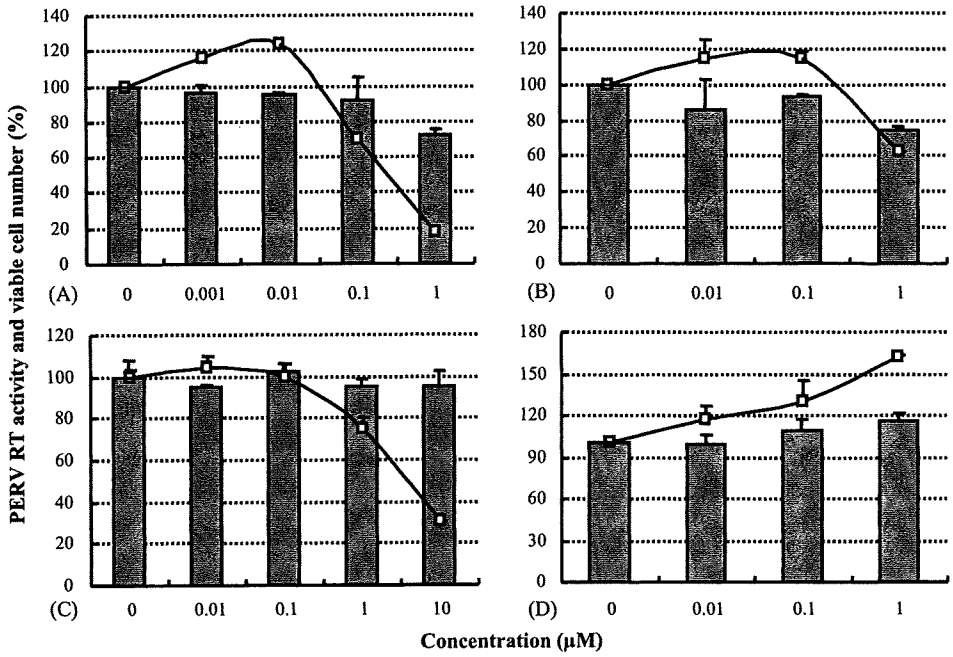

Fig. 2. Inhibitory effects of the test compounds on PERV replication in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation period, the culture supernatants were collected and mixed with 22% (w/v) polyethylene glycol 6000 solution for 5 h. PERV particles were harvested by centrifugation of the mixture. The viral pellets were resuspended in lysis buffer and subjected to RT assay (lines). The viable cell number was determined by a tetrazolium dye cell proliferation assay (bars). Both the RT activity and cell proliferation assays were performed in duplicate. The data represent means plus ranges. Representative results for two independent experiments are shown.

bition of Tat functions. Furthermore, K-37 was reported to inhibit the gene expression of human T-lymphotropic virus type 1 (HTLV-1) in persistently infected cells (Wang et al., 2002a). Although the target molecule of K-37 still remains to be determined, the present observations for PERV suggest that K-37 may interact with a cellular factor or factors that play an important role in retroviral gene expression. It is assumed that K-37 inhibits an early stage of transcriptional elongation of viral RNA (Okamoto et al., unpublished observations). EM2487 is a substance produced from a *Streptomyces* species and a potent and selective inhibitor of HIV-1 replication in acutely and chronically infected cells (Baba et al., 1999). Like K-37, EM2487 could inhibit HTLV-1 gene expression without affecting host cellular functions (Wang et al., 2002b). The chemical structures of K-37 and EM2487 are totally different from each other

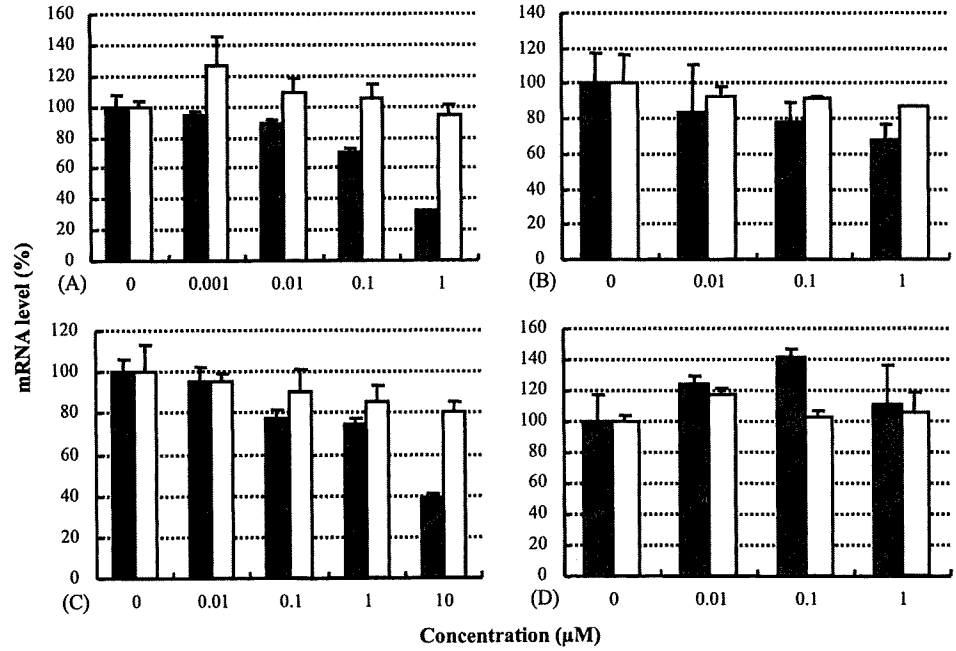

Fig. 3. Inhibitory effect of the test compounds on PERV mRNA synthesis in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation, the cells were collected, and total RNA was extracted. Quantitative real-time RT-PCR was performed to determine the amount of PERV mRNA in PK15 cells using a primer pair and probe specific to the PERV *pol* gene (gray columns). The inhibitory effect of the test compounds on host cellular mRNA synthesis was determined by quantitative RT-PCR for 18S mRNA (white columns). All experiments were performed in triplicate. The data represent means plus standard deviations. Representative results for two independent experiments are shown.

Table 1
Inhibitory effect of test compounds on PERV antigen production and mRNA synthesis in PK15 cells[a].

Compounds	PERV[b]			HIV-1[c]		HTLV-1[d]	
	EC_{50} (μM)	IC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
K-37	0.35 ± 0.04	0.34 ± 0.05	4.63 ± 1.62	0.033 ± 0.012	2.1 ± 0.3	0.44 ± 0.13	5.7 ± 1.0
EM2487	5.44 ± 1.40	4.36 ± 0.30	>10	0.075 ± 0.032	12.5 ± 4.1	3.6 ± 0.6	30.6 ± 3.5
JTK-101	>1	>1	2.18 ± 0.63	0.0014 ± 0.0005	3.8 ± 0.2	N.D.[e]	N.D.
Cepharanthine	>1	>1	4.39 ± 1.99	0.028 ± 0.016	1.3 ± 0.3	>3.0	3.0 ± 0.4

[a] Each experiment was carried out in duplicate or triplicate, and all data represent means ± ranges for two independent experiments.

[b] EC_{50}: 50% effective concentration based on the inhibition of PERV antigen production (RT) in culture supernatants of PK15 cells. IC_{50}: 50% inhibitory concentration based on the inhibition of PERV mRNA synthesis. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation.

[c] EC_{50}: 50% effective concentration based on the inhibition of HIV-1 antigen production (p24) in chronically infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2007) for K-37 and JTK-101, Baba et al. (1999) for EM2487, and Baba et al. (2001) for cepharanthine.

[d] EC_{50}: 50% effective concentration based on the inhibition of HTLV-1 antigen production (p19) in infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2002a) for K-37 and cepharanthine and Wang et al. (2002b) for EM2487.

[e] Not determined.

(Fig. 1), nevertheless they appear to share some common properties in antiretroviral activity and mechanism of action.

JTK-101 is a novel naphthalene derivative that inhibits HIV-1 replication in cell cultures (Wang et al., 2007). This compound was found to be highly active against HIV-1 in chronically infected cells but much less active in acutely infected cells. Studies of its mechanism of action suggested that JTK-101 exerted its anti-HIV-1 activity through the inhibition of CDK9/cyclin T1. Cepharantine is a plant alkaloid that has been shown to inhibit HIV-1 replication in a certain chronically infected cell line at low concentrations through the inhibition of NF-κB (Okamoto et al., 1998). Cepharanthine could also suppress stimulation-induced production of proinflammatory cytokines in human macrophages (Okamoto et al., 2001). This compound did not inhibit PERV replication in PK15 cells or even slightly enhanced it at the highest concentration tested (Fig. 2D).

The viral gene expression inhibitors K-37 and EM2487 may be able to keep PERV silent in porcine organs thereby reducing the risk of PERV transmission to recipients, which is never attainable with RT inhibitors. On the other hand, RT inhibitors are capable of inhibiting De Novo infection of recipients with PERV derived from porcine organs. Thus, an ideal strategy to prevent PERV transmission to organ recipients may be the combined treatment with an RT inhibitor for recipients and a gene expression inhibitor for donor organs. Unfortunately, the current gene expression inhibitors, such as K-37 or EM2487, may be toxic to human recipients at concentrations that completely suppress PERV production from donor cells or organs. Therefore, the optimization of their chemical structures would be required for the inhibition of PERV replication in vivo without generating serious side effects. Although the risk of PERV transmission upon xenotransplantation is supposed to be lower than initially thought, optimized retroviral gene expression inhibitors may be worth further pursuing for their potential efficacy in the clinical setting.

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Design of Peptide-based Inhibitors for Human Immunodeficiency Virus Type 1 Strains Resistant to T-20*[S]

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Enfuvirtide (T-20) is a fusion inhibitor that suppresses replication of human immunodeficiency virus (HIV) variants with multi-drug resistance to reverse transcriptase and protease inhibitors. It is a peptide derived from the C-terminal heptad repeat (C-HR) of HIV-1 gp41, and it prevents interactions between the C-HR and the N-terminal HR (N-HR) of gp41, thus interfering with conformational changes that are required for viral fusion. However, prolonged therapies with T-20 result in the emergence of T-20-resistant strains that contain primary mutations such as N43D in the N-HR of gp41 (where T-20 and C-HR bind) that help the virus escape at a fitness cost. Such variants often go on to acquire a secondary mutation, S138A, in the C-HR of gp41 region that corresponds to the sequence of T-20. We demonstrate here that the role of S138A is to compensate for the impaired fusion kinetics of HIV-1s carrying primary mutations that abrogate binding of T-20. To preempt this escape strategy, we designed a modified T-20 variant containing the S138A substitution and showed that it is a potent inhibitor of both T-20-sensitive and T-20-resistant viruses. Circular dichroism analysis revealed that the S138A provided increased stability of the 6-helix bundle. We validated our approach on another fusion inhibitor, C34. In this case, we designed a variant of C34 with the secondary escape mutation N126K and showed that it can effectively inhibit replication of C34-resistant HIV-1. These results prove that it is possible to design improved peptide-based fusion inhibitors that are efficient against a major mechanism of drug resistance.

HIV-1[2] entry into the target cells is mediated by two envelope glycoproteins, gp120 and gp41, that form a trimeric gp120·gp41 complex. After binding of gp120 to the CD4 receptor and CCR5 (or CXCR4) coreceptor on the surface of the target cell, the gp41 trimer forms an extended conformation of the three helices that allows a hydrophobic fusion peptide to be inserted into the target cell membrane, generating an intermediate that is anchored to both cellular and viral membranes. After this step, the gp41 is believed to start refolding to a more stable 6-helix bundle composed of the α-helical trimer of the N-terminal heptad repeat (N-HR) folded into an anti-parallel conformation with the three C-terminal heptad repeats (C-HR) (1, 2). This refolding brings the viral and cellular membranes together to catalyze fusion.

The transition of the extended intermediate to the 6-helix bundle can be inhibited by the addition of exogenous peptides derived from gp41 C-HR (Fig. 1*A*) that prevent the formation of the 6-helix bundle and inhibit the HIV-1 fusion with the target cells (3–6). T-20, a 36-amino acid peptide derived from C-HR, effectively suppresses *in vivo* replication of HIV-1 resistant to inhibitors of reverse transcriptase and protease (7, 8). However, HIV-1 variants resistant to T-20 have recently emerged carrying primary mutations in the Leu-33–Leu-45 region of the N-HR domain (9–15). Among them, V38A and N43D seem to be major primary mutations for T-20 resistance. Meanwhile, a secondary mutation at the C-HR region (S138A) has been reported to enhance T-20 resistance with an as yet undefined mechanism (9, 14, 15) (Fig. 1*B*).

