancement phenotype on NL4-3. Our data suggest a novel adaptation mechanism of HIV-1 to NFV, in which coevolution of Gag and PR genes generates a variant that replicates more efficiently in the cellular environment in the presence of NFV than without the drug.

Human immunodeficiency virus type 1 (HIV-1) protease (PR) is essential for maturation of virus particles. During or after budding of virus particles from the plasma membrane, PR cleaves Gag and Gag-Pol precursor proteins into the multifunctional Gag proteins (matrix [MA; p17], capsid [CA; p24], and nucleocapsid [NC; p7]) and three enzymes (reverse transcriptase [RT], integrase, and PR), which are indispensable for de novo retroviral replication (13, 26). Protease inhibitors (PIs) are designed to bind into the active site of the PR homodimer, a functional entity of PR, and block its catalytic activity. Consequently, PI treatment of infected cells results in accumulation of morphologically immature, replication-incompetent viral particles, leading to prevention of de novo infection. Various PIs, such as indinavir, saquinavir (SQV), ritonavir, amprenavir, and nelfinavir (NFV), are now widely used clinically as the main drug in highly active antiretroviral therapy (HAART), in combination with other classes of anti-HIV-1 drugs.

When suppression of HIV-1 replication by PI-containing therapy is incomplete, variants with reduced sensitivity to the PI can emerge by accumulating nonsynonymous mutations in the PR gene (4, 14, 17, 20), causing a serious reduction in the clinical efficacy of HAART (1, 5, 10). PI resistance-associated mutations often affect the substrate specificity of PR (8) and can impair enzyme function, resulting in reduction of the replicative capacity of the variants (2, 7, 16–18, 28). In some in vivo infection cases, however, the impaired growth capacity recovers partially by accumulation of secondary mutations at the cleavage sites in the Gag-Pol precursor (6, 16, 21, 29) or at non-cleavage sites in Gag (11). Thus, under selective pressures of PIs, HIV-1 seems to evolve through stepwise accumulation of amino acid substitutions to increase the replicative advantages under the PI environment.

While the assumption is conceivable, no study has thus far addressed a variant(s) that replicates better in the presence of a PI than without the drug. Here, we report a remarkable HIV-1 NFV-resistant case, in which HIV-1 had evolved after prolonged administration of an NFV-containing regimen to display not only a high level of NFV resistance but also enhanced replication under subinhibitory concentrations of NFV. Molecular characterization of the variant suggested that coevolution of Gag and PR genes had provided the predecessor virus in the host the ability to replicate better in the presence of NFV than in the absence of the drug. Our data illustrate a novel mechanism, i.e., NFV-dependent replication enhancement, for HIV-1 adaptive evolution under the selective pressure of NFV.

MATERIALS AND METHODS

Clinical specimens and ethical considerations. A Japanese homosexual man infected with HIV-1 had consulted the AIDS Clinical Center, International Medical Center of Japan, since 1997. Plasma samples and clinical isolates were obtained serially from the patient. The patient had been treated with various

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anti-HIV agents, including zidovudine, zalcitabine (ddC), and SQV, before treatment with lamivudine plus stavudine combined with NFV. The Institutional Ethics Committee approved this study (IMCJ-H13-80), and a written informed consent was obtained from the patient.

Virus isolates. Clinical HIV-1 isolates CL-1, CL-2, CL-3, and CL-4 were obtained from the serial plasma samples obtained from our patient by using a CCR5-expressing HeLa/CD4$^+$ cell clone 1-10 (MAGIC-5) (12). Briefly, MAGIC-5 cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in a 48-well plate for 24 h were incubated with 1 ml of fresh plasma. The culture medium was changed every 2 or 3 days until a cytopathic effect was observed. Spread of HIV infection in the culture was confirmed by staining the cells with 5-bromo-4 chloro-3-indoyl-β-D-galactopyranoside (X-Gal) and measuring HIV-1 p24 antigen in the culture supernatant. The virus isolates were kept at −80°C until use.

Cells. Peripheral blood mononuclear cells (PBMCs) obtained from HIV-1-seronegative healthy donors were stimulated with 1 μg of phytohemagglutinin (PHA)/ml for 72 h and grown in RPMI 1640 with 10% FCS and 10 U of interleukin-2 (Gibco-BRL, Grand Island, N.Y.) per ml for 24 h before infection. Transformed T-cell lines (MT-2 and PM-1 [15]) were maintained in RPMI 1640 with 10% FCS.

Drug susceptibility assay with MAGIC-5 cells. NFV was kindly provided by the Japan Tobacco Co. (Tokyo, Japan). The drug susceptibility of the virus isolates to NFV was determined with MAGIC-5 cells (12). Briefly, MAGIC-5 cells (10^4) were infected with the diluted virus stock (300 blue cell-forming units [BFU]) in increasing concentrations of NFV (0, 0.001, 0.01, 0.1, and 1 μM) and incubated for 78 h. The culture supernatant was transferred to a new well containing MAGIC-5 cells without NFV and incubated for 48 h, fixed and stained with X-Gal, and counted under a microscope to assess the magnitude of de novo infection. The 50% inhibitory concentration (IC_{50}) of NFV was calculated based on the dose-response curve. This experiment was performed in triplicate and repeated twice.

Sequence analyses of *gag* and *pol* genes. Viral RNA was extracted from HIV-1 isolates with a High-Pure viral RNA kit (Boehringer, Mannheim, Germany), followed by RT-PCR with a One-Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan) to amplify the HIV-1 *gag-pol* DNA segment (2,341 bp). The first RT-PCR was conducted with a F641-R2982 primer pair (F641, 5′-GCCCGAACAGGGACTTGAAAGCG, pNL4-3 primer binding site at position 641 to 662; R2982, 5′-GATATCTAATCCCTGGTGTCT, pNL4-3 *pol* at position 2961 to 2982). The second PCR was performed with a F671-R2961 primer pair (F671, 5′-CCAGAGGAGATCTCTCGACGC, pNL4-3 noncoding positions 671 to 692; R2961, 5′-TCTTGTTTATACTAGGTATG, pNL4-3 *pol* position 2940 to 2961). The PCR products were purified with SUPREC-02 (TaKaRa Shuzo) and subjected to direct sequencing with an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif.). The primers used for the sequencing reaction were F671, F990 (5′-CCTTCAGACAGGATCAGAAG, pNL4-3 *gag* position 990 to 110), F1283 (5′-GCCCAGAAGTAATACCCATG, pNL4-3 *gag* position 1283 to 1302), F1741 (5′-ACAGAAACCTTGTTGGTCCA, pNL4-3 *gag* position 1741 to 1760), F2012 (5′-CTAGGAAAAAGGGCTGTTGG, pNL4-3 *gag* position 2012 to 2031), and DRPR3 (5′-AGCAGGAGACGATAGACAAGG, pNL4-3 *gag* position 2228 to 2248). Amino acid sequences were deduced with the Genetyx-Win program version 4.1 (Software Development, Tokyo, Japan).

