Journal of Bioscience and Bioengineering
VOL. 122 No. 2, 183–187, 2016

www.elsevier.com/locate/jbiosc

Optimization of RNA-based c-di-GMP fluorescent sensors through tuning their structural modules

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Received 4 December 2015; accepted 26 January 2016
Available online 9 March 2016

Cyclic diguanylate (c-di-GMP) is a second messenger of bacteria and its detection is an important issue in basic and applied microbiology. As c-di-GMP riboswitch ligand-binding domains (aptamer domains) capture c-di-GMP with high affinity and selectivity, they are promising platforms for the development of RNA-based c-di-GMP sensors. We analyzed two previously reported c-di-GMP sensor RNAs derived from the Vc2 riboswitch. We also designed and tested their variants, some of which showed improved properties as RNA-based c-di-GMP sensors.

[**Key words:** c-Di-GMP; Fluorescent sensor; RNA-based sensor; Riboswitch; Spinach RNA]

Cyclic diguanylate (c-di-GMP), which acts as a second messenger in a variety of bacteria, has attracted considerable attention because it participates in cellular signaling processes leading to a wide range of biological responses in bacterial cells (1–5). Several distinct types of c-di-GMP binding proteins have been identified as key players in bacterial c-di-GMP signaling (6,7). Some of these proteins have been employed as components for c-di-GMP sensors (8–10), the importance of which has grown in basic and applied microbiology. More recently, it has been shown that RNA serves as another class of biopolymer that can recognize c-di-GMP and plays direct roles in c-di-GMP-dependent control of gene expression (11–13). Naturally occurring RNAs that selectively recognize c-di-GMP belong to a class of functional RNAs designated as riboswitches (14–17). These riboswitches are usually located in the 5′-untranslated region (5′-UTR) of bacterial mRNAs and specifically recognize their target ligands. Ligand binding triggers conformational changes in the 5′-UTR of mRNA that regulate expression of their downstream open reading frames (ORFs). The riboswitch has a modular structure consisting of a domain that directly recognizes its target ligand (aptamer domain) and a second domain that transduces a stimulus from the aptamer domain to cause changes in gene expression (14–17).

Aptamer domains of riboswitch have been regarded as promising platforms for RNA-based sensor systems because aptamer domains bind their target ligands with high specificity (18). In the case of c-di-GMP riboswitches, the aptamer domain of the Vc2 c-di-GMP riboswitch has been employed to develop fluorescent c-di-GMP sensors by connecting the domain with spinach RNA aptamer (19,20). Spinach RNA forms a complex with 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), through which the RNA strongly enhances fluorescence of DFHBI (21). Using this modular assembly approach, two similar c-di-GMP sensor RNAs (designated as 1b and 2a in this study, see Fig. 1) have been constructed. While the two sensor RNAs have highly similar secondary structures and also have high selectivity for c-di-GMP inherited from the parent Vc2 aptamer domain (19,20), 1b and 2a also have some structural differences, which would be related to differences in their ability to sense c-di-GMP (19, 20; see also Figs. 3 and S2). In this study, we compared the two previously reported c-di-GMP sensor RNAs to evaluate the contributions of their structural elements to their sensor abilities. This modular dissection also allowed us to design a series of variant sensor RNAs, from which improved variants of this class of sensor RNA may be identified.

MATERIALS AND METHODS

RNA preparation RNAs were prepared by *in vitro* transcription. Template DNAs for *in vitro* transcription reactions of sensor RNAs were prepared by PCR. We used appropriate plasmid DNAs or oligonucleotides bearing the sequences of the Vc2 riboswitch aptamer domain or its derivatives as templates for PCR (11,22). PCR was carried out using a set of primers with which the linker element, the spinach module, and T7 promoter sequence could be added to the aptamer domain sequences. After *in vitro* transcription with T7 RNA polymerase, RNAs were purified by electrophoresis on 9% denaturing polyacrylamide gels. The concentrations of RNAs were calculated from the absorption at 260 nm (A_{260}). The DSL ribozyme and its variants were also prepared by *in vitro* transcription with T7 RNA polymerase. PCR products bearing the T7 promoter sequence were amplified from plasmids bearing the sequences of the respective ribozyme RNAs (22,23). RNAs were purified by electrophoresis on 5% denaturing polyacrylamide gels.

