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RNA-Based Fluorescent Biosensors for Live Cell Imaging of Second Messengers Cyclic di-GMP and Cyclic AMP-GMP

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Supporting Information

ABSTRACT: Cyclic dinucleotides are an important class of signaling molecules that regulate a wide variety of pathogenic responses in bacteria, but tools for monitoring their regulation *in vivo* are lacking. We have designed RNA-based fluorescent biosensors for cyclic di-GMP and cyclic AMP-GMP by fusing the Spinach aptamer to variants of a natural GEMM-I riboswitch. In live cell imaging experiments, these biosensors demonstrate fluorescence turn-on in response to cyclic dinucleotides, and they were used to confirm *in vivo* production of cyclic AMP-GMP by the enzyme DncV.

Cyclic dinucleotides are a newly expanded class of second messengers that mediate intracellular signaling pathways in bacteria.[1] Cyclic di-GMP (c-di-GMP) has been shown to regulate physiological processes such as biofilm formation, motility, and virulence response.[2] More recently, two more cyclic dinucleotides were identified as natural products. Cyclic di-AMP (c-di-AMP) is involved in regulating sporulation, cell size, and cell wall stress tolerance,[3,4] and cyclic AMP-GMP (c-AMP-GMP) has been implicated in affecting intestinal colonization by bacteria.[5] In addition, there is evidence that these cyclic dinucleotides stimulate the innate immune response in mammalian cells.[6−8] Thus, cyclic dinucleotides are of further interest as potential small molecule adjuvants for vaccine development.[9]

As with other second messengers, cyclic dinucleotide signaling appears to be tightly temporally and spatially controlled. Thus, there is a critical need for *in vivo* biosensors that can monitor their dynamics in order to understand how physiological changes are signaled. A Förster resonance energy transfer (FRET)-based biosensor recently has been employed to monitor c-di-GMP in *Caulobacter crescentus*.[10] It exhibits a modest decrease in net FRET signal upon ligand binding. There are as yet no reported biosensors for c-di-AMP or c-AMP-GMP.

The use of RNA as a tool for *in vivo* molecular sensing has long been under-developed despite the natural ability of RNAs, such as riboswitches, to selectively recognize small molecules. Paige et al. recently showed that fluorescence turn-on of 3,5-difluoro-4-hydroxybenzylidene (DFHBI) by the Spinach RNA aptamer[11] can be activated by ligands binding to aptamers inserted within the Spinach sequence.[12] Here we show that a selective and sensitive fluorescent biosensor for c-di-GMP can be generated by fusing a natural GEMM-I riboswitch aptamer to Spinach. Furthermore, mutation of the ligand binding pocket of the riboswitch enables recognition of both c-AMP-GMP and c-di-GMP. Finally, we demonstrate the utility of RNA-based biosensors for detecting the activity of cyclic dinucleotide producing enzymes in live cells and validate the cellular activity of the first discovered c-AMP-GMP synthase, DncV.

Binding of the conditionally fluorescent molecule DFHBI by the Spinach aptamer is highly dependent on formation of its second stem loop.[12] We replaced this stem loop with the aptamer domain of the Vc2 GEMM-I class riboswitch that binds c-di-GMP with very high affinity (K_d ∼ 0.01 nM) and specificity (Figure 1a,b).[13,14] The natural P1 stem of Vc2 was used directly as the transducer stem, since published structural probing experiments were consistent with P1 stabilization upon ligand binding.[13] We found that this RNA construct, called