Construction of *gag-pro* recombinant DNA clones. pUC18 containing the *Sac*I-*Sse*8387I fragment (2,357 bp) of pNL4-3 (pUC18-NL4-3-SaSs) was constructed first to facilitate molecular cloning of the *gag-pro* segment. The DNA fragments amplified by PCR from the primary isolates were digested with *Bss*HII and *Bal*I (*Bss*HII-*Bal*I; 1,908 bp), and the fragment covering the entire *gag* and PR gene was cloned into pUC18-NL4-3-SaSs. A subclone designated p17PRmt-BsBa, with a sequence identical to that of each clinical isolate determined by the direct-sequencing method, was selected as a representative clone of the virus isolate. Subsequently, the *Eco*T22I-*Bal*I fragment (1,372 bp) and the *Apa*I-BalI fragment (615 bp) of p17PRmt-BsBa covering the *gag* p24-PR and PR genes, respectively, were cloned into pUC18-NL4-3-SaSs. These two clones were designated p24PRmt-EcBa and PRmt-ApBa. Lastly, three pUC18-NL4-3-SaSs constructs carrying cloned p17PRmt-BsBa, p24PRmt-EcBa, and PRmt-ApBa were digested with *Bss*HII and *Sse*8387I. Then, the digests (2,133 bp) were cloned back into pNL4-3 to generate full-length HIV-1 molecular clones of NL4-3PRmt, NL4-3p24PRmt, and NL4-3p17PRmt. The nucleotide sequences of the PCR-amplified fragments and around the recombinant sites of p17PRmt, p24PRmt, and PRmt were verified with an automatic sequencer.

Preparation of cell-free virus stocks of *gag-pro* recombinants by transfection. HeLa cells (5×10^5 cells) were grown in DMEM with 10% FCS in a T25 flask for 24 h and transfected with 3 μg each of pNL4-3, pNL4-3PRmt, pNL4-3p24PRmt, and pNL4-3p17PRmt plasmid DNA using FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). The cells were incubated at 37°C for 24 h, washed once with DMEM, and cultured in 3 ml of DMEM containing 10% FCS. The culture supernatant containing the chimera virus was collected at 48, 72, and 96 h after transfection, respectively, filtered (0.45-μm pore size), analyzed for RT activity (27), and kept at −80°C until use.

Effects of NFV on HIV replication. The method used to infect cells has been described previously (23–25). Briefly, PHA-stimulated PBMCs (2×10^5 cells), MT-2 cells (2×10^4 cells), and PM-1 cells (2×10^4 cells) were infected with 0.2 ml of cell-free supernatant containing HIV-1 (2×10^5 ^{32}P cpm of RT activity) in the absence or presence of NFV (0.1 and 1 μM) at 37°C for 16 h, washed once, and cultured in 0.2 ml of culture medium with the same concentration of NFV. In all infections, half of the culture medium volume was changed every 2 or 3 days, and the supernatant was kept at −80°C until use. Each experiment was carried out in duplicate and repeated three times.

Western blot analysis. HeLa cells were transfected with 3 μg each of pNL4-3, pNL4-3p17PRmt, or pNL4-3PRmt plasmid DNA in the absence or presence of 0.1 μM NFV. The culture supernatant was harvested at 48 h after transfection and centrifuged at 37,800 × *g* for 90 min at 4°C to pellet virus particles. Transfected HeLa cells were washed once with phosphate-buffered saline and prepared for protein analysis as described previously (22). The virion pellet (6×10^5 cpm of RT activity) and cellular protein (25 μg of protein) resolved in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, Calif.) were fractionated with sodium dodecyl sulfate gradation gel (10 to 20%) electrophoresis (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane (Millipore, Bedford, Mass.). The membrane was incubated with serum from an HIV-1-seropositive patient and hybridized with anti-protein A antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The immune complex was visualized with an ECL system (Amersham Pharmacia Biotech) according to the instructions provided by the manufacturer. The level of p24 in the loading sample was measured using Lumipulse Ortho HIV-1/2 (Fuji-Rebio, Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence data reported here have been submitted to the DDBJ database under the accession numbers AB083565 through AB083568.

RESULTS

Identification of an HIV-1 variant CL-4 exhibiting NFV-dependent enhancement of replication. We have recently established a rapid and simple assay for assessing the drug susceptibility of HIV-1 using the CCR5-expressing HeLa/CD4$^+$ cell clone 1-10 (MAGIC-5) (12). In conducting the phenotypic anti-HIV-1 drug resistance assay using this system for a large set of clinical virus isolates, we noticed that some PI-resistant variants appeared to replicate better in the presence of the corresponding PI used in the treatment (data not shown). To better understand the underlying mechanism(s) of this observation, we selected a representative case treated with an NFV-containing regimen for the present study.

The clinical history and phenotypic drug resistance profile of the patient are summarized in Table 1. The nadir of the CD4-positive T-cell count was 68/μl, and the plasma HIV-1 RNA level was 2.1×10^5 copies/ml 8 months before commencement of treatment with the NFV-containing regimen. At the time of obtaining clinical isolate 1 (CL-1), the patient was being treated with ddC and SQV. Clinical isolates 2 (CL-2), 3 (CL-3), and 4 (CL-4) were obtained 11, 23, and 32 months after commencement of HAART, respectively. Although CD4 counts were increased to >100/μl, suppression of the viral load was incomplete during such treatment. Coinciding with a sustained high viral load, CL-3 exhibited high levels of resistance to SQV and NFV (increases in IC_{50}s of SQV and NFV were more than 100-fold). After receiving 9 months of the same NFV-containing regimen, a variant CL-4 was isolated from the patient which was found to be extremely resistant to NFV, as

TABLE 1. Clinical data and time of isolation of clinical isolates[a]

Isolate	Regimen	Mos after NFV	CD4/μl	Viral load (copies/ml)	Fold resistance (IC_{50}/μM)	
					NFV	SQV
—[b]	AZT	−8	68	2.1×10^5	NT	NT
CL-1	ddC, SQV	−2	84	8.5×10^4	NT	NT
CL-2	d4T, 3TC, NFV	11	246	1.6×10^4	NT	NT
CL-3	d4T, 3TC, NFV	23	192	1.9×10^4	107 (0.22)	156 (0.59)
CL-4	d4T, 3TC, NFV	32	166	3.6×10^4	600 (>1)	128 (0.48)

[a] The phenotypic drug resistance assay was performed using MAGIC-5 cells. Fold resistance was calculated by dividing the IC_{50} of the clinical isolate by the IC_{50} of NL4-3. AZT, zidovudine; ddC, zalcitabine; SQV, saquinavir; d4T, stavudine; 3TC, lamivudine; NFV, nelfinavir; NT, not tested.
[b] —, no isolate employed.

evidenced by the significantly increased IC_{50} (from 107- to 600-fold). On the other hand, the IC_{50} of SQV remained similar during this period (from 156- to 128-fold).

In the drug resistance assay system using MAGIC-5 cells (12), CL-3 displayed a typical dose-response curve to NFV, similar to that usually seen in most PI-resistant clinical isolates (Fig. 1A). In contrast, the dose-response curve of CL-4 to NFV was quite unique. Counts of blue cells derived from HIV-1 infection were consistently high in repeated experiments in the presence of 0.001 to 0.1 μM NFV, reaching up to 119% of that without NFV, suggesting that CL-4 replicates better in the presence of subinhibitory concentrations of NFV than in the absence of the drug in CCR5-expressing HeLa/$CD4^+$ cells.

To assess the effects of NFV on CL-4 replication in a more physiologically relevant system, PHA-stimulated PBMCs were infected with CL-4 and replication kinetics were monitored in the absence or presence of NFV (0.1 and 1.0 μM) by measuring RT activity released into the culture supernatant (Fig. 1B). In the absence of NFV, the kinetics of CL-4 replication were similar to those of the drug-sensitive control virus NL4-3, although the level of RT activity of CL-4 at the peak of infection (day 7) was about half that of NL4-3. In the presence of 0.1 μM NFV, replication of NL4-3 was completely suppressed, whereas variant CL-4 showed very efficient replication. In three repeated experiments, the replication kinetics of CL-4 was constantly fast, and the level of RT activity at the peak of infection was consistently higher in the presence of 0.1 μM NFV than without the drug (Fig. 1B, CL-4), suggesting that the subinhibitory concentration of NFV could potentiate replication of variant CL-4 in PBMCs, as well as in CCR5-expressing HeLa/$CD4^+$ cells. Such enhancement of replication was not seen with other PIs, such as SQV and amprenavir, under the same experimental conditions. It was also not seen with variant CL-3, the predecessor virus isolate of CL-4 (data not shown), suggesting that the phenomenon is specific to CL-4 and NFV.

