In Vitro Selection of N-Peptide-Binding RNA on a Quartz-Crystal Microbalance to Study a Sequence-Specific Interaction between the Peptide and Loop RNA

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KEYWORDS:

peptides · quartz-crystal microbalance · RNA · RNA recognition · selection

RNA-binding proteins play a key role in fundamental cellular processes such as translation of mRNA, and in viral processes involved in infection by RNA viruses.[1] Investigation of specific interactions between an RNA-binding protein and a structuralized RNA molecule is important for the design of drugs to inhibit infections by RNA viruses. The RNA-binding domains of these proteins can be grouped into families characterized by features such as a ribonucleoprotein motif, a double-stranded RNA binding domain,[2] and an arginine-rich motif.[3] The 1 – 22 amino acid region of the transcriptional antitermination N protein (107 amino acids) from bacteriophage λ as an argine-rich motif has a simple α-helical structure and can alone recognize the hairpin-pentaloop RNA (GAAGA) called boxB RNA without a large part of the protein (Figure 1).[4] The general GNRNA-type pentaloop RNA (GNRNA; N = A, C, G, or T; R = A or G) has a stable structure and has also been found in native RNA structures.[5–7] Interaction of the N peptide and the boxB RNA could act as a simple model for the study of peptide – loop-RNA interaction mechanisms. NMR spectroscopy structural analysis of the complex revealed that Trp18, the 18th amino acid residue from the Met residue of the N terminus of the N peptide, stacks with the second adenine base from the 5′ end of the pentaloop of the boxB RNA and stabilizes the interaction between the protein and RNA loop structures (Figure 1).[8] However, contribution of the Trp18 residue on the N peptide to sequence-specific RNA recognition has not been established.

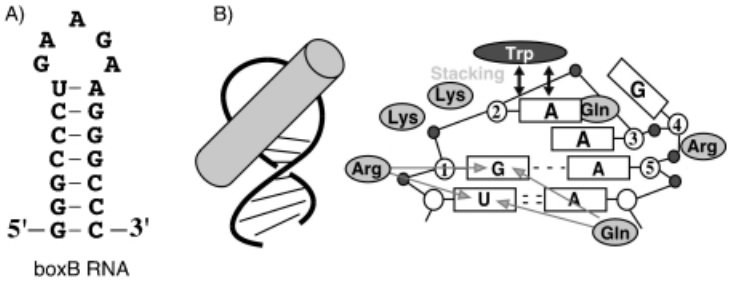

Figure 1. A) Sequence of boxB RNA. B) Schematic illustrations of a specific interaction between amino acid residues of N peptide and a loop structure of boxB RNA (adapted from ref. [8]).

In this study, we used an N-peptide-immobilized 27-MHz quartz-crystal microbalance (QCM) for in vitro selection of boxB RNA. This in vitro selection allows us to identify the optimal molecule for binding to the target. In conventional in vitro selection methods, peptides are immobilized on beads and radioisotope- or fluorescent-labeled random RNAs are selected as they pass through a column. Evaluation of the binding ability of the selected RNAs must be carried out separately with time-consuming gel-shift or ELISA assays. A 27-MHz QCM is a highly sensitive mass measuring device whose resonance frequency decreases linearly with increasing mass on the QCM electrode at the nanogram level in aqueous solutions.[9, 10] We were able both to monitor a selection process from a random RNA pool as a mass change and to evaluate the association constant of the selected RNA quantitatively on the same QCM plate, without recourse to radioactive labeling or fluorescent probes.[11, 12] We immobilized the N peptide or a modified peptide that has Trp18 replaced by Ala18 on a QCM plate and performed in vitro selections with each peptide-binding RNA molecule and a pentaloop-randomized RNA (G*NRN*A) library in which the molecules were designed to have a hairpin-pentaloop structure based on that of boxB RNA. Our aim was to study the recognition activity of Trp18 for the loop region of various RNA sequences. The RNA sequences that bind to each peptide were identified and compared.