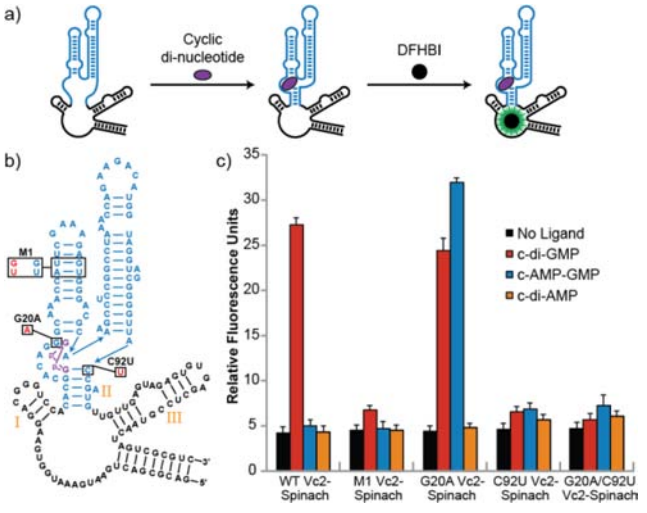

Figure 1. RNA-based fluorescent biosensors detect cyclic dinucleotide second messengers. (a) Design scheme for a fluorescent biosensor that detects cyclic dinucleotides. Ligand binding to the Vc2 riboswitch aptamer (blue) enables the Spinach aptamer (black) to bind and activate the conditionally fluorescent molecule DFHBI. (b) Sequence and secondary structure model of the Vc2-Spinach construct. Mutants analyzed in the study are boxed, c-di-GMP is shown in purple, and Spinach stem loops are numbered in orange. (c) *In vitro* screen of Vc2-Spinach biosensor variants for fluorescence activation in response to cyclic dinucleotides. Error bars represent the standard deviation of three independent experiments with duplicate samples.

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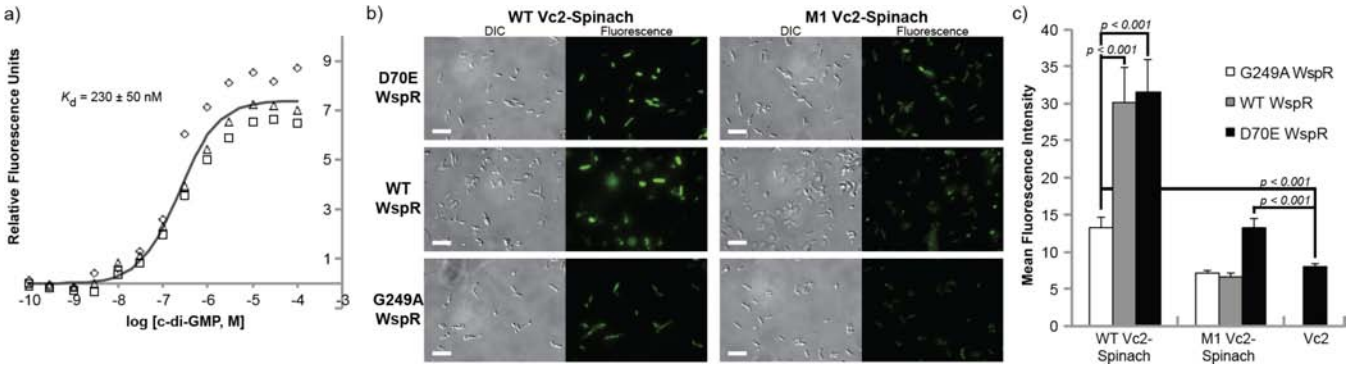

Figure 2. WT Vc2-Spinach is a sensitive biosensor for c-di-GMP in live cells. (a) *In vitro* analysis of WT Vc2-Spinach binding affinity for c-di-GMP. Data from three independent replicates and the best-fit curve are shown. Background fluorescence (without c-di-GMP) was subtracted from all data points. (b) Differential interference contrast (DIC) and fluorescence images of *E. coli* cells expressing Vc2-Spinach tRNAs and WspR enzyme variants after incubation with DFHBI. Cells expressing WT WspR were observed to have more cellular debris compared to other samples. Scale bars represent 10 μm. (c) Quantitation of mean fluorescence intensity of cells. Error bars indicate SEM for at least 50 cells. *P*-values from student's *t*-test comparisons for fluorescence changes discussed in text are shown.

wild-type (WT) Vc2-Spinach, exhibits selective fluorescence activation in response to c-di-GMP (Figures 1c and S1). As expected, the related RNA construct M1 Vc2-Spinach, which harbors a disruptive mutation in the riboswitch aptamer structure,[13] exhibits very little fluorescence activation in response to c-di-GMP (Figure 1b,c).

