Communication

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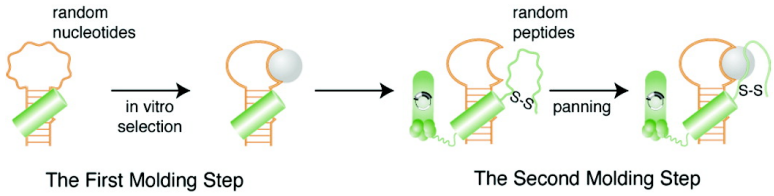

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Stepwise Molding of a Highly Selective Ribonucleopeptide Receptor

Shin-ichi Sato,† Masatora Fukuda,† Masaki Hagihara,† Yukiko Tanabe,† Katsutoshi Ohkubo,† and Takashi Morii*,†,‡

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

Received September 8, 2004; E-mail: t-morii@iae.kyoto-u.ac.jp

The marvelous functions of RNA−protein complexes in cells are exemplified by the action of ribosomes, which catalyze peptide-bond formation.[1] Three-dimensional structures of RNA−protein complexes solved by X-ray crystal analysis or NMR spectroscopy provide a sense of the remarkable diversity of RNA−protein complexes[2] and suggest that the RNA−protein complex serves as a novel framework for design of receptors and enzymes.

We have reported on a ribonucleopeptide (RNP) that binds ATP[3] by employing a structure-based design that provides a stable RNP scaffold of a Rev-RRE (*R*ev *R*esponsive *E*lement) complex[4] and successive in vitro selection[5] steps. The structural characteristics of the RNP are suitable for molding of a ligand-binding pocket of the RNP in a stepwise manner (Figure 1). The first step involves molding of the RNA subunit by in vitro selection of an RNP pool originating from an RNA library and the Rev peptide.[3] The second step involves selection from an RNP library consisting of Rev peptides with randomized amino acid residues and the RNA subunit selected in the first molding. Together, these steps afford an expanded ligand-binding surface consisting of both RNA and peptide subunits. We report here a stepwise molding strategy to tailor an ATP-binding pocket in the RNP. The ATP-binding pocket produced by sequential molding of RNA and peptide subunits shows higher affinity and specificity to ATP than the ATP-binding RNP receptor in which only the RNA subunit was molded.

The RNA subunit RRE30N was designed to consist of two functionally separated domains, namely a ligand-binding domain with 30 randomized nucleotides and an adjacent stem region derived from RRE RNA that serves as a specific binding site for the Rev peptide (Figure 1). RNP receptors for ATP were first isolated from RNP complexes of RNA and the Rev peptide by the in vitro selection method as described.[3] The RRE30N RNA transcribed from this initial library and the Rev peptide were mixed together in a 1:1 ratio to form specific RNPs for the initial selection. After 10 rounds of selection, nucleotide sequences of the RNA were determined to reveal consensus sequences that are positioned at various locations within the 30-nucleotide ATP-binding loop (Figure 2A).

The peptide-based molding of the ATP-binding RNP was carried out next by using a peptide-derived RNP library constructed by the phage display technique,[6] along with an RNA subunit of the ATP-binding RNP obtained from the first RNA-based library selection. An LpRev peptide was designed to possess seven randomized amino acids (Cys-X_7-Cys) at the N-terminus of Rev (Figure 2B) to facilitate efficient formation of a binding surface.[7] The random LpRev peptides were expressed at the amino terminus of the pIII protein of the filamentous bacteriophage and were expected to form a loop structure upon disulfide bond formation. Phages from the LpRev library were pooled and mixed with the

