TABLE 1. Antiviral activity of HIV-1 gp41-derived peptides against gp120 and/or gp41 recombinant viruses[a]

Virus or substitution	EC_{50} (nM)			
	ddC	T-20	N36	C34
$HIV\text{-}1_{WT}$[b]	264	12	51	2.1
ΔFNSTW[c]	98 (0.4)	18 (1.5)	50 (1.0)	9.5 (4.6)
A30V	205 (0.8)	6.3 (0.5)	38 (0.7)	7.0 (3.4)
D36G	173 (0.7)	0.92 (0.1)	90 (1.7)	1.6 (0.8)
D36S[e]	166 (0.6)	6.6 (0.6)	89 (1.7)	3.7 (0.6)
I37T	284 (1.1)	156 (13)	40 (0.8)	23 (11)
I37K	326 (1.2)	2,482 (212)	99 (1.9)	27 (13)
V38M[e]	223 (0.8)	305 (26)	94 (1.8)	31 (15)
Q39H[d]	330 (1.3)	1.9 (0.2)	165 (3.2)	5.3 (2.6)
N126K[d]	380 (1.4)	23 (1.9)	137 (2.7)	14 (6.8)
L204I	247 (0.9)	13 (1.1)	105 (2.0)	4.4 (2.1)
D36S/V38M[e]	294 (1.1)	60 (5.1)	46 (0.9)	16 (7.7)
I37T/N126K	292 (1.1)	158 (14)	54 (1.1)	22 (11)
I37K/N126K	309 (1.2)	1,570 (134)	51 (1.0)	57 (28)
A30V/I37K/N126K	409 (1.5)	198 (17)	119 (2.3)	22 (10)
D36G/I37K/N126K	329 (1.2)	269 (23)	156 (3.0)	148 (72)
D36G/I37K/N126K/L204I[d]	209 (0.8)	117 (10)	41 (1.2)	112 (54)
ΔFNSTW/D36G/I37K/N126K/L204I[d]	213 (0.8)	746 (64)	54 (1.0)	171 (83)

[a] Anti-HIV activity was determined with the MAGI assay. The data shown are mean values obtained from the results of at least three independent experiments, and resistance (*n*-fold) in EC_{50} for recombinant viruses compared to $HIV\text{-}1_{WT}$ is shown in parentheses.
[b] $HIV\text{-}1_{NL4\text{-}3}$ was used was a wild-type virus.
[c] ΔFNSTW is the deletion of 5 amino acids at positions 364 to 368 in the gp120 V4 region of $HIV\text{-}1_{NL4\text{-}3}$.
[d] Mutant viruses observed during induction of C34 resistance variants in vitro (Fig. 1B).
[e] D36S/V38M has been reported for T-20-resistant HIV-1 variants (26).

Peptide binding affinity. To clarify the effect of the substitutions on the interaction of N-HR and C-HR, the binding affinity of the peptides in vitro was examined with the synthesized peptides (Table 2). The affinity between $N36_{D36G/I37K}$ and C34 was unstable even at 37°C, indicating that the peptide inhibitor C34 hardly bound to $N36_{D36G/I37K}$. However, it is still unclear whether it is a direct effect of the N-HR mutations decreasing the affinity of C34 binding or an indirect effect of the N-HR mutations destabilizing the N-HR trimer formation. In contrast, $C34_{N126K}$, with the substitution responsible for the resistance, showed enhanced binding affinity not only to N36 but also to $N36_{D36G/I37K}$. Thus, there are two implications of mutations in gp41 for conferring C34 resistance: the decreased affinity of C34 for $N36_{D36G/I37K}$ and the increased affinity of $C34_{N126K}$ for both $N36_{WT}$ and $N36_{D36G/I37K}$. In other words, the D36G and I37K substitutions in the N-HR interfere with the binding of the peptide inhibitors, such as T-20 and C34, and N126K in the C-HR enhances the intra-gp41 binding of N-HR and C-HR compared with the peptide inhibitors.

Replication kinetics of C34-resistant variants. To determine the effects of the identified deletion and mutations on HIV-1 replication, we first examined the replication kinetics of HIV-1 variants by p24 production in the culture supernatants. The p24 production by the variants ranged from 14 to 34% of that of $HIV\text{-}1_{WT}$ ($HIV\text{-}1_{I37T/N126K}$, 14%; $HIV\text{-}1_{N126K}$, 30%; and $HIV\text{-}1_{A30V/I37T/N126K}$, 34%) as determined at day 8 (Fig. 2). Next, the replication levels of variants with representative substitutions were compared by CHRA. The resistances (*n*-fold) of the variants are also shown in Fig. 2. Since $HIV\text{-}1_{Q39H}$ was considered to be one of the polymorphisms and appeared only transiently, we first compared the replication of $HIV\text{-}1_{WT}$ and $HIV\text{-}1_{N126K}$ and found an impaired replication profile for $HIV\text{-}1_{N126K}$. The variant with a combination including the I37T substitution, $HIV\text{-}1_{I37T/N126K}$, which was not observed during selection, showed the slowest replication profile. To develop an $HIV\text{-}1_{A30V/I37T/N126K}$ variant, the A30V substitution was introduced first, and then the I37T substitution was introduced (Fig. 1B). This was consistent with the results of the CHRA that the replication profile of $HIV\text{-}1_{A30V/I37T/N126K}$ was

TABLE 2. Binding affinity of wild and mutated peptides[a]

Peptide	T_m (°C)
N36/C34	49.5
$N36_{D36G/I37K}$/C34	38.5
N36/$C34_{N126K}$	55.0
$N36_{D36G/I37K}$/$C34_{N126K}$	45.0

[a] The binding affinity of the peptides is shown with T_m values.

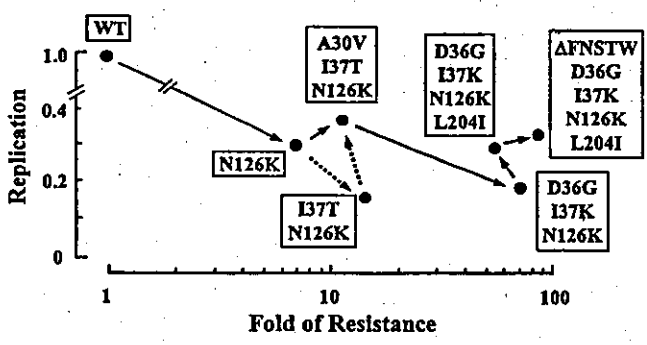

FIG. 2. Replication kinetics of the resistant variants. The replication kinetics determined by p24 antigen production and the CHRA are summarized. The data are depicted as the resistance (*x* axis) and replication (*y* axis) compared with those of $HIV\text{-}1_{WT}$. Variants observed (continuous arrows) and not observed (dashed arrows) in the selection are shown in the order of their emergence.

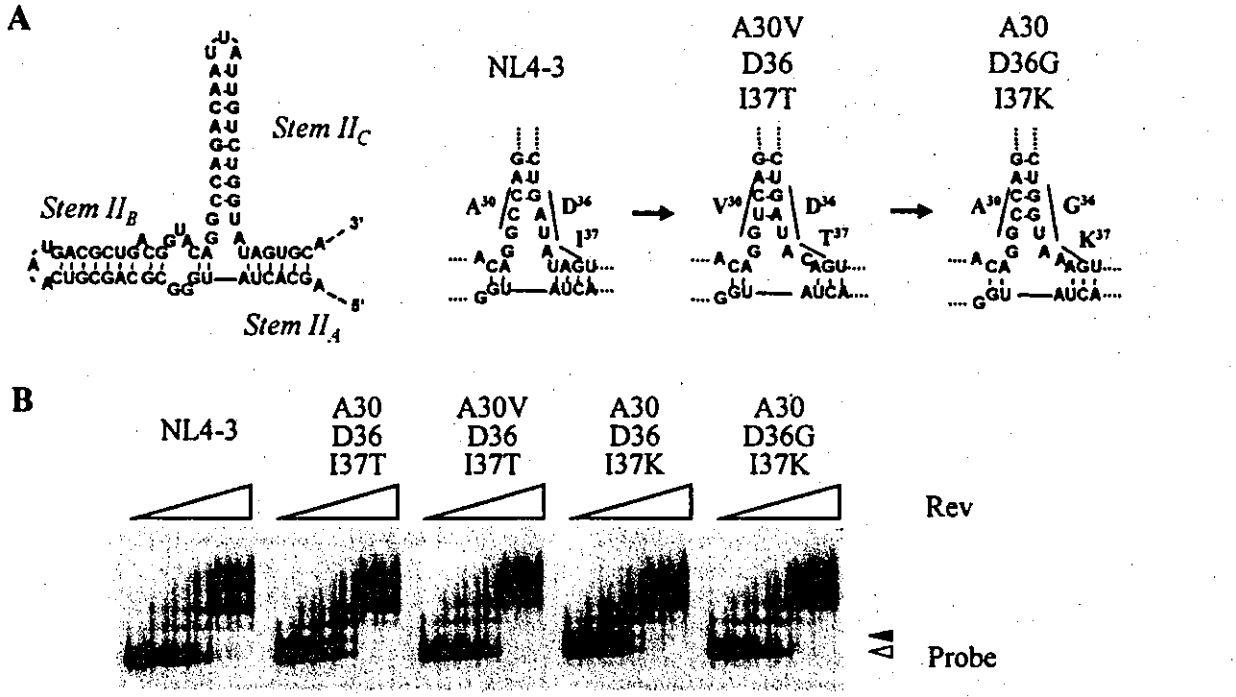

FIG. 3. Putative secondary structure of the RRE and locations of the nucleotides corresponding to the substitutions. The II_A, II_B, and II_C stems and the effects of the nucleotide substitutions are shown (A). A30V (GCC to GTC) to D36 (GAT) and A30 (GCC) to D36G (GAT to GGT) are located complementary to each other in the stem II_B (underlined). The effects of the nucleotide substitutions on the Rev-RRE interaction were examined by gel shift assays (B). The RRE of HIV-1_{I37T} and HIV-1_{I37K} displayed two signals. The amounts of Rev used were 0, 20, 40, 80, 160, 240, and 320 nM (left to right).

greater than that of HIV-$1_{I37T/N126K}$, suggesting that the A30V substitution improved the impaired replication profile of HIV-$1_{I37T/N126K}$. Since both HIV-1_{I37K} and HIV-$1_{I37K/N126K}$ could replicate for only a few passages, these variants could not be studied. For I37K/N126K substitutions, D36G instead of A30V substitution seemed to be suitable in order to maintain resistance and replication, although the replication of HIV-$1_{D36G/I37K/N126K}$ still remained impaired. The L204I substitution improved the replication kinetics of HIV-$1_{D36G/I37K/N126K}$. The deletion in the V4 region contributed to both the increased resistance and replication. These results indicate that among the deletion and the substitutions, I37T/K and N126K, and especially I37K, are related to resistance, while A30V, D36G, and L204I are associated with improvement of replication and the deletion in the V4 region had modest effects on increased resistance and replication.

Rev and RRE interactions. The nucleotides for the substitutions A30V, D36G, and I38T/K also encode the RRE, and the nucleotides for A30 and D36 are located complementary to each other in stem II_C (Fig. 3A). A30V emerged along with I37T and intact D36, and D36G was found with I37K and intact A30. Thus, these mutations were thought to maintain the positioning of stems II_A and II_B in the tertiary structure of the RRE. To examine the effect of these nucleotide substitutions on the Rev-RRE interaction, we performed a gel shift assay. The secondary structure of the RRE that contained the I37T or I37K substitutions showed two forms (Fig. 3B). Introduction of A30V, which could improve the replication of HIV-1_{I37T}, changed the two RRE structures into a structure identical to that observed in the wild type. D36G observed with I37K also changed the separated RRE structure. However, little difference (less than 10%) in the Rev binding caused by the substitutions was observed between the wild-type and the mutated RRE (Fig. 3B). These results indicate that substitutions at A30 or D36 maintain the stability of the RRE secondary structure that is affected by nucleotide substitutions at I37.

DISCUSSION

After adsorption to cells followed by conformational changes of gp120, HIV fusion takes place by interaction and binding of the N-HR and C-HR of gp41. C-HR-derived peptides, e.g., T-20 or C34, inhibit HIV replication as a decoy for C-HR (2, 30). Previous reports showed that mutations in N-HR (19) were associated with resistance to T-20 (9, 25, 26, 28). In this study, we showed that competition between C34 and C-HR also plays a key role in acquiring resistance. However, in order to develop high resistance to C34, changes in the binding affinity of N-HR, as observed in T-20 resistance, are insufficient on their own, and an additional mutation in C-HR is necessary. According to the crystal structure of gp41 (2), none of the substitutions observed in this study are located on the binding surface of the N-HR or C-HR. Even one of the primary mutations that directly contribute to the resistance, I37K, is not located on the binding surface of N-HR. We also identified a primary mutation, N126K, that is located outside of the binding surface of C-HR. Although the significance of these locations still remains to be defined at present, further structural analysis may provide insights into the mechanism of binding

enhancement. Our observations also provide evidence that small compounds interacting directly with the binding surface seem to inhibit HIV replication efficiently, since HIV would hardly be mutated on the binding surface.

