Available online at www.sciencedirect.com
SCIENCE DIRECT
Antiviral Research 70 (2006) 51–59
Antiviral Research
www.elsevier.com/locate/antiviral

Cooperative contribution of gag substitutions to nelfinavir-dependent enhancement of precursor cleavage and replication of human immunodeficiency virus type-1

Saori Matsuoka-Aizawa [a,b], Hiroyuki Gatanaga [a,*], Hironori Sato [c], Kazuhiko Koike [b], Satoshi Kimura [a], Shinichi Oka [a]

[a] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[b] *Graduate School of Medicine, University of Tokyo, Japan*
[c] *Division of Molecular Genetics, National Institute of Infectious Diseases, Tokyo, Japan*

Received 9 June 2005; accepted 11 January 2006

Abstract

We previously described a clinical human immunodeficiency virus type-1 (HIV-1) isolate, CL-4, which showed nelfinavir (NFV)-dependent enhancement of replication (Matsuoka-Aizawa, S., Sato, H., Hachiya, A., Tsuchiya, K., Takebe, Y., Gatanaga, H., Kimura, S., Oka, S, 2003. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J. Virol. 77, 318–327.). To identify the responsible region(s) of HIV-1 proteins for such replication enhancement, we constructed a panel of recombinant HIV-1 clones harboring portions of the Gag and protease of CL-4 and analyzed their replication capabilities and Gag processing patterns. Our data suggested that the substitutions in the matrix and N-terminal half of capsid of CL-4 were indispensable for the NFV-dependent enhancement of replication and that NFV facilitated the cleavage between the matrix and capsid of the Gag precursor harboring these substitutions. The substitutions in C-terminal half of capsid rather decreased the cleavability of Gag precursor and NFV counteracted such negative impact. Efficient replication enhancement with NFV can be observed only in the presence of the substitutions in entire Gag and protease of CL-4.

Keywords: Human immunodeficiency virus type 1; Nelfinavir-resistant; Gag mutation

1. Introduction

Under the selective pressure of antiretroviral agents, the human immunodeficiency virus type-1 (HIV-1) evolves and acquires drug-resistance-associated mutations. The major protease inhibitor (PI)-resistance-associated mutations are located in the active sites of HIV-1 protease and impair its enzymatic functions (Bleiber et al., 2001; Croteau et al., 1997; Martinez-Picado et al., 1999). In order to compensate such impaired enzymatic function, PI-resistant HIV-1 further acquires mutations not only in protease but also in one of its substrate, Gag, resulting in full recovery of replication ability (Doyon et al., 1996; Gatanaga et al., 2002; Tamiya et al., 2004; Zhang et al., 1997). We previously described a unique clinical HIV-1 isolate, CL-4, which replicated more efficiently in the presence of sub-inhibitory concentrations of nelfinavir (NFV) (0.001–0.1 μM) (Matsuoka-Aizawa et al., 2003). CL-4 had a total of 56 amino acid substitutions in *gag-pro* genes compared with NL4-3; 22 substitutions had emerged in the matrix, SP1, and protease during administration of NFV-containing therapy, and 34 other substitutions had already existed before the introduction of the therapy (Matsuoka-Aizawa et al., 2003). In that study, we constructed three HIV-1 clones including, p17PRmt, PRmt, and p24PRmt, and found that only p17PRmt, which possessed the entire Gag and protease segment of CL-4, showed NFV-dependent enhancement of replication. Therefore, we concluded that the substitutions in matrix are indispensable for replication enhancement (Matsuoka-Aizawa et al., 2003). However, it is still unknown whether the substitutions in matrix alone are sufficient or whether other Gag substitutions are necessary for the replication enhancement with NFV. In this study we constructed four more recombinant HIV-1 clones and characterized their replica-

* Corresponding author. Tel.: +81 3 5273 5193; fax: +81 3 5273 5193.
E-mail address: higatana@imcj.acc.go.jp (H. Gatanaga).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.01.004

tion kinetics and Gag processing in the absence and presence of NFV.

2. Materials and methods

2.1. Cells and antiretroviral agents

HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transformed T cell lines, MT-2, PM-1, and H9 cells were maintained in RPMI-1640 with 10% FCS. NFV was kindly provided by the Japan Tobacco Co. (Tokyo, Japan).

2.2. Plasmid construction and preparation of gag-pro recombinant HIV-1 clones

Clinical HIV-1 isolates CL-1, CL-2, CL-3, and CL-4 were sequentially obtained from the same patient before and during NFV-containing treatment (Matsuoka-Aizawa et al., 2003). Direct sequences of these four clinical isolates and sub-cloning