The N peptide and the Ala-substituted N peptide (23 aa) were prepared by a single stepwise manual solid-phase peptide synthesis with 9-fluorenylmethoxycarbonyl amino acids. The G*NRN*A pentaloop-randomized RNAs (61-mers) were obtained by reverse transcription PCR of randomized DNAs (Figure 2A). A schematic illustration of the experimental setup used for in vitro selection on a QCM is shown in Figure 2B. Each peptide was immobilized on the one side of an Au electrode (area: 5 mm^2) of a 27-MHz QCM plate, through a Cys-SH group with a long poly(ethylene oxide) spacer. The peptide immobilized on the QCM was soaked in a buffer solution (10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.5, 0.1 M NaCl) and the frequency decrease (mass increase) response to the addition of random single-stranded RNA (ssRNA) was monitored in the aqueous solution. After monitoring of the selection process, the QCM surface was washed with the selection buffer described above and the selected ssRNA was recovered with the elution buffer (10 mM HEPES, pH 7.5, 1.0 M NaCl). The ssRNA was reverse-transcribed to give DNA, which was amplified with PCR. The double-stranded DNA (dsDNA) was transcribed to RNA and used for the next selection process.[11–16] Each selection process was repeated 1 – 5 times.

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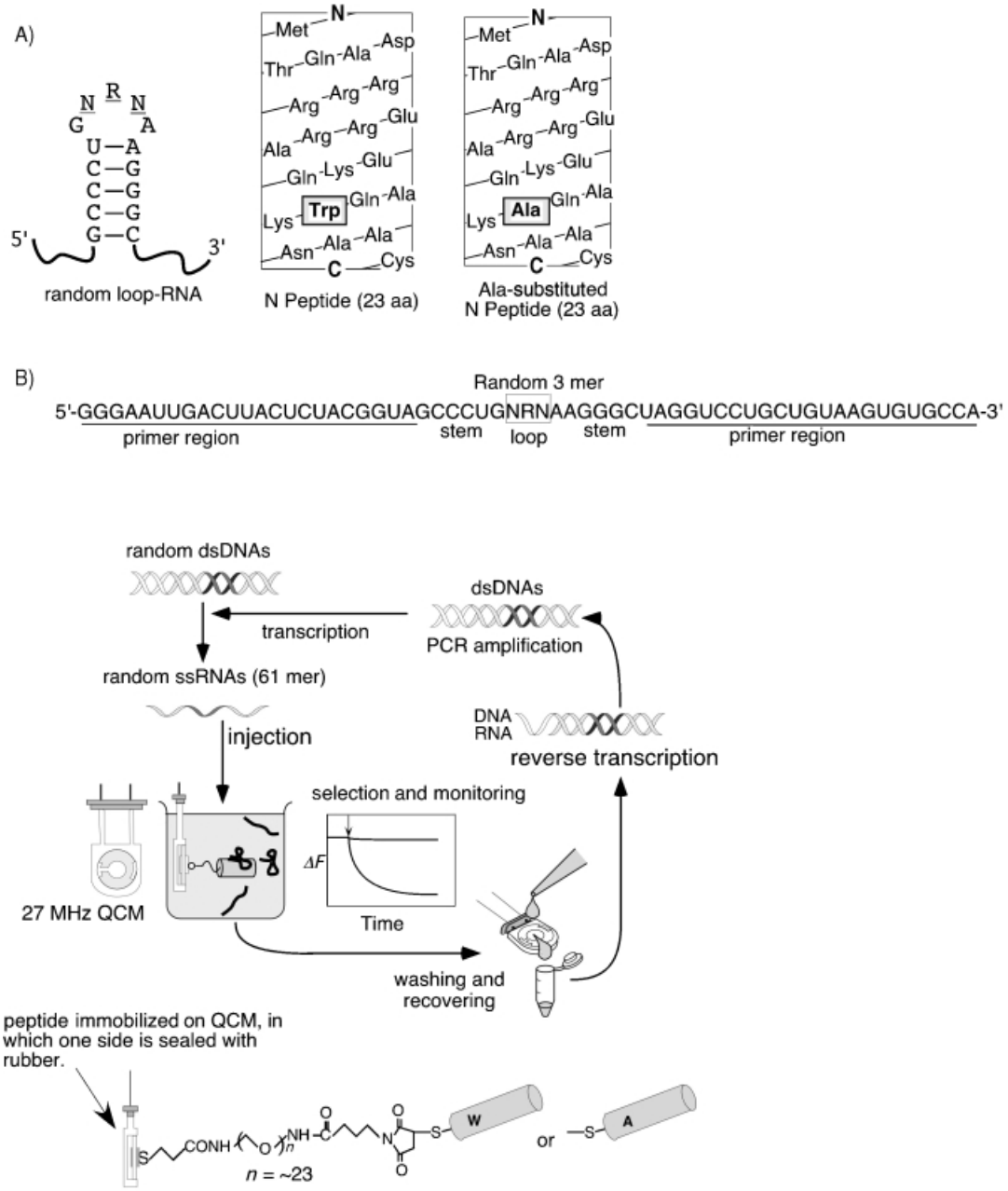

