J|A|C|S
ARTICLES
Published on Web 04/06/2002

In Vitro Selection of ATP-Binding Receptors Using a Ribonucleopeptide Complex

Takashi Morii,*,†,‡ Masaki Hagihara,† Shin-ichi Sato,† and Keisuke Makino†

Contribution from the Institute of Advanced Energy, Kyoto University, and PRESTO, Japan Science and Technology Corporation, Uji, Kyoto 611-0011, Japan

Received July 10, 2001

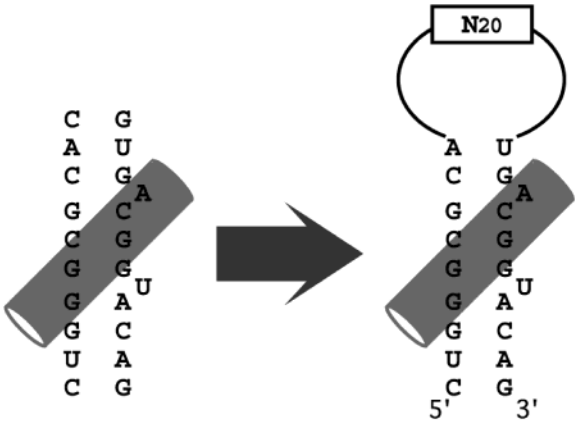
Abstract: A recently described three-dimensional structure of the ribosome provides a sense of remarkable diversity of RNA−protein complexes. We have designed a new class of scaffold for artificial receptors, in which a short peptide and RNA with a randomized nucleotide region form a stable and specific complex. The randomized nucleotide region was placed next to the HIV-1 Rev response element to enable the formation of "ribonucleopeptide" pools in the presence of the Rev peptide. In vitro selection of RNA oligonucleotides from the randomized pool afforded a ribonucleopeptide receptor specific for ATP. The ATP-binding ribonucleopeptide did not share the known consensus nucleotide sequence for ATP aptamers and completely lost its ATP-binding ability in the absence of the Rev peptide. The ATP-binding activity of the ribonucleopeptide was increased by a substitution of the N-terminal amino acid of the Rev peptide. These results demonstrate directly that the peptide is incorporated in the functional structure of RNA and suggest that amino acids outside the RNA-binding region of the peptide modulate the ATP-binding of ribonucleopeptide. Our approach would provide an alternative strategy for the design of "tailor-made" ribonucleopeptide receptors and enzymes.

Introduction

In vitro selection provides one of the most powerful strategies for obtaining functional RNA molecules.[1] The selection and evolution technique has produced RNA aptamers that specifically bind small molecules[1,2] or ribozymes that catalyze a variety of chemical reactions[1,3] from randomized pools of oligoribonucleotides. Recent advances in the in vitro selection method have provided signaling nucleic acid aptamers[4] applicable for biosensors and allosteric ribozymes regulated by both ligands and proteins.[5] The key feature of RNA to perform such a variety of function has been attributed to the fact that a random pool of RNA provides a large number of different shapes that facilitate a chance to select RNA molecules with desired function.[6] RNA performs a wide range of functions in conjugation with proteins in all cells. One of such examples is found in the three-dimensional structures of RNA−protein complexes, particularly in that of the ribosome.[7] The ribosome is the ribonucleoprotein that performs the remarkable task of selecting the amino acids to be added to the growing polypeptide chain by reading successive messenger RNA codons. It has been considered that RNA, not protein, is responsible for catalysis of the peptide transferase activity and that the ribosomal proteins stabilize and orient the RNA into a specific, active structure.[6,8] Such protein cofactors are required by many RNA enzymes, including RNase P and most group I and group II introns, for catalysis under physiological conditions.[9] The remarkable diversity of RNA−protein complexes suggests that its miniature analogue, a ribonucleopeptide, serves as an alternative framework for the design of tailor-made receptors and enzymes. We report here a strategy that allows assembly of a ribonucleopeptide receptor by means of the structure-based design[10] and the in vitro selection method.

* To whom correspondence should be addressed. E-mail: t-morii@iae.kyoto-u.ac.jp.
† Kyoto University.
‡ PRESTO, JST.

