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Truncated form of the title: Mechanism of c-di-GMP signaling

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Summary

Cyclic dimeric guanosine monophosphate (c-di-GMP) is a common, bacterial second messenger that regulates diverse cellular processes in bacteria. Opposing activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) control c-di-GMP homeostasis in the cell. Many microbes have a large number of genes encoding DGCs and PDEs that are predicted to be part of c-di-GMP signaling networks. Other building blocks of these networks are c-di-GMP receptors which sense the cellular levels of the dinucleotide. C-di-GMP receptors form a more diverse family, including various transcription factors, PilZ domains, degenerate DGCs or PDEs, and riboswitches. Recent studies revealing the molecular basis of c-di-GMP signaling mechanisms enhanced our understanding of how this molecule controls downstream biological processes and how c-di-GMP signaling specificity is achieved.

Introduction

A key regulator of bacterial physiology is the nucleotide-based, second messenger c-di-GMP which controls the transition from a free-living, motile lifestyle to a biofilm one. C-di-GMP also controls other cellular processes including virulence, cell-cell signaling, and cell cycle regulation (Figure 1). Diguanylate cyclase (DGC) proteins containing the GGDEF domain produce c-di-GMP [1,2], while phosphodiesterase (PDE) proteins bearing the EAL [3,4] or HD-GYP [5] domains degrade it. Many of the early studies on c-di-GMP focused on the analysis of enzymatic activities of individual DGC and PDE proteins and biological responses involving c-di-GMP. While recent genome-wide studies have revealed redundancies and specificities of DGCs and PDEs on a broader scale, much less is known regarding c-di-GMP circuitries and signaling mechanisms. Here, we focus in particular on recent efforts to elucidate c-di-GMP signaling systems and the effectors that directly couple c-di-GMP production and cellular responses.

Regulation of active DGCs and PDEs

Many GGDEF, EAL and HD-GYP domains occur in diverse multi-domain signaling proteins and as part of multi-component signaling systems, suggesting a role for c-di-GMP

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in sensing environmental cues [6]. While input signals for the majority of these proteins remain to be discovered, recent studies have begun to shed light on the mechanisms of enzyme activation by environmental signals. The environmental regulation of c-di-GMP homeostasis is achieved by the incorporation of one of several domains into GGDEF and EAL domain-containing proteins. These domains include those that react to light (BlrP1 from *Klebsiella pneumonia* [7], BphG1 from *Rhodobacter sphaeroides* [8]) or gases (DosC and DosP from *E. coli* [9], BpeGReg from *Bordetella pertussis* [10], AxDGC2 and AxPDEA1 from *Acetobacter xylinum* [11,12]). Common domains for these types of regulation are Per-Arnt-Sim (PAS), Light-Oxygen-Voltage (LOV), Blue Light Using FAD (BLUF) photoreceptor, and globin domains, which use cofactors, such as flavin adenine dinucleotide (FAD) or heme. Protein-protein interactions involving DGCs and PDEs with proteins containing sensory domains have also been shown to control enzymatic activities. In *Legionella pneumophila*, the activity of a DGC is inhibited by the NO receptor H-NOX1 (a Heme Nitric oxide/Oxygen (H-NOX) domain-containing protein) in the NO-ligated state [13]. In *Rhodopseudomonas palustris*, light-dependent activation of PapB, a photoreceptor with a BLUF domain [14], regulates the PDE activity of PapA.

Alternatively, response regulator enzymes with receiver domains occur as modules in multi-component signaling systems. Examples are the *Borrelia burgdorferi* Rrp1 [1], *V. cholerae* PDE VieA [15], the *P. aeruginosa* DGC WspR [16] and the *P. aeruginosa* PDE SadR (also known as RocR) [17,18]. Sensing of the environment in these systems is achieved by transmembrane sensor proteins that react to surface growth conditions or other external stimuli. Molecular mechanisms for the activation of DGCs and PDEs by response regulator domains are well established. Pioneering work on *Caulobacter crescentus* PleD, a response regulator DGC involved in polar development, established key concepts in c-di-GMP signaling, including the dimerization of GGDEF domains by phosphorylated receiver domains and the presence of a widely used, inhibitory c-di-GMP-binding site (I-site) [19,20]. In essence, these regulatory mechanisms are conserved in *P. aeruginosa* WspR [21].