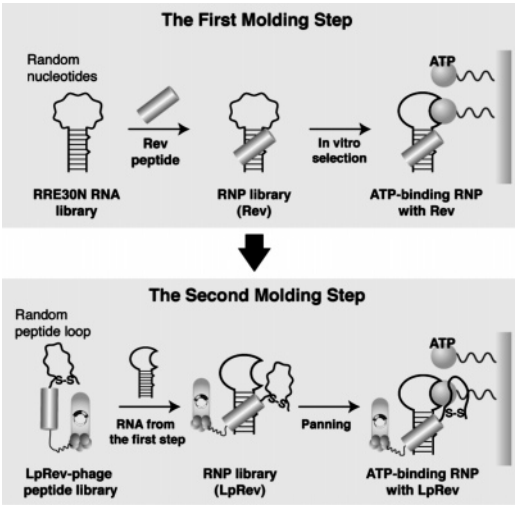

Figure 1. Schematic illustration shows the stepwise molding strategy to generate ribonucleopeptide (RNP) receptors for ATP. The first molding step utilizes in vitro selection of an RNP library originating from a randomized RNA nucleotide pool (RRE30N).[3] Combination of the RNA subunit of RNP selected in the first molding and a phage peptide library of the Rev peptide containing seven randomized amino acid residues affords a peptide-diverged RNP library, which enables the selection in the second molding step of improved ATP-binding RNPs.

A

```
08                  GUGUA UAGGG   UA GU CU UGUGCUUCGUCUUU
18 CUUAUGUGCGGCGUC GUGUA UGGGG   UA AU C
30          AUCUGG GUGUA UUAGGG   UA UU CU CCUCUU
33        UUCUCGU GUGUA UGGGG     UA UG CU GCGUUGG
```

B

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Rev                          TRQARRNRRRRWRERQR
LpRev AC       X7       CTRQARRNRRRRWRERQR-GGGGS-pIII

