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In Vitro Selection of Allosteric Ribozymes that Sense the Bacterial Second Messenger c-di-GMP

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Abstract

Recently, a number of study have shown the ligand-dependent allosteric ribozymes can be harnessed as biosensors, high-throughput screening, and agents for the control of gene expression in vivo, called artificial riboswitches. In this chapter, we describe how in vitro selection can be used to create an allosteric ribozyme that senses bacterial second messenger cyclic-di-GMP (c-di-GMP). A hammerhead ribozyme was joined to a natural c-di-GMP class I riboswitch aptamer via communication modules. Both c-di-GMP-activating and -inhibiting ribozyme can be obtained by this approach.

Keywords

Allosteric ribozyme; Aptamer; Hammerhead ribozyme; In vitro selection; Cyclic di-GMP

1 Introduction

Allosteric ribozymes are RNA enzymes whose activity is modulated by the binding of an effector molecule to an aptamer domain [1, 2]. Most allosteric ribozymes are engineered by conjugating an aptamer to a ribozyme through a communication module, that is, ligand binding to the aptamer induces a conformational change, which is transmitted to the ribozyme, the activity of which is modified. Our group [3–8] and others [9–13] have been applied directed evolution strategies to engineer allosteric ribozymes or artificial riboswitches. Also, our group recently found that in nature cells use allosteric ribozymes, i.e., cyclic di-GMP class II (c-di-GMP-II) riboswitch, to regulate gene expression [14]. In *Clostridium difficile* strains, a c-di-GMP-II aptamer is found adjacent to a group I intron. Group I introns are ribozymes that catalyze the splicing of the RNA molecule in which they are embedded. In this case, the outcome of the splicing reaction catalyzed by the intron is controlled by the natural allosteric ribozyme in response to c-di-GMP levels.

c-di-GMP is a bacterial second messenger produced by the GGDEF domain-containing diguanylate cyclase and regulates many biological processes including the planktonic traits (motility) and communal or sessile traits (synthesis of bacterial surface components such as biofilm, exopolysaccharide, pili, and stalks) of several bacterial groups by its intracellular concentration [15]. Due to the widespread functions of c-di-GMP, the method to quantify the level of this second messenger is required to understand a wide range of bacterial phenomena. High-performance liquid chromatography (HPLC) is a conventional method to

monitor the concentration of bacterial metabolites; however, it requires multiple semi-purification steps to remove other metabolites that have similar chemical properties to the target molecule. The allosteric ribozyme is an attractive alternative to HPLC due to its high affinity and specificity to c-di-GMP. The natural c-di-GMP-II riboswitch might be useful for quantitative analysis of c-di-GMP. However, it is not a versatile approach because of low rate constant of the group I intron and requirement of guanosine 5'-triphosphate (GTP) for its catalytic activity.

We have used the method described below to create an engineered allosteric ribozyme resulting from the fusion of a c-di-GMP-I aptamer [16] to a hammerhead ribozyme for simple monitoring of c-di-GMP levels (Fig. 1). Several engineered allosteric ribozymes derived from the hammerhead ribozyme show equal rate constant to the minimum ribozyme core, suggesting that they could be used for rapid quantification of ligands in chemical or biological samples. Moreover, allosteric ribozymes could be used for high-throughput screening to facilitate the rapid discovery of compounds that target the aptamer [17–19].

2 Materials

2.1 Construction of the Starting Pool

1. Chemically synthesized oligonucleotides for DNA templates to prepare the RNA pool shown in Fig. 1a. Oligonucleotide 1: 5'-
TAATACGACTCACTATAGGCGTAGCCTGATGAGNNNN-
GCACAGGGCAAACCATTCGAAAGAGTGGGACGCAAAGCCTCCGGCCT
AAACCAGAAGACATGGTAG-3'. Oligonucleotide 2: 5'-
GGCGCAGCTACGTGGCTTTCACC ACGTTTC-G NNNNN
GTAACCCCGCTACCTACCATGTCTTCTGGTTTAG-3'. Underlined
nucleotides are complementary each other at their 3' ends. T7 promoter
sequences are shown in italic.
2. Chemically synthesized oligonucleotides for DNA templates to prepare the RNA pool shown in Fig. 1b. Oligonucleotide 3: 5'-
TAATACGACTCACTATAGGCACCTGATGAG NNNNN N GCACAGG-
GCAAACCATTCGAAAGAGTGGGACGCAAAGCCTCCGGCCTAAACCAG
AAGACATGGTAG-3'. Oligonucleotide 4: 5'-*CTTAGGCACTACGTGGCT*
TTCACCACGTTTCGNNNNNNNG-
TAACCCCGCTACCTACCATGTCTTCTGGTTTAG-3'. Underlined nucleotides
are complementary each other at their 3' ends. T7 promoter sequences are
shown in italic.
3. 5× reverse transcription buffer: 250 mM Tris-HCl, pH 8.3 at 23 °C, 375 mM KCl, 15 mM MgCl₂.
4. 100 mM dithiothreitol (DTT).
5. 10× deoxynucleoside 5'-triphosphate (dNTP) mix: 2 mM each of deoxyguanosine 5'-triphosphate (dGTP), deoxyadenosine 5'-triphosphate (dATP), deoxythymidine 5'-triphosphate (dTTP), deoxycytidine 5'-triphosphate (dCTP).

