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Rev-derived peptides inhibit HIV-1 replication by antagonism of Rev and a co-receptor, CXCR4

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ABSTRACT

Rev, a viral regulatory protein of HIV-1, binds through its arginine-rich domain to the Rev-responsive element (RRE), a secondary structure in transcribed HIV-1 RNA. Binding of Rev to RRE mediates export of singly spliced or unspliced mRNAs from the nucleus to the cytoplasm. It has been previously shown that a certain arginine-rich peptide exhibits not only RRE-binding ability but also cell permeability and antagonism of CXCR4, one of the major coreceptors of HIV-1. Here we designed and synthesized arginine-rich peptides derived from the RNA-binding domain of Rev (Rev_{34-50}) and evaluated their anti-HIV-1 activities. Rev_{34-50}-A_4C, comprising Rev_{34-50} with AAAAC at the C-terminus to increase the α-helicity, inhibited HIV-1 entry by CXCR4 antagonism and virus production in persistently HIV-1-infected PM1-CCR5 cells. Interestingly, similar motif of human lymphotropic virus type I Rex (Rex_{1-21}) also exerted moderate anti-HIV-1 activity. These results indicate that arginine-rich peptide, Rev_{34-50}-A_4C exerts dual antagonism against CXCR4 and Rev.

1. Introduction

HIV-1 attaches to its target cells through a major receptor, CD4, and then interacts with coreceptors such as chemokine receptors to enter the cells (Este and Telenti, 2007). The T-cell-line-tropic (X4) and macrophage-tropic (R5) HIV-1 strains mainly use CXCR4 and CCR5 as coreceptors, respectively. HIV-1 completes its infection by integration of reverse-transcribed double-stranded cDNA into the host genome. Expression of HIV-1 genes is enhanced by the viral transactivator protein Tat, while regulation of the expressed genes through RNA splicing is tightly controlled by the viral regulatory protein Rev. Some expressed RNAs are protected against RNA splicing by binding of Rev to a Rev-responsive element (RRE), comprising an RNA secondary structure co-encoded with gp41, resulting in singly spliced (e.g., for *env* mRNA) or unspliced (e.g., for *gag-pol* mRNA or viral progeny genomes) RNAs, which are exported to the cytoplasm by Rev and used for viral protein synthesis and/or progeny genomes (Felber et al., 1989; Malim et al., 1989b).

Rev contains several functional signals, such as a nuclear localization signal (NLS; aa 35–50), which also serves as an RNA-binding domain (Malim and Cullen, 1991; Olsen et al., 1990), and a nuclear export signal (NES; aa 75–84) as shown in Fig. 1 (Fischer et al., 1995; Szilvay et al., 1995). The RNA structure of the RRE in these singly and unspliced RNAs plays an important role in the interaction with Rev (Charpentier et al., 1997; Dayton et al., 1989). Rev mainly binds to stem-loop IIB of the RRE and oligomerization of Rev on the RRE is required for sufficient RNA export (Daugherty et al., 2008; Kjems et al., 1992; Mann et al., 1994; Zapp et al., 1991). Another human retrovirus, human T-cell leukemia virus type I (HTLV-I), also has a regulatory protein, Rex, which is the counterpart of HIV-1 Rev. Rex interacts with not only a Rex-responsive element (RxRE) but also with the HIV-1 RRE via its RNA-binding domain (aa 1–16), which also acts as an NLS (Bogerd et al., 1991; Hammes and Greene, 1993; Siomi et al., 1988). Functionally, Rex is also involved in the nuclear export of RNAs containing an RxRE or RRE (Rimsky et al., 1988). However, Rex and Rev interact with the RRE in different manners, since Rev mainly binds to stem-loop IIB while Rex mainly binds to the stem-loop III/IV/V region (Ahmed et al., 1990; Bogerd et al., 1991; Charpentier et al., 1997; Kjems et al., 1992).

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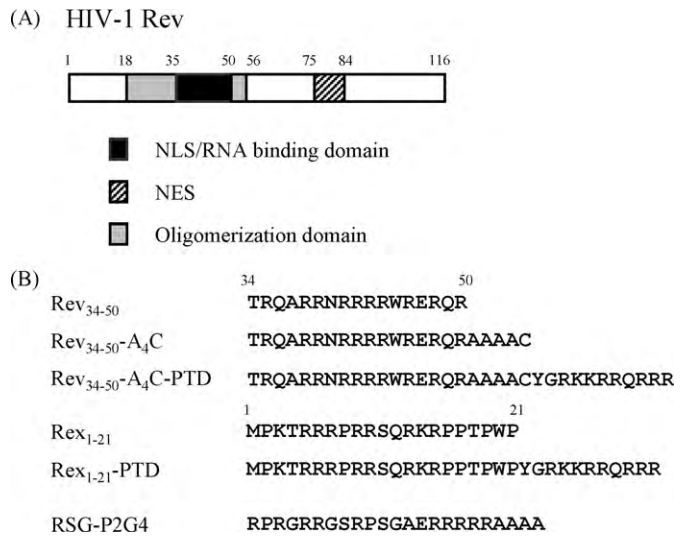

