

Guanylate cyclase in *Dictyostelium discoideum* with the topology of mammalian adenylate cyclase

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The core of adenylate and guanylate cyclases is formed by an intramolecular or intermolecular dimer of two cyclase domains arranged in an antiparallel fashion. Metazoan membrane-bound adenylate cyclases are composed of 12 transmembrane spanning regions, and two cyclase domains which function as a heterodimer and are activated by G-proteins. In contrast, membrane-bound guanylate cyclases have only one transmembrane spanning region and one cyclase domain, and are activated by extracellular ligands to form a homodimer. In the cellular slime mould, *Dictyostelium discoideum*, membrane-bound guanylate cyclase activity is induced after cAMP stimulation; a G-protein-coupled cAMP receptor and G-proteins are essential for this activation. We have cloned a *Dictyostelium* gene, *DdGCA*, encoding a

protein with 12 transmembrane spanning regions and two cyclase domains. Sequence alignment demonstrates that the two cyclase domains are transposed, relative to these domains in adenylate cyclases. *DdGCA* expressed in *Dictyostelium* exhibits high guanylate cyclase activity and no detectable adenylate cyclase activity. Deletion of the gene indicates that *DdGCA* is not essential for chemotaxis or osmo-regulation. The knock-out strain still exhibits substantial guanylate cyclase activity, demonstrating that *Dictyostelium* contains at least one other guanylate cyclase.

Key words: cGMP, cloning, chemotaxis, mutant, structure.

INTRODUCTION

The eukaryotic amoeba *Dictyostelium discoideum* grows as a unicellular organism in the presence of a food source, such as bacteria. Starvation induces a developmental programme leading to cell aggregation and the formation of a fruiting body, which is composed of a stalk with a spore head. Chemotaxis plays a pivotal role during both growth and development. Growing cells utilize metabolites secreted by bacteria, such as folic acid, to sense and move towards their food source. Upon starvation, *Dictyostelium* amoebae start secreting cAMP; other amoebae detect this signal and move towards the source of cAMP, initiating the formation of a multicellular structure.

Both cAMP and folic acid bind to G-protein-coupled receptors. The activation of these receptors leads to dissociation of heterotrimeric G-proteins into $G\alpha$ and $G\beta\gamma$ subunits. $G\beta\gamma$ is thought to mediate the activation of the adenylate cyclase, *DdACA* [1]. This activation also requires a cytosolic regulator of adenylate cyclase (*DdCRAC*) [2], a Ras guanine nucleotide exchange factor (*aimless*) [3], *Pianissimo* [4], and a mitogen-activated protein kinase (*DdERK2*) [5]. In addition to *DdACA*, the activation of cAMP and folic acid receptors also leads to the stimulation of guanylate cyclase and phospholipase C activity. The analysis of knock-out cell lines of *DdACA* and *Dictyostelium* phospholipase C has shown that these two enzymes are not directly involved in chemotaxis to cAMP and folic acid [6,7]. In contrast, analysis of a collection of non-chemotactic cell lines with chemically induced mutations has indicated that almost all mutants are misregulated in one or more components of their cGMP signal transduction pathway. One of these mutants, *KI-8*, has very low guanylate cyclase activity [8]. Another mutant,

stmF, is mutated in its cGMP-specific phosphodiesterase, and shows a prolonged cGMP response after chemoattractant stimulation and prolonged movement [9]. These observations suggest that cGMP is a second messenger in *Dictyostelium*, mediating chemotaxis.

After stimulation of the cAMP or folic acid receptor, guanylate cyclase is activated, leading to a transient increase in intracellular cGMP levels. The activation of guanylate cyclase *in vivo* is dependent on a heterotrimeric G-protein pathway, since disruption of the single $G\beta$ subunit that is known to exist in *Dictyostelium* results in abolition of the receptor-induced stimulation of guanylate cyclase [1]. The observation that guanosine 5'-[γ -thio]triphosphate (GTP[S]) stimulates guanylate cyclase activity *in vitro* supports a role for G-proteins in the regulation of guanylate cyclase [10], but the precise mechanism is unclear. Besides chemoattractants, guanylate cyclase is also activated by osmotic shock *in vivo* (e.g. 0.3 M glucose), a pathway that does not require the presence of $G\beta$ [11–13]. This activation is relatively slow, resulting in maximal cGMP levels at about 15 min after stimulation, compared with 10 s after stimulation with cAMP or folic acid.