Signaling networks incorporating catalytically inactive GGDEF and EAL domain-containing effectors

Several bioinformatic, structural and functional studies determined sequence requirements for active DGCs and PDEs, but also highlighted the existence of catalytically inactive GGDEF and EAL domain-containing proteins in large numbers. Degenerate GGDEF and EAL domains are a major class of c-di-GMP effectors. Signal output often involves features that were maintained from active enzymes, such as the presence of I-site motifs and domain oligomerization. These degenerate GGDEF and/or EAL domain-containing proteins form central modules in signaling networks. Two of the more extensively studied systems are involved in the control of the cell cycle in *C. crescentus* and the commitment to stable cell adhesion in *P. fluorescens* biofilms. These are discussed below in more detail.

One example of an enzymatically inactive c-di-GMP-binding protein is PopA, which contains two receiver domains and a GGDEF domain with a conserved I-site and degenerate active site [22]. In *C. crescentus*, PopA regulates cell cycle progression. *C. crescentus* undergoes asymmetric cell division, forming a motile swarmer cell and sessile stalked cell [22]. A coordinated integration of c-di-GMP and two-component signaling ensures proper progression through the division cycle [23]. During the G1-to-S phase transition, PopA is sequestered to the old pole, where it is involved in recruiting proteins required for the degradation of the master cell-cycle regulator CtrA, which controls cell cycle-related genes and directly binds the chromosomal origin of replication, occluding replication initiation factors. Polar localization of PopA is mediated by binding of c-di-GMP to PopA's I-site [22]. C-di-GMP levels are regulated at the flagellated pole by the opposing activities of a

PDE, PdeA, and the DGC, DgcB, until the G1-S transition. Transition into S phase is marked by PdeA degradation and by the concomitant phosphorylation and polar localization of another response regulator DGC, PleD. DgcB and PleD activity subsequently promote entry of the cell into S-phase and morphogenesis of the stalked pole [23].

Similarly to PopA, *P. aeruginosa* PelD, a protein that regulates production of PEL exopolysaccharide and biofilm formation, requires c-di-GMP binding for its function [24]. Secondary structure predictions indicate some structural similarity of PelD with GGDEF domains. More importantly, binding of c-di-GMP depends on a conserved RxxD motif resembling an I-site found in active DGCs [24]. Another example of this family of c-di-GMP receptors appears to be CdgG of *V. cholerae*. Although it remains to be determined whether CdgG can bind to c-di-GMP, genetic and enzymatic analysis show that this protein is not a DGC and its RxxD motif is critical for its function [25], *i.e.*, the negative regulation of biofilm formation through a yet unknown mechanism. Together, these studies highlight the mechanistic conservation of inactive GGDEF domains as signal integrators.

Similar to proteins with degenerate GGDEF domains, many enzymatically inactive PDEs retain their ability to bind to c-di-GMP and regulate cellular processes. Interestingly, several proteins contain both inactive GGDEF and EAL domains, such as *P. aeruginosa* FimX [26,27] or *P. fluorescens* LapD [28]. LapD was identified as a new class of c-di-GMP-binding transmembrane proteins that control the cell-surface localization of the large cell surface adhesin LapA via an inside-out signal transduction mechanism, and thus regulating biofilm formation [28,29]. Binding of c-di-GMP to LapD's cytoplasmic EAL domain alters the conformation of the receptor, and the signal is propagated from the GGDEF-EAL module to the output domain through a HAMP (Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases) domain [30]. As a consequence, LapD sequesters the periplasmic protease LapG, which prevents LapA processing and ensures stable anchorage of cells via the intact adhesin [29,30]. Cellular c-di-GMP levels are inversely regulated by the PDE RapA [31], whose expression is regulated on a transcriptional level in response to phosphate availability, and a subset of three DGCs [32]. Whether signaling specificity is achieved by direct protein-protein interactions that are similar to the network architecture described for *Caulobacter* cell cycle regulation remains to be shown.

FimX of *P. aeruginosa*, a GGDEF-EAL domain-containing protein with little to no activity, also senses c-di-GMP via a degenerate EAL domain. Mutation of the degenerate GGDEF or EAL domains of a fluorescently tagged FimX construct alters the single polar localization of FimX to a bi-polar distribution, and regulates the assembly of type IV pili and twitching motility [26]. Qi and colleagues has shown that c-di-GMP binding to the EAL domain induces long-range conformational changes, establishing a potential mechanism for its function as a c-di-GMP receptor [33].

c-di-GMP signaling at the transcriptional level

C-di-GMP also regulates gene expression, using a diverse group of specific receptors. One example is FleQ, which activates expression of flagella biosynthesis genes and represses transcription of genes involved in exopolysaccharide biosynthesis in *P. aeruginosa* [34]. FleQ is an AAA domain-containing protein that has been classified as a sigma⁵⁴-dependent transcriptional activator. C-di-GMP binding to FleQ results in the disassembly of FleQ•DNA complexes [34]. The mechanism by which c-di-GMP binding alters FleQ function remains to be determined.