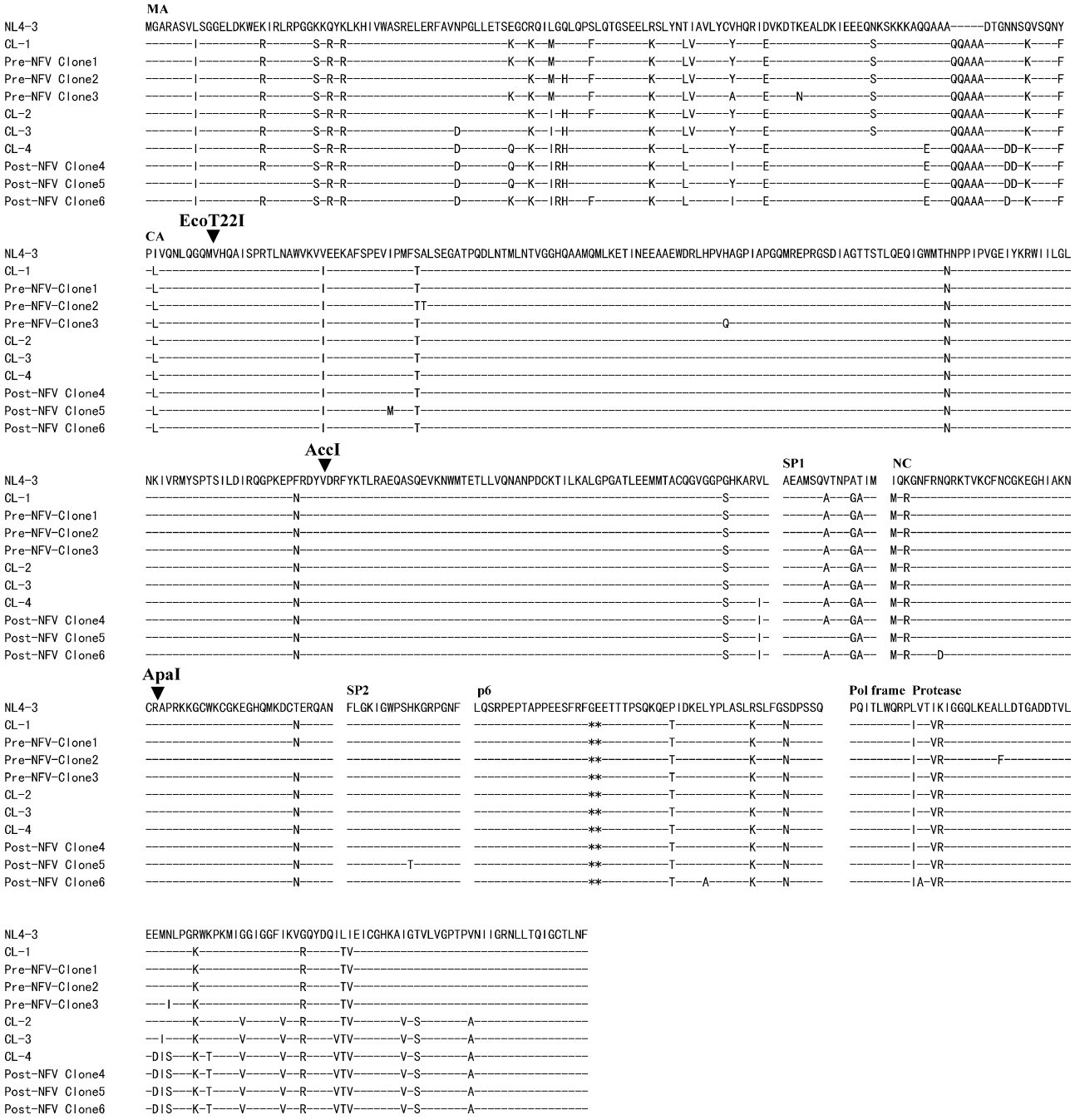

Fig. 1. Direct and sub-clonal sequences of clinical HIV-1 isolates. Direct and sub-clonal amino acid sequences of whole Gag and protease of HIV-1 isolates are shown. Pre-NFV Clone 1–3 and Post-NFV Clone 4–6 were derived from CL-1 and CL-4, respectively. The amino acid sequence of HIV-1_{NL4-3} is shown at the top as reference. The identical amino acids with those of HIV-1_{NL4-3} are indicated with dashes and the star shows deletion compared with HIV-1_{NL4-3} sequence. The restriction sites used in the construction of recombinant HIV-1 plasmids are also shown. MA, matrix; CA, Capsid; NC, nucleocapsid; and PR, protease.

sequences of CL-1 and CL-4 indicated that 11 and 10 amino acid substitutions accumulated in Gag and protease during PI-containing treatment, respectively (Fig. 1). Post-NFV Clone 4 (Fig. 1) was used in the construction of CL-4-derived recombinant HIV-1 plasmid. The pNL4-3-based plasmids of PRmt (HIV-1 carrying only the substitutions in protease of CL-4), p24PRmt (carrying the substitutions in capsid and protease of CL-4), and p17PRmt (carrying the substitutions in whole Gag and protease of CL-4) were constructed as previously described (Matsuoka-Aizawa et al., 2003) (Fig. 2), and the plasmids of MAmt (carrying only the substitutions in the matrix of CL-4) and MA + PRmt (carrying the substitutions in the matrix and protease of CL-4) were constructed by using the same restriction enzyme sites (Figs. 1 and 2). The plasmids of NCAmt (carrying the substitutions in matrix, N-terminal half of capsid, and protease of CL-4) and CCAmt (carrying the substitutions in matrix, C-terminal half of capsid, and protease of CL-4) were constructed by using AccI site. Originally, pNL4-3 has two AccI site between *gag* and protease region, one in the matrix, and the other in the capsid. However, since the one in the matrix was extinct due to natural substitution in CL-4, the other in the capsid was unique in *gag* and protease region.

HeLa cells (5×10^5 cells) were grown in DMEM with 10% FCS for 24 h and transfected with 3 μg of pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNAs by using FuGINE 6 transfection reagent (Roche Diagnosis, Basel, Switzerland). The cells were incubated for 24 h, washed once with PBS, and cultured in 5 ml of culture medium. The culture supernatant containing virus was collected at 48 h after transfection, filtered, analyzed for RT activity (10432–17162 cpm/μM), and kept at −80 °C until use. The virus titer used for infection and Western blot analysis was adjusted with RT activity.

2.3. HIV-1 replication kinetics

The methods used to infect cells were described previously (Matsuoka-Aizawa et al., 2003). Briefly, MT-2, PM-1, and H9 cells (2×10^4) were infected with 200 μl 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) for 16 h, washed once, and cultured in 200 μl of culture medium with the same concentration of NFV. A half volume of culture medium was changed every 2 or 3 days, and the supernatant was kept at −80 °C for measurement of RT activity. Each experiment was carried out in duplicate and repeated three times.

