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２．学会発表

なし

Ｈ．知的財産権の出願・登録状況

なし

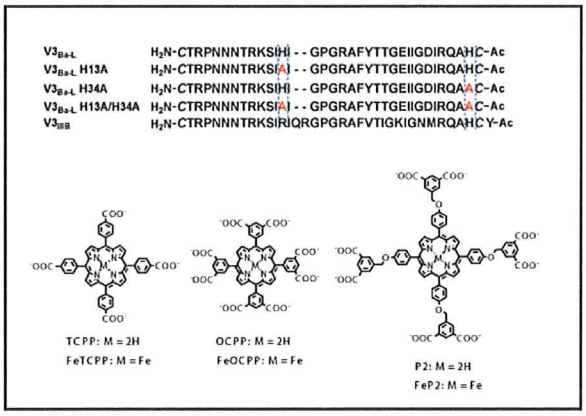

Figure 1. Amino acid sequences of the V3Ba-L, V3Ba-LH13A, V3Ba-LH34A, V3Ba-LH13A/H34A, and V3IIIB peptides (A) and multivalent anionic porphyrins and their iron complexes used in this work (B).

Table 1. Anti-HIV activity of porphyrin derivatives

Inhibitor	EC_{50} [a]		CC_{50} [b]
	HIV-1_{Ba-L}	HIV-1_{IIIB}	
		μM	
AZT	0.034 ± 0.009	0.023 ± 0.010	>10
DS5000 [(c)]	0.27 ± 0.021	0.36 ± 0.021	>21
AMD3100	>0.2	0.0034 ± 0.0002	>0.2
TCPP	4.7 ± 0.15	2.6 ± 0.23	40 ± 5
FeTCPP	0.89 ± 0.21	1.4 ± 0.30	>100
OCPP	1941 ± 196	1699 ± 562	36 ± 5
FeOCPP	353 ± 25	488 ± 187	>100
P2	187 ± 29	205 ± 46	35 ± 5
FeP2	116 ± 16	59 ± 14	>100

[a]The EC_{50} values were determined by the MAGI assay.
[b]The CC_{50} values were determined by the MTT method.

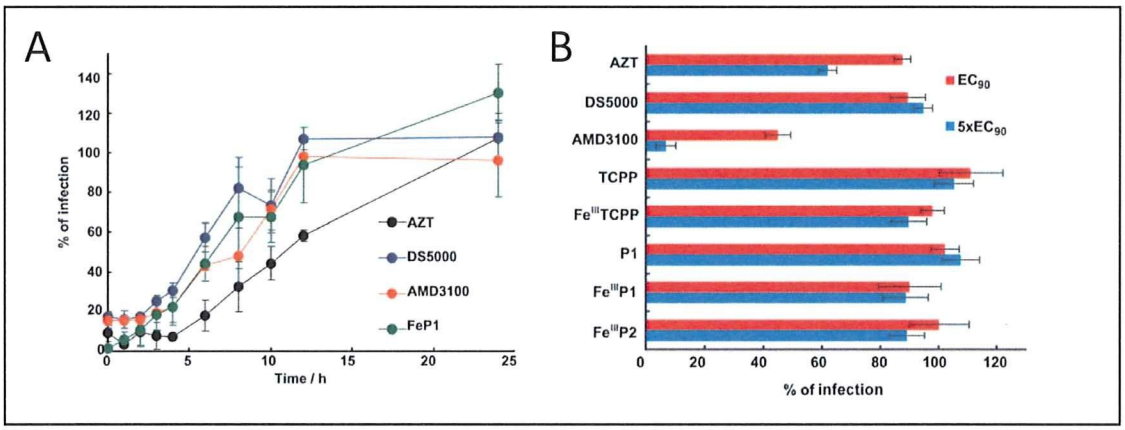

Figure 2. Time of drug-addition profiles for infection of the HIV-1_{IIIB} strain to HeLa-CD4/CCR5-LTR/β-gal cells (A) and preincubation effects on the anti-HIV-1 activity (B). Each drug with the concentrations of EC_{90} and 5xEC_{90} was preincubated with HeLa-CD4/CCR5-LTR/β-gal cells for 2 h and the preincubated samples were washed 5 times with phosphate buffer solution (PBS) before inoculation with the HIV-1_{IIIB} strain.

