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Engineered Allosteric Ribozymes that Sense the Bacterial Second Messenger c-di-GMP

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Abstract

A series of allosteric ribozymes that respond to the bacterial second messenger cyclic di-guanosyl-5'-monophosphate (c-di-GMP) have been created by using *in vitro* selection. An RNA library was generated by using random-sequence bridges to join a hammerhead self-cleaving ribozyme to an aptamer from a natural c-di-GMP riboswitch. Specific bridge sequences, called communication modules, emerged through two *in vitro* selection efforts that either activate or inhibit ribozyme self-cleavage upon ligand binding to the aptamer. Representative RNAs were found that exhibit EC₅₀ (half maximal effective concentration) values for c-di-GMP as low as 90 nM, and IC₅₀ (half maximal inhibitory concentration) values as low as 180 nM. The allosteric RNAs display molecular recognition characteristics that mimic the high discriminatory ability of the natural aptamer. Some engineered RNAs operate with ribozyme rate constants approaching that of the parent hammerhead ribozyme. Using these allosteric ribozymes, cytoplasmic concentrations of c-di-GMP in three mutant strains of *Escherichia coli* were quantitatively estimated from cell lysates. Our findings demonstrate that engineered c-di-GMP-sensing ribozymes can be used as convenient tools to monitor c-di-GMP levels from complex biological or chemical samples. Moreover, these ribozymes could be employed in high-throughput screens to identify compounds that trigger c-di-GMP riboswitch function.

The second messenger c-di-GMP regulates many physiological processes in bacteria, including conversion between motile and biofilm lifestyles^{1,2} and the transition to virulence in some pathogens³. Under conditions favoring the motile lifestyle, cells transition to a planktonic state by lowering intracellular c-di-GMP levels through degradation of the second messenger by using specific phosphodiesterase (PDE)^{4–6} enzymes. Conversely, production of c-di-GMP results from the fusion of two GTP molecules by the action of diguanylate cyclase (DGC)³ enzymes. When c-di-GMP concentrations are increased, genes

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Supporting Information

Supporting Information Available: Additional information as noted in the text (Materials and Methods; Figures S1 to S16; Table S1 to S3) is available free of charge via the Internet at <http://pubs.acs.org>.

such as those involved in extracellular polysaccharides biosynthesis are upregulated, thus leading to biofilm formation. In many organisms, the genes coding for enzymes that catalyze c-di-GMP degradation or synthesis are controlled by two distinct classes of riboswitches that selectively sense and respond to this second messenger^{7,8}.

Many of the additional cellular effects of c-di-GMP can be predicted based on the associations of c-di-GMP riboswitches with genes for other physiological processes. These associations and other experimental analyses have revealed a surprising diversity of effects of c-di-GMP on bacterial cells, including its involvement in cell differentiation⁹, quorum sensing¹⁰ and cAMP signaling¹¹ pathways. Because of the widespread effects of c-di-GMP, monitoring cellular levels of this second messenger is of great interest to those seeking to understand a wide range of bacterial phenomena. Existing technologies such as high performance liquid chromatography (HPLC) can be used to detect c-di-GMP and evaluate its concentration in biological samples. However, analyses of complex mixtures of compounds can be problematic due to similar molecular weights and chemical properties of other metabolites. A quantitative sensor and reporter for cellular c-di-GMP that ignores all other natural metabolites would provide an attractive alternative method for establishing its concentration even in complex chemical or biological mixtures.

Aptamers are attractive alternatives to HPLC because of their ease of synthesis, their ease of manipulation, and their high affinity and high specificity binding characteristics. RNA molecular switches, including allosteric ribozymes that incorporate both aptamer and ribozyme domains, have been engineered to trigger only in the presence of their corresponding ligands. Ligand-binding aptamers previously have been demonstrated to function as molecular recognition components of novel array-based biosensor devices¹². Furthermore, using modular rational design^{13–15} and *in vitro* selection¹⁶, a series of allosteric ribozymes that are sensitive to various effector molecules such as ATP, flavin mononucleotide (FMN), theophylline, and the second messengers cGMP and cAMP^{13,15,17,18} have been created. These engineered allosteric ribozymes have been used in prototype multiplex biosensor arrays¹⁹. More recently²⁰, riboswitch aptamers have been grafted onto ribozymes to create allosteric ribozymes.

