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Construction of Peptides with Nucleobase Amino Acids: Design and Synthesis of the Nucleobase-Conjugated Peptides Derived from HIV-1 Rev and their Binding Properties to HIV-1 RRE RNA

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Abstract—In order to develop a novel molecule that recognizes a specific structure of RNA, we have attempted to design peptides having L-α-amino acids with a nucleobase at the side chain (nucleobase amino acid (NBA)), expecting that the function of a nucleobase which can specifically recognize a base in RNA is regulated in a peptide conformation. In this study, to demonstrate the applicability of the NBA units in the peptide to RNA recognition, we designed and synthesized a variety of NBA-conjugated peptides, derived from HIV-1 Rev. Circular dichroism study revealed that the conjugation of the Rev peptide with an NBA unit did not disturb the peptide conformation. RNA-binding affinities of the designed peptides with RRE IIB RNA were dependent on the structure of the nucleobase moieties in the peptides. The peptide having the cytosine NBA at the position of the Asn40 site in the Rev showed a higher binding ability for RRE IIB RNA, despite the diminishing the Asn40 function. Furthermore, the peptide having the guanine NBA at the position of the Arg44 site, which is the most important residue for the RNA binding in the Rev, bound to RRE IIB RNA in an ability similar to $Rev_{34–50}$ with native sequence. These results demonstrate that an appropriate NBA unit in the peptide plays an important role in the RNA binding with a specific contact such as hydrogen bonding, and the interaction between the nucleobase in the peptide and the base in the RNA can enhance the RNA-binding affinity and specificity.

Introduction

RNA–protein interaction plays important roles in nature. Many cellular processes, including transcription, RNA splicing, and translation, depend on the specific interaction of proteins and RNA.[1] RNAs can fold into a wide range of tertiary structures, and then RNA-binding proteins recognize the specific structures of RNA including secondary structural domains such as hairpin loops, internal loops, and bulges.[2] In many cases, an RNA-binding domain of protein forms a suitable conformation such as an α-helix and a β-sheet to recognize a structured RNA,[3–5] and amino acids orientated exactly in the protein structure make specific contacts to RNA backbone and bases.[2] Especially, the hydrogen bonding between the amino acid residues in a protein and the nucleobases in an RNA is important to increase the binding affinity and specificity of the protein to the RNA.[2]

On the other hand, interaction between nucleobases is important to form a specific conformation of DNA and RNA. Since the recognition ability between bases is highly selective, this ability can be used to make a novel compound that binds to DNA and RNA specifically. Peptide nucleic acid (PNA), developed by Nielsen and co-workers, is a DNA mimic with nucleobases on a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units.[6,7] A PNA molecule has an ability of efficient and sequence specific binding both single-stranded DNA and RNA as well as double-stranded DNA. Although PNA is an useful item to recognize the sequence of DNA and RNA, a simple PNA molecule itself may not discriminate a highly structured RNA with high specificity, because PNA alone would not

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have a high ability to form various conformations as proteins or peptides do.

The study for design and synthesis of molecules that recognize a specific structure of RNA including secondary or tertiary structure might lead to a way of drug discovery targeting RNA. For the goal of developing such a novel molecule, we have attempted to design peptides containing L-α-amino acids with a nucleobase at the side chain (nucleobase amino acid (NBA)), expecting that the function of a nucleobase capable of specifically recognizing a base in RNA is regulated in a peptide conformation such as an α-helix. The use of chiral NBA units should have more advantage than the use of achiral PNA units in respect of keeping the peptide conformation on RNA-binding.[8] We did not select the L-α-amino-β-(nucleobase)-propanoic acids such as L-Willardiine,[9] which carries a uracil base at the β-position of alanine, but L-α-amino-γ-(nucleobase)-butyric acids[10,11] as NBA units, expecting the possibility of a more flexible ethylene linker in an effective RNA recognition. Furthermore, as an advantage of NBA, it can be introduced at any position of peptides the same manner as the natural amino acids by use of the solid-phase peptide synthesis.

To demonstrate the applicability of NBA units in peptides, we have chosen an active part of the regulatory protein of virion expression (Rev) of human immunodeficiency virus type-1 (HIV-1)[12–15] among many examples of the proteins that bind to RNA specifically. The Rev protein binds to the corresponding responsive region of HIV-1 mRNA (RRE),[16–18] and this protein–RNA interaction plays a key role in the HIV-1 virus replication.[14,15] The arginine-rich domain (34–50) of the Rev protein binds specifically to the stem-loop IIB region of RRE RNA (Fig. 1c) by forming an α-helix conformation.[19] NMR structural analyses revealed that the Rev α-helix penetrates deeply into the major groove widened by two non-Watoson-Crick base pairs, G47–A73 and G48-G71.[3] The α-helix potential of the $Rev_{34–50}$ peptide affects the binding affinity and specificity of the peptide to RRE IIB RNA.[19,20] We have reported that the peptide having cytosine NBA (C_{NBA}) instead of Gln36 in $Rev_{34–50}$ was successfully designed and synthesized, and the peptide bound to RRE IIB RNA with higher affinity and specificity than $Rev_{34–50}$.[11] In this study, we designed and synthesized a series of peptides conjugated with one or two NBA units at various positions of Rev (Fig. 1), and evaluated the effects of NBA conjugation with the Rev peptide on the binding affinity and specificity to HIV-1 RRE IIB RNA.

Results and Discussion

Design and synthesis of the peptides with NBA

Design of a series of NBA-conjugated peptides was carried out based on the structure of HIV-1 $Rev_{34–50}$

(a)

Rev_{34-50}	suc-TR**Q**ARRN**N**RRRRWRERQR-NH_2
	Asn, Gln mutants
$N40C_{NBA}$	suc-TRQARR**C_{NBA}**RRRRWRERQR-NH_2
$N40T_{NBA}$	suc-TRQARR**T_{NBA}**RRRRWRERQR-NH_2
$N40C_{PNA}$	suc-TRQARR**C_{PNA}**RRRWRERQR-NH_2
N40A	suc-TRQARR**A**RRRRWRERQR-NH_2
$Q36C_{NBA}$	suc-TR**C_{NBA}**ARRNRRRRWRERQR-NH_2
$Q36C_{NBA}$·$N40C_{NBA}$	suc-TR**C_{NBA}**ARR**C_{NBA}**RRRRWRERQR-NH_2
$Q36C_{NBA}$·$N40T_{NBA}$	suc-TR**C_{NBA}**ARR**T_{NBA}**RRRRWRERQR-NH_2

Arg mutants

R35 mutants	suc-T**X**QARRNRRRRWRERQR-NH_2
R39 mutants	suc-TRQAR**X**NRRRRWRERQR-NH_2
R44 mutants	suc-TRQARRNRRR**X**WRERQR-NH_2

X	R35	R39	R44
G_{NBA}	$R35G_{NBA}$	$R39G_{NBA}$	$R44G_{NBA}$
A_{NBA}	$R35A_{NBA}$	$R39A_{NBA}$	$R44A_{NBA}$
C_{NBA}	$R35C_{NBA}$	$R39C_{NBA}$	$R44C_{NBA}$
T_{NBA}	$R35T_{NBA}$	$R39T_{NBA}$	$R44T_{NBA}$
Ala	R35A	R39A	R44A