(1) (a) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−822. (b) Ellington, A. D. *Curr. Biol.* **1994**, *4*, 427−429. (c) Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. *Annu. Rev. Biochem.* **1995**, *64*, 763−797. (d) Osborne, S. E.; Ellington, A. D. *Chem. Rev.* **1997**, *97*, 349−370. (e) Wilson, D. S.; Szostak, J. W. *Annu. Rev. Biochem.* **1999**, *68*, 611−647.
(2) (a) Sassanfar, M.; Szostak, J. W. *Nature* **1993**, *364*, 550−553. (b) Burgstaller, P.; Famulok, M. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1084−1087. (c) Burke, D, H.; Gold, L. *Nucleic Acids Res.* **1997**, *25*, 2020−2024.
(3) (a) Bartel, D. P.; Szostak, J. W. *Science* **1993**, *261*, 1411−1418. (b) Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924−1927.
(4) (a) Potyrailo, R. A.; Conrad, R. C.; Ellington, A. D.; Hieftje, G. M. *Anal. Chem.* **1998**, *70*, 3419−3425. (b) Jhaveri, S.; Rajendran, M.; Ellington, A. D. *Nat. Biotechnol.* **2000**, *18*, 1293−1297. (c) Brody, E. N.; Gold, L. *J. Biotechnol.* **2000**, *74*, 5−13. (d) Yamamoto, R.; Baba, T.; Kumar, P. K. *Genes Cells* **2000**, *5*, 389−396. (e) Lee, M.; Walt, D. R. *Anal. Biochem.* **2000**, *282*, 142−146. (f) Hamaguchi. N.; Ellington, A. D.; Stanton, M. *Anal. Biochem.* **2001**, *294*, 126−131.
(5) (a) Tang, J.; Breaker, R. R. *Chem. Biol.* **1997**, *4*, 453−459. (b) Tang, J.; Breaker, R. R. *Nucleic Acids Res.* **1998**, *26*, 4214−4221. (c) Araki, M.; Okuno, Y.; Hara, Y.; Sugiura, Y. *Nucleic Acids Res.* **1998**, *26*, 3379−3384. (d) Koizumi, M.; Soukup, G. A.; Kerr, J. N. Q.; Breaker, R. R. *Nat. Struct. Biol.* **1999**, *6*, 1062−1071. (e) Robertson, M. P.; Ellington, A. D. *Nucleic Acids Res.* **2000**, *28*, 1751−1759.
(6) Fontana, W.; Konings, D. A. M.; Stadler, P. F.; Schuster, P. *Biopolymers* **1993**, *33*, 1389−1404.
(7) (a) Ban, N.; Nissen, P.; Hansen, J.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 905−920. (b) Nissen, P.; Hansen, J.; Ban, N.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 920−930.
(8) Puglisi, J. D.; Blanchard, S. C.; Green, R. *Nat. Struct. Biol.* **2000**, *7*, 855−861.
(9) Caprara, M. G.; Nilsen, T. W. *Nat. Struct. Biol.* **2000**, *7*, 831−833.
(10) (a) Pomerantz, J. L.; Sharp, P. A.; Pabo, C. O. *Science* **1995**, *267*, 93−96. (b) McColl, D. J.; Honchell, C. D.; Frankel, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9521−9526.



Ac-Rev: Ac-TRQARRNRRRRWRERQR-NH2

Figure 1. Schematic illustrations showing the structures of Rev−RRE complex[9] (left) and Rev−RRE20N ribonucleopeptide complex (right). An amino acid sequence for the Rev peptide (34−50, Ac-Rev) is shown at the bottom.

Results and Discussion

Design and in Vitro Selection of Ribonucleopeptide Complexes. On the basis of the *R*ev *r*esponse *e*lement (RRE) RNA and HIV-1 Rev peptide complex,[11] an RNA subunit, referred to as RRE20N, was designed to consist of two functionally separated regions, a possible ligand-binding region with 20 randomized nucleotides and an adjacent stem region that served as a binding site for the Rev peptide (Figure 1). Gel mobility shift assay of the RRE20N and the Rev peptide (Ac-Rev) confirmed an RNA−peptide complex formation, indicating that introduction of the additional ligand binding region did not interfere with the specific complex formation between RRE20N and the Rev peptide.

Ribonucleopeptide receptors for ATP were isolated from a pool of RNA sequences (4^{20}) by the in vitro selection method. In each round of selection RNA pools were incubated with immobilized ATP in the presence of the Rev peptide (Ac-Rev), unbound sequences were washed away, and then fractions were eluted with free ATP. The eluted fractions were collected, reverse transcribed, and applied to successive RT-PCR amplification to generate new DNA pools. DNA templates were transcribed, and the resulting RNA pools were subjected to the next round of selection. After nine rounds of selective amplification in the presence of the Rev peptide, the fraction of RRE20N−peptide complex eluting with free ATP increased to 15%. The RNA pool thus enriched in the presence of the Rev peptide, termed P9, showed considerably low ATP-binding activity (2%) in the absence of the peptide. As a control, selective amplifications of RRE20N in the absence of the peptide were performed in a separate run. With RNA pool enriched after six rounds, termed R6, the fraction of RRE20N eluting with free ATP increased to 19%. Both P9 and R6 were converted to DNA, cloned, and sequenced for further analysis.