Figure 2. A) Sequence of random loop RNAs and amino acid sequences of N peptide and the Ala-substituted peptide. B) Experimental procedure for in vitro selection of RNA that shows a high affinity for the peptide immobilized on a 27-MHz QCM plate.

Selection processes were monitored as mass changes by using a QCM and the results are summarized in Figure 3. Binding saturation was observed within 10 min, which indicates that 30 min is long enough for selection operations. In Round 1, the random RNA hardly bound to the N peptide on the QCM plate. The amount of RNA bound increased with each selection round up to Round 3 (Figure 3 A). Since the frequency change was constant for Rounds 3 – 5, selection was stopped after Round 5. In the case of the Ala-substituted N peptide, the amount of RNA bound increased smoothly with selection rounds; however, the amount bound at Round 5 was half that for the N peptide. This result suggests that the Ala-substituted N peptide may have a lower ability for binding RNAs than the N peptide. Selection by the QCM system gives us useful information even during selection process, in contrast to the conventional beads and column method.

The selected RNA for each peptide was reverse-transcribed to DNA, which was PCR-amplified and cloned by the T/A cloning method, and the sequence was determined by a standard dideoxynucleotide method.[11, 12] The sequences obtained in the randomized region of the RNAs in the pentaloop library after selection Round 5 are summarized in Table 1. Binding of some cloned RNAs to the N peptide was quantified with the peptide immobilized on the QCM. Saturation binding behavior showing the simple Langmuir adsorption pattern was observed with increasing concentration of added RNA, as shown in Figure 4.

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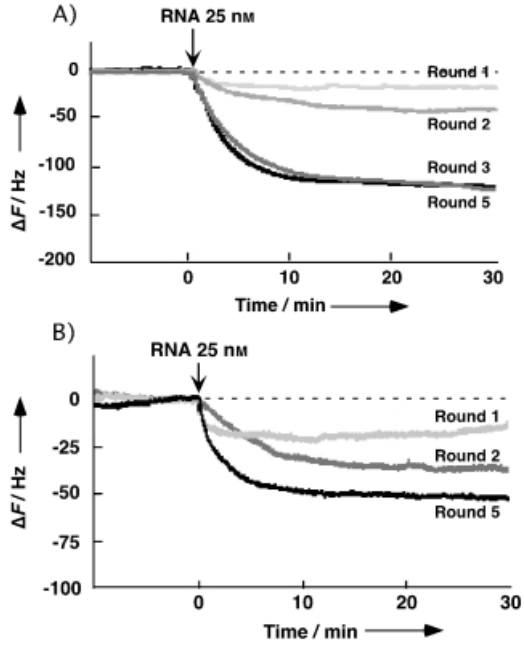

Figure 3. Results obtained by monitoring the selection of randomized loop RNAs that bind to (A) N peptide and (B) Ala-substituted N peptide immobilized on a QCM (10 mM HEPES, pH 7.5, 0.1 M NaCl, 20 °C).

