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HIV Rev Peptides Conjugated with Peptide Nucleic Acids and Their Efficient Binding to RRE RNA

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Abstract—HIV Rev peptides conjugated with peptide nucleic acids (PNAs) were designed and synthesized to develop a designing approach for a novel RNA-binding molecule. The binding affinities of PNA-peptides with the Rev responsive element (RRE) RNA were determined by the competition assay using a rhodamine-labeled Rev. The peptide conjugated with an antisense PNA (TGCGC) bound RRE RNA more efficiently than the molecule without the PNA or the peptide sequence.

Introduction

RNA–protein interaction plays crucial roles in organisms from virus to eucaryote. Specific interactions of proteins and RNAs control many cellular functions, including transcription, RNA splicing, and translation.[1] RNAs form a variety of 2-D and 3-D structures such that proteins do. Then, RNA-binding proteins mostly interact with secondary structural domains such as hairpin loops, internal loops, and bulges of RNA for recognition, but rarely target fully double-stranded portions of RNA.[1] RNA-binding domains of proteins form suitable conformations such as α-helix and β-strand to recognize structured RNAs, and amino acids orientated exactly in protein structures make specific contacts to RNA bases, resulting in the high affinity and high specificity in RNA binding. Construction of molecules that specifically recognize not only RNA sequences but also 2-D or 3-D structures of RNA is useful for giving the promising information of designing highly specific probes and drug candidates against RNA. For this aim, it is necessary to pay attention to the 2-D and 3-D structures on the design of peptides.

On the other hands, peptide nucleic acid (PNA) is a DNA mimic with the nucleobases on a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units.[2–4] PNAs have an ability of efficient and sequence specific binding both single-stranded DNA and RNA as well as double-stranded DNA. In order to develop such a novel RNA-binding molecule described above, we have attempted to combine peptides capable of forming a structure such as an α-helix on RNA binding and PNAs that can specifically recognize bases in RNA.[5,6] Conjugating PNA with the RNA-binding peptide has revealed that PNA can be more effectively applied to the terminal use of peptide than the use at the middle positions owning to the conformation-disturbing nature of the flexible molecule. In order to expand the feasibility of conjugating a peptide with PNAs for RNA recognition, we report here the design and synthesis of a series of PNA-conjugated peptides, derived from the regulatory protein of virion expression (Rev) of human immunodeficiency virus type 1 (HIV-1),[7–10] and the effect of introduction of PNAs on the RNA binding.

Results and Discussion

The arginine-rich domain of the $Rev_{34–50}$ peptide (17 residues) binds specifically to the stem-loop IIB region of Rev-responsive element (RRE) RNA (Fig. 1).[11,12] Rev has an almost disordered structure in aqueous solution, but forms an α-helix conformation when it binds to the RNA. Therefore, the α-helix potential of

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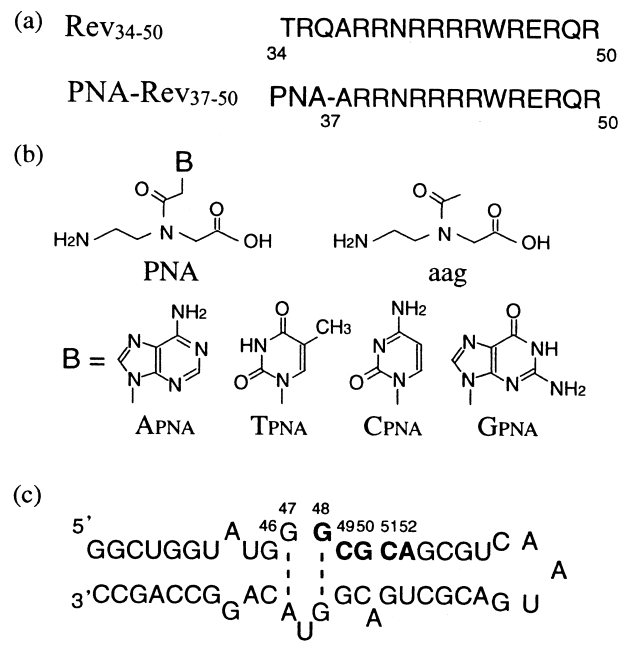

Figure 1. (a) Sequences of $Rev_{34–50}$ and PNA-$Rev_{37–50}$ peptides; (b) structures of PNAs including acetyl(2-aminoethyl)glycine (aag); and (c) 2-D structure of RRE IIB RNA (47nt).