Analysis of the nucleotide sequences of clones from P9 revealed three distinct consensus sequences (Figure 2). Among the 26 clones for P9, 15 revealed a nine-nucleotide consensus sequence 5′-GUGUA-UA-CU-3′, denoted as class I. Two other

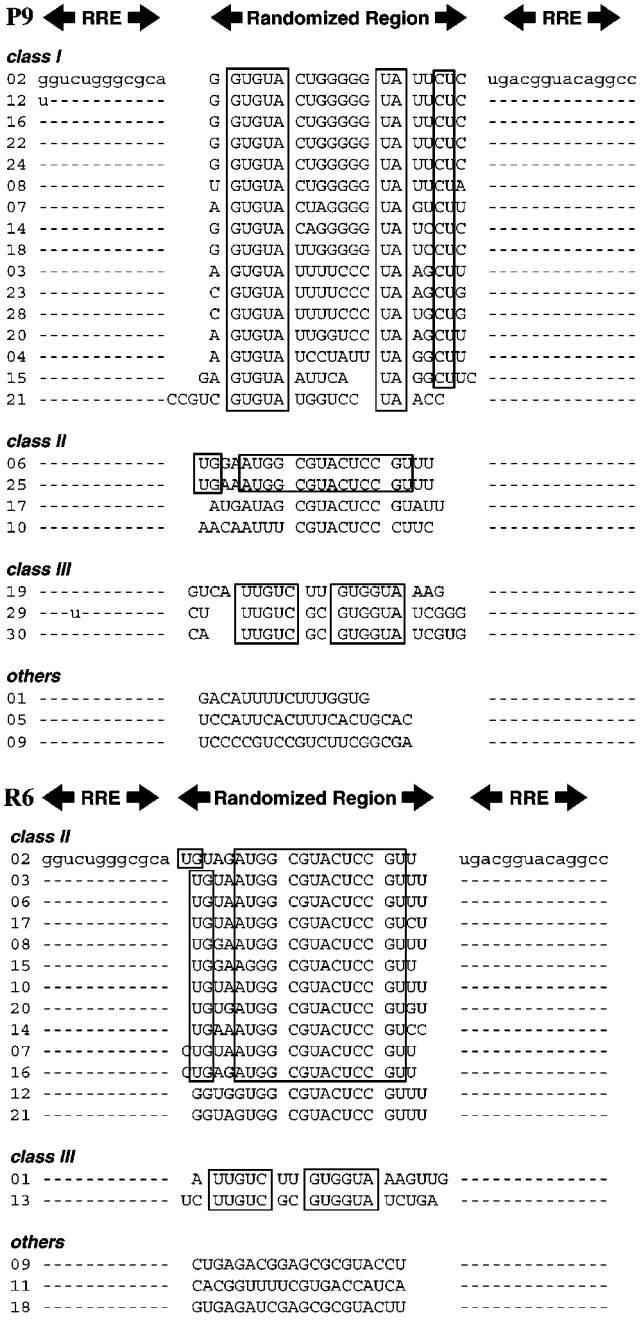


Figure 2. Sequences identified in the clones of selected by in vitro selection in the presence (P9, top) or absence (R6, bottom) of the Ac-Rev peptide. The characteristic consensus sequences in the N20 randomized region are marked by boxes. RRE denotes the nucleotide sequence of the Rev binding region.

types of consensus sequences were identified for the four and three clones with 5′-UG-AUGG-CGUACUCC-GU-3′ (class II) and 5′-UUGUC-GUGGUA-3′ (class III) consensus sequences, respectively. The class II sequence was dominantly found in 13 out of 18 clones from R6. Two clones from R6 revealed the class III consensus sequence, while none of the clones from R6 contained the class I consensus sequence. RNA aptamers for ATP,[2a] for nicotinamide adenine dinucleotide,[2b] and for *S*-adenosyl-methionine[2c] have been isolated previously, and the highly conserved sequence 5′-GGAAGA-3′ has been shown to contact directly with the adenosine moiety.[12] The RNA obtained in this study did not share a nucleotide sequence exactly matched

(11) Battiste, J. L.; Mao, H.; Rao, N. S.; Tan, R.; Muhandiram, D. R.; Kay, L. E.; Frankel, A. D.; Williamson, J. R. *Science* **1996**, *273*, 1547−1551.