Standard assay conditions for fluorogenic detection of c-di-GMP by sensor RNAs An aqueous solution containing c-di-GMP sensor RNA was heated to 80°C for 5 min and cooled to 26°C. c-Di-GMP and 10× concentrated buffer were added to this RNA solution. The solution was kept for 30 min at 26°C followed by addition of DFHBI solution. The resulting solution contained 1 μM sensor RNA, 1 μM c-di-GMP, and 10 μM DFHBI in a buffer consisting of 40 mM HEPES-KOH (pH 7.5), 125 mM KCl, and 3 mM $MgCl_2$. The resulting solution was incubated for 10 min and then fluorescence emission of the solution was measured using a plate reader (Tecan Infinite F200-pro) with excitation and emission at 485 nm and 535 nm,

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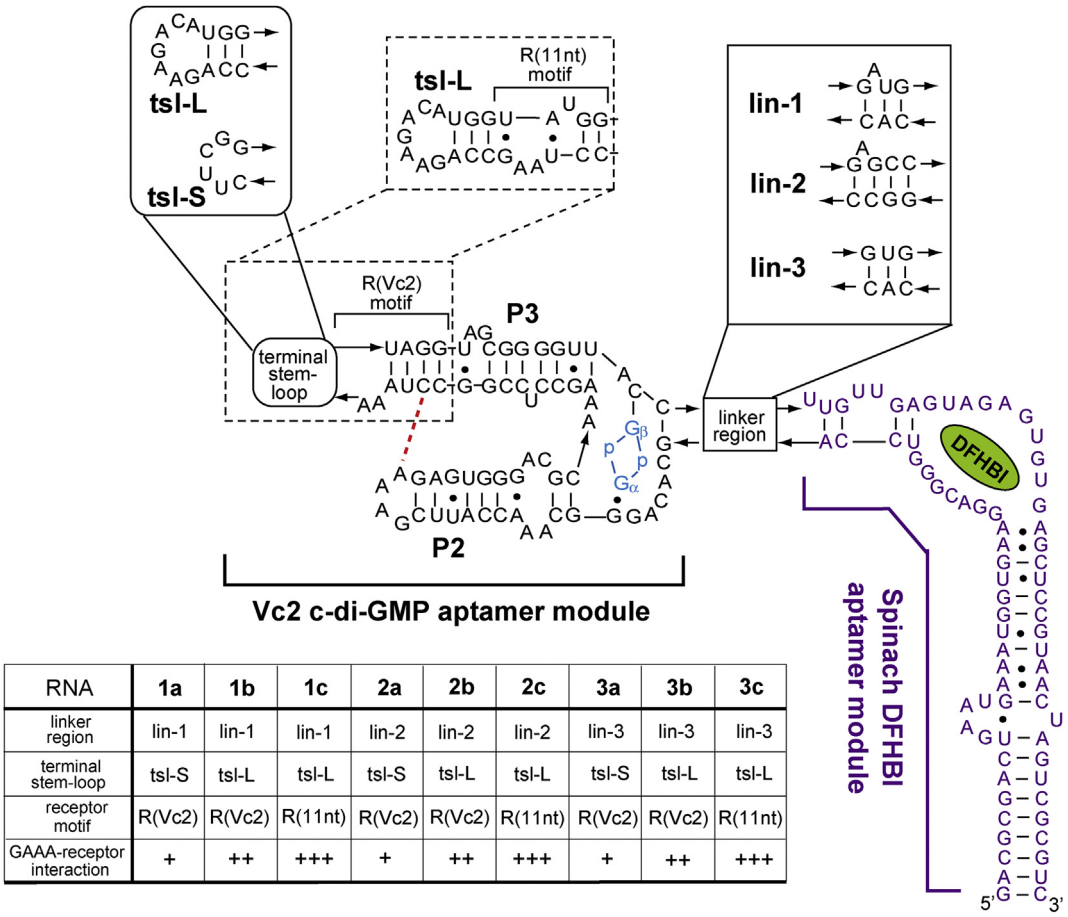
http://dx.doi.org/10.1016/j.jbiosc.2016.01.011