6. Reverse transcriptase (e.g., SuperScript II, Invitrogen).
7. 10× transcription buffer: 500 mM HEPES, pH 7.5 at 23 °C, 150 mM MgCl₂, 20 mM spermidine and 50 mM DTT.
8. 3 M sodium acetate, pH 5.2.
9. 100 % ethanol.
10. 5× nucleoside 5'-triphosphate (NTP) mix: 12.5 mM each of guanosine 5'-triphosphate (GTP), adenosine 5'-triphosphate (ATP), thymidine 5'-triphosphate (TTP), cytidine 5'-triphosphate (CTP).
11. T7 RNA polymerase (T7 RNAP).
12. [α -³²P] UTP, 3,000 Ci/mmol (PerkinElmer), and appropriate equipment for handling ³²P.
13. Crush-Soak buffer: 10 mM HEPES, pH 7.5 at 23 °C, 200 mM NaCl, and 1 mM ethylenediaminetetraacetate (EDTA).
14. 2× denaturing gel-loading buffer: 32 % (w/v) sucrose, 0.16 % (w/v) sodium dodecyl sulfate, 0.08 % (w/v) bromophenol blue, 0.08 % (w/v) xylene cyanol, 7.25 M urea, 144 mM Tris-HCl, 144 mM boric acid, and 200 mM EDTA.
15. Reagents and apparatus for denaturing 8 M urea polyacrylamide gel electrophoresis.

2.2 Selection

1. Cyclic-diguanosyl 5'-monophosphate (c-di-GMP, Biolog).
2. 2× selection buffer: 100 mM Tris-HCl, pH 7.5 at 23 °C, 200 mM NaCl.
3. 100 mM MgCl₂.
4. X-ray film and reagents for developing the X-ray film.
5. PhosphorImager cassettes and PhosphorImager (e.g., Storm PhosphorImager, GE HealthCare).

2.3 Reverse Transcription and Polymerase Chain Reaction

1. Chemically synthesized oligonucleotides for the amplification of the cleaved products. Primer A: 5'-TAATACGACTCACTATAGGCGTAGCCTGATGAG-3'. Primer B: 5'-GGCGCAGCTACGTGGCTTTCACCACGTTTCG-3'. Primer A': 5'-TAATACGACTCACTATAGGCACCTGATGAG-3'. Primer B': 5'-CTTAGGCACTACGTGGCTTTCACCACGTTTCG-3'.
2. Reverse Transcriptase (e.g., SuperScript II, Invitrogen).
3. 10× PCR buffer: 100 mM Tris-HCl, pH 8.3 at 23 °C, 400 mM KCl, 15 mM MgCl₂, and 0.1 % gelatin.
4. *Taq* DNA polymerase.

5. Reagent and apparatus for agarose gel electrophoresis.

3 Methods

3.1 General Selection Strategy

The construct used for the selection of allosteric ribozymes that respond to c-di-GMP was created by linking the P1 stem of the c-di-GMP class I aptamer derived from *Vibrio cholera* [16] to stem II of the hammerhead ribozyme via a communication module (CM) (Fig. 1). The P1 stem of the aptamer was chosen for CM since the stability of P1 stem is highly involved in the affinity between the aptamer and the ligand. Also, there is an important tertiary interaction between stems P2 and P3 [20, 21], so that using these stems for CM might disrupt key structural contacts within the aptamer. The CM also replaces a majority of the stem II of the hammerhead ribozyme—a structural element that is a critical determinant of the ribozyme activity [22, 23]. The CM within the resulting tripartite construct will provide a sampling of alternative stem II elements that might respond to c-di-GMP binding in the adjacent aptamer domain and confer either positive or negative allosteric control on the adjoining ribozyme domain (*see* Note 1). This strategy has been applied to the selection of various allosteric ribozymes (6–8). We have first used the construct shown in Fig. 1a for both c-di-GMP-inhibited and -induced ribozymes. However, the selection for the induced ribozymes was unsuccessful probably because shorter CM (four and five randomized nucleotides) disfavors allosteric induction due to steric clash between important nucleotides of the aptamer and ribozyme. Therefore, we redesigned the construct by elongating randomized sequences (Fig. 1b). This second construct allowed us to obtain the c-di-GMP-induced ribozymes (*see* Note 2).