The Clp transcriptional regulators from the plant pathogens *Xanthomonas campestris* [35] and *Xanthomonas axonopodis* [36] have been shown to bind c-di-GMP. These CRP (cAMP Receptor Protein)/FNR (regulator of fumarate and nitrate reduction) superfamily members

comprise an N-terminal β -barrel-containing domain and C-terminal DNA binding domain [37]. In contrast to previously characterized CRP-like proteins, Clp binds to its target DNA sites in the absence of any ligand. However, Clp undergoes structural rearrangements and loses its DNA binding ability upon incubation with c-di-GMP [37]. Identification of a CRP family protein that functions as a c-di-GMP receptor protein whose DNA binding ability is allosterically inhibited is an elegant example in evolution of nucleotide-based signaling systems. In contrast, *Burkholderia cenocepacia* Bcam139, another c-di-GMP-binding CRP/FNR family protein, shows enhanced DNA binding in the presence of the dinucleotide [38].

The transcriptional regulator VpsT from *V. cholerae* is an example of a novel c-di-GMP receptor [39]. VpsT inversely regulates expression of motility and matrix production genes in a c-di-GMP-dependent manner. VpsT consists of a non-canonical, N-terminal receiver and a C-terminal helix-turn-helix domain. A crystal structure of VpsT revealed that, unlike previously described receiver domains, VpsT has an additional helix ($\alpha 6$) at its C-terminus. Dimerization utilizing this helix is facilitated by binding of two intercalated c-di-GMP molecules to the base of the VpsT dimer. VpsT binds c-di-GMP using a 4-residue-long, conserved sequence, W[F/L/M][T/S]R [39]. Mutations in the conserved c-di-GMP binding motif or the dimerization interface abolish c-di-GMP binding and the ability of VpsT to bind to its target sequences.

A recent study has shown that *Klebsiella pneumoniae* MrkH, a PilZ domain-containing transcriptional activator, also binds to its target promoters only in the presence of c-di-GMP [40], although the exact molecular mechanism remains to be determined.

c-di-GMP signaling at a post-translational level via PilZ domains

PilZ domain-containing proteins were first identified by computational studies as potential c-di-GMP receptors and have wide phylogenetic distribution [41]. PilZ domains occur either as single domain proteins or in conjunction with other domains predicted to have enzymatic, regulatory or transport functions [41]. Most genomes encode more than one of these potential receptors, with YcgR from *E. coli* and BcsA from *Gluconacetobacter xylinus* being the first experimentally verified c-di-GMP receptors of this family [42].

Structures of several PilZ domain proteins alone and in complex with c-di-GMP have been determined recently. These include YcgRN-PilZ family proteins, represented by VCA0042/PlzD [43] and PP4397 [44], which contains an N-terminal YcgR-like domain, and PP4608 [45], a single-domain PilZ protein. While the fold of the different proteins is very similar, some of the molecular details, such as c-di-GMP binding stoichiometry, affinity, and dinucleotide-induced changes in quaternary structure, appear to be different for the individual proteins. A common feature of PilZ domains is a seven-residue loop, designated the —c-di-GMP switch, which invariably undergoes a conformational change upon c-di-GMP binding. In addition, the conserved RxxxR and D/NxSxxG motifs of the PilZ domains are critical for c-di-GMP binding [43–45]. These properties have been exploited in the design of the first genetically encoded c-di-GMP sensor [46], which allows detection of changes in the cellular c-di-GMP concentration in a diverse set of bacteria, highlighting its potential to further studies of c-di-GMP on a cellular and potentially sub-cellular level.

In general, PilZ proteins studied to date are activated by c-di-GMP and most of them function at the post-translational level via protein-protein interactions. One of the best studied examples is YcgR from *E. coli*, which binds c-di-GMP and interacts with components of the flagellar motor to affect directional switching and possibly speed [47–49].

PilZ domain proteins impact diverse cellular processes. In *V. cholerae*, mutations in three of the five PilZ domain proteins impact motility, biofilm formation and intestinal colonization [50]. At least one of the seven *P. aeruginosa* PilZ proteins (PilZ) is required for twitching motility, while another (Alg44) is required for alginate production [51]. It remains to be seen whether similar mechanisms control other PilZ-type c-di-GMP receptors that affect production of exopolysaccharide, virulence.