Upon receptor activation, a transient rise in cGMP is established within the cell, which presumably activates a cGMP-dependent protein kinase. This kinase directly or indirectly phosphorylates myosin light-chain-specific protein kinase and myosin heavy-chain-specific protein kinase C, which in turn phosphorylate the different myosins, resulting in rearrangements of myosin filaments necessary for movement [14–19].

In order to study the cGMP-dependent pathways in *Dictyostelium* chemotaxis, we have begun to isolate the genes encoding enzymes involved in the synthesis, degradation and action of

Abbreviations used: *DdGCA*, *Dictyostelium* guanylate cyclase; *DdACA*, *Dictyostelium* adenylate cyclase; ORF, open reading frame; GCE, mammalian membrane-bound guanylate cyclase type E; GTP[S], guanosine 5'-[γ -thio]triphosphate).

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cGMP in *Dictyostelium*. In the present study, we used degenerate primers, based upon conserved regions within the guanylate cyclase family, to clone a cDNA coding for a unique guanylate cyclase from *D. discoideum* (*DdGCA*). The deduced amino acid sequence shows a topology typical of adenylate cyclases, with 12 transmembrane spanning regions and two cyclase homology domains. The enzyme is at least 30-fold more specific for GTP than for ATP. Knock-out analyses suggest that *DdGCA* is not the sole guanylate cyclase involved in *Dictyostelium* chemotaxis.

MATERIALS AND METHODS

Strain and culture conditions

AX3 (wild-type), *ddaca*⁻ and *ddgca*⁻ cells were grown in HG5 medium. When cells were grown with selection, HG5 medium was supplemented with 10 µg/ml blasticidine S or 10 µg/ml geneticin (neomycin). Cells were starved for up to 5 h by shaking in 10 mM phosphate buffer, pH 6.5, at a density of 10⁷ cells/ml for the times indicated. For longer starvation periods, cells were deposited on non-nutrient agar plates [1.5% (w/v) agar in phosphate buffer, pH 6.5] and incubated at 22 °C for the indicated times. To study morphogenesis, cells were incubated at 22 °C on non-nutrient agar at various densities (2 × 10⁶ cells/cm², 4 × 10⁵ cells/cm² or 8 × 10⁴ cells/cm²). Transfection of cells with DNA was performed by electroporation [20]. Chemotaxis towards cAMP and folic acid was measured by the small-population assay [21].

Isolation of *DdGCA* cDNA

Degenerate primers, based upon conserved amino acid regions within the guanylate cyclase family, were used in a PCR with *Dictyostelium* genomic DNA as a template. Primers encoded the motifs R{I/V}G{T/L/V}HTG (forward primer AG3: CATCGAAGCTTGWRTHGGHRTHCAYACHGG) and AGVVGL (reverse primer GC3'B: GAAGCTTARDCCDACDACCDCDGC). With these primers, we obtained a fragment containing 10 bp between the primers. Based upon this sequence, a new reverse primer was designed: AG6 (CATCGGATTTCDACA-CCAGCAATAACAGGACC), which, in combination with a degenerate primer based upon the conserved amino acids YKVET{I/V}GD (forward primer GC5'P: CATCGAAGCTT-AYAARGTHGAAACHRTHGGHGA), resulted in a 216 bp PCR fragment. This fragment was used to screen a *Dictyostelium* cDNA library (kindly provided by Dr R. A. Firtel, Center for Molecular Genetics, University of California, San Diego, CA, U.S.A.), which resulted in several partial cDNA clones and one clone with a full-length open reading frame (ORF).

Expression of *DdGCA* in *Dictyostelium*

For overexpression of *DdGCA*, the complete cDNA was cloned into pMB12N, a *Dictyostelium* extra-chromosomal plasmid containing a blasticidine-resistance cassette [22], creating plasmid A¹⁵-GCA (in which expression is driven by the actin15 promoter). To subclone the coding region of the *DdGCA* cDNA, a primer was designed containing *SacI* and *BamHI* restriction sites followed by the start methionine (dGC8: GCGAGCTCGGATCCATGATGTTTGGTAAT). This primer was used together with an internal guanylate cyclase-specific primer in a PCR. The PCR fragment was digested using the newly introduced *SacI* restriction site and an internal *DdGCA* *Clal* restriction site. The fragment obtained was ligated into *SacI/Clal*-digested *DdGCA*

in pGEM7, and the Δ5'GCA obtained was excised with *BamHI* and cloned in the *BglII* site of pMB12N, resulting in the expression plasmid A¹⁵-Δ5'GCA. A third overexpression construct was made using the method described above with a slightly modified primer, dGC8b, which contained five additional adenosines in front of the start codon (creating a preferable *Dictyostelium* Kozak-like sequence). The latter *Dictyostelium* expression plasmid was named A¹⁵-Δ5'A₅-GCA.