We next tested whether specific mutations to the ligand binding pocket would confer responsiveness to the other cyclic dinucleotides of interest. The X-ray crystal structure of the Vc2 aptamer bound to c-di-GMP revealed that nucleobases C92 and G20 (numbered using the riboswitch aptamer sequence, Figure 1b) form a Watson−Crick and Hoogsteen base pair, respectively, with each of the guanine nucleotides of the ligand.[14,15] It has been shown that the single mutant C92U binds c-AMP-GMP (K_d = 19 ± 1.7 nM)[16] and the double mutant G20A/C92U binds c-di-AMP (K_d = 1,200 ± 130 nM),[14] albeit in each case nonselectively and with much poorer affinity than the WT aptamer for c-di-GMP. The single mutant G20A also has been shown to bind c-di-GMP (K_d = 0.21 ± 0.07 nM),[17] but its affinity for the other cyclic dinucleotides was not determined. These three variants of Vc2-Spinach were assayed for fluorescence activation by cyclic dinucleotides. It was observed that G20A Vc2-Spinach responds robustly to c-di-GMP and c-AMP-GMP but not to related compounds (Figure S2), while the other variants exhibit little to no fluorescence activation by any of the cyclic dinucleotides (Figure 1c). Therefore, we focused the remainder of our analysis on the WT and G20A Vc2-Spinach RNA constructs.

In order to model physiological conditions, *in vitro* experiments were carried out at 37 °C in buffer containing 3 mM $MgCl_2$, which is in the range of the estimated free Mg^{2+} in the *E. coli* cytosol.[18,19] Under these conditions, it was found that WT Vc2-Spinach has an apparent K_d of 230 ± 50 nM for c-di-GMP (Figure 2a). This binding affinity is much poorer than the previously reported value for the Vc2 aptamer alone and can be attributed to two effects. First, WT Vc2-Spinach exhibits increased sensitivity for c-di-GMP (K_d of 8 ± 1 nM) at 25 °C, 10 mM $MgCl_2$ (Figure S3), the conditions used in the previous study.[14] In parallel, greater fluorescence turn-on is observed (Figure S4), consistent with stabilization of the RNA fold under these conditions. The remaining difference in binding affinity appears to be due to placement of the Vc2 aptamer within the context of Spinach, as we observe the same affinity trends for G20A Vc2-Spinach (Figures 3a and S5). Thus, the apparent insensitivity of C92U and G20A/C92U biosensors is consistent with comparable reductions in binding affinities leading to K_d values much larger than the ligand concentration (100 μM) used in the assay. Another WT Vc2-Spinach construct with an artificial transducer stem in place of the second stem loop of Spinach has been described that displays weak binding affinity to c-di-GMP even at 25 °C.[20] In this case, the difference in affinity relative to our WT construct is likely due to alteration of the Vc2 aptamer P1 stem sequence, which reduces ligand binding.[17]

Since the concentration of c-di-GMP appears to range from <50 nM to a few micromolar in bacteria,[2] WT Vc2-Spinach should be capable of detecting c-di-GMP at biologically relevant concentrations. Furthermore, the dynamic range of the RNA-based biosensor is larger than that of the previously mentioned protein-based biosensor, which binds c-di-GMP cooperatively as a dimer.[10] The *in vitro* fluorescence signal of WT Vc2-Spinach changes from 10% to 90% between 25 and 2000 nM c-di-GMP (Figure 2a). In comparison, the protein-based biosensor exhibits similar affinity as the RNA biosensor, but its FRET signal *in vitro* changes from 90% to 10% between 67 and 560 nM c-di-GMP.[9] The RNA-based biosensor does display relatively slow activation and deactivation rates (Figure S6), so it would need to be improved if it is necessary to monitor rapid dynamics.