Genetic changes of *gag-pro* genes during HAART. The above clinical data and phenotypic profiles of the clinical isolates suggested that PI-resistant HIV-1 variants emerged first, from which the CL-4 variant with the NFV-dependent replication enhancement phenotype had evolved. To assess the genetic changes in HIV-1 during antiretroviral therapy in our patient, we determined the nucleotide sequences of the *gag-pro* genes of CL-1, CL-2, CL-3, and CL-4 by direct sequencing of amplified DNA.

Comparison of the PR sequences showed a stepwise accumulation of amino acid substitutions during 32 months of treatment with an NFV-containing regimen (Table 2). With regard to PI resistance-associated mutations in the PR region, the CL-1 variant, which was isolated 2 months before the use of the NFV-containing regimen, already possessed a single mutation (Leu10→Ile), which might have developed during the preceding SQV-containing regimen. After 11 months of treatment with NFV, the patient harbored variant CL-2, which possessed four amino acid substitutions: L10I (Leu10→Ile), G48V, I54V, and V82A. CL-3 and CL-4 gained another substitution, M36I.

Interestingly, other substitutions (E35D, N37S, K43T, I62V, I72V, and T74S), whose contributions to PI resistance have not been described previously, also accumulated gradually in a manner dependent on the duration of NFV therapy. In particular, while CL-4 showed no further substitutions compared with CL-3 at the known drug resistance-associated mutation sites, three novel substitutions (E35D, N37S, and K43T) had accumulated in the N terminal of the PR.

Similarly, comparison of Gag sequences revealed several stepwise changes that occurred most remarkably in the Gag p17 peptide (Table 3). A total of nine amino acid substitutions (N47D, K55Q, M61R, G62R, F66S, V82I, S109N, Q117E, and N129D) accumulated gradually and sporadically through the p17 region of variant CL-4 during 32 months of NFV-containing antiretroviral therapy. In contrast, other regions of Gag were highly conserved during this period. This conservation was also noticed around the cleavage site of the Gag p55 precursor, and only a single substitution was found in CL-4 (Table 4).

Roles of genetic changes in conferring the biological phenotype of CL-4. To assess the roles of the mutations described above in shaping the biological phenotype of CL-4, several recombinant molecular clones were constructed based on the pNL4-3 genetic background (Fig. 2). NL4-3PRmt, NL4-3p24PRmt, and NL4-3p17PRmt carried the cloned p1-p6-PR, p24-p2-p7-p1-p6-PR, and p17-p24-p2-p7-p1-p6-PR segments, respectively, from CL-4 or CL-3 virus isolates in the backbone of pNL4-3. They were used to assess the role of sporadic mutations in the corresponding regions of clinical isolates with respect to the genetic backbone of a drug-sensitive HIV-1.

PHA-stimulated PBMCs, MT-2, and PM-1 cells were infected with an amount of virus corresponding to 2×10^5 cpm of RT activity (26), and virus replication was monitored in the absence or presence of 0.1 and 1 μM NFV (Fig. 3). In the absence of NFV, all recombinant viruses tested retained replication competence in all tissue culture infection systems

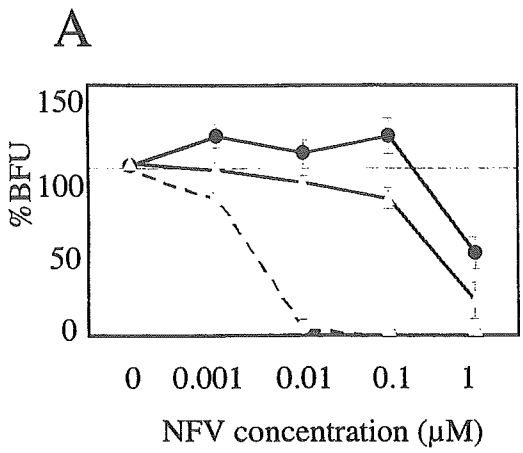

FIG. 1. Identification of HIV-1 variant CL-4 that exhibits NFV-dependent enhancement of replication. (A) Effect of NFV on HIV-1 infectivity of MAGIC-5 cells (12). CCR5-expressing HeLa/CD4$^+$ cells (MAGIC-5) were infected with 300 BFU of HIV-1 in the presence of the indicated concentrations of NFV for 78 h. The infectious titer was measured in culture supernatants of MAGIC-5 cells, and the percentages of BFU in NFV-treated cultures relative to those in NFV-free cultures were determined. Data are mean ± standard deviation values of six determinations. ○, NL4-3; △, CL-3; ●, CL-4. (B) Effects of NFV on HIV-1 replication in PBMCs. PHA-stimulated PBMCs (2×10^5 cells) were infected with NL4-3 and CL-4 (2×10^5 ^{32}P cpm of RT activity) in the absence (○) or presence (●) of 0.1 μM NFV and cultured in the same concentration of NFV. Progeny virion production was monitored by RT activity (27) released into the culture medium at the indicated time points. Each experiment was carried out in duplicate using three different batches of donor PBMCs (panels a, b, and c).

