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Dictyostelium uses the prokaryote messenger c-di-GMP to trigger stalk cell differentiation

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Abstract

Cyclic di-(3′:5′)-guanosine monophosphate (c-di-GMP) is a major prokaryote signalling intermediate, which is synthesized by diguanylate cyclases and triggers sessility and biofilm formation[1,2]. We detected the first eukaryote diguanylate cyclases (*DgcAs*) in all major groups of Dictyostelia. Upon food depletion, *Dictyostelium discoideum* amoebas collect into aggregates, which first transform into migrating slugs and next into sessile fruiting structures. These structures consist of a spherical spore mass that is supported by a column of stalk cells and a basal disk. A polyketide, DIF-1, was isolated earlier, which induces stalk-like cells *in vitro*[3]. However, its role *in vivo* proved recently to be restricted to basal disk formation[4]. Here we show that *Dictyostelium* DgcA produces c-di-GMP as the morphogen responsible for stalk cell differentiation. *D.discoideum* DgcA synthesized c-di-GMP in a GTP-dependent manner and was expressed at the slug tip, the site of stalk cell differentiation. Disruption of the *DgcA* gene blocked the transition from slug migration to fructification and the expression of stalk genes. Fructification and stalk formation were restored by exposing *dgca*- slugs to wild-type secretion products or to c-di-GMP. Moreover, c-di-GMP, but not c-di-AMP, induced stalk gene expression in dilute cell monolayers. Apart from identifying the long elusive stalk-inducing morphogen, our work also identifies the first role for c-di-GMP in eukaryotes.

Prokaryote diguanylate cyclases (DGCs) contain a signature GGDEF domain[1], which was recognized during annotation of the *D.discoideum* (*Ddis*) genome[5]. *Ddis* resides in group 4 of the four major groups of Dictyostelia[6]. Query of genomes of species that represent the other three groups yielded single genes in *D.lacteum* (*Dlac*, group 3), *Polysphondylium pallidum* (*Ppal*, clade 2B), *Acytostelium subglobosum* (*Asub*, clade 2A) and 13 homologous genes in *D.fasciculatum* (*Dfas*, group 1). In addition to the GGDEF domain, most proteins also contain a putative transmembrane (TM) helix (Figure 1A). Alignment of the Dictyostelid GGDEF domains with the structurally resolved GGDEF domain of the *Caulobacter crescentus* (*Ccre*) DGC PleD[7] shows conservation of all residues that are essential for catalysis and binding to the substrate Mg^{2+}-GTP, except for a G(glycine) to S(serine) substitution in the GGDEF domain (Supplementary figure S1A). The closest homolog of the Dictyostelid sequences is a DGC from the prokaryote *Gallionella capsiferriformans (Gcap)*. However, phylogenetic inference showed that the *Gcap* DGC was still more similar to *Ccre* PleD and that the Dictyostelid proteins formed a separate clade (Fig. 1A). The 13 *Dfas* GGDEF domain proteins are monophyletic and are most likely derived by gene duplications from a single ancestor (Figure S1C).

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To identify a role for a putative DGC in Dictyostelid physiology or development, we disrupted *Ddis DgcA* by homologous recombination (Supplementary Figure S2). The *dgca-* mutant showed normal growth, aggregation and formation of migrating slugs, but could not form fruiting bodies (Fig. 1B, supplementary movie 1). Its slugs continued migration until they were exhausted. Fructification was fully restored by transforming *Ddis dgca-* cells with either the *Ddis* or *Ppal DgcA* coding sequence (Fig. 1C). This indicates that the fructification-deficient phenotype is due to loss of the *DgcA* gene and that *Ddis* and *Ppal DgcA* are functionally equivalent. We also tested the ability of a number of truncated *dgcA* constructs to restore the *dgca-* phenotype. Constructs lacking the putative transmembrane helix and low complexity regions flanking the helix still restored fructification in *dgca-* cells (Supplementary Figure S3A). However, constructs lacking either the GGDEF domain and/or 60 amino acids N-terminal of this domain did not, indicating that the GGDEF domain is essential for DgcA function. The full-length gene fused to the YFP reporter was expressed throughout the cytosol (Fig. S3B), confirming that the putative transmembrane helix did not function as such.