the $Rev_{34–50}$ peptide affects the binding affinity and specificity of the peptide with RRE IIB RNA.[11,13] Because PNA is a flexible molecule, it perturbed the α-helical property of peptide.[5,6] In this study, therefore, the strategy combining PNAs at the terminal extension of the peptide was selected and a series of PNA-conjugated peptides was designed (Table 1). It is proposed that the glutamine residue at the 36-position (Q36) is close to the guanine base at the 48-position (G48) in the bulge of RRE IIB RNA.[12] For the complementary interaction with G48, cytosine PNA (CPNA) was introduced at the N-terminal of $Rev_{37–50}$ peptide (CRev) in which three residues at the N-terminal (T34, R35, Q36) of $Rev_{34–50}$ peptide were deleted. Moreover, other PNA (APNA, TPNA or GPNA) was added at the N-terminal. As a reference, acetyl(2-aminoethyl)glycine (aag) was also used. To increase the stability of the α-helix structure, the N- and C-terminals of the peptides were succinylated and amidated, respectively. The PNA peptides were synthesized by the solid-phase method using Fmoc-strategy.[14] Synthesized peptides were purified with HPLC, and identified by matrix assisted laser desorption ionization time-of-flight mass spectrometry[15] and amino acid analysis. RRE IIB RNA (47nt) was prepared in vitro using T7 RNA polymerase and a synthetic oligonucleotide template, and then purified by polyacrylamide gel electrophoresis.[5,6,16]

The binding affinities of the peptides with RRE IIB RNA were determined by the competition assay[5,6,17] using the 5-carboxytetramethylrhodamine-labeled Rev peptide (Rhod-Rev) as a fluorescence tracer. The binding of Rhod-Rev with the RNA enhanced the fluorescence anisotropy, showing that the tracer bound to the RNA with a dissociation constant (K_d) of 2.1 nM. The competition experiments were performed to determine the binding affinities of the designed peptides with RRE IIB RNA. In the mixture of Rhod-Rev (10 nM) and RRE IIB RNA (25 nM), fluorescence anisotropy values were decreased by the addition of the peptides as a competitor, affording the free Rhod-Rev (Fig. 2). The dissociation constants of the peptides with RNA were calculated by the equation assumed as a 1:1 stoichiometry.[5,6] These competition experiments revealed that $Rev_{37–50}$ bound RRE IIB RNA with a dissociation constant of 86 nM (Table 1). In contrast, the addition of CPNA at the N-terminal of $Rev_{37–50}$ (CRev) showed a higher binding affinity (K_d = 31 nM, Fig. 3). Other peptides containing one PNA unit (ARev, TRev, GRev and aagRev) also showed affinities (K_d = 30–44 nM) comparable to CRev (Table 1, Fig. 3). These results indicated that there is no effect of nucleobase addition but a contribution of peptide elongation as demonstrated in aagRev.

Table 1. Sequences and dissociation constants (K_d) with RRE IIB RNA and α-helicities of PNA-peptides

Compound	Sequence[a]	K_d (nM)	α-Helicity (%)	K_d^{Rev}/K_d[b]
$Rev_{37–50}$	suc-Rev(37–50)-NH_2	86±4	40	1.0
aagRev	suc-**aag**Rev(37-50)-NH_2	44±2	51	2.0
ARev	suc-$\mathbf{A_{PNA}}$Rev(37–50)-NH_2	36±2	52	2.4
TRev	suc-$\mathbf{T_{PNA}}$Rev(37–50)-NH_2	39±3	50	2.2
CRev	suc-$\mathbf{C_{PNA}}$Rev(37–50)-NH_2	31±1	52	2.8
GRev	suc-$\mathbf{G_{PNA}}$Rev(37–50)-NH_2	30±3	50	2.9
ACRev	suc-$\mathbf{A_{PNA}C_{PNA}}$Rev(37–50)-NH_2	28±3	52	3.1
TCRev	suc-$\mathbf{T_{PNA}C_{PNA}}$Rev(37–50)-NH_2	27±2	52	3.2
CCRev	suc-$\mathbf{C_{PNA}C_{PNA}}$Rev(37–50)-NH_2	28±5	50	3.1
GCRev	suc-$\mathbf{G_{PNA}C_{PNA}}$Rev(37–50)-NH_2	16±2	49	5.4
AGCRev	suc-$\mathbf{A_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	15±3	51	5.7
TGCRev	suc-$\mathbf{T_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	17±3	48	5.1
CGCRev	suc-$\mathbf{C_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	9.6±0.4	49	9.0
GGCRev	suc-$\mathbf{G_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	8.7±0.7	50	9.9
CCCRev	suc-$\mathbf{C_{PNA}C_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	34±5	47	2.5
GCGCRev	suc-$\mathbf{G_{PNA}C_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	5.7±1.6	45	15
TGCGCRev	suc-$\mathbf{T_{PNA}G_{PNA}C_{PNA}G_{PNA}C_{PNA}}$Rev(37–50)-$NH_2$	5.4±0.7	48	16
TGCGC	ac-$\mathbf{T_{PNA}G_{PNA}C_{PNA}G_{PNA}C_{PNA}}$K-$NH_2$	>100 μM	—	—