We then tested the activity of WT Vc2-Spinach as a fluorescent biosensor to detect c-di-GMP levels in live cells (Figures 2b,c and S7). All RNA constructs were inserted into a tRNA scaffold to improve stability upon expression in *E. coli*.[21] The tRNA scaffold has a negligible effect on ligand binding affinities (Figures S3 and S5). Plasmids encoding WT Vc2-Spinach tRNA and variants of the diguanylate cyclase WspR from *Pseudomonas fluorescens* were cotransformed into cells. We verified that the expression levels of RNA constructs were unaffected by expression of different enzymes (Figure S8). Besides WT WspR,[22] we analyzed the D70E mutant, which mimics phosphorylation at that site and is constitutively active, and the G249A mutant, which knocks out the conserved GGDEF domain and is constitutively inactive.[22] Thus, cells expressing the WT biosensor and G249A WspR represent the fluorescence signal for endogenous levels of c-di-GMP in *E. coli*. As shown, the average fluorescence of these cells is 1.6-fold

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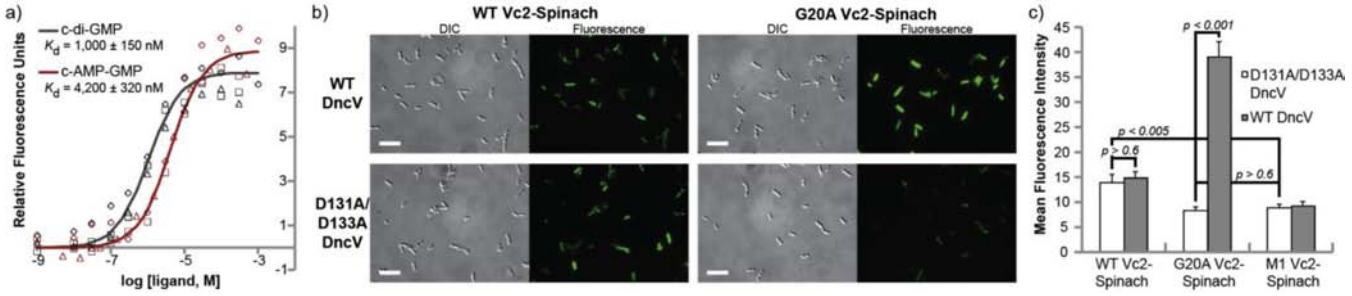

Figure 3. Analysis of the enzymatic activity of DncV in live cells using Vc2-Spinach biosensors. (a) *In vitro* analysis of G20A Vc2-Spinach binding affinity for c-di-GMP (black) and c-AMP-GMP (red). Data from three independent replicates each and the best-fit curves are shown. Background fluorescence (without ligand) was subtracted from all data points. (b) DIC and fluorescence images of *E. coli* cells expressing Vc2-Spinach tRNAs and DncV enzyme variants after incubation with DFHBI. Scale bars represent 10 μm. (c) Quantitation of mean fluorescence intensity of cells. Error bars indicate SEM for at least 50 cells. *P*-values from student's *t*-test comparisons are shown.

above the nonspecific background, which is represented by cells expressing Vc2 tRNA, a construct that does not contain the Spinach aptamer (Figure S9). In comparison, cells expressing the biosensor and WT or D70E WspR exhibit roughly the same increases in average fluorescence (2.3- and 2.4-fold, respectively) above the signal corresponding to endogenous levels. These data are consistent with production of additional c-di-GMP by the WT and constitutively active enzymes.