We next studied the expression of *DgcA* during *Ddis* development. Figure 2A shows that *DgcA* mRNA was absent from growing cells, but increased upon starvation to reach a maximum at 10 h when cells are forming slugs. The *dgca-* cells did not express *DgcA* mRNA, confirming disruption of the gene. To investigate which cells express DgcA, we transformed wild-type cells with a gene fusion of the ~ 1 kb *DgcA* 5′ intergenic region and the *LacZ* reporter, and stained developing structures for β-galactosidase activity. Figure 2B shows that *DgcA* is first expressed in cell scattered throughout the aggregate. In slugs, *DgcA* expression is strongest at the anterior tip region (Fig. 2C), whereas in fruiting bodies the tip and stalk show high expression (Fig. 2D). This expression pattern suggests that DgcA exerts its function in tip and stalk cells.

To assess whether the requirement of DgcA for fructification is cell-autonomous, *dgca-* cells were developed intermixed with increasing numbers of wild-type cells. 10% wild-type cells were sufficient to fully restore fructification of *dgca-* cells (Fig. 3A), suggesting that the *dgca-* phenotype could be due to lack of production of a secreted stimulatory signal. This was tested directly by incubating *dgca-* cells on agar that was preconditioned by secretion products from developing *dgca-*, wild-type and DgcA overexpressing cells. Supplementary figure S4A shows that agar preconditioned by wild-type cells or *DgcA* overexpressors, but not by *dgca-* cells, restored fructification of the *dgca-* mutant. The secreted stimulatory signal is likely to be c-di-GMP, the natural product of DGC. c-di-GMP and structurally related compounds were deposited as droplets on top of *dgca-* slugs at 20 h of development. c-di-GMP fully restored fruiting body formation (Fig. 3A) but solvent, c-di-AMP, cGMP, 5′GMP, GTP and GDP had no effect (Figure S4B).

Fructification initiates when the slug projects its tip and lays down a cellulose tube at its centre. The anterior prestalk cells then move into the tube and differentiate into stalk cells[8]. The *dgca-* slugs do project up occasionally, but do not follow this up with stalk formation, suggesting that they either fail to form the stalk tube, or to differentiate into stalk cells. Coordination of cellulose synthesis was the first established role of bacterial DGCs[9], and although the *Ddis* cellulose synthase, DcsA is more similar to bacterial than eukaryote enzymes[10], it does not have the PilZ domain that mediates regulation by c-di-GMP, negating a role for c-di-GMP in cellulose deposition. We therefore focussed on expression of marker genes for stalk and spore differentiation in the *dgca-* mutant. The prespore gene pspA[11] was somewhat overexpressed in the *dgca-* mutant, while the prestalk gene *ecmA*[12] was initially normally expressed, but failed to be downregulated after 20 h as occurred in wild-type cells. The spatial expression pattern of both genes in the slug stage was the same in *dgca-* and wild type cells (Supplementary Figure S5). In contrast, the spore gene *SpiA*[13] was not expressed

at all (Fig. 3B), while the stalk/basal disk gene *ecmB*[12] was expressed very poorly. These data suggests that c-di-GMP could have a role in induction of stalk cell differentiation. Spores only mature when stalk formation is almost completed. The lack of *SpiA* expression could therefore be a derived effect.

To test acute effects of c-di-GMP on stalk gene expression, we used cells transformed with a gene fusion of the β-galactosidase reporter gene with the proximal stalk-specific region of the *ecmB* promoter (ST-gal), which was defined previously[14,15]. Aggregates were dissociated into single cells, which were incubated with increasing concentrations of c-di-GMP. Figure 3C shows that c-di-GMP activates ST-gal expression within 8 hours, with halfmaximal induction occurring at ~200 nM. C-di-AMP had little effect up to 10 μM, while cAMP partially inhibited ST-gal induction by c-di-GMP. c-di-GMP also activated ST-gal expression in *dmtA*- cells that cannot synthesize DIF-1[16], indicating that c-di-GMP does not exert its effects by activating DIF-1 synthesis.