Several engineered allosteric ribozymes derived from the self-cleaving hammerhead ribozyme display greater than 100-fold modulation of the ribozyme rate constant upon binding their corresponding effectors. In some cases, the maximum rate constants ($k_{\max}$) are near the observed $k_{\max}$ of the minimal hammerhead ribozyme core¹⁷. These findings suggest that additional engineered allosteric ribozymes could be generated and used for rapid detection of ligands in chemical or biological samples using biosensors like those described previously^{19,21}. In addition, allosteric ribozymes could be used for high-throughput screening to facilitate the rapid discovery and development of compounds that target riboswitch aptamers^{22–24}. *In vivo* applications for engineered allosteric ribozymes may need to make use of full-length hammerhead constructs that exhibit higher rate constants for RNA cleavage due to the presence of additional tertiary interactions^{25,26}.

Most allosteric ribozymes have been created by joining preexisting ligand-binding aptamer domains with ribozyme domains to produce ligand-responsive self-processing RNA

constructs. Similarly, we sought to create allosteric ribozymes that could be used to sense c-di-GMP. Recently, a class of natural RNA switches was identified that regulates gene expression in response to c-di-GMP binding⁷. The aptamer from one member of this c-di-GMP-I riboswitch class present in *Vibrio cholera* exhibits a K_D of ~10 pM²⁷. Moreover, atomic-resolution structural models for this RNA aptamer bound to c-di-GMP have been generated based on x-ray crystallography data^{27,28}. We exploited a pre-existing c-di-GMP aptamer and knowledge of its key structural features to engineer allosteric ribozymes for c-di-GMP with characteristics suitable for biosensor applications.

RESULTS AND DISCUSSION

In vitro selection of allosteric ribozymes

RNA constructs carrying the *V. cholera* c-di-GMP-I aptamer^{7,27,28} and a self-cleaving hammerhead ribozyme^{29,30} were created by linking the P1 stem of the aptamer to stem II of the hammerhead ribozyme via two random-sequence regions (Figures 1A and 1B). Atomic-resolution structure models of a natural c-di-GMP-I riboswitch^{27,28} reveal the presence of important tertiary contacts between stems P2 and P3, which guided our decision to use a connection to P1 and avoid possible disruption of key structural contacts within the aptamer. Furthermore, it was known that P1 stem stability is enhanced upon ligand binding by the aptamer⁷, and this structural stabilization was expected to facilitate the isolation of allosteric ribozymes. The formation of hammerhead stem II is critical for ribozyme activity^{31,32} and therefore sequence variants of the bridge between P1 and stem II that allow ligand binding to influence the stability of the fused stem should facilitate allosteric self-cleaving ribozyme function. This general design strategy has been used previously to create numerous allosteric hammerhead ribozymes via *in vitro* selection^{17,18}.

The bridges were derived from two random-sequence regions of four and five nucleotides in length (Figure 1A) that yield a population of 262,144 possible variants, or two random-sequence regions of six and seven nucleotides (Figure 1B) that yield over 67 million possible variants. Initially, two RNA pools each containing approximately 1.2×10^{13} molecules of the shorter bridge construct were subjected to *in vitro* selection⁷ (Figure S1) either for c-di-GMP-dependent allosteric inhibition (Figure S2A) or allosteric induction (Figure S2B). Later, the longer bridge construct was used for the allosteric induction selection (Figure S2C).

To isolate variants that direct the allosteric inhibition of ribozymes, the initial RNA population was prepared by *in vitro* transcription in the presence of c-di-GMP, and the full-length precursor RNAs were purified by denaturing 8% PAGE. The precursor RNAs were subjected to a “negative selection” reaction in the presence of c-di-GMP under reaction conditions that are otherwise permissive for ribozyme function (reaction buffer: 50 mM Tris-HCl [pH 7.5 at 23°C], 100 mM NaCl, 10 mM MgCl₂). Again, uncleaved precursors were purified by PAGE and subjected to “positive selection” by brief incubation under the permissive reaction conditions in the absence c-di-GMP. The resulting 5' fragments generated by self-cleavage were isolated by PAGE and amplified by reverse transcription and polymerase chain reaction (RT-PCR). This selection and amplification process was

repeated until the population was enriched for variants that undergo robust allosteric inhibition by c-di-GMP.