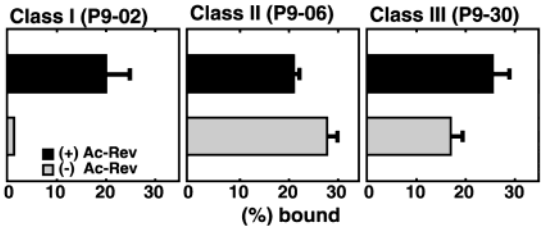


Figure 3. ATP binding activities of class I (P9-02), class II (P9-06), and class III (P9-30) ribonucleopeptides. Binding activities are represented by the fractions of ATP bound to RNAs in the presence (solid bar) or absence (shaded bar) of the Ac-Rev peptide.

to the previously reported ATP-binding consensus. However, clones from R6 preserved a homologous 5′-G(G/U)AAUGG-3′ sequence adjacent to the 5′-CGUACUCC-3′ consensus for the class II RNA sequence. Interestingly, we found the 5′-GUGUA-3′ class I consensus in the cyclic AMP-binding region of the cyclic AMP-response ribozyme,[5d] although its exact binding mode to cyclic AMP has not been determined.

ATP Binding of Ribonucleopeptide Complexes. Individual RNA clones from class I (P9-02), class II (P9-06), and class III (P9-30) were transcribed from the respective DNA sequences and tested for their ATP-binding activity. The fraction of each RNA-bound ATP was determined in the presence or absence of the Ac-Rev peptide (Figure 3). All the RNA−peptide complexes bound ATP with similar affinity. The class I RNA P9-02 obtained from the selective amplification in the presence of the Rev peptide completely lost its ATP-binding activity in the absence of the peptide. The class III RNA P9-30 also bound ATP more efficiently in the presence of the Ac-Rev peptide. In contrast, the affinity of class II RNA to ATP was slightly increased in the absence of the peptide. The Rev peptide affected differently the ATP-binding of each class of ribonucleopeptide, which in turn suggested that the ATP-binding pocket of the particular class of ribonucleopeptide was formed in unique geometry and distance to the RNA-bound peptide.

To obtain quantitative insights on the ATP binding of the ribonucleopeptide receptor, equilibrium dissociation constants of the class I RNA−peptide complex were determined. Formation of the Ac-Rev−class I RNA complex was confirmed by gel mobility shift assay in the absence or presence of ATP (Figure 4). A 1:1 class I RNA−peptide complex was formed with an equilibrium dissociation constant of 1.4 nM in the absence of ATP, and 1.2 nM in the presence of 2 mM ATP.

The stability of the ATP complex of the class I ribonucleopeptide was next analyzed by measuring the fraction of RNA bound to ATP−agarose at a range of immobilized ATP concentrations. A ^{32}P-end-labeled class I RNA−peptide complex was applied to an ATP−agarose resin, and unbound ribonucleopeptide was removed by washing with the binding buffer, and specifically bound ribonucleopeptide was competitively eluted off the immobilized ligand with a buffer containing ATP. The fraction of ribonucleopeptide specifically eluted at each ATP concentration yields a binding saturation curve (Figure 5). For comparison, a binding saturation curve for class II RNA to the immobilized ATP was obtained. An equilibrium dissociation constant (K_D = 32.8 ± 4.0 μM) for the complex of class I ribonucleopeptide to ATP was determined from the saturation curve by nonlinear least-squares analysis using the standard binding equation as described in the Experimental Section. Because the formation of RNA−peptide complex is quite efficient, the ribonucleopeptide complex is expected to form almost completely in the condition used for the ATP binding of the class I RNA and the Ac-Rev peptide. Though class II RNA in the absence of peptide revealed a higher affinity (K_D = 3.6 ± 0.1 μM) to ATP, the class I ribonucleopeptide formed an ATP complex with reasonable stability.

Specific Binding of Class I Ribonucleopeptide. To investigate the ATP-recognition mode of ribonucleopeptide receptors, competitive binding assays were performed by using ligands structurally related to ATP. The class I ribonucleopeptide preferentially bound ATP over other ribonucleotide triphosphate UTP, CTP, and GTP, demonstrating that the adenine base moiety actually participated in the selective binding (Figure 6A). As previously reported for the cyclic AMP aptamer,[13] 8-bromoadenosine triphosphate (8-BrATP) formed a more stable complex with the ribonucleopeptide than ATP did, suggesting that ATP bound to the class I ribonucleopeptides in the syn-conformation. The phosphate charge of ATP also contributed to the specific binding complex formation of the class I ribonucleopeptide (Figure 6B). Adenosine monophosphate (AMP) bound the class I ribonucleopeptide less efficiently than ATP did. Adenosine diphosphate (ADP) showed an affinity between ATP and AMP to class I ribonucleopeptide. The class I ribonucleopeptide failed to discriminate dATP from ATP efficiently, indicating that the 2′-OH group of ribose moiety did not contribute to the recognition of ATP. These results indicate that the adenine base is a key determinant for the specific ATP binding of the class I ribonucleopeptide. The adenine base would bind in the interior of binding pocket in the *syn*-conformation, while the triphosphate moiety may be either exposed to the solvent or contacted by the peptide subunit.