Creating a *ddgca*⁻ cell

Two plasmids were constructed for the disruption of the *DdGCA* gene; in these plasmids a blasticidine resistance cassette replaced part of the *DdGCA* cDNA [a *Clal/HindII* fragment (nt 654–1425, deletion starting from amino acid 111), or a *HindII/SnaBI* fragment (nt 1425–3247, deletion starting from amino acid 368)]. These plasmids were used as a template in a PCR to produce linear DNA for homologous recombination. For the plasmid with the *Clal/HindII* substitution, primers dGC8 (GCGAGCTCGGATCCATGATGTTTGGTAAT) and dGCmasE_K (TGT-TTAACTTTTGTCACTCC) were used, and for the *HindII/SnaBI* substitution, primers dGC1 (GATATTCGTAATACTCTG) and dGCmasL_S (TCTTTGATCTTTATCTGATAA-TCTACCAT) were used. In these PCR products, the blasticidine cassette is flanked by 200 to 800 bp derived from the *DdGCA* cDNA. After purifying the PCR fragment, to avoid any contaminating plasmid, a second PCR was performed to obtain sufficient amounts for electroporation of *Dictyostelium* (2–10 µg of the purified PCR product). After clonal selection and Southern-blot analysis, two knock-out clones were selected (*gca*^{-b} and *gca*^{-e} from the *Clal/HindII* and *HindII/SnaBI* replacements respectively).

Northern- and Southern-blot analysis

Vegetative cells were obtained from a confluent 10-cm dish (about 2 × 10⁷ cells), and 3 × 10⁷ starved cells were obtained from a non-nutrient agar dish or from a shaking suspension. RNA isolation was performed using the Rneasy minikit (Qiagen) with the manufacturer's protocol for animal cells. After separation of the RNA samples on a formaldehyde-agarose gel (1% agarose with 0.65% formaldehyde present), the samples were transferred to a Nytran filter (Schleicher & Schuell). Genomic DNA was purified using a protocol described previously [23]. Approx. 0.25 µg of genomic DNA was digested with the indicated restriction enzymes, the products were separated on a 0.7% (w/v) agarose gel and transferred to a Nytran filter.

Northern and Southern blots were pre-incubated for 5 h at 65 °C in hybridization solution [0.5 M sodium phosphate buffer (pH 7.0), 7% (w/v) SDS, 0.2 mg/ml BSA]. Hybridization was carried out as above, except that a radioactive probe was included in the hybridization solution. Probes were ³²P-labelled using the random primer method (High Prime; Boehringer Mannheim). Blots were washed at high stringency using wash solution 1 (0.5 M phosphate buffer/1% SDS, pH 7.0) and wash solution 2 (0.1 M phosphate buffer/1% SDS, pH 7.0), and exposed using a phosphorimager.

Guanylate and adenylate cyclase assays

Guanylate cyclase assays were performed as described previously [24]. In brief, 10⁸ cells/ml in lysis buffer [40 mM Hepes/NaOH (pH 7.5), 6 mM MgSO₄, 6 mM EGTA] containing 0.1 mM GTP[S] were lysed by forced filtration through a Nucleopore filter (pore size 3 µm). After taking a zero time sample, the enzyme reactions were started at 30 s after lysis by adding an

equal volume of assay mixture (10 mM dithiothreitol, 1 mM GTP). Reactions were terminated at the indicated time points by the addition of an equal volume of 3.5% (v/v) perchloric acid. The cGMP content was measured using an isotope dilution assay [24]. To determine the effect of forskolin or YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (Alexis Biochemicals)] these compounds were added to the assay at a final concentration of 100 μ M.

The adenylate cyclase assay was similar to the guanylate cyclase assay, except that the assay mixture contained 1 mM ATP instead of GTP. The cAMP content was measured using an isotope dilution assay as described previously [24].

In vivo responses

In order to measure the response to folic acid and cAMP, cells were starved for the appropriate period of time (1 h and 5 h respectively) and resuspended in phosphate buffer (pH 6.5) at 10^8 cells/ml. Cells were stimulated with 10 μ M folic acid or 0.1 μ M cAMP, and reactions were terminated by the addition of an equal volume of 3.5% perchloric acid.