2.4. Competitive HIV-1 replication assay

H9 cells (2×10^5 cells) were incubated with two HIV-1 clones (each of 100 $TCID_{50}$) simultaneously for 16 h, washed with PBS twice, and cultured in the absence or presence of 0.1 μM NFV for 7 days. These infection periods were defined as a single passage. At the end of each passage, H9 cells were harvested and the culture supernatants were used to infect fresh

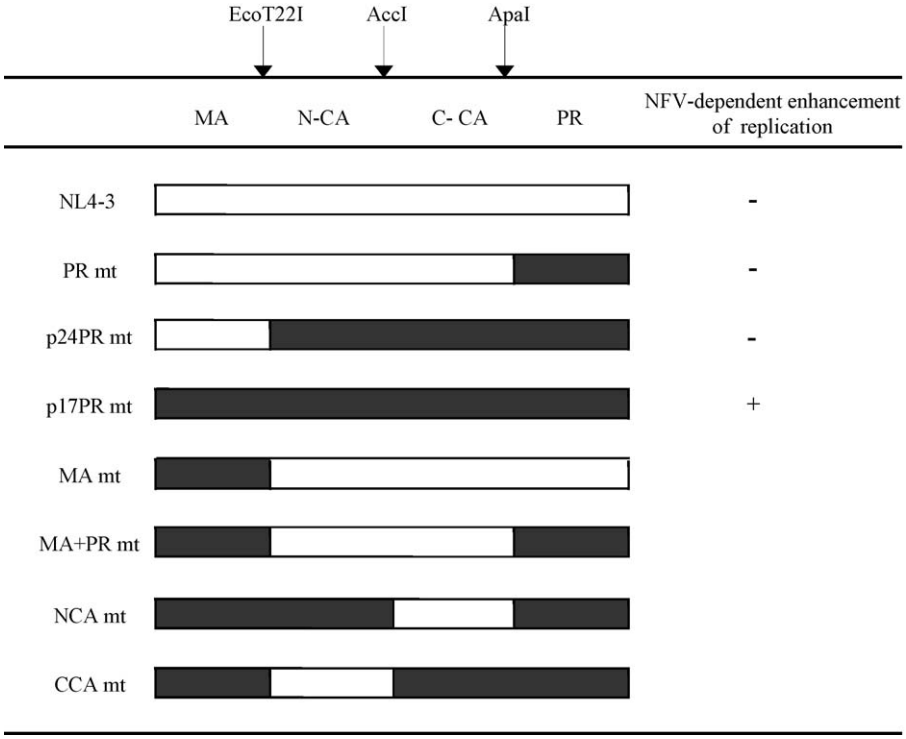

Fig. 2. Previously and newly constructed recombinant HIV-1s. The recombinant molecular clones were constructed based on pNL4-3 as a genetic backbone. The Gag-PR region of HIV-1 was segmented into four areas, MA (BssHII-EcoT22I fragment), N-terminal half of CA (NCA) (EcoT22I-AccI fragment), C-terminal half of CA (CCA) (AccI-ApaI fragment), and PR (ApaI-BalI fragment). Originally, pNL4-3 has two AccI sites between the gag and PR region, in MA and CA. However, because the one in MA was extinct in CL-4 due to natural substitution, the other AccI site in CA was unique for gag-PR gene of CL-4. Open boxes indicate the NL4-3-originated fragments, and closed boxes indicate fragments that were derived from CL-4 variants. The NFV-dependent replication enhancement of previously analyzed clones was also shown and indicated as (+). MA, matrix; CA, capsid; and PR, protease.

uninfected H9 cells. The cells harvested at each passage were subjected to PCR for amplification of HIV-1 *gag* region and direct DNA sequencing was performed. The viral populational changes were determined by relative peak height on sequence electrophoregram (Kosalaraksa et al., 1999).

2.5. HIV-1 susceptibility to NFV

MT-2 cells were infected with 500 $TCID_{50}$ of each virus in the absence and the presence of 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.316, 1, and 3.16 μM of NFV, and cultured in triplicate for 7 days. At the end of culture, the amounts of p24 in the supernatants were measured and 50% inhibitory concentrations (IC_{50}) of NFV were determined by referring to the dose–response curve.

2.6. Western blot analysis of HIV-1 virions

HeLa cells were transfected with pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNA in the absence and presence of 0.1 μM NFV. The culture supernatant was harvested at 48 h after transfection, centrifuged at 37,000 × *g* for 90 min to pellet virus particles. The virion pellet (6×10^5 cpm of RT activity) was applied to an SDS gradient gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with anti-HIV-1 p24 antisera (Advanced Biotechnology, Columbia, USA) and HIV-1-infected patients' serum, respectively, and hybridized with anti-protein A antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The immune complex was visualized with an ECL Plus system (Amersham Pharmacia Biotech) according to the manufactures' description.

The percent signal density of Gag products was analyzed on a Windows computer by using the ImageJ Program (developed at the U.S. National Institutes of Health ⟨http://www.rsb.info.nih.gov/ij/⟩) and the percent density of p24 was determined by the following formula: percent density of p24 = 100 × (the density of p24 signal)/(the cumulated density of all *Gag* signals) (Tamiya et al., 2004).

3. Results

3.1. Whole capsid substitutions necessary for NFV-enhanced replication

MAmt, carrying only the substitutions in the matrix (Fig. 2), grew well in the absence of NFV (Fig. 3). In the presence of NFV, however, it did not grow at all, indicating that matrix substitutions were not sufficient to confer NFV resistance. MA + PRmt, carrying substitutions in the matrix and protease (Fig. 2), replicated as efficiently as PRmt (carrying only the substitutions in protease), both in the absence and presence of 0.1 μM NFV, though its replication was not enhanced with NFV, indicating that the substitutions in matrix and protease were not sufficient for NFV-dependent enhancement of replication. As reported in our previous study (Matsuoka-Aizawa et al., 2003), p17PRmt replicated more efficiently in the presence of 0.1 μM NFV than in the absence of NFV. Therefore, some of the substitutions in the capsid should be responsible for such unique phenotype of CL-4 strain. The HIV-1 capsid contains two domains, a C-terminal oligomerization domain and N-terminal core domain, which function differently in viral assembly (Turner and Summers, 1999). Therefore, we divided the EcoT22I—ApaI segment of CL-4 into two segments at ACC I site, named them the N-terminal half of the capsid (NCA) and the C-terminal half of the capsid (CCA), and constructed two recombinant HIV-1 clones, NCAmt and CCAmt, which possessed all the substitutions in the matrix and protease of CL-4, and the substitutions in NCA and CCA, respectively (Fig. 2). NCAmt and CCAmt grew efficiently both in the absence and presence of 0.1 μM NFV, and only NCAmt showed weak replication enhancement with 0.1 μM NFV in PM-1 and MT-2 cells though it was not so efficient as that of p17PRmt, suggesting that the substitutions in CCA, contributed to the efficient replication enhancement of p17PRmt (Fig. 3). CCAmt did not show the p17PRmt's phenotype, indicating that the substitutions in NCA were indispensable for replication enhancement. As we reported previously (Matsuoka-Aizawa et al., 2003), p24PRmt lacking the substitutions in matrix did not show replication enhancement by NFV. Taken together, the substitutions in the whole matrix, capsid, SP1, and the N-terminal end of nucleocapsid of CL-4 were indispensable for efficient replication enhancement of p17PRmt.