[a]Rev(37–50): ARRNRRRRWRERQR.
[b]K_d^{Rev}/K_d is the ratio of the dissociation constants between $Rev_{37–50}$ and the PNA-peptide.

To target the RNA bases G48C49G50 with the PNA-Rev peptides according to the NMR structure of Rev-RRE,[12] the N-terminal PNA sequence was elongated as GPNACPNA or CPNAGPNACPNA to give GCRev and CGCRev (Table 1). ACRev, TCRev and CCRev, and also AGCRev, TGCRev and GGCRev were examined for comparison of the RNA-binding activity. Among the peptides with two PNA units, GCRev showed the strongest affinity to the RNA ($K_d = 16$ nM), which was 5.4-fold stronger than that of $Rev_{37–50}$ and ca. 2-fold stronger than that of single-PNA peptides (Fig. 3). The addition of other PNAs (A, T, or C) into CRev was not effective for the binding improvement ($K_d = 27–28$ nM). These findings suggest that the elongated PNAs in GCRev can interact specifically to G48C49 in the bulge region of RRE IIB RNA in an antiparallel fashion. In the addition of the third PNA, both CGCRev and GGCRev showed considerably higher abilities ($K_d = 9.6$ and 8.7 nM, respectively, ca. 10-fold higher than $Rev_{37–50}$) than AGCRev ($K_d = 15$ nM) and $TGCRev_{37–50}$ ($K_d = 17$ nM). Introduction of CPNA or GPNA in GCRev was effective but APNA or TPNA was not. These results imply that the PNA part of CGC may recognize G48C49G50 sequence of the RNA and that both the CPNA or GPNA at the N-terminal can interact with the G50 base so as to make hydrogen bond(s) such as those in the C:GC and G:GC triplets.[18] It is noteworthy that the activity of CCCRev that targets G48G47G46 in the RNA was rather low ($K_d = 34$ nM).

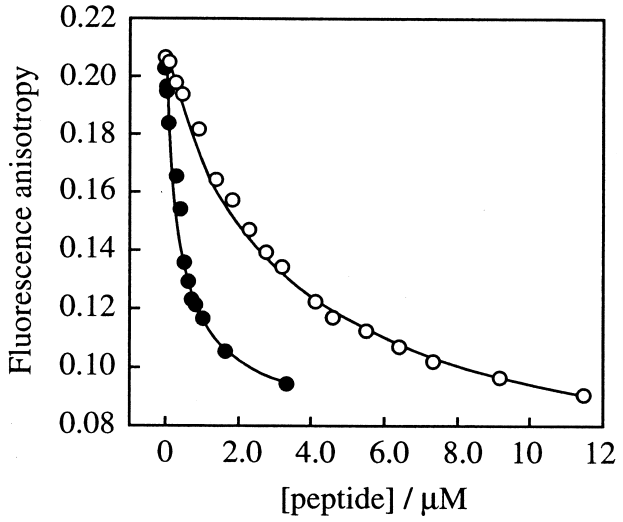

Figure 2. Fluorescence anisotropy change of Rhod-Rev (10 nM) with RRE IIB RNA (25 nM) as a function of CGCRev (solid circle) and $Rev_{37–50}$ (open circle) concentration in buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA) at 25 °C ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 580$ nm).