厚生労働科学研究費補助金（政策創薬総合研究事業）
分担研究報告書

HIV 吸着・膜融合過程を標的とする多剤耐性克服型耐性克服型 HIV 化学療法剤の開発

研究代表者：京都大学ウイルス研究所　　松岡雅雄
研究分担者：国立感染症研究所エイズ研究センター　村上　努

研究要旨: 本分担研究の目的は、CXCR4 阻害剤を材料として試験管内で耐性誘導を行い、耐性変異のパターンや耐性機構を解析することにより、より耐性の出にくい薬剤をデザインしたり、耐性変異パターンを予測することである。平成 18 年秋に開始した KRH-3955 と KRH-3148 を用いた PM1/CCR5-NL4-3 の感染系による薬剤耐性誘導実験はそれぞれ培養開始時の約 5 倍、約 3 5 倍の薬剤耐性ウイルスが得られており、それらの Env 領域に蓄積された変異を解析中である。さらに、平成 21 年度は、CXCR4 阻害剤 KRH-3955 を用いて異なる Donor からの PBMC への感染防御活性の比較、その持続的阻害活性について検討した。また、カニクイサル AIDS モデルにおいて事前内服による感染予防効果があるか否かについても評価し、KRH-3955 事前内服が末梢血 CD4 陽性細胞数の保持に一定の効果が認められた。

A.　研究目的

本研究班の課題である HIV 吸着・膜融合過程を標的とする多剤耐性克服型 HIV 化学療法剤の開発に関して、共同研究者（株）クレハが開発した KRH-1636 の誘導体で、経口吸収性を示す CXCR4 阻害剤 KRH-3955 に関して行った研究成果を報告する。本研究の目的は、新しい作用機序を有する HIV 阻害剤として期待される CXCR4 阻害剤に対する耐性 HIV-1 を誘導し、その耐性変異のパターンや耐性機構を解析することによって、より耐性の出にくい薬剤をデザインしたり、耐性変異パターンを予測することである。 材料としては、共同研究者（株）クレハが開発した経口吸収性を示す 2 種類の高活性 CXCR4 阻害剤 KRH-3955、 KRH-3148 を使用した。また、KRH-3955 と KRH-3148 を用いた PM1/CCR5-NL4-3 の感染系による薬剤耐性誘導実験は、AMD3100、AMD070 を対照薬剤として使用して行い、現在も継続中である。

B.　研究方法

（1）抗 HIV-1 活性測定：固定化抗 CD3 抗体で刺激し、IL-2 存在下で増殖させた PBMC を標的細胞として HIV-1 を MOI=0.001 で感染させ、種々の濃度の薬剤存在下で 7 日から 10 日培養した。抗 HIV-1 活性は培養上清中の p24 抗原量を市販の ELISA を用いて測定後算出した。

（2）CCR5 阻害剤との併用効果：KRH-3955 と CCR5 阻害剤 SCH-D の併用によって、PBMC への X4 HIV-1(NL4-3)と R% HIV-1(JR-CSF) 同時感染を防御できるかを検討した。

（3）異なる donor からの PBMC への感染防御試験： KRH-3955 が、8 人から分離した PBMC への NL4-3 の感染をばらつきなく抑制するかを検討した。

（4）KRH-3955 による抗 CXCR4 抗体 12G5 結合の持続的阻害活性の測定：Molt-4 細胞を on ice で薬剤処理した後細胞を洗浄し（または洗浄せず）、抗体反応後結合した CXCR4 抗体量を FACS にて定量した。また、薬剤処理した後細胞を洗浄したのち、37C で一定時間（0, 3, 6 時間）細胞を静置し、12G5 結合阻害活性を測定した。