In contrast, cells expressing the M1 biosensor do not exhibit average fluorescence above background except in the case where D70E WspR is coexpressed. M1 Vc2-Spinach has very weak binding to c-di-GMP, as we observed *in vitro* (Figure 1c), so it appears that levels of c-di-GMP that are endogenous or produced by WT WspR are below the threshold of detection. However, D70E WspR produces c-di-GMP at extremely high concentrations, estimated to be around 3 mM,[23] and so elicits a small fluorescence response from the mutant biosensor. For the WT biosensor, the lack of a significant difference in fluorescence with overexpression of the active WspR variants suggests that each produces enough c-di-GMP to saturate the biosensor and give maximal fluorescence signal. These data demonstrate that WT Vc2-Spinach is a sensitive biosensor that gives a measurable fluorescence response to endogenous c-di-GMP and increased signal upon elevation of c-di-GMP concentrations.

In order to analyze the G20A Vc2-Spinach aptamer for use *in vivo*, its relative affinities for c-di-GMP and c-AMP-GMP were determined. The G20A Vc2-Spinach aptamer has an apparent K_d of 1000 ± 150 nM for c-di-GMP and 4200 ± 320 nM for c-AMP-GMP at 37 °C, 3 mM $MgCl_2$ (Figure 3a). The G20A biosensor appears to be not sensitive enough to detect endogenous c-di-GMP but, as expected, gives a fluorescence signal upon expression of WT and D70E WspR (Figure S10). In this case, the fluorescence signal with WT WspR is not saturated and is clearly less than the D70E WspR signal. While G20A Vc2-Spinach by itself cannot distinguish between c-di-GMP and c-AMP-GMP, we considered that comparing the ratio of fluorescence activation between the G20A and WT biosensors may allow us to differentiate between responses to the two cyclic dinucleotides.

The dinucleotide cyclase DncV from *V. cholerae* was recently shown to synthesize c-di-GMP, c-di-AMP, and c-AMP-GMP *in vitro*, but it preferentially makes c-AMP-GMP in the presence of all four nucleotide triphosphates.[5] Also, mass spectrometry analysis of cell lysates supports that c-AMP-GMP is produced *in vivo* by WT DncV but not by the catalytically inactive D131A/D133A DncV mutant.[6] However, it was not possible to ascertain whether c-di-GMP also was being produced by this enzyme *in vivo*. We found that cells expressing the WT biosensor and D131A/D133A DncV have an average fluorescence 1.6-fold above background, which is the same as observed with inactive WspR and corresponds to detection of endogenous c-di-GMP (Figure 3b,c). Expression of WT DncV does not significantly change the average fluorescence of the WT biosensor, providing direct evidence that this enzyme does not produce c-di-GMP *in vivo* under these conditions.

Cells expressing the G20A biosensor and D131A/D133A DncV exhibit an average fluorescence that is similar to background, represented by M1 Vc2-Spinach (Figures 3b,c, S11, and S12). This result agrees with G20A Vc2-Spinach having a reduced sensitivity for c-di-GMP and *E. coli* cells not making c-AMP-GMP. In contrast, cells expressing the G20A biosensor and WT DncV exhibit greater than 4-fold increase in fluorescence above background. Given the results from the WT biosensor, this observation is consistent with fluorescence activation of the G20A biosensor due to production of c-AMP-GMP by WT DncV. Thus, we have engineered a biosensor that can detect c-AMP-GMP, a newly discovered second messenger, in live cells.

In summary, we have developed two different fluorescent biosensors for live cell imaging of cyclic dinucleotides. We demonstrate the ability to alter specificity of the RNA-based biosensor by exploiting rational mutations to the ligand binding pocket instead of by inserting distinct aptamers. Ongoing work focuses on improving the binding characteristics of these first-generation biosensors and on developing a fluorescent biosensor for c-di-AMP. We aim to use our biosensors to accurately quantitate changes in the intracellular levels of cyclic dinucleotides upon different physiological stimuli, similar to sensors for cGMP.[24] It is envisioned that sensitive and specific biosensors for each cyclic dinucleotide will help elucidate how these different signals are integrated and transduced by bacteria.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods, supplementary experiments, and characterization of c-AMP-GMP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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