The mechanism of resistance to C34, another C-HR peptide-based inhibitor of HIV fusion, has been the subject of multiple studies (13, 16). Because of a 22-amino acid overlap between the T-20 and C34 peptides (Fig. 1*B*), HIV-1 has developed primary mutations for C34 resistance *in vitro* at the identical Leu-33–Leu-45 region of the peptides. During *in vitro* selection of C34 resistance, we identified a mutation in the C-HR domain, N126K, that is also observed in some T-20-resistant clinical variants (10, 15, 17). We showed that N126K conferred resistance to C34 by compensating for the impaired intra-gp41 inter-

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[2] The abbreviations used are: HIV, human immunodeficiency virus; T-20, enfuvirtide; HR, heptad repeat; MAGI, multinuclear activation of galactosidase indicator; EC_{50}, 50% effective concentration; T_m, melting temperature; CD, circular dichroism; shRNA, short hairpin RNA; WT, wild-type.

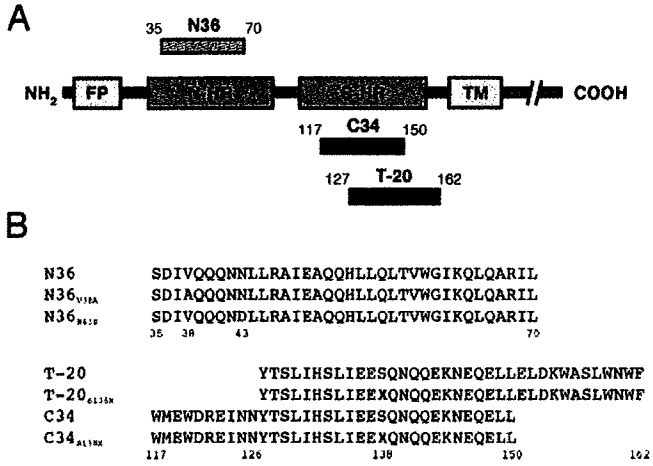

FIGURE 1. **Schematic view of gp41 and peptide sequence.** *A*, structure of HIV-1 gp41 and locations of N-HR or C-HR peptides (*FP*, fusion peptide; *TM*, transmembrane domain). *B*, amino acid sequences of peptides used in this study. Only the amino acid located at Ser-138 was substituted with all physiological amino acids (*X*), as Asn-126 lies outside of the amino acid sequence of T-20.

action by a primary mutation, I37K (13). N126K was initially identified in background of V38A, another primary mutation, for T-20 resistance *in vivo* (17). Baldwin *et al.* (17, 18) demonstrated a striking T-20-dependent replication phenotype in the V38A/N126K variant and proposed that T-20 acts as a safety pin to prevent premature formation of helical bundle, as N126K enhanced binding capacity of the introduced C-HR to N36 with V38A. Taken together, these studies suggest that mutations in the C-HR serve as secondary mutations.

In this study we show that the main role of secondary mutations that follow the appearance of primary mutations during treatment with peptide-based fusion inhibitors is to compensate for the impairment in replication kinetics that is caused by the primary mutations (supplemental Fig. 1). Based on this finding we hypothesized that analogs of T-20 carrying substitutions corresponding to secondary T-20 resistance mutations should be active against both wild-type and T-20-resistant viruses containing primary mutations. Indeed, our results confirmed our hypothesis and showed that T-20 with the S138A substitution ($T\text{-}20_{S138A}$) has a strong anti-HIV-1 activity even against T-20-resistant clones. Moreover, we demonstrate that this restoration is concomitant to improved binding of $C\text{-}HR_{S138A}$ to $N\text{-}HR_{N43D}$, suggesting that our approach utilizing the resistance-associated mutations to design peptides may provide useful broad insights into effective peptide-based therapies.

EXPERIMENTAL PROCEDURES

Cells and Viruses—MT-2 cells were grown in RPMI 1640 medium. 293T cells were grown in Dulbecco's modified Eagle's medium-based culture medium. HeLa-CD4-LTR-β-gal cells were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (Bethesda, MD) and were used for the drug susceptibility assay as described previously (13, 19, 20). An HIV-1 infectious clone, pNL4–3 (21), was used for generation of HIV-1 variants.

Antiviral Agents—The peptides used in this study were synthesized as described previously (6).

Determination of Drug Susceptibility of HIV-1—The peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as described previously (13). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat microtiter culture plates. On the following day the cells were inoculated with the HIV-1 clones (60 MAGI unit/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration, (EC_{50}).

Generation of Recombinant HIV-1 Clones—Recombinant infectious HIV-1 clones, carrying various mutations, were generated as described previously (13). Each molecular clone was transfected into 293T cells with *Trans*IT® (Madison, WI). After 48 h, the supernatants were harvested and stored at −80 °C until use.

Circular Dichroism Spectroscopy—Each peptide (10 μM) was mixed with 10 mM phosphate-buffered saline, pH 7.4, and the data were collected using a Jasco spectrometer (Model J-710; Jasco, Tokyo, Japan) equipped with a thermoelectric temperature controller. The thermal stability was assessed by monitoring the change in the circular dichroism signal at 222 nm. The midpoint of the thermal unfolding transition (melting temperature, (T_m) of each complex was determined as described previously (6).

Viral Replication Kinetics Assay—MT-2 cells (10^5 cells/3 ml) were infected with each virus preparation (1000 MAGI unit) for 16 h. The infected cells were then washed and cultured in a final volume of 3 ml. The culture supernatants were harvested after infection on days 2–7, and the levels of p24 antigen were determined (22).

For each competitive HIV-1 replication assay, two infectious clones of interest that had been previously titrated were mixed and added to MT-2 cells (10^5 cells/3 ml) as described previously (13, 22) with minor modifications. To ensure that the two infectious clones being compared were of approximately equal infectivity, a fixed amount (500 MAGI unit) of one infectious clone was mixed with three different amounts (250, 500, and 1000 MAGI unit) of the other infectious clone. On day 1, one-third of the infected MT-2 cells were harvested and washed twice with phosphate-buffered saline, and the cellular DNA was extracted. The purified DNA was subjected to nested PCR and then direct DNA sequencing. The HIV-1 co-culture, which best approximated a 50:50 mixture on day 1, was further propagated. Every 3–4 days, the co-culture supernatant (100 μl) was transmitted to new uninfected MT-2 cells (5×10^5 cells/3 ml). The cells harvested at the end of each passage were subjected to direct sequencing, and the viral population change was determined.

Structure Modeling of gp41 S138A Mutant Core—The gp41 core model was built using the coordinates of crystal structure of the N36/C34 complex (23) (PDB code 1AIK). The coordi-

TABLE 1

Antiviral activity of T-20-derived peptides against T-20-resistant gp41 recombinant viruses

Anti-HIV activity was determined with the MAGI assay. The data shown are the mean values and S.D. that were obtained from the results of at least three independent experiments. Shown in parentheses are the -fold increases in resistance (increase in EC_{50} value) calculated by comparison to a reference virus. Increases of >10-fold are indicated in bold.

	EC_{50}			
	HIV-1_{WT}[a]	HIV-1_{V38A}	HIV-1_{N43D}	HIV-$1_{N43D/S138A}$
		nM		
T-20	2.4 ± 0.6	23 ± 8.2 (9.6)	49 ± 10 (20)	84 ± 16 (35)
Small				
T-20_{S138G}	1.3 ± 0.5 (0.5)	65 ± 8.8 (27)	141 ± 26 (59)	185 ± 68 (77)
T-20_{S138A}	0.6 ± 0.1 (0.3)	3.6 ± 1.7 (1.5)	3.5 ± 0.9 (1.5)	3.2 ± 1.0 (1.3)
Hydrophobic				
T-20_{S138V}	0.4 ± 0.2 (0.2)	31 ± 14 (13)	22 ± 3.5 (9.2)	23 ± 5.7 (9.6)
T-20_{S138L}	0.7 ± 0.1 (0.3)	13 ± 6 (5.4)	2.9 ± 0.7 (1.2)	2.2 ± 0.4 (0.9)
T-20_{S138I}	0.5 ± 0.1 (0.2)	4.9 ± 2 (2)	2.9 ± 0.8 (1.2)	2.4 ± 0.6 (1)
T-20_{S138M}	0.7 ± 0.2 (0.3)	4.4 ± 0.1 (1.8)	1.7 ± 0.5 (0.7)	1.2 ± 0.4 (0.5)
T-20_{S138P}	446 ± 167 (186)	>1000 (>416)	>1000 (>416)	>1000 (>416)
Nucleophilic				
T-20_{S138T}	0.9 ± 0.2 (0.4)	39 ± 8.5 (16)	161 ± 35 (67)	124 ± 43 (52)
Aromatic				
T-20_{S138F}	9.4 ± 2.6 (4)	203 ± 89 (85)	393 ± 119 (164)	478 ± 116 (200)
T-20_{S138Y}	25 ± 9 (10)	516 ± 223 (215)	>1000 (>416)	>1000 (>416)
T-20_{S138W}	29 ± 14 (12)	>1000 (>416)	>1000 (>416)	>1000 (>416)
Amide				
T-20_{S138N}	19 ± 4 (8)	>1000 (>416)	>1000 (>416)	>1000 (>416)
T-20_{S138Q}	34 ± 11 (14)	>1000 (>416)	>1000 (>416)	>1000 (>416)
Acidic				
T-20_{S138D}	210 ± 94 (88)	>1000 (>416)	>1000 (>416)	>1000 (>416)
T-20_{S138E}	283 ± 80 (118)	>1000 (>416)	>1000 (>416)	>1000 (>416)
Basic				
T-20_{S138H}	210 ± 85 (88)	>1000 (>416)	>1000 (>416)	>1000 (>416)
T-20_{S138K}	708 ± 145 (295)	>1000 (>416)	>1000 (>416)	>1000 (>416)
T-20_{S138R}	362 ± 114 (150)	>1000 (>416)	>1000 (>416)	>1000 (>416)

[a] To improve the replication kinetics, D36G mutation, observed in the majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (reference virus).

nates of the water molecules were removed. Additionally, the hydrogen atoms were placed in optimal positions and refined by the energy minimization with the AMBER9 program (24) using the FF99 force field. Ser-138 in the gp41 core model was replaced with alanine (replacement of -OH with -H), and the positions of the hydrogen atoms were refined as described above. The S138A mutant core model (N36/$C34_{S138A}$ complex) was further optimized by the energy minimization using the FF99 force field with the restraints on each of the three residues of N and C termini and the backbone atoms. The restraint weight was 5.0 kcal/mol $Å^2$.

RESULTS

Effect of Amino Acid Substitutions at 138 on Antiviral Activities—We chemically synthesized peptide analogs of T-20 with all natural amino acid substitutions at the 138 position (T-20_{S138X}) and evaluated them for their ability to inhibit three major T-20-resistant clones using the MAGI assay (13) (Table 1). The results indicated that only T-20_{S138A} inhibited replication of T-20-resistant clones as efficiently as the wild-type clone. Substitution to glycine enhanced T-20 activity, but unlike T-20_{S138A}, T-20_{S138G} reduced its activity against T-20-resistant clones by ~2–3-fold as compared with the parental peptide, T-20. Substitutions to hydrophobic amino acids leucine, isoleucine, and methionine maintained their anti-HIV-1 activity; however, those to valine reduced anti-HIV-1 activity to T-20-resistant clones. The proline substitution drastically decreased the anti-HIV-1 activity of the peptide inhibitors.

Nucleophilic amino acid at position 138 of T-20 (T-20_{S138T}) showed similar profiles. Conversely, aromatic and amide substitutions reduced the anti-HIV-1 activity of T-20 against HIV-1_{WT}- and T-20-resistant clones. Other amino acid substitutions, especially acidic and basic amino acids, decreased the anti-HIV-1 inhibitory activity even against HIV-1_{WT}. These results suggest that smaller hydrophobic (Ala > Leu, Ile) or more flexible (Met > Thr) residues are preferred in this position. Furthermore, the α-helical structure is important for the interaction, as a mutation to proline which is expected to disrupt the helix (25) resulted in an inactive T-20 analog.