To isolate variants that undergo allosteric activation by c-di-GMP, the starting pool was transcribed in the absence of ligand. Uncleaved RNAs were isolated and subjected to negative selection in the absence of c-di-GMP, and RNA precursors that remained uncleaved were isolated and subjected to positive selection in the presence of c-di-GMP. The 5' cleavage fragments were isolated and amplified by RT-PCR and the process was repeated until the population exhibited robust allosteric activation by c-di-GMP.

After seven rounds of selective amplification (G7) for c-di-GMP inhibition, the population displayed considerable difference in activity in the absence versus the presence of 100 μ M c-di-GMP (Figure S2A). To favor ribozymes with high sensitivity to ligand, the concentration of c-di-GMP in the transcription and negative selection steps was decreased from 100 μ M (G0-G7) to 1 μ M (G8-G9), and then to 0.3 μ M (G10-G13). Similarly, to favor ribozymes with high rate constants for RNA cleavage, the positive selection incubation times were reduced from 15 min (G0, G1) to 5 min (G2-G5) and eventually to 1 min (G6 to G13). Both the G8 and G14 populations were cloned, sequenced, and assayed for allosteric inhibition function (Figures 2, 3, S3–9).

The parallel selection to isolate ribozymes that are activated by c-di-GMP was less successful and only a modest difference in self-cleavage activity was obtained by G7 (Figure S2B). Individual RNA clones isolated from the G8 RNA pool showed only weak ligand induction (data not shown). We hypothesized that our shorter bridge construct (Figure 1A) might disfavor allosteric activation possibly due to a steric clash between key substructures of the aptamer and ribozyme domains. Tertiary contacts between stems I and II are essential for stabilizing their parallel orientation and for the ribozyme to achieve maximal activity.^{25,26} Steric hindrance between stems I and II might occur in our constructs when the relatively large c-di-GMP aptamer is fused to stem II of the ribozyme. This would prevent ribozyme stems I and II from adopting a parallel orientation and preclude the isolation of allosteric c-di-GMP-induced ribozymes with robust activity. To reduce the potential for steric hindrance and favor the isolation of c-di-GMP-induced ribozymes, a modified aptamer-ribozyme fusion construct was prepared with additional random-sequence nucleotides in the two linker regions (Figure 1B). These longer bridge sequences further separate the ribozyme core from the aptamer to reduce steric clash potential. Moreover, the increased number of sequence variants creates greater opportunities for variants that are robustly modulated by ligand binding.

The possibility for steric clash between the aptamer and ribozyme was further reduced by shortening the length of stem I to five base pairs (Figure 1B). The RNA population isolated after five rounds of selection (G5) exhibited higher sensitivity to c-di-GMP (Figure S2C) compared to that isolated from the original pool (Figure S2B). Representative variants from this G5 population were cloned, sequenced, and assayed for allosteric induction function (Figures 2, 3, 4, S10–14).

Sequences of Bridge Elements and Allosteric Modulation of Ribozymes

Among the 50 clones sequenced from each of the G8 and G14 populations from the allosteric inhibition selection, five classes were identified and designated as “inhibition elements” I through V (Figure S3). All representatives within these different classes exhibited allosteric inhibition upon addition of c-di-GMP (Figure S4). The best representatives from G8 (clone 8⁻I) and G14 (clone 14⁻II) were selected for further kinetic analyses (Figure S5).

Both RNAs exhibit distinct rate constants for self-cleavage in the absence (k_{obs}^-) versus the presence (k_{obs}^+) of ligand (Figures 2, S5–6; Table S1). The k_{obs}^- value of 1.0 min^{-1} for 8⁻I approaches the maximum k_{obs} (1.1 min^{-1}) for a minimized hammerhead ribozyme¹⁷, while the k_{obs}^- for 14⁻II is approximately 0.22 min^{-1} . These RNAs also exhibit greater than 100-fold allosteric inhibition ($k_{\text{obs}}^-/k_{\text{obs}}^+$) (Table S1), which is similar in magnitude to the kinetic modulation seen with previously engineered allosteric ribozymes¹⁷ and some natural allosteric proteins.