Regulation of gene expression by c-di-GMP-binding riboswitches

The discovery of new classes of riboswitches—mRNA segments controlling gene expression, which specifically bind c-di-GMP—has revealed a completely new level of regulation that involves this dinucleotide [52,53]. Thus far, two main classes, the c-di-GMP I and II riboswitches, respectively, have been identified in computational searches and validated experimentally. Crystallographic analyses of the c-di-GMP-bound aptamers has elucidated the overall structures and mode of ligand binding [54–56]. While both riboswitches bind c-di-GMP in an asymmetric fashion involving somewhat similar molecular interactions, the sequences and structural motifs of the RNA aptamers that accommodate c-di-GMP are quite different, suggesting that they evolved independently. These regulatory motifs are widely used and can occur in large numbers indicating the major potential of aptamer-based c-di-GMP signaling.

Conclusion

Since the discovery of c-di-GMP 26 years ago as an allosteric regulator of cellulose synthase [57], c-di-GMP has become one of the most studied signaling molecules, controlling diverse cellular processes in bacteria. There remain many unsolved questions regarding the mechanism of c-di-GMP signaling. Various systems studied in detail thus far suggest that both general and more localized signals can be generated and integrated to control different cellular processes. Future studies will need to address the overall mechanism of localization of signaling reactions, correlation of the global c-di-GMP pool and affinities of c-di-GMP receptors within signaling networks ultimately revealing c-di-GMP signaling specificity. Genetic and biochemical studies suggest that other types of c-di-GMP receptors exist. Although the identities of some receptors are known [58], the identities of many other receptors have yet to be determined. Regulation of c-di-GMP homeostasis at transcriptional, translational and post-translational levels, the building principles of c-di-GMP-dependent signaling cascades, and the integration of these pathways in other environmental signaling networks is being elucidated [59,60], but we have a limited understanding of regulatory interplays. On the protein level, we are only beginning to appreciate the extent to which GGDEF, EAL and HD-GYP domain-containing proteins are functionally diversified. As discussed above, some have evolved to function as c-di-GMP effector proteins, while others have regulatory functions independent of c-di-GMP signaling. It also remains to be determined whether some of the mechanistic principles play a role in signaling and overall function of c-di-AMP, a recently discovered dinucleotide similar to c-di-GMP [61]. Such studies will lead to a better understanding of c-di-GMP signaling mechanisms and c-di-GMP signal transduction pathways that are critical for biology of a broad range of bacteria.

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•• Of outstanding interest

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Figure 1.

c-di-GMP is a common, bacterial second messenger that controls the transition from a free-living, motile lifestyle to a biofilm one. c-di-GMP is produced by DGC proteins containing the GGDEF domain and degraded by PDE proteins bearing the EAL or HD-GYP domains. c-diGMP is sensed by receptor proteins or RNAs from either the PilZ, degenerate GGDEF or EAL domain, transcriptional factors or riboswitch families. Typically, receptor proteins then interact with a downstream target to affect a particular cellular function.

Table 1

C-di-GMP-specific effectors.

Protein	Organism	Mechanism	Biological output	Reference
Transcription factors				
FleQ	<i>P. aeruginosa</i>	Release from DNA	Biofilm formation Motility	[34]
VpsT	<i>V. cholerae</i>	Dimerization-induced DNA binding	Biofilm formation Motility	[39]
Clp	<i>Xanthomonas campestris</i> <i>Xanthomonas axopodis</i>	Release from DNA	virulence	[35,36]
Bcam1349	<i>Burkholderia cenocepacia</i>	Ligand-enhanced DNA binding	Biofilm formation Virulence	[38]
PilZ domain-containing proteins				
YcgR	<i>E. coli</i>	Protein-protein interaction	Direction of flagellar switching	[47–49]
DcgR	<i>C. crescentus</i>	Protein-protein interaction	Motility	[62]
Alg44	<i>P. aeruginosa</i>	Protein-protein interaction (?)	Alginate production	[51]
Plz	<i>Vibrio cholerae</i>	unknown	Motility Biofilm formation Virulence	[50]
MrkH	<i>Klebsiella pneumoniae</i>	DNA binding	Type 3 Fimbriae expression and biofilm formation	[40]
GGDEF and/or EAL domain-containing proteins				
FimX	<i>P. aeruginosa</i>	Conformational change	Twitching motility	[33]
LapD	<i>P. fluorescens</i>	Disruption of an autoinhibited conformation	Biofilm formation	[30]
PeiD	<i>P. aeruginosa</i>	unknown	Exopolysaccharide production	[24]
PopA	<i>C. crescentus</i>	Spatial redistribution in the cell	Cell cycle regulation	[23]
Others				
PNPase	<i>E. coli</i>	Enzyme activation	RNA processing	[58]
Riboswitches				
Class I	<i>V. cholerae</i> Vc2(tfoX)	RNA compaction, Structural rearrangement	Gene expression	[53]
Class II	<i>Clostridium difficile</i>	Activation of ribozyme self-splicing	Translational control	[52]