We also tested whether c-di-GMP could induce fully differentiated stalk cells in monolayers of *D.discoideum* V12M2 cells[17]. Figure 3D shows that after 30 h of c-di-GMP treatment, V12M2 cells had formed the central large vacuole (arrow) that characterizes stalk cells. In the V12M2 test system, DIF-1 also induces stalk cell differentiation, but additionally requires the presence of 1-5 mM cAMP. However, this is not the case for c-di-GMP (Fig. 3E), where cAMP, if anything, has an inhibitory effect, as was also evident in the ST-gal induction assay (Fig. 3C). The fact that DIF-1 requires cAMP as a co-factor probably reflects that it actually induces basal disk cells[4], which are morphologically similar to stalk cells. In normal development, basal disk cells are derived from the prespore cell population, which themselves require cAMP for differentiation[18]. Because the spore gene SpiA was also not expressed in the *dgca*- mutant, we tested whether c-di-GMP was required for or could induce spore formation in monolayers. However, neither proved to be the case (supplementary figure S6), highlighting the specific role for c-di-GMP in stalk formation.

Induction of ST-gal gene expression provided us with a bioassay to ascertain that *Ddis* DgcA has diguanylate cyclase activity. A fusion protein of YFP and the essential C-terminal region of DgcA (ΔTL-DgcA-YFP, supplementary figure S3) was purified from *Ddis* cell lysates by immunoprecipitation with YFP antibody. The immunoprecipitate was incubated with the DGC substrate GTP/Mg^{2+} for 60 minutes and then tested for ST-gal induction activity. Figure 4 shows that the ΔTL-DgcA-YFP immunoprecipitate synthesized a stalk gene inducing factor in a time, GTP and Mg^{2+} dependent manner. The factor was confirmed to be c-di-GMP by mass spectrometry (supplementary figure S7).

We showed that species representing all major groups of Dictyostelia contain one or more conserved diguanylate cyclases that were previously only found in eubacteria. *Ddis* DgcA is essential for stalk formation and this requirement can be replaced by secretion products of wild-type cells, by c-di-GMP and by a DgcA from a distantly related Dictyostelid. *DgcA* is expressed in the organising tip of structures, where stalk cell differentiation is initiated. Combined, these data indicate that c-di-GMP is an apically secreted conserved signal for induction of stalk cell differentiation in Dictyostelia. The polyketide DIF-1, which was identified after painstaking isolation from *Ddis* cells, has been for 30 years the primary candidate for a stalk-inducing factor[3]. However, identification and abrogation of its synthetic enzymes by the same team revealed that it was required for formation of the basal disk, but not for the stalk[4,16].

c-di-GMP and its synthetic enzyme DGC appeared more recently at the forefront of bacterial signal transduction. c-di-GMP has a principal role in the loss of motility and the synthesis of adhesins and exopolysaccharide matrix components by bacteria, which mark their transition

from a swarming phase to a sessile biofilm associated life style. In *C.crescentus* this also involves formation of a stalk[1,2]. In Dictyostelia, c-di-GMP also triggers the transition from motile slugs into sessile fruiting bodies and the intriguing question arises whether this represents either convergent evolution or deep ancestral connections between Dictyostelid stalk formation and bacterial sessility. In the latter case DGCs should also be present in other Amoebozoa, such as the polyphyletic Protosteloid amoebas, which differentiate into a single spore sitting on its own stalk[19]. Unfortunately, no protostelid genome sequences are yet available to address this issue. Of immediate interest are the questions how Dictyostelid DGCs are themselves regulated and how the c-di-GMP signal is processed to activate stalk gene expression. These questions will be the topic of our future research.