（5）事前内服による感染予防効果の検討(共同研究者：エイズ研究センター仲宗根主任研究官)：カニクイサルに SHIV-KS661$_C$ を感染させる AIDS モデルにおいて KRH-3955 を 100 mg/kg で感染 24 時間前に投与（対照薬剤：TDF/FTC は、30/20 mg/kg を感染 24 時間前と感染直前の 2 回投与）後、10 AID50 の SHIV-KS661$_C$ を経直腸感染させ、12 週にわたって観察する。

主なモニター項目は、CBC、CD4/CD8 細胞数、ウイルス量、などである（図１）。

（6）CXCR4 阻害剤耐性 HIV-1 誘導実験

PM1/CCR5 細胞(共同研究者　熊本大・前田先生分与）を標的細胞として、X4 株である NL4-3 をウイルスとして用い、KRH-3955、KRH-3148、AMD3100、AMD070 の４薬剤について平成 18 年 10 月に開始した。各薬剤の EC50 付近の濃度を初発濃度としてウイルス培養を開始し、薬剤耐性ウイルスの出現を CPE や培養上清中の p24 濃度で判断して、添加する薬剤濃度を段階的に増加させる。

（倫理面での配慮）

本研究で使用を予定する複製可能な組換えウイルスは大臣確認を得ている（大 19-6 および大 17-23）ほか、組換え DNA 実験の申請は本研究所に機関承認されている（機 19-63 ほか）。

C.　研究結果

（１）CCR5 阻害剤との併用効果：４ nM KRH-3955+4 nM SCH-D と 20 nM KRH-3955+20 nM SCH-D の併用によって、PBMC への X4 HIV-1(NL4-3) と R5 HIV-1(JR-CSF) 同時感染(感染価として 1:1)によるウイルス複製は、それぞれ 91%、96%抑制された。

（２）異なる donor からの PBMC への感染防御試験：KRH-3955 が、８人から分離した PBMC への NL4-3 の感染をばらつきなく抑制するかを検討した結果、ウイルス複製量(培養上清上の p24 濃度)は、17-120 ng/ml とバラツキが認められたが、KRH-3955 のウイルス複製阻害活性は、EC50 で 0.23-1.3 nM、EC90 で 2.7-3.5 nM といずれの PBMC においても強力な効果を示した。

（３）KRH-3955 による抗 CXCR4 抗体 12G5 結合の持続的阻害活性の測定：Molt-4 細胞を on ice で薬剤処理した後細胞を洗浄し（または洗浄せず）、抗体反応後結合した CXCR4 抗体量を FACS にて定量した。KRH-3955 (10 nM)では、薬剤処理した後細胞を洗浄し、遊離した薬剤を除去した環境においても、12G5 結合を顕著に阻害した。対照的に、AMD3100 では未洗浄ではほぼ完全に抗体結合を阻害した 1000 nM 処理においても細胞の洗浄によってその阻害活性をほぼ失っていた。そこで次に、KRH-3955 処理した細胞を洗浄後、細胞を薬剤無添加の培地中で 37C にて一定時間（0, 3, 6 時間）細胞を静置し、12G5 結合阻害活性を測定した。その結果、洗浄後 3、6 時間においてもそれぞれ 50、40%の 12G5 結合阻害活性を保持していた。

（４）事前内服による感染予防効果の検討：KRH-3955 を 100 mg/kg で感染 24 時間前に投与した３頭の末梢血 CD4 数は、感染後３ヶ月経ってもウイルス感染時のレベルを維持していた。一方、対照薬剤の TDF/FTC では、感染後３ヶ月において３頭中２頭の末梢血 CD4 数はウイルス感染時のレベルを維持していたが、１頭は感染後１ヶ月で急激な末梢血 CD4 数の減少を起こした（図２）。