To measure cGMP responses to osmotic stimuli, cells were starved for 1 h, resuspended in phosphate buffer (pH 6.5) at 10^8 cells/ml and stimulated with 300 mM glucose. The levels of cGMP were determined using the isotope dilution assay. Survival after osmotic shock was measured by diluting cells in phosphate buffer (pH 6.5), plating 150 cells on to a 1/3 SM plate with *Escherichia coli*, and counting the number of colonies several days later.

RESULTS

Cloning of DdGCA

Using *Dictyostelium* genomic DNA and degenerate primers based upon conserved regions in guanylate cyclases, we performed a PCR and obtained a DNA fragment of 72 bp (containing 62 bp from the primers). The non-primer sequence was used to design a specific primer, which, in combination with a third degenerate primer, yielded a PCR fragment of 216 bp. Using this fragment, a cDNA library was screened, and a full-length clone was obtained. The cDNA clone is 4810 bp long and contains an ORF of 4458 bp, which is preceded by stop codons in all reading frames. The ORF starts with a methionine codon at position 332 and ends with a stop codon at position 4790.

Translation of the deduced ORF resulted in a protein consisting of 1486 amino acids with a calculated mass of 168 kDa. Analysis of the amino acid sequence revealed that the protein consisted of two regions that share homology with each other as well as with cyclase domains of guanylate and adenylate cyclases, the cyclase homology domain. BLAST analysis of the first cyclase homology domain, C₁, gave the highest score with a soluble guanylate cyclase β -3 from tobacco hornworm (*Manduca sexta*) (40% identity and 63% similarity), while the second cyclase homology domain, C₂, showed greatest similarity with a C-type natriuretic peptide receptor guanylate cyclase precursor from spiny dogfish (*Squalus acanthias*) (48% identity and 65% similarity). Hydrophathy analysis indicated the existence of two stretches consisting of six putative transmembrane-spanning regions each. This suggests that the encoded protein possesses a topology which is typical for the family of mammalian adenylate cyclases (Figure 1). As shown below, the cDNA identified here encodes a guanylate cyclase without detectable adenylate cyclase activity. The gene was therefore named *DdGCA*.

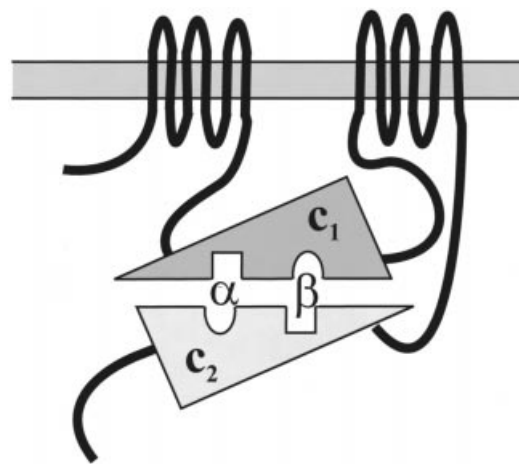


Figure 1 Putative topology of *D. discoideum* guanylate cyclase DdGCA

Two hydrophobic stretches, each consisting of six transmembrane-spanning regions, are intersected and followed by two cyclase homology domains, C₁ and C₂, respectively. The two cyclase homology domains form an antiparallel dimer. The catalytic site is located at the interface of the two domains, but not in the centre. Therefore, there are two potential catalytic sites, α and β ; the α site is defined as the site that is more proximal to the N-terminus of C₁ in the X-ray structure, whereas the β site is closer to the N-terminus of C₂. In a homodimer, such as in membrane-bound mammalian guanylate cyclase, the α and β catalytic sites are identical. However, in heterodimers, α and β could be different. In mammalian adenylate cyclases, ATP is converted in the β site and the α site has evolved into a forskolin-binding site. In contrast, sequence alignment indicates that in *Dictyostelium* DdGCA, GTP is hydrolysed in the α catalytic site, whereas the β site is presumably inactive.