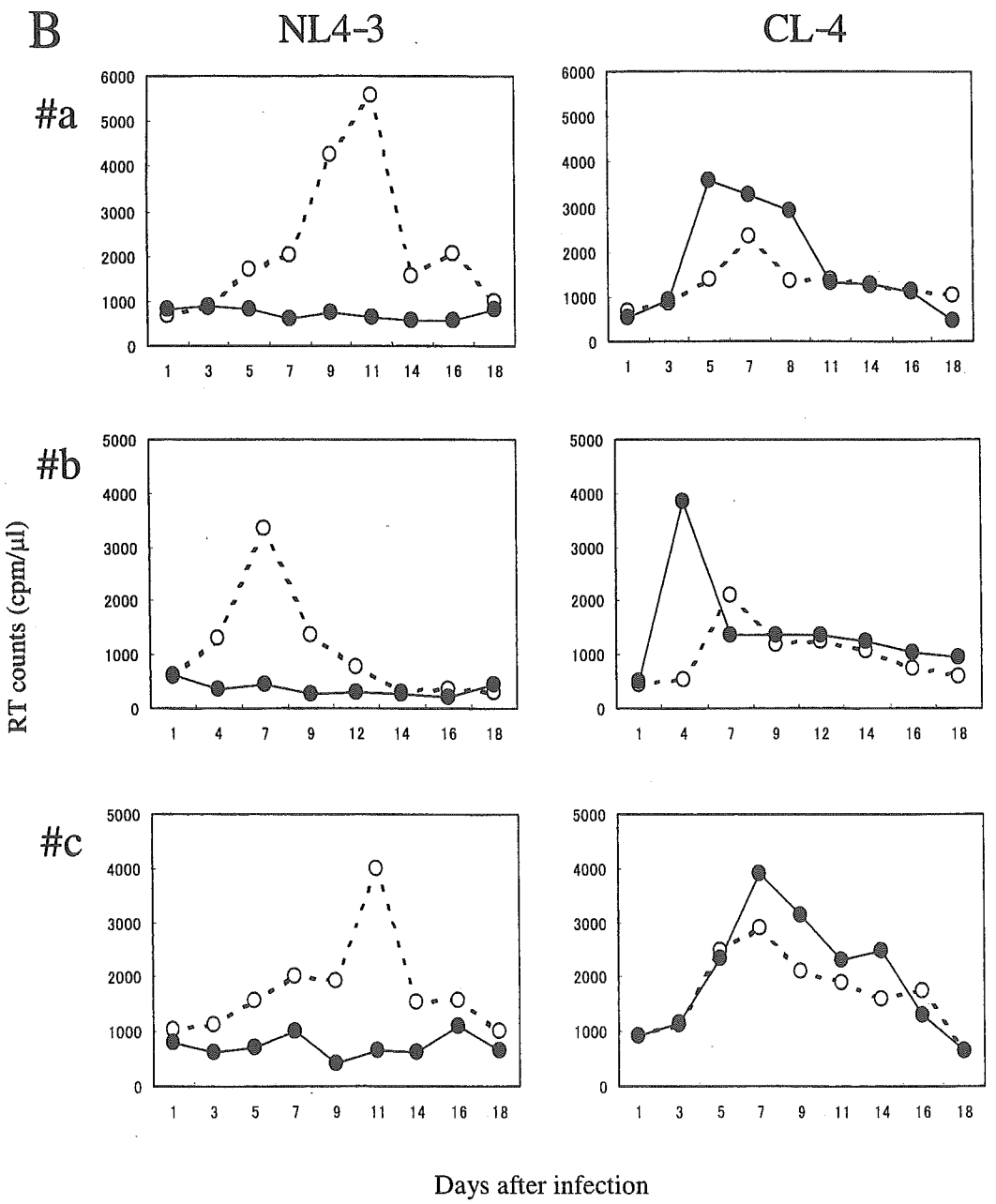

TABLE 2. Amino acid substitutions in PR[a]

Isolate	Resistance-associated mutations					Other mutations					
	L10	M36	G48	I54	V82	E35	N37	K43	I62	I72	T74
CL-1	I	—	—	—	—	—	—	—	—	—	—
CL-2	I	—	V	V	A	—	—	—	—	V	S
CL-3	I	I	V	V	A	—	—	—	V	V	S
CL-4	I	I	V	V	A	D	S	T	V	V	S

[a] For each amino acid residue, the letter in the top lane indicates the amino acid associated with NL4-3. A dash indicates identity with NL4-3, and a single letter indicates the amino acid substitution. For example, CL-1 had an amino acid substitution at Leu10→Ile (L10I).

tested. The replication kinetics in PBMCs were comparable among parental and each recombinant NL4-3, whereas those in PM-1 and MT-2 were significantly slower with the recombinant NL4-3 carrying the p17-PR segment of CL-3 or CL-4 than with others (Fig. 3). These data suggest the presence of intrinsic impairment of the Gag-PR segment of CL-3 and CL-4 that becomes apparent only on replication in transformed T-cell lines.

As expected, the parental NL4-3 did not grow at all in the presence of NFV, whereas all CL-4 recombinants retained replicative capacity under such an environment. The recombinant carrying the C-terminal portion of Gag and the entire PR (NL4-3PRmt of CL-4) grew efficiently in the presence of 0.1 μM NFV in all cells tested (Fig. 3A, PR), suggesting that mutations in these segments alone are sufficient to confer NFV resistance on NL4-3.

Of note was the recombinant carrying the entire *gag* and PR genes of CL-4 (NL4-3p17PRmt of CL-4). In the presence of 0.1 μM NFV, the recombinant replicated with significantly faster kinetics to higher titers than the same amount of virus did in the absence of the drug (Fig. 3A, p17PR). The NFV-dependent replication enhancement was observed in all cells tested in three repeated experiments. In contrast, the phenomenon was not seen in other recombinants of CL-4 (Fig. 3A, PR and p24PR), or in a recombinant carrying the entire *gag-pro* genes of CL-3 (Fig. 3B, p17PR). These data suggest that mutations in the Gag p17 segment of CL-4 are indispensable for generating the phenotype of the original CL-4 virus isolate.

Western blot analyses of the Gag processing pattern in the presence of NFV. To obtain further insight into the role of NFV in modulating viral infectivity, the Gag processing pattern was assessed in the absence or presence of NFV by Western blot analysis (Fig. 4). After transfection of equal amounts of the parental and recombinant NL4-3 DNAs into HeLa cells, the cells were cultured with or without NFV for 48 h. Virions corresponding to 6×10^5 cpm of RT activity (Fig. 4A) and cell lysate containing 25 μg of protein (Fig. 4B) were loaded in each lane for electrophoresis.

In the absence of NFV in virions (Fig. 4A), the Gag p55 precursor of the NL4-3 control was efficiently cleaved into the Gag p24 CA peptide (Fig. 4A, lane 1). In contrast, processing of the recombinant carrying the PR of CL4 was less efficient, as evidenced by the presence of p55 and p41 Gag uncleaved products (Fig. 4A, lane 3), suggesting that the CL-4 PR mutant altered the substrate specificity and that the NL4-3 Gag precursor was not an efficient substrate. Interestingly, the processing of the recombinant carrying the entire Gag and PR of CL4 was almost as efficient as that of the parental NL4-3 (Fig. 4A, lane 5), suggesting that mutations in CL-4 Gag compensate for the impairment of the PR mutant.

In the presence of NFV (0.1 μM), NL4-3 virions only had the p55 precursor (Fig. 4A, lane 2), confirming that the concentration of NFV used in the present replication experiment could completely block the PR function of the PI-sensitive clone. In contrast, processing of the recombinant carrying the PR of CL4 was not significantly affected by NFV (Fig. 4A, lane 4). The amount of p55 Gag precursor was completely cleaved in the recombinant virus carrying p17PR4 (Fig. 4A, lane 6).

The Western blot analysis of virions failed to reveal cleavage enhancement by NFV in p17PR4 carrying recombinant virus (Fig. 4A, lanes 5 and 6). Therefore, we further analyzed the cleavage pattern in the transfected HeLa cells. As expected, p41 Gag was efficiently cleaved in NL4-3 in the absence of NFV (Fig. 4B, lane 1). In contrast, such a cleavage was inhibited by NFV (Fig. 4B, lane 2). In PR4 carrying recombinant virus, p41 Gag was visible both in the absence and presence of NFV (Fig. 4B, lanes 3 and 4), suggesting that the cleavage efficiency was partially complicated but not affected by NFV. In the absence of NFV, the cleavage efficiency of the p17PR4-carrying recombinant virus was still impaired, as suggested by the presence of visible p41 Gag (Fig. 4B, lane 5). However, in the presence of NFV, p41 Gag was cleaved as efficiently as NL4-3 (wild type) (Fig. 4B, lane 6).

TABLE 3. Amino acid substitutions in Gag p17

Isolate	Amino acid substitution in Gag p17[a]									
CL-1	N47	K55	M61	G62	F66	V82	S109	Q117	N129	N130
CL-2	—	E	I	—	S	—	—	—	—	D
CL-3	D	E	I	—	S	—	—	—	—	D
CL-4	D	Q	I	R	S	I	N	E	D	—

[a] The letter and number in the top lane indicate the amino acid residue and its position in the Gag region associated with CL-1. A dash indicates identity with CL-1, and a single letter indicates the amino acid substitution. For example, CL-3 had an amino acid substitution at Asn47→Asp (N47D).

DISCUSSION

In the present study, (i) we identified a unique NFV-resistant variant of HIV-1 (CL-4) by applying our simple drug resistance assay system (12); (ii) we classified, based on sequence comparisons, the mutations potentially involved in conferring the CL-4 phenotype, which were determined by using the pNL4-3-based chimeric viruses harboring the gene segment responsible for conferring the CL-4 phenotype; and (iii) we assessed the roles of CL-4 Gag mutations in compensating Gag cleavage defects of the PR mutant. Based on the above analyses, we showed in the present study (i) the existence of an HIV-1 variant that replicated more efficiently under NFV than in the absence of this drug (Fig. 1); (ii) gradual accumulation of point mutations in the PR and Gag p17 during treatment with an NFV-containing regimen, but lack of any such change in most cleavage sites of the Gag precursor (Tables 2, 3, and 4); (iii) mutations in the CL4 Gag p6-PR segment alone are sufficient to confer NFV resistance, while those in the CL-4 Gag p17 are indispensable for generating the CL-4 phenotype (Fig.