Fig. 1. Domain structure of HIV-1 Rev and sequences of Rev- and Rex-derived peptides. (A) Diagrammatic representation of HIV-1 Rev. The functional domains and regions of HIV-1 Rev are depicted. (B) Sequences of Rev-derived, Rex-derived and RSG-P2G4 peptides. The Rev- and Rex-derived peptides were synthesized based on the HIV-1_{NL4-3} (GenBank Accession No. AF324493) and HTLV-I MT-2 (GenBank Accession No. L03561) sequences, respectively. The protein transduction domain (PTD; YGRKKRRQRRR), an amino acid sequence derived from HIV-1_{NL4-3} Tat protein (aa 47–57), was added to the C-terminus of the peptides.

At least three properties seem to be required to antagonize Rev function: cell permeability, nuclear localization and specific binding to HIV-1 RRE or Rev. In this regard, Rev M10, a Rev mutant lacking the nuclear export activity, efficiently suppresses HIV-1 replication through a dominant-negative effect on Rev function (Malim et al., 1989a). However, it has several obstacles to overcome, such as immunogenicity, chemical synthesis and administration, for clinical application. In contrast, small peptides may be ideal for overcoming such obstacles. Harada et al. (1996, 1997) designed and examined artificial arginine-rich peptides as Rev antagonists in biochemical binding assays. The crystal structure of Rev/RRE has been reported (Jain and Belasco, 2001). Based on this structure (Jain and Belasco, 2001; Jiang et al., 1999), we designed peptides of 17 and 21 amino acids derived from the RRE-binding domains of Rev and Rex proteins, designated Rev_{34-50} and Rex_{1-21}, respectively, and examined their anti-HIV-1 activities. These peptides contain an arginine-rich domain, which not only binds to the RRE but also imparts cell permeability and nuclear localization (Futaki et al., 2001). Notably, it has been reported that arginine-rich peptides, e.g., ALX40-4C (N-α-acetyl-nona-D arginine), show anti-HIV-1 activity via antagonistic effects against CXCR4 (Doranz et al., 1997). We hypothesized that the arginine-rich RNA-binding domains of Rev and Rex inhibit both the entry of HIV-1 through CXCR4 and the production of progenitor HIV-1 by interrupting the function of Rev in the early and late phases of the replication cycle.

In this study, we tested this hypothesis by synthesizing Rev- and Rex-derived peptides based on their RNA-binding domains, as well as RSG-P2G4, an arginine-rich peptide that interacts with the RRE (Harada et al., 1997), and by evaluating their antiviral activities as bifunctional inhibitors that target Rev and CXCR4.

2. Materials and methods

2.1. Reagents

AZT was purchased from Sigma (St. Louis, MO). Amprenavir (APV) was kindly provided by Dr. H. Mitsuya (Kumamoto University, Kumamoto, Japan). AMD3100 was a kind gift from Dr. S. Shigeta (Fukushima Medical University, Fukushima, Japan). TAK-779 was obtained from the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD). All peptides used in this study were chemically synthesized by Fmoc-solid-phase peptide synthesis on a Rink amide resin as reported previously (Futaki et al., 1997). A phycoerythrin (PE)-conjugated mouse anti-human CXCR4 MAb (12G5) was purchased from BD Bioscience Clontech (San Jose, CA).

2.2. Cells and viruses

Two laboratory strains, HIV-1_{IIIB} and HIV-1_{Ba-L}, that mainly interact with CXCR4 and CCR5 as coreceptors, respectively, were used in this study.

MT-2 cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO). PM1-CCR5 cells, which are CCR5-transduced PM1 cells, and PM1-CCR5/IIIB and PM1-CCR5/Ba-L cells, which are PM1-CCR5 cells persistently infected with HIV-1_{IIIB} or HIV-1_{Ba-L}, respectively, were maintained in RPMI 1640 medium supplemented with G418 disulfide (0.5 mg/ml; Nacalai Tesque, Kyoto, Japan). HeLa CD4/CCR5/LTR-β-galactosidase (MAGI/CCR5) cells (Vodicka et al., 1997) were kindly provided by Dr. J. Overbaugh through the AIDS Research and Reference Program. MAGI/CCR5, 293T and NP-2 cells were maintained in Dulbecco's modified Eagle's medium (Sigma). All media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin.

2.3. Determination of anti-HIV-1 activity

The anti-HIV-1 activities of the peptides in the early phase of the replication cycle were determined by the MAGI assay as described previously (Nameki et al., 2005). The inhibitory effects of the peptides in the late phase were evaluated using PM1-CCR5 cells persistently infected with HIV-1. Briefly, PM1-CCR5/IIIB or PM1-CCR5/Ba-L cells were washed three times and resuspended at 4×10^4 cells/ml with the peptides in the RPMI 1640-based medium. After 48 h, the amounts of viral p24 antigen present in the culture supernatants were measured using a RETRO-TEK HIV-1 p24 Antigen ELISA Kit (ZeptoMetrix Corporation, Buffalo, NY). The cytotoxicities of the peptides were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously (Kodama et al., 2001).