Table 1. *Sequences of selected and random ssRNAs.*[a]

A[b] Loop GNRNA		B[b] Loop GNRNA	C[c] Loop GNRNA	
GCGCA	$4.9 \times 10^8\,M^{-1}$	GCACA	GUGCA	
GCGCA		GCACA	GUGCA	
GCGCA		GCACA	GAAGA	
GCGCA		GCGCA	GAAGA	
GCGCA		GCGCA	GCGCA	
GCGCA		GAAGA	GAGGA	
GCGCA		GAAGA	GAGCA	
GCGCA		GUACA	GAGUA	
GAAGA		GUACA	GUAAA	
GAAGA	$3.7 \times 10^8\,M^{-1}$ (boxB RNA)	GGACA	GCGAA	
GAAGA		GGACA	GCAGA	
GCACA		GCAUA	GUGUA	
GCACA		GCAUA	GUGGA	
GAACA		GGGCA	GGACA	
GAACA		GAAUA	GNRNA	$1.1 \times 10^8\,M^{-1}$
GCGUA		GAGAA		
G N R N A		G N R N A	G N R N A	

[a] The heights of the letters at the bottom of the table indicate the ratios: (number of sequences with A, C, G, or T present at that position in the loop)/(total number of sequences considered). Binding constants (K_a) of the selected or random RNAs to the N peptide were obtained from the data presented in Figure 4. [b] Sequences of ssRNAs after the fifth round of selection from the randomized loop RNAs with (A) N peptide or (B) Ala-substituted N peptide. [c] Sequences of the randomized RNAs. Randomized sequence positions are underlined.

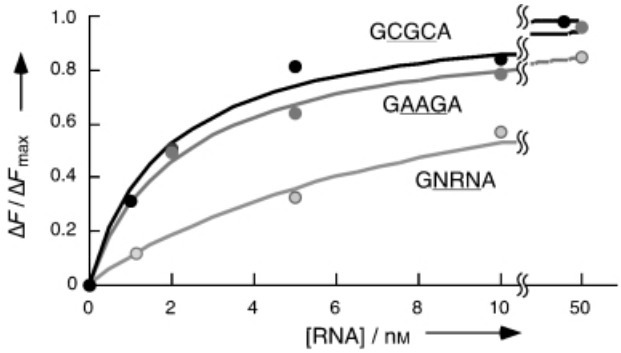

Figure 4. Saturation binding behavior of the selected RNA clones when binding to the N peptide immobilized on the QCM plate (10 mM HEPES, pH 7.5, 0.1 M NaCl, 20 °C).

Binding constants (K_a) were calculated from reciprocal plots and are summarized in Table 1.

The G*CGC*A loop RNA was selected as a consensus sequence with the ratio 8/16 (50%; ratio = number of G*CGC*A clones/number of total sequenced clones), and the G*AAG*A loop (boxB RNA, a recognition site of a native N protein) was only obtained at a ratio of 3/16 (19%). These selection results agree well with the larger K_a value of the G*CGC*A loop ($4.9 \times 10^8\,M^{-1}$) for binding to the N peptide compared to that of the GAAGA loop ($3.7 \times 10^8\,M^{-1}$). Thus, the GCGCA pentaloop should be an optimized RNA sequence for N peptide binding.

On the contrary, the consensus sequences for the N peptide in which we replaced the Trp residue with an Ala residue were not obtained at a ratio of more than 3/16 (19%; Table 1B). The N peptide prefers a C or A base as the second base from the 5′ end in the pentaloop, whereas the Ala-substituted N peptide did not select a specific base at this position (see also Figure 1B). This difference is derived from the replacement of Trp18 with an Ala residue. The bases at the third position of the RNA loop sequences can be either G or A for the N peptide, because the selection ratios of the G and A bases are similar to the proportions of these bases present in the RNA sequences in the random library. In the fourth base position of the selected RNAs the C base was favored for both peptides, as compared with the bases in the random library sequences. The loop-out fourth base may interact with another residue of the peptide (see Figure 1B). Thus, the Trp residue is significant for recognition of a specific RNA loop sequence at the second position of this sequence, which is consistant with previous reports that the Trp residue stacks with the adenine residue at the second position.[8]

This study shows that the N peptide with Trp18 prefers C to A as the base at the second position of the RNA target, but does not accept a G base in this position. We suppose that the interaction of Trp18 and the C or A base is not only stabilized by an aromatic stacking effect, but also contributes to optimal stacking of π orbitals by alignment of an indole group with an aromatic purine or pyrimidine base.