To define further the role of substitutions in the matrix, NCA, and CCA, viral replication efficiency was compared among the HIV-1 clones described above in the absence and presence of NFV using competitive HIV-1 replication assay (Kosalaraksa et al., 1999). MA + PRmt outgrew PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4a), and MAmt was outgrown by NL4-3 in the absence of NFV (Fig. 4b), suggesting that the substitutions in the matrix of CL-4 reduced the replication of HIV-1 harboring wild-type protease, but compensated the replication of HIV-1 harboring NFV-resistant protease of CL-4. NCAmt outgrew MA + PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4c), suggesting that the substitutions in NCA were compensatory for the replication of HIV-1 harboring protease and matrix of CL-4. However, CCAmt was outgrown by MA + PRmt in the absence of NFV, but its replication in the presence of 0.1 μM NFV was comparable with that of MA + PRmt under similar condition (Fig. 4d), suggesting that the substitutions in CCA reduced the replication capability of MA + PRmt, while NFV compensated the mutation effect. Sub-cloning analyses of proviral sequences at both of the passages 3 and 4 in competitive HIV-1 replication assay in the presence of 0.1 μM NFV showed that five of 10 clones were derived from CCAmt and the other five clones were derived from MA + PRmt, which confirmed that CCAmt and MA + PRmt had comparable replication ability in the presence of 0.1 μM NFV (Fig. 4d). MA + PRmt readily outgrew p17PRmt in the absence of NFV, but was outgrown by p17PR in the presence of 0.1 μM NFV (Fig. 4e), suggesting that the substitutions in NCA and CCA reduced the replication capability of MA + PRmt, while NFV counteracted the mutation effect and rather enhanced replication ability at sub-inhibitory concentration (Fig. 3, p17PRmt).