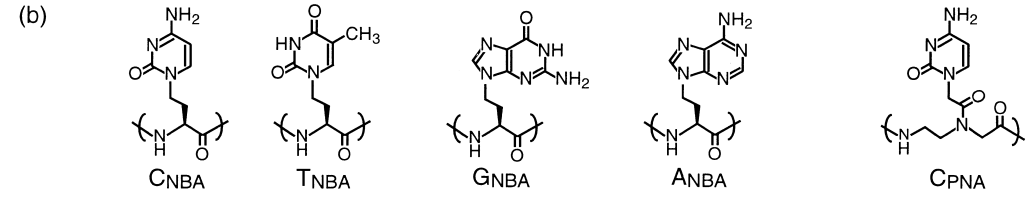

Figure 1. (a) Amino acid sequences of $Rev_{34–50}$ and designed NBA-peptides; (b) structures of nucleobase amino acids (NBA), C_{NBA}, T_{NBA}, G_{NBA}, and A_{NBA}, and a peptide nucleic acid, C_{PNA}; (c) the secondary structures of wild type RRE IIB RNA, and G48A and C46G74 mutant RNAs.

that binds to RRE IIB RNA specifically. In many cases of DNA or RNA binding proteins, Asn and Arg residues play an important role in making hydrogen bonds with some nucleic acid bases. It has been proposed by NMR structural analyses that the amide group of Asn40 residue in the $Rev_{34–50}$ interacts with the major groove edge of both bases of the G47–A73 base pair (Fig. 1c) through formation of a pair of hydrogen bonds.[3] In order to examine whether the use of a different NBA unit, such as C_{NBA} or T_{NBA}, can modulate the RNA-binding activity, we designed and synthesized two peptides, $N40C_{NBA}$ and $N40T_{NBA}$, in which L-α-amino-γ-cytosine-butyric acid (C_{NBA}) or L-α-amino-γ-thymine-butyric acid (T_{NBA}) was, respectively, introduced in place of Asn40 in $Rev_{34–50}$ (Fig. 1a). It is expected that the cytosine or thymine moiety in the peptide could be substituted for the Asn40 in $Rev_{34–50}$ owing to the structural similarity. Furthermore, the chiral C_{NBA} and T_{NBA} units possibly give a rigid conformation in the peptides. For the evaluation of structural importance of NBA, $N40C_{PNA}$, in which Asn40 and Arg41 were replaced by Nielsen type cytosine PNA (C_{PNA}),[6] was also prepared. The two amino acids were replaced by C_{PNA}, because a PNA monomer has the length equivalent to two amino acids in the main chain. N40A having Ala instead of Asn40 was prepared to clarify the effect of the nucleobase moiety in $N40C_{NBA}$ and $N40T_{NBA}$. Moreover, the peptides with two NBA units at the Gln36 and Asn40 positions were designed and examined for the first step to construct multi-NBA peptides. We have already found that the peptide, $Q36C_{NBA}$, in which Gln36 is replaced by C_{NBA}, binds to RRE IIB RNA with a high affinity ($K_d = 1.7$ nM).[9] On this basis, $Q36C_{NBA}$•$N40C_{NBA}$, in which two C_{NBA} residues were introduced in places of Gln36 and Asn40, and $Q36C_{NBA}$•$N40T_{NBA}$, in which C_{NBA} and T_{NBA} were introduced at the 36th and 40th positions, respectively, were designed. It is expected that the second addition of the C_{NBA} residue at the Gln36 site into the single mutants, $N40C_{NBA}$ and $N40T_{NBA}$, is effective in the RNA binding.

Arg residues in RNA-binding proteins play key roles in the RNA binding with not only electrostatic interaction to a phosphate backbone but also hydrogen bonding to a specific base in RNA. The NMR structural analyses revealed that three out of 10 Arg residues (35th, 39th, 44th positions) in the Rev peptide interacted with the bases of RRE IIB RNA in a specific manner mainly by making the hydrogen bonds.[3] Therefore, the mutation of the Arg residues to Ala and Lys decreased significantly the binding affinity and specificity to RRE IIB RNA.[19,20] In order to examine whether the introduction of various NBA units at the positions of important Arg residues in the Rev peptide can modulate the RNA-binding ability, we designed and synthesized the NBA-conjugated peptides, in which three Arg residues, Arg35, Arg39, and Arg44 of $Rev_{34–50}$, were replaced by the guanine, adenine, cytosine, and thymine NBA units (Fig. 1a). It is expected that the peptides having an NBA with guanine (G_{NBA}) bind to the RNA more strongly than the other peptides, because the guanine moiety contains functionality comparable to the guanidium group of the Arg side chain and can interact with the guanine on the G–C pair through hydrogen bonds. Furthermore, it is also expected that the RNA-binding specificity of the peptides with RRE IIB RNA is enhanced by the conjugation of the G_{NBA} unit due to diminishing the effect of non-specific interaction such as an electrostatic one. To evaluate the effect of the NBA-conjugation, the binding affinity of NBA-peptides to RRE IIB RNA is compared with the Ala-mutant peptides, because the cationic Arg residues have an ability of the electrostatic interaction with the phosphate backbone but the NBA units do not have it. To increase the stability of the α-helix structure, the N-terminal amino and C-terminal carboxyl groups of the peptides including $Rev_{34–50}$ were succinylated and amidated, respectively.[19,20]

Fmoc-protected NBA units were synthesized according to the reported method[10] with some modifications. The exocyclic amino group of cytosine and adenine moieties were protected by the Z group to prevent the side reaction in the peptide synthesis. Peptides were synthesized by the Fmoc solid-phase method.[21] Introduction of the NBA units did not affect the coupling efficiency. The peptides were purified by semi-preparative reversed-phase HPLC (RP-HPLC) to give a high purity (>98% on analytical RP-HPLC). The peptides were identified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). The amino acid analysis was also utilized for determination of the peptide concentration.