METHODS SUMMARY

Expression constructs

The full length *Ddis* and *Ppal* DgcA coding regions were amplified by PCR from *Ddis* and *Ppal* genomic DNA, using primer pairs DgcF/DgcR and PpDgcF/PpDgcR (Supplementary Table S1), respectively. PCR products were digested with BamHI and XhoI and inserted into vector pB17S-EYFP[20], which was transformed into *Ddis dgca-* cells.

DgcA promoter-LacZ construct

The 968 bp DgcA 5′ intergenic region was amplified from genomic DNA with primers DgcApr5′ and DgcApr3′ (Table S1) and inserted into pDdGal-17[21]. After transformation in *Ddis*, β-galactosidase activity was visualized with X-gal in developing structures[22].

RNA analysis

RNA was extracted using RNeasy kits (Qiagen) with on-column DNA digestion, and transcribed into cDNA using ImPromII reverse transcriptase (Promega). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Gene expression levels were normalized to expression of the constitutively expressed *IG7* gene.

Gene induction assay

dgca- cells, transformed with ST-gal[14,15], were incubated with variables for up to 10 h. Cells were lysed and spectrophotometrically assayed for β-galactosidase activity[23].

Immunoprecipitation

ΔTL-DgcA-YFP transformed cells (Supplemenary Figure S3) were filter-lysed in DGC assay buffer[24]. 500 μl of cleared supernatant was incubated with 20 μl GFP-Trap_A beads (Chromotek) for 60 min. After 3 washes with 150-500 mM NaCl, beads were resuspended in 20 μl assay buffer.

DGC assay

5 μl of immunoprecipitate was incubated at 22°C with 95 μl of 0.1 mM GTP and 10 $MgCl_2$[24] in DGC assay buffer. Reactions were terminated by boiling. 10 μl of the reaction mixture was incubated for 8 h with 90 μl of *dgca-*/ST-gal cells and assayed for β-galactosidase activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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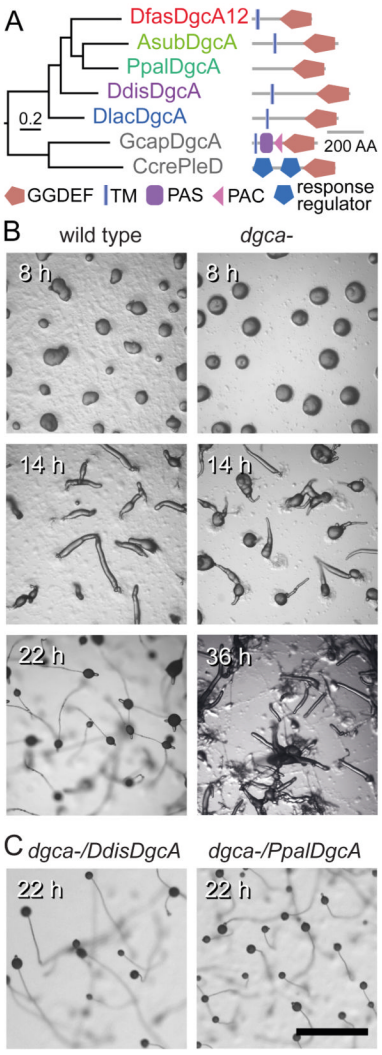

Figure 1. Identification and disruption of diguanylate cyclases

A. The *Ddis* genome[5] contained a single gene with GGDEF domain. BLAST search identified orthologs in *Dlac*, *Ppal*[25] and *Asub*, and 13 monophyletic genes in *Dfas*[25]. A phylogenetic tree was constructed from aligned Dictyostelid and prokaryote GGDEF sequences and annotated with the domain architecture of the proteins.

B. *Ddis DgcA* was ablated by homologous recombination. *Dgca-* and wild-type cells were incubated on non-nutrient agar to follow developmental progression.