TABLE 4. Amino acid substitutions at cleavage sites of Gag precursor

Isolate	Amino acid substitutions at cleavage sites of Gag precursor[a]				
	MA/CA	CA/p2	p2/NC	NC/p1	p1/p6
CL-1	SQNF/PIVQ	ARVL/AEAM	GAIM/MQRG	RQAN/FLGK	PGNF/LQSR
CL-2	----/----	----/----	----/----	----/----	----/----
CL-3	----/----	----/----	----/----	----/----	----/----
CL-4	----/----	--I-/----	----/----	----/----	----/----

[a] Flanking amino acid residues at cleavage sites of Gag precursor are listed. Each amino acid is associated with CL-1. A dash indicates identity with CL-1. MA, matrix (p17); CA, capsid (p24); NC, nucleocapsid (p7).

3); and (iv) mutations in the CL-4 Gag can compensate for Gag cleavage defects caused by PR mutations (Fig. 4).

During acquisition of the CL-4 phenotype, three amino acid substitutions (E35D, N37S, and K43T), a single substitution (V to I), and seven substitutions (E55Q, G62R, V82I, S109N, Q117E, N129D, and D130N) accumulated in a stepwise fashion in the PR, the Gag CA/p2 cleavage site, and the Gag p17, respectively (Tables 2, 3, and 4). In contrast, other regions remained highly conserved. Our data suggest that some or all of these mutations, in concert with preexisting mutations, culminated in the formation of the CL-4 phenotype of HIV-1 during the 9-month NFV-based therapy. In particular, the substitutions in Gag p17 are essential, because only the p17PR segment of CL-4, but not of CL-3, or the p24PR segment or PR segment could confer the CL-4 phenotype of the drug-sensitive virus (Fig. 3). It is possible, however, that substitutions localized to the α-helix of the C-terminal domain of Gag p17 might interact with a p24 mutation and alter the exposure of the MA-CA cleavage site in the Gag precursor. Further studies involving site-directed mutagenesis are necessary to determine the precise set of mutations conferring the NFV-dependent replication enhancement phenotype.

The underlying molecular mechanism(s) of the NFV-dependent replication enhancement was not identified in the present study. It is possible that in the case of the mutant PR of CL-4, NFV acts as an allosteric effector and regulator of the enzyme function, instead of acting as a competitive inhibitor. Modulation of binding affinity to a substrate by binding of different

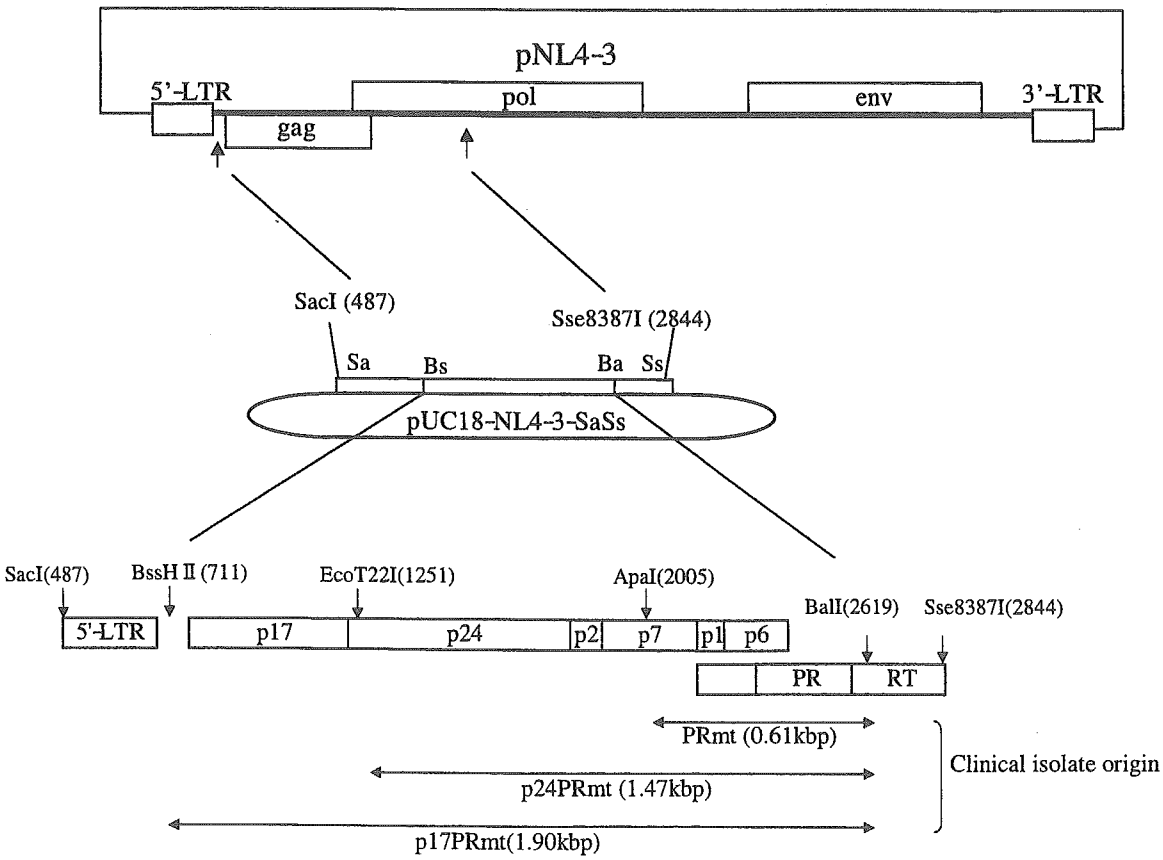

FIG. 2. Construction of pNL4-3-based *gag-pro* recombinants. The HIV-1 *gag-pro* DNA segment was amplified by RT-PCR from the CL-3 or CL-4 virus isolate and replaced with the *Bss*HII-*Bal*I fragment of pUC18-NL4-3-SaSs. Subsequently, the *Bss*HII-*Sse*8387I fragment of pUC18-NL4-3-SaSs was cloned into pNL4-3 to reconstitute full-length HIV-1 molecular clones.

