Supporting Information for

Molecular basis for discrimination of cGAMP linkage isomers by a c-di-GMP-II riboswitch variant

Xiaochen Xu[1,†], Wang Cheng[2,†], Huiqin You[1], Zejia Hu[1], Xinyue Bao[1], Hongcheng Li[1], Jinzhu Zhang[1], Dinshaw J. Patel[2,*] and Aiming Ren[1,*]

[1] Department of Cardiology of The Second Affiliated Hospital and Life Sciences Institute and School of Medicine, Zhejiang University, Hangzhou 310058, China

[2] Structural Biology Program, Memorial Sloan-Kettering Center, New York, New York 10065, USA.

Corresponding Author: Aiming Ren and Dinshaw J. Patel

Email: aimingren@zju.edu.cn and pateld@mskcc.org

This PDF file includes:

Supporting text

SI References

Extended Methods

Compounds

The ligands 2′,3′-cGAMP and 3′,3′-cGAMP used in this study were purchased from InvivoGen.

RNA preparation

The BhP1-5delC were generated through assembly Polymerase Chain Reaction (PCR). The BhP1-5delC mutants were generated through mutation PCR based on the wild-type BhP1-5delC. All constructs were cloned into pUT7 vector with an upstream T7 promoter for initiating transcription and a downstream Hammerhead ribozyme for keeping homogenous 3' terminals. DNA templates were generated through PCR following by alcohol precipitation. In vitro transcription was performed for 4.5 h at 37℃ with DNA template, nucleoside triphosphate and T7 polymerase. The transcribed samples were purified by 15% denaturing urea polyacrylamide gel electrophoresis (PAGE). Target RNA were excised from the gel, crushed up, soaked in 0.5×TAE buffer (20 mM Tris-acetic acid, 0.5 mM EDTA, pH 8.0) and precipitated with ethanol. Finally, the RNA samples were dissolved by diethyl pyrocarbonate (DEPC) treated double-distilled water.

Crystallization

0.4 mM BhP1-5delC aptamer or BhP1-5delC mutants were refolded in presence of 2 mM 2′,3′-cGAMP or 3′,3′-cGAMP by annealing at 70℃ for 5 min, 37℃ for 5 min and incubating on ice for 30 min in crystallization buffer (50 mM HEPES pH 7.0, 50 mM KCl, 5 mM $MgCl_2$). Crystallization trials were performed at 16°C using a Gryphon-LCP crystallization robot employing the sitting-drop vapor-diffusion method. Each trial mixed 0.2 μL RNA-ligand complex with 0.2 μL of reservoir solution. The crystals of BhP1-5delC or BhP1-5delC mutants in complex with 2′,3′-cGAMP or 3′,3′-cGAMP appeared within one week under specific crystallization buffer. Specifically, the high-resolution crystals of BhP1-5delC or BhP1-5delC mutants in complex with 2′,3′-cGAMP

grew in the solution containing 0.2 M ammonium acetate, 0.1 M Tris 8.5, 45% MPD, while the crystals of BhP1-5delC or BhP1-5delC mutants in complex with 3′,3′-cGAMP grew in the solution containing 0.2 M magnesium formate, 20% PEG3350. All crystals were cryoprotected by brief soaking in reservoir solution supplemented with 20% glycerol and flash-cooled in liquid nitrogen for X-ray diffraction data collection at the Shanghai Synchrotron Radiation Facility (SSRF).

Structure determination and refinement

All structures of BhP1-5delC or BhP1-5delC mutants in complex with 2′,3′-cGAMP or 3′,3′-cGAMP were solved by molecular replacement method using the structure of c-di-GMP-II riboswitch (PDB code: 3Q3Z) as the initial model in the CCP4 suite (1). The Matthew's coefficient suggested that there were two RNA molecules present in one asymmetric unit of 2′,3′-cGAMP bound BhP1-5delC or its mutants, while one RNA molecule was present in one asymmetric unit of 3′,3′-cGAMP bound BhP1-5delC or its mutants. The RNA structures were built in COOT (2) and refined in Phenix (3). 2′,3′-cGAMP or 3′,3′-cGAMP was introduced in the structures at the last several refinements. The X-ray data collection and refinement statistics of the crystals were listed in *Dataset S01*.

Isothermal titration calorimetry (ITC)

RNA-ligand binding affinities were measured by a Microcal PEAQ-ITC at National Center for Protein Science-Shanghai (NCPSS). Prior to experiments, RNA samples were dialyzed overnight against ITC buffer (50 mM HEPES pH 7.0, 50 mM KCl, 10 mM $MgCl_2$), followed by refolding via annealing (70℃ for 5 min, 37℃ for 5 min and incubating on ice for 30 min). The ligands were diluted in ITC buffer. ITC experiments were performed at 25°C, with 0.02 mM RNA was loaded into the sample cell and 0.18 mM ligand was loaded into the syringe. Each experiment consisted of 19 consecutive injections (2 μL per injection) of ligand into the RNA solution, with a 120 s interval between injections. The collected data were fitted to a one set of sites binding model with MicroCal PEAQ-ITC software. All the binding constants and thermodynamic

values are listed in *Dataset S02*.

SI References

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