During the selection of the C34-resistant variants, the substitutions were introduced in the following order: a substitution (N126K) associated with susceptibility to C34 was introduced first, followed by a substitution (A30V) associated with replication. L204I also improved the replication kinetics of HIV-$1_{D36G/I37K/N126K}$. It is likely that substitutions that are associated with resistance usually impair the replication kinetics, resulting in selection of HIV-1 variants containing substitutions that improve the replication disadvantages. This hypothesis has been proved with analyses of replication kinetics of T-20-resistant variants described previously (20). Moreover, such substitution patterns have previously been observed in multi-dideoxynucleoside-resistant variants (15, 21). However, the mechanism of replication improvement in multi-dideoxynucleoside-resistant variants remains unknown. In this study, I37 is one of the key amino acids for C34 resistance, and it is located in an important region for the Rev-RRE interaction (13). The significance of the secondary mutations, A30V and D36G, for improvement of the RRE structural stability impaired by I37T or I37K is thought to be that they maintain both gp41 and RRE functions. In contrast to the C34 resistance mutations, nucleotides encoding some T-20 resistance mutations, L33S and N43K (9), are located in a single-stranded bulge region of the stem II_C loop top (UUA to UCA) (Fig. 3A) and in the bulge region of stem III, indicating that the structural changes to the RRE would be minimal, while other T-20 resistance mutations (20), such as G36D, G36S, I37K, and possibly V38A and V38 M, appear to alter the stability of the RRE stem II_C structure and to impair the replication kinetics. To date, the Rev-RRE interaction has mainly been examined for stem II_B, since Rev directly binds to it (10, 13). Although the functions of stems II_A and II_C remain to be defined, it is possible that the secondary mutations in stem II_C influence the Rev-RRE interaction, since we have shown that the secondary mutations were introduced simultaneously with the primary mutations and improved replication. These results also indicate that the conformation of the RRE is essential for the Rev-RRE interaction and not just the nucleotide sequence of the RRE itself.

NL4-3 gp41 contains four N-glycan attachment sites, N-X-S/T, located at N100-A-S, N105-K-S, N114-M-T, and N126-Y-T. These four sites are highly conserved in various HIV strains (11, 16). Mutational analysis revealed that each substitution of the N glycosylation sites had a modest effect on HIV replication, whereas some combined substitutions severely impaired replication (11). Although the effect of N126-glycan on binding of the N-HR and C-HR remains unknown, it would be possible that N126-glycan plays some roles for C34 resistance. Different effects of N126K substitution on susceptibility to T-20 and C34 (1.9- and 6.8-fold, respectively) were observed (Table 1). This result might be accounted for by the finding that N126 locates at −1 (outside) from the N terminus of T-20, whereas it locates inside (+10) of C34 (Fig. 1A).

It has been reported that a tyrosine-based sorting signal in the gp41 cytoplasmic domain, Y201-X-X-L, was involved in trafficking and targeting to the plasma membrane of the gp41 (3). The motif is highly conserved among various HIV strains (16, 19). Although the role of Y201 for infectivity has been studied in detail (3, 19), that of L204 remains to be defined. In the present study, we showed that the L204I substitution enhanced viral replication, suggesting that L204, as well as Y201, plays an important role for viral replication.

pNL4-3 was established as a molecular clone of wild-type HIV-1 (1) and is widely used in HIV research. However, it represents only one of the wild-type HIV-1 variants. In fact, even in the absence of C34, we still observed several substitutions in NL4-3 that were identified in the C34 selection, e.g., A30V and Q39H. These substitutions are also observed in some treatment-naïve clinical isolates (16). It is well known that HIV reverse transcriptase makes several nucleotides miss incorporation during the reverse transcription, suggesting that each HIV isolate, even in the wild-type population, contains several substitutions in the integrated DNA genome. D36 is identified only in pNL4-3-derived clones, although the G36/I37/V38 motif is well conserved, not only in HIV-1 but also in HIV-2 and simian immunodeficiency virus strains (16). Furthermore, the 5-amino-acid deletion in gp120 was reported not only in a fusion inhibitor, T-20 (9), but also in CD4-gp120-binding inhibitors DS5000 (8) and AR177 (Zintevir) (7), CXCR4 antagonists, bicyclams JM2763 and SID791 (6), and SDF-1α-resistant variants (27). In these reports, pNL4-3-derived viruses were also used for the selection of the resistant variants. Only pNL4-3 has the 5-amino-acid tandem sequence FNSTWFNSTW in the gp120 V4 region. Therefore, this deletion is thought to be specific for HIV-1_{NL4-3}, although the 5-amino-acid deletion conferred weak C34 resistance. These results indicate that we should be careful before concluding that such substitutions are involved in the resistance or replication kinetics.

In conclusion, HIV acquires resistance against C34 by mutations in both N-HR and C-HR. However, mutations in N-HR are restricted by Rev-RRE and/or gp120-gp41 interactions, suggesting that HIV-1 fusion is one of the most attractive targets for blocking HIV infection.

ACKNOWLEDGMENTS

We thank Hiroaki Mitsuya for helpful suggestions and Ayako Yoshioka for manuscript preparation.

This work was supported in part by a grant for the Promotion of AIDS Research from the Ministry of Health and Welfare of Japan (M.M.), a grant for Research for Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation (E.K.), and a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.K.). D.N. is supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of the acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59:284–291.
2. **Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89:263–273.
3. **Day, J. R., C. Munk, and J. C. Guatelli.** 2004. The membrane-proximal tyosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. J. Virol. 78:1069–1079.
4. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. 74:8358–8367.

5. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, Z. Zhang, W. A. O'Brien, L. Ratner, G. M. Shaw, and E. Hunter.** 2001. Sensitivity of human immunodeficiency virus type 1 to fusion inhibitors targeted to the gp41 first heptad repeat involves distinct regions of gp41 and is consistently modulated by gp120 interactions with the coreceptor. J. Virol. 75:8605–8614.
6. **De Vreese, K., V. Kofler-Mongold, C. Leutgeb, V. Weber, K. Vermeire, S. Schacht, J. Anne, E. De Clercq, R. Datema, and G. Werner.** 1996. The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. J. Virol. 70:689–696.
7. **Este, J. A., C. Cabrera, D. Schols, P. Cherepanov, A. Gutierrez, M. Witvrouw, C. Pannecouque, Z. Debyser, R. F. Rando, B. Clotet, J. Desmyter, and E. De Clercq.** 1998. Human immunodeficiency virus glycoprotein gp120 as the primary target for the antiviral action of AR177 (Zintevir). Mol. Pharmacol. 53:340–345.
8. **Este, J. A., D. Schols, K. De Vreese, K. Van Laethem, A. M. Vandamme, J. Desmyter, and E. De Clercq.** 1997. Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. Mol. Pharmacol. 52:98–104.
9. **Fikkert, V., P. Cherepanov, K. Van Laethem, A. Hantson, B. Van Remoortel, C. Pannecouque, E. De Clercq, Z. Debyser, A.-M. Vandamme, and M. Witvrouw.** 2002. *env* chimeric virus technology for evaluating human immunodeficiency virus susceptibility to entry inhibitors. Antimicrob. Agents Chemother. 46:3954–3962.
10. **Henderson, B. R.** 1997. Interaction between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-β. J. Mol. Biol. 274:693–707.
11. **Johnson, W. E., J. M. Sauvron, and R. C. Desrosiers.** 2001. Conserved, N-linked carbohydrates of human immunodeficiency virus type 1 gp41 are largely dispensable for viral replication. J. Virol. 75:11426–11436.
12. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. 66: 2232–2239.
13. **Kjems, J., M. Brown, D. D. Chang, and P. A. Sharp.** 1991. Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element. Proc. Natl. Acad. Sci. USA 88:683–687.
14. **Kodama, E. I., S. Kohgo, K. Kitano, H. Machida, H. Gatanaga, S. Shigeta, M. Matsuoka, H. Ohrui, and H. Mitsuya.** 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. 45:1539–1546.
15. **Kosalaraksa, P., M. F. Kavlick, V. Maroun, R. Le, and H. Mitsuya.** 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.
16. **Kuiken, C., B. Foley, B. Hahn, P. Marx, F. McCutchan, J. Mellors, S. Wolinsky, and B. Korber (ed.).** 2001. HIV sequence compendium 2001. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.M.
17. **Lalezari, J. P., K. Henry, M. O'Hearn, J. S. Montaner, P. J. Piliero, B. Trottier, S. Walmsley, C. Cohen, D. R. Kuritzkes, J. J. Eron, Jr., J. Chung, R. DeMasi, L. Donatacci, C. Drobnes, J. Delehanty, and M. Salgo.** 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N. Engl. J. Med. 348:2175–2185.
18. **Lazzarin, A., B. Clotet, D. Cooper, J. Reynes, K. Arasteh, M. Nelson, C. Katlama, H. J. Stellbrink, J. F. Delfraissy, J. Lange, L. Huson, R. DeMasi, C. Wat, J. Delehanty, C. Drobnes, and M. Salgo.** 2003. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N. Engl. J. Med. 348:2186–2195.
19. **Lodge, R., J.-P. Lalonde, G. Lemay, and E. A. Cohen.** 1997. The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. EMBO J. 16:695–705.
20. **Lu, J., P. Sista, F. Giguel, M. Greenberg, and D. Kuritzkes.** 2004. Relative replicative fitness of human immunodeficiency virus type 1 mutants resistant to enfuvirtide (T-20). J. Virol. 78:4628–4637.
21. **Maeda, Y., D. J. Venzon, and H. Mitsuya.** 1998. Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance. J. Infect. Dis. 177: 1207–1213.
22. **Malashkevich, V. N., D. C. Chan, C. T. Chutkowski, and P. S. Kim.** 1998. Crystal structure of the simian immunodeficiency virus (SIV) gp41 core: conserved helical interactions underlie the broad inhibitory activity of gp41 peptides. Proc. Natl. Acad. Sci. USA 95:9134–9139.
23. **Matthews, T., M. Salgo, M. Greenberg, J. Chung, R. DeMasi, and D. Bolognesi.** 2004. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat. Rev. Drug Discov. 3:215–225.
24. **Otaka, A., M. Nakamura, D. Nameki, E. Kodama, S. Uchiyama, S. Nakamura, H. Nakano, H. Tamamura, Y. Kobayashi, M. Matsuoka, and N. Fujii.** 2002. Remodeling of gp41-C34 peptide leads to highly effective inhibitors of the fusion of HIV-1 with target cells. Angew. Chem. Int. Ed. Engl. 41:2937–2940.
25. **Poveda, E., B. Rodes, C. Toro, L. Martin-Carbonero, J. Gonzalez-Lahoz, and V. Soriano.** 2002. Evolution of the gp41 env region in HIV-infected patients receiving T-20, a fusion inhibitor. AIDS 16:1959–1961.
26. **Rimsky, L. T., D. C. Shugars, and T. J. Matthews.** 1998. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J. Virol. 72:986–993.
27. **Schols, D., J. A. Este, C. Cabrera, and E. De Clercq.** 1998. T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1α contains mutations in the envelope gp120 but does not show a switch in coreceptor use. J. Virol. 72:4032–4037.
28. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46:1896–1905.
29. **Weiner, M. P., G. L. Costa, W. Schoettlin, J. Cline, E. Mathur, and J. C. Bauer.** 1994. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. Gene 151:119–123.
30. **Wild, C., T. Oas, C. McDanal, D. Bolognesi, and T. Matthews.** 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc. Natl. Acad. Sci. USA 89:10537–10541.

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Virology 327 (2004) 215–224
VIROLOGY
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Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naïve patients

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Received 27 April 2004; accepted 2 July 2004
Available online 9 August 2004

Abstract

Several reports have recently shown that drug-resistant human immunodeficiency virus type 1 (HIV-1) is often isolated from treatment-naïve patients. We phenotypically analyzed HIV-1 strains isolated from 44 treatment-naïve individuals and found two strains highly resistant (69- and >310-fold) against nevirapine (NVP). Direct sequencing showed these two isolates had a novel mutation, K238S, in reverse transcriptase (RT), but did not have any reported NVP resistance-associated mutation.

A 48-h culture in the presence of NVP, however, selected HIV-1 carrying NVP resistance-associated mutations, V106A, V108I, or both, suggesting that minor viral populations of these two isolates had harbored these mutations. Replication kinetic studies of recombinant HIV-1 clones suggested that K238S conferred a significant resistance against NVP, especially when accompanied with V106A (530-fold) or V108I (56-fold). Our study identified a novel NVP resistance-associated mutation, K238S, which could be persistently detected by genotypic assay longer than V106A and V108I during off-treatment period.