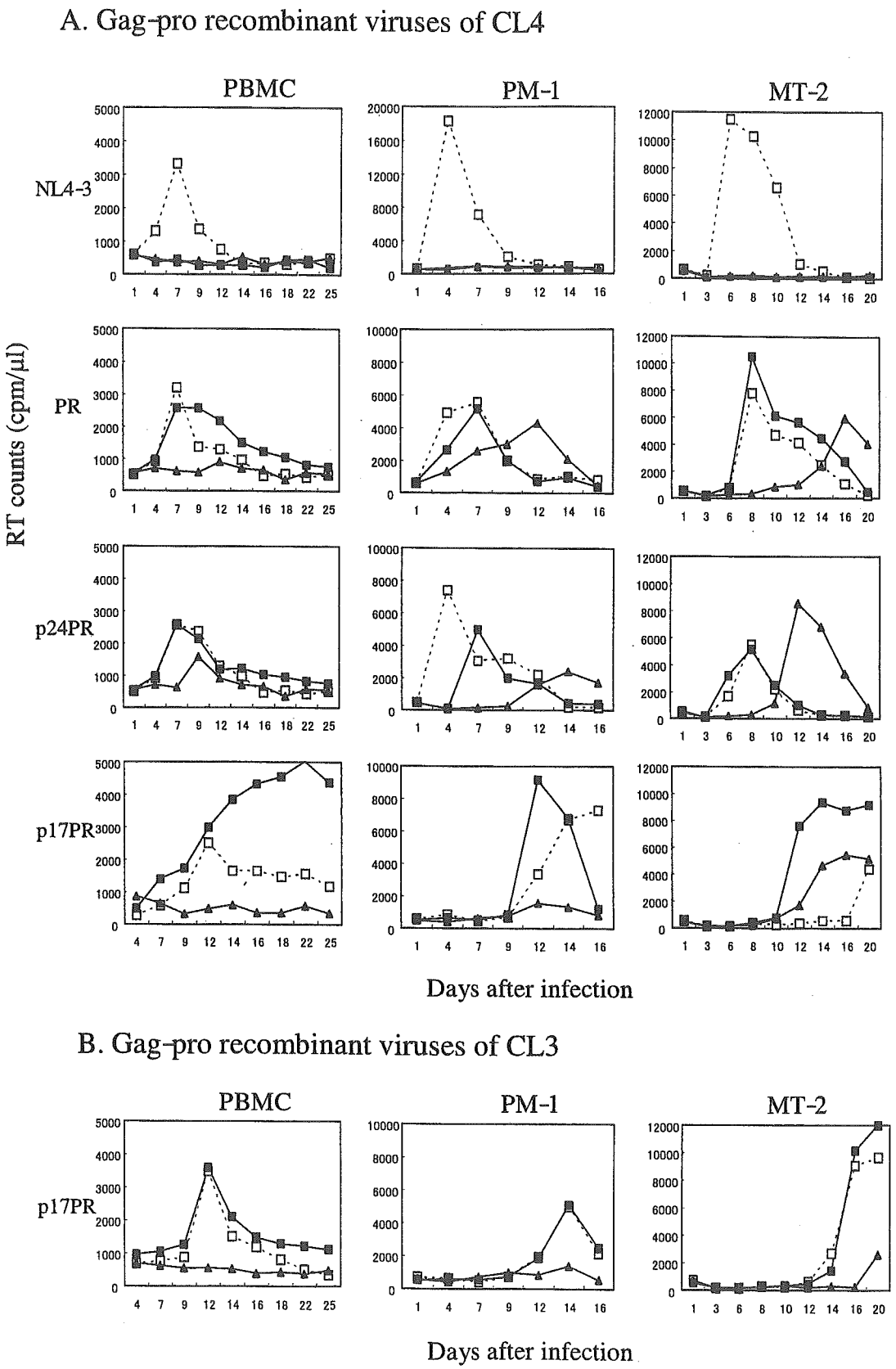

FIG. 3. Effects of NFV on replication of *gag-pro* recombinant viruses in PBMCs, PM-1, and MT-2 cells. (A) Replication kinetics of NL4-3 and NL4-3PRmt, NL4-3p24PRmt, and NL4-3p17PRmt of CL-4 were examined with PBMCs, PM-1, and MT-2 in the absence (□) or presence of 0.1 μM (■) and 1 μM (▲) NFV. (B) Replication kinetics of NL4-3p17PRmt of CL-3 were examined under the same conditions.

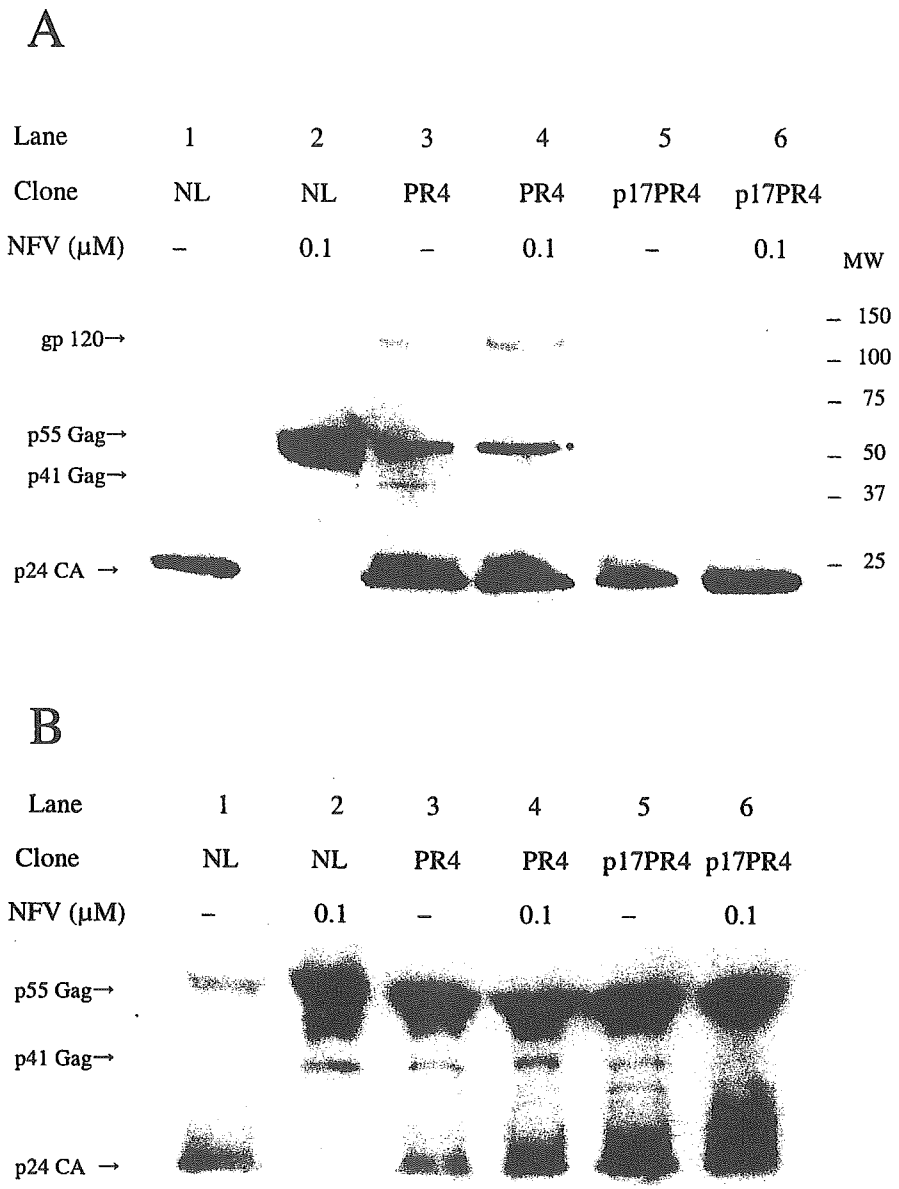

FIG. 4. Western blot analyses in the absence or presence of NFV. HeLa cells were transfected by full-length molecular clones and cultured in the absence or presence of NFV (0.1 μM). At 48 h posttransfection, virions in culture supernatants (A) and cells (B) were harvested and subjected to Western blot analysis. HIV Gag protein was visualized using serum from an HIV-1-seropositive patient. Lanes 1 and 2, pNL4-3; lanes 3 and 4, pNL4-3PRmt; lanes 5 and 6, pNL4-p17PRmt.

low-molecular-weight ligands is commonly seen in multisubunit proteins for metabolic control and is an important mechanism for regulating enzyme activity. Although no study has reported such an allosteric feature for the HIV-1 PR homodimer, prolonged selective pressures of NFV during HAART, combined with a high level of tolerance of the HIV-1 PR to the sequence variation, might have generated the PR mutant possessing an allosteric binding site to NFV. In this context, mutations of p17 on the Gag-pol precursor of CL-4 could be critical in enhancing the cleavage of the Gag-pol polypeptide by the NFV-bound PR.