Furthermore, to address the PNA part to the extended RNA region (G48C49G50C51A52) in the antiparallel manner, GCGCPNA and TGCGCPNA were added at the N-terminal of $Rev_{37–50}$. Further elongation of GPNA and TGPNA enhanced the RNA-binding ability ($K_d = 5.7$ and 5.4 nM, respectively) to afford ca. 15-fold stronger binders to the RNA than $Rev_{37–50}$. These PNA-conjugates can be potential inhibitors of Rev-RRE interaction comparable to $Rev_{34–50}$. The elongated PNA part potentially recognizes the RNA sequence from G48 to A52 and the recognition can enhance the binding ability of the peptide. The PNA interaction could compensate the roles of peptide sequence TRQ in $Rev_{34–50}$.

Circular dichroism (CD) studies revealed that the PNA-peptides possessed the ability of forming an α-helix structure. Each peptide showed a typical α-helix pattern with double negative maxima at 208 and 222 nm in tri-

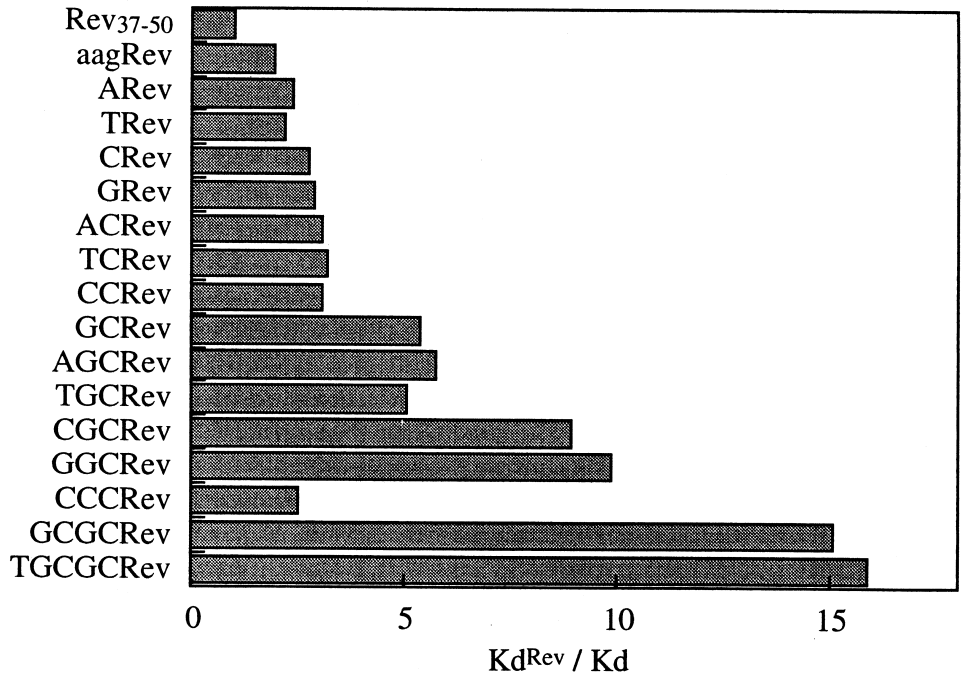

Figure 3. The relative activity of PNA-peptides. K_d^{Rev}/K_d is the ratio of the dissociation constants between $Rev_{37–50}$ and the PNA-peptide.

fluoroethanol at 25 °C. From the ellipticity at 222 nm, the α-helicity of each peptide was estimated (Table 1).[19] All PNA-peptides showed comparable abilities of the α-helix formation (ca. 50% as a helix content), although the $Rev_{37–50}$ peptide had a potential slightly lower (40%) than the PNA peptides, probably due to the lack in the elongated peptide bonds. These results suggest that introduction of the first PNA backbone including aag at the N-terminal of $Rev_{37–50}$ increases the α-helical potential and may affect increases in the binding affinity of the peptides. These results also indicate that the conjugation of PNAs at the N-terminal of the peptide does not disturb the α-helix conformation.[5]

The PNA-conjugated Rev peptides were successful to enhance the RNA-binding activity of the peptide $Rev_{37–50}$ with the elongation of PNA sequence. Moreover, only the PNA, TGCGC-Lys, was not able to dissociate the Rev-RRE complex at the concentration up to 100 μM. Thus, neither PNA in the same size nor the peptide $Rev_{37–50}$ was able to show the higher ability. These results demonstrated the feasibility of conjugation of PNA with the peptide at the terminal on the RNA recognition. This strategy combining PNA and peptide will lead to an approach of de novo designing of novel RNA binding molecules and inhibitors of virus protein and RNA.

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