2.4. Flow cytometric analysis of the antagonistic effects on CXCR4

MT-2 cells were resuspended in phosphate-buffered saline (PBS) containing 1% FCS and preincubated with peptides at 4 °C for 30 min. PE-conjugated anti-CXCR4 MAb (12G5) was then added and incubated at 4 °C for a further 30 min. The cells were washed three times, fixed with 1% formaldehyde and analyzed using an EPICS XL flow cytometer (Beckman Coulter, Miami Lakes, FL).

2.5. Evaluation of the anti-HIV-1 effects of intracellular expression of Rev peptides

We constructed pCI-Rev-IG vectors encoding various Rev mutants, an internal ribosomal entry site (IRES) and neomycin phosphotransferase ($G418^r$) under the control of a cytomegalovirus (CMV) promoter as shown in Fig. 4. The pCI-Rev-IG plasmids were transfected into MT-2 cells using the TransIT transfection reagent (Mirus Bio, Madison, WI). After 16 h, the transduced MT-2 cells were subjected to G418 selection, and further cultured in the presence of G418 (0.5 mg/ml).

Rev-transduced MT-2 cells were resuspended in 96-well plates (3×10^3 cells/well) and incubated with HIV-1_{IIIB}. After 5 days, the

cytopathic effects were determined by the MTT assay as described previously (Kodama et al., 2001).

2.6. Subcellular localizations of Rev and Rex peptides

To evaluate the subcellular localizations of Rev and Rex peptides, we constructed pRev$_{peptide}$-EGFP and pRex$_{peptide}$-EGFP vectors expressing C-terminally EGFP-fused peptides using pEGFP N1 (Clontech Laboratories Inc., Palo Alto, CA). The plasmids were transfected into 293T and NP-2 cells using the TransIT transfection reagent. After 48 h, the cells were fixed with 1% formaldehyde and examined with a BioZERO fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

3. Results

3.1. Arginine-rich peptides inhibit HIV-1$_{IIIB}$ infection

The amino acid sequences of the peptides are shown in Fig. 1. The peptides were examined for their anti-HIV-1 activities by the MAGI assay (Table 1), which only detects anti-HIV-1 activity in the early phase of the replication cycle up to the point of Tat interaction with the long terminal repeat (LTR) within 48 h post viral inoculation (Uchida et al., 1997). The reverse transcriptase inhibitor zidovudine (AZT) inhibited both HIV-1$_{IIIB}$ and HIV-1$_{Ba-L}$, which use CXCR4 and CCR5 as coreceptors, respectively, while AMD3100 and TAK-779, which are specific antagonists of CXCR4 or CCR5 (Baba et al., 1999; Donzella et al., 1998), only inhibited HIV-1$_{IIIB}$ and HIV-1$_{Ba-L}$, respectively. All peptides showed anti-HIV-1 activity against HIV-1$_{IIIB}$, but not HIV-1$_{Ba-L}$. Addition of the 5 amino acids AAAAC (A_4C), which stabilizes the α-helical structure (Lin et al., 2004), to the C-terminus of Rev$_{34-50}$ (Rev$_{34-50}$-A_4C) enhanced the activity against HIV-1$_{IIIB}$ compared with the parental Rev$_{34-50}$. The anti-HIV-1 activity of Rev$_{34-50}$-A_4C was slightly enhanced by addition of the protein transduction domain of HIV-1 Tat (Tat-PTD) to the C-terminus (see Fig. 1B), which increases the intracellular delivery of the conjugated protein or peptide (Nagahara et al., 1998). Rex$_{1-21}$ partially inhibited infection by HIV-1$_{IIIB}$ at 10 μM (approximately 40%) and its anti-HIV-1 activity was increased by addition of Tat-PTD. Another arginine-rich peptide, RSG-P2G4 (Harada et al., 1997), also only inhibited HIV-1$_{IIIB}$ infection.

Table 1
Anti-HIV-1 activities of Rev- and Rex-derived peptides[a].

Compound	EC_{50}[b] (μM)	
	HIV-1$_{IIIB}$	HIV-1$_{Ba-L}$
Rev$_{34-50}$	1.7 ± 0.25	>10
Rev$_{34-50}$-A_4C	0.35 ± 0.07	>10
Rev$_{34-50}$-A_4C-PTD	0.37 ± 0.09	>10
Rex$_{1-21}$	>10	>10
Rex$_{1-21}$-PTD	2.5 ± 0.76	>10
RSG-P2G4	2.2 ± 0.51	>10
AZT	0.022 ± 0.005	0.055 ± 0.009
AMD3100	0.0038 ± 0.0011	>100
TAK-779	>100	0.0030 ± 0.0019

[a] Anti-HIV-1 activity was determined by the MAGI assay that detects early phase of HIV replication (Uchida et al., 1997). HIV-1$_{IIIB}$ and HIV-1$_{Ba-L}$ are one of representative strains for CXCR4 and CCR5 tropic HIV-1.
[b] EC_{50}, 50% effective concentration. Data represent the means ± SD of at least three independent experiments.