In this regard, Western blot analyses suggested that the cleavage efficiency of the Gag C-terminus p6 of this mutant can be enhanced in the presence of NFV, which is consistent with the above possibility. The possible allosteric effects of NFV on the CL-4 PR should be most effective for CL-4 Gag substrates, because only the chimera possessing the entire Gag of CL-4 exhibited a detectable NFV effect on virus replication (Fig. 3). Biochemical and structural studies of the CL-4 PR are necessary in order to assess each of these issues.

Several studies have suggested coevolution of *gag* and *pro* genes during treatment with PI-containing regimens (6, 16, 21, 29). It is conceivable that the HIV-1 PR and its substrates evolve coordinately to generate the correct processing products, thereby assuring production of infectious progeny virions. Most of the *gag* mutations for PI resistance reported so far are

located around the cleavage sites of the Gag p55 precursor (6, 16, 21, 29), whereas the roles of mutations at the Gag non-cleavage sites have been poorly addressed. Because highly PI-resistant viruses often lack cleavage site mutations and their compensatory effects on the impaired PR function appear to be partial (16), and because mutations apart from the cleavage sites can affect the cleavability of the precursor in the three-dimensional structure, it is important to evaluate the roles of non-cleavage site mutations in PI resistance.

The present findings evoke an argument regarding continuation of incomplete HAART. When HAART results in incomplete suppression of virus replication due to emergence of variants resistant to the available drugs, some clinical trials currently recommend continuation of therapy, because any interruption could result in increased plasma viral load and low CD4 cell counts (3, 9, 19). In general, it is believed that continuation of HAART under such circumstances can still produce some clinical benefits because of the reduced replication capacity of the PI-resistant virus (9). The present findings, however, suggest that in some cases continuation of incomplete HAART may allow viral replication, resulting in the generation of variants of phenotypes similar to those described in the present study. Thus, phenotypic drug resistance assays appear to deserve more attention, particularly for patients who fail to respond to HAART but must continue treatment using the same regimen.

In conclusion, we have described in the present study a novel mechanism, NFV-dependent replication enhancement, for HIV-1 adaptive changes. Our results suggested that coevolution of Gag and PR genes was a key event for adaptation of HIV-1 to survive the strong pressure of NFV-containing therapy in this particular patient. The present findings have clinical implications that may have an impact on HAART.

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Emergence of Protease Inhibitor Resistance–Associated Mutations in Plasma HIV-1 Precedes That in Proviruses of Peripheral Blood Mononuclear Cells by More Than a Year

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Abstract: HIV-1 genotype assay using plasma viruses has been widely applied for detection of resistant viruses in infected individuals, whereas there are only a few reports about proviral genotype in peripheral blood mononuclear cells (PBMCs). To determine which sample, plasma or PBMC, should be used for early detection of drug-resistant viruses during antiretroviral treatment, we analyzed 275 plasma-derived and 211 PBMC-derived HIV-1 protease sequences obtained from HIV-1–infected patients during protease inhibitor (PI) therapy. In 70 of 107 pairs (65.4%) of plasma and PBMC samples taken from the same blood draws, the numbers of PI resistance–associated mutations in the plasma-derived genotype were different from those in the PBMC-derived genotype. Plasma viruses had more PI resistance–associated mutations than PBMC proviruses (P = 0.0004). Analysis of serial samples showed that plasma-derived genotype assay could detect primary mutations about 425 days earlier than PBMC-derived genotype when the plasma viral load was less than 10^4 copies/mL. Our data suggest that genetic turnover of PBMC proviruses is slower than that of plasma viruses and that time lag between emergence of mutations in plasma-derived and PBMC-derived genotypes correlates inversely with viral load. Plasma viruses should be the material of choice for early detection of drug resistance during antiretroviral treatment.

Key Words: HIV-1 genotype, protease inhibitors, resistance mutations, plasma viruses, peripheral blood mononuclear cell proviruses, genotypic discordance

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It has been possible to control the viral load below detection limits in most HIV-1–infected individuals by highly active antiretroviral therapy (HAART). In a considerable number of patients, however, the viral load remains above the detection limits even during continuous administration of HAART. In these patients, drug-resistant viruses can emerge and invalidate the antiretroviral agents.[1–3] Therefore, early detection of resistant viruses is essential to change invalidated drugs to efficient ones before acceleration of the viral replication process.

Genotype assay using direct sequencing of plasma viruses has been applied for detection of resistant viruses, because plasma viruses turn over rapidly[4,5] and are considered to represent actively replicating HIV-1. In genotype assay of plasma viruses, it is often difficult to obtain results, however, due to the detection limit of gene amplification when the plasma viral load is less than 10^3 copies/mL.[6] Considering that replication-competent HIV-1 can be recovered from peripheral blood mononuclear cells (PBMCs) even from infected individuals whose plasma viral loads have remained below the detection limit for a long period,[7–11] analysis of HIV-1 proviral sequences in PBMCs during therapy may allow early detection of resistant viruses. Nevertheless, given that there is a genetic discordance between viruses in plasma and proviruses in PBMCs,[12–17] sequences of PBMC proviruses may yield different information.

In this study, we compared HIV-1 protease gene sequences in plasma viruses and PBMC proviruses in serial samples obtained during therapy (including protease inhibitors [PIs]), analyzed their genetic discordances, and assessed the spreading pattern of resistant viruses in plasma and PBMCs of infected individuals.

MATERIALS AND METHODS

Patients

In our clinic, the AIDS Clinical Center (ACC), International Medical Center of Japan (IMCJ), almost all HIV-1–infected patients agree to participate in retrospective clinical

studies, and their plasma and PBMC stocks from residues of routine blood examinations for future studies are maintained in an ACC-IMCJ sample bank for varying intervals after obtaining signed informed consent.

Measurements of HIV-1 viremia (Amplicor HIV-Monitor, Roche, NJ) and CD4 and CD8 lymphocyte cell counts (monoclonal antibodies and flow cytometry) were performed at each blood sampling. For patients with viral loads persistently over 400 copies/mL in spite of continuous administration of HAART, genotypes of plasma HIV-1 were routinely assayed. In total, 22 HIV-1–infected patients who met the following criteria were enrolled in the present study (Table 1): (1) viruses in plasma must have acquired at least 1 PI resistance–associated mutation[6] and (2) PBMC samples taken at a minimum of 5 time points must be available in the ACC-IMCJ sample bank. The Institutional Ethics Committee approved this study (IMCJ-H13-80), and written informed consent for this study was obtained from each patient.