（５）CXCR4 阻害剤耐性 HIV-1 誘導実験：PM1/CCR5 細胞を標的細胞として、NL4-3 を親株とした薬剤耐性株誘導実験を 2007.10.11 に開始した。実験開始時の薬剤濃度は EC_{50} よりやや低い濃度に設定し、ほぼ４日おきに 1:5 に培養物を継代した。ウイルス感染による CPE が培養全体に観察されるようになった時点で薬剤濃度を 1.5 倍上昇させた。なお、コントロールとして薬剤無添加での感染細胞の継代培養（この場合は、CPE でほぼ完全に細胞が死滅するので培養上清のみを継代した）も併行して行った。2009 年 12 月下旬の時点での薬剤濃度は、KRH-3955、KRH-3148、AMD3100、AMD070 でそれぞれ 15、750、3000、4500 nM である。現在、それぞれの薬剤に対する耐性ウイルスについてそれらの Env 領域に蓄積した変異を解析中である（図３、図４）。

D.　考察

これまでの実験から、KRH-3955 が経口吸収性を有する強力な CXCR4 阻害剤であり、X4 HIV-1 の複製阻害剤として有望であると考えられる。今回行った 12G5 結合阻害の持続的効果の結果から、一番よく研究されている CXCR4 阻害剤 AMD3100 とは明らかに CXCR4 への親和性が異なっていた。耐性誘導実験において Env 領域に出現する変異パターンもこの２薬剤間

では異なることが予想される。

E. 結論

経口投与可能な CXCR4 阻害剤 KRH-3955 が抗 HIV-1 剤として有望であることを示すことができた。薬剤としての有効性や CXCR4 への作用様式をさらに明らかにするためまた臨床応用への可能性を評価するため耐性誘導実験を継続中である。

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G. 知的財産権の出願・登録状況 (予定を含む)

該当事項なし。

図1

薬剤投与法（前日投与）

- 投与回数：単回、２回(TDF/FTC)
- 投与ルート：経口（経鼻カテーテル）
- 剤型：3ml蒸留水に溶解
- 投与量：
  - ○ KRH-3955=100mg/kg
  - ▲ TDF/FTC= 30/20mg/kg

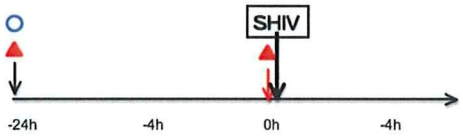


図2

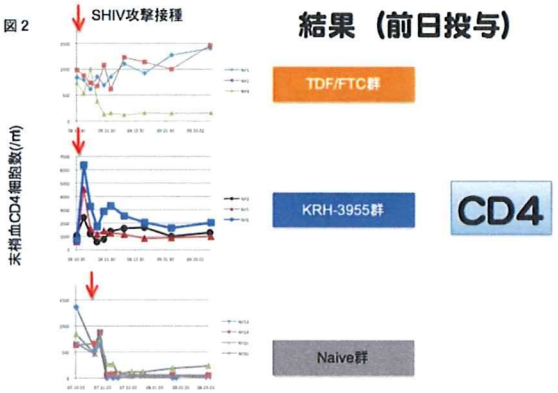


図3

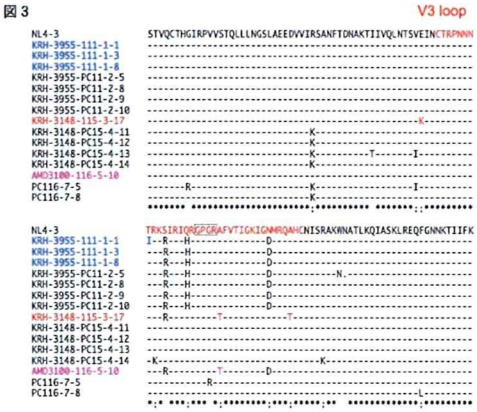
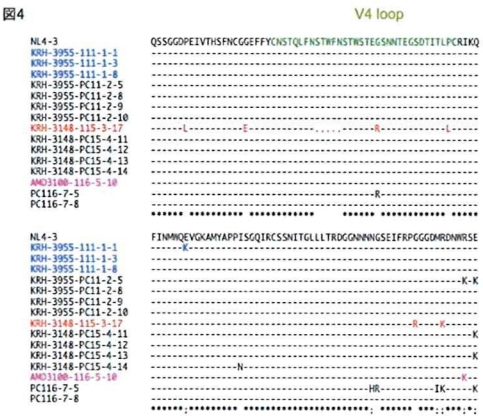