Circular dichroism study of NBA-peptides

Circular dichroism (CD) spectra of a series of the NBA-conjugated peptides were examined in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA or trifluoroethanol (TFE) solution at 25 °C. In the buffer solution, the conformations of the peptides, including $Rev_{34–50}$, were almost in a random structure (data not shown). It was reported that the Rev peptide bound to RRE IIB RNA by forming an α-helical conformation.[3,19,20] These results suggest that the peptides may not form stable hydrogen bonds for α-helix structure in aqueous solution by the effect on the charge repulsion of the cationic Rev and NBA-peptides, but the interaction of the peptides with the RNA induces the α-helical conformation of the peptides. However, it was difficult to measure the CD spectra of the peptide–RRE IIB RNA complex, because the conformation of the RNA was also changed by the peptide binding.[20]

In contrast, in TFE solution, which is known to be an α-helix forming solvent,[22] the NBA-peptides, $N40C_{NBA}$ and $N40T_{NBA}$, and $Rev_{34–50}$ showed a typical α-helix CD pattern at the amide region (Fig. 2a). The α-helicities of the peptides in TFE were estimated from the ellipticity at 222 nm as shown in Table 1.[23] The α-helix contents of $N40C_{NBA}$ and $N40T_{NBA}$ were estimated as 63% and 66%, respectively, slightly higher than that of $Rev_{34–50}$ (59%). These results suggest that the C_{NBA} and T_{NBA} units have a potential of forming an α-helix structure slightly higher than Asn having an α-helix

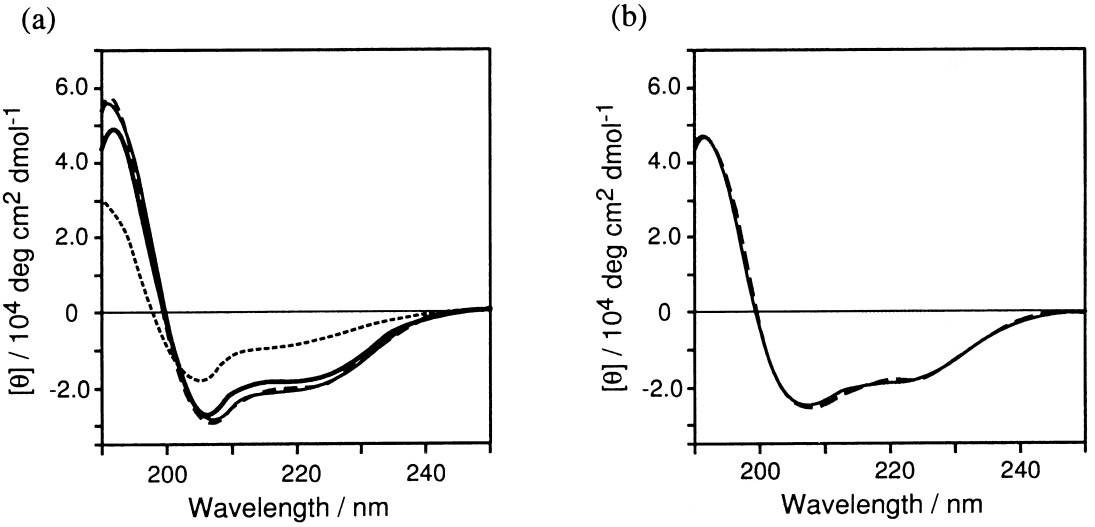

Figure 2. (a) CD spectra of $N40C_{NBA}$ (——), $N40T_{NBA}$ (– – – –), $N40C_{PNA}$ (- - - - - -), and $Rev_{34–50}$ (———) in TFE at 25 °C; (b) CD spectra of $Q36C_{NBA}$·$N40C_{NBA}$ (——), $Q36C_{NBA}$·$N40T_{NBA}$ (- - - -) in TFE at 25 °C. [Peptide] = 20 μM.

breaking nature.[24] Furthermore, the α-helicities of double mutants, $Q36C_{NBA}$·$N40C_{NBA}$ and $Q36C_{NBA}$·$N40T_{NBA}$, were estimated as 54 and 57%, respectively, ca. 10% lower than $N40C_{NBA}$ and $N40T_{NBA}$. These results indicate that the introduction of the second NBA unit slightly decreases the α-helix potential of the peptides. However, the α-helix potential of the double mutants was kept as similar level to that of $Rev_{34–50}$. In contrast, the α-helicity of $N40C_{PNA}$ was decreased to 30%. We have reported that the α-helix structure is very much lowered by the introduction of the flexible and achiral PNA unit in the middle position of the peptides, although the introduction of a PNA unit at the C-terminal of the Rev peptide does not interrupt the α-helix formation.[8] These results indicate that the NBA unit on the α-helix peptides is superior to the PNA unit in regard to keeping the peptide conformation.

In the case of Arg-mutant peptides, the NBA-conjugated peptides instead of Arg35, Arg39, or Arg44 also showed a typical α-helix CD pattern in TFE solution. The α-helicities of the peptides were estimated as about 50–70% (Table 1). The peptides having a cytosine and thymine moieties generally showed higher α-helicities than those having guanine and adenine, although the differences were dependent on the NBA positions. These findings indicate that the bulky purine rings on the peptides inhibit slightly the forming of the α-helical conformation compared with the pyrimidine rings. However, the introduction of a NBA unit in the peptide at each position does not strongly disturb the conformational properties of the peptides.

The binding activity of NBA-peptides at Asn40 with RRE IIB RNA

The binding properties of the peptides with RRE IIB RNA were evaluated by the competition assay[25] using the Rev peptide modified with 5-carboxytetramethylrhodamine at the N-terminal (Rhod-Rev) as a fluorescence tracer. All the experiments were carried out in 10 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA at 25 °C. Fluorescence anisotropy of Rhod-Rev was increased by the addition of RRE IIB RNA. The significant increase of anisotropy is attributed to the difference in molecular size and conformation between the free and bound Rhod-Rev with RRE IIB RNA. The dissociation constant of 2.1 nM was calculated from the increasing anisotropy for Rhod-Rev using an equation with 1:1 stoichiometry.

On the basis of this result, competition experiments were carried out in the mixture of Rhod-Rev (10 nM) and RRE IIB RNA (25 nM) by the addition of a peptide solution as a competitor. Fluorescence anisotropy values were decreased by the addition of $Rev_{34–50}$ and the NBA-peptides, affording the free Rhod-Rev (Fig. 3). The dissociation constant was also calculated by an equation with 1:1 stoichiometry from the anisotropy decrease. This competition assay revealed that $Rev_{34–50}$

Table 1. α-Helix contents of the peptides in TFE solution

Peptide	α-Helicity (%)[a]	Peptide	α-Helicity (%)
Rev_{34-50}	59	$R35G_{NBA}$	59
		$R35A_{NBA}$	55
$N40C_{NBA}$	63	$R35C_{NBA}$	67
$N40T_{NBA}$	66	$R35T_{NBA}$	63
N40A	59	R35A	65
$N40C_{PNA}$	30		
		$R39G_{NBA}$	60
$Q36C_{NBA}$[b]	60	$R39A_{NBA}$	54
$Q36T_{NBA}$	56	$R39C_{NBA}$	65
$Q36C_{NBA}$·$N40C_{NBA}$	54	$R39T_{NBA}$	59
$Q36C_{NBA}$·$N40T_{NBA}$	57	R39A	55
		$R44G_{NBA}$	54
		$R44A_{NBA}$	67
		$R44C_{NBA}$	66
		$R44T_{NBA}$	67
		R44A	51

[a]α-Helix contents were estimated from $[\theta]_{222}$ according to the method of ref 23.
[b]Data from ref 11.