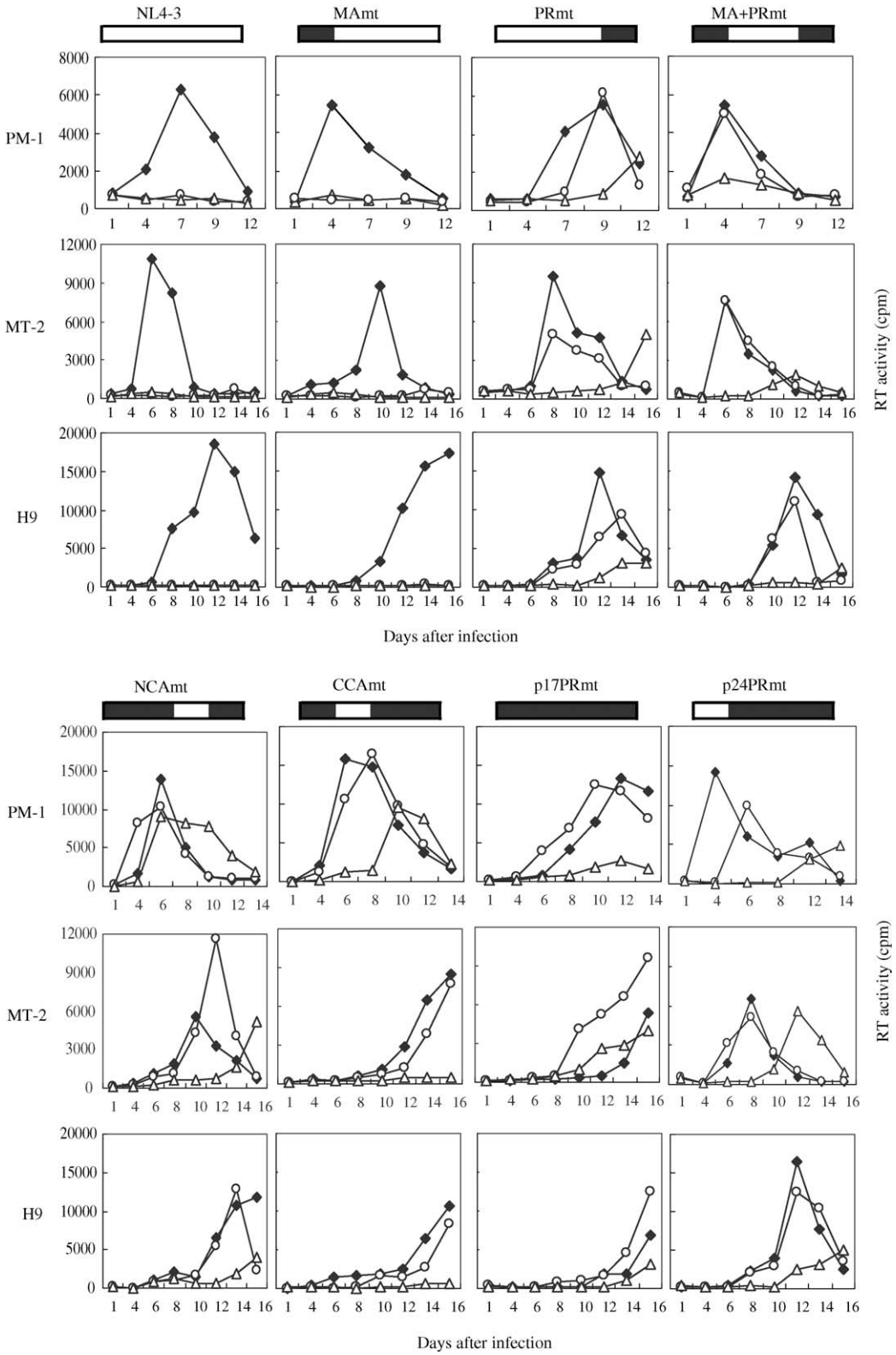

Fig. 3. Effects of NFV on replication capability of recombinant HIV-1s. PM-1, MT-2, and H9 cells (2×10^4 cells) were exposed to 0.2 ml of cell-free supernatant containing each HIV-1 clone (2×10^5 ^{32}P cpm of RT activity), washed once, and cultured in 0.2 ml of medium in the absence (closed diamonds) and presence of NFV (0.1 μM; open circles, 1 μM; open triangles). Half volume of the culture medium was changed every 2 or 3 days, and the supernatant was kept at −80 °C until the measurement of RT activity. Each experiment was carried out in duplicate and repeated three times, and representative data are shown.

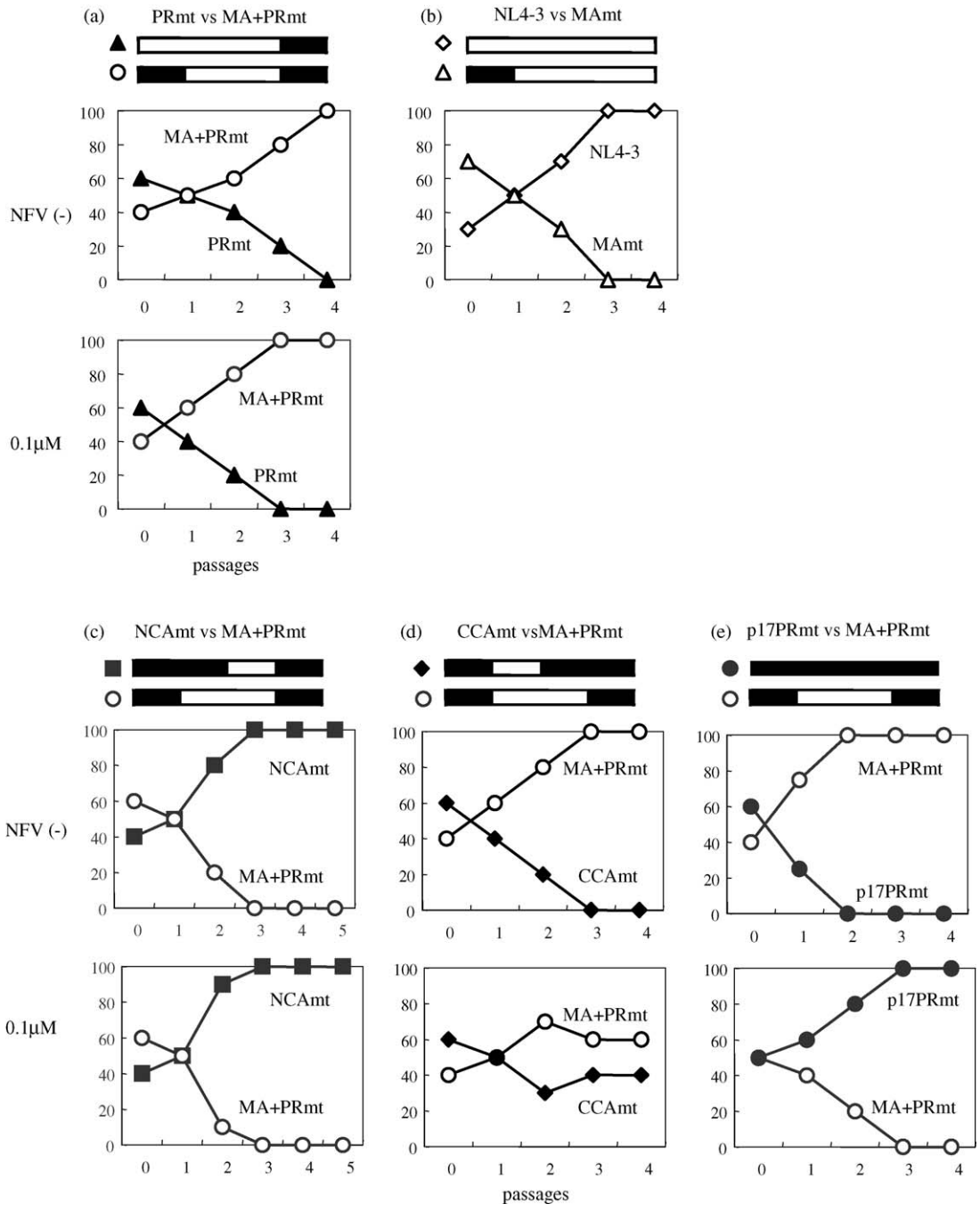

Fig. 4. One-to-one competitive HIV-1 replication assay. H9 cells (2×10^5 cells) were incubated with two recombinant HIV-1s (each of 100 $TCID_{50}$) simultaneously at 37 °C for 16 h, washed with PBS twice, and cultured in the absence and presence of 0.1 μM of NFV. At 7 days post-infection, the culture supernatant was used to infect fresh uninfected H9 cells. The cells harvested at each passage were subjected to direct DNA sequencing, and the viral population changes were determined by the relative peak height in the sequencing electrophoregram. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay. (a) PRmt vs. MA + PRmt; (b) NL4-3 vs. MAmt; (c) NCAmt vs. MA + PRmt; (d) CCAmt vs. MA + PRmt; (e) p17PRmt vs. MA + PRmt.