Roles of the Peptide Subunit for the ATP Binding of Class I Ribonucleopeptide. In an effort to elucidate the role of peptide in the recognition of ATP by class I ribonucleopeptide, the N-terminal portion of the acetylated Rev peptide (Ac-Rev) was modified by removing the acetyl group (N-Rev), or by adding charged amino acids Arg (R-Rev) or Glu (E-Rev). These peptides were allowed to form ribonucleopeptide complexes with the class I P9-02 RNA, and analyzed for the ATP-binding (Figure 7). Interestingly, ribonucleopeptides with N-Rev and R-Rev showed higher affinity to ATP with equilibrium dissociation constants of 16.4 ± 0.9 and 19.0 ± 0.3 μM, respectively. Ribonucleopeptide with E-Rev bound ATP with slightly lower affinity (K_D = 38.2 ± 1.4 μM) than the original Ac-Rev ribonucleopeptide did. Because the stability of these class I ribonucleopeptide P9-02 complexes judged by gel shift titration assay were similar to each other, these results suggest that the N-terminal region of Rev peptide participates in the formation of the binding pocket for ATP. Other possibilities, such as a slight change in the overall structure of the ribonucleopeptide, would also explain the observed difference in the ATP binding affinity.

Conclusions

Structure-based design using the Rev-RRE−RNA complex[9] followed by the in vitro selection method afforded a peptide−

(12) (a) Jiang, F.; Kumar, R. A.; Jones, R. A.; Patel, D. J. *Nature* **1996**, *382*, 183−186. (b) Dieckmann, T.; Suzuki, E.; Nakamura, G. K.; Feigon, J. *RNA* **1996**, *2*, 628−640. (c) Dieckmann, T.; Butcher, S. E.; Sassanfar, M.; Szostak, J. W.; Feigon, J. *J. Mol. Biol*. **1997**, *273*, 467−478.

(13) Koizumi, M.; Breaker, R. R. *Biochemistry* **2000**, *39*, 8983−8992.

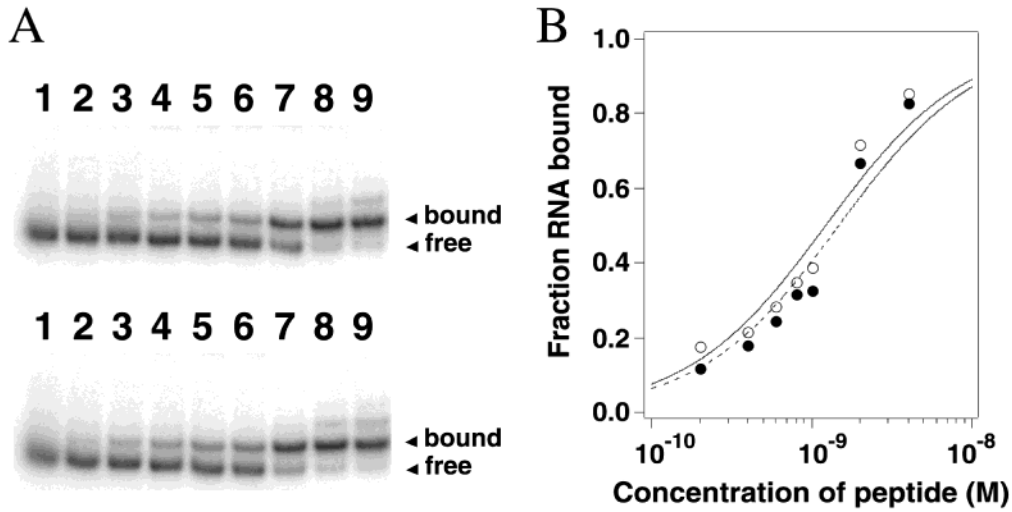


Figure 4. (A) Autoradiograms of the gel shift assay show class I RNA forms a complex with the Ac-Rev peptide in the absence (top) or presence (bottom) of 2 mM ATP in a buffer containing 10 mM Tris-HCl (pH7.6), 100 mM KCl, 5 mM $MgCl_2$, 0.005% Tween 20, and 6% sucrose at 4 °C. Peptide concentrations for lanes 1 to 9 were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, and 6.0 nM. (B) Semilogarithmic plots show the fraction of ^{32}P-labeled RNA bound to the Ac-Rev peptide in the absence (solid circles) or presence (open circles) of 2 mM ATP.