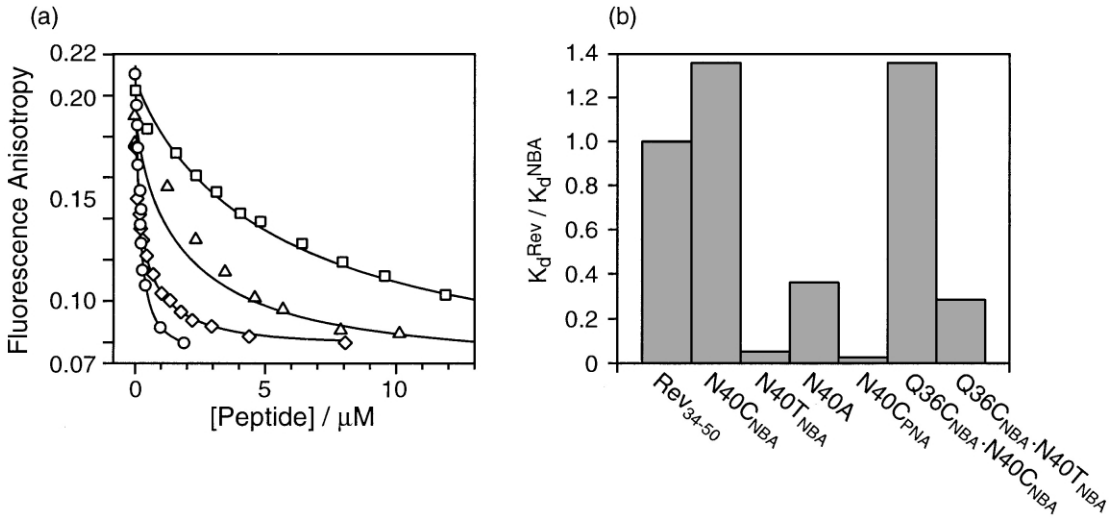

Figure 3. (a) Fluorescence anisotropy of Rhod-Rev with RRE IIB RNA as a function of $N40C_{NBA}$ (○), $N40T_{NBA}$ (△), $N40C_{PNA}$ (□), and N40A (◇) concentrations in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA at 25 °C. [Rhod-Rev] = 10 nM and [RRE IIB] = 25 nM. (b) Relative binding affinities between the NBA-peptides and Rev_{34-50} to RRE IIB RNA. K_d^{Rev}/K_d^{NBA} is the ratio of the dissociation constants of Rev_{34-50} and NBA-peptides.

bound to RRE IIB RNA strongly with a dissociation constant of 3.4 nM (Table 2). The binding affinity of $N40C_{NBA}$ to the RNA was as strong as that of Rev_{34-50} ($K_d = 2.5$ nM). N40A bound to the RNA with $K_d = 9.4$ nM, ca. 4-fold weaker than $N40C_{NBA}$ did. These results suggest that the cytosine moiety of $N40C_{NBA}$ contributes to the RNA binding, that is, the cytosine base in the peptide may interact with the G47–A73 base pair in the RNA such that the Asn40 residue in Rev_{34-50} does.[3] In contrast, the binding affinity of $N40T_{NBA}$ to RRE IIB RNA was greatly diminished to $K_d = 67$ nM, ca. 20-fold weaker than Rev_{34-50} did, even though $N40T_{NBA}$ has an α-helix potential similar to $N40C_{NBA}$. Although N40A decreased the RNA-binding affinity, ca. 3-fold weaker compared with Rev_{34-50} by removing the function of the Asn residue, the thymine base in $N40T_{NBA}$ significantly weakened the RNA-binding affinity more than Ala in N40A. These findings indicate that the thymine moiety hardly makes a specific contact such as hydrogen bonding to the RNA, and also that the existence of the thymine base, which possesses the bulky and hydrophobic methyl group, in the peptide further prevents the binding of the peptide with the RNA. The binding affinity of $N40C_{PNA}$, which has also the cytosine moiety in the peptide, to RRE IIB RNA was rather low at $K_d = 140$ nM. This result suggests that the decrease of the binding affinity is attributed to not only the deletion of the charged residue, Arg41, but also the lower α-helix potential of $N40C_{PNA}$ (30% in TFE).[8,11,19]

The binding activity of NBA-peptides at Gln36 and Asn40 with RRE IIB RNA

In the cases of the peptides conjugated with two NBA units, the dissociation constant of $Q36C_{NBA}{\cdot}N40C_{NBA}$ with RRE IIB RNA was 2.5 nM as same as that of $N40C_{NBA}$ (Fig. 3b and Table 2), even though the α-helix potential of $Q36C_{NBA}{\cdot}N40C_{NBA}$ was lower than that of $N40C_{NBA}$. These results indicate that two cytosine moieties, substituted for Gln36 and Asn40, may interact with the bases, namely G48 base and G47–A73 base pair, respectively,[3] resulting in keeping the RNA-binding affinity as similar to that of $N40C_{NBA}$ and wild-type Rev_{34-50}. $Q36C_{NBA}{\cdot}N40T_{NBA}$ bound to the RNA with $K_d = 12$ nM, ca. 5-fold stronger than the single mutant $N40T_{NBA}$ did. This result strongly suggests that the additional interaction by the cytosine moiety in $Q36C_{NBA}{\cdot}N40T_{NBA}$ effectively compensates for the lower RNA-binding affinity of $N40T_{NBA}$.

Furthermore, for the elucidation of the binding specificity of $Q36C_{NBA}{\cdot}N40C_{NBA}$ and $Q36C_{NBA}{\cdot}N40T_{NBA}$ for RRE IIB RNA, a mutant G48A RNA, in which the guanine-48 base of RRE IIB RNA was replaced by adenine, was also prepared (Fig. 1c). We have reported that the designed peptide $Q36C_{NBA}$ bound to the mutant RNA with $K_d = 5.5$ nM (Table 3), 3.2-fold weaker than that of the wild type RNA ($K_d = 1.7$ nM). In contrast, Rev_{34-50} bound to the mutant RNA with $K_d = 4.6$ nM,

Table 2. Dissociation constants (K_d) of the NBA-peptides at Gln36 and Asn40 with wt RRE IIB RNA

Peptide	K_d (nM)	K_d^{Rev}/K_d^{NBA}[a]
Rev_{34-50}	3.4±0.3	1.00
$N40C_{NBA}$	2.5±0.2	1.36
$N40T_{NBA}$	67±7.6	0.05
N40A	9.4±0.2	0.36
$N40C_{PNA}$	140±6.2	0.02
$Q36C_{NBA}$[b]	1.7±0.1	2.00
$Q36T_{NBA}$	5.4±0.1	0.63
$Q36C_{NBA}{\cdot}N40C_{NBA}$	2.5±0.3	1.36
$Q36C_{NBA}{\cdot}N40T_{NBA}$	12.0±1.2	0.28

[a] K_d^{Rev}/K_d^{NBA} is the ratio of dissociation constants of Rev_{34-50} and NBA-peptides with wt RRE IIB RNA.
[b] Data from ref 11.

comparable to that of the wild type RRE IIB RNA ($K_d = 3.4$ nM).[11] That is, the cytosine moiety in $Q36C_{NBA}$ may interact with guanine-48 in RRE IIB RNA, and the interaction enhances the binding specificity to the wild type RRE IIB RNA more than Gln36 in $Rev_{34–50}$. It is expected that the cytosine moiety replacing Gln36 in $Q36C_{NBA}$·$N40C_{NBA}$ also enhances the binding specificity to the wild type RNA. $Q36C_{NBA}$·$N40C_{NBA}$ bound to the mutant G48A RNA with a dissociation constant of 10.0 nM, 4.0-fold weaker than that of the wild type RNA (Table 3). That is, the RNA mutation at guanine-48 in RRE IIB RNA decreases the binding ability of $Q36C_{NBA}$·$N40C_{NBA}$, but does not change the ability of $Rev_{34–50}$. The binding affinity of $Q36C_{NBA}$·$N40T_{NBA}$ to the mutant G48A RNA was also decreased to $K_d = 28.0$ nM, 2.3-fold weaker than that of the wild type RNA. These results indicate that the cytosine moiety at the 36th position can interact specifically with the guanine-48 in RRE IIB RNA, and the cytosine at the 40th position also has contacts with the G47–A73 base pair, resulting in that the binding affinity and specificity was enhanced more than that of $Rev_{34–50}$ and $Q36C_{NBA}$.