C. *Ddis dgca-* cells were transformed with fusion constructs of the A15 promoter with either *DdisDgcA* or *PpalDgcA* and developed for 22 h. Bar: 1 mm.

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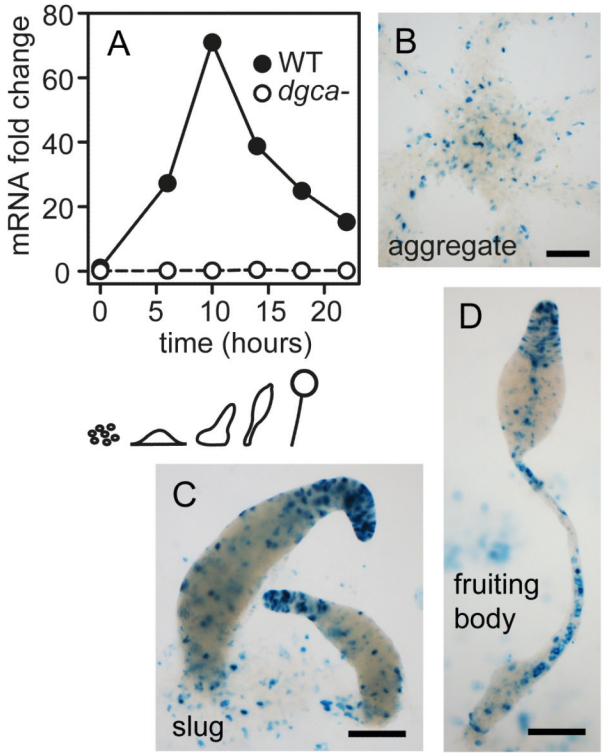

Figure 2. *DgcA* expression pattern

A. Total RNA was isolated during development of *Ddis* wild-type (WT) and *dgca*- cells on non-nutrient agar. *DgcA* RNA levels were measured by qRT-PCR with *DgcA* specific primers DgcAf and DgcAr (Supplementary Table S1).

B-D. *Ddis* cells were transformed with a fusion of 1 kb DgcA 5′ intergenic sequence and the *LacZ* reporter gene. β-galactosidase activity was visualized with Xgal in fixed aggregates (B), slugs (C) and fruiting bodies (D). Bar: 100 μm.