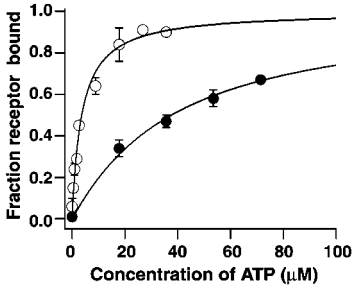


Figure 5. Saturation curves for the binding of the class I ribonucleopeptide complex (solid circles) and the class II RNA (open circles) to immobilized ligand as described in the Experimental Section. The class I ribonucleopeptide complex was formed with RNA (clone P9-02) and the Ac-Rev peptide, and clone P9-06 was used for the class II RNA.

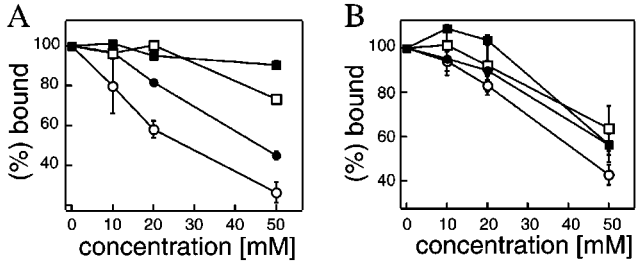


Figure 6. Competition analysis of the class I ribonucleopeptide with (A) nucleotide triphosphates, 8-BrATP (open circles), ATP (solid circles), UTP (open squares), and CTP (solid squares), and with (B) nucleotide phosphates, ATP (open circles), ADP (solid circles), AMP (open squares), and dATP (solid squares) revealed a specific binding of class I ribonucleopeptide. The class I ribonucleopeptide complex was formed with RNA (clone P9-02) and the Ac-Rev peptide.

RNA complex, a ribonucleopeptide, which specifically bound ATP. The most abundant class of ATP-binding ribonucleopeptide, termed as class I, shares a novel nucleotide sequence as compared to the previously reported ATP-binding RNA aptamers. The class I ribonucleopeptide binds ATP only upon formation of the RNA−peptide complex, suggesting that the ATP−ribonucleopeptide complex forms in a cooperative manner. Such a ternary complex formation is reminiscent of the reaction profile for ligand-regulated ribozymes.[5] As depicted for the allosteric ribozyme, the peptide subunit would play a

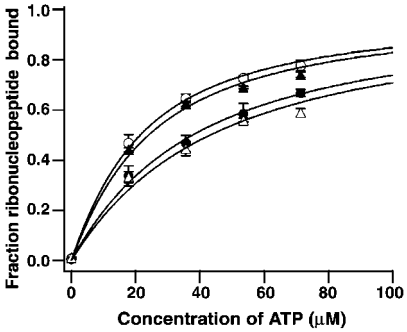


Figure 7. N-terminal modification of the Rev peptide affected for the ATP binding of class I ribonucleopeptide. Saturation curves for the binding of the class I ribonucleopeptide complex formed with N-Rev (open circles), Ac-Rev (solid circles), E-Rev (open triangles), and R-Rev (solid triangles) to immobilized ligand were obtained as described in the Experimental Section.

crucial role in stabilizing the active conformation of class I ribonucleopeptide. The class I ribonucleopeptide originally afforded from the in vitro selection binds ATP with moderate affinity. However, further modification at the N-terminal of the peptide increased the ATP-binding affinity of the class I ribonucleopeptide. Successive selection by using a peptide library of Rev extended at the N-terminal could introduce higher selectivity or affinity or even a chemical reactivity to the ribonucleopeptide. For example, one of the proteins in the spliceosome is suggested to play a role in the chemical event of splicing.[14] The ATP-binding ribonucleopeptide complex reported here consists of two subunits. It would be possible to assemble a larger number of binding units, that is, by creating additional binding sites for a peptide within RNA. Such multisubunit approaches using the ribonucleopeptide scaffold would open a new possibility for the design of small artificial receptors and enzymes with desired specificity and chemical activity.

Experimental Section

Materials. ATP−agarose (immobilized on cross-linked 4% beaded agarose, 2.3 μmol ATP/ml packed gel) and glucose−agarose

(14) Collins, C. A.; Guthrie, C. *Nat. Struct. Biol.* **2000**, *7*, 850−854.