The binding activity of NBA-peptides at Arg35, Arg39, and Arg44 with RRE IIB RNA

In the series of Arg35 mutant peptides, R35A having Ala instead of Arg35 of $Rev_{34–50}$ bound to RRE IIB RNA with a dissociation constant of 36 nM, 10-fold weaker than $Rev_{34–50}$ did ($K_d = 3.4$ nM). Since Arg35 residue in $Rev_{34–50}$ interacted not only with the phosphate backbone in RRE IIB RNA but also with the base (guanine-67) in the RNA specifically,[3] deletion of the Arg residue decreased significantly the binding affinity to the RNA. The NBA-peptides, $R35G_{NBA}$ and $R35A_{NBA}$, bound to the RNA with $K_d = 12$ and 9.9 nM, respectively, ca. 3- or 4-fold stronger than R35A did (Table 4). In contrast, the binding affinities of $R35C_{NBA}$ and $R35T_{NBA}$ to the RNA were rather low as $K_d = 29$ and 42 nM, a similar affinity to R35A. These results suggest that the guanine and adenine moieties of the peptides at the 35th position are successful in the RNA binding, but the cytosine and thymine moieties are not effective for the RNA binding. It seems that the purine ring can reach the bases in the RNA but the pyrimidine ring is difficult to interact with the RNA bases.

In the series of Arg39 mutants, R39A bound to RRE IIB RNA with $K_d = 38$ nM, ca. 10-fold weaker than $Rev_{34–50}$ did. $R39G_{NBA}$ bound to the RNA with $K_d = 14$ nM, ca. 3-fold stronger than R39A did, and $R39A_{NBA}$ bound to the RNA with $K_d = 18$ nM, ca. 2-fold stronger than R39A (Table 4). These results suggest that the introduction of the guanine and adenine at the position of Arg39 was also effective for the RNA binding as similar to the cases of Arg35 mutants. Interestingly, $R39C_{NBA}$ and $R39T_{NBA}$ showed the dissociation constants of 81 and 70 nM, ca. 2-fold weaker than R39A. This result indicates that the introduction of the cytosine and thymine moieties at the position of Arg39 disturbs the RNA binding. The RNA-binding abilities of the peptides were significantly dependent on the structure of the nucleobase moieties in the peptides.

Next, the binding of the Arg44 mutant peptides with RRE IIB RNA was elucidated. The dissociation constant of R44A with the RNA was 109 nM, ca. 30-fold weaker than $Rev_{34–50}$. This result suggests that Arg44 of the Rev peptide is most important for the binding to RRE IIB RNA, owing to the significantly specific interaction of the guanidium group on the Arg side chain with the base in the RNA. Interestingly, $R44G_{NBA}$ bound to the RNA with $K_d = 6.9$ nM, ca. 16-fold stronger than R44A did (Fig. 4 and Table 4). This RNA-binding affinity of $R44G_{NBA}$ was almost comparable to that of $Rev_{34–50}$ (2-fold weaker), despite diminishing the cationic Arg side chain. In contrast, the binding affinities of $R44A_{NBA}$, $R44C_{NBA}$, and $R44T_{NBA}$ were small as $K_d = 20$, 90, and 81 nM, respectively. That is, only the guanine base is replaceable at the position of Arg44 so as to maintain the high binding affinity. These results suggest that the guanine moiety in $R44G_{NBA}$ can make a specific contact such as hydrogen bonding with the RNA.

For the elucidation of the binding specificity of $R44G_{NBA}$ to RRE IIB RNA, a mutant RNA C46–G74, in which G46–C74 base pair was replaced by C46–G74, was also prepared (Fig. 1c). It was reported by the NMR structural analyses that the Arg44 side chain in the Rev peptide interacts with the U45 and is close to the G46 base in RRE IIB RNA,[3] and replacing G46–C74 with the C46–G74 base pair in RRE IIB RNA decreased the RNA-binding affinity of the Rev peptide.[19,20] We have expected that the introduction of the G_{NBA} unit at the Arg44 site increases the binding specificity to RRE IIB RNA, due to the specific interaction of the guanine moiety in the peptide with the base in RNA.

$Rev_{34–50}$ bound to the mutant C46G74 RNA with a dissociation constant of 12 nM, 3.5-fold weaker than

Table 3. Dissociation constants (K_d) of the NBA-peptides at Gln36 and Asn40 with the G48A mutant RNA

peptide	K_d (nM) for G48A	K_d^{G48A}/K_d^{wt} [a]
$Rev_{34–50}$	4.5±0.3	1.3
$Q36C_{NBA}$	5.5±0.2	3.2
$Q36C_{NBA}$·$N40C_{NBA}$	10.0±1.0	4.0
$Q36C_{NBA}$·$N40T_{NBA}$	28.0±4.2	2.3

[a] K_d^{G48A}/K_d^{wt} is the ratio of the dissociation constants of peptides with G48A RNA and wt RRE IIB RNA.

Table 4. Dissociation constants (K_d) of the NBA-peptides at Arg35, Arg39 and Arg44 with RRE IIB RNA

	K_d (nM) (K_d^{Ala}/K_d^{NBA})[a]		
Peptide	Arg35	Arg39	Arg44
G_{NBA}	12±1 (3.00)	14±1 (2.71)	6.9±0.4 (15.8)
A_{NBA}	9.9±0.4 (3.63)	18±1 (2.11)	20±2 (5.45)
C_{NBA}	29±2 (1.24)	81±5 (0.47)	90±5 (1.21)
T_{NBA}	42±3 (0.86)	70±3 (0.54)	81±3 (1.35)
Ala	36±2 (1.00)	38±3 (1.00)	109±5 (1.00)

[a] K_d^{Ala}/K_d^{NBA} is the ratio of the dissociation constants of the peptides having Ala and NBA-peptides with RRE IIB RNA.