We noticed that the second strands of the bridge domain for all c-di-GMP-inhibited ribozymes are identical (5'-CCUGCCC-3'). Further examination of the nucleotide sequence and possible secondary structures revealed that some nucleotides within this bridge domain are complementary to the 5' end of stem I of the ribozyme domain (Figure S16). The close positioning of stems I and II, aided by this possible base pairing, might explain the high rate constants for cleavage for the c-di-GMP-inhibited ribozymes tested in the absence of c-di-GMP. Tertiary contacts between stem I and stem II substructures commonly exist for natural hammerhead ribozymes^{25,26}, and these contacts are necessary for high speed function at low (physiological) divalent magnesium ion concentrations.

Among the 50 clones sequenced from the G5 population of the allosteric induction selection with the longer bridge construct, four different bridge sequences were identified (Figure S10). The fastest self-cleaving clone 5⁺III (Figure S11) was chosen for further kinetic analyses (Figure S12–13). As with the engineered ribozymes characterized above, the k_{obs}^- value of 0.0045 min^{-1} and k_{obs}^+ value of 0.44 min^{-1} yield nearly 100-fold allosteric modulation ($k_{\text{obs}}^+/k_{\text{obs}}^-$) (Figure 2).

Dose-response and Dynamic Range Characteristics

Our goals for this study were to obtain allosteric ribozymes that respond to low concentrations of ligand (high ligand sensitivity) and high rate constants for RNA cleavage (short assay time). The rate constants noted above yield substantial amounts of RNA cleavage during reactions that last only minutes. To assess the apparent binding affinity for each allosteric ribozyme construct, we sought to determine the concentration of c-di-GMP needed to half-maximally modulate ribozyme activity (IC_{50} or EC_{50}). If ligand binding directly modulates ribozyme activity, then this ligand concentration might approximate the dissociation constant (K_D) that the aptamer exhibits for c-di-GMP.

We conducted assays to reveal the dose-response curves for the allosterically inhibited ribozymes 8⁻I (Figures S5, S7) and 14⁻II (Figures 3C, S5, S7), and for the allosterically activated ribozyme 5⁺III (Figures 3D, S12). IC_{50} values for ribozymes 8⁻I and 14⁻II were

determined by establishing the effector concentrations that produce half maximal inhibition of ribozyme yield during a 6 minute reaction (Figures 3C, S5, S7). The IC_{50} values of 2 μ M for 8^{-I} and 0.18 μ M for 14^{-II} suggest that a 10-fold improvement in c-di-GMP binding affinity allowed 14^{-II} to become more enriched during *in vitro* selection with lower ligand concentration (Figure S7, Table S1).

Similarly, the EC_{50} value for ribozyme 5^{+III} was determined by establishing the effector concentration that produces a half maximal induction response during a 10 minute reaction (Figures 3D, S12; Table S2). Because the selection of c-di-GMP-induced ribozymes with the modified construct started with a low concentration of effector (0.3 μ M), ribozyme 5^{+III} exhibits an EC_{50} of 90 nM (Figure 3D), which is as low as the IC_{50} of 14H-II. This highlights the need to employ a low concentration of effector for the selective amplification of highly sensitive ribozymes.

We also estimated the IC_{50} and EC_{50} values by comparing the rate constants for ribozyme cleavage at various ligand concentrations. Half maximal rate constants 14^{-II} and 5^{+III} were observed at 70 nM and 500 nM c-di-GMP, respectively (Figures 3A, 3B, S6, S13). These values are close to the 300 nM concentration of effector used for *in vitro* selection. Moreover, the dose-response curves for these two allosteric ribozymes reveal dynamic ranges for effector-induced ribozyme control. Consistent with our preliminary analyses (Figure 2), ribozymes 14^{-II} and 5^{+III} exhibit dynamic ranges of about two orders of magnitude (Figures 3A, 3B). Although the IC_{50} and EC_{50} values are poorer than the corresponding K_D value for the parent c-di-GMP aptamer when examined in isolation, losses in binding affinity have been observed previously for aptamers when integrated as components of allosteric ribozymes³³. This is likely due to competition between alternative structures formed by the allosteric ribozyme.