The outline of the selection scheme for the c-di-GMP-activated allosteric ribozyme is shown in Fig. 2. The RNA population of the initial generation (G0) was generated by primer extension of two synthetic oligonucleotides with reverse transcriptase (Fig. 2a), followed by transcription of about 20 pmol (10–20 copies) of the resulting double-strand DNA (Fig. 2b, *see* Note 3). After the purification by denaturing PAGE, the full-length RNA precursors were subjected to a negative selection in the absence of c-di-GMP (Fig. 2c). RNAs that remained uncleaved during this reaction were isolated by PAGE and subsequently subjected to a positive selection for self-cleavage in the presence of c-di-GMP (Fig. 2d). The resulting cleavage products were isolated by PAGE and amplified by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 2e, *see* Note 4). The DNA from this stage were transcribed to generate next generation of RNA population, which were then subjected to additional rounds

¹A typical communication module ranges from a few to a dozen nucleotides. The two constructs (Fig. 1) used in our study contains 9 and 13 random nucleotides, respectively, in the domain of the communication module. These should give us the anticipated RNA libraries with great diversity (262,144 (4⁹) and 67,108,864 (4¹³) pieces of different RNA sequences, respectively).

²When the hammerhead self-cleaving motif is fused with the large c-di-GMP-I aptamer to create the c-di-GMP-dependent allosteric ribozymes, there may be some steric hindrance between the two moieties that could possibly affect the isolation of active allosteric ribozymes. To minimize the potential steric clash, a longer communication module and truncated stem I of the hammerhead ribozyme was used as shown in Fig. 1b.

³Considering that a typical run-off transcription produces about 10–20 copies of each DNA sequence, including 20 pmol of DNA template in the initial transcription step should give us more than 200 pmol of the RNA library with guaranteed sequence diversity.

⁴When engineering the RNA constructs for allosteric selection, make sure the anticipated difference in size between the full-length RNA precursor and the cleaved RNA product is sufficient (no less than eight nucleotides) to be resolved by denaturing PAGE gels. Also ensure that there are enough fixed sequences flanking the random domain (no less than 13 nucleotides) to allow the efficient PCR amplification of the cleaved product with the appropriate primers.

of selective amplification by repeating stages B to E in Fig. 2. The selection for the c-di-GMP-inhibited allosteric ribozyme was done in a same way except for adding c-di-GMP to the transcription and negative selection steps and removing c-di-GMP from positive selection step.

3.2 Construction of the Starting Pool

1. To prepare the double-stranded DNA template for transcription, incubate 40 pmol of each of the two oligonucleotides in 50 μ L of 1 $\times$ reverse transcription buffer supplemented with 10 mM DTT, 200 μ M of the four dNTPs, and 8 U/ μ L reverse transcriptase.
2. Incubate at 37 $^{\circ}$ C for 2 h.
3. Run a small aliquot out on a 2 % agarose gel to confirm that double-strand DNA was synthesized and is the correct size.
4. Precipitate the double-stranded DNA by adding 0.1 vol of 3 M sodium acetate and 2.5 vol of cold ethanol. Pellet the mixture by centrifugation at 15,000 $\times g$ for 20 min.
5. Resuspend the DNA to 50 μ L of 1 $\times$ transcription buffer supplemented with 2 mM of the four NTPs, 1,000 units of T7 RNA polymerase, and 10 μ Ci of [α - 32 P]UTP. For the c-di-GMP-inhibited ribozyme selection, 100 μ M c-di-GMP was added to the mixture to favor isolation of ribozymes that are inactive when bound to c-di-GMP.
6. Incubate at 37 $^{\circ}$ C for 2 h (*see* Note 5).
7. Add 2 $\times$ denaturing loading buffer to the reaction mixture.
8. Separate full-length precursor RNAs from 3' cleavage products, premature transcription products, and unincorporated [α - 32 P]UTP by denaturing 8 % PAGE.
9. Use an autoradiogram of the gel to excise the gel band containing the precursor RNAs.
10. Elute the RNA from the diced gel slices overnight at 4 $^{\circ}$ C using crush soak buffer.
11. Transfer the solution containing the eluted RNAs into a fresh tube.
12. Concentrate the sample by ethanol precipitation and centrifuge as in **step 4**.
13. Resuspend the RNA in sterile deionized water.