Circular Dichroism—To clarify the mechanism by which the substitutions at Ser-138 influence the antiviral activity of T-20 derivatives, we examined the binding affinities of these peptides to N-HR using circular dichroism (CD) analysis (Fig. 2). CD spectra reveal the presence of stable α-helical structure of the 6-helix bundle that is a requisite for biological activity and is thought to be mechanistically and thermodynamically correlated with HIV-1 fusion (26). Therefore, CD spectra typically at 222 nm indicate interaction of N-HR (N36) and C-HR (T-20 or C34). Because T-20 does not interact significantly *in vitro* with the N36 peptide, which is derived from amino acids 35–70 of N-HR, we used a derivative of C34, a peptide that overlaps with T-20 and also inhibits HIV fusion by the same mechanism. The C34 derivative contained the analogous T-20 substitutions described above (Fig. 1*B*). Consistent with antiviral activities, a mixture of N36 and $C34_{S138P}$ or $C34_{S138W}$ showed no apparent or reduced α-helicity, respectively. For binding with $N36_{V38A}$

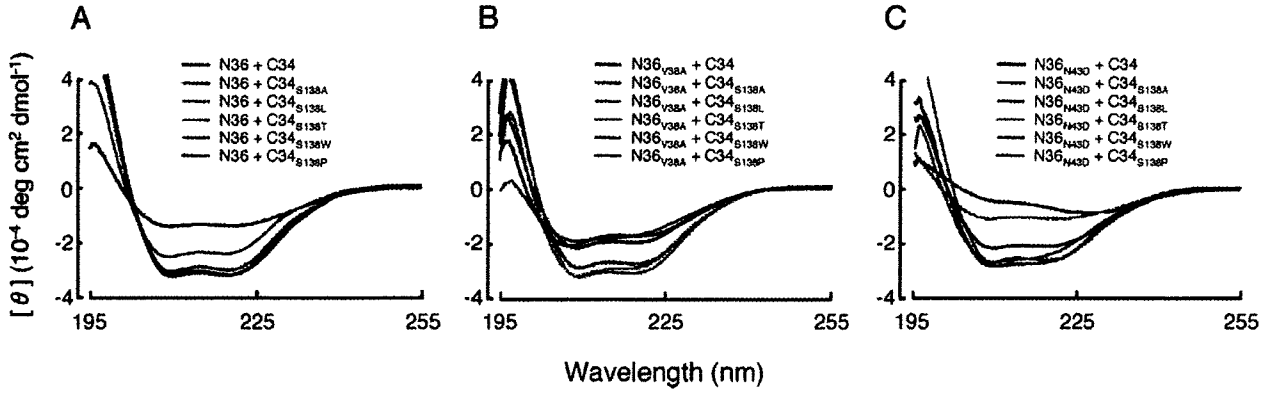

FIGURE 2. **CD spectra of $C34_{S138X}$ complexes with N36 (*A*), $N36_{V38A}$ (*B*), and $N36_{N43D}$ (*C*) are shown.** Equimolar amounts (10 μM) of the N- and C-HR peptides were incubated at 37 °C for 30 min in phosphate-buffered saline. The CD spectra of each mixture were then collected at 25 °C using a Jasco (Model J-710) spectropolarimeter.

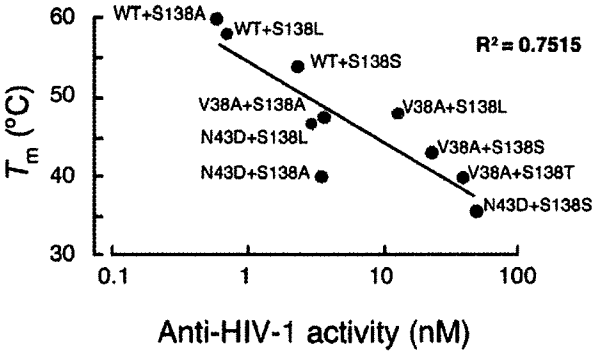

FIGURE 3. **Correlation of T_m values of complexes formed from N36 and C34 peptides (Fig. 2) and anti-HIV-1 activities of T-20_{S138X} (Table 1).**

or $N36_{N43D}$, sufficient α-helicity at 25 °C was observed only in $C34_{S138A}$, $C34_{S138L}$, and $C34_{S138T}$ or $C34_{S138A}$, $C34_{S138L}$, and $C34_{S138W}$, respectively (Fig. 2, *A*–*C*).

To determine the thermal stability of the helical complexes formed from the N36 and C34 peptides, we measured the melting temperature (T_m) of each complex (supplemental Table 1). The sigmoidal transition of the CD signal at 222 nm correlates with the thermal stability of the helical complexes formed from the N36 and C34 peptides, which in turn are indicative of the binding affinity of these peptides. The melting temperature (T_m) indicating the 50% disruption of 6-helix bundle was comparatively evaluated. Complexes of N36 and C34 containing the S138A or S138L substitutions ($N36/C34_{S138A}$ or $N36/C34_{S138L}$) showed high thermal stability, comparable with that of the wild-type N36/C34 complex. Similarly, the addition of the S138A or S138L also improved the thermal stability of the $N36_{N43D}$/C34 complex. These results reveal a striking correlation between the thermal stability and the anti-HIV-1 activity of the complexes (R^2 = 0.75, Fig. 3). The low T_m value of the complex formed from $N36_{N43D}$ and C34 suggests that virus containing the N43D mutation shows high resistance to T-20, likely due to less favorable thermodynamics that are expected to drive the formation of the 6-helix bundles containing T-20 inhibitor.

Antiviral Activity of Substituted C34 at Ser-138—To confirm that binding of C34 to N-HR is indeed representative of T-20 binding to N-HR, we examined the anti-HIV-1 activities of

TABLE 2

Antiviral activity of $C34_{N126K}$ peptides against C34-resistant gp41 recombinant viruses

Anti-HIV activity was determined by the MAGI assay. The data shown are the mean values and S.D. that were obtained from the results of at least three independent experiments. Shown in parentheses are the -fold increases in resistance (increase in EC_{50} value) calculated by comparison to a reference virus. The increase of >10-fold is indicated in bold.

	EC_{50}	
	HIV-1_{WT}[a]	HIV-$1_{\Delta V4/I37K/N126K/L204I}$[b]
	nM	
C34	1.6 ± 0.35	114 ± 29 (71)
$C34_{N126K}$	0.95 ± 0.22 (0.6)	1.1 ± 0.5 (0.7)

[a] To improve the replication kinetics, the D36G mutation, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (reference virus).

[b] C34-resistant HIV-1 was constructed with the reference virus as described (13). ΔV4 indicates 5 amino acids deletion (FNSTW) in the V4 region of gp120.

C34-derived peptides that have S138A substitutions. The $C34_{S138A}$ and $C34_{S138L}$ peptides showed potent anti-HIV-1 activities, similar to T-20_{S138A} and T-20_{S138L} (supplemental Table 2). Based on these findings, we conclude that the stability of complexes comprised of modified C34s and N36s containing T-20 resistance mutations offers a good measure of the binding affinity of T-20_{S138X} to N-HR.

Antiviral Activity of C34 with N126K—We have recently identified another mutation at the N-HR of gp41 (N126K) during exposure of HIV-1 to C34 *in vitro* (13). The N126K has been occasionally observed after prolonged T-20-containing therapy (10, 15). Here we have confirmed that the $C34_{N126K}$ peptide can also suppress a C34-resistant clone containing several mutations: I37K/N126K/L204I (Table 2). Therefore, peptides designed to have compensatory mutations seem to have potent antiviral activity. However, because residue 126 is located outside the amino acid sequence of T-20 (Fig. 1*B*), we could not examine the effect of N126K substitution on T-20 activity.

Replication Kinetics of Ser-138-substituted HIV-1—To evaluate the effect of Ser-138 substitutions on viral replication, we constructed molecular clones introducing several Ser-138 and determined their replication kinetics by measuring p24 gag antigen production in the culture supernatant. Single nucleotide changes to the TCA codon for Ser-138 may generate 4 amino acid substitutions, Ala, Thr, Leu, Pro, and Trp. As expected, the compensative substitution, S138A, in the T-20

resistance mutation N43D background enhanced replication kinetics of the N43D-containing clone as shown in supplemental Fig. 1. However, in the WT background the S138A appeared to decrease production of p24 as compared with HIV-1_{WT} (Fig. 4). Other substitutions also reduced their replication kinetics. Interestingly, the S138W substitution did not show measurable p24 production. Syncytia induction and single cycle replication kinetics of the Ser-138-substituted HIV-1 were also examined (supplemental Fig. 2). Sizes of syncytia of each virus formed in the MAGI cells (supplemental Fig. 2, *panels A–E*) were associated with p24-normalized single-cycle infectivities (supplemental Fig. 2, *panel F*) and multicycle replication kinetics (Fig. 4). These results suggest that substitutions at Ser-138 are not likely to appear in the absence of T-20 therapy or the emergence of N43D mutation.

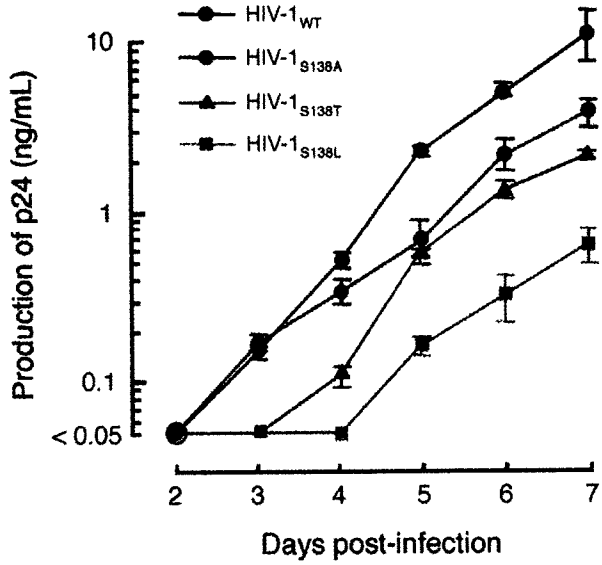

FIGURE 4. **Replication kinetics of HIV-1_{S138X} variants (*X*, any natural amino acid).** HIV-1_{S138A} (*bright red circles*) showed replication kinetics comparable with those seen for HIV-1_{WT} (*blue circles*). Replication of HIV-1_{S138T} (*emerald green triangles*) was reduced, somewhat surprisingly, as both threonine and serine are β-hydroxy amino acids, albeit with different hydrophobicity and torsional flexibility. HIV-1_{S138L} (*orange squares*) also showed reduced replication kinetics. Note that HIV-1_{S138P} and HIV-1_{S138W} failed to replicate (data not shown). Results shown are representative of three independent experiments. An identical order of replication kinetics was observed. Productions of p24 antigen on days 4–7 between HIV-1_{WT} and HIV-1_{S138A} were significant (*t* test, $p < 0.05$).

Structure Modeling—The side chain of amino acid 138 (Ser or Ala) closely contacts with the hydrophobic pocket formed by Leu-44 and Leu-45 in the N-HR. The mutation from Ser to Ala increases hydrophobicity and may help to stabilize the N-HR/C-HR complex related with the potency of the HIV-1 fusion inhibitors (Fig. 5). Larger hydrophobic substitutions such as S138W, S138L, or S138I are likely to sterically interfere with efficient packing of the N-HR and C-HR helices. Similarly, introduction of charged residues at this region of the interface would also disrupt the hydrophobic environment and result in destabilized helix bundles, consistent with the biochemical and virological findings (Figs. 2–4 and Table 1).

Based on crystallographic studies (27, 28), we observe that the T-20 resistance N43D mutation should affect interactions between helices in the 6-helix bundle. Specifically, residue 46 of N-HR is proximal to residue Glu-137 of the C-HR helix of another molecule in the 6-helix bundle. We believe that this increase in proximal negative charges and juxtaposition of Asp-36 next to Glu-137 may destabilize the formation of the 6-helix bundle in a way that results in reduced efficiency of fusion and reduced replication kinetics. Increase of the hydrophobic interactions by introduction of the S138A mutation should help overcome the negative effects of the N43D mutation.

DISCUSSION

In this study we demonstrate that by introducing a secondary resistance mutation into the sequence of peptide-fusion inhibitors such as C34 and T-20, we can suppress efficiently replication of wild-type and of fusion inhibitor-resistant HIV-1. Our circular dichroism analysis revealed that C-HR-based fusion inhibitors that carry secondary resistance mutations can form tight 6-helix bundles with N-HR that contains primary resistance mutations responsible for T-20 resistance. A similar approach has been applied for the development of short hairpin RNA (shRNA) sequences that inhibit HIV-1 replication (29). The synthesized shRNA with mutations that confers resistance to the parental shRNA effectively suppressed replications of shRNA resistant HIV-1 but not wild-type HIV-1. Therefore, it is possible to gain valuable insights from the resistance information and directly apply it to design new peptides or oligonucleotides in the case of shRNA that preempt the viral escape mechanism and suppress resistant variants. Moreover, this strategy should not result in more adverse effect than those that might be obtained during use of the original peptide or oligonucleotide reagents.