図4



研究成果の刊行に関する一覧表

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雑誌

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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-XX-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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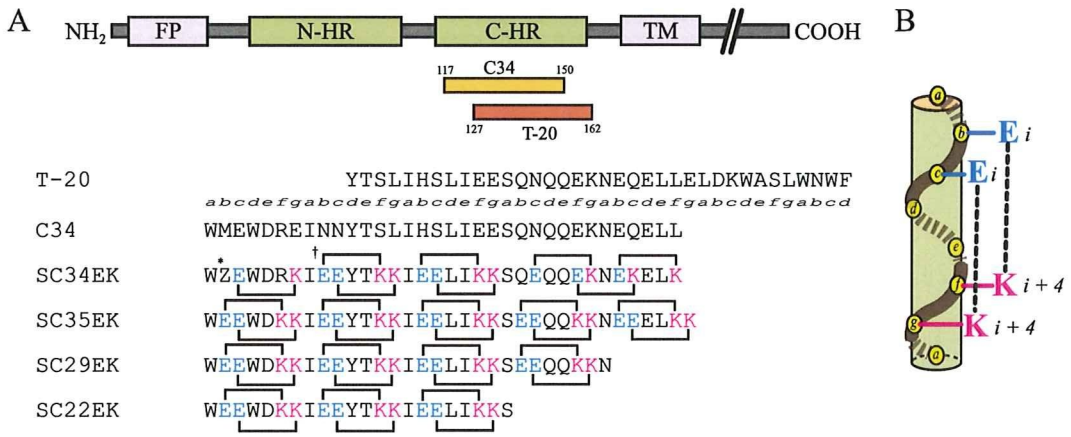


FIG. 1. (A) Schematic diagram of HIV-1 gp41 and sequences of C-HR-derived peptides. FP, fusion peptide; TM, transmembrane domain. The residues at each position in the helical turns are denoted in italics. *Z, an artificial amino acid, norleucine, instead of methionine, to avoid oxidation of the side 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

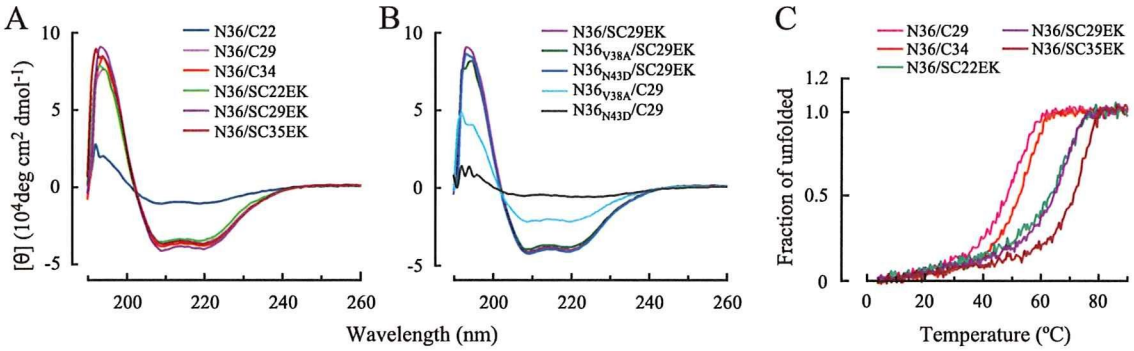
Virus	EC_{50}[a] (nM)					
	T-20	SC22EK	C29	SC29EK	C34	SC34EK
$HIV\text{-}1_{NL4\text{-}3}$	15 ± 1 (6.3)	217 ± 41 (0.3)	245 ± 42 (4.7)	2.4 ± 0.1 (1.3)	2.3 ± 0.1 (1.0)	1.6 ± 0.2 (0.7)
$HIV\text{-}1_{D36G}$	2.4 ± 0.6	686 ± 94	52 ± 18	1.9 ± 0.0	2.3 ± 0.6	2.4 ± 1.0
$HIV\text{-}1_{D36G/V38A}$	23 ± 8 (9.6)	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		T_m (°C)
N-HR	C-HR	
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