⁵A white precipitate or cloudy solution may be seen during the course of in vitro transcription, resulting from the formation of an insoluble compound between Mg^{2+} and inorganic pyrophosphate, which indicates that a large amount of RNA has been produced. The insoluble compound can be easily removed by PAGE purification.

3.3 Selection of Allosterically Inhibited Ribozymes

1. As a negative selection, incubate 300 pmol of an initial pool of RNA precursors (internally³² P-labeled RNA) at 23 °C for 2 h in 1× selection buffer in the presence of 10 mM MgCl₂ and 100 μM c-di-GMP (*see* Notes 6 and 7).
2. To concentrate the RNAs from the reactions, precipitate with ethanol and centrifuge as in Subheading 3.2, **step 4**.
3. Resuspend the RNAs in sterile deionized water and combine with an equal volume of 2 × denaturing gel-loading buffer.
4. Separate the precursor RNAs that resisted cleavage during incubation by denaturing 8 % PAGE. A cleavage marker (full-length precursor) should be run in parallel (*see* Note 8).
5. Collect the precursor RNAs as in Subheading 3.2, **steps 9–12**.
6. Resuspend the resulting RNAs in the 1× selection buffer and 10 mM MgCl₂ at 23 °C for appropriate time in the absence of c-di-GMP (positive selection) (*see* Notes 9 and 10).
7. To concentrate the RNAs from the reactions, precipitate with ethanol and centrifuge as in Subheading 3.2, **step 4**.
8. Resuspend the RNAs in sterile deionized water and combine with an equal volume of 2 × denaturing gel-loading buffer.
9. Separate the cleaved products by denaturing 8 % PAGE. A cleavage marker (e.g., cleaved products in the negative selection) should be run in parallel.
10. Image the resulting gel using a phosphorimager and calculate the fraction of the RNAs that are cleaved (*see* Notes 11 and 12).

⁶To select for c-di-GMP-inhibited ribozymes, 100 μM c-di-GMP was included in the mixture to ensure that the active allosteric ribozymes did not cleave in the transcription step. The starting concentration of c-di-GMP in the selection reaction is influenced by its binding affinity to the aptamer. The c-di-GMP-I aptamer used in this study has a reported K_D value in the picomolar range. Hence, it is very likely to recover all possible c-di-GMP-dependent allosteric ribozymes by using micromolar to nanomolar concentrations of c-di-GMP, which is significantly above its K_D value.

⁷The order of addition of the components is critical for the selection. In the negative selection for c-di-GMP-inhibited ribozymes, RNA was firstly mixed with the assay buffer that does not contain Mg²⁺. Then c-di-GMP was added and the mixture was annealed to allow the RNAs to prefold in the presence of 100 mM NaCl in the assay buffer. Last, 10 mM Mg²⁺ (final concentration) was added to the mixture to initiate the negative selection.

⁸It is better to collect the cleaved product band in the negative selection step, for use as a size marker in the following positive selection step. However, one must pay attention not to cross-contaminate the samples. To avoid the possible cross-contamination during PAGE purification, it is suggested keeping a one- or two-lane distance between the marker and the selection product as the samples are loaded into the gel.

⁹In the positive selection for c-di-GMP-inhibited ribozymes, the same order was performed except that no c-di-GMP was mixed with RNA. Also, the same order was applied to select for c-di-GMP-induced ribozymes, except that c-di-GMP was removed in the negative selection and included in the positive selection.

¹⁰In order to select for the most active effector-dependent ribozymes, the incubation time for the positive selection reaction was reduced after several rounds of selection. Usually in the early rounds of selection, the RNA population was incubated for relatively long time (15 min) to increase the recovery of c-di-GMP-dependent self-cleaving RNAs. As more and more rounds of selection were conducted, the duration of the incubation time was gradually reduced to 1 min.

¹¹In the early (typically two to three) rounds of selection, the fraction RNAs cleaved in both the positive and negative selection reactions should be at the similar level, because the effector-independent RNAs still dominate the early populations.

¹²Usually the radioactive signal of the cleaved product is sufficient to allow detection on an autoradiogram after an exposure for 10–30 min. Using fresh [α -³² P] UTP to internally label RNA can enhance the radioactive signal of the cleaved product for better detection.

11. Collect the cleaved products as in Subheading 3.2, **steps 9–13** (*see* Note 13).

3.4 Selection of Allosterically Induced Ribozymes

Selection of ribozymes that undergo c-di-GMP induction was conducted using identical method as allosterically inhibited ribozymes (Subheading 3.3), except that c-di-GMP was included in the positive selection reaction, but was excluded in both the transcription and the negative selection reactions (*see* Note 14).