RNA	1a	1b	1c	2a	2b	2c	3a	3b	3c
linker region	lin-1	lin-1	lin-1	lin-2	lin-2	lin-2	lin-3	lin-3	lin-3
terminal stem-loop	tsl-S	tsl-L	tsl-L	tsl-S	tsl-L	tsl-L	tsl-S	tsl-L	tsl-L
receptor motif	R(Vc2)	R(Vc2)	R(11nt)	R(Vc2)	R(Vc2)	R(11nt)	R(Vc2)	R(Vc2)	R(11nt)
GAAA-receptor interaction	+	++	+++	+	++	+++	+	++	+++

FIG. 1. Secondary structures of RNA-based c-di-GMP fluorescent sensors. The Vc2 riboswitch aptamer domain was used as a c-di-GMP binding module and the spinach RNA aptamer was used as a fluorescent module to achieve enhanced DFHBI emission upon complex formation with RNA.

respectively. Experimental values were derived from two or more independent experiments.

***In vitro* activity assay of the DSL ribozymes** Aqueous solutions containing the DSL ribozyme or its variants were denatured by incubation at 80°C for 5 min, followed by incubation at 26°C for 3 min. RNA folding was initiated by adding 5× concentrated reaction buffer at 26°C. After incubation of the mixture for 2 min, 10× concentrated carboxyfluorescein (FAM)-labeled substrate (5′-FAM-CGTACACGTACTCACGCGTATACA-rGrUrCrCrArC-3′, where the first 24 and the last 6 nucleotides were DNA and RNA, respectively) was added to initiate the reaction. The final concentrations of the ribozyme and substrate were 2.0 μM and 0.5 μM, respectively. The reactions were carried out at 30°C in the presence of 30 mM Tris–HCl (pH 7.5) and 60 mM $MgCl_2$. At given time points, aliquots were taken and treated with an equal volume of stop solution consisting of 80% formamide, 90 mM EDTA, and 0.1% xylene cyanol. Samples were separated on 9% denaturing polyacrylamide gels and quantified using a fluoroimager (BioRad Pharos FX). Experimental values were derived from two independent experiments.

RESULTS AND DISCUSSION

Modular construction of two sets of c-di-GMP sensor RNAs Based on the structural comparison of two RNA sensors (1b and 2a) reported previously (19,20), we attempted modular engineering to improve this type of RNA sensor. We prepared two sets (set-1 and set-2) of variant RNA sensors based on structural difference of the linker region. Set-1 and set-2, which were derived from 1b and 2a, respectively (Fig. 1), shared the lin-1 and lin-2 linkers, respectively. As both the number of base pairs and G-C content were higher in the lin-2 linker than the lin-1 linker, sensor RNAs belonging to set-2 may share a residual ability to capture DFHBI without ligand-induced stabilization of the linker duplex.

In addition to the linker region, RNA 1b and 2a have structural difference in the P3 element of the c-di-GMP aptamer module (Fig. 1). In the P3 element, the R(Vc2) motif is embedded as a module that recognizes a GAAA tetraloop in the P2 element. Crystal structure analysis of the Vc2 aptamer domain revealed an atomic-level architecture with which the R(Vc2) motif recognizes the GAAA tetraloop in the P2 element (24,25). In RNA 1b, the R(Vc2) motif is closed by 13 nucleotides, which are capable of forming three base pairs capped by a heptaloop (termed tsl-L in this study). On the other hand, the corresponding region of RNA 2a is composed only of six nucleotides (5′-CUUCGG-3′) (termed tsl-S in this study). Such differences in the structure of P3 may affect the structural stability of the neighboring R(Vc2) motif and also GAAA binding ability of the receptor motif.

To compare the ability of the R(Vc2) motif capped by tsl-L and tsl-S to recognize a GAAA loop, we incorporated them into the DSL-ligase ribozyme (Fig. 2A) (23). Due to the structural modularity of tetraloop-receptor (L/R) interactions, it has been shown that relative binding affinity of L/R interactions is commonly adaptable among different structural scaffolds without disturbing their order (26–29). The relative position and orientation between the GAAA loop and the receptor motif in the DSL ribozyme is also nearly same to that in the Vc2 riboswitch (29). The DSL ribozyme has been employed as an assay system, with which L/R interactions can be characterized conveniently because its ribozyme activity is strongly dependent on its L/R interaction that docks the P1 substrate module to the P3 catalyst module (22,23). Two variants possessing the R(Vc2) motif were less active than the parent DSL ribozyme, whose