Sequence Analysis of Protease Gene of HIV-1

Plasma and PBMC samples ($1.0–14.4 \times 10^6$ copies/sample) were obtained from a 7-mL EDTA-treated blood sample and stocked at −80°C. Total RNA extracted from 200 μL of each plasma sample was subjected to reverse transcriptase (RT) and the first polymerase chain reaction (PCR) with a primer pair of 01 and 02 using the One Step RNA PCR Kit (TaKaRa, Kyoto, Japan), followed by the second PCR with a primer pair of 03 and 04 to amplify HIV protease gene.[18,19] If the viral load was less than 1000 copies/mL or nested PCR could not amplify DNA sufficiently, an increased volume (500 μL) of plasma sample was used for RNA extraction. Total DNA extracted from a 10th of stocked PBMCs was also subjected to the nested PCR with the same sets of primers. Each PCR procedure consisted of 30 cycles of 94°C denaturing, 50°C elongation, and 72°C annealing. Primer sequences were as follows: sense primer 01 5′-CCA ACA GCC CCA CCA GA-3′ (2152–2168), antisense primer 02 5′-ATT TTC AGG

TABLE 1. Clinical Data and Sequenced Samples of 22 HIV-1–Infected Patients

Patient	Study Period (Months)	Used Protease Inhibitor[a]	Viral Load (Copies/mL)	CD4 (Cells/μL)	Number of Sequenced Samples		
					Plasma	PBMC's	Pairs[b]
1	20	N	5.6×10^2–2.0×10^4	215–321	11	8	3
2	28	I, R/S	3.0×10^3–1.0×10^5	80–243	12	6	4
3	27	S, I, R/S	3.9×10^3–3.2×10^5	55–115	12	6	4
4	13	S, N	1.0×10^5–6.0×10^5	7–210	6	6	5
5	39	N, R/S	1.3×10^4–6.5×10^5	203–809	16	9	5
6	56	S, N, A	<400–1.8×10^4	414–643	18	7	2
7	42	S, N, R/S	1.9×10^4–9.8×10^5	116–460	21	10	8
8	36	S, N, R/S	5.9×10^3–9.3×10^4	264–385	14	12	7
9	38	N, R/S	1.5×10^3–2.8×10^4	172–257	9	10	4
10	20	N, A	<400–5.9×10^4	103–277	6	8	1
11	50	S, N, L	<50–1.8×10^5	193–286	15	7	6
12	55	S, N, A	5.1×10^1–1.7×10^5	6–172	23	17	12
13	41	N, A/R	<400–2.9×10^5	30–283	12	13	4
14	45	N, S	<50–3.2×10^4	306–529	8	12	3
15	41	S, N, R/S	2.4×10^3–1.9×10^4	45–130	14	13	5
16	19	N, R/S	1.8×10^3–1.8×10^5	203–499	13	8	6
17	45	N, R/S	<50–1.5×10^5	98–372	14	18	6
18	31	I, R/S, A	6.4×10^2–7.2×10^5	60–433	7	8	4
19	29	I, R/S	<400–4.1×10^4	138–319	9	5	2
20	26	N, A/R	4.3×10^3–1.0×10^6	173–291	14	11	10
21	18	N, R/S	7.2×10^1–1.2×10^4	208–982	7	9	3
22	30	N	6.3×10^2–2.0×10^5	326–643	14	8	3
Total					275[c]	211	107

PBMCs, peripheral blood mononuclear cells; N, nelfinavir; I, indinavir; R, ritonavir; S, saquinavir; A, amprenavir; L, lopinavir.
[a]The protease inhibitors before and after a slash were administered together.
[b]Pairs of plasma and PBMC samples taken at the same time.
[c]Reverse-transcriptase polymerase chain reaction-negative samples are not included.

CCC ATT TTT TGA-3′ (2711–2691), sense primer 03 5′-AGC AGG AGA CGA TAG ACA AGG-3′ (2213–2233), and antisense primer 04 5′-CTG GCT TTA ATT TTA CTG GTA-3′ (2592–2572). The numbering parallels the HIV-1_{MN} sequence. Direct sequencing was performed bidirectionally with sense primer 03 and antisense primer 04 by an automatic sequencer (model 377; Applied Biosystems, Foster City, CA). A heterozygous base sequence was identified when the chromatogram showed a minor peak at >50% of the major peak.

Estimation of Time Lag Between Emergence of Mutations in Plasma Viruses and Peripheral Blood Mononuclear Cell Proviruses

The latency between the emergence of mutation in plasma viruses and PBMC proviruses was estimated as the difference between the sampling dates at which the mutation was first detected in plasma and PBMCs during the study period (34 ± 12 months [mean ± SD], range: 13–56 months). When the PI(s) was discontinued or changed to other PI(s) while genotypic discordance between plasma viruses and PBMC proviruses on the mutation was persistently noted, the time lag was estimated to have continued until the day of discontinuance or change of PI(s). Such genotypic lag times of all the mutations that had emerged in plasma viral genotype during the study period were analyzed.

RESULTS

Protease Inhibitor Resistance–Associated Mutations of Plasma Viruses Outnumber Those of Peripheral Blood Mononuclear Cell Proviruses

We analyzed 293 plasma and 211 PBMC samples from 22 HIV-1–infected patients enrolled in the study. All samples were collected during treatment that included 1 or more PIs. After the RNA extraction from the plasma samples, nested PCR following RT (RT-PCR) was performed for amplification of HIV-1 protease gene. RT-PCR failed to amplify HIV-1 protease gene from 18 plasma samples, however, all of which contained less than 400 copies/mL HIV-1 RNA (these samples are not included in Table 1). In our assay, when the plasma viral load was less than 400 copies/mL, the success ratio of RT-PCR was 5.3%. On the other hand, enough DNA was amplified by nested PCR from all PBMC samples, even when amplification from the plasma sample taken at the same time failed. Therefore, we analyzed 275 plasma-derived and 211 PBMC-derived HIV-1 samples by direct sequencing (see Table 1) and counted the number of observed PI resistance–associated mutations in each sequence (Table 2).

One hundred seven pairs of plasma-derived and PBMC-derived sequences were obtained from the same blood draws (see Table 1). In 70 of such 107 pairs (65.4%), the numbers of PI resistance–associated mutations were different between plasma-derived and PBMC-derived HIV-1 protease sequences, suggesting that the majority of the viruses in plasma were produced from a small portion of HIV-1–infected PBMCs or noncirculating cell(s).

The average number of such mutations in paired samples (plasma and PBMC, respectively) was calculated in each patient first, and such averages were then analyzed statistically to eliminate possible biasing of data due to the unequal number of samples available for each patient. In 17 of 22 patients, the average number of PI resistance–associated mutations was larger in plasma than in PBMCs, and in total of 22 patients, it was also larger in plasma than in PBMCs, with statistical significance (Table 3, bottom column). When the patients were divided according to geometric mean of plasma viral load during the collection period of paired samples, plasma samples still had significantly larger number of mutations than PBMC samples in both groups. The P value (Wilcoxon signed rank test) was smaller in the patients with a viral load $<10^4$ copies/mL than in the patients with a viral load $>10^4$ copies/mL (see Table 3, middle columns), however, which suggests that the difference between the number of PI resistance–associated mutations in plasma and that in PBMCs was larger in the patients with a lower viral load. To confirm the relation between the difference in the number of such mutations in 2 compartments and mean viral load, the ratio of average of mutation counts in PBMCs and that in plasma was plotted against mean viral load in each patient (Fig. 1). In the patients with a mean viral load $>3 \times 10^4$ copies/mL (patients 3, 4, 5, 7, and 13), the ratios of mutation counts were 1.0 ± 0.1. In contrast, the ratios in the patients with a mean viral load $<5 \times 10^3$ copies/mL (patients 6, 14, 17, 18, and 19) were less than 0.4. The ratio was significantly correlated with mean viral load (Spearman rank correlation coefficient, $P = 0.0021$). The number of mutations was smaller in PBMCs compared with that in plasma, especially in the patients with a lower viral load. This finding suggests that PI resistance–associated mutations appear in plasma-derived genotypic assay earlier than in PBMC-derived assay during therapy including PIs for patients with a low viral load.

Low Viral Load Is Associated with a Longer Time Lag

Plasma viruses had more PI resistance–associated mutations than PBMC proviruses during PI treatment, especially in the patients with a low viral load. To delineate how the difference in mutations emerged between plasma and PBMCs, we focused on the time lag of appearance of PI resistance–associated mutations in plasma viral and PBMC proviral genotypic assays. During the 34-month (1020-day) study period, a total of 58 PI resistance–associated mutations, including 27 (46.6%) primary mutations for some PIs, emerged in plasma viruses of 20 participants (see Table 2). Primary and secondary mutations emerged in 17 and 16 participants, respectively. The