3.2. Gag substitutions conferring NFV resistance

To analyze the role of the substitutions in the matrix, NCA, and CCA in NFV resistance, IC_{50}s of NFV for the HIV-1 clones described above were determined by using MT-2 cells (Zhang et al., 1997). MAmt (IC_{50}: 9.4 nM) showed 1.9-fold resistance against NFV compared with NL4-3 (5.0 nM), and p17PRmt (893 nM) showed 3.3-fold resistance compared with p24PRmt (273 nM) (Table 1), indicating that the substitutions in the matrix may make a small contribution to the viral resistance against NFV. NCAmt (483 nM) had 2.1-fold resistance against NFV compared with MA + PRmt (229 nM), and p17PRmt had 4.1-fold resistance compared with CCAmt (217 nM), indicating that the substitutions in NCA gave positive impact on viral resistance. Interestingly, CCAmt showed 0.95-fold resistance against NFV compared with MA + PRmt, indicating that substitutions in CCA

Table 1
NFV resistance of recombinant HIV-1s

HIV-1	IC_{50} (nM)	Fold-resistance
NL4-3	5.0 ± 0.4	1.0
PRmt	241 ± 34	48
p24PRmt	273 ± 13	55
p17PRmt	893 ± 28	179
MAmt	9.4 ± 3.3	1.9
MA + PRmt	229 ± 21	46
NCAmt	483 ± 26	97
CCAmt	217 ± 32	43

The concentrations of drug added to the growth medium for calculation of the IC_{50}s were 0, 1, 3.16, 10, 31.6, 100, and 316 nM and 1 and 3.16 μM NFV, and the IC_{50}s were derived from plots of percent of inhibition of p24 production in culture supernatant versus NFV concentration.

may give small negative impact on viral resistance in the absence of the substitutions in NCA. p17PRmt, however, had 1.8-fold resistance compared with NCAmt, indicating the substitutions in CCA may give a small but positive contribution to viral resistance in the presence of the substitutions in NCA. The role of the substitutions in CCA in viral resistance was altered by the presence of the substitutions in NCA.

3.3. Gag substitutions facilitating cleavage between matrix and capsid

To further delineate the impact of each substitution, the Gag processing pattern was assessed in the absence and presence of NFV by Western blot analysis using anti-p24 monoclonal antibody (Fig. 5A1–2 and B1–2). As expected, 0.1 μM of NFV effectively blocked cleavage of the Gag p55 precursor of NL4-3 (percent density of p24; 4.7% versus 87.5% in Fig. 5A1; 4.2% versus 83.3% in Fig. 5A2). In contrast, NFV gave only a small influence on the cleavage patterns of the p55 precursor of MA + PRmt (percent density of p24; 65.5% versus 87.4% in Fig. 5A1; 77.8% versus 92.6% in Fig. 5A2), which is consistent with the indistinguishable replication kinetics of this mutant in the absence and presence of NFV (Fig. 3). Interestingly, NFV enhanced the cleavability of the p55 precursor of p17PRmt (percent density of p24; 94.8% versus 74.3% in Fig. 5A1; 72.2% versus 54.1% in Fig. 5A2), which was paralleled with NFV-dependent replication enhancement of this mutant (Fig. 3). NFV also gave a small positive effect on the cleavability of the p55 precursor of NCAmt (percent density of p24; 97.1% versus 94.6% in Fig. 5B1; 97.5% versus 96.2% in Fig. 5B2), which was paralleled with the partial enhancement of replication with NFV (Fig. 3). Furthermore, percent density of p24 of NCAmt was increased compared with that of MA + PRmt (percent density of p24; 94.6% and 96.2% versus 87.4% and 92.6% in the absence of NFV; 97.1% and 97.5% versus 65.5% and 77.8% in the presence of 0.1 μM NFV), suggesting that the substitutions in NCA play a significant role in Gag cleavability. Finally, NFV decreased percent density of p24 of CCAmt (percent density of p24; 68.9% versus 78.2% in Fig. 5B1; 45.3% versus 79.0% in Fig. 5B2), which was paralleled with NFV-induced delay of replication kinetics (Fig. 3). For further confirmation, the Gag processing pattern of NCAmt and CCAmt was also assessed by Western blot analysis using HIV-1-infected patient's serum (Fig. 5B3). As expected, NFV slightly increased cleavability of the p55 precursor of NCAmt (percent density of p24; 96.9% versus 94.5% in Fig. 5B3), and gave a negative impact on Gag cleavage of CCAmt (percent density of p24; 41.9% versus 74.3% in Fig. 5B3), which were well compatible with the cleavage pattern analyzed by using anti-p24 monoclonal antibody (Fig. 5B1, 2). In summary, NFV induced enhanced cleavability of Gag precursors of p17PRmt and NCAmt, which was well paralleled with NFV-induced enhancement of replication capability of these mutants.

4. Discussion

We previously reported that the substitutions in p6-protease segment alone are sufficient to confer NFV resistance while those in matrix are indispensable for the replication enhancement of CL-4 by NFV (Matsuoka-Aizawa et al., 2003). In the present study, we found that not only the matrix substitutions but the mutations in N-terminal half of capsid also played critical role in the enhancement and that the full potential of the enhancement phenotype was achieved only with the cooperation of mutations in the entire Gag and protease region of CL-4. The substitutions in matrix and those in N-terminal half of capsid compensated the otherwise compromised viral replication in the absence and presence of NFV (Fig. 4a and c). Probably, these substitutions cooperatively altered the tertiary structure of the Gag precursor and made the cleavage site between matrix and capsid more accessible to mutant protease harboring multiple resistance-associated mutations. The cleavage pattern analyzed by Western blot analysis supported the idea that the substitutions in N-terminal half of capsid improved the Gag cleavage. Percent density of p24 of NCAmt was increased compared with that of MA + PRmt in the absence of NFV (Fig. 5A1–2 and B1–2; 94.6% and 96.2% versus 87.4% and 92.6%). It is worth noting that CL-4 had a total of 56 amino acid substitutions in *gag-pro* genes compared with NL4-3; 22 substitutions had emerged during NFV-containing therapy, and 34 other substitutions had already existed before the introduction of the therapy, and that all the substitutions in N-terminal half of capsid of CL-4 were pre-existing before NFV-therapy (Fig. 1), suggesting that certain polymorphic amino acid residues seen in HIV-1 clinical isolates were associated with drug resistance. Interestingly, the amino acid insertion at the same site of the matrix of CL-4 compared with NL4-3 (Fig. 1; amino acids 121–125 in MA, QQAAA) was reported to increase viral replication harboring mutant protease by improving otherwise impaired Gag processing (Tamiya et al., 2004). Gatanaga et al. also reported that a polymorphic substitution in N-terminal half of capsid was indispensable for the development of high multitude of resistance against PIs (Gatanaga et al., 2002), though CL-4 did not harbor the same substitution. It is also known that certain drug-resistance-conferring amino acid substitutions found in one subtype HIV-1 isolated from patients under therapy may be detected in HIV-1 of other subtypes from untreated individuals (Cornelissen et al., 1997; Quinones-Mateu et al., 1998). More-