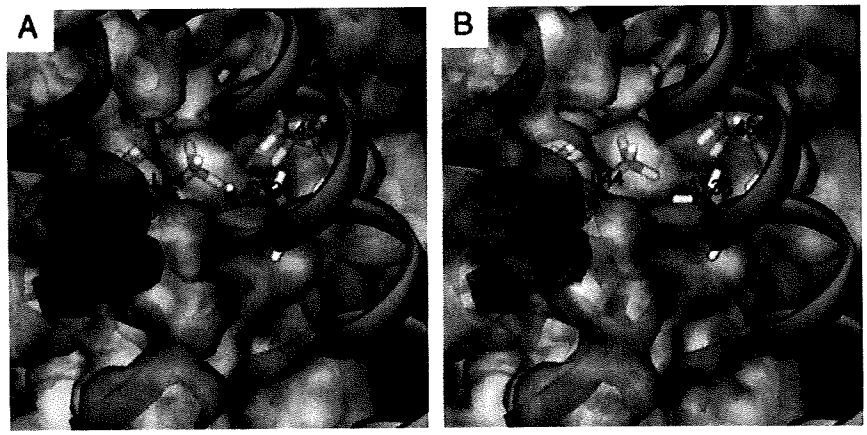

FIGURE 5. **Structure of gp41 at the region near position 138 in the C-HR.** *A*, crystal structure of the N36/C34 complex (PDB code 1AIK). *B*, computational structure modeling of the S138A mutant (N36/$C34_{S138A}$ complex). N-HR and C-HR helices are colored *green* and *orange*, respectively. The van der Waals surface of only N-HR is shown and colored according to the electrostatic potential.

Recently we (6, 30, 31) and others (5) reported that hydrophilic amino

acid substitutions stabilized the α-helix of C-HR peptides and increased their binding affinity to N-HR, thus providing potent anti-HIV activity. This property may be one of the key attributes of the recently developed potent peptide inhibitors, SC34EK (6, 30), T-20EK (31), or T-2429 (5), that have been reported to efficiently inhibit T-20 resistant variants. However, the S138A substitution on T-20 in the present study had little effect on the random coil structure, as judged by CD (data not shown), indicating that $T\text{-}20_{S138A}$ increases its binding affinity not by simply enhancing the α-helicity of this region (5, 6). Our approach of introducing substitutions selected on the basis of the mutation(s) that appears in resistant viruses significantly improved the affinity with N-HR. This approach may complement the effects of enhancing helical stability and may help generate more potent and effective fusion inhibitors for resistant HIV-1 variants.

Other methods have also been employed to improve the potency of HIV fusion inhibitors. For example, T-1249 is a peptide that is based on the T-20 sequence and has improved binding properties (32, 33). It contains 17 changes compared with T-20 (3 additional residues and 14 substitutions to increase the α-helicity/binding affinity according to amino acid sequences of HIV-2 and simian immunodeficiency virus). T-2635 is another efficient peptide fusion inhibitor that was recently developed and is also modified extensively (19 substitutions in 38 amino acids) (5). Also, SC34EK is an electrostatically constrained peptide that also suppresses replication of T-20-resistant variants, and it required 12 substitutions in the original C34 inhibitor (6, 30). Hence, it is possible to improve the potency of existing peptide inhibitors through intense modeling and iterative testing in *in vitro* studies that could lead to the design and synthesis of improved peptide drugs. However, the approach we followed in the design of the $T\text{-}20_{S138A}$ inhibitor is considerably simpler and involves a smaller number of sequence changes (1 residue changed, compared with 19 and 12 in the cases of T-2635 and SC34EK, respectively; see above). It takes advantage of information obtained from the viral evolution under drug pressure and uses the resistance information to design improved inhibitors. In addition, we believe that this approach may be applicable to other targets even when the interactions do not involve helical bundles or detailed information on related systems is not available. Importantly, whenever possible, a combination of the two approaches would likely generate even more effective peptide inhibitors that can suppress replication of resistant variants.

α-Helical structure is a significant factor not only in HIV-1 fusion but also in other examples of protein-protein interactions. Peptide-based drugs have to overcome multiple obstacles, including poor oral bioavailability, less permeability into the target cells, and high cost. Several modifications, such as using arginine-rich peptide tags (34, 35), and chemical treatments (36) have been used to overcome the cell permeability problem. At any rate, peptide-based reagents can be an important tool in the discovery and validation of novel therapeutic targets through *in vitro* experiments. For example, it has been shown that the function of a target protein can be inhibited by designing synthetic peptides that have the amino acid sequence of a domain which is important for the protein function. In such cases the peptides may act as decoys that have antagonistic/agonistic or competitive effects, leading to inhibition of the protein function. Similarly, screening through peptide sequences of proteins may be useful for the identification of functionally important domains that could become future targets for peptide-based or small molecule-based drug development.

In this study we designed peptides tailored to suppress T-20-resistant HIV-1 strains. To our knowledge, this is the first report of direct application of resistance information in drug design and may be applicable to other, unrelated systems. For example, a BH3 domain of the anti-apoptotic protein Bcl-2 has been targeted by an α-helical domain mimic peptide (37, 38). The resulting hydrocarbon-stapled peptide, $SAHB_A$, penetrates into cells via endocytosis pathway and inhibits the function of Bcl-2, inducing apoptosis in transplanted leukemia cells in mice. However, during prolonged therapy with such peptides, leukemic cells could develop resistance to the peptides through substitutions in the Bcl-2 region in the selection process for survival reminiscent of HIV-1. One can envision that our strategy of using mutational resistance information to overcome drug resistance might help in the design of substituted peptides that suppress the resistant variants more efficiently, thus contributing to broader applications of successful peptide-based therapies.

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Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide

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ABSTRACT

Primary mutations in HIV-1 that are directly involved in the resistance to enfuvirtide have been well documented. However, secondary mutations that are associated with primary mutations and contribute little to the resistance still remain to be elucidated. This study reveals that synonymous mutations at gp41 Q41 (CAG to CAA) or L44 (UUG to CUG) act as secondary mutations. Complementary mutations in the nucleotide level are located in the Rev responsive element (RRE) of the HIV-1 RNA-genome and maintain the replication kinetics of HIV-1 through increasing the structural stability of stem-loop III in the RRE. Therefore, synonymous mutations in the gp41/RRE sequence improve the viral replication impaired by the primary mutations and play a key role as secondary (complementary) mutations.

1. Introduction

Enfuvirtide (T-20), an HIV-1 fusion inhibitor which has been approved for the treatment of HIV-1 infected patients, successfully suppresses the replication of HIV-1 even in strains resistant to various reverse transcriptase and protease inhibitors (Fung and Guo, 2004; Lalezari et al., 2003; Lazzarin et al., 2003; Manfredi and Sabbatani, 2006). However, HIV-1 variants resistant to T-20 have emerged after prolonged T-20 therapy (Lu et al., 2006; Marcelin et al., 2004; Sista et al., 2004; Wei et al., 2002). The majority of resistant variants develop primary mutations to T-20, including V38A and N43D, in the consensus sequence, GIVQQQNNLL (DIVQQQNNLL for NL4-3) motif of the gp41 within amino acid positions 36–45 (Cabrera et al., 2006; Fikkert et al., 2002; Lu et al., 2006; Marcelin et al., 2004; Menzo et al., 2004; Mink et al., 2005; Poveda et al., 2002; Sista et al., 2004; Su et al., 2006; Wei et al., 2002). Few studies have so far addressed secondary mutations for resistance to T-20.

Primary and secondary mutations were recently well-defined for C34, a peptide derived from the gp41 carboxyl terminus heptad repeat (C-HR) in vitro (Nameki et al., 2005). A30V and D36G in the gp41 act as secondary mutations which enhance the replication kinetics impaired by primary mutations. Interestingly, these mutations are complementarily located in stem-loop IIA and C (Fig. 1) of the Rev responsive element (RRE) which is an essential RNA structure for transporting non- and singly spliced viral RNA to the cytoplasm from the nucleus (Olsen et al., 1990; Zapp and Green, 1989). Most recently, T18A and V38A complementarily located in the stem IIA of the RRE has been detected in clinical isolates treated with T-20 (Svicher et al., 2008). Co-presence of T18A and V38A is associated with high level of viral load. These data support our recent in vitro results that these secondary mutations in stem II maintain HIV-1 replication through substitutions at the nucleotide as well as the amino acid level (Nameki et al., 2005). This study analyzed the nucleotide sequences of T-20 resistant variants deposited in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and revealed that synonymous mutations in stem-loop III of the RRE maintain HIV-1 replication impaired by N43D, one of primary mutations for T-20 resistance.

2. Materials and methods

2.1. Antiviral agents and cells

T-20 was synthesized as described previously (Otaka et al., 2002). 2′,3′-Dideoxycytidine (ddC) was purchased from Sigma (St.

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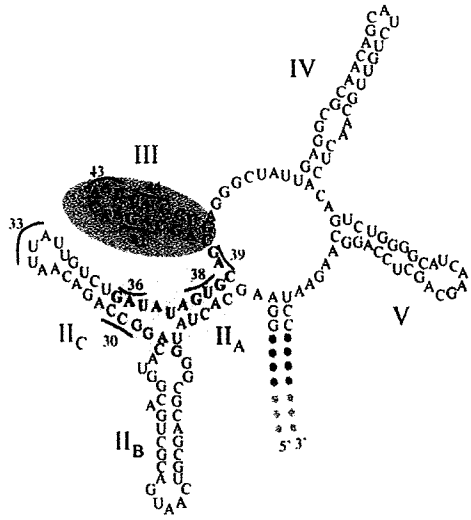
(A)



(B)

Mutations: Amino acid	Mutations: Nucleotide	Secondary structure of stem loop III	ΔG (kcal/mol)
None (WT: NL4-3)	None		-6.5
Q39Q	CAG → CA**A**		-6.8
Q41Q	CAG → CA**A**		-6.4
L44L	UUG → **C**UG		-9.1
L44L	UUG → **UU**A		0.1
N43D	AAU → **G**AU		-6.5
N43D L44L	AAU → **G**AU UUG → **C**UG		-9.0
Q39Q N43D	CAG → CA**A** AAU → **G**AU		-6.8
Q41Q N43D	CAG → CA**A** AAU → **G**AU		-6.4
N43K	AAU → AA**A**		-4.9
N43K	AAU → AA**G**		-5.4
N43K L44L	AAU → AA**A** UUG → **C**UG		-7.7
N43K L44L	AAU → AA**G** UUG → **C**UG		-7.9
Q40H L45M	CAG → CA**T** CUG → **A**UG		-4.5

(C)

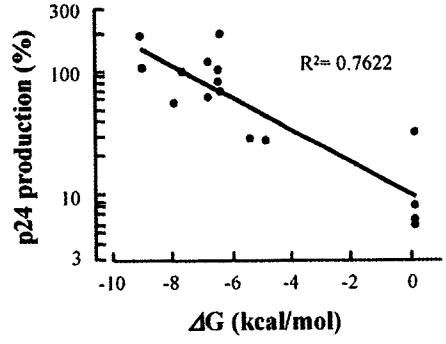


Fig. 1. (A) Secondary structure of RRE depicted based on references (Olsen et al., 1990; Zapp and Green, 1989). T-20-resistant mutations are accumulated between amino acid position from 36 to 45 of gp41 and nucleotides coding for these amino acids, are given in bold. Stem-loop II (light gray region) was examined as described previously (Nameki et al., 2005) and stem-loop III (dark gray region) was analyzed in this study. (B) Amino acid substitutions in the gp41 with their nucleotide changes and putative secondary structures and their stabilities of stem-loop III are shown. Nucleotide substitutions are indicated in bold and circle. Newly generated nucleotide pairs are indicated by the bold line. The stability of stem-loop III defined by ΔG value was calculated using the MFold program version 3.2 (Mathews et al., 1999; Zuker, 2003). (C) Correlation of the amounts of p24 production and stability of stem-loop III (ΔG value) is shown. Plots of Q41H and L45M were included (see text) but that of V38A was excluded since V38A is located in stem IIA. Since replication of N43D containing clones were extremely low, p24 productions obtained from D36G combined N43D or K containing clones, were used. When excluding data of D36G combined with N43D or N43K and including that of N43K without D36G, correlation coefficient (R^2) was 0.72.

Louis, MO). HeLa-CD4-LTR-β-gal cells were used for the drug susceptibility assay (MAGI assay) as described (Nameki et al., 2005). MT-2 and 293T cells were grown in RPMI1640- and Dulbecco's modified Eagle's medium (DMEM)-based culture medium, respectively.

2.2. Generation of recombinant viruses

An HIV-1 infectious clone, pNL4-3, which was kindly provided by Dr. H. Sakai, Institute for Virus Research, Kyoto University (Kyoto, Japan), was used for the construction and the production of HIV-1 variants. To generate major T-20-resistent molecular clones, desired mutations were introduced into the Nhe I-BamH I region (1221 bp) of pSLgp41WT, which encoded nucleotides 7250–8470 of pNL4-3, by site-directed mutagenesis as described (Nameki et al., 2005). The Nhe I-BamH I fragments were then inserted into pNL4-3, generating various molecular clones with the desired mutations. Viruses were recovered from the supernatant of the transfected 293T cells and stored at −80 °C until use. A wild type HIV-1, HIV-1WT, was generated by transfection of pNL4-3 into 293T cells.