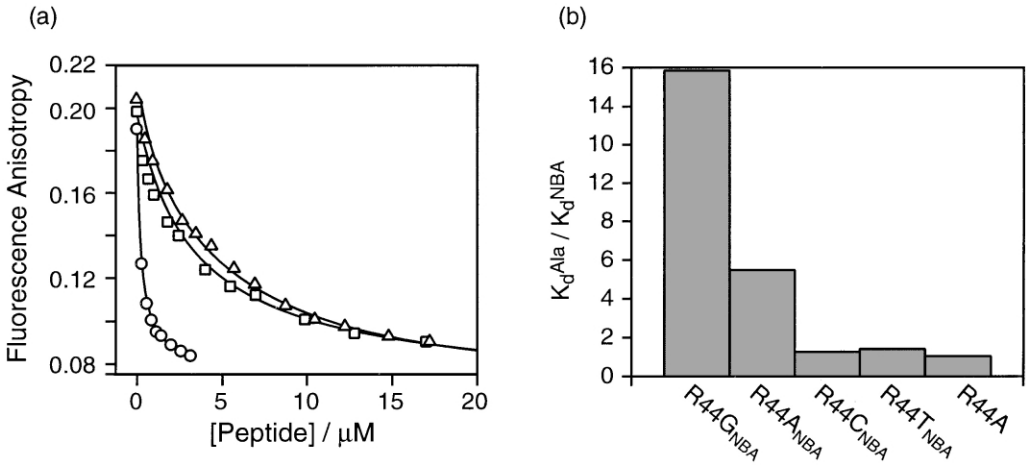

Figure 4. (a) Fluorescence anisotropy of Rhod-Rev with RRE IIB RNA as a function of $R44G_{NBA}$ (○), $R44C_{NBA}$ (□), and R44A (△) concentrations in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA at 25 °C. [Rhod-Rev] = 10 nM and [RRE IIB] = 25 nM. (b) Relative binding affinities of the Arg44-mutant peptides with RRE IIB RNA. K_d^{Ala}/K_d^{NBA} is the ratio of dissociation constants of R44A and NBA-peptides.

that to the wild type RRE IIB RNA (Table 5). R44A also bound to the mutant RNA with $K_d = 168$ nM, 1.5-fold weaker than that to the wild type RNA. These results indicate that the interaction of the Arg side chain with the bases in the RNA enhances the binding specificity to the wild type RNA. Interestingly, $R44G_{NBA}$ and $R44A_{NBA}$ bound to the mutant RNA with $K_d = 44$ and 122 nM, 6.4- and 6.1-fold, respectively, weaker than those to the wild type RNA. From the comparison of the dissociation constants of $Rev_{34–50}$, $R44G_{NBA}$, and $R44A_{NBA}$ it is apparent that the mutation of the G46–C74 pair to C46–G74 decreases the binding affinity of $R44G_{NBA}$ and $R44A_{NBA}$ more than that of $Rev_{34–50}$. That is, the binding specificity of $R44G_{NBA}$ and $R44A_{NBA}$ to the RNA is superior to that of $Rev_{34–50}$. On the contrary, binding affinities of $R44C_{NBA}$ and $R44T_{NBA}$ to the mutant RNA were slightly decreased to $K_d = 156$ and 224 nM, respectively, ca. 2-fold lower than those to the wild type RNA. Those analyses with the mutant RNA imply that the guanine moiety of $R44G_{NBA}$ can make a specific interaction with the U45 or G46 base in RRE IIB RNA, and the interaction enhances the binding specificity of the peptide with the RNA. In the case of $R44A_{NBA}$, the adenine moiety of $R44A_{NBA}$ can also make a specific interaction with the base in the RNA; however, the introduction of the adenine moiety may slightly disturb the structure of the peptide–RNA complex, resulting in the result that $R44A_{NBA}$ was not a superior binder to $R44G_{NBA}$.

Table 5. Dissociation constants (K_d) of NBA-peptides with the C46G74 mutant RNA

Peptide	K_d (nM)	K_d^{C46G74}/K_d^{wta}
Rev_{34-50}	12±2	3.5
$R44G_{NBA}$	44±4	6.4
$R44A_{NBA}$	122±10	6.1
$R44C_{NBA}$	156±18	1.7
$R44T_{NBA}$	224±17	2.3
R44A	168±29	1.5

[a] K_d^{C46G74}/K_d^{wt} is the ratio of the dissociation constants of peptides with C46G74 RNA and RRE IIB RNA.

Conclusion

For the aim to construct novel RNA-binding molecules using the function of nucleobase moieties on the rigid conformation, we have demonstrated that the Rev-peptides, conjugated with L-α-amino acids with a nucleobase, were successfully designed and synthesized, and bound specifically to the RRE IIB RNA. CD studies of the designed peptides revealed that the NBA-peptides have a potential to fold in an α-helix structure, although introduction of the PNA unit at the middle position inhibits extremely the formation of an α-helix conformation. The NBA unit on the α-helix peptide is superior to the PNA unit in regard to keeping the peptide conformation.

The first series of the peptides, in which C_{NBA} and T_{NBA} were introduced instead of Asn40 in the $Rev_{34–50}$, showed unique RNA-binding properties depending on the type of the NBA units. The peptide $N40C_{NBA}$ bound to RRE IIB RNA as a similar level to $Rev_{34–50}$ despite diminishing the Asn residue. In contrast, the binding affinity of the peptide $N40T_{NBA}$ for the RNA was lowered much more than N40A. These findings indicate that not the thymine but the cytosine moiety in the peptide can interact with the bases in RRE IIB RNA in a specific manner such as Asn in $Rev_{34–50}$. Furthermore, the peptides having two cytosine NBA units at the positions of Gln36 and Asn40 also showed the RNA-binding affinity similar to native $Rev_{34–50}$ and a single mutant $N40C_{NBA}$. Both cytosine moieties in the peptide were successful in interacting with the bases in RRE IIB RNA for keeping the binding activity and enhancing the binding specificity. If the cytosine moiety of the peptides may compensate for the Asn function,[3]

exocyclic NH_2 proton and N3 atom of cytosine make hydrogen bonds with O6 oxygen of G47 and exocyclic NH_2 proton of A73, respectively, in the RNA.

The second series of the peptides, in which C_{NBA}, T_{NBA}, G_{NBA}, and A_{NBA}, were introduced instead of the Arg35, Arg39, or Arg44 of the $Rev_{34–50}$, also showed the RNA-binding properties depending on the NBA type. In the Arg35- and Arg39-mutant peptides, the binding affinities of the peptides having the G_{NBA} and A_{NBA} for RRE IIB RNA were higher than those having C_{NBA}, T_{NBA} and Ala. Interestingly, in the Arg44-mutant peptides, only the G_{NBA} mutant, $R44G_{NBA}$, bound to the RNA comparable to $Rev_{34–50}$, although the binding affinities of the other peptides were significantly decreased. The Arg44 residue of the Rev peptide is the most important residue for the binding with RRE IIB RNA. Moreover, only G_{NBA} can replace the Arg function in $Rev_{34–50}$, probably due to its specific contacts such as hydrogen bonding with the bases in the RNA.[3]

In summary, the NBA units in the α-helix-forming peptide, derived from HIV-1 Rev, can be effectively utilized for the specific binding with RRE IIB RNA. The function of the NBA units orientated on peptide structures will be further demonstrated for the binding to other RNAs. This study can lead to a new strategy applicable to the construction of molecules that specifically recognize structured RNAs using the various NBA units on peptide 2D/3D structures.