(immobilized on cross-linked 4% beaded agarose, 1 mL of gel will bind 15 mg of concanavalin A) were purchased from Sigma-Aldrich. Nucleotides (ATP, ADP, AMP, dATP, UTP, CTP, GTP, 8-bromo-ATP) were purchased from Sigma-Aldrich. *N*-α-Fmoc-protected amino acids and HATU were from Nova Biochem and Applied Biosystems, respectively.

Synthesis of Oligopeptides. Oligopeptides were synthesized on a MilliGen/Bioserch 9050 peptide synthesizer according to the Fmoc chemistry protocols by using Fmoc-PAL-PEG resin (0.2 mmol/g, Applied Biosystems), protected Fmoc-amino acids, and HATU, purified by a reversed phase HPLC, and characterized by Voyager MALDI-TOF spectrometry (Applied Biosystems). The C-termini of peptides were amidated, and the N-terminal of Ac-Rev was acetylated. Ac-Rev calculated for [M^+] 2478.8, found 2480.5; N-Rev calculated for [M^+] 2436.8, found 2438.4; R-Rev calculated for [M^+] 2593.0, found 2594.6; E-Rev calculated for [M^+] 2565.9, found 2567.6. Peptide concentrations were determined using tryptophan absorbance with $\epsilon_{280} = 5500\ M^{-1}\ cm^{-1}$.

Nucleic Acids Preparation. The original double-stranded DNA pools were constructed by Klenow polymerase (New England Biolabs) reaction from a synthesized oligonucleotide containing 20 random nucleotides (5′-GGAATAGGTCTGGGCGCA-N20-TGACGGTACAG-GCCGAAAG-3′) and a 3′-DNA primer (5′-CTTTCGGCCTGTAC-CGTCA-3′), followed by PCR amplification to add the promoter for T7 RNA polymerase using Pyrobest DNA polymerase (TaKaRa) with the 3′-DNA and a 5′-DNA primer (5′-TCTAATACGACTCACTAT-AGGAATAGGTCTGGGCGCA-3′: T7 RNA promoter is underlined).

RNA transcription was performed using AmpliScribeT7 Kit (Epicentre) for 3 h at 37 °C according to a supplier's recommended protocols. The resulting RNA was phenol/chloroform extracted, precipitated with ethanol, and pelleted by centrifugation. The RNA was suspended in water and passed through an NAP-5 column (Amersham Pharmacia Biotech) to remove unincorporated nucleotides and recovered. Concentrations of RNAs were quantitated by UV-spectroscopy.

In Vitro Selection Procedure. Ribonucleopeptide complexes that bound ATP were selected as follows: RNA was heated at 80 °C for 3 min and cooled to room temperature for 2 h for proper secondary structure. A binding buffer (200 μL) [10 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$] containing 10 μM RNA, 15 μM Ac-Rev, and 50 μL vol of ATP−agarose was incubated to allow a formation of a specific ribonucleopeptide complex for 30 min on ice. RNA−peptide−ATP resin complexes were washed three times with 300 μL (6 vols of resin) of binding buffer to remove unbound RNA−peptide complexes and eluted three times with 150 μL (3 vols of resin) of binding buffer containing 10 mM ATP. Prior to ATP selection, the RNA−peptide complexes were incubated with a glucose-immobilized agarose to prevent the selection of RNA populations that bound to the agarose matrix. Recovered ribonucleopeptide complexes were precipitated with ethanol and resuspended in TE buffer. After reverse transcription with AMV reverse transcriptase (Promega) of the selected RNA using the 3′-DNA primer used in PCR amplification and successive PCR amplification (RT-PCR) using the 5′- and 3′-DNA primer, DNA templates were transcribed, and the resulting RNAs were subjected to the next round of selection.

Sequencing Analysis of Selected RNA. Selected RNA pools were converted to DNA and PCR-amplified to introduce *Bam*HI, *Eco*RI restriction sites by using primers 5′-GCGGGATCCTTTCGGCCTG-TACCGTCA-3′ and 5′-CGGAATTCTAATACGACTCACTATAGG-3′. After enzymatic digestion (New England Biolabs), DNAs were cloned into the pUC19 vector using Ligation Kit Ver. 2 (TaKaRa) and sequenced using a BigDyeTerminator Cycle Sequencing Kit (Applied Biosystems) with a model 377 DNA sequencer (Applied Biosystems).