3.2. Antagonism against CXCR4

It has been demonstrated that arginine-rich peptides bind to CXCR4, resulting in inhibitory effects on the binding of the HIV-1 gp120 V3 loop to CXCR4 (entry inhibition) (Doranz et al., 1997; Murakami et al., 1999). Our synthesized peptides contained an arginine-rich motif in their sequences and blocked early-phase infection only by the CXCR4-tropic HIV-1$_{IIIB}$ virus. Therefore, we investigated whether the anti-HIV-1 activities of these peptides in the early phase were produced by CXCR4 antagonism using flow cytometric analysis with an anti-CXCR4 monoclonal antibody (MAb; 12G5). All the peptides, including Rev$_{34-50}$-A_4C, Rev$_{34-50}$-

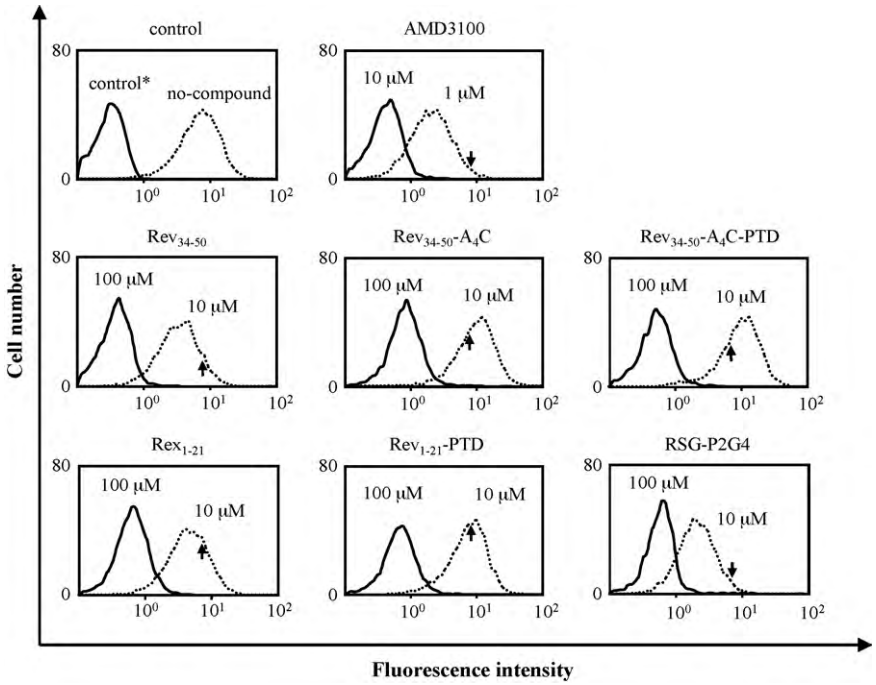

Fig. 2. Effects of the peptides on binding of a MAb against CXCR4 expressed on the MT-2 cell surface. MT-2 cells were resuspended in PBS containing 1% FCS and preincubated with the peptides at 4 °C for 30 min. PE-conjugated anti-CXCR4 MAb (12G5) was then added and incubated at 4 °C for a further 30 min. The cells were washed three times, fixed with 1% formaldehyde, and analyzed using a flow cytometer. A CXCR4 antagonist, AMD3100, was used a positive control. The results represent one of three independent experiments. (*) Fluorescence without PE-conjugated 12G5 is shown. Arrowhead indicates the mean fluorescence intensity of the no compound control.

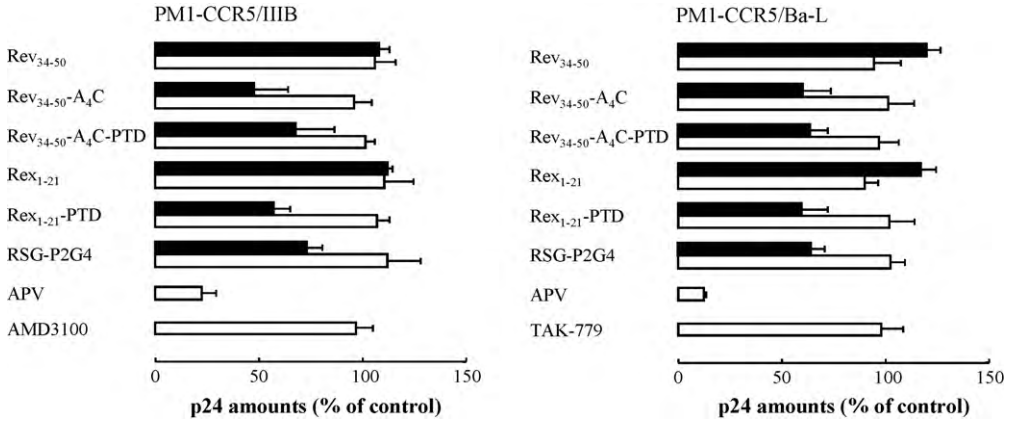

Fig. 3. Inhibitory effects of the peptides on p24 production from persistently HIV-1-infected PM1-CCR5 cells. PM1-CCR5 cells persistently infected with HIV-1_{IIIB} or HIV-1_{Ba-L} (PM1-CCR5/IIIB or PM1-CCR5/Ba-L cells, respectively) were washed and resuspended at 4×10^4 cells/ml with the peptides (black bars: 10 μM; white bars: 1 μM), APV as a positive control or AMD3100, TAK-779 or medium alone (control) as negative controls. After 48 h, the amounts of viral p24 in the culture supernatants were measured. The results are expressed as the percentages of p24 relative to that of the control. The data represent the means + SD of three independent experiments.