2.3. Determination of titer and drug susceptibility of HIV-1

The titer and peptide-sensitivity of infectious clones was determined by the MAGI assay with some modifications (Kimpton and Emerman, 1992; Kodama et al., 2001; Maeda et al., 1998). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the supernatant of 293T cells transfected with HIV-1 clones and the titrated HIV-1 clones in the presence of various concentrations of drugs in fresh medium, for determination

of titer and drug susceptibility of HIV-1, respectively. Forty-eight hours after the viral exposure, all the blue cells stained with X-Gal were counted in each well. Viral titer was determined with the MAGI assay as blue cell forming units (BFUs). The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (EC_{50}).

2.4. Viral replication kinetics assay

MT-2 cells (10^5 cells) were infected with each virus preparation (500 BFUs) derived from molecular-constructed clone for 4 h. The infected cells were washed and cultured in a volume of 3 ml. The culture supernatants were harvested on day 4 after infection during the linear replication phase and p24 antigen production was determined (Hachiya et al., 2008). For competitive HIV-1 replication assays (CHRA), the two titrated infectious clones were mixed and added to MT-2 cells as described previously (Nameki et al., 2005). To ensure that the two infectious clones being compared were of approximately equal infectivity, a fixed amount (500 BFUs) of one infectious clone was mixed with three different amounts (250, 500 and 1000 BFUs) of the other infectious clone. On day 1, one-third of the infected MT-2 cells were harvested, and subjected to DNA extraction. The purified DNA was used for nested PCR and then direct sequencing. Every 4–5 days, the viral population change was also determined, and the cell-free supernatant of the virus coculture (1 ml) was transmitted to new uninfected MT-2 cells. The cells harvested at the end of each passage were subjected to direct sequencing, and the viral population change was determined.

2.5. GenBank accession numbers

All sequences of clinical isolates referred in this study are available under GenBank accession nos. AF500084 to AF500093, AJ964904 to AJ964940, AY185366 to AY185492, AY436381 to AY436401, AY523979 to AY523991, AY750998 to AY751078, AY768582 to AY768660, and AY785131 to AY785135.

3. Results

3.1. T-20 susceptibility of HIV-1 clones

V38A, N43D, and a combination of Q40H and L45M conferred strong resistance to T-20 (more than 48-fold) while the Q40H, N43K, and L45M substitutions conferred moderate resistance (5.4–13-fold) in comparison to NL4-3 (wild type, WT) (Table 1). These results are consistent with those of a previous report (Labrosse et al., 2006; Lu et al., 2004; Menzo et al., 2004; Pérez-Alvarez et al., 2006; Wei et al., 2002) and demonstrate that these substitutions act as a primary mutation for T-20 resistance. All synonymous mutations at 39, 41, and 44 conferred little T-20 resistance by themselves and even in combination with other substitutions, including N43D and N43K (Table 1). Since Q39, located adjacent to stem-loop III, has no complementary partner, Q39 was used for further experiments as a control for synonymous mutations. Therefore, the gp41 amino acid sequence solely confers T-20 resistance, while the RNA sequence or the structure itself may not markedly contribute to T-20 resistance.

Although the D36G substitution located in the stem-loop II is observed in the vast majority of HIV-1 strains, only NL4-3 strain contains D36 at this position. The introduction of D36G into NL4-3 based T-20 resistant clones enhanced T-20 susceptibility by approximately 10-fold (Table 1). The D36G partially or completely restored T-20 susceptibility attenuated by N43D or K in NL4-3 viruses, respectively. Therefore, D36 may actually enhance T-20 resistance caused by N43D or N43K in the vast majority of the HIV-1 strains. In fact, D36 is frequently detected in T-20 experienced patients and is associated with T-20 resistance (Cabrera et al., 2006). However, N43K containing variants with D36G background seem to be insufficient for resistance to T-20 (only 4-fold resistance), which is consistent with low frequency of emergence of N43K containing variants in T-20-experienced patients (Lu et al., 2006; Morozov et al., 2007; Si-Mohamed et al., 2007).

3.2. Replication kinetics

In D36 background (pNL4-3 derived virus), replication of HIV-1 with primary mutation(s) was much attenuated (Table 1). It is well consistent with rare frequency of simultaneous introduction of V38A and/or N43D with D36 in vivo (Cabrera et al., 2006). Synonymous substitutions at amino acids, Q41 and L44 were frequently observed in T-20-resistant clinical isolates with N43 substitutions deposited in the GenBank, suggesting that these synonymous mutations may work as secondary mutations. To prove this hypothesis, N43D mutants were generated with synonymous mutations, L44L-CUG: UUG → CUG, Q39Q-CAA: CAG → CAA and Q41Q-CAA: CAG → CAA, designated as HIV-$1_{N43D/L44L-CUG}$, HIV-$1_{Q39Q-CAA/N43D}$ and HIV-$1_{Q41Q-CAA/N43D}$, respectively, and their replication kinetics were determined. The p24 production of all of the mutants remained less than 2% in comparison to that of HIV-1WT (Table 1). Replication kinetics were compared based on CHRA, demonstrating that combination of synonymous and complementary mutations, Q41Q-CAA and L44L-CUG restored replication kinetics impaired by N43D, while that of a simple synonymous mutation (not complementary), Q39Q-CAA, did not (Table 2). However, the D43N (GAU → AAU) substitution, which reverts to WT sequence, was detected in the virus population as early as on day 10 during the CHRA, when HIV-1N43D was used (Fig. 2). N43D (GAU) is the putative target site of apobec3F or 3G deamination that is involved in innate immunity to HIV-1 infection (Bishop et al., 2004; Harris et al., 2003; Liddament et al., 2004; Mangeat et al., 2003; Wiegand et al., 2004; Zhang et al., 2003). The MT-2 cells used in this study express both apobec 3G and 3F as confirmed by reverse transcription 165 coupled PCR (data not shown). Therefore, N43D might be reverted to N43 (identical sequence of HIV-1WT) by the deaminases, thus resulting in the appearance of NL4-3 strains.

Since D36G increases the level of replication by controlling the fusogenic activity (Kinomoto et al., 2005) and stability of RRE structure (Nameki et al., 2005), the generated N43D mutants were combined with D36G (HIV-$1_{D36G/N43D}$, HIV-$1_{D36G/N43D/L44L-CUG}$, HIV-$1_{D36G/Q39Q-CAA/N43D}$ and HIV-$1_{D36G/Q41Q-CAA/N43D}$). These recombinant viruses showed comparable replication kinetics with HIV-1WT or HIV-1_{D36G} (Table 1) and revealed an identical order of replication observed in the N43D containing HIV-1s by the CHRA (Table 2). It is likely that N43D mainly impaired replication kinetics through altered fusion kinetics, since reduced replication kinetics by N43D can be partially restored by introduction of S138A (Cabrera et al., 2006; Marcial et al., 2006; Mink et al., 2005; Xu et al., 2005) and the ΔG value is identical to that of WT (Fig. 1B). It is also possible that nucleotide sequence of N43D-GAT may influence Rev binding to stem III.

In N43K mutants, the synonymous mutation, L44L-CTG, also enhanced the replication kinetics (Table 2). The L44L-CTG enhancement was greater in N43K-AAA mutant. Taken together, synonymous and complementary mutations restore HIV-1 replication impaired by introduced primary mutations, suggesting that these mutations act as secondary mutations.

3.3. Stability of stem-loop III

The structural stability of stem-loop III for N43K with codons AAA or AAG, were both comparable (Fig. 1) when calculated using

Table 1
Drug susceptibility[a] and viral replication[b] of HIV-1 clones with primary mutations or synonymous mutations.

Mutation(s)	EC_{50}[c] (μM)		p24(%)
	ddC	T-20	
WT[d]	0.51 ± 0.089	0.021 ± 0.0093	100
D36G	0.66 ± 0.22 (1.3)[e]	0.0026 ± 0.0012 (0.1)	95 ± 15
Primary mutations			
V38A	0.65 ± 0.12 (1.3)	>1.0 (>48)	3.3 ± 1.7
Q40H	0.47 ± 0.11 (0.9)	0.21 ± 0.087 (10)	31 ± 11
N43D	0.48 ± 0.13 (0.9)	>1.0 (>48)	<2
$N43K_{AAA}$	0.22 ± 0.005 (0.4)	0.28 ± 0.024 (13)	21 ± 11
$N43K_{AAG}$	0.32 ± 0.009 (0.6)	0.11 ± 0.019 (5.4)	30 ± 16
L45M	0.68 ± 0.12 (1.3)	0.27 ± 0.11 (13)	5.4 ± 0.8
Q40H/L45M	0.37 ± 0.2 (0.7)	>1.0 (>48)	7.7 ± 3.7
Synonymous mutations			
$Q_{CAG}39Q_{CAA}$	0.62 ± 0.077 (1.2)	0.015 ± 0.006 (0.7)	115 ± 16
$Q_{CAG}41Q_{CAA}$	0.58 ± 0.13 (1.1)	0.054 ± 0.011 (2.6)	192 ± 53
$L_{UUG}44L_{CUG}$	0.62 ± 0.089 (1.2)	0.030 ± 0.018 (1.5)	191 ± 34
$L_{UUG}44L_{UUA}$	0.64 ± 0.18 (1.3)	0.014 ± 0.0026 (0.7)	5.9 ± 1.7
N43D series			
N43D[f]	0.48 ± 0.13 (0.9)	>1.0 (>48)	<2[g]
$N43D/L44L_{CUG}$	0.35 ± 0.050 (0.7)	>1.0 (>48)	<2
$Q39Q_{CAA}/N43D$	0.34 ± 0.17 (0.7)	>1.0 (>48)	<2
$Q41Q_{CAA}/N43D$	0.62 ± 0.097 (1.2)	>1.0 (>48)	<2
D36G/N43D	0.56 ± 0.18 (1.1)	0.13 ± 0.056 (6.1)	81 ± 10
$D36G/N43D/L44L_{CUG}$	0.63 ± 0.19 (1.2)	0.10 ± 0.045 (4.9)	103 ± 34
$D36G/Q39Q_{CAA}/N43D$	0.76 ± 0.12 (1.5)	0.14 ± 0.057 (6.8)	59 ± 23
$D36G/Q41Q_{CAA}/N43D$	0.74 ± 0.14 (1.5)	0.14 ± 0.066 (6.8)	67 ± 16
N43K series			
$N43K_{AAA}$[f]	0.22 ± 0.005 (0.4)	0.28 ± 0.024 (13)	21 ± 11
$N43K_{AAG}$[f]	0.32 ± 0.009 (0.6)	0.28 ± 0.077 (13)	30 ± 16
$N43K_{AAA}/L44L_{CUG}$	0.64 ± 0.087 (1.3)	0.22 ± 0.082 (11)	76 ± 18
$N43K_{AAG}/L44L_{CUG}$	0.59 ± 0.10 (1.2)	0.20 ± 0.067 (10)	37 ± 15
$D36G/N43K_{AAA}$	0.61 ± 0.086 (1.2)	0.0069 ± 0.0004 (0.3)	26 ± 18
$D36G/N43K_{AAG}$	0.60 ± 0.078 (1.2)	0.0087 ± 0.0008 (0.4)	27 ± 12
$D36G/N43K_{AAA}/L44_{CUG}$	0.28 ± 0.14 (0.6)	0.0057 ± 0.0016 (0.3)	96 ± 19
$D36G/N43K_{AAG}/L44L_{CUG}$	0.69 ± 0.14 (1.4)	0.0069 ± 0.0003 (0.3)	53 ± 8.1

[a] Antiviral activity was determined by the MAGI assay. The data shown are mean values and standard deviations obtained from the results of at least three independent experiments.

[b] Viral replication was determined by p24 ELISA. The data shown are mean values and standard deviations obtained from the results of at least three independent experiments. Absolute p24 value for $HIV\text{-}1_{WT}$ was 1.8 ± 0.2 ng/ml.

[c] 50% effective concentration was determined with the MAGI assay (Nameki et al., 2005).

[d] $HIV\text{-}1_{NL4\text{-}3}$ was used as a wild type virus.

[e] Fold change in EC_{50} of the gp41 recombinant molecular clone compared with that of $HIV\text{-}1_{WT}$.

[f] Also described in the *primary mutations* section of Table 1.