22 AC       PRSKPSV CTRQARRNRRRRWRERQR
03 AC    DE PRTHE   CTRQARRNRRRRWRERQR
19 AC    FT PRDAD   CTRQARRNRRRRWRERQR
04 AC    ST PREVL   CTRQARRNRRRRWRERQR
10 AC HASTAPR       CTRQARRNRRRRWRERQR
18 AC  YRTAPRW      CTRQARRNRRRRWRERQR
11 AC  VPTIPRS      CTRQARRNRRRRWRERQR
```

Figure 2. (A) Nucleotide sequences obtained for the randomized region of RRE30N after the first molding of ATP-binding RNP receptors show consensus sequences. Only four nucleotide sequences are shown. (B) Amino acid sequences for Rev, LpRev and sequences of the randomized seven amino acid residues of LpRev obtained by the second molding step are shown. A binding buffer (10 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, pH 7.6, 100 μL) containing 1 μM RNA, ~2 nM phage, and 50 μL ATP-agarose (137 μM) was incubated for 30 min on ice. After the resin was washed with the binding buffer, bound phage was eluted with a binding buffer containing 4 mM ATP at ambient temperature. The elutant was used to infect freshly prepared *Escherichia coli* SOLR cells. After 12 rounds of selection, phage pools were sequenced.

RNA obtained in the first molding step to form a library of RNPs before starting selection against ATP-agarose resin. By using the four kinds of RNAs obtained in the first molding step (RNA08,

† Kyoto University.
‡ Japan Science and Technology Corporation.

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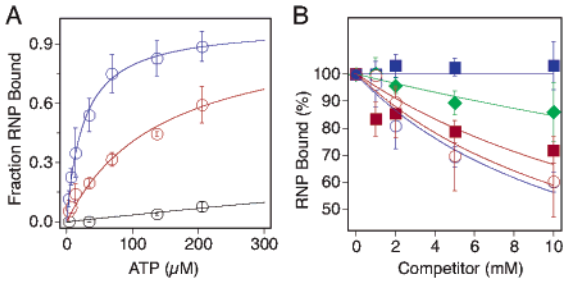

Figure 3. (A) Saturation curves for the binding of RNP33/LpRev19 (open blue circles), RNP33/Rev (open red circles), and RRE33 (open black circles) to immobilized ATP, showing that RNP33/LpRev19 binds ATP with higher affinity. (B) Competition analysis of the ATP complex of RNP33/LpRev19 with dATP (filled blue squares) or ATP (open blue circles) and the ATP complex of RNP33/Rev with dATP (filled red squares) or ATP (open red circles), showing that RNP33/LpRev19 discriminates ATP against dATP. RNP with a modified LpRev (RNP33/LpRev19Ser) revealed less efficient dATP discrimination (filled green diamonds).

-18, -30, and -33), four different peptide-diverged RNP pools were generated. Panning against the ATP resin was carried out for each RNP pool. An RNP pool containing RNA33 revealed a characteristic amino acid sequence for the LpRev peptide after 12 rounds of selection cycles (Figure 2B). Out of the 30 clones sequenced from the 12th round pool, seven contained a Pro-Arg sequence within the random-loop region.

RNPs consisting of LpRev03, LpRev04, LpRev11, or LpRev19 and RNA33 were compared for ATP-binding with that of the RNP33/Rev complex consisting of RNA33 and the original Rev peptide (Figure 3A). The two-step receptor, RNP33/LpRev19, bound ATP with a dissociation constant (K_D) of 25.7 ± 1.9 μM, compared to a 6-fold lower affinity for ATP by the one-step receptor, RNP33/Rev (K_D = 153.6 ± 11.3 μM). RNP33/LpRev03, RNP33/LpRev04, and RNP33/LpRev11 bound ATP with dissociation constants of 113.7 ± 4.5, 121.2 ± 5.4, and 164.8 ± 8.6 μM, respectively.

Selectivity for ATP binding by RNP 33/LpRev19 was further studied by competitive binding assays. The ATP-binding RNP obtained from the first RNA-based library RNP33/Rev discriminated ATP against other NTPs or AMP. Such specificity was maintained in the high-affinity RNP complex RNP33/LpRev19 (Figure S1, Supporting Information). Interestingly, RNP33/LpRev19 showed distinct selectivity for ATP versus dATP, whereas the original RNP33/Rev bound almost equally to ATP and dATP (Figure 3B). These results suggest that the loop region of LpRev19 contributes to ATP binding by expanding the recognition surface for ATP. To confirm the role of the loop structure of LpRev19 in the ATP-binding RNP complex, a modified RNP consisting of the RNA33 and LpRev19Ser, in which the two cysteine residues of LpRev19 were replaced with serine residues, was analyzed in a competitive binding assay (Figure 3B). By replacing Cys residues with Ser residues, the postulated loop structure of LpRev is expected to be destabilized. In contrast to RNP33/LpRev, the modified RNP consisting of RNA33 and LpRev19Ser failed to establish selectivity for ATP over dATP. Thus, a stable loop structure of LpRev19 likely represents an expanded recognition surface for the sugar moiety of ATP. Out of the seven amino acid residues in the loop, the central five amino acid residues are almost identical for LpRev19 (F-*TPRDA*-D) and LpRev04 (S-*TPREV*-L). Nonetheless, RNP33/LpRev19 shows higher affinity for ATP than RNP33/LpRev04, and a distinct selectivity for ATP over dATP is observed only for RNP33/LpRev19. Further studies are in progress to establish the mechanism for the selectivity.

In summary, we have developed a stepwise molding strategy to tailor a specific binding pocket for small molecules by using RNP. The RNA-based RNP library was first utilized to mold a binding pocket for ATP, and successive selection from the peptide-based RNP library enabled expansion of the recognition surface to enhance the selectivity for ATP. As the power of directed evolution is appreciated more in the design of receptors, the limitation in the size of the initial library has become an important issue for selecting receptors for any ligand on demand. Our approach of the stepwise molding described here offers the advantage of increasing the diversity of the RNP library by utilizing characteristics of different biopolymers. Alternatively, it would be possible to develop further the RNP system to construct dynamic combinatorial libraries[8] by taking advantage of the noncovalent assembly of RNP. For instance, combination of independent libraries of 10^{18} RNAs (30 randomized nucleotides) and 10^9 peptides (7 randomized amino acid residues) could afford an RNP library with a final estimated diversity of 10^{27}. The ribonucleopeptide-based multi-subunit approach, also extendable to other biomacromolecular assemblies, may yield artificial receptors and enzymes with increased specificity and more diverse chemical activities.

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Supporting Information Available: Results of ATP-binding assay of RNP33/LpRev complexes (Figure S1) and experimental details for each molding step (PDF). This material is available free of charge via the Internet at http://pubs.asc.org.

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