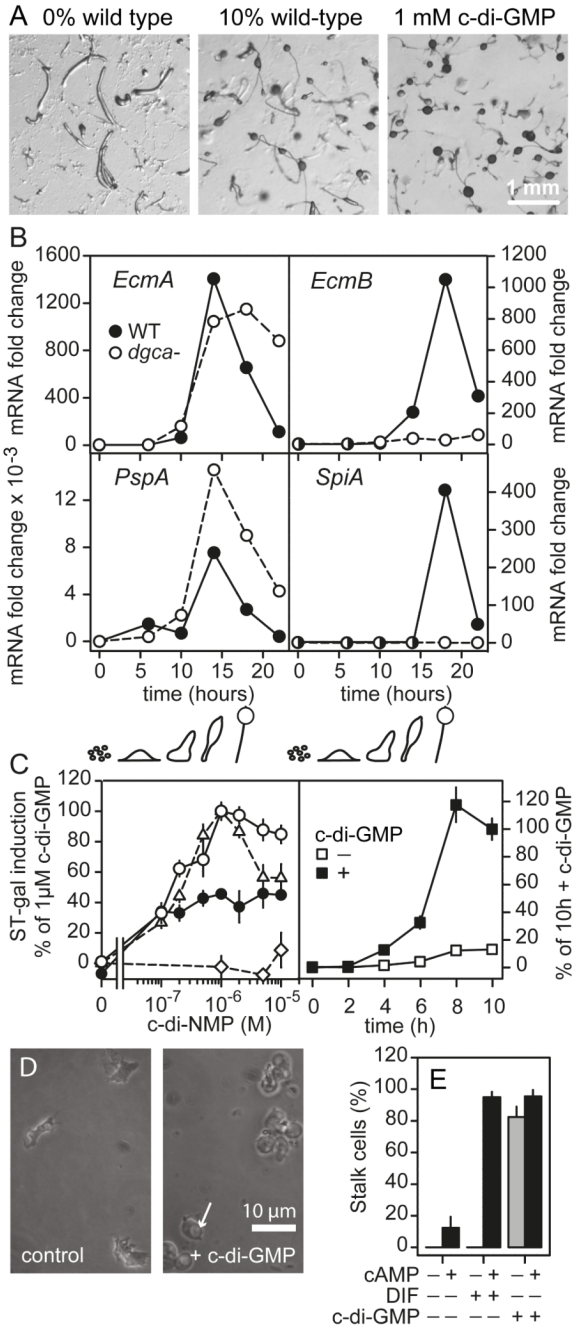

Figure 3. Biological role of c-di-GMP

A. *DgcA*- cells were mixed with 0 or 10% wild-type cells and developed for 24 h, or *dgca*- slugs were exposed to 1 mM c-di-GMP and observed after 10 h.

B. The wild-type and *dgca*- RNA time series (Fig. 2A) was used to amplify *ecmA, ecmB, pspA* and *SpiA* by qRT-PCR using gene-specific primers (Table S1).

C. Dissociated aggregates of *dgca*- (open circles, diamonds, filled circles) or *dmta*- (triangles), transformed with ST-gal, were incubated at 10^6 cells/ml for 8 h with increasing concentrations of c-di-AMP (diamonds), c-di-GMP (open circles, triangles) or c-di-GMP and 1 mM cAMP (filled circles), followed by β-galactosidase assay. *DgcA*-/ST-gal was

additionally incubated for variable time periods with (filled squares) or without (open squares) 1 μM c-di-GMP before assay. Means and s.e.m. (n=9)
D/E. *Ddis* V12M2 cells were incubated in monolayers[17] with 10 μM c-di-GMP, 100 nM DIF and/or 1 mM cAMP. After 30 h cells were photographed (D) and the proportion of stalk cells to total cells was determined (E). Means and SD (n=2).

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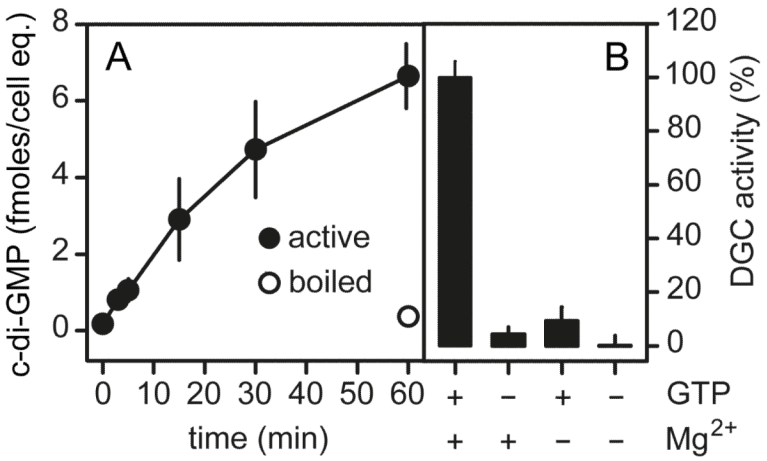

Figure 4. Bioassay of DGC activity

A. Preboiled or active immunoprecipitates of the ΔTL-DgcA-YFP fusion protein were incubated with 0.1 mM GTP and 10 mM $MgCl_2$ at 22°C. After boiling, reaction mixtures were tested for ST-gal induction activity. c-di-GMP concentrations in the mixtures were estimated by comparison with a dose-response curve of ST-gal induction by c-di-GMP, and standardized on the amount of cells from which the immunoprecipitate was derived.

B. DGC activity was measured for 60 min with combinations of 0.1 mM GTP and 10 mM $MgCl_2$ as indicated. Data represent means and s.e.m. of two experiments assayed in triplicate.

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