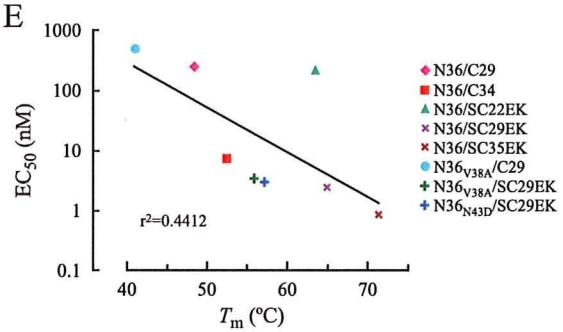


FIG. 2. Analysis of N36 and various C-HR-derived peptides complexes by CD spectroscopy. CD spectra for N36- and C-HR-derived peptide complexes (A) and mutated N36-C29 or SC29EK complexes (B). (C) Temperature-dependent transitions of the dissociation degree of N36 and various C-HR-derived peptide complexes. (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.

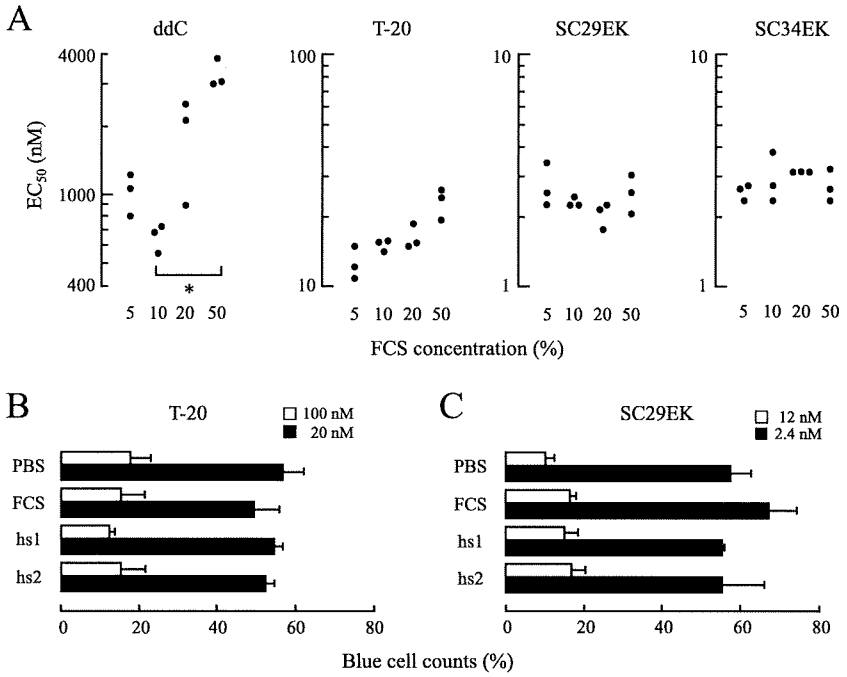


FIG. 3. Effect of serum components on antiviral activity. Antiviral activities in the presence of serum at various concentrations (5, 10, 20, and 50%) were determined by the MAGI assay. (A) Black dots indicate EC_{50}s (nM), each of which was determined three times independently (*, $P = 0.01$ by Student's 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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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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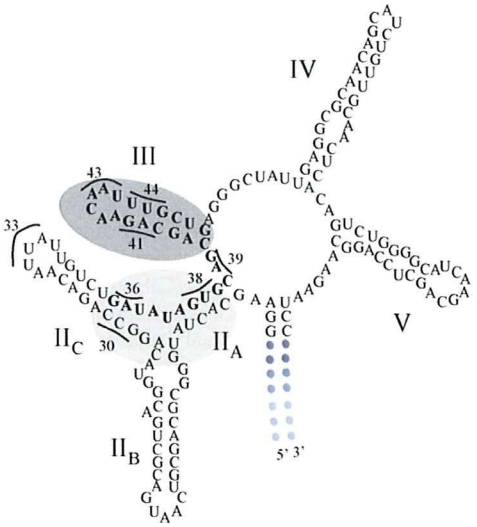
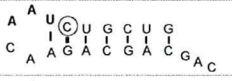
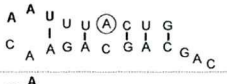
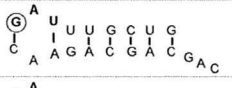
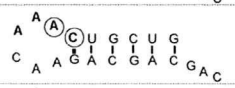
(A)