Keywords: Human immunodeficiency virus type 1; K238S; Drug-naïve; Nevirapine-resistant

Introduction

The emergence of human immunodeficiency virus type 1 (HIV-1) variants resistant to antiretroviral agents is one of the most common causes for therapeutic failure in infected individuals. Fortunately, the availability of drug-resistance testing has substantially improved the ability of clinicians to deal knowledgeably with drug-resistant HIV-1 variants. Various guidelines for therapy of HIV-1 infection recommend that each individual therapy should be optimized by choosing efficient agents based on the results of drug-resistance testing. Numerous studies have recently reported that significant proportion of newly diagnosed HIV-1 infection cases are infected with viral strains resistant to at least one antiretroviral agent, justifying drug-resistance testing even in treatment-naïve patients (Briones et al., 2001; Duwe et al., 2001; Grant et al., 2002; Little et al., 2002; Salomon et al., 2000; Simon et al., 2002). However, it is important to recognize that the ability to detect resistant viruses may decrease as a function of time from initial infection because there is generally a shift to wild type over time during off-treatment (Devereux et al., 1999; Miller et al., 2000; Verhofstede et al., 1999). The usefulness of phenotypic assay could be enhanced if the virus is propagated in the presence of drug, because such a condition could propagate resistant viral strains that might have been outgrown by wild-type viruses in the absence of drug pressure. In this study, we screened 44 clinical strains isolated from newly infected individuals by using phenotypic assay and identified two highly nevirapine (NVP)-resistant isolates that could not be detected by genotypic

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0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.07.001

assay. Phylogenetic analyses showed these two isolates were genetically closely related and probably originated from a single patient. Furthermore, we identified a novel NVP resistance-associated mutation, K238S, in these isolates and determined its role in viral replication and resistance using newly generated recombinant HIV-1 clones.

Results

NVP-resistant HIV-1 from treatment-naïve patients

From January 2000 through December 2001, a total of 44 patients, who had had clinical evidence of recent HIV-1 infection (seroconversion or increased bands of western blotting against HIV-1 antigens), visited our clinic and all of them gave written informed consent to the participation in this study. To determine the prevalence of drug resistance in recent HIV-1 infection, susceptibility of HIV-1 isolates derived from these patients to six antiretroviral agents including zidovudine (AZT), lamivudine (3TC), stavudine (d4T), abacavir (ABC), NVP, and efavirenz (EFV) was examined phenotypically by using MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001). As expected, most of such isolates were sensitive to all tested antiretroviral agents. However, two isolates (4.5%; isolated from Cases 33 and 23) showed significantly greater resistance (69-fold and more than 310-fold compared with NL4-3) to NVP, though their resistance to EFV was not high (Fig. 1, Table 1).

To delineate the mechanism of the NVP resistance in the above two isolates, direct sequencing of HIV-1 reverse transcriptase (RT) gene from plasma samples was performed. Both plasma-derived sequences did not have any non-nucleoside RT inhibitor (NNRTI) resistance-associated mutations listed in resistance table of the International AIDS Society (Johnson et al., 2003) but had six-amino-acid substitutions including V35I, R83K, I135T, T200K, R211K, and K238S, compared with HXBII sequences, and the plasma-derived sequence in Case 23 had a mixture of K102 and K102R (Table 2). We postulated that there might be a minor viral population harboring NNRTI resistance-associated mutations in both plasma samples, which could not be detected by direct sequencing. To detect such a minor NVP-resistant viral population, the clinical isolates were propagated in MAGIC-5 cells in the presence of NVP at various concentrations and the RT gene of HIV-1 obtained from a 48-h culture supernatant was analyzed. In the HIV-1 isolated from Case 33, V108I, known as a NVP resistance-associated mutation, emerged in the presence of NVP (1 μM) although it could not be detected in direct sequencing of plasma (Table 2). In the HIV-1 isolated from Case 23, V106A, and V108I emerged in the presence of NVP (1 and 10 μM). Interestingly, clonal sequencing analysis of HIV-1 culture (NVP 1 μM) isolated from Case 23 revealed that K102R and V106A always coexisted on the same molecules and that V108I did not coexist with K102R and V106A (Table 2). All clones analyzed had V35I, R83K, I135T, T200K, R211K, and K238S. Four of these mutations (V35I, R83K, I135T, and R211K) are known as polymorphic mutations and were frequently observed in HIV-1 isolated from treatment-naïve individuals in our cohort (72%, 18%, 27%, and 36%, respectively, $n = 11$). T200K and K238S had not been reported previously and might be associated with NVP resistance.

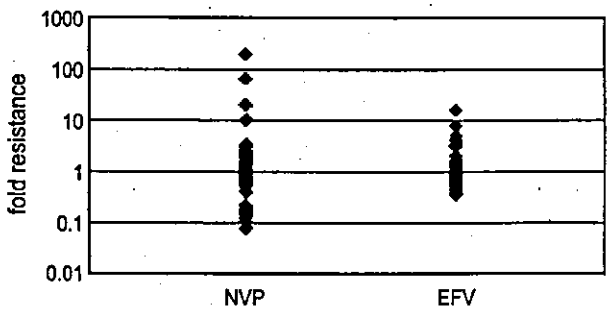

Fig. 1. Results of drug resistance assay using MAGIC-5 cells in treatment-naïve individuals. Fold resistances compared with NL4-3 are shown.

Phylogenetic analysis of clinical HIV-1 isolates

Because the clinical HIV-1 isolates derived from Cases 33 and 23 had unusually high resistance against NVP and had exactly the same amino acid substitutions (V35I, R83K, I135T, T200K, R211K, and K238S), we suspected that these two patients had acquired HIV-1 infection from a single infected patient. Then, we phylogenetically analyzed the direct sequences of *env* region and RT gene of the two HIV-1 isolates derived from Cases 33 and 23, and then compared their sequences with four clinical isolates derived from other patients (Cases 2, 3, 26, and 29) and five referential unrelated strains [U63632, HXB2CG, OY1, RF, and simian immunodeficiency virus (SIV)]. In both phylogenetic trees using the sequences of the *env* region and RT gene, Cases 33- and 23-derived sequences formed a tight cluster separated from other sequences by the tree branch with high bootstrap probabilities (99% and 100%) (Figs. 2A and B), suggesting that the isolates from Cases 33 and 23 were genetically closely related and probably originated from a single source. It was noteworthy that the *env* sequences derived from Cases 33 and 23 were closely related but not identical, which excludes the possibility of contamination during the procedures of viral culture and PCR.

Three-dimensional positions of mutations and NVP-binding pocket in HIV-1 RT

To delineate the positional relationship between NVP-binding pocket and the mutations in RT described above, a structural model of HIV-1 RT complexed with NVP was

Table 1
Results of phenotypic drug resistance testing in two untreated patients

HIV-1	IC_{50}[a] [μM] (fold resistance[b])					
	AZT	3TC	d4T	ABC	NVP	EFV
Case 33	0.04 ± 0.03 (1.2)	0.61 ± 0.20 (1.8)	1.7 ± 0.10 (0.77)	3.6 ± 0.87 (2.3)	2.2 ± 0.29 (69)	0.011 ± 0.003 (4.2)
Case 23	0.12 ± 0.03 (3.5)	0.81 ± 0.15 (2.4)	3.2 ± 0.95 (1.5)	3.3 ± 0.87 (2.1)	>10 (>310)	0.023 ± 0.00002 (8.8)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (AZT, 0.034 μM; 3TC, 0.34 μM; d4T, 2.2 μM; ABC, 1.6 μM; NVP, 0.032 μM; EFV, 0.0026 μM).

prepared based on the published crystal structures (Fig. 3) (Ren et al., 2000). The Val residues at 106 and 108 codons of RT (V106 and V108), the site of NNRTI resistance-associated mutations, V106A and V108I, respectively, were in close contact with NVP, packed in the hydrophobic pocket of the palm domain. Five residues, V35, R83, I135, T200, and R211, were located distantly from the hydrophobic pocket. Two residues, K102 and K238, were located outside the frame of hydrophobic pocket and the substitutions of these amino acids, such as K102R and K238S, were thought to produce some structural changes of the pocket and might be associated with NNRTI resistance.

Role of K102R and K238S in HIV-1 replication and resistance against NVP

Because K238S has not been reported previously and K102 and K238 were located on the frame of NVP-binding pocket, we chose to construct seven recombinant HIV-1 strains, HIV-1_{K102R}, HIV-1_{V106A}, HIV-1_{V108I}, HIV-1_{K238S}, HIV-$1_{V106A/K238S}$, HIV-$1_{V108I/K238S}$, and HIV-$1_{K102R/V106A/K238S}$, to delineate the effects of K102R and K238S on viral susceptibility against NVP. K102R did not confer significant resistance against NVP and EFV (Table 3), while V106A and V108I, both of which were known as NVP resistance-associated mutations, conferred 97- and 3.8-fold resistance against NVP, respectively. However, these mutations did not confer significant resistance against EFV. K238S conferred a significant resistance against NVP as did V108I. Interestingly, the combination of V106A and K238S (V106A/K238S) conferred surprisingly high-level resistance (530-fold) against NVP, and the combination of V108I and K238S (V108I/K238S) also conferred high resistance (56-fold) only against NVP but not against EFV (Table 3). Furthermore, the triple combination of K102R, V106A, and K238S (K102R/V106A/K238S), which were found in 15 of 21 clones derived from Case 23 (Table 2), also conferred high resistance (340-fold) against NVP, although its effect on viral resistance against EFV was not significant (Table 3). These data suggest that K238S is strongly associated with NVP resistance in the combination with V106A or V108I.

We next analyzed the effects of K102R and K238S on viral replication kinetics in the absence or presence of NVP. The addition of K238S to HIV-1WT (HIV-1_{K238S}) reduced the replication of HIV-1_{WT} in the absence of NVP, and the addition of V108I to HIV-1_{K238S} (generating HIV-$1_{V108I/K238S}$) further reduced the viral replication (Fig. 4A). On the other hand, in the presence of NVP (1 μM), K238S made HIV-1 replication-competent (HIV-1_{WT} could not replicate), and HIV-$1_{V108I/K238S}$ had facilitated viral replication (Fig. 4B). The combination of V106A to K238S (HIV-$1_{V106A/K238S}$) severely compromised viral replication and the addition of K102R (HIV-$1_{K102R/V106A/K238S}$) did

Table 2
Sequences of HIV-1 RT-coding region of clinical samples and isolates

	Sequenced sample	Amino acid residue														
		35	83	100[a]	102	103[a]	106[a]	108[a]	135	181[a]	188[a]	190[a]	200	211	230[a]	238
HXBII		V	R	L	K	K	V	V	I	Y	Y	G	T	R	M	K
Case 33	plasma	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	–	–	–	V/I	T	–	–	–	K	K	–	S
Case 23	plasma	I	K	–	K/R	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	K/R	–	V/A	V/I	T	–	–	–	K	K	–	S
	10 μM[b]	I	K	–	K/R	–	A	V/I	T	–	–	–	K	K	–	S
	Number of clones															
Isolate from	15	I	K	–	R	–	A	–	T	–	–	–	K	K	–	S
Case 23	3	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
(1 μMb)	2	I	K	–	–	–	–	I	T	–	–	–	K	K	–	S
	1	I	K	–	–	R	–	–	T	–	–	–	K	K	–	S

Amino acids identical to HXBII (top column) are indicated with dashes.
[a] Reported residue associated with NVP resistance (Johnson et al., 2003).
[b] Cultured HIV-1 isolate in the presence of NVP at the indicated concentrations.

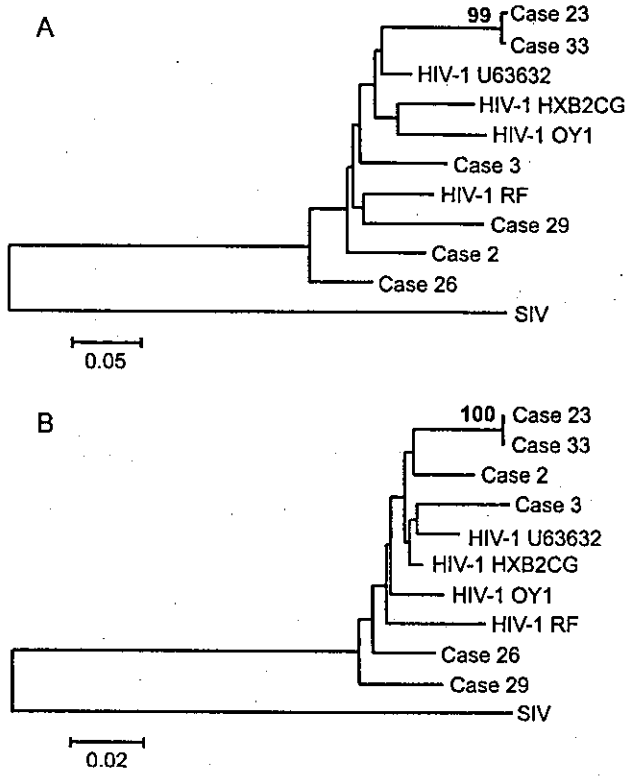

Fig. 2. Phylogenetic analysis of *env* sequences (A) and RT genes (B) of six clinical isolates and five referential strains. Bootstrap probabilities of important tree branches are shown as percentages.