Table 1 Overexpression of DdGCA in AX3 cells using various expression constructs

The AX3 strain was transfected with various plasmids. Control, empty plasmid MB12N; A¹⁵-GCA, a construct containing the complete DdGCA cDNA cloned behind the actin-15 promoter of MB12N; A¹⁵- Δ 5'-GCA, the same plasmid as A¹⁵-GCA, except that the 5'-untranslated region of the DdGCA cDNA has been deleted, leaving a *Bam*HI site before the start ATG; A¹⁵- Δ 5'A₅-GCA, the same plasmid as A¹⁵- Δ 5'-GCA, except that, between the *Bam*HI site and the start ATG, five adenines were introduced to create a better *Dictyostelium* Kozak sequence. Cells were washed in lysis buffer (see the Materials and methods section) and lysed. At 30 s after lysis the guanylate cyclase reaction was started by the addition of GTP and dithiothreitol; samples were quenched before and after GTP addition with perchloric acid. cGMP levels were measured in the neutralized extracts. The basal level of cGMP was measured immediately before the addition of GTP. Guanylate cyclase activity was measured in the presence of 0.1 mM GTP[S], 1 mM GTP, 1 mM dithiothreitol, 3 mM MgSO₄, 3 mM EGTA and 20 mM HEPES/NaOH, pH 7.0. Data shown are the means \pm S.E.M. of at least three independent experiments.

	Basal cGMP (pmol/10 ⁷ cells)	Guanylate cyclase activity (pmol cGMP/min per 10 ⁷ cells)
Control	12.5 \pm 5.6	167 \pm 23
A ¹⁵ -GCA	21.5 \pm 1.9	201 \pm 11
A ¹⁵ - Δ 5'-GCA	85.3 \pm 23.8	436 \pm 108
A ¹⁵ - Δ 5'A ₅ -GCA	149 \pm 62	470 \pm 70

Guanylate and adenylate cyclase activity

To study the substrate specificity of the putative cyclase, the cDNA was cloned into a *Dictyostelium* extrachromosomal plasmid under the control of the actin-15 promoter. The plasmid was transformed to *Dictyostelium* AX3 cells and to *aca*⁻ cells that lack the major adenylate cyclase activity. With the complete cDNA (A¹⁵-GCA) we obtained only a very weak overexpression (1.2-fold) as deduced from basal cGMP levels or guanylate

cyclase activity in cell lysates, compared with control cells transfected with the empty plasmid, MB12N (Table 1). Therefore an expression construct lacking the 5' non-coding sequence was constructed, resulting in the construct A¹⁵-Δ5'-GCA. Transfection of AX3 cells lead to a 2.6-fold increase in guanylate cyclase activity. *In vivo* basal cGMP levels of the A¹⁵-Δ5'-GCA-transfected cell line were also substantially greater (almost 7-fold) compared with control cells. In an attempt to increase the guanylate cyclase activity further, we made a construct A¹⁵-Δ5'A₅-GCA, containing the DdGCA ORF preceded by a *Dictyostelium* Kozak sequence. This resulted in a 12-fold greater cGMP level *in vivo* compared with control cells, indicating a further increase of guanylate cyclase activity *in vivo*. However, this did not result in higher guanylate cyclase activity *in vitro*, since this was still 2.8-fold higher than the activity of control cells. The enzyme activity was determined at different substrate concentrations in the presence of 50 μM GTP[S] and linear Michaelis–Menten kinetics with a K_m of 88 μM GTP were observed (Figure 2).

To investigate if the expressed protein also exhibits adenylate cyclase activity we transfected *aca*⁻ cells with A¹⁵-Δ5'-GCA. The assay conditions used were identical to the conditions for detection of guanylate cyclase activity, except that the substrate is ATP instead of GTP. The experiments revealed that the *aca*⁻ cells transformed with A¹⁵-Δ5'-GCA had no detectable adenylate cyclase activity (Table 2). Two control experiments were performed (results not shown), indicating that the *aca*⁻ cells transformed with A¹⁵-Δ5'-GCA showed enhanced guanylate cyclase activity, and that adenylate cyclase activity was clearly detectable in AX3 cells starved for 5 h. These results showed that the adenylate cyclase activity from the transfected *aca*⁻ cell line is less than 10 pmol cAMP/min per 10⁷ cells, compared with the enhanced guanylate cyclase activity of about 300 pmol cGMP/min per 10⁷ cells. Thus the cloned cDNA codes for a cyclase which is at least 30-fold more specific for GTP conversion than for ATP conversion. We therefore conclude that DdGCA is a guanylate cyclase with a topology that is typical for mammalian adenylate cyclases.

In heterodimers, regulatory compounds may bind in the non-catalytic site. Mammalian adenylate cyclases are activated by forskolin and soluble guanylate cyclases are assumed to be activated in a similar way by YC-1 [25,26]. We therefore examined if DdGCA was activated by one of these compounds in the presence or absence of GTP[S]. Forskolin and YC-1 at a concentration of 100 μM did not affect guanylate cyclase activity (results not shown), implying that these compounds do not stimulate GCA by more than 10%.