(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)

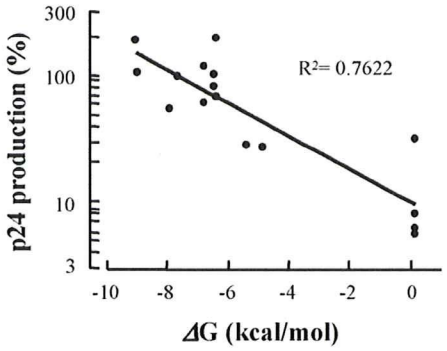


Fig. 1. (A) Secondary structure of RRE depicted based on references (Olsen et al., 1990; Zapp and Green, 1989). T-20-resistant mutations are accumulated between amino acid position from 36 to 45 of gp41 and nucleotides coding for these amino acids, are given in bold. Stem-loop II (light gray region) 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-$1_{N43D/L44L\text{-}CUG}$, HIV-$1_{Q39Q\text{-}CAA/N43D}$ and HIV-$1_{Q41Q\text{-}CAA/N43D}$, 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-$1_{D36G/N43D/L44L\text{-}CUG}$, HIV-$1_{D36G/Q39Q\text{-}CAA/N43D}$ and HIV-$1_{D36G/Q41Q\text{-}CAA/N43D}$). 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.

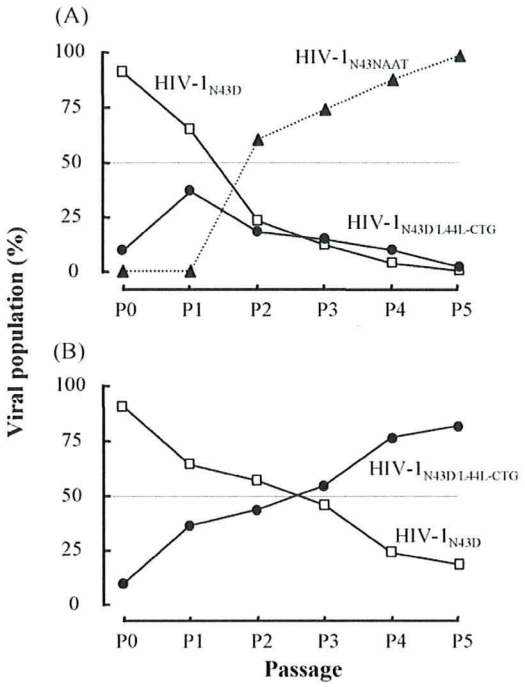


Fig. 2. Emergence of HIV-$1_{N43N\text{-}AAT}$ (HIV-1_{WT}) during CHRA for HIV-1_{N43D} and HIV-$1_{N43D/L44L\text{-}CTG}$ are shown. Open square, closed circle, and closed triangle represent HIV-1_{N43D}, HIV-$1_{N43D/L44L\text{-}CTG}$, and HIV-$1_{N43N\text{-}AAT}$, respectively. Absolute viral populations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