[g] Only insufficient replication of $HIV\text{-}1_{N43D}$ in MT-2 cells was observed. However, we could determine initial titer of the $HIV\text{-}1_{N43D}$ obtained from freshly transfected 293T cells in the MAGI cells, since the MAGI assay only detects first round of viral infection up to Tat expression. Therefore, it may be ideal for determination of accurate inhibitory effect compared to other assays that allow multiple replications.

the MFold program version 3.2 (http://frontend.bioinfo.rpi.edu/applications/mfold/) (Mathews et al., 1999; Zuker, 2003). The replication kinetics of HIV-1N43K-AAA and HIV-1N43K-AAG were comparable, in combination of L44L-CTG, however, that of HIV-1N43K-AAA was greater by the CHRA. It is possible that the nucleotide sequence itself may affect binding affinity of Rev to the RRE, although detailed mechanism of the difference between N43K-AAA and -AAG remains to be elucidated. Introduction of D36G to N43K-containing clones did not improve the replication kinetics of N43K-containing clones but restored their susceptibilities to T-20 (Table 1). These results indicate that synonymous mutations thus maintain HIV-1 replication.

A combination of primary mutations, namely Q40H and L45M (both ΔG values are 0.1 but in combination that is −4.5), which are complementarily located and stabilize stem III structure (Fig. 1B) and observed in vivo (Cabrera et al., 2006; Marcial et al., 2006; Mink et al., 2005; Xu et al., 2005), may alter the replication kinetics at either the nucleotide or amino acid level. Recent studies also highlight strong co-presence of Q40H and L45M in clinical isolates treated with T-20 (Svicher et al., 2008). These results sug-

Table 2
Effect of synonymous mutations on replication of HIV-1.

Mutation background	Order of replication
N43D	$HIV\text{-}1_{N43D/L44L\text{-}CUG} = HIV\text{-}1_{Q41Q\text{-}CAA/N43D} > HIV\text{-}1_{N43D} = HIV\text{-}1_{Q39Q\text{-}CAA/N43D}$
D36G/N43D	$HIV\text{-}1_{D36G/N43D/L44L\text{-}CUG} = HIV\text{-}1_{D36G/Q41Q\text{-}CAA/N43D} > HIV\text{-}1_{D36G/N43D} > HIV\text{-}1_{D36G/Q39Q\text{-}CAA/N43D}$
N43K	$HIV\text{-}1_{N43K\text{-}AAA/L44L\text{-}CUG} > HIV\text{-}1_{N43K\text{-}AAG/L44L\text{-}CUG} > HIV\text{-}1_{N43K\text{-}AAA} = HIV\text{-}1_{N43K\text{-}AAG}$
Synonymous mutations	$HIV\text{-}1_{L44L\text{-}CUG} = HIV\text{-}1_{Q41Q\text{-}CAA} > HIV\text{-}1_{WT} = HIV\text{-}1_{Q39Q\text{-}CAA}$

Competition of HIV-1 replication assay (CHRA) was performed in MT-2 cells. At least two independent CHRAs were performed.

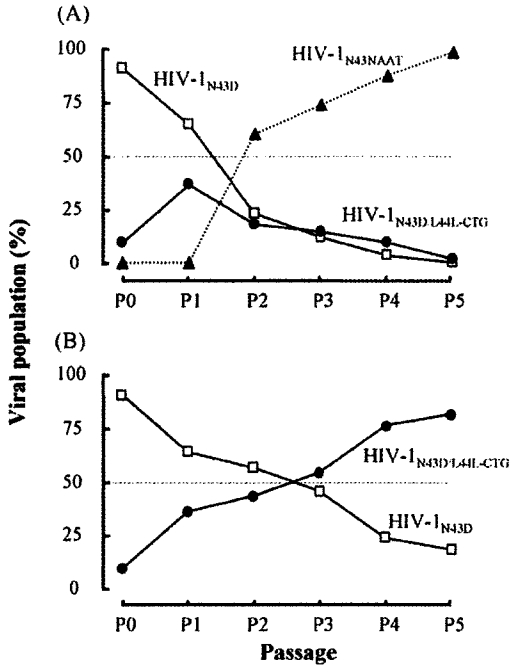


Fig. 2. Emergence of HIV-$1_{N43N\text{-}AAT}$ (HIV-1_{WT}) during CHRA for HIV-1_{N43D} and HIV-$1_{N43D/L44L\text{-}CTG}$ are shown. Open square, closed circle, and closed triangle represent HIV-1_{N43D}, HIV-$1_{N43D/L44L\text{-}CTG}$, and HIV-$1_{N43N\text{-}AAT}$, respectively. Absolute viral populations of each HIV-1s (A) and relative viral populations of HIV-1_{N43D} and HIV-$1_{N43D/L44L\text{-}CTG}$ (B) were shown.

gest that introduction of the primary mutations in the nucleotide level are affected by the RRE stability, indicating that amino acid and nucleotide substitutions in the gp41 and the RRE, respectively, co-operatively play a role.

3.4. Effect of synonymous mutations

As expected, the synonymous mutations (Q39Q-CAA, Q41Q-CAA, and L44L-CUG) solely affect viral replication but not T-20 susceptibility (0.7–2.6-fold in Table 1). They displayed an order of replication of HIV-1L44L-CUG = HIV-1Q41Q-CAA > HIV-1WT = HIV-1Q39Q-CAA, also demonstrating that only synonymous and complementary mutations, Q41Q-CAA and L44L-CTG, enhance replication kinetics. Finally, HIV-1L44L-UUA that is not detected in vivo was constructed and its replication kinetics was examined. As shown in Fig. 1, the third nucleotide for L44 raises the ΔG value, thus indicating that structure of the stem-loop III is unstable. The variants displayed impaired replication kinetics (Table 1). These results indicate that, in addition to the influence on gp41 function by amino acid substitutions, the structural stability of stem-loop III is one of the major determinants of the replication kinetics of mutated clones.

4. Discussion

This study demonstrated that synonymous mutations in the stem-loop III of RRE play an important role in the improvement of HIV-1 replication without affecting T-20 susceptibility. The structural stability of stem-loop III defined by ΔG value was strongly correlated with the replication kinetics ($R^2 = 0.76$, Fig. 1C), while the susceptibility, based on the EC_{50} value, was less ($R^2 < 0.3$ excluding clones that showed over 48-fold resistance, data not shown). Although it is likely that the nucleotide sequence of stem-loop III as well as the structural stability may also influence the RRE functions, including Rev binding, the current results indicate that the structural stability of RRE as well as gp41 amino acid substitutions seem to be a determinant for replication kinetics. At present, however, it is impossible to conclude that the pre-existence of such synonymous mutations in the RRE predicts on how T-20 resistance mutations are acquired.

Armand-Ugón et al initially isolated and reported resistance to C34 in vitro (Armand-Ugón et al., 2003), although we previously demonstrated that some of mutations for C34 resistance are involved in the RRE function as described (Nameki et al., 2005). The variants contained L33S or V38E mutations in the gp41 and both showed strong (more than 500-fold resistance) C34 resistance (Armand-Ugón et al., 2003). These mutations in the nucleotide level are also located in RRE (Fig. 1A); the nucleotide substitutions for L33S and V38E are located in the loop of stem IIB and the middle of stem IIC, respectively. Interestingly, V38E emerged in the HxB2-derived strain containing D36G polymorphism that stabilizes stem IIB structure, while L33S, which might have little effect on stem IIC stability due to its location, was observed in the NL4-3-derived resistant strain. These results also suggest that introduction of some mutations in the gp41 is restricted by RRE function. Armand-Ugón et al. (2003) failed to identify the secondary mutations for L33S or V38E. It is likely that the effect on RRE function may be tolerable for viral replication. Alternatively, relative short induction periods (maximum 17 passages) might also influence efficient introduction of the secondary mutations.

Functional analysis for Rev, RRE, and/or both seems to be important to reveal a detailed effect on viral replication. In this regard, we have previously demonstrated that effect of mutations for C34 resistance located in stem-loop II on binding of Rev to RRE was not apparent (less than 10% in the gel shift assay (Nameki et al., 2005)). It is possible that other factors including nuclear export and host factors, which may be influenced by the mutations, are involved in viral replication through interaction of Rev/RRE in HIV infected cells. Therefore, in the present study, we constructed an artificial mutant L44-TTA, which destabilizes or disrupts the stem III structure, and examined the effect on viral replication (Table 1). Replication of L44-TTA containing mutant showed much reduced replication kinetics even without gp41 amino acid substitutions, again indicating that stem III also plays an important role in viral replication.

So far, no information concerning Rev and Tat nucleotide substitutions is available in T-20 experienced patients. It is possible that the altered function of RRE may induce Rev mutation(s). In this regard, the entire sequence of Rev coding region of a C34 resistant variant was determined, however, no mutations were observed (Nameki et al., 2005). Most of the coding region of Rev also encodes Tat and gp41, thus indicating that Rev mutation(s) would alter these functions. This suggests that, even for single amino acid substitution, the genetic barrier for T-20 resistance seems to be relatively high when synonymous mutations are required to be introduced with the primary mutations and further fusion inhibitors that target the N-helical region thus appear to be promising.

In conclusion, this study provides valuable insight into the functional importance of RRE in HIV-1 with T-20 resistance for the replication kinetics. To reveal the function of gp41, experiments with artificial amino acid substitutions, *e.g.*, alanine scanning, which can be used to rapidly identify residues important for protein function by alanine substitution, should be carefully conducted. Further studies will reveal the functional significance of the RNA and protein function in this region.

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SC29EK, a Peptide Fusion Inhibitor with Enhanced α-Helicity, Inhibits Replication of Human Immunodeficiency Virus Type 1 Mutants Resistant to Enfuvirtide[▽]

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Peptides derived from the α-helical domains of human immunodeficiency virus (HIV) type 1 (HIV-1) gp41 inhibit HIV-1 fusion to the cell membrane. Enfuvirtide (T-20) is a peptide-based drug that targets the step of HIV fusion, and as such, it effectively suppresses the replication of HIV-1 strains that are either wild type or resistant to multiple reverse transcriptase and/or protease inhibitors. However, HIV-1 variants with T-20 resistance have emerged; therefore, the development of new and potent inhibitors is urgently needed. We have developed a novel HIV fusion inhibitor, SC34EK, which is a gp41-derived 34-amino-acid peptide with glutamate (E) and lysine (K) substitutions on its solvent-accessible site that stabilize its α-helicity. Importantly, SC34EK effectively inhibits the replication of T-20-resistant HIV-1 strains as well as wild-type HIV-1. In this report, we introduce SC29EK, a 29-amino-acid peptide that is a shorter variant of SC34EK. SC29EK blocked the replication of T-20-resistant HIV-1 strains and maintained antiviral activity even in the presence of high serum concentrations (up to 50%). Circular dichroism analysis revealed that the α-helicity of SC29EK was well maintained, while that of the parental peptide, C29, which showed moderate and reduced inhibition of wild-type and T-20-resistant HIV-1 strains, was lower. Our results show that the α-helicity in a peptide-based fusion inhibitor is a key factor for activity and enables the design of short peptide inhibitors with improved pharmacological properties.

The envelope proteins of human immunodeficiency virus (HIV) type 1 (HIV-1) exist as functional trimeric complexes of gp120-gp41 heterodimers and play an important role in viral entry into host cells. Interactions of gp120 with CD4 molecules expressed on the cell surface cause structural changes that allow further interactions with the CXCR4 or CCR5 coreceptor. These interactions also induce a conformational change in gp120 that initiates gp41-mediated membrane fusion that leads to viral entry (4). In the process of fusion, the amino-terminal heptad repeat (N-HR) of gp41 trimer interacts with the carboxyl-terminal heptad repeat (C-HR) of gp41 trimer to form a six-helix bundle that makes viral and cell membranes accessible (3).

Peptides derived from N-HR or C-HR, such as N36 (3, 18) and enfuvirtide (T-20) (30), suppress the six-helix bundle formation, resulting in the inhibition of membrane fusion. T-20 blocks the entry of various HIV-1 strains, even those resistant to inhibitors of reverse transcriptase and/or protease (15, 16). However, T-20-resistant HIV-1 variants, which frequently show mutations in gp41, such as V38A and N43D, have emerged (14, 25, 26, 28, 32). Therefore, novel fusion inhibitors that suppress the replication of T-20-resistant variants are urgently needed.