Table 3
Drug resistance of recombinant HIV-1s

Recombinant HIV-1	IC_{50}[a] [μM] (fold resistance[b])	
	NVP	EFV
K102R	0.047 ± 0.02 (1.5)	0.002 ± 0.001 (0.77)
V106A	3.1 ± 0.36 (97)	0.002 ± 0.0003 (0.77)
V108I	0.12 ± 0.03 (3.8)	0.001 ± 0.0001 (0.38)
K238S	0.14 ± 0.04 (4.4)	0.002 ± 0.0003 (0.77)
V106A/K238S	17 ± 4.6 (530)	0.004 ± 0.001 (1.5)
V108I/K238S	1.8 ± 0.66 (56)	0.001 ± 0.0001 (0.38)
K102R/V106A/K238S	11 ± 3.1 (340)	0.002 ± 0.0001 (0.77)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (NVP; 0.032 μM, EFV; 0.0026 μM).

not seem to rescue its replicability in the absence of drugs (Fig. 4C). In the presence of NVP (1 μM), the combination of V106A and K238S (HIV-$1_{V106A/K238S}$) rendered HIV-1 replicable, and the addition of K102R to V106A/K238S (HIV-$1_{K102R/V106A/K238S}$) did not significantly alter the replication (Fig. 4D). These data showed that V108I/K238S and V106A/K238S can confer viral replicability in the presence of NVP, although the role of K102R when combined with V106A/K238S remained to be determined.

To further define the significance of each mutation, the viral replicability was compared among molecular infectious HIV-1 clones described above in the presence and absence of NVP using competitive HIV-1 replication assay (CHRA) (Kosalaraksa et al., 1999). Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells, and their population changes through passages were determined by the relative peak height on sequencing electrogram. In the absence of NVP, HIV-1_{WT} readily outgrew HIV-

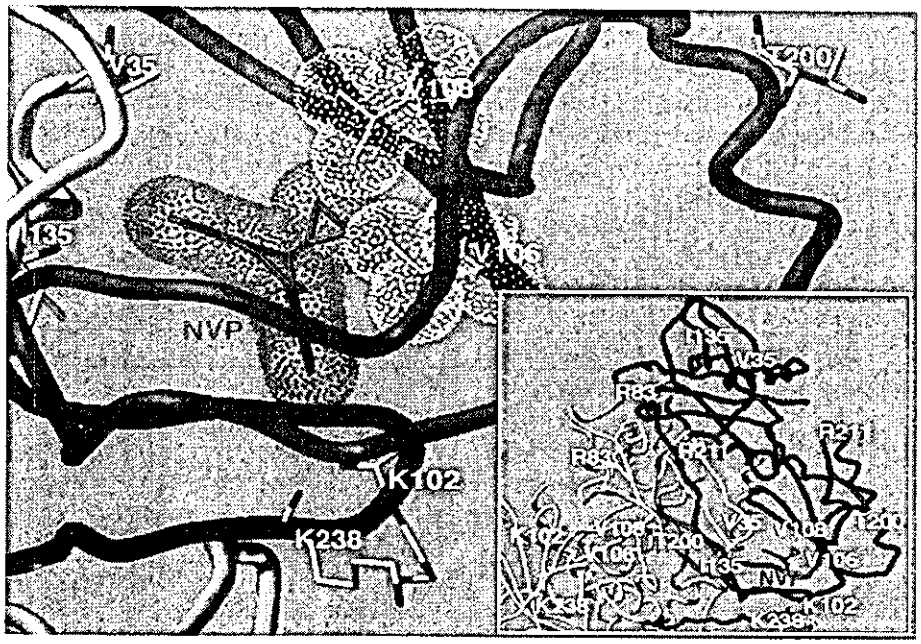

Fig. 3. The structure of the HIV-1 RT complexed with NVP. A view of the RT complexed with NVP, generated using SYBYL 6.7 and the coordinate set 1FKP.pdb obtained from the Protein Data Bank (Ren et al., 2000). The fingers, palm, thumb, and connection subdomains of the p66 subunit are colored blue, red, green, and yellow, respectively. The p51 subunit is colored white. Residues of the p66 subunit are yellow-colored, while those of the p51 subunit are white-colored. The van der Waals volumes of side chain of V106 and V108 (white) are shown to interact with NVP (orange).

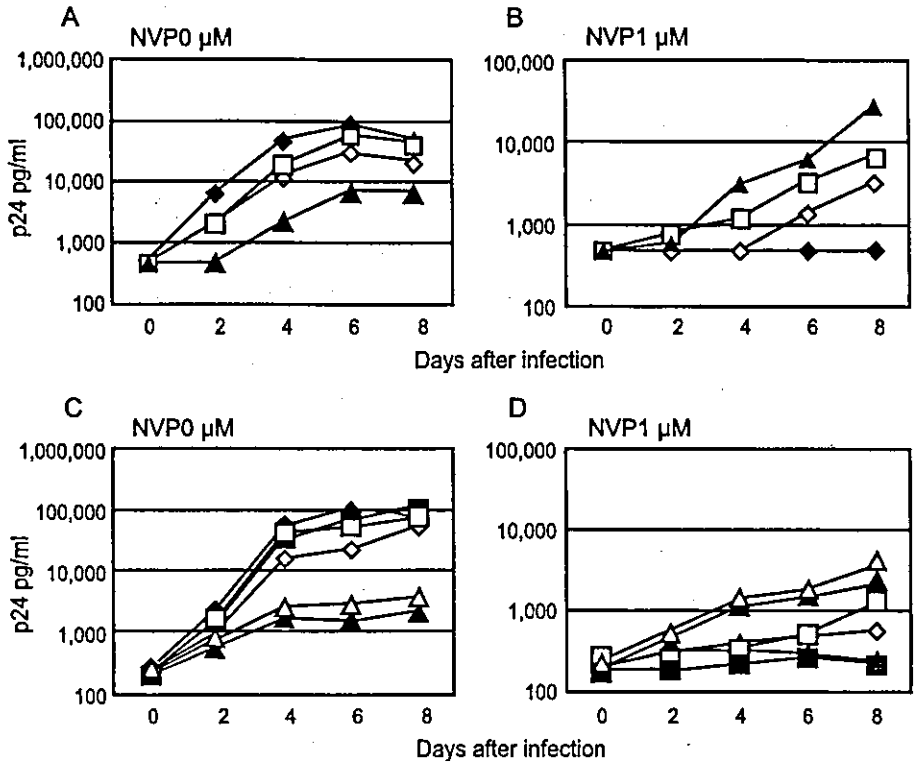

Fig. 4. Replication kinetics of recombinant HIV-1s. Graphs A and B show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{V108I} (open squares), HIV-1_{K238S} (open diamonds), and HIV-$1_{V108I/K238S}$ (solid triangles) in the absence and presence of NVP (1 μM), respectively. Graphs C and D show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{K102R} (solid squares), HIV-1_{V106A} (open squares), HIV-1_{K238S} (open diamonds), HIV-$1_{V106A/K238S}$ (solid triangles), and HIV-$1_{K102R/V106A/K238S}$ (open triangles) in the absence and presence of NVP (1 μM), respectively. The results shown are representative of three independent experiments.

1_{K238S} (Fig. 5A). In the presence of NVP (0.1 μM), however, HIV-1_{K238S} predominated over HIV-1_{WT} (Fig. 5B), suggesting that K238S compromised the viral replication in the absence of NVP but conferred resistance against NVP. Next, we analyzed the effect of V106A and V108I on HIV-1_{K238S} replication. In comparison between HIV-1_{K238S} and HIV-$1_{V106A/K238S}$, HIV-1_{K238S} predominated over HIV-$1V_{106A/K238S}$ in the absence of NVP (Fig. 5C), but was outgrown by HIV-$1_{V106A/K238S}$ in the presence of NVP (1 μM) (Fig. 5D). In comparison between HIV-1_{K238S} and HIV-$1_{V108I/K238S}$, HIV-1_{K238S} predominated over HIV-$1_{V108I/K238S}$ in the absence of NVP (Fig. 5E), but was outgrown by HIV-$1_{V108I/K238S}$ in the presence of NVP (1 μM) (Fig. 5F). Taken together, these data suggest that each of V106A and V108I compromised the viral replication in the absence of NVP but conferred resistance against NVP. Finally, we analyzed the effect of K102R on replication of HIV-1_{WT} and HIV-$1_{V106A/K238S}$. HIV-1_{K102R} was slowly overcome by HIV-1_{WT} in the absence of NVP (Fig. 5G). In comparison between HIV-$1_{V106A/K238S}$ and HIV-$1_{K102R/V106A/K238S}$, HIV-$1_{K102R/V106A/K238S}$ predominated over HIV-$1_{V106A/K238S}$ both in the absence (Fig. 5H) and presence (1 μM) (Fig. 5I) of NVP. Considered together, these results suggest that K102R compromised the replication of HIV-1_{WT} but compensated the replication ability of HIV-$1_{V106A/K238S}$ regardless of the presence of NVP.

Discussion

Several studies reported the recent spread of drug-resistant HIV-1 in the developed countries and among treatment-naïve individuals as well, which has been a menace for clinicians. Therefore, for certain treatment-naïve patients, such as cases of acute or recent HIV-1 infection, drug-resistance assay is recommended (Hirsch et al., 2003). However, resistant HIV-1 can be replaced with wild-type HIV-1 rapidly in plasma in the absence of treatment, and it may be difficult to detect resistant HIV-1 in the treatment-naïve patients even if they harbored resistant HIV-1. In this regard, phenotypic assay may be superior to genotypic assay using direct sequencing, because in phenotypic assay, resistant viruses could be propagated during culture in the presence of antiretroviral agents, while direct sequencing can only detect relatively major viral population in plasma. In fact, in the present study, the phenotypic assay using MAGIC-5 cells detected two highly NVP-resistant isolates in treatment-naïve patients, in whom direct sequencing failed to detect the presence of minor population of V106A and V108I.

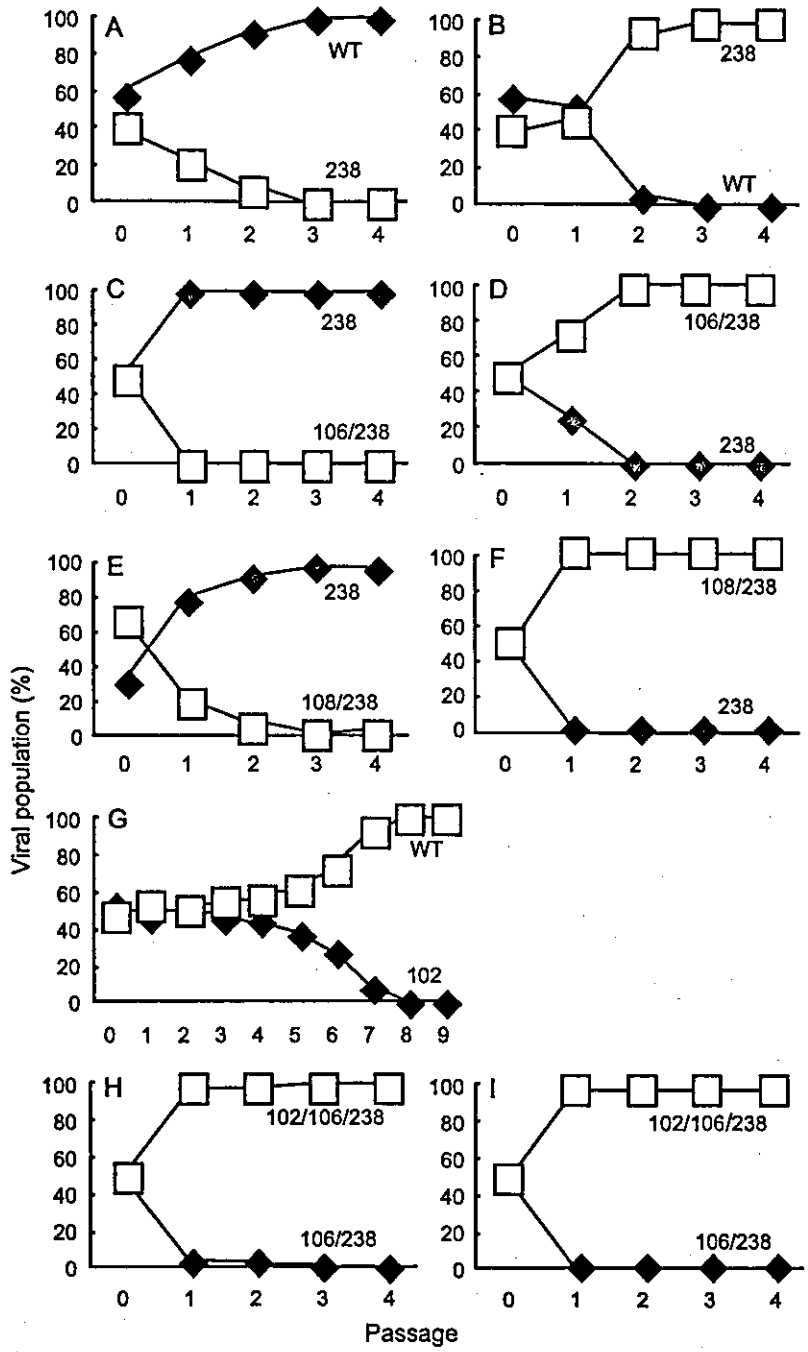

Fig. 5. Competitive HIV-1 replication assay for recombinant HIV-1s. Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells in the absence (A, C, E, G, and H) and presence of NVP (B: 0.1 μM; D, F, and I: 1 μM). The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells at the end of each passage were subjected to nucleotide sequencing, and proportion of Lys and Ser at position 238 (Graphs A and B), those of Val and Ala at position 106 (Graphs C and D), those of Val and Ile at position 108 (Graphs E and F), and those of Lys and Arg at position 102 (Graphs G–I) were determined.