Experimental

Materials and methods

All chemicals and solvents were of reagent or HPLC grade. Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical. MALDI-TOFMS was measured on a Shimadzu MALDI III mass spectrometer by using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. HPLC was carried out on a YMC ODS A-302 5C18 column (YMC) (4.6×150 mm) or a YMC ODS A-323 5C18 column (10×250 mm) by employing a Hitachi L-7000 HPLC system. Amino acid analyses were performed by using the phenyl isothiocyanate (PTC) method on a Wakopak WS-PTC column (Wako chemical).

Synthesis of Fmoc-protected α-amino-γ-(Z-cytosine, thymine, guanine, and Z-adenine)-butyric acids (Fmoc-C_{NBA}(Z)-OH, Fmoc-T_{NBA}-OH, Fmoc-G_{NBA}-OH, and Fmoc-A_{NBA}(Z)-OH)

Fmoc-protected-γ-nucleobase amino acids were synthesized according to the reported method[10] with some modifications. *tert*-Butyl 4-bromo-2*S*-butoxycarbonylaminobutyrate (Boc-NH-CH(CH_2CH_2Br)-COO*t*-Bu) was obtained by the reaction (3 h at 0 °C) of the Boc-Glu-O*t*-Bu (26 mmol) and 2-mercaptopyridine *N*-oxide (27 mmol) in the presence of DCC (27 mmol) and dimethylaminopyridine (2.6 mmol) in a mixture of THF (10 mL) and $CBrCl_3$ (10 mL) followed by irradiation with a 100W lamp for 2 h at 20 °C and purification by column chromatography on silica (80% yield). Boc-NH-CH(CH_2CH_2Br)–COO*t*-Bu (4.6 mmol) was reacted with N^4-benzyloxycarbonylcytosine[26] (6.4 mmol) in the presence of K_2CO_3, Cs_2CO_3, and tetrabutylammonium iodide (TBAI; 0.58 mmol) in DMF at 50 °C. Purification by silica gel chromatography gave *tert*-butyl 4-(N^4-benzyloxycarbonylcytosin-1-yl)-*2S*-butyloxycarbonylaminobutyrate (Boc-C_{NBA}(Z)–O*t*-Bu) (50% yield). Deprotection of the Boc and *t*-Bu groups in Boc-C_{NBA}(Z)–O*t*-Bu was carried out with TFA in CH_2Cl_2 solution at 0 °C, and protection of α-amino group was reacted with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) to give final material 4-(N^4-benzyloxycarbonylcytosin-1-yl)-*2S*-(fluoren-9-ylmethoxycarbonyl)-aminobutyric acid (Fmoc-C_{NBA}(Z)–OH) (80% yield). Reaction of Boc-NH-CH(CH_2CH_2Br)–COO*t*-Bu with N^3-benzoylthymine[27] in the presence of K_2CO_3, Cs_2CO_3, and TBAI in DMF at 50 °C gave *tert*-butyl 4-(N^3-benzoylthymin-1-yl)-*2S*-butyloxycarbonylaminobutyrate (Boc-T_{NBA}(Bz)–O*t*-Bu) (90% yield). Deprotection of the benzoyl, Boc, and *t*-Bu groups in Boc-T_{NBA}(Bz)–O*t*-Bu was carried out with 30% HBr in acetic acid at 0 °C, and protection of α-amino group was reacted with Fmoc-OSu to give 4-(thymin-1-yl)-2*S*-(fluoren-9-ylmethoxycarbonyl)aminobutyric acid (Fmoc-T_{NBA}-OH) (63% yield). Reaction of Boc-NH-CH(CH_2CH_2Br)-COO*t*-Bu with 2-amino-6-chloropurine in the presence of K_2CO_3, Cs_2CO_3, and TBAI in DMF at 50 °C gave *tert*-butyl 4-(2-amino-6-chloropurin-9-yl)-*2S*-butyloxycarbonylaminobutyrate (Boc-G_{NBA}(Cl)-O*t*-Bu) (62% yield). Deprotection of the Boc and *t*-Bu groups and hydrolysis at position 6 of the purine ring in Boc-G_{NBA}(Cl)-O*t*-Bu were carried out with 1 N HCl solution for 2 h at 90 °C. The protection of α-amino group was reacted with Fmoc-OSu to give 4-(guanin-9-yl)-2*S*-(fluoren-9-ylmethoxycarbonyl)aminobutyric acid (Fmoc-G_{NBA}-OH) (76% yield). Reaction of Boc-NH-CH(CH_2CH_2Br)-COO*t*-Bu with N^6-N^6-benzyloxycarbonyladenine[26] in the presence of K_2CO_3, Cs_2CO_3, and TBAI in DMF at 50 °C gave *tert*-butyl 4-(N^6-benzyloxycarbonyladenin-9-yl)-2*S*-butyloxycarbonylaminobutyrate (Boc-A_{NBA}(Z)-O*t*-Bu) (47% yield). After treatment with TFA and protection by the Fmoc group, 4-(N^6-benzyloxycarbonyladenin-9-yl)-2*S*-(fluoren-9-yl-methoxycarbonyl) aminobutyric acid (Fmoc-A_{NBA}(Z)-OH) was obtained (37% yield). The guanine ring was not protected, since the amino group on the guanine was hardly modified under the reaction of elongation of peptides. These amino acid derivatives were identified by ^1H NMR spectra; ^1H NMR (500 MHz, (D_6)DMSO, 25 °C): Fmoc-C_{NBA}(Z)-OH 10.76 (br, 1H), 7.92–7.86 (m, 3H), 7.72 (m, 2H), 7.43–7.32 (m, 10H), 6.98 (d, 1H), 5.18 (s, 2H), 4.32 (m, 2H), 4.24 (m, 1H), 3.93 (m, 1H), 3.83 (m, 2H), 2.15 (m, 1H), 1.94 (m, 1H); Fmoc-T_{NBA}-OH 11.23 (s, 1H), 7.90 (d, 2H), 7.73 (d, 2H), 7.46–7.30 (m, 6H), 4.31 (m, 2H), 4.24 (m, 1H), 3.95 (m, 1H), 3.69 (m, 2H), 2.18 (m, 1H), 1.88 (m, 1H), 1.72 (s, 3H); Fmoc-G_{NBA}-OH 10.58 (s, 1H), 7.90 (d, 2H), 7.78 (d, 1H), 7.72 (d, 2H), 7.62 (s, 1H), 7.43–7.30 (m, 5H), 6.43 (br, 2H), 4.33 (m, 2H), 4.25 (m, 1H), 4.20–3.94 (m, 2H), 3.86 (m, 1H), 2.25 (m, 1H), 2.00 (m, 1H); Fmoc-A_{NBA}(Z)–OH 10.61 (s, 1H), 8.57 (s, 1H), 8.36 (s, 1H), 7.88 (d, 2H), 7.73 (d,

2H), 7.46–7.30 (m, 10H), 5.38 (s, 2H), 4.68–4.40 (m, 5H), 3.87 (m, 1H), 2.40 (m, 1H), 2.14 (m, 1H).