C34, a C-HR-derived peptide (Fig. 1A), also inhibits fusion in vitro and does so much more efficiently than T-20 (3, 18, 22). Previously, we remodeled C34 by introducing amino acid substitutions that resulted in highly soluble and active derivatives (24). We replaced amino acids at the solvent-accessible site of the helical bundle with glutamate (E) and lysine (K) and maintained those at the interactive site, as these are critical for the interaction with N-HR. In an α-helical heptad repeat, residues separated by three positions (position i versus position $i + 4$) are located on the same side of the helix and are closely positioned in space (Fig. 1B). Hence, we introduced consecutive EK motifs separated by three residues (E at positions i and $i + 1$ and K at positions $i + 4$ and $i + 5$) of the solvent-accessible site of C34, which resulted in a repeat of the following type: X-EE-XX-KK (where X indicates the original amino acid in HIV-1). A C34 derivative, SC34EK, which has two complete and three incomplete X-EE-XX-KK motifs (Fig. 1), showed enhanced anti-HIV-1 activity compared with that of the parental peptide, C34 (24). A similar result was obtained with T-20EK, the peptide derived by introducing this motif into T-20 (23). Circular dichroism (CD) analysis revealed that both the α-helicity of SC34EK and the thermal stability of the N36-SC34EK complex were enhanced. Interestingly, the antiviral activity of SC35EK, which has five complete X-EE-X X-KK motifs, was comparable to that of SC34EK (24), indicating that five complete X-EE-XX-KK motif repeats are not

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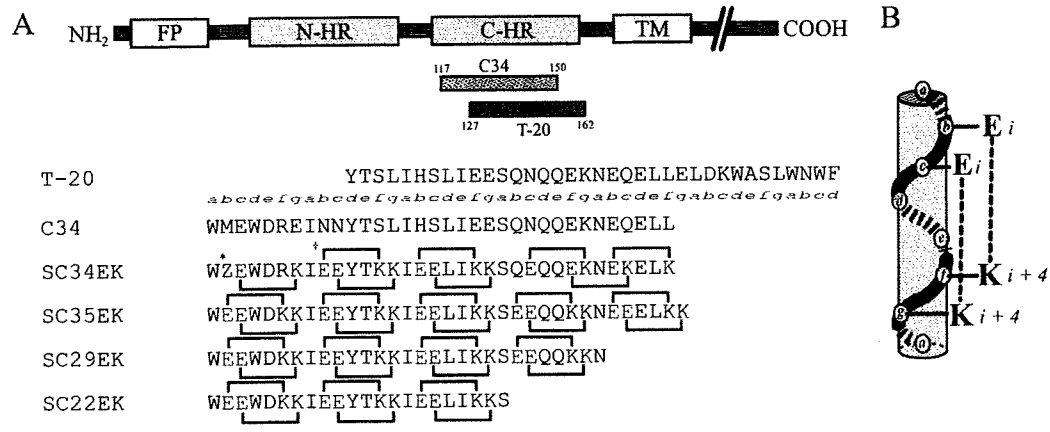


FIG. 1. (A) Schematic diagram of HIV-1 gp41 and sequences of C-HR-derived peptides. FP, fusion peptide; TM, transmembrane domain. The residues at each position in the helical turns are denoted in italics. *Z, an artificial amino acid, norleucine, instead of methionine, to avoid oxidation of the side chain of methionine; †, possible electrostatic interactions indicated by lines and correlating amino acids. (B) One heptad helical turn.

required for strong anti-HIV-1 activity. To address how many complete X-EE-XX-KK motifs are involved in the potent antiviral activity of SC34EK, we synthesized SC29EK and SC22EK (Fig. 1), which contain four and three complete repeats of X-EE-XX-KK, respectively, and evaluated them for their activities against T-20-resistant viruses.

MATERIALS AND METHODS

Cells. HeLa CD4/LTR-β-galactosidase cells obtained from M. Emerman through the AIDS Research and Reference Reagent Program (Germantown, MD) and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, and 50 μg/ml streptomycin.

Viruses. An HIV-1 infectious clone, pNL4-3 (1), was used for the construction and production of HIV-1 clones. Clones with a certain resistance mutation(s) were introduced by site-directed mutagenesis (29) into the pNL4-3 construct. Although the vast majority of HIV-1 strains have a glycine (G) at position 36 in gp41, the NL4-3 strain used in this study has an aspartic acid (D) residue, which results in impairment of the fusion kinetics of HIV-1 (13, 17). Therefore, in this study, we first constructed a D36G clone, pNL4-3_{D36G}, and used this as a template for the introduction of T-20-resistant mutations, as described previously (21, 31). We constructed three T-20-resistant clones, HIV-$1_{D36G/V38A}$, HIV-$1_{D36G/N43D}$, and HIV-$1_{D36G/N43D/S138A}$ (8), and two C34-resistant clones, HIV-$1_{D36G/N126K}$ and HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$ (ΔV4 indicates a five-amino acid [FNSTW] deletion in the V4 region of gp120) (22). Infectious HIV-1 clones were generated by transfection of plasmid clones into 293T cells.

Antiviral agents. Peptide-based fusion inhibitors, including T-20, were synthesized by standard 9-fluorenylmethoxy carbonyl-based solid-phase techniques (24). High-pressure liquid chromatography purification of crude materials on a preparative Cosmosil 5C18 AR-II column with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid gave the desired peptide samples for biological tests. 2′,3′-Dideoxycytidine (ddC) was purchased from Sigma-Aldrich (St. Louis, MO).

Determination of efficacies of antiviral agents. The efficacies of the antiviral agents were determined by multinuclear activation of galactosidase indicator (MAGI) assays (12, 22). Briefly, 10^4 HeLa CD4/LTR-β-galactosidase cells per well were plated in flat 96-well culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI U/well, which gave 60 blue cells after 48 h of incubation) and were cultured in the presence of various concentrations of drugs in fresh medium. After incubation for 48 h after virus inoculation, all of the blue cells that were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside in each well were counted. The activities of the test compounds were determined as the concentration that blocked HIV-1 replication by 50% (the 50% effective concentration [EC_{50}]).

Effect of sera on anti-HIV activity. The effect of the FCS concentration on antiviral activity was measured by MAGI assays with FCS at several concentrations (5, 10, 20, and 50%). The effect of serum components on antiviral activity was assessed by MAGI assays. Briefly, T-20 or SC29EK was dissolved in phosphate-buffered saline (PBS), FCS, or serum freshly prepared from HIV-seronegative healthy volunteers at 4 μM; and the mixtures were incubated for 2 h at 37°C. The mixture was diluted to a concentrations of about 1× or 5× the EC_{50} by using a DMEM-based complete medium supplemented with 10% FCS and was subjected to the MAGI assay.

CD analysis. N36- and C-HR-derived peptide complexes were incubated at 37°C for 30 min (final peptide concentration, 10 μM in PBS). The CD spectra were acquired on a spectropolarimeter (model J-710; Jasco Inc., Tokyo, Japan) at 25°C as the average of eight scans. Thermal stability was assessed by monitoring the change in the CD signal at 222 nm as a function of temperature. Thermal unfolding at intervals of 0.5°C was performed after a 15-min equilibration at the desired temperature and an integration time of 1.0 s. The midpoint of the thermal unfolding transition (melting temperature [T_m]) of each complex was determined from the maximum of the first derivative, with respect to the reciprocal of the temperature, of the $[\theta]_{222}$ values.

RESULTS

Antiviral activities of peptides into which EK is introduced. Because W117, W120, and I124, which are crucial for binding to N-HR (2, 3), are located in the N terminus of C34, we deleted the C-terminal region of SC35EK to produce short peptides. SC29EK, which has four complete X-EE-XX-KK motifs, inhibited HIV-1_{NL4-3} infection at a level comparable to that at which SC34EK did (Table 1). As was observed with SC34EK, SC29EK also maintained an inhibitory effect toward T-20-resistant clones. Although SC34EK blocked the replication of C34-resistant clone HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$, SC29EK failed to do so. On the other hand, C29, with a 5-amino-acid deletion from the C terminus of C34, exerted drastically reduced antiviral activity. SC22EK, which consisted of three X-EE-XX-KK motifs, also showed much reduced antiviral activity compared with the activities of SC29EK and SC34EK. A native peptide corresponding to SC22EK, C22, exhibited no activity against HIV-1_{NL4-3} at concentrations up to 10 μM (data not shown). Thus, to inhibit the physiological interaction of N-HR and C-HR, a peptide 22 amino acids in length, without modification, may be insufficient. The D36G substitution enhanced the susceptibility of HIV-1 to T-20 (28) but not to C34 or its derivatives (Table 1). These results suggest that four X-EE-XX-KK motifs are required to maintain

TABLE 1. Activities of HIV-1 gp41-derived peptides against T-20-resistant mutants

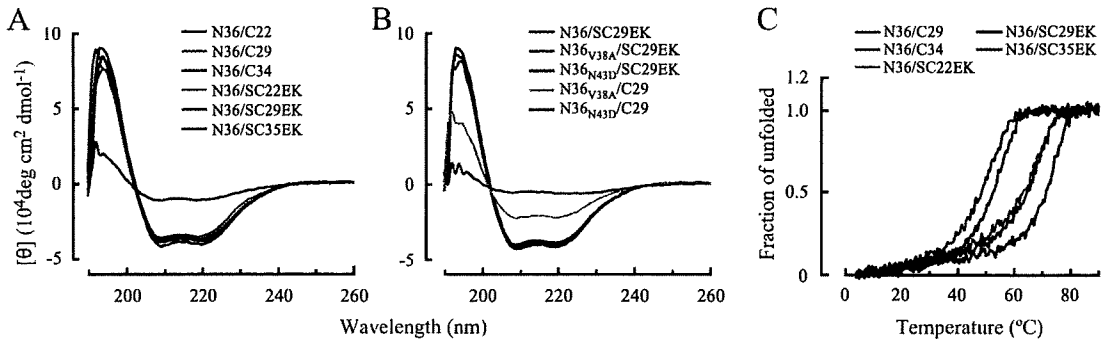
Virus	EC_{50}[a] (nM)					
	T-20	SC22EK	C29	SC29EK	C34	SC34EK
$HIV\text{-}1_{NL4\text{-}3}$	15 ± 1 (6.3)	217 ± 41 (0.3)	245 ± 42 (4.7)	2.4 ± 0.1 (1.3)	2.3 ± 0.1 (1.0)	1.6 ± 0.2 (0.7)
$HIV\text{-}1_{D36G}$	2.4 ± 0.6	686 ± 94	52 ± 18	1.9 ± 0.0	2.3 ± 0.6	2.4 ± 1.0
$HIV\text{-}1_{D36G/V38A}$	23 ± 8 (9.6)	289 ± 84 (0.4)	504 ± 193 (9.7)	3.0 ± 0.6 (1.6)	4.4 ± 1.4 (1.9)	2.2 ± 0.4 (0.9)
$HIV\text{-}1_{D36G/N43D}$	49 ± 10 (20)	114 ± 36 (0.2)	>1,000 (>19)	4.1 ± 0.6 (2.2)	7.9 ± 0.9 (3.4)	1.6 ± 0.4 (0.7)
$HIV\text{-}1_{D36G/N43D/S138A}$	84 ± 16 (35)	>1,000 (>1.5)	>1,000 (>19)	3.4 ± 0.9 (1.8)	15 ± 2 (6.4)	1.5 ± 0.3 (0.6)
$HIV\text{-}1_{D36G/N126K}$	3.4 ± 0.6 (1.4)	>1,000 (>1.5)	192 ± 22 (3.7)	2.7 ± 0.1 (1.4)	7.0 ± 2.0 (3.0)	12 ± 1 (5.0)
$HIV\text{-}1_{\Delta V4/D36G/I37K/N126K/L204I}$[b]	390 ± 155 (163)	252 ± 71 (0.4)	>1,000 (>19)	50 ± 11 (26)	171 ± 15 (74)	3.0 ± 0.2 (1.3)

[a] Antiviral activity, shown as the EC_{50}, was determined by the MAGI assay. Each EC_{50} represents the mean ± standard deviation obtained from at least three independent experiments. The values in parentheses indicate relative changes (*n*-fold) in the EC_{50} compared with the EC_{50} in the presence of the D36G substitution.
[b] ΔV4 indicates a 5-amino-acid deletion (FNSTW) in the V4 region of gp120.

the inhibitory effect of the peptides on the membrane fusion of HIV-1 strains resistant to T-20, as well as $HIV\text{-}1_{NL4\text{-}3}$.

α-Helicity of the six-helix bundle. To elucidate the mechanism by which SC29EK exerts strong anti-HIV activity, we performed CD analysis of the N36-SC29EK complex. The CD spectrum for the N36-SC29EK complex revealed an α-helix conformation with a characteristic double minimum at 208 nm and 222 nm, similar to the conformations of the N36-C34 and N36-SC35EK complexes. The N36-C29 complex showed an α-helical conformation, while a complex of N36 with C22 showed decreased α-helical spectra (Fig. 2A), in direct correlation to moderately and severely decreased antiviral activities of C29 and C22, respectively. The CD spectra of complexes of N36 peptides containing T-20 resistance-associated mutations with SC29EK, $N36_{V38A}$-SC29EK and $N36_{N43D}$-SC29EK, were almost identical to the CD spectrum of N36 with SC29EK, indicating that SC29EK retains binding affinity for the mutated N36 peptides (Fig. 2B). On the other hand, the mutated N36 peptides and C29 complexes showed little α-helical conformation. These results indicate that introduction of the X-EE-X X-KK motif increases the binding affinity of SC29EK for the mutated N36 peptides.