The use of NVP has markedly increased worldwide because a single formulation of NVP combined with d4T and 3TC is a generic drug widely used in resource-limited situations (Cohen, 2003; Penzak et al., 2003). The use of NVP in pregnant women is also one of the main strategies to prevent mother-to-child transmission of HIV-1 (Guay et al., 1999; Marseille et al., 1999). Therefore, understanding of the mechanism of NVP resistance development is urgently needed.

In the present study, we identified a novel mutation, K238S, which was associated with a high multitude of NVP resistance when it was coupled with V106A or V108I. Interestingly, direct sequencing identified only K238S in the two patients infected with highly NVP-resistant HIV-1.

These results showed that V106A and V108I were reverting to their wild-type V106 and V108, respectively, in major viral populations in the plasma. Furthermore, the results indicate that K238S can remain longer than V106A and V108I in the absence of NVP treatment, partly because at least two nucleotide substitutions are required for K238S while V106A and V108I can occur with only a single nucleotide substitution.

M230 is located on the opposite side of Q151 in relation to the incoming dNTP, forming the primer grip of RT and contacting the primer strand (Huang et al., 1998; Sarafianos et al., 1999), and several mutations around this site were reported to occur in patients during NNRTI treatment. Parkin et al. (2000) observed the emergence of F227L and M230L during NVP-containing combination therapy and it was associated with the loss of NVP susceptibility of their clinical isolates. Demeter et al. (2000) reported that P236L occurred in subjects receiving delavirdine monotherapy. With regard to K238, Demeter et al. (1998) observed the emergence of K238T in one patient treated with atevirdine (an NNRTI) and AZT. Considering these reports, the region containing M230 and K238 is important for drug susceptibility and there may be more resistance-associated mutations around these sites.

Phylogenetic analysis showed a close relation between the isolates from Cases 33 and 23 (Fig. 2), suggesting that the two patients had acquired HIV-1 infection from the same source, although they did not know each other and interviewing them could not identify the source patient. This source patient presumably had been under antiretroviral treatment including NVP, which had failed to suppress his viral load because of the sets of mutations, K102R/V106A/K238S and V108I/K238S. It remains unknown whether K238S had existed as a polymorphism before the introduction of antiretroviral treatment, or it had emerged during NVP treatment in the source patient. Considering that a single dose of NVP to prevent mother-to-child transmission can induce resistant strains (Morris et al., 2003; Sullivan, 2003), some polymorphic mutations in treatment-naïve population could be associated with NVP resistance.

Materials and methods

Reagents and cells

AZT, d4T, and ABC were purchased from Sigma Co. (Tokyo, Japan). 3TC, NVP, and EFV were generously provided by Nippon Glaxo-Smithkline (Tokyo, Japan), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Merck Co. Inc. (Rahway, NJ), respectively.

H9 cells and COS-7 cells were grown in RPMI 1640 and Dulbecco's modified eagle medium (DMEM), respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001, 2003) were grown in DMEM supplemented with 10% FCS, hygromycin B (100 μg/ml) (Invitrogen Co., Carlsbad, CA), and blasticidin (1 μg/ml) (Funakoshi, Tokyo). Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated by phytohemagglutinin (PHA) in RPMI 1640-based medium containing interleukin-2 (20 U/ml) (R & D Systems, Minneapolis, MN) for 2 days before HIV-1 exposure.

Patients and clinical isolates

Clinical strains were isolated from fresh plasma of the study participants attending the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, by using MAGIC-5 cells as described previously (Hachiya et al., 2001). The obtained isolates were stored at −80 °C until use and the infectivity was measured as blue cell-forming unit (BFU) of MAGIC-5 cells (Hachiya et al., 2001). The Institutional Review Board approved this study (IMCJ-H13-80) and a written informed consent was obtained from all the participants.

Case 33 was a 33-year-old homosexual man. He suffered from high fever and severe headache, probably symptoms of primary HIV-1 infection, in April 2000. On May 15, Western blotting showed faint bands against HIV-1 gp160, p68, p55, p25, and p18 antigens. One week after, these bands became bold and new bands against HIV-1 gp110, p40, and p34 antigens appeared. The CD4 count was 401/μl and HIV-1 viral load was 2.0×10^5/ml.

Case 23 was a 23-year-old homosexual man. He also suffered from severe headache and was hospitalized in January, 2001. Western blotting showed one faint band against HIV-1 gp160 antigen. In April, 2001, the band became bold and new bands against gp110, p68, p55, p52, gp41, p40, p34, p25, and p18 antigens appeared. The CD4 count was 210/μl and HIV-1 viral load was 7.1×10^4/ml.

Sequence analyses of HIV-1 RT and V3 region

Viral RNA was extracted from plasma and clinical isolates with Smi-test nucleic acid extraction kit (Genome Science, Fukushima, Japan). The HIV-1 RT gene was amplified by RT-PCR using One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan). For plasma-derived RNA, nested PCR was conducted subsequently to amplify enough DNA for direct sequencing. The sequences of primer sets for the first PCR (T1 and T4) and the second PCR (T12 and T15) were as follows (Gatanaga et al., 1999; Hachiya et al., 2001, 2003): T1, 5′-AGGGGGAATTGGAGGTTT (RF positions, 1910 to 1927); T4, 5′-TTCTGTTAGTGCTTTGGTT (RF positions, 2939–2921); T12, 5′-CCAGTAAAATTAAAGCCAG (RF positions, 2091–2109); and T15, 5′-TCCCACTAACTTCTGTATGTC (RF positions, 2852–2832). The gp120 V3 domain of several HIV-1 isolates was amplified by RT-

PCR and nested PCR. The sequences of primer sets for the first PCR (ES1 and EA1) and the second PCR (ES2 and EA2) were as follows (Ida et al., 1997): ES1, 5′-AATGTCAGCACAGTACAATGTACAC (RF positions, 6502–6526); EA1, 5′-ACAATTTCTGGGTCCCCTCCT-GAGGA (RF positions, 6890–6865); ES2, 5′-ATGGAAT-TAGGCCAGTAGTG (RF positions, 6527–6546); EA2, 5′-CTCCTAATTTTGTAACTAC (RF positions, 6829–6811). Specific precautions, including physical separation of processing areas, were taken to avoid template and amplified product carryover. Stringent quality control to prevent PCR contamination was employed to protect against cross-contamination of product DNA. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) and model 3700 automated DNA sequencer (Applied Biosystems). Amino acid sequences were deduced with the Genetyx-Win program version 4.1 (Software Development, Tokyo). For some PCR products, molecular cloning was performed with pT7 Blue T-Vector (Novagen, CA) and their sequences were analyzed.

For the phylogenetic analysis, RT gene and V3 sequences were aligned by Clustal-W program with reference sequences from the Los Alamos sequence database. The results of the alignment were then analyzed by the neighbor-joining method and the distance matrixes were generated with Kimura two-parameter model.

Bootstrap resampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree.

Construction of recombinant HIV-1 clones

Recombinant infectious HIV-1 clones carrying various mutations in RT were constructed with a site-directed mutagenesis. Briefly, the desired mutations were introduced into *Xma*I–*Nhe*I region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (Kodama et al., 2001). The *Xma*I–*Nhe*I fragment was inserted into pNL101-based plasmid (a kind gift from Jeang Kuan-The, NIAID/NIH, Bethesda, MD), generating various molecular clones with the desired mutations. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each molecular clone (10 μg/ml as DNA) was transfected into COS-7 cells (4×10^5 cells/100-mm-diameter dish) by Fugene transfection reagent (Roche Diagnosis, Basal, Switzerland). After 48 h, culture supernatants were harvested and stored at −80 °C until use. The infectivity of the viruses was determined as BFU in MAGIC-5 cells.

Drug susceptibility assay with MAGIC-5 cells

HIV-1 drug susceptibility to various RTIs was determined in triplicate by using MAGIC-5 cells as described previously (Hachiya et al., 2001). Briefly, MAGIC-5 cells were infected with adjusted virus stock (300 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (Takara Shuzo). The blue-stained cells were counted under a light microscope. Drug concentrations that inhibited 50% of the stained cells of drug-free control (IC_{50}) were determined by referring to the dose–response curve.

Viral replication kinetic assays

PHA-stimulated PBMCs were exposed to each infectious virus preparation (300 BFU) in a final volume of 200 μl for 2 h. Infected cells were then washed and cultured in a volume of 200 μl in the absence or presence of NVP (1 μM). Assays were performed in triplicate and repeated at least three times using independently generated virus preparations. On days 0, 2, 4, 6, and 8 of infection, 100-μl aliquots of culture supernatants were filtered and stocked for measurements of p24 antigen concentration and replaced with equal amount of fresh medium with or without NVP. The concentrations of p24 in the supernatants were determined by chemiluminescence enzyme immunoassay (CLEIA) kit (Fuji-Rebio, Tokyo, Japan).

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed to virus preparations (300 BFU) to be examined for their replication ability and cultured in the presence or absence of NVP as described previously (Gatanaga et al., 2002; Kosalaraksa et al., 1999; Yoshimura et al., 1999). On day 1 in culture, one-third of the infected H9 cells were harvested and washed twice with PBS, and cellular DNA was extracted with Smi-test nucleic acid extraction kit. Purified DNAs were subjected to nested PCR for sequencing HIV-1 RT gene. Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at each passage were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Structural analysis of mutations in HIV-1 RT

To examine the interaction of HIV-1 RT with NVP, the SYBYL 6.7 (http://www.tripos.com/, Tripos Associates, St. Louis, MO) on a Silicon Graphics Octane2 workstation was employed. The site of the enzyme ligand complex was constructed based on the previously reported X-ray structure of the covalently trapped catalytic complex of HIV-1 RT with NVP (Protein Data Bank entry 1FKP) (Ren et al., 2000).

Acknowledgments

The authors thank Yukiko Takahashi and Fujie Negishi for sample preparation. This study was supported in part by the Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001) and by the Organization of Pharmaceutical Safety and Research (01-4).

References

Briones, C., Perez-Olmeda, M., Rodriguez, C., del Romero, J., Hertogs, K., Soriano, V., 2001. Primary genotypic and phenotypic HIV-1 drug resistance in recent seroconverters in Madrid. J. Acquired Immune Defic. Syndr. 26, 145–150.

Cohen, J., 2003. Thailand's do-it-yourself therapy. Science 301, 1662.

Demeter, L.M., Meehan, P.M., Morse, G., Fischl, M.A., Para, M., Powderly, W., Leedom, J., Holden-Wiltse, J., Greisberger, C., Wood, K., Timpone Jr., J., Wathen, L.K., Nevin, T., Resnick, L., Batts, D.H., Reichman, R.C., 1998. Phase I study of atevirdine mesylaye (U-87201E) monotherapy in HIV-1-infected patients. J. Acquired Immune Defic. Syndr. 19, 135–144.

Demeter, L.M., Shafer, R.W., Meehan, P.M., Holden-Wiltse, J., Fischl, M.A., Freimuth, W.W., Para, M.F., Reichman, R.C., 2000. Delavirdine susceptibilities and associated reverse transcriptase mutations in human immunodeficiency virus type 1 isolates from patients in a phase I/II trial of delavirdine monotherapy (ACTG 260). Antimicrob. Agents Chemother. 44, 794–797.

Devereux, H.L., Youle, M., Johnson, M.A., Loveday, C., 1999. Rapid decline in detectability of HIV-1 drug resistance mutations after stopping therapy. AIDS 13, F123–F127.

Duwe, S., Brunn, M., Altmann, D., Hamouda, O., Schmidt, B., Walter, H., Pauli, G., Kucherer, C., 2001. Frequency of genotypic and phenotypic drug-resistant HIV-1 among therapy-naïve patients of German Seroconverter Study. J. Acquired Immune Defic. Syndr. 26, 266–273.

Gatanaga, H., Oka, S., Ida, S., Wakabayashi, T., Shioda, T., Iwamoto, A., 1999. Active HIV-1 redistribution and replication in the brain with HIV encephalitis. Arch. Virol. 144, 29–43.