A_4C-PTD and Rex_{1-21}-PTD (data not shown), inhibited binding of 12G5 in a dose-dependent manner as observed for AMD3100 (Fig. 2). Although Rex_{1-21} showed only weak inhibitory activity against HIV-1_{IIIB} at 10 μM (data not shown), it blocked the binding of 12G5 sufficiently at 100 μM (Fig. 2). Relatively high concentrations of peptides were required to block binding of 12G5. It is likely that numerous functional and non-functional CXCR4 for HIV-1 infection may be expressed on the cell surface. In the CCR5 case, the antagonism observed in less than 20% of cell surface expressed CCR5 appeared to be sufficient for inhibition of HIV infection (Maeda et al., 2004), since only that to functional CCR5 as an HIV-1 receptor, including co-localization with CD4, appears to be required. However, for a flow cytometer analysis, monoclonal antibodies and small agents including peptides can recognize both functional and non-functional, therefore, high concentration seems to be needed. Actually, another peptide CXCR4 antagonist, T134 was also required high concentration to inhibit 12G5 binding in a flow cytometry (Arakaki et al., 1999). These results indicate that the arginine-rich peptides may inhibit infection by HIV-1_{IIIB} through antagonism for CXCR4.

3.3. Inhibitory effects against viral production

In addition of the CXCR4 antagonism, the inhibitory effects of the peptides in the late phase were independently evaluated using persistently HIV-1-infected PM1-CCR5 cells. AMD3100, TAK-779 and the protease inhibitor APV were used as controls. The specific CXCR4 or CCR5 antagonists AMD3100 and TAK-779, respectively, had no inhibitory effects on viral production, whereas APV (1 μM) effectively inhibited the production of viral p24 antigen from both PM1-CCR5/IIIB and PM1-CCR5/Ba-L cells (Fig. 3). Rev_{34-50}, which specifically binds to the RRE similar to intact Rev *in vitro* (Kjems et al., 1992), showed no inhibitory effects on the production of viral p24, whereas Rev_{34-50}-A_4C inhibited the production of p24 from both PM1-CCR5/IIIB and PM1-CCR5/Ba-L cells. Addition of Tat-PTD to Rev_{34-50}-A_4C, which provides cell permeability, hardly enhanced the inhibitory effect. On the other hand, although Rex_{1-21} had no effect on the production of viral p24, Rex_{1-21}-PTD inhibited the production of viral p24 from both PM1-CCR5/IIIB and PM1-CCR5/Ba-L cells. RSG-P2G4, which interacts with the RRE more tightly than Rev_{34-50} (Harada et al., 1997), also inhibited the production of p24 from both PM1-CCR5/IIIB and PM1-CCR5/Ba-L cells. However, the antiviral activities of Rev_{34-50}-A_4C, Rev_{34-50}-A_4C-PTD and Rex_{1-21}-PTD were comparable to that of RSG-P2G4. None of the peptides showed any cytotoxicity for 5 days, as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (data not shown).

3.4. Infectivity of secreted HIV-1

To address whether HIV-1 secreted from the persistently infected PM-1 treated with peptides, maintains its infectivity, we examined the infectivity of HIV-1 particles in the supernatant. To avoid effect of CXCR4 antagonism by the peptides, we used HIV-1_{Ba-L} strain for the experiments. PM1-CCR5/Ba-L cells were washed 3 times and exposed to each peptide (10 μM) for 24 h. The supernatant, which contains newly produced and secreted HIV-1_{Ba-L}, was harvested and subjected to the titration with the MAGI cells and the p24 assay. $Rev_{3450A4C}$ attenuated the infectivity compared to the anticipated one by p24, while other peptides, Rev_{3450} and P2G4 showed moderately reduced infectivity (Table 2). However, both Rex-derived peptides showed little changes in actual and anticipated infectivity. These results indicate that the peptides, especially $Rev_{34-50}A_4$C, might sustain its binding to the RRE even in the virion and/or during RT reaction after the next round of the new infection, led to the reduced infectivity.

3.5. Anti-HIV-1 effects on intracellular expression of Rev peptides

The arginine-rich peptides inhibited the production of p24 in PM1-CCR5 cells persistently infected with HIV-1 (Fig. 3). Next, we evaluated the effects of intracellular expression of various Rev mutants in MT-2 cells against HIV-1 infection using MTT assays.

Table 2
Peptide effect on infectivity of HIV-1_{Ba-L}.