Peptide synthesis

The peptides were synthesized by the solid-phase method according to the procedure using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole hydrate (HOBt·H_2O) as a coupling reagent.[21] Fmoc derivatives of the following amino acids were used: Arg(Mtr), Asn(Trt), Gln(Trt), Glu(O*t*-Bu), and Thr(*t*-Bu) (Mtr; 4-methoxy-2,3,6-trimethylbenzenesulphonyl, Trt; triphenylmethyl). To synthesize succinylated-peptide resin, the Fmoc-deprotected peptide resin was treated with succinic anhydride (10 equiv×2) in 2-methylpyrolidone (NMP) for 30 min. To remove the resin and protecting groups, the peptide resin was stirred with 1 M trimethylsilyl bromide (TMSBr; 0.70 mL) in TFA (4.0 mL) in the presence of thioanisole (0.36 mL), m-cresol (0.06 mL), and ethanedithiol (0.18 mL) as a scavenger for 1.5 h at 0 °C.[28] The resin was filtered and washed three times with TFA. The solvent was removed by the flow of N_2 gas and solidified with diethyl ether on ice-bath to give crude peptides. The Z groups on the adenine and the cytosine moieties in the peptides were stable during the treatment with 1 M TMSBr. The Z groups were removed with 1 M trimethylsilyl trifluoromethanesulphonate (TMSOTf; 0.93 mL) in TFA (3.0 mL) in the presence of thioanisole (0.57 mL), m-cresol (0.10 mL), and ethanedithiol (0.20 mL) for 1.5 h at 0 °C.[29] The product was solidified with diethyl ether on ice-bath. All crude peptides were purified with RP-HPLC on a YMC ODS A-323 column (10×250 mm) using a linear gradient of 5–35% acetonitrile/0.1% TFA (30 min) to give purified peptides. Peptides were identified by molecular ion peak $(M+H)^+$ on MALDI-TOFMS; $N40C_{NBA}$, *m/z* 2617.2 ($(M+H)^+$) (calc. = 2617.9); $N40T_{NBA}$, *m/z* 2632.0 ($(M+H)^+$) (calc. = 2633.0); $N40C_{PNA}$, *m/z* 2518.0 ($(M+H)^+$) (calc. = 2518.8); $Q36C_{NBA}$·$N40C_{NBA}$, *m/z* 2685.1 ($(M+H)^+$) (calc. = 2684.0); $Q36C_{NBA}$·$N40T_{NBA}$, *m/z* 2698.4 ($(M+H)^+$) (calc. = 2699.0); $R35G_{NBA}$, *m/z* 2615.9 ($(M+H)^+$) (calc. = 2615.9); $R35A_{NBA}$, *m/z* 2599.1 ($(M+H)^+$) (calc. = 2599.9); $R35C_{NBA}$, *m/z* 2575.9 ($(M+H)^+$) (calc. = 2575.9); $R35T_{NBA}$, *m/z* 2591.4 ($(M+H)^+$) (calc. = 2590.9); $R39G_{NBA}$, *m/z* 2617.0 ($(M+H)^+$) (calc. = 2615.9); $R39A_{NBA}$, *m/z* 2600.3 ($(M+H)^+$) (calc. = 2599.9); $R39C_{NBA}$, *m/z* 2575.5 ($(M+H)^+$) (calc. = 2575.9); $R39T_{NBA}$, *m/z* 2591.4 ($(M+H)^+$) (calc. = 2590.9); $R44G_{NBA}$, *m/z* 2616.4 ($(M+H)^+$) (calc. = 2615.9); $R44A_{NBA}$, *m/z* 2599.3 ($(M+H)^+$) (calc. = 2599.9); $R44C_{NBA}$, *m/z* 2575.9 ($(M+H)^+$) (calc. = 2575.9); $R44T_{NBA}$, *m/z* 2590.7 ($(M+H)^+$) (calc. = 2590.9).

Preparation of RRE IIB and the mutant RNA

Double-stranded DNA corresponding to RRE IIB RNA was prepared by the PCR strategy using 5′-AATTTAATACGACTCACTATAGGCTGGTATGGGCGCAGCGTCAATGACGCTGACGGTACAGGCCAGCC-3′ synthetic 68-mer ssDNA (Amersham Pharmacia Biotech). The two PCR primers used were 5′-AATTTAATACGACTCACTATA-3′ (primer 1; T7 promoter sequence, 21-mer) and 5′-GGCTGGCCTGTACCGTCAGCG-3′ (primer 2, 21-mer). PCR was carried out at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and the product was purified by ethanol precipitation. RRE IIB RNA was prepared by transcription[30] of the DNA using the RiboMAX™ large scale RNA production system-T7 (Promega). The template DNA was degraded by RNase-free DNase I, and then RNA was purified by Sephadex G-50 gel filtration and by polyacrylamide gel electrophoresis. Other mutant RRE IIB RNAs, G48A and C46G74 were also prepared by the same method described above. Concentrations of RNA were determined by the absorbance at 260 nm.

CD measurements

CD spectra were recorded on a Jasco J-720WI spectropolarimeter using a quartz cell with 1.0 mm pathlength in the amide region (190–250 nm). Peptides were dissolved in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA or trifluoroethanol (TFE) solution in peptide concentration of 20 μM.

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were performed on a Shimadzu RF-5300PC spectrofluorophotometer using a quartz cell with 1.0 cm pathlength at 25 °C in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM EDTA. Fluorescence anisotropy was calculated by the intensities at 580 nm excited at 540 nm.

Determination of dissociation constants between peptides and RRE IIB RNA

The affinities of the peptides to RRE IIB RNA were determined by the competition assay using fluorescence anisotropy change of Rhod-Rev as a tracer. Mixture of Rhod-Rev (10 nM) and RRE IIB RNA (25 nM) in the buffer solution was titrated with peptide. After each addition of peptide, samples were stirred for 1 min and equilibrated for 5 min at 25 °C, then fluorescence anisotropy was measured. The anisotropy change with increasing peptide concentrations was corrected for dilution. The dissociation constants of the peptides with RNA were calculated by eq (1) assumed as a 1:1 stoichiometry using Kaleida Graph (Synergy Software);

$$(P)_0 = \left(K'_d/(K_d(A - A_f)/(A_b - A)) + 1\right) \times \left((R)_0 - (K_d(A - A_f)/(A_b - A)) - (T)_0(A - A_f)/(A_b - A_f)\right) \quad (1)$$

where $(P)_0$, $(R)_0$, and $(T)_0$ represent the initial concentrations of the peptide, RNA, and Rhod-Rev as a tracer, respectively, and A, A_b, A_f are the anisotropy values of each solution, the bound Rhod-Rev, and the free Rhod-Rev, respectively. K'_d and K_d are the dissociation constants of Rhod-Rev and the peptide with RNA, respectively.

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