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.

Grant, R.M., Hecht, F.M., Warmerdam, M., Liu, L., Liegler, T., Petropoulos, C.J., Hellmann, N.S., Chesney, M., Busch, M.P., Kahn, J.O., 2002. Time trends in primary HIV-1 drug resistance among recently infected persons. JAMA 288, 181–188.

Guay, L.A., Musoke, P., Fleming, T., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Cucar, C., Deseyve, M., Emel, L., Mirochnick, M., Fowler, M.G., Mofenson, L., Miotti, P., Dransfield, K., Bray, D., Mmiro, F., Jackson, J.B., 1999. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomized trial. Lancet 354, 795–802.

Hachiya, A., Aizawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gatanaga, H., Hirabayashi, Y., Kojima, A., Tatsumi, M., Oka, S., 2001. Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 by using CCR5-expressing HeLa/CD4$^+$ cell clone 1-10 (MAGIC-5). Antimicrob. Agents Chemother. 45, 495–501.

Hachiya, A., Matsuoka-Aizawa, S., Tsuchiya, K., Gatanaga, H., Kimura, S., Tatsumi, M., Oka, S., 2003. "All-in-One Assay", a direct phenotypic anti-human immunodeficiency virus type 1 drug resistance assay for three-drug combination therapies that takes into consideration in vivo drug concentrations. J. Virol. Methods 111, 43–53.

Hirsch, M.S., Brun-Vezinet, F., Clotet, B., Conway, B., Kuritzkes, D.R., D'Aquila, R.T., Demeter, L.M., Hammer, S.M., Johnson, V.A., Loveday, C., Mellors, J.W., Jacobsen, D.M., Richman, D.D., 2003. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 Recommendations of an international AIDS society-USA panel. Clin. Infect. Dis. 37, 113–128.

Huang, H., Chopra, R., Verdine, G.L., Harrison, S.C., 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science 282 (5394), 1669–1675.

Ida, S., Gatanaga, H., Shioda, T., Nagai, Y., Kobayashi, N., Shimada, K., Iwamoto, A., Oka, S., 1997. HIV type 1 V3 variation dynamics in vivo: long-term persistence of non-syncytium-inducing genotypes and transient presence of syncytium-inducing genotypes during the course of progressive AIDS. AIDS Res. Hum. Retroviruses 13, 1597–1609.

Johnson, V.A., Brun-Vezinet, F., Clotet, B., Conway, B., D'Aquila, R.T., Demeter, L.M., Kuritzkes, D.R., Pillay, D., Schapiro, J.M., Telenti, A., Richman, D.D., International AIDS Society-USA Drug Resistance Mutations Group, 2003. Drug resistance mutations in HIV-1. Top. HIV Med. 11, 215–221.

Kodama, E.I., Kohgo, S., Kitano, K., Machida, H., Gatanaga, H., Shigeta, S., Matsuoka, M., Ohrui, H., Mitsuya, H., 2001. 4′-ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. 45, 1539–1546.

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.

Little, S.J., Holte, S., Routy, J.P., Daar, E.S., Markowitz, M., Collier, A.C., Koup, R.A., Mellors, J.W., Connick, E., Conway, B., Kilby, M., Wang, L., Whitcomb, J.M., Hellmann, N.S., Richmann, D.D., 2002. Antiretroviral-drug resistance among patients recently infected with HIV. N. Engl. J. Med. 347, 385–394.

Marseille, E., Kahn, J.G., Mmiro, F., Guay, L., Musoke, P., Fowler, M.G., Jackson, J.B., 1999. Cost effectiveness of single-dose nevirapine regimen for mothers and babies to decrease vertical HIV-1 transmission in sub-Saharan Africa. Lancet 354, 803–809.

Miller, V., Sabin, C., Hertogs, K., Bloor, S., Martinez-Picado, J., D'Aquila, R., Larder, B., Lutz, T., Gute, P., Weidmann, E., Rabenau, H., Phillips, A., Staszewski, S., 2000. Virological and immunological effects of treatment interruptions in HIV-1 infected patients with treatment failure. AIDS 14, 2857–2867.

Morris, L., Pillay, C., Chezzi, C., Lupondwana, P., Ntsala, M., Levin, L., Venter, F., Martinson, N., Gray, G., McIntyre, J., 2003. Low frequency of the V106M mutation among HIV-1 subtype C-infected pregnant women exposed to nevirapine. AIDS 17, 1698–1700.

Parkin, N.T., Deeks, S.G., Wrin, M.T., Yap, J., Grant, R.M., Lee, K.H., Heeren, D., Hellmann, N.S., Petropoulos, C.J., 2000. Loss of antiretroviral drug susceptibility at low viral load during early virological failure in treatment-experienced patients. AIDS 14, 2877–2887.

Penzak, S.R., Acosta, E.P., Turner, M., Tavel, J.A., Masur, H., 2003. Analysis of generic nevirapine products in developing countries. JAMA 289, 2648–2649.

Ren, J., Milton, J., Weaver, K.L., Short, S.A., Stuart, D.I., Stmmers, D.K., 2000. Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. Structure Fold Des. 8, 1089–1094.

Salomon, H., Wainberg, M.A., Brenner, B., Quan, Y., Rouleau, D., Cote, P., LeBlanc, R., Lefebvre, E., Spira, B., Tsoukas, C., Sekaly, R.P., Conway, B., Mayers, D., Routy, J.P., 2000. Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec primary infection study. AIDS 14, F17–F23.

Sarafianos, S.G., Das, K., Ding, J., Boyer, P.L., Hughes, S.H., Arnold, E., 1999. Touching the heart of HIV-1 drug resistance: the fingers close down on the dNTP at the polymerase active site. Chem. Biol. 6, R137–R146.

Simon, V., Vanderhoeven, J., Hurley, A., Ramratnam, B., Louie, M., Dawson, K., Parkin, N., Boden, D., Markowitz, M., 2002. Evolving patterns of HIV-1 resistance to antiretroviral agents in newly infected individuals. AIDS 16, 1511–1519.

Sullivan, J.L., 2003. Prevention of mother-to-child transmission of HIV—What next? J. Acquired Immune Defic. Syndr. 34, S67–S72.

Verhofstede, C., Wanzeele, F.V., Van Der Gucht, B., De Cabooter, N., Plum, J., 1999. Interruption of reverse transcriptase inhibitors or a switch from reverse transcriptase to protease inhibitors resulted in a fast reappearance of virus strains with a reverse transcriptase inhibitor-sensitive genotype. AIDS 13, 2541–2546.

Yoshimura, K., Feldman, R., Kodama, E., Kavlick, M.F., Qiu, Y.L., Zemlicka, J., Mitsuya, H., 1999. In vitro induction of human immunodeficiency virus type 1 variants resistant to phosphoralaninate prodrugs of *z*-methylenecyclopropane nucleoside analogues. Antimicrob. Agents Chemother. 43, 2479–2483.

JOURNAL OF MOLECULAR RECOGNITION
J. Mol. Recognit. 2005; **18**: 169–174
Published online 8 October 2004 in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/jmr.725

RNase S complex bearing arginine-rich peptide and anti-HIV activity

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Basic peptide-mediated protein delivery into living cells is becoming recognized as a potent approach for the understanding of cellular mechanisms and drug delivery. We have prepared the conjugates of the S-peptide (1–15) derived from RNase S with membrane-permeable basic peptides, octaarginine and the human immunodeficient virus (HIV)-1 Rev (34–50). The RNase S complexes, formed among these S-peptide (1–15)–basic peptide conjugates and the S-protein and having a dissociation constant in the range of 10^{-5} M, efficiently penetrated into the HeLa cells. These RNase S complexes exerted an anti-HIV replication activity. The time-of-drug-addition assay suggested that the site of action for these complexes would reside in the stages between the viral entry into the cells and reverse transcription. The present study exemplified the applicability of the arginine-rich peptides to the intracellular targeting of non-covalent protein complexes and supramolecular assemblies for the research in chemical and cellular biology. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: **arginine-rich peptide; drug delivery; protein design; cell membrane; anti-HIV activity; protein transduction; HIV-1 Tat; HIV-1 Rev; RNase**

Received 21 June 2004; revised 30 July 2004; accepted 17 August 2004

INTRODUCTION

Basic peptide-mediated protein delivery into living cells has been emphasized as a novel and promising technology for the understanding and modulation of cellular events with therapeutic potential (for reviews see Futaki *et al.*, 2003; Snyder and Dowdy, 2004; Wright *et al.*, 2003; Vivès, 2003). Among the peptides having such an activity is the arginine-rich peptide derived from HIV-1 Tat (positions 48–60; Vivès *et al.*, 1997); HIV-Rev (34–50) and octaarginine (R_8) peptides show an equivalent ability (Futaki *et al.*, 2001a, 2003; Suzuki *et al.*, 2002; Wright *et al.*, 2003). Although these peptides are highly hydrophilic, they easily translocate through cell membranes. By chemical conjugation or genetic fusion with the Tat segment, many proteins, as well as oligonucleotides, chelating molecules and magnetic beads, successfully entered cells while retaining their biological activity.

On the other hand, there are many non-covalent protein complexes or supramolecular assemblies that have great potential for intracellular applications. Although delivery of the Tat-biotin–avidin complex has been reported (Lee and Pardridge, 2001), the binding is quite strong and substantially irreversible. It has never been demonstrated whether a protein assembly with a dissociation constant in the range 10^{-5}–10^{-6} M is able to cross the membrane with retention of its structure.

In this report, we clearly show that such a non-covalent protein assembly was successfully introduced into cells using the RNase S bearing an arginine-rich segment as a model. We also describe how these protein complexes exerted anti-HIV activity. These internalized RNase S complexes retained their non-covalent assembly structure, suggesting minimal structural change in protein structure or unfolding is required for basic-peptide mediated protein translocation into cells.

METHODS

Preparation of arginine peptide–S-peptide conjugates

Preparation of the Rev-S peptide conjugate has already been reported (Futaki *et al.*, 2001b). R_8–S peptide conjugate was prepared similarly to the Rev-S peptide. Briefly, N-terminal thioglycolated basic peptide segments and the N-terminal

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Contract/grant sponsor: Japan Science and Technology Agency.

Abbreviations used: AZT, 3′-azido-3′-deoxythymidine; DS, dextran sulfate; EC_{50}, 50% effective concentration; EC_{90}, 90% effective concentration; ESIMS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HIV, human immunodeficient virus; LTR, long terminal repeat; MAGI, multinuclear activation of the galactosidase indicator; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; α-MEM, alpha-minimum essential medium; PBS, phosphate buffered saline; RRE, Rev response element; TAR, *trans*-activation responsive region; Tris, tris(hydroxymethyl)aminomethane.

chloroacetylated S-peptide (1–15) segment were prepared by Fmoc-solid-phase peptide synthesis followed by conjugation with each other by reacting these segments overnight in 6 M guanidine HCl–0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0). The fidelity of the peptides was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) [calculated for $(M+H)^+$, 3111.6; found, 3111.5].

Rhodamine labeling

Rhodamine labeling of the S-protein was conducted by incubating the S-protein (Sigma; 1 mg) with tetramethylrhodamine succinimidyl ester (Molecular Probes) (45 μg, 1 eq.) in PBS (100 μl) at room temperature for 1 h. The mixture was applied to a Sephadex G-25 column (1 × 5 cm) and eluted with 1 M acetic acid. The first major peak was collected and lyophilized to yield 0.7 mg of a mixture of the non-labeled, mono-labeled, and di-labeled S-proteins at a ratio of 7:2.5:1. These ratios were estimated from the peak areas of the electrospray ionization mass spectrometry (ESIMS) performed on an Applied Biosystems API-3000. R_8 peptide (NH_2-R_8-$CONH_2$) was similarly labeled with tetramethylrhodamine and purified by HPLC.

Preparation of the leucine zipper peptide derived from GCN4 and its conjugates

Peptide **4** was constructed with Fmoc-solid-phase peptide synthesis, followed by treatment with trifluoroacetic acid-ethanedithiol (95:5) and HPLC purification [MALDI-TOFMS: calculated for $(M+H)^+$, 4081.7; found, 4081.7]. Peptide **4** was labeled with 5-maleimidofluorescein diacetate (Sigma) to give peptide **5** as reported (Futaki *et al.*, 2001a) [MALDI-TOFMS: calculated for $(M+H)^+$, 4593.1; found, 4593.0]. Conjugate **6** was prepared by cross-linking the peptide **4** with the R_8 peptide (NH_2-R_8-$CONH_2$), using *N*-(4-maleimidobutyryloxy)succinimide ester as reported (Futaki *et al.*, 2001a) [MALDI-TOFMS: calculated for $(M+H)^+$, 5513.4; found, 5513.5].