Compound	% of non-treated control		Infectivity/p24
	Infectivity	p24	
Rev_{34-50}	74 ± 11	119 ± 16	0.62
Rev_{34-50}-A_4C	24 ± 1	67 ± 8	0.36
Rex_{1-21}	121 ± 43	93 ± 24	1.3
Rex_{1-21}-PTD	74 ± 30	82 ± 15	0.97
RSG-P2G4	72 ± 32	92 ± 5	0.78

Infectivity and amount of p24 in the supernatant of PM1-CCR5/Ba-L cells exposed to peptides (10 μM) were determined by the MAGI assay and a commercially available p24 kit, respectively. Each value was normalized by that of simultaneously performed non-treated control as 100%. Data represent the means ± SD of three independent experiments.

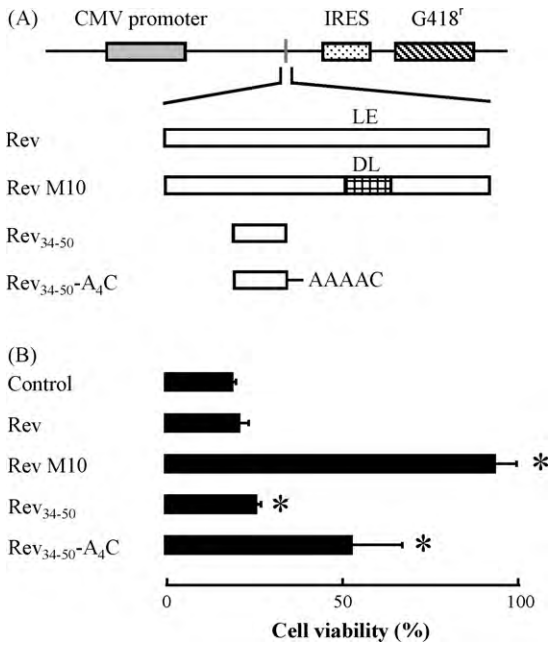

Fig. 4. Anti-HIV-1 activities of various Rev-transduced MT-2 cells. (A) Structures of the pCI-Rev-IG expression vectors encoding various Rev mutants. The fragments encoding Rev, Rev M10 ($D_{78}L_{79}$), Rev_{34-50} and Rev_{34-50}-A_4C were inserted into the upstream of IRES-$G418^r$ under the control of the CMV promoter. Amino acid substitutions at $L_{78}E_{79}$ (wild type Rev) to $D_{78}L_{79}$ (Rev dominant-negative mutant, Rev M10) diminish nuclear export of HIV mRNA (Malim and Cullen, 1991). (B) Effects of various Rev mutant transductions on HIV-1. MT-2 cells were transfected with various Rev mutant-encoding vectors and subjected to G418 selection. The Rev-transduced cells were infected with $HIV\text{-}1_{IIIB}$. On day 5 post-infection, the cell viabilities were determined by the MTT assay. The assays were performed in triplicate and the means + SD are shown. The results are representative of three independent experiments. $^*p < 0.05$, significant difference to the control by Student's *t*-test.

To obtain such MT-2 cells, the cells were transfected with various Rev expression vectors and then selected by G418. Although the expression of Rev hardly affected the cell death caused by HIV-1 infection, transduction of Rev M10, a dominant-negative mutant of Rev lacking NES activity, dramatically conferred resistance against HIV-1 infection (Fig. 4). Transduction of Rev_{34-50} moderately protected the cells. However, Rev_{34-50}-A_4C-transduced cells became more resistant to HIV-1 infection than Rev_{34-50}-transduced cells. These results for the transduced cells are consistent with those obtained for the persistently HIV-1-infected cells (Fig. 3). Therefore, the intracellular expression of Rev peptides conferred resistance against HIV-1 infection.

3.6. Subcellular localizations of Rev and Rex peptides

To elucidate the inhibitory mechanism of the arginine-rich peptides in the late phase of the replication cycle, we constructed $pRev_{peptide}$-EGFP and $pRex_{peptide}$-EGFP vectors expressing C-terminally EGFP-fused Rev and Rex peptides and examined the subcellular localizations of the EGFP-fused Rev and Rex peptides. In 293T cells, EGFP alone was equally distributed throughout the cells (Fig. 5, the 1st panel Control). In contrast, Rev and Rev M10 were mostly detected in nucleoli as previously reported (Dundr et al., 1995; Kalland et al., 1994; Stauber et al., 1995). Rev_{34-50}, Rev_{34-50}-A_4C and Rex_{1-21} were moderately detected in the cytoplasm, but dominantly accumulated in the nucleus. Similar results were observed in NP-2 cells (data not shown). These results indicate that Rev peptides exert antiviral activity in the same manner as Rev M10.