Discrimination Between c-di-GMP and Several Analogs

We were also interested in whether the engineered c-di-GMP-sensing ribozymes could be used for high-throughput screening of chemical libraries to identify analogs that might trigger riboswitch function in cells. As an initial proof of concept of this application, we tested whether the selected ribozymes could discriminate against c-di-GMP analogs.

Three linear analogs of c-di-GMP, GpG, dGpG and d(GpG) (Figure S8) were obtained and each was used in ribozyme assays with constructs 14^{-II} (Figure S9) and 5^{+III} (Figure S14). In the concentration range we tested (0–300 μ M), self-cleavage of both ribozymes was activated (for 5^{+III}) or inhibited (for 14^{-II}) by GpG, although a much higher concentration was needed compared to c-di-GMP. Ribozyme 5^{+III} did not exhibit (< 5%) allosteric induction upon the addition of up to 300 μ M dGpG or d(GpG). To quantitatively compare the binding affinities of each ribozyme to c-di-GMP and the three analogs, the inhibition and induction responses versus the logarithm of the concentration of ligands were plotted (Figures 3C, 3D). The IC_{50} and EC_{50} values of the four compounds for ribozymes 14^{-II} and 5^{+III}, respectively, were derived from the plots and are summarized in Table S2. Apparent K_D values of the four compounds for the natural c-di-GMP riboswitch aptamer were determined by using in-line probing (data not shown) and are also listed in Table S2.

The trend of K_D values for these compounds matches the trend of IC_{50} and EC_{50} values of the four effectors, indicating that the aptamer component of the engineered allosteric ribozymes maintains its ability to discriminate between the effector and the analogs examined. However, ribozyme 5⁺III discriminates more strongly against the three analogs compared to 14⁻II, even though its activity is two-fold (Table S2) more sensitive to c-di-GMP. This finding demonstrates that the bridge sequences do affect the extent of discrimination despite the fact that they largely reside outside the core of the aptamer.

Atomic-resolution structural models of a class I c-di-GMP riboswitch aptamer bound to c-di-GMP^{27,28} based on x-ray crystallography data reveal that the both of the 2'-hydroxyl groups of the ligand are involved in molecular recognition by forming hydrogen bonds with nucleotides in the ligand binding pocket of the aptamer. The observation that both allosteric ribozymes become less sensitive to the ligand when analogs containing two (GpG), one (dGpG), or no (d(GpG)) 2' hydroxyl groups supports the hypothesis that these effectors are likely to be recognized in the same way in our engineered constructs as it is in nature.

Quantitative Estimation of Cytoplasmic c-di-GMP Levels

The engineered ribozymes 14⁻II and 5⁺III have allosteric characteristics that should permit the use of these RNAs as tools to monitor c-di-GMP levels from complex biological or chemical samples. To demonstrate this capability, ribozyme 5⁺III was used to estimate the cytoplasmic c-di-GMP levels in four strains of *E. coli*. We speculated that the diverse constituents of a cell lysate might adversely affect the rate constant for ribozyme cleavage, affect RNA-ligand interactions, or alter the allosteric interplay between aptamer and ribozyme. Therefore, we first assessed the performance of internally ³²P-labeled ribozyme 5⁺III in the absence (Figure 4A) or presence (Figure 4B) of bacterial cell lysate (1/3rd volume of cell lysate added to the reaction) and various concentrations of c-di-GMP. Cell lysate was prepared from WT *E. coli* (strain BW25113) grown in Luria-Bertani (LB) medium³⁴ (see Materials and Methods in Supporting Information for details).