Cell culture

Human cervical cancer-derived HeLa cells were maintained in alpha-minimum essential medium (α-MEM) (Invitrogen) with 10% heat-inactivated calf serum (Invitrogen). Cells were grown on 60 mm dishes and incubated at 37 °C under 5% CO_2 to approximately 70% confluence. A sub-culture was performed every 3–4 days.

Protein internalization and microscopic observation

For each assay, 2×10^5 cells were plated on 35 mm glass-bottomed dishes (Iwaki) and cultured for 48 h. After complete adhesion, the culture medium was changed. Prior to incubating with cells, the S-peptide–arginine peptide conjugate was mixed with the tetramethylrhodamine-labeled S-protein in a molar ratio of 2:1 and allowed to stand at room temperature for 10 min. The stipulated protein concentration was based on that of the added S-protein. The cells were then incubated at 37 °C for 1 h with the fresh medium (200 μl) containing the protein complexes (10 μM). For comparison, cells were treated with tetramethylrhodamine-labeled R_8 peptide (1 μM) at 37 °C for 1 h. Cells were then washed five times with phosphate-buffered saline (PBS). Distribution of the fluorescence-labeled peptides was analyzed using a confocal scanning laser microscope LSM 510 (Zeiss) equipped with a × 40 lens without fixing cells.

Flow cytometry

To analyze the internalization of the RNase S complexes **1** and **3** by FACS, 1.5×10^5 HeLa cells in a fresh culture medium (1.5 ml) were plated on a 12-well microplate (Iwaki) and cultured for 48 h. After complete adhesion, the culture medium was exchanged (with α-MEM containing 10% heat-inactivated calf serum) and the cells were incubated with S-peptide–R_8 peptide conjugate or the S-peptide (1–15) mixed with tetramethylrhodamine-labeled S-protein in a molar ratio of 2:1 and allowed to stand for 10 min. The protein concentration (10 μM) was based on that of the added S-protein. The 1 mol of the S protein was found to be labeled with 0.43 mol of tetramethylrhodamine by ESIMS. Tetramethylrhodamine-labeled R_8 peptide (4.3 μM) was therefore used for the comparison of the internalization efficiency. The cells were incubated at 37 °C for 1 h with fresh medium (400 μl) containing the protein complexes prior to washing for 2 × 3 min with PBS. The cells were treated with 0.01% trypsin (Invitrogen, 400 μl) at 37 °C for 10 min prior to adding 600 μl of PBS. The cells were centrifuged at 2000 rpm for 5 min and, after removing the supernatant, they were washed with 1 ml of PBS and centrifuged at 2000 rpm for 5 min. After repeating this washing cycle, the cells were suspended in 1.5 ml of fresh culture medium and subjected to fluorescence analysis using flow cytometry. This was performed with a FACScalibur (BD Biosciences) flow cytometer using 488 nm laser excitation and a 564–606 nm emission filter. Usually tetramethylrhodamine is not used for FACS analysis because of its low excitability at 488 nm, but the dye was used here to analyze the internalization efficiency under the condition as close as that used for microscopic observation.

MAGI assay

The assay was conducted with some modification using the viral preparation titrated as previously reported (Kodama *et al.*, 2001). Briefly, target cells (HeLa CD4-LTR/β-gal; 10^4 cells/well), which both express high levels of CD4 and contain a single integrated copy of a β-galactosidase gene under the control of a truncated HIV-1 long terminal repeat (LTR), were plated in 96-well flat microtitier culture plates. On the following day, the medium was aspirated, and the cells were infected with HIV-1 (III_B) (70 MAGI units/well) and cultured in the presence of various concentrations of the protein complexes in fresh medium. Forty-eight hours after

viral exposure, all the blue cells in each well were counted. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Design of RNase S complexes bearing arginine-rich segments

RNase S is a protein complex comprising a non-covalent assembly of two segments, the S-peptide (positions 1–20 of bovine pancreatic RNase A) and the S-protein (positions 21–124). These respective segments do not retain the RNase activity by themselves, but when mixed together they fold into a complex and exert RNase activity. Interestingly, the S-peptide and the S-protein form an active structure even when the S-peptide is conjugated with other exogenous proteins (Karpeisky *et al.*, 1994).

The design of RNase S bearing an arginine-rich peptide is illustrated in Fig. 1(A). Octaarginine (R_8), and Rev (34–50) were employed as membrane-permeable peptides (Futaki *et al.*, 2001a). These peptides were conjugated with the

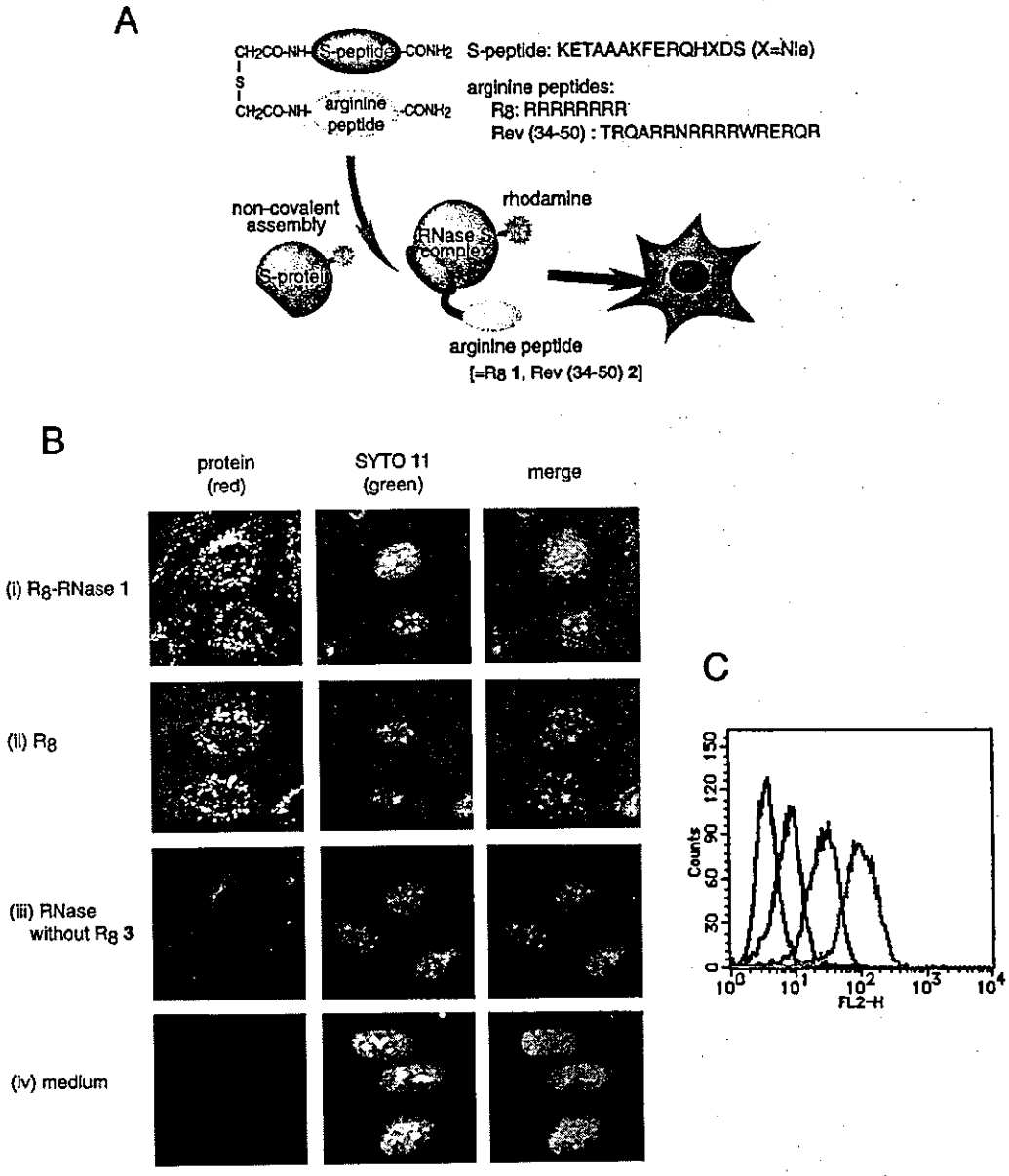

Figure 1. (A) The schematic representation of RNase S complexes bearing arginine-rich segments, and (B) confocal laser microscopic observations of HeLa cells treated at 37 °C for 1 h with the rhodamine-labeled R_8 peptide (1 μM), RNase S bearing the R_8 peptide **1** (10 μM), and RNase S without an arginine-rich segment **3** [the complex of the S protein with S-peptide (1–15) (NH_2-KETAAAKFERQHXDS-$CONH_2$, X = norleucine), 10 μM], respectively. Note that the rhodamine was attached only on the S-protein in the case of RNase complexes. Nucleus was stained with SYTO (no. 11; 5 μM). (C) FACS analysis of internalization efficiency of RNase S complexes **1** and **3**. HeLa cells were treated with RNase S complexes bearing R_8 peptide (**1**, red), RNase S complex without R_8 peptide (**3**, blue) and R_8 peptide (orange), respectively, as described in the methods. Control cells (untreated) are shown in black.

S-peptide (1–15), the segment of which sufficiently forms a complex with the S-protein without loss of the RNase activity (Karpeisky *et al.*, 2004). The S-protein was labeled with the tetramethylrhodamine for microscopic observation.

Internalization of the RNase S complexes bearing arginine-rich peptides was assessed in HeLa cells. Figure 1(B) shows confocal microscopic observation after incubation of the protein complex (10 μM) with HeLa cells in the presence of medium at 37 °C for 1 h. Internalization of the complex was clearly observed in almost all the cells treated with RNase S bearing the respective arginine-rich peptides and resulted in intracellular distributions similar to those of the R_8 peptide [Fig. 1(B) (i) and (ii)]. On the other hand, much less efficient internalization was observed for RNase S without the arginine-rich peptides [a complex of the S-protein and S-peptide (1–15), NH_2-KETAAAKFERQH XDS-$CONH_2$ (X = norleucine, Nle)] **3** [Fig. 1(B) (iii)]. Note that the fluorescent probe was attached only on the S-protein, suggesting that the S-protein was internalized with retention of its complex structure. FACS analysis showed that, by conjugation with R_8 peptide, the internalization efficiency of the RNase complex **1** was almost five times as much as that for the RNase complex without having an arginine segment **3**. On the other hand, that for complex **1** was approximately 25% of that for the R_8 peptide [Fig. 1(C)]. When the cells were treated with a 1:1 mixture of the S-peptide–R_8 conjugate and the S-protein, the internalization efficiency was about 80% of that of the 2:1 mixture described above. Co-incubation of the complex **3** with two equivalents of R_8 peptide (NH_2-R_8-$CONH_2$, 20 μM) gave no increase in the cellular uptake of **3** (data not shown).

Recently, internalization of HIV-1 Tat (48–60) has become interpreted as being mediated by an endocytic process (Richard *et al.*, 2003; Vivès, 2003). Although detailed mechanisms of Tat entry awaits clarification, translocation of the peptides from endocytic vesicular compartments to the cytosol would be necessary for the delivered molecules to work in living cells.

Anti-HIV replication activity of the RNase S complexes

The Rev segment was originally identified as an RNA-binding peptide which recognizes a specific structure of the RNA derived from HIV (Tan and Frankel, 1995). Moreover, we have previously shown that the RNase S bearing the Rev peptide cleaved an RNA corresponding to the Rev binding site (the Rev response element, RRE; Futaki *et al.*, 2001b). It has also been reported that a peptide composed of nine residues of arginine (R_9) also binds to the *trans*-activation responsive region (TAR) of HIV-1 RNA (Calnan *et al.*, 1991). Therefore, it may be possible that the R_8 segment could bind to the TAR or other specific sites of the viral RNA. These facts motivated us to examine the anti-HIV activity of the RNase S bearing these arginine-rich segments. The activity of RNase S complexes bearing the R_8 and Rev peptides, represented as the concentration that blocks HIV-1 replication by 50% (EC_{50}), was 0.67 and 1.7 μM, respectively [Fig. 2(A)], determined by the multinuclear activation of the galactosidase indicator (MAGI) assay (Kodama *et al.*, 2001). In this assay, HIV-1 infection in HeLa CD4/LTR-β-gal cells leads to the activation of

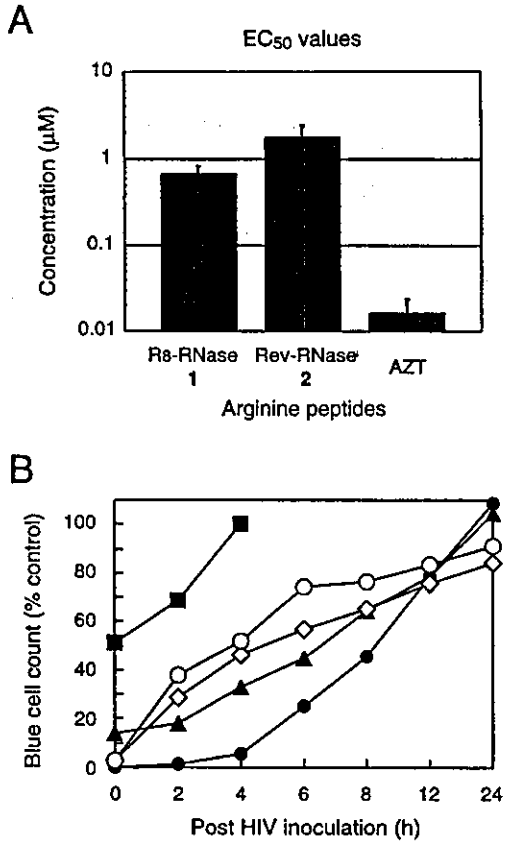

Figure 2. (A) Anti-HIV replication activity of RNase S complexes bearing arginine-rich peptides **1** and **2** in MAGI assay (Kodama *et al.*, 2001). HIV-1 infection in HeLa CD4/LTR-β-gal cells leads to the activation of β-galactosidase gene, which is visualized through the formation of blue cells in 48 h of culture. The anti-HIV activity is demonstrated by the decrease in the number of blue cells. EC_{50} of RNase S without having an arginine segment **3** was judged to be >100 μM. (B) Anti-HIV activity of RNase S complexes **1** and **2** in time-of-drug-addition assays. The RNase S complexes were added to the MAGI (HeLa CD4/LTR-β-gal) cells at the indicated points after viral inoculation, and blue cells produced were counted at the completion of the 48 h period of incubation. Protein complexes at the concentration corresponding to the EC_{90} (MAGI assay) were employed: RNase S complex bearing R_8 **1** (open circle, 7 μM) and Rev **2** (open diamond, 10 μM), respectively. DS (solid square, 200 ng/ml), AZT (solid triangle, 500 nM), MKC442 (solid circle, 500 nM).