4. Discussion

4.1. Rev- and Rex-derived peptides act as dual antagonists toward CXCR4 and Rev

In the present study, we have demonstrated that arginine-rich peptides corresponding to the RRE-binding domains of HIV-1 Rev and HTLV-I Rex have dual inhibitory effects, namely CXCR4 antagonism and a dominant-negative effect for Rev function. Addition of A_4C, which stabilizes the α-helical structure (Lin et al., 2004), to Rev_{34-50} enhanced the inhibitory effects toward both CXCR4 antagonism and Rev function. It has been demonstrated that Rev_{34-50} forms an α-helical structure (Battiste et al., 1996; Tan et al., 1993) and binds to the RRE more efficiently when its α-helicity is increased (Tan et al., 1993). Short α-helical peptides (>20 amino acids) are known to have unstable helical structures and their helical structure is stabilized as their length increases (Marqusee et al., 1989). Hence, it is likely that the expected increase in α-helicity of Rev_{34-50} by the addition of A_4C resulted in enhanced CXCR4 antagonism and inhibition of p24 production at a post-transcriptional step for HIV-1 genes. The Rev peptides lacked NES activity, similar to the case of Rev M10, which is a trans-dominant repressor of Rev function (Malim et al., 1989a; Malim and Cullen, 1991). Therefore, the peptides may inhibit HIV-1 replication by competing with Rev for binding to the RRE, similar to the case of Rev M10. We observed that the Rev peptides, which contained a NLS, accumulated in the nucleoli. Expression of Rev M10 inhibited the cell death caused by HIV-1 significantly more efficiently than Rev_{34-50}-A_4C. This may be the result of Rev_{34-50}-A_4C being able to inhibit only the Rev-RRE binding step, whereas Rev M10 can inhibit multiple Rev functions

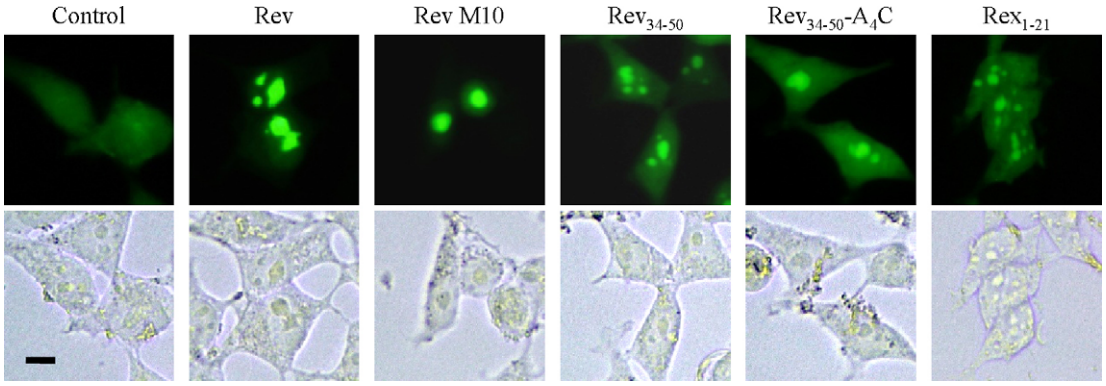

Fig. 5. Subcellular localizations of Rev and Rex peptides. 293T cells were transfected with $pRev_{peptide}$-EGFP and $pRex_{peptide}$-EGFP plasmids expressing Rev-, Rev M10-, Rev_{34-50}-, Rev_{34-50}-A_4C- and Rex_{1-21}-EGFP fusion proteins and EGFP alone as a control. After 48 h, the cells were fixed and examined using a fluorescence microscope (excitation: 488 nm). Fluorescent (upper panels) and corresponding phase-contrast (lower panels) images are shown. Scale bar: 10 μm.

by forming Rev-Rev M10 hetero-oligomers lacking NES activity (Stauber et al., 1995). However, despite the higher potency of Rev M10, its clinical applications are limited because it only differs from Rev by two amino acids. In this regard, the short peptide Rev_{34-50}-A_4C is more well suited for clinical applications. In fact, enfuvirtide (T-20), a similar 36-amino acid long peptide which inhibits HIV-1 fusion has been approved for the treatment of HIV infections and its clinical efficacy and utility has been demonstrated (Lalezari et al., 2003; Lazzarin et al., 2003).

Although addition of Tat-PTD may enhance the cell permeability of the peptides, its effects on cell permeability may be limited because the Rev and Rex peptides showed efficient cell permeability, as previously described (Futaki et al., 2001). It has been reported (Futaki et al., 2001) that peptides containing 6 and 8 arginine residues show efficient cell permeability. It is likely that addition of Tat-PTD to Rev_{34-50}-A_4C or Rex_{1-21}-A_4C hardly affected their cell permeability, as they already contained a large number of arginines. Notably, Tat-PTD does not interact with the RRE (Harada et al., 1996). On the other hand, Rex_{1-21} itself has sufficient cell permeability (Futaki et al., 2001), and addition of Tat-PTD to Rex_{1-21} enhanced its inhibitory activity likely through interactions with the CXCR4 coreceptor. Specifically, although Rex_{1-21} showed a weak antagonistic effect against CXCR4 by itself, addition of Tat-PTD to Rex_{1-21} enhanced its antagonistic effect because Tat-PTD contains an arginine-rich motif. These results indicate that the secondary structure and/or arginine content of peptides may be important for CXCR4 antagonism.