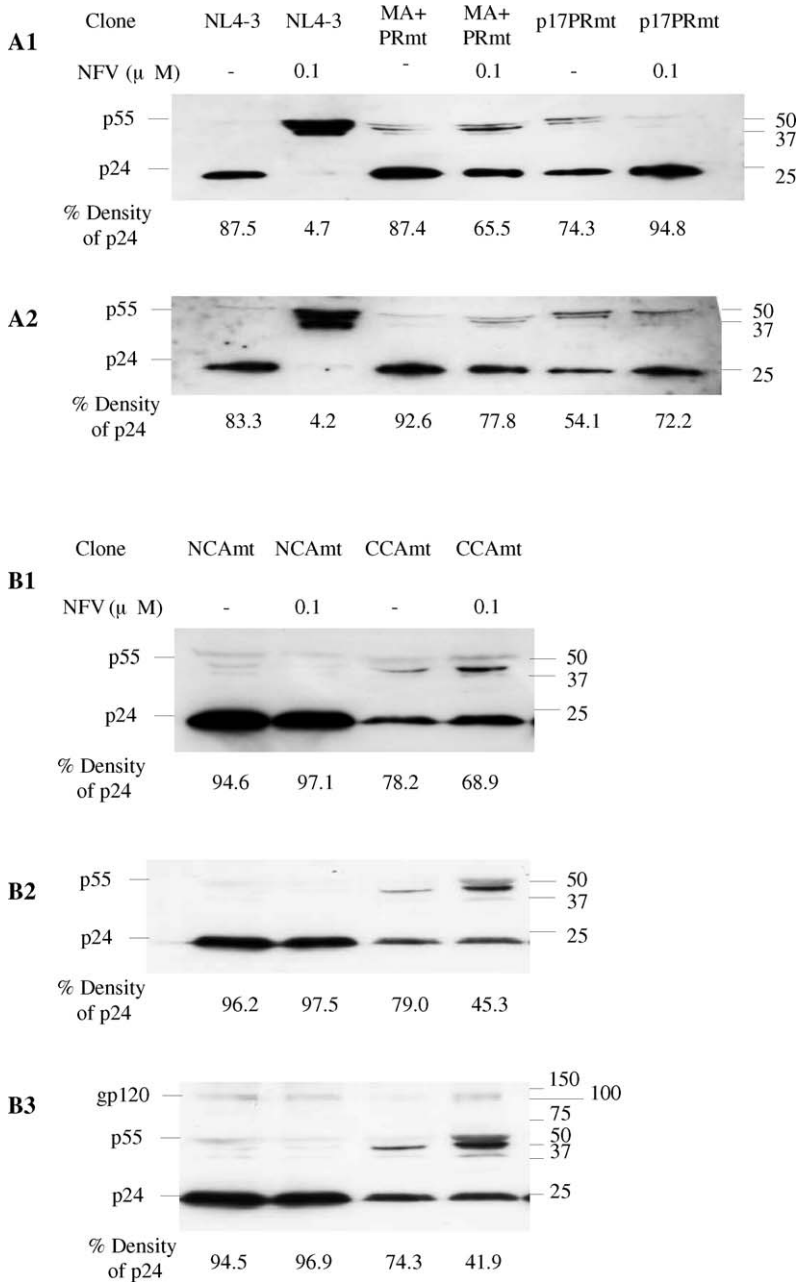

Fig. 5. Western blot analysis in the absence and presence of NFV. HeLa cells were transfected with each of full-length molecular clones and cultured in the absence and presence of 0.1 μM NFV. At 48 h post-transfection, virions in the culture supernatant (6×10^5 cpm of RT activity) were harvested and subjected to Western blot analysis. Gag proteins were visualized by using anti-p24 monoclonal antibody (A1-2, B1-2) and HIV-1-infected patient's serum (B3). Percent density of p24 was calculated as 100 × (p24 signal density/total Gag product signal densities) in a Western blot.

over, a recent study of Colson et al. revealed that HIV-2 strains harbor specific patterns of natural polymorphism and resistance (Colson et al., 2004). HIVs seem to acquire drug-resistance by utilizing the pre-existing polymorphic mutations and by coordinating the development of multiple substitutions.

Furthermore, the substitutions in N-terminal half of capsid of CL-4 altered the effect of NFV on viral replication. Sub-inhibitory concentration (0.1 μM) of NFV slightly accelerated the Gag precursor cleavage of NCAmt (percent density of p24; 97.1% versus 94.6% in Fig. 5B1; 97.5% versus 96.2% in Fig. 5B2; 96.9% versus 94.5% in Fig. 5B3), which was paralleled with the partial replication enhancement with NFV (Fig. 3), though it showed inhibitory effect in Gag processing of MA + PRmt (percent density of p24; 65.5% versus 87.4% in Fig. 5A1; 77.8% versus 92.6% in Fig. 5A2). Therefore, one of the mechanisms of viral replication enhancement with NFV is the improved processing of Gag harboring the substitutions in N-terminal half of capsid of CL-4 cooperated with the substitutions in the matrix. On the other hand, the role of the substitutions in C-terminal half of capsid seemed different, though they were also indispensable for the full potential of replication enhancement with NFV. They impaired the cleavability of Gag precursor