A reduction in allosteric activation by c-di-GMP is observed in a 10 minute reaction when cell extract is added to the assay (Figures 4B, 4C) relative to those reactions that contain the same amounts of c-di-GMP but no extract (Figure 4A). Moreover, the WT strain has a reported cytoplasmic c-di-GMP concentration of 1.1 μ M in logarithmically growing cells³⁵. Therefore, some allosteric activation should have occurred even in the absence of supplemented c-di-GMP. The possibility that components of the cell extract were chelating the essential Mg^{2+} cofactor for ribozyme was examined by increasing the concentration of Mg^{2+} in the reaction buffer from 10 mM to 30 mM (Figure S15). However, this adjustment had no effect on the activity of the 5⁺III allosteric ribozyme, and therefore the inhibitory effects of cell extract cannot be overcome simply by including additional Mg^{2+} .

To overcome the adverse effects of extract on the accuracy of the c-di-GMP detection assay, we established a standard curve (Figure 4D) for c-di-GMP-dependent RNA cleavage yields for ribozyme 5⁺III in the presence of WT lysate (Figure 4B). The levels of ribozyme cleavage in the presence of extract from WT cells and from three *E. coli* variants with disabling mutations in genes encoding c-di-GMP-specific phosphodiesterases (PDE)⁴⁻⁶ were assessed, and these values were used to estimate the levels of the second messenger in

these strains. Specifically, *E. coli* strains JW1484-4 (*dosP*), JW-1278-1 (*gmr*) and JW5943-1 (*yhjK*) all bear a deletion in one of the genes that encode c-di-GMP-specific phosphodiesterases and therefore are expected to accumulate higher internal c-di-GMP concentrations.

The concentrations of c-di-GMP in the lysates relative to WT lysate were estimated by comparing the induction (%) of RNA cleavage in the presence of same volume of each cell lysate (Figure 4C) with the standard curve (Figure 4D). For example, incubation of ribozyme 5⁺III for 10 min in a reaction containing 1/3 volume of *E. coli* *yhjK* cell lysate resulted in 67% cleavage of the RNA (Table S3). In this example, the level of RNA cleavage reflects the presence of an extra 1.1 μM of c-di-GMP in the reaction mixture relative to that in the WT extract (Table S3). Because the cell lysate was diluted to one-third its original concentration, this indicates the cell extract contains an additional 3.3 μM of c-di-GMP relative to WT cell extract (Table S3).

Based on the total volume of cell lysate (400 μL) and the total number of cells ($\sim 2.80 \times 10^{12}$) in the culture (see Materials and Methods in Supporting Information for details), the extra number of molecules of c-di-GMP per cell generated in the *yhjK* strain was calculated to be 284 ± 58 (Table S3). A typical *E. coli* cell will have an internal volume of $\sim 2 \times 10^{-16}$ liters³⁶. Thus, one molecule per cell will give a concentration of about 10 nM. Three replicate assays reveal that the deletion of *yhjK* increases the level of c-di-GMP in cells by $2.84 \pm 0.58 \mu\text{M}$ compared to that of WT (Figure 4E, Table S3).

Similarly, the *dosP* and *gmr* strains were determined to yield a small increase in cytoplasmic c-di-GMP levels (0.12 ± 0.03 and $0.15 \pm 0.04 \mu\text{M}$, respectively) relative to WT (Figure 4E, Table S3). The small increases in cytoplasmic c-di-GMP for these strains could be due to the normally low expression of *dosP* and *gmr* genes under the conditions used in this study. For example, *dosP* is a gene encoding the oxygen-sensing c-di-GMP phosphodiesterase. In a previous study⁶, it was determined that over 80% of *dosP* expression occurred only in solutions that exceed 30% of oxygen saturation (oxygen concentrations greater than 75 μM).