Since Rex_{1-21} accumulated in the nucleoli and Rex binds to the stem-loop III/IV/V region of the RRE, which is different from the recognition site of Rev (stem-loop IIB) (Ahmed et al., 1990; Kjems et al., 1992), Rex_{1-21}-PTD probably inhibited the production of viral p24 by interfering with an event after the Rev-stem-loop IIB interaction, possibly Rev oligomerization. It is possible that the increased size of Rex_{1-21}-PTD over Rex_{1-21} results somehow to increased steric hindrance that prevents REV oligomerization. It is also possible that the increased size of Rex_{1-21}-PTD over Rex_{1-21}- confers higher stability to the secondary structure of the peptide for binding to the RRE or competing with the Rev function in Rex_{1-21}. Binding of Rex_{1-21}-PTD to the stem-loop III/IV/V region, which is different from the binding of Rev (Ahmed et al., 1990), also inhibited p24 production, indicating that not only stem-loop IIB but also the whole RRE structure may represent potential drug targets. Moreover, it provides opportunities for combination therapies of peptides that target different domains of RRE.

RSG-P2G4, another arginine-rich peptide which permeates the cell membrane efficiently, (Harada et al., 1997), also inhibited viral p24 production. RSG-P2G4 binds to stem-loop IIB of the RRE (Harada et al., 1997), and therefore inhibited p24 production as efficiently as the Rev peptides.

4.2. Potential of Rev antagonists as chemotherapeutic agents

In the early phase of the HIV-1 replication cycle, Tat protein promotes production early proteins such as Tat, Rev and Nef. Since Tat enhances viral transcription without controlling splicing, the early proteins accumulate in the cells. One of the early proteins, Rev, inhibits splicing of the transcripts and exports them to the cytoplasm to generate late proteins including Gag, Pol, Env and progenitor genomes, resulting in suppression of Tat and Rev expression (Felber et al., 1990; Seelamgari et al., 2004). These decreases subsequently increase Tat expression, such that Tat and Rev cooperate for efficient viral gene expression with a certain balance. This cooperation plays an important role in the generation of progeny viruses, suggesting that interference with this cooperation may be sufficient for suppression of viral replication. Limited loss of Rev function without full suppression of its activity may cause an imbalance of Tat/Rev functions, resulting in dysregulation of viral expression and the formation of incomplete viruses. Therefore, only partial inhibitory effects of Rev inhibitors, rather than the full inhibition observed for reverse transcriptase and protease inhibitors, may be sufficient for effective viral suppression. In the state of Tat dominance, infected cells will keep producing disproportionate amounts of early proteins, such as Tat, Rev and Nef. Accumulation of such proteins may be detrimental to an antigen-presenting cell, as it may result in targeting by the host immune system, and elimination of infected cells. This would be a novel strategy for eliminating HIV-infected cells using peptide-based therapies.

As with all potential anti-HIV therapeutics a concern regarding Rev inhibitors is the development of resistance. However, it should be noted that in this case there would be additional constraints that are likely to affect the barrier to resistance. Specifically, introduction of mutations into the Rev coding region also influences the Tat and/or Env sequences and possibly their functions, as both proteins are simultaneously encoded with Rev. Furthermore, it is likely that successful introduction of mutations might interfere with the balance of Rev/Tat, which would also reduce viral replication and lead to possible elimination of the infected cells by the host immune system. In the case of RRE mutations, we have clearly demonstrated that the RRE and the gp41 N-heptad repeat (N-HR), one of the key helical domains for virus fusion, are simultaneously encoded and that mutations in the N-HR influenced RRE structure clearly affect the replication kinetics of the virus (Nameki et al., 2005; Ueno et al., 2009). These observations highlight a number of difficulties that HIV-1 would have to overcome in order to develop resistance against Rev inhibitors. Even if HIV-1 does acquire resistance, this may result in deteriorating replication kinetics, since it will simultaneously alter the functions of other viral proteins, such as Tat and gp41, and the RRE. Therefore, genetic barrier for resistance against Rev inhibitors is expected to be high and Rev inhibitors may be able to sustain antiviral activity during prolonged use.

5. Conclusion

We have shown that arginine-rich peptides derived from the RNA-binding domain of HIV-1 Rev can act as dual-target inhibitors that inhibit HIV-1 entry and viral production in the early and late phases of replication. Targeting Rev for chemotherapy is a promising strategy because it is entirely distinct from current drug targets. In addition, acquisition of resistance to Rev inhibitors may be limited because the Rev and RRE encoding regions simultaneously encode Tat and/or Env. Furthermore, the Rev peptides inhibit a completely different step of HIV-1 infection, the coreceptor CXCR4, and may, therefore, exert synergistic effects. Moreover, inhibiting Rev function may induce elimination of infected cells by the host immune system, suggesting that Rev peptides may represent the first inhibitors to enhance vaccine efficacy. Taken together, the dual-target Rev peptides are attractive agents and our present observations support their development as dual-target inhibitors for the prevention of HIV-1 infection.

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