of MA + PRmt (Fig. 5A1–2 and B1–2; percent density of p24; CCAmt versus MA + PRmt = 78.2% and 79.0% versus 87.4% and 92.6%) and NCAmt (Fig. 5A1–2 and B1–2; percent density of p24; p17PRmt versus NCAmt = 74.3% and 54.1% versus 94.6% and 96.2%) in the absence of NFV, which were parallel with viral replication data (CCAmt versus MA + PRmt, Fig. 4d; p17PRmt versus NCAmt, Fig. 3). The effects of NFV on Gag cleavage pattern were different between CCAmt and p17PRmt; sub-inhibitory concentration (0.1 μM) of NFV facilitated the Gag cleavability of p17PRmt (percent density of p24; 94.8% versus 74.3% in Fig. 5A1; 72.2% versus 54.1% in Fig. 5A2), though it decreased the cleavability of CCAmt Gag (percent density of p24; 68.9% versus 78.2% in Fig. 5B1; 45.3% versus 79.0% in Fig. 5B2; 41.9% versus 74.3% in Fig. 5B3), which was also parallel with viral replication data showing enhancement only in p17PRmt but not in CCAmt (Fig. 3). Considering together, the substitutions in C-terminal half of capsid compromised viral replication by impairing the Gag preprocessing, and NFV could counteract the negative impact only in the presence of the substitutions in N-terminal half of capsid. In the absence of the substitutions in N-terminal half of capsid, only partial counteraction was seen (Fig. 4d). In summary, NFV-induced viral replication enhancement of CL-4 was caused by two mechanisms; NFV facilitates the processing of Gag harboring the substitutions in the matrix and N-terminal half of capsid of CL-4, and NFV counteracts the impaired Gag cleavage caused by the substitutions in C-terminal half of capsid of CL-4 only in the presence of the substitutions in the matrix and N-terminal half of capsid of CL-4. Therefore, the full potential of the enhancement phenotype was achieved only with the cooperation of mutations in the entire Gag and protease region of CL-4.

Notably, we found several other PI-resistant isolates with the phenotype of PI-dependent replication enhancement (data not shown), suggesting that HIV-1 can evolve to acquire capability to replicate better with the drugs. Such replication enhancement with antiretroviral agents presents formidable challenge in the therapy of HIV-1 infection. Future studies of structural analyses of Gag precursor(s) harboring substitutions of these mutants are warranted to clarify the underlying mechanism(s).

Acknowledgments

The authors thank A. Hachiya, K. Tsuchiya, Y. Suzuki, and Y. Hirabayashi for their helpful suggestions and continuous discussions throughout this study. We are also indebted to Y. Takahashi and F. Negishi for their technical assistance. This study was supported by a Grant-in-Aid for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001), by the Organization of Pharmaceutical Safety and Research (01-4), and by the Japanese Foundation for AIDS Prevention.

References

Bleiber, G., Munoz, M., Ciuffi, A., Meylan, P., Telenti, A., 2001. Individual contributions of mutant protease and reverse transcriptase to viral infectivity, replication, and protein maturation of antiretroviral drug-resistant human immunodeficiency virus type 1. J. Virol. 75, 3291–3300.

Colson, P., Henry, M., Tourres, C., Lozachmeur, D., Gallais, H., Gastaut, J.A., Moreau, J., Tamalet, C., 2004. Polymorphism and drug-selected mutations in the protease gene of human immunodeficiency virus type 2 from patients living in southern France. J. Clin. Microbiol. 42, 570–577.

Cornelissen, M., van den Burg, R., Zorgdrager, F., Lukashov, V., Goudsmit, J., 1997. pol gene diversity of five human immunodeficiency virus type 1 subtypes: evidence for naturally occurring mutations that contribute to drug resistance, limited recombination patterns, and common ancestry for subtypes B and D. J. Virol. 71, 6348–6358.

Croteau, G., Doyon, L., Thibeault, D., McKerche, G., Pilote, L., Lamarre, D., 1997. Impaired fitness of human immunodeficiency virus type 1 variants with high-level resistance to protease inhibitors. J. Virol. 71, 1089–1096.

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., Lamarre, D., 1996. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J. Virol. 70, 3763–3769.

Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M.F., Nagashima, K., Gorelick, R.J., Mardy, S., Tang, C., Summers, M.F., Mitsuya, H., 2002. Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. J. Biol. Chem. 277, 5952–5961.

Kosalaraksa, P., Kavlick, M.F., Maroun, V., Le, R., Mitsuya, H., 1999. Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an *in vitro* competitive HIV-1 replication assay. J. Virol. 73, 5356–5363.

Martinez-Picado, J., Savara, A.V., Sutton, L., D'Aquila, R.T., 1999. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. J. Virol. 73, 3744–3752.

Matsuoka-Aizawa, S., Sato, H., Hachiya, A., Tsuchiya, K., Takebe, Y., Gatanaga, H., Kimura, S., Oka, S., 2003. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J. Virol. 77, 318–327.

Quinones-Mateu, M.E., Albright, J.L., Mas, A., Soriano, V., Arts, E.J., 1998. Analysis of pol gene heterogeneity. Viral quasispecies, and drug resistance in individuals infected with group O strains of human immunodeficiency virus type 1. J. Virol. 72, 9002–9015.

Tamiya, S., Mardy, S., Kavlick, M.F., Yoshimura, K., Mistuya, H., 2004. Amino acid insertions near Gag cleavage sites restore the otherwise compromised replication of human immunodeficiency virus type 1 variants resistant to protease inhibitors. J. Virol. 78, 12030–12040.

Turner, B.G., Summers, M.F., 1999. Structural biology of HIV. J. Mol. Biol. 285, 1–32.

Zhang, Y.M., Imamichi, H., Imamichi, T., Lane, H.C., Falloon, J., Vasudevachari, M.B., Salzman, N.P., 1997. Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. J. Virol. 71, 6662–6670.