The order of cytoplasmic c-di-GMP levels (WT $\approx$ *dosP* $\approx$ *gmr* < *yhjK*) measured by our allosteric ribozyme does not perfectly correlate with our previous report (WT < *dosP* *yhjK* < *gmr*)³⁷, in which the estimation was based on biofilm formation and a genetic reporter assay. Several factors could contribute to this difference. For example, the allosteric ribozyme estimates average total concentration of cytoplasmic c-di-GMP while the biofilm assay and the reporter assay may be influenced by c-di-GMP concentration differences between subcellular locations³⁸. Also, small volumes of *E. coli* cell cultures were examined during exponential phase growth when examining biofilm formation and reporter gene expression. In contrast, much larger volumes (200 mL) of *E. coli* cell cultures were grown to stationary phase in order to concentrate cytoplasmic c-di-GMP for our allosteric ribozyme assays. These differences in volume size could lead to the difference in oxygen saturation in cell cultures. It has been reported that the growth conditions (temperature, oxygen level, etc.) and the growth phases (stationary or exponential) substantially affect cytoplasmic c-di-GMP levels^{4,6,39,40}.

CONCLUSIONS

The simultaneous use of modular rational design and *in vitro* selection is a powerful strategy for engineering allosteric ribozymes with desired kinetic characteristics. Both the ligand-binding and ribozyme domains are typically retained without substantive mutation by the selected RNAs. This focuses the original construct design and *in vitro* selection processes on the creation and isolation of relatively short bridge domains that are the appropriate length and sequence to functionally couple ligand binding and ribozyme functions.

For some previous engineered allosteric ribozymes, allosteric regulation appears to be achieved through localized base-pairing changes within the bridge domain¹⁷, which exploits binding energy derived from ligand-aptamer complex formation to generate a shift in base-paired substructures. The mechanism of regulation can be more easily predicted if the structural states of the ligand-bound aptamer and the ribozyme are well understood. However, the precise mechanism of regulation for some allosteric ribozymes can remain obscure. For example, ribozymes that respond to the second messengers cGMP and cAMP have been successfully generated by using a modified version of allosteric selection¹⁸, wherein novel aptamers are generated by *in vitro* selection rather than included as preexisting domains. Less is known about the new aptamers that result from the selection, and so more hypotheses regarding the mechanism of allosteric control may need to be experimentally assessed.

Given that the original construct design (Figure 1A) failed to yield a robust population of ribozyme that were activated by c-di-GMP, we speculated that there might be some steric clash between the aptamer and ribozyme domains that precludes the selection of ligand-induced ribozymes. Regardless of the source of the problem, the use of a more diverse population of constructs carrying longer bridging sequences bypassed the problem and resulted in the successful isolation of c-di-GMP-induced ribozymes.

All engineered c-di-GMP-dependent ribozymes isolated in this study that are inhibited by ligand (8⁻I to V, 14⁻I to V) carry a nucleotide sequence of CUGCC in the second portion of the original random-sequence bridge domain. This suggests that each representative may exploit the same strategy for at least a portion of their allosteric control mechanism. In contrast, a greater diversity of bridge sequences emerged from the selections for allosterically activated ribozymes (5⁺I to IV). Therefore, these ribozymes appear to function by different mechanisms.

The cytoplasmic c-di-GMP concentrations of bacterial strains were established by analyzing the activities of a representative allosteric ribozyme when incubated with cell lysate. This assay is similar to that used previously involving immobilized engineered ribozymes to monitor levels of naturally produced 3',5'-cyclic adenosine monophosphate (cAMP) in bacterial culture media¹⁹. In this previous study, an engineered allosteric ribozyme for cAMP was used to establish ligand concentrations ranging from ~1 to 1,000 μ M when present in complex biological mixtures. The c-di-GMP-dependent RNA switches engineered in this study (14⁻II & 5⁺III) can be used to detect the concentration of c-di-GMP ranging from ~0.01 to 3 μ M (Figures S5, S13). The substantial increase in sensitivity for the new

allosteric ribozymes is likely due to a combination of the high sensitivity of the natural aptamer used to design the parent construct and the use of low concentrations of ligand during *in vitro* selection.

Ribozyme 14^{-II} is triggered by GpG and dGpG with IC₅₀ values of 10 and 90 μ M, respectively (Figure 3C; Table S2). For high-throughput screening, the concentration of each molecule tested is around 30 μ M. Therefore, an allosteric ribozyme assay based on our engineered ribozymes should be useful for identifying novel compounds that are bound by the c-di-GMP aptamer and allosterically modulate ribozyme activity.

