Nuclease-resistant c-di-AMP derivatives that differentially recognize RNA and protein receptors

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ASSOCIATED CONTENT

Supporting Information

Supplemental results, including four figures, two tables, and one scheme as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

c-di-AMP, (3’-5’)-cyclic diadenosine monophosphate; c-di-GMP, (3’-5’)-cyclic diguanosine monophosphate; c-AMP-GMP, (3’-5’)-cyclic adenosine monophosphate-guanosine monophosphate; DAC, diadenylate cyclase; PDE, phosphodiesterase; PDB, Protein Data Bank.

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Figure 1. c-di-AMP bound to macromolecular receptors (A) LmPC, (B) KtrA, and (C) $PstA_{SA}$ (ref. 32,33,37). c-di-AMP is colored by atom with carbon in white, oxygen in red, nitrogen in blue, and phosphorous in orange. Protein binding sites are shown as green cartoons. (D) Conformations adopted by c-di-AMP when bound to downstream targets (ref. 32,33,37,45-47). C-di-AMP is colored in green for *ydaO*, grey for $PstA_{SA}$, orange for LmPC, and purple for KtrA. The structures are superimposed at a single adenosine, with the remaining atoms floating.

Figure 2. c-di-AMP recognition by the *ydaO* riboswitch (ref. 45-47). (A) Structure of the *ydaO* aptamer from *B. subtilis* bound to two molecules of c-di-AMP. c-di-AMP is colored in red. Conserved Watson-Crick G-C pairs involved in type-I A-minor interactions with c-di-AMP are shown in purple for Site 1 and green for Site 2. Bases that stack with the adenines are shown in blue. Important nucleotides are labeled. (B and C) c-di-AMP-A2α recognition by the *ydaO* motif. c-di-AMP is colored by atom with carbon in white, oxygen in red, nitrogen in blue, and phosphorous in orange. Hydrogen-bonding nucleotides of *ydaO* are colored as described, with carbons in teal. Hydrogen bonds are shown as black dashed lines. (B) Recognition of the Watson-Crick and Hoogsteen faces of the c-di-AMP nucleobases featuring the type-1 A-minor interaction. (C) Backbone recognition of c-di-AMP by the *ydaO* riboswitch. These c-di-AMP recognition motifs are observed with each adenosine of the ligand.

Figure 3. K_d measurements of c-di-AMP by gel-shift and its various analogs using the competition gel-shift assay with radiolabeled c-di-AMP. (A) Representative gel-shift experiment for measuring the K_d of c-di-AMP for the *ydaO* RNA by direct binding. (B) c-di-AMP binding curve for the

ydaO riboswitch. (C) Representative competition gel-shift experiment. RNA, radiolabeled c-di-AMP, and increasing concentrations of competitor analog are incubated until equilibrium is achieved. Free c-di-AMP is separated from RNA-bound c-di-AMP by native PAGE. (D) Sample binding curve from the competition gel-shift assay with c-di-dAMP. Data are fit to an equation for competitive binding to determine the analog K_d.

Figure 4. Structures of c-di-AMP analogs used in this study. X indicates where modifications were made in each series. (A) Base, (B) Ribose, and Phosphate-modified analogs.

Figure 5. Monitoring the enzymatic hydrolysis of c-di-AMP by HPLC. (A) Domain architecture of GdpP-family proteins (1-659) and the protein construct used for enzymatic assay (84-659). (B) HPLC traces showing elution of pApA and c-di-AMP. Peaks corresponding to each of these compounds are labeled. After 60 min under the experimental conditions used, c-di-AMP is completely degraded. (C) The amount of pApA formed was measured to determine the initial velocity of c-di-AMP degradation. Analog degradation was measured and analyzed in the same manner.

Figure 6. Recognition and enzymatic hydrolysis of c-di-AMP by PDE proteins (ref. 28). (A) c-di-AMP bound to the active site of HD domain protein PgpH from *L. monocytogenes* (PDB entry 4S1B). c-di-AMP is colored by atom with carbon in white, oxygen in red, nitrogen in blue, and phosphorous in orange. Ligand binding residues are colored in purple, metal coordinating residues

are in green, and the catalytic histidine and aspartic acid are in cyan. Hydrogen-bonding interactions are shown as dashed lines (red) and metal-coordinating interactions are displayed as solid lines (black). Iron metals are shown as orange spheres and the nucleophilic water is a red sphere. (B) Proposed mechanism of c-di-AMP cleavage. In the presence of a PDE protein, a nucleophilic water molecule attacks one of the c-di-AMP phosphodiester bonds to produce the linear 5'-pApA.

Figure 7. Degradation of c-di-AMP by GdpP. (A) Steady-state kinetic analysis of the enzyme-catalyzed hydrolysis of c-di-AMP by $GdpP_{84-659}$. (B) Fraction of pApA formed from degradation of radiolabeled c-di-AMP over time in the presence of unlabeled c-di-AMP analogs.