Phylogenetics

To place the unusual DdGCA in a phylogenetic context, we selected a number of amino acid sequences as representatives of the different groups of adenylate and guanylate cyclases. We included all potential *Dictyostelium* adenylate or guanylate cyclases, and a selection of bacterial class III cyclases as well as metazoan soluble and membrane-bound adenylate and guanylate cyclases. With these sequences, an alignment was made using the CLUSTAL W program [27] together with some manual adaptations; a selection of the data is shown in Figure 3. The alignment revealed that most amino acids known to be involved in the binding of ATP or GTP are conserved in DdGCA. These amino acids are spread over the two domains, as in other cyclases that function as a heterodimer. It is remarkable that, in DdGCA, the two domains appear to be transposed; thus DdGCA-C₁

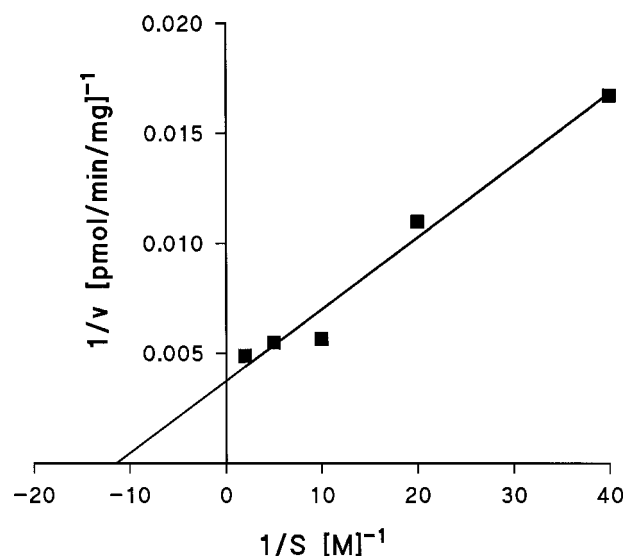


Figure 2 Lineweaver–Burk plot of guanylate cyclase activity in cells overexpressing DdGCA

Guanylate cyclase activities *in vitro* for wild-type cells overexpressing A¹⁵-Δ5'-GCA was determined, as described in Table 1, at different GTP concentrations (25–500 μM).

Table 2 Overexpression of DdGCA in *aca*⁻ cells, lacking adenylate cyclase activity

Shown are the adenylate cyclase activities of *aca*⁻ cells, *aca*⁻ cells expressing the plasmid A¹⁵-Δ5'-GCA and, as a control, AX3 cells which had been starved in buffer for 5 h. Adenylate cyclase activity was measured as described for guanylate cyclase activity (see Table 1), except that GTP was replaced by ATP. No significant adenylate cyclase activity was detected in *aca*⁻ cells expressing A¹⁵-Δ5'-GCA; the lower limit of the assay was approx. 10 pmol cAMP/min per mg of protein. Data shown are the means ± S.E.M. of at least three independent experiments.

	Adenylate cyclase activity (pmol cAMP/min per 10 ⁷ cells)
AX3	218 ± 76
<i>aca</i> ⁻	2.9 ± 4.0
<i>aca</i> ⁻ + A ¹⁵ -Δ5'-GCA	-1.3 ± 2.3

bears the amino acids conserved in the C₂ domains of other cyclases, and DdGCA-C₂ bears those conserved in C₁.

The aligned sequences were used as basis for a phylogenetic analysis with ProtDist and Fitch (PHYLP 3.5), resulting in the phylogenetic tree shown in Figure 4(a). Three major groups can be distinguished in this tree, a group containing mainly bacterial cyclases, a group containing yeast adenylate cyclases and a group containing the other eukaryotic cyclases. The group of the bacterial cyclases also harbours a recently cloned rat soluble adenylate cyclase [28]. In addition, scrutinizing the database of the *Dictyostelium* genomic sequencing project, we found nucleotide sequences which encode the recently cloned adenylate cyclase R (*acrA*) [29] and a protein containing two cyclase homology domains which also groups with the bacterial cyclases and has a high level of similarity with rat soluble adenylate cyclase.

DdGCA belongs to the group of eukaryotic cyclases. A refined bootstrapped analysis of these cyclases revealed four subgroups