Previously we showed that engineered allosteric ribozymes could be altered to function as bimolecular constructs¹⁷ to cleave substrate RNAs in response to ligand binding. Separation of catalytic and substrate domains permits multiple-turnover ribozyme function, which can increase the sensitivity of ligand detection. The use of fluorescent-tagged substrates (*e.g.*, FRET pairs) would allow the convenient evaluation of allosteric ribozyme activity²². Furthermore, immobilization of the allosteric ribozyme constructs would also permit reuse of the assay matrix by addition of fresh substrate. Implementing a combination of these adaptations would yield a highly sensitive and cost-effective system for measuring c-di-GMP concentrations in many samples, or would facilitate the identification of riboswitch ligand analogs by high-throughput screening. These features provide advantages over other methods such as HPLC-based analysis for measuring natural ligand concentrations or for discovering novel ligands for aptamers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Constructs used for the selection of allosteric ribozymes that respond to c-di-GMP. (A) Ribozyme construct consisting of a hammerhead ribozyme joined to a class I c-di-GMP-binding aptamer via regions of four and five random-sequence (N) nucleotides. The three stems that form the hammerhead ribozyme 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. The random-sequence nucleotides are expected to form the fused P1 and stem II structures. (B) Modified construct for the selection of c-di-GMP-activated ribozymes. In this modified design, the bridge domain carries a total of thirteen random-sequence nucleotides. Also, stem I of the ribozyme was truncated to five base pairs

and four unpaired nucleotides were present at the 3' terminus to increase the size difference between the precursor and the 5' cleaved product. Other details are as described in (A).

Figure 2.

Bridge sequences and ligand-dependent activities for three representative allosteric ribozymes. Plots depict ribozyme cleavage rate constants in the absence (open circle) or presence (filled circle) of 3 μM c-di-GMP.

Figure 3.

Characteristics of allosteric modulation of engineered hammerhead ribozymes by c-di-GMP. The logarithm of the rate constant for ribozyme cleavage versus the logarithm of the allosteric effector concentration is plotted for ribozymes 14⁻II (A) and 5⁺III (B). The apparent IC_{50} and EC_{50} values for ribozyme 14⁻II and 5⁺III are 70 nM and 500 nM, respectively. Percent cleavage versus the logarithm of the allosteric effector concentration is plotted for ribozymes 14⁻II (C) and 5⁺III (D). Percent inhibition values for ribozyme 14⁻II were determined by comparing the level of inhibition at a given concentration of effector in a six minute reaction versus the level of maximal inhibition at saturating levels of c-di-GMP.

Similarly, percent induction values for ribozyme 5⁺III were determined for 10 minute reactions. The lines depicted simulate that expected for a one-to-one binding interaction between RNA and ligand.

Figure 4.

Quantitative estimation of cytoplasmic c-di-GMP levels in four *E. coli* strains. (A) Cleavage of ribozyme 5⁺III induced by c-di-GMP at various concentrations. Ribozyme 5⁺III was incubated with c-di-GMP in HEPES buffer (pH 7.5 at 23°C) and 10 mM MgCl₂ for 10 min. Filled arrowheads (denoted Pre) identify bands corresponding to uncleaved internally ³²P-labeled RNA precursor RNAs. Open arrowheads (denoted Clv) identify bands corresponding to 5' cleavage fragments. (B) Cleavage of ribozyme 5⁺III induced by c-di-GMP at various concentrations in buffer containing cell lysate from WT *E. coli*. Other details are as described for (A). (C) Cleavage of ribozyme 5⁺III in buffer containing cell lysate of WT and various other strains of *E. coli*. Each cell lysate was derived from an approximately equivalent number of cells. Other details are as described for (A). (D) The induction response of 5⁺III ribozyme cleavage for the extracts of various strains of *E. coli* are compared to a standard curve for responses to c-di-GMP added to reactions containing WT *E. coli* extract. The standard curve (filled circles) from the data in (B) is overlaid with a line simulating that expected for a one-to-one binding interaction between RNA and ligand.

(E) Plot of the estimated cytoplasmic c-di-GMP levels for the three strains relative to WT. The standard deviation in (D) and (E) was generated from three replicate assays.