β-galactosidase gene, which is visualized through the formation of blue cells after 48 h of culture. The anti-HIV activity is demonstrated by the decrease in the number of blue cells. Although the activity of the RNases was lower than that of 3′-azido-3′-deoxythymidine (AZT; EC_{50} value 0.016 μM), these RNase complexes actually exerted anti-HIV activity. These facts clearly indicated that these intracellular RNase S complexes retained their active structure. On the other hand, the EC_{50} of the RNase complex without the arginine segment **3** was >100 μM.

An oligoarginine peptide, acetyl-NH-(D-Arg)$_9$-$CONH_2$ (ALX40-4C) has been reported to inhibit interaction of HIV-1 gp120 and CXCR4, which is used as a co-receptor for T-tropic HIV, and the eventual viral entry to the cells (Doranz *et al.*, 1997). We have confirmed that the R_8 peptide also has a similar activity to prevent the viral entry to the cells using syncytium formation assay (Mitsuya *et al.*, 1998;

data not shown). In order to elucidate the interaction stage of these RNase S complexes with the HIV replicative cycle, a time-of-drug-addition experiment (De Clercq *et al.*, 1992) was conducted [Fig. 2(B)]. HeLa CD4-LTR/β-gal cells were infected with HIV-1 (III_B) (70 MAGI units/well) at time zero. The test protein complexes at concentrations that block the HIV-1 replication by 90% (EC_{90}; obtained from MAGI assay) were then added at the indicated time points (0, 2, 4, 6, 8, 12 or 24 h). Blue cells produced were counted at the completion of the 48 h period of incubation. If complexes were added before the viral replication cycle reached their target replication stage, they were effective without significant loss of activity. In comparison of the delay time for the sample with that for the drug of known mechanism, the site of action could be estimated [Fig. 2(B)]: dextran sulfate (DS) (MW 5000) inhibited the adsorption of HIV on the cell surface, which showed activity only when it was added early after viral exposure. MKC442 is a non-nucleoside reverse transcriptase inhibitor, which directly binds and inhibits reverse transcriptase, and the addition was effective up to ~4 h after viral inoculation. AZT is a nucleoside reverse transcriptase inhibitor. Activation of the drug requires its phosphorylation in the cells. Thus, addition of AZT was effective up to ~2 h after viral inoculation.

The effect of RNase S bearing these arginine-rich peptides decreased more slowly than that of DS and faster than MKC442. The result suggested that the action stage of these RNase S complexes is not the adsorption of the virus on the cell surface as in the case of R_8 peptide, but should reside after this stage and before the start of reverse transcription. Thus, the RNase complexes showed anti-HIV activity only while the virus stayed in the RNA form. Considering the difference in translocation efficiency (~5 fold) and EC_{50} values (>100 fold) between the RNase complexes with and without arginine segments, it would be possible that the arginine segments not only accelerated the internalization of the RNase complexes but also increased their affinities to HIV RNA.

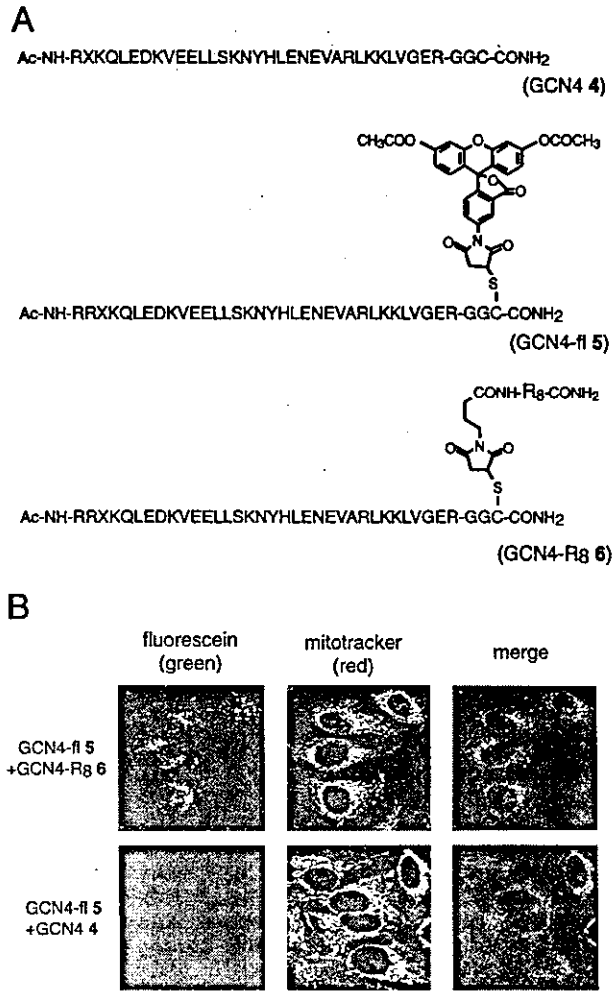

Figure 3. (A) Structures of GCN4 leucine–zipper segments bearing fluorescein (5) and R_8 (6) moieties (X = norleucine). (B) Internalization of GCN4 leucine zipper complex into HeLa cells. The cells were incubated with the peptide mixture for 3 h and subjected to confocal microscopic observation. MitoTracker (Molecular Probes; 0.5 μM) was used to outline the shapes of the cells.

General applicability of this approach to the delivery of other non-covalent protein complexes

The dissociation constant of the RNase S bearing the R_8 segment was determined to be 1.8×10^{-5} M using the procedure of Woodfin and Massey (1968). This value is very close to that for RNase S without the arginine peptide (2.5×10^{-5} M), suggesting that conjugation with the arginine-peptides had little effect on the stability of the RNase complexes. This result indicates that even a non-covalent complex with a dissociation constant in this range can go through the membranes while retaining its structure. Similar results were obtained from the study using a leucine-zipper segment of yeast transcription factor GCN4. This leucine zipper peptide has been well characterized to form a homodimer with a dissociation constant of 4.7×10^{-7} M (Wendt *et al.*, 1994).

For the assessment of internalization of the leucine–zipper complex, the peptide segment 4 shown in Fig. 3(A) was synthesized. The peptide design was based on GCN4-p1C peptide, which has already been shown to form a stable homodimer (O'Shea *et al.*, 1989). The C-terminus cysteine of peptide 4 was labeled with 5-maleimidofluorescein diacetate, which fluoresces after hydrolysis of the acetyl moieties by cellular esterases (Guilbault and Kramer, 1964), to give peptide 5. Alternatively, peptide 4 was cross-linked with the R_8 peptide (NH_2-R_8-$CONH_2$) using *N*-(4-maleimidobutyryloxy)succinimide ester to give conjugate 6.

Internalization of the GCN4 leucine–zipper peptides with the aid of R_8 was observed by confocal microscopy after treating HeLa cells with a mixture of 5 and 6 (20 μM each) at 37 °C for 1 h [Fig. 3(B)]. Significant internalization was not observed for the cells treated with a mixture of peptides 4 and 5 (20 μM each). In the above experiments, the homodimer of each peptide segment should also be formed. However, because the efficiency of internalization for the homodimer of 5 was estimated to be very low by the latter experiments, it was judged that only the complex of segments 5 and 6 was observable in the cells. These results clearly showed that the fluorescein-labeled GCN4 peptide

was efficiently brought into the cells with the help of the GCN4-R_8 peptide.

CONCLUSIONS

In this report, we have demonstrated that non-covalent protein complexes were efficiently incorporated into the cells while retaining their active structure. The results suggested that many supramolecules having dissociation constants in this range can be introduced into cells using this strategy. This opens new technological avenues for studies of mechanism and control of cellular functions.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors thank Dr A.T. Jones, Cardiff University for reading the manuscript.

REFERENCES

Calnan BJ, Tidor B, Biancalana S, Hudson D, Frankel AD. 1991. Arginine-mediated RNA recognition: the arginine fork. *Science* **252**: 1167–1171.

De Clercq E, Yamamoto N, Pauwels R, Baba M, Schols D, Nakashima H, Balzarini J, Debyser Z, Murrer BA, Schwartz D, Thornton D, Bridger G, Fricker S, Henson G, Abrams M, Piker D. 1992. Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc. Natl Acad. Sci. USA* **89**: 5286–5290.

Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, Daar ES, Doms RW, O'Brien WA. 1997. A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J. Exp. Med.* **186**: 1395–1400.

Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y. 2001a. Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* **276**: 5836–5840.

Futaki S, Araki M, Kiwada T, Nakase I, Sugiura Y. 2001b. A 'cassette' RNase: site-selective cleavage of RNA by RNase S equipped with RNA-recognition segment. *Bioorg. Med. Chem. Lett.* **11**: 1165–1168.

Futaki S, Goto S, Sugiura Y. 2003. Membrane permeability commonly shared among arginine-rich peptides. *J. Mol. Recognit.* **16**: 260–264.

Guilbault GG, Kramer DN. 1964. Fluorometric determination of lipase, acylase, α-, and γ-chymotrypsin and inhibitors of these enzymes. *Anal. Chem.* **36**: 409–412.

Karpeisky MYa, Senchenko VN, Dianova MV, Kanevsky VYu. 1994. Formation and properties of S-protein complex with S-peptide-containing fusion protein. *FEBS Lett.* **339**: 209–212.

Kodama E-I, Kohgo S, Kitano K, Machida H, Gatanaga H, Shigeta S, Matsuoka M, Ohrui H, Mitsuya H. 2001. 4'-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants *in vitro*. *Antimicrob. Agents Chemother.* **45**: 1539–1546.

Lee HJ, Pardridge WM. 2001. Pharmacokinetics and delivery of tat and tat-protein conjugates to tissues *in vivo*. *Bioconjug. Chem.* **12**: 995–999.

Mitsuya H, Looney DJ, Kuno S, Ueno R, Wong-Staal F, Broder S. 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to $CD4^+$ cells. *Science* **240**: 646–649.

O'Shea EK, Rutkowski R, Kim PS. 1989. Evidence that the leucine zipper is a coiled coil. *Science* **243**: 538–542.

Richard JP, Melikov K, Vivès E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. 2003. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **278**: 585–590.

Snyder EL, Dowdy SF. 2004. Cell penetrating peptides in drug delivery. *Pharm. Res.* **21**: 389–393.

Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, Sugiura Y. 2002. Possible existence of common internalization mechanisms among arginine-rich peptides. *J. Biol. Chem.* **277**: 2437–2443.

Tan R, Frankel AD. 1995. Structural variety of arginine-rich RNA-binding peptides. *Proc. Natl Acad. Sci. USA* **92**: 5282–5286.

Vivès E. 2003. Cellular uptake of the Tat peptide: an endocytosis mechanism following ionic interactions. *J. Mol. Recognit.* **16**: 265–271.

Vivès E, Brodin P, Lebleu B. 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **272**: 16010–16017.

Wendt H, Baici A, Bosshard HR. 1994. Mechanism of assembly of a leucine zipper domain. *J. Am. Chem. Soc.* **116**: 6973–6974.

Woodfin BM, Massey V. 1968. Spectrophotometric determination of the dissociation constant of ribonuclease S'. *J. Biol. Chem.* **243**: 889–892.

Wright LR, Rothbard JB, Wender PA. 2003. Guanidinium rich peptide transporters and drug delivery. *Curr. Protein Pept. Sci.* **4**: 105–124.