In conclusion, the QCM system could be a useful tool for in vitro selection of RNAs because it can monitor both selection and evaluation processes in situ without any labeling techniques

and thus allows investigation of the ability of an RNA-binding peptide to recognize various RNA sequences.

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External Regulation of Hairpin Ribozyme Activity by an Oligonucleotide Effector

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The hairpin ribozyme belongs to the class of self-cleaving nucleases that are found in plant viroids, virussoids or viral satellite RNAs.[1] The 50-nucleotide-long minimal sequence (Figure 1) catalyses the reversible specific cleavage of a suitable 14-nucleotide-long RNA substrate. The secondary structure of the hairpin ribozyme – substrate complex is composed of two independently folded domains, A and B, each of which consists of an internal loop (loops A and B) flanked by two helices (H-1, H-2 in domain A; H-3, H-4 in domain B). The substrate is bound to the ribozyme by Watson – Crick base pairing, which generates helices 1 and 2, and cleavage takes place within loop A to produce characteristic products with a 2′,3′-cyclic phosphate group and a free 5′-OH group.

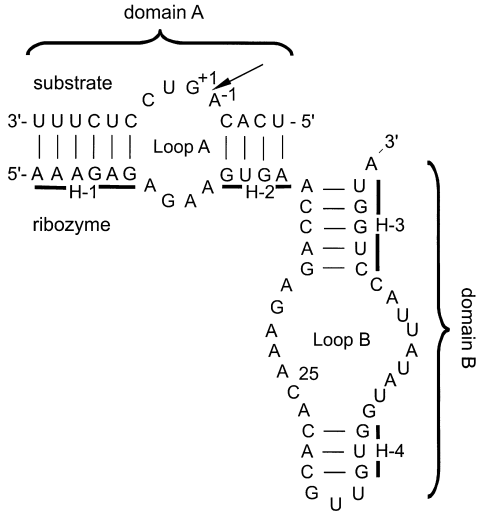

Figure 1. *Secondary structure of the hairpin ribozyme – substrate complex. The arrow denotes the cleavage site. The four Watson – Crick helices are marked (H-1 to H-4). Nucleotides discussed in the text are numbered.*

Herein we report on the external regulation of hairpin ribozyme activity by an oligonucleotide effector. RNA folding is a hierarchical process and thus formation of RNA secondary structure is an important prerequisite for tertiary folding into a functional conformation. Changes in RNA secondary structure may therefore influence the three-dimensional folding pattern and consequently the function of an RNA molecule. This fact allows for the development of activators or effectors, which assist the folding of an inactive ribozyme derivative into a catalytically competent conformation by restoring a required secondary structure. Over the past few years a number of allosteric RNA catalysts have been developed.[2] Most allosteric ribozymes described in the literature are derived from the hammerhead ribozyme, which, like the hairpin ribozyme, belongs to the group of small self-cleaving endonucleases. These hammerhead ribozymes usually contain an additional aptamer domain that can bind specific ligands, such as adenosine triphosphate,[3] flavin mononucleotide,[4] or Theophyllin.[4c, e, 5] Hammerhead ribozymes that are activated by oligonucleotide effectors have also been described. These ribozymes were developed either by rational design[6] or by in vitro selection from an RNA pool.[7] Ohtsuka and co-workers very recently introduced the first hairpin ribozyme that can be activated with short oligonucleotides.[8] In vitro selection was carried out to yield an allosteric hairpin ribozyme that showed cleavage activity only in the presence of an oligonucleotide that binds to an allosteric binding site and thus triggers a structural change of a hairpin loop to form an active conformation.

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