3.5 Reverse Transcription and Polymerase Chain Reaction

1. Add 10 pmol of the 3' primer (primer A or A') to the resuspended cleaved products.
2. Reverse transcribe the recovered RNAs in 100 μ L of 1 $\times$ reverse transcribe buffer supplemented with 10 mM DTT, 200 μ M of the four dNTPs, and 8 U/ μ L reverse transcriptase.
3. Incubate at 37 $^{\circ}$ C for 2 h.
4. Prepare PCR mixture by adding 5–10 μ L of the reverse transcription reaction and 20–50 pmol each of primers (primers A and B for allosterically inhibited ribozymes, primers A' and B' for allosterically induced ribozymes) to a 100 μ L PCR reaction containing 1 $\times$ PCR buffer, 200 μ M each of the four dNTPs, and 1 U/ μ L *Taq* DNA polymerase (*see* Note 15).
5. Amplify full-length double-stranded DNAs using the following PCR cycling parameters: segment 1: 94 $^{\circ}$ C for 1 min; segment 2: 94 $^{\circ}$ C for 30 s, 48 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s; segment 3: 72 $^{\circ}$ C for 5 min (*see* Note 16).
6. Analyze a small aliquot of the PCR reaction on a 2 % agarose gel to check the amount and size of the PCR products.
7. Use the amplified DNAs as templates to transcribe the RNA pool for the next round of selection, as in Subheading 3.2, **step 5**. Save a portion of the PCR products.
8. Sequence the individual clones after several rounds of selection (*see* Notes 17 and 18).

¹³The efficient recovery of cleaved RNAs from the positive selection step is important in the early rounds of selection because the occurrence of c-di-GMP-dependent sequences is rare. To avoid losing most molecules during the ethanol precipitation step, glycogen (10 μ g) was added as a carrier to help improve the recovery yield.

¹⁴In order to select for the effector-dependent ribozymes with the best binding affinity, the concentration of c-di-GMP was decreased as more rounds of selection were conducted. Initially 100 μ M c-di-GMP was included in the selection to ensure the maximal recovery of c-di-GMP-dependent self-cleaving RNAs. After several rounds of selection, the concentration of c-di-GMP was reduced to 0.3 μ M to favor the isolation of the allosteric ribozymes that bind c-di-GMP tightly. However, drastically decreasing the effector concentration may result in the loss of the most sensitive effector-dependent ribozymes. It is suggested that in the later rounds of selection, a 5- to 10-time decrease in the effector concentration is performed each time.

¹⁵During the first couple rounds of selection, it is recommended to amplify all of the cDNA. This will increase the probability that even the rarest of the active molecules will be amplified and propagated in subsequent generations.

¹⁶The number of cycles required for complete amplification depends on the amount of input template DNA. For each round of selection, the number of PCR cycles is estimated from the quantity of input cDNA, which in turn is estimated from the fraction of cleaved RNA in the positive selection step. Over-amplifying cDNA with more cycles than needed will generate undesired higher molecular weight products due to mispriming.

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¹⁷To clone the PCR products, it is very convenient to work on a TOPO TA cloning kit (Invitrogen) because this method does not require restriction sites at both the two ends of the PCR amplified product.

¹⁸To generate DNA templates for the transcription of individual clones for further analysis, either PCR amplifying the plasmid DNA or chemically synthesizing the oligo-strands can be conducted.

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Fig. 1.

(a) Constructs used for the selection of allosteric ribozymes responding to c-di-GMP. The ribozyme consists of a hammerhead ribozyme (HHR) joined to a class I c-di-GMP aptamer via communication module (CM) consisting of random sequence (N) nucleotides. The three stems that form the HHR are designated I, II, and III and the three stems that form the aptamer are labeled P1, P2, and P3. An *arrowhead* identifies the site of hammerhead-mediated cleavage. (b) Modified construct for the selection of c-di-GMP-activated ribozyme

Fig. 2.

The selection scheme for the c-di-GMP-activated allosteric ribozyme. (A) Two synthetic oligonucleotides are extended with reverse transcriptase to generate double-strand DNA encoding randomized sequence. (B) Transcription with T7 RNA polymerase. (C) The full-length RNAs (precursor) are purified by PAGE, and subject to negative selection in the absence of c-di-GMP. The cleavage products are removed by PAGE. (D) The precursor are subjected to positive selection in the presence of c-di-GMP. (E) The PAGE-purified cleavage products are reverse transcribed and amplified by PCR