ATP-Binding Assay. Double-stranded DNA templates were prepared by PCR amplification from individual clones by using primers 5′-GAATTCTAATACGACTCACTATAGG-3′ and 5′-CTTTCGGC-CTGTACCGTCA-3′, and these templates were transcribed as described above. Individual RNAs were labeled at the 3′-terminus with T4 RNA ligase (New England Biolabs) and [^{32}P]-pCp (Amersham Pharmacia Biotech). The ATP-binding assay was performed as follows. A binding buffer (100 μL) [10 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$] containing 10 μM RNA, 15 μM peptide, and a 50-μL vol of ATP−agarose were incubated for 30 min on ice. The resulting RNA−peptide−ATP complexes were washed three times with 300 μL (6 vols of resin) of binding buffer to remove unbound RNA−peptide complexes and eluted three times with 150 μL (3 vols of resin) of binding buffer containing 10 mM ATP. The fractions of RNA bound to the ATP resin are quantitated by Cerenkov counting in a scintillation counter.

Gel Mobility Shift Assays. The binding reaction was performed in the presence of the indicated amount of the protein with 20 pM 3′-^{32}P-labeled oligonucleotide in the binding mixture containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 0.005% Tween 20, and 6% sucrose at 4 °C. The increase of the mobility-shifted band was quantitated by a STORM phosphor imager (Amersham Pharmacia). The fraction-bound RNA was obtained by dividing the intensity of the mobility-shifted band with the sum of the intensities of both bound and unbound bands.

Determination of the Equilibrium Dissociation Constants. The affinity of the ribonucleopeptide complexes and RNA aptamer for ATP was determined by measuring the fraction of ribonucleopeptide or RNA aptamer bound to ATP−agarose at a range of immobilized ligand concentrations in a binding buffer containing 10 mM Tris-HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, and 0.005% Tween 20 as described previously.[15] The concentration of ATP available for binding was determined by saturating ATP-agarose with excess amounts of ^{32}P-end-labeled P9-06 RNA aptamer and measuring the amount of RNA specifically eluted upon washing with elution buffer containing 10 mM Tris-HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.005% Tween 20, and 10 mM ATP. The available ATP concentration on the ATP agarose was estimated at 89.1 ± 8 nmol/mL undiluted matrix by assuming that a single RNA bound per ATP molecule.

For binding studies, an indicated amount of ATP-agarose, 10 μM of labeled RNA and peptide complex (class I) or labeled RNA (class II, ∼1 nM) were added to a 0.2-μm spin filter column and allowed to equilibrate for 30 min. The binding mixtures were centrifuged for 1 min and immediately washed with 200 μl of binding buffer. Specifically bound RNA was eluted from the matrix with 500 μl of elution buffer (10 mM ATP in binding buffer), followed by an additional wash with 150 μl of elution buffer. These elutes were combined and quantitated by Cerenkov counting in a scintillation counter.

The fraction of RNA (class II) specifically eluted as a function of immobilized ATP concentration was plotted and fitted by nonlinear regression to an equation:

$$f = \frac{f_0[\mathrm{ATP}]}{[\mathrm{ATP}] + K_\mathrm{D}}$$

where f is the fraction of input RNA bound to the matrix, K_D is the apparent dissociation constant, and f_0 is the fraction of input RNA bound to the ligand.

The fraction of class I ribonucleopeptide specifically eluted as a function of immobilized ATP concentration was plotted and fitted by nonlinear regression to a function of the form:

$$f = ([\mathrm{RNP}] + [\mathrm{ATP}] + K_\mathrm{D} - (([\mathrm{RNP}] + [\mathrm{ATP}] + K_\mathrm{D})^2 - 4[\mathrm{RNP}][\mathrm{ATP}])^{1/2})/2[\mathrm{RNP}]$$

where f is the fraction of bound ribonucleopeptide to input ribonucleopeptide, [RNP] is the concentration of ribonucleopeptide, and K_D is the dissociation constant of ribonucleopeptide for ATP.

(15) Wilson, C.; Nix, J.; Szostak, J. *Biochemistry* **1998**, *37*, 14410−14419.

ATP-Binding Competition Assay. The competition assay was performed as follows. A binding buffer (100 μL) (10 mM Tris-HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$) containing 10 μM RNA, 15 μM peptide, and a 50-μL vol of ATP−agarose was incubated for 30 min on ice in the presence of competitive ligand. Ribonucleopeptide−ATP complexes were washed three times with 300 μL (6 vols of resin) of binding buffer to remove unbound ribonucleopeptides and eluted three times with 150 μL (3 vols of resin) of binding buffer containing 10 mM ATP. The fractions of RNA bound to the ATP resin are quantitated by Cerenkov counting in a scintillation counter.

Acknowledgment. This work was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan to T.M. (No. 12680590) and to K.M. (No. 11101001).

JA016569X