The thermal stabilities of these complexes were assessed by monitoring the shift in $[\theta]_{222}$ (Fig. 2C). A relatively low T_m (48.5°C) (Fig. 2D) and approximately 80% α-helicity at 37°C (Fig. 2C) were observed with the N36-C29 complex, consistent with its moderate antiviral activity (Table 1). The T_ms of N36- and C-HR-derived peptides into which a X-EE-XX-KK motif was introduced were higher than the T_m of the N36-C34 complex (Fig. 2D), while the T_ms of peptides with the native se-



D

peptide N-HR	peptide C-HR	T_m (°C)
N36	C29	48.5
N36	C34	52.5
N36	SC22EK	63.5
N36	SC29EK	65
N36	SC35EK	71.5
$N36_{V38A}$	C29	41
$N36_{V38A}$	SC29EK	56
$N36_{N43D}$	C29	NA
$N36_{N43D}$	SC29EK	57

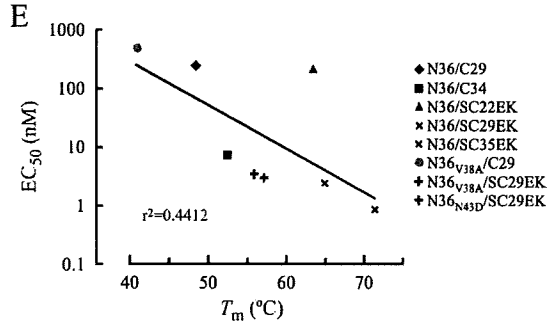


FIG. 2. Analysis of N36 and various C-HR-derived peptides complexes by CD spectroscopy. CD spectra for N36- and C-HR-derived peptide complexes (A) and mutated N36-C29 or SC29EK complexes (B). (C) Temperature-dependent transitions of the dissociation degree of N36 and various C-HR-derived peptide complexes. (D) T_ms of complexes of various N-HR peptides and C-HR peptides. NA, not available. (E) Relation between EC_{50}s of C-HR-derived peptides and T_ms of N36 and various C-HR-derived peptide complexes. The strength of the correlation between EC_{50}s and T_ms is increased ($r^2 = 0.8002$) when the data for SC22EK are excluded.

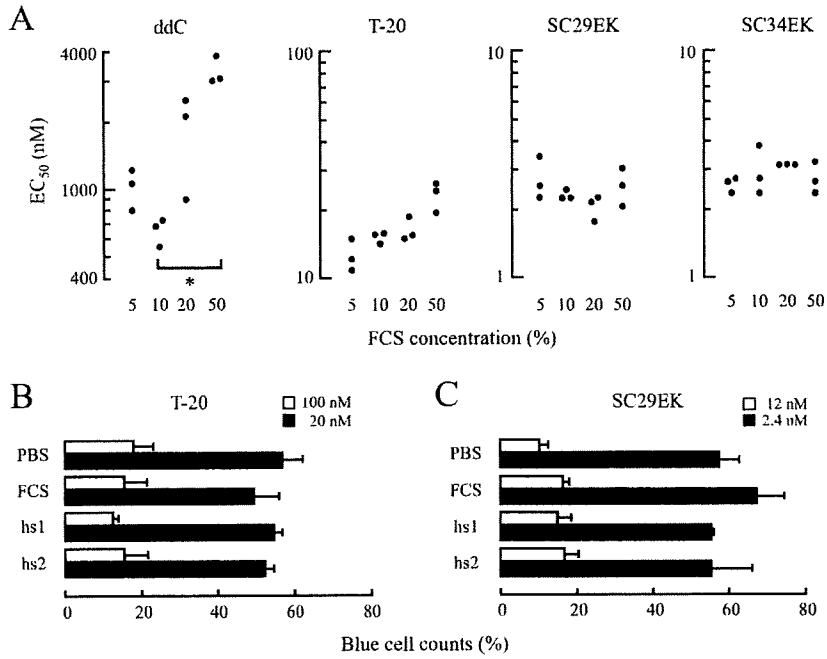


FIG. 3. Effect of serum components on antiviral activity. Antiviral activities in the presence of serum at various concentrations (5, 10, 20, and 50%) were determined by the MAGI assay. (A) Black dots indicate EC_{50}s (nM), each of which was determined three times independently (*, $P = 0.01$ by Student's t test). Antiviral activities of T-20 (B) and SC29EK (C) in human HIV-seronegative sera (hs1 and hs2) were assessed by counting the number of blue cells. Bars indicate the percentages of blue cell counts in PBS, FCS, and human serum compared with the count obtained with no antiviral agents (control, for which the value was 100%). Error bars represent the standard deviation of each mean.

quence but without the introduced motif were lower. The relationships between the EC_{50}s of C-HR-derived peptides and their T_ms are shown in Fig. 2E. The correlation between the EC_{50} and the T_ms was weak ($r^2 = 0.4412$); however, with the exclusion of the data for SC22EK, which showed weak antiviral activity, despite its high T_m, the strength of this correlation was increased ($r^2 = 0.8002$), suggesting that other factors, including solubility and intrapeptide interactions, may be involved in the enhanced antiviral activity of EK-containing peptides.

Effect of serum on antiviral activity. Finally, we assessed the anti-HIV activity of SC29EK in the presence of a high concentration of FCS and in fresh human serum. The activities against HIV-1_{NL4-3} in the presence of various concentrations of FCS (5, 10, 20, and 50%) were determined. A reverse transcriptase inhibitor, ddC, was used as a control. The antiviral activity of ddC was decreased in a concentration-dependent manner (Fig. 3A). In the presence of 50% FCS, the reduction in the EC_{50} of ddC was statistically significant ($P = 0.01$). Similarly, but to a much lesser extent, the EC_{50} of T-20 appeared to be reduced with the FCS concentration in a concentration-dependent manner. Even in the presence of 50% FCS, the mean EC_{50} was comparable to the EC_{50}s in 5% and 10% FCS ($P = 0.082$ and 0.075, respectively). However, the effects of SC29EK and SC34EK were less affected by increased FCS concentrations.

For further evaluation, sera freshly isolated from two HIV-seronegative healthy volunteers were prepared. T-20 and SC29EK incubated for 2 h at 37°C in fresh human serum, FCS, or PBS were diluted with a DMEM-based complete medium supplemented with 10% FCS and were subjected to the MAGI assay. The final FCS concentrations in the various sera that included FCS in these diluted mixtures ranged from 9.75 to 12.2%. Because the antiviral activities of T-20 and SC29EK were not significantly influenced by the FCS concentration (Fig. 3A), it is unlikely that the differences in the FCS concentrations in this experiment had any effect on their antiviral activities. Compared with the inhibitory effects of the PBS-treated peptides, small changes in the inhibitory effects of both T-20 and SC29EK treated with FCS and human sera were observed (Fig. 3B and C). Taken together, these findings suggest that SC29EK stably exerts its strong anti-HIV-1 activity in vivo in the same manner that T-20 does.

DISCUSSION

We show here that SC29EK inhibits the membrane fusion of T-20-resistant HIV-1 strains, suggesting that four X-EE-X X-KK motifs are sufficient to inhibit the fusion of T-20-resistant variants. As revealed by the EC_{50}s and T_ms (Table 1 and Fig. 2), resistance-associated mutations in the N-HR region, such as V38A and N43D, seem to decrease the binding affinity of C-HR-derived peptides for N-HR. Therefore, HIV-1 strains with V38A or N43D show resistance to T-20. However, the anti-HIV-1 activity of SC29EK was less affected by these mutations, because at the physiological temperature for HIV-1 replication, SC29EK showed a stable interaction with N36 peptides containing mutations conferring resistance to T-20. The activity of SC29EK against the C34-resistant clone HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$ was decreased, while SC34EK maintained its activity. One of the primary mutations underlying C34 resistance, I37K, is located close to but outside of the

putative binding site of SC29EK. Previously, we reported that an N126K substitution in C-HR enhances the intra-gp41 binding of N-HR and C-HR (22); therefore, we hypothesized that the activity of SC29EK might be decreased by competition with C-HR with the N126K mutation. However, SC29EK also inhibits the entry of HIV-$1_{D36G/N126K}$. Although no structural analysis of the mutated six-helix bundle was performed, it is possible that mutations conferring C34 resistance might induce some structural changes at or adjacent to the SC29EK binding site, because a peptide shortened by a further 7 amino acids, SC22EK, suppressed the entry of the C34-resistant clone.

C34 itself did not have an α-helical spectrum, while SC29EK did (data not shown). SC29EK may achieve its strong antiviral activity by forming an α-helix as a result of E/K substitutions on the solvent-accessible site (Fig. 1). CD analysis shows that HIV-1 builds up resistance to T-20 by introducing certain mutations in N-HR, such as V38A and N43D, which reduce the binding affinity between N-HR and C-HR. SC29EK can efficiently inhibit the fusion of these mutant HIV-1 strains, suggesting that the ability of SC29EK to bind to mutated N-HR and its weak affinity for C-HR are maintained. On the other hand, the D36G, N126K, and S138A mutations increase viral fusion activity (13) by enhancement of the binding affinity of C-HR for N-HR (22, 31). SC29EK effectively suppresses the replication of viruses that have these mutations, such as HIV-1_{D36G}, HIV-$1_{D36G/N43D/S138A}$, and HIV-$1_{D36G/N126K}$. This indicates that the binding capacity of SC29EK is stronger than that of mutated C-HR containing the N126K or the S138A mutation. Therefore, the monomeric α-helical form may inhibit the interactions of N-HR and C-HR with mutations that affect their binding affinity and thus the formation of the six-helix bundle.

Although SC22EK has enhanced α-helicity and a high T_m, it has less antiviral activity than SC29EK. In the interaction between N-HR- and C-HR-derived peptides, while the cavity-forming region (from L54 to Q66) of the C terminus of N-HR (the "pocket") and the cavity-binding region (side chains of W117, W120, and I124) of the N terminus of C-HR (the "knob") play an important role (2, 3, 10), another region of C-HR may also be required. A constrained 14-residue peptide (C14linkmid), which corresponds to the knob region, shows chemical cross-linking and contains amino acid substitutions (27), and it is about 15,000-fold less active than SC29EK, which contains proximal regions in addition to the knob region. These findings also suggest that the knob region of C-HR is important but not sufficient for the formation of a stable complex. Another possible explanation of the weak activity of C14linkmid is that because not only the binding of N-HR and C-HR but also dynamic structural changes are easily anticipated during fusion, it would be difficult to maintain tight binding to the target N-HR due to its rigid constrained form. To maintain the binding of C-HR to N-HR despite such drastic conformational changes during fusion, there may be some unknown interaction, besides the interaction between the pocket and the knob regions, that is necessary for membrane fusion. At present, we cannot conclude whether (i) the length of the peptide itself is crucial, (ii) some other domain has a role, or (iii) a combination of both is important. Further experiments will be needed to clarify the mechanism of inhibition. Such information will be valuable for the generation of effective short peptide inhibitors or small molecules. To generate effective small-molecule inhibitors, if the second possibility is correct, a combination of two agents, one of which interacts with the pocket and the other of which interacts with an unidentified domain, should provide enhanced efficacy. To date, only a limited number of small-molecule compounds that inhibit the six-helix bundle formation with marginal activities have been reported (5, 9, 11), although among the peptide-based inhibitors, several effective peptides have been developed, including T1249 (7), SC34EK (24), T2635 (6), and T-20EK (23).

The T_m of the N36-SC29EK complex was higher than that of the N36-C29 complex, suggesting that EK substitutions reinforced the affinity of binding to N-HR through enhanced α-helicity. It has been considered that the enhanced α-helical structure is maintained by intrahelical salt bridges formed by the introduction of EK substitutions (19). We recently revealed that an electrostatic interaction formed by the EK alignment is involved in enhanced α-helicity (22a), indicating that the strong α-helical stability of SC29EK is probably provided by a mechanism identical to that for SC34EK. Similar peptides with substitutions of glutamate and arginine provided to increase α-helicity have been reported (6). These peptides also increase the stability of the helix and have activity against T-20-resistant HIV-1. Moreover, these peptides were relatively stable in an in vivo model. It is possible that enhanced binding affinity confers nonspecific binding to other α-helical regions of cellular proteins, for example, human serum albumin, which contains 31 α-helical regions (20). However, this effect will be minimal, because the antiviral activity of SC29EK was highly stable in the presence of higher concentrations of FCS and was less affected by human serum.

In this study, we demonstrated that a 29-amino-acid short peptide, SC29EK, suppresses the replication of T-20-resistant variants. SC29EK maintained its activity in the presence of high concentrations of sera, indicating that SC29EK is a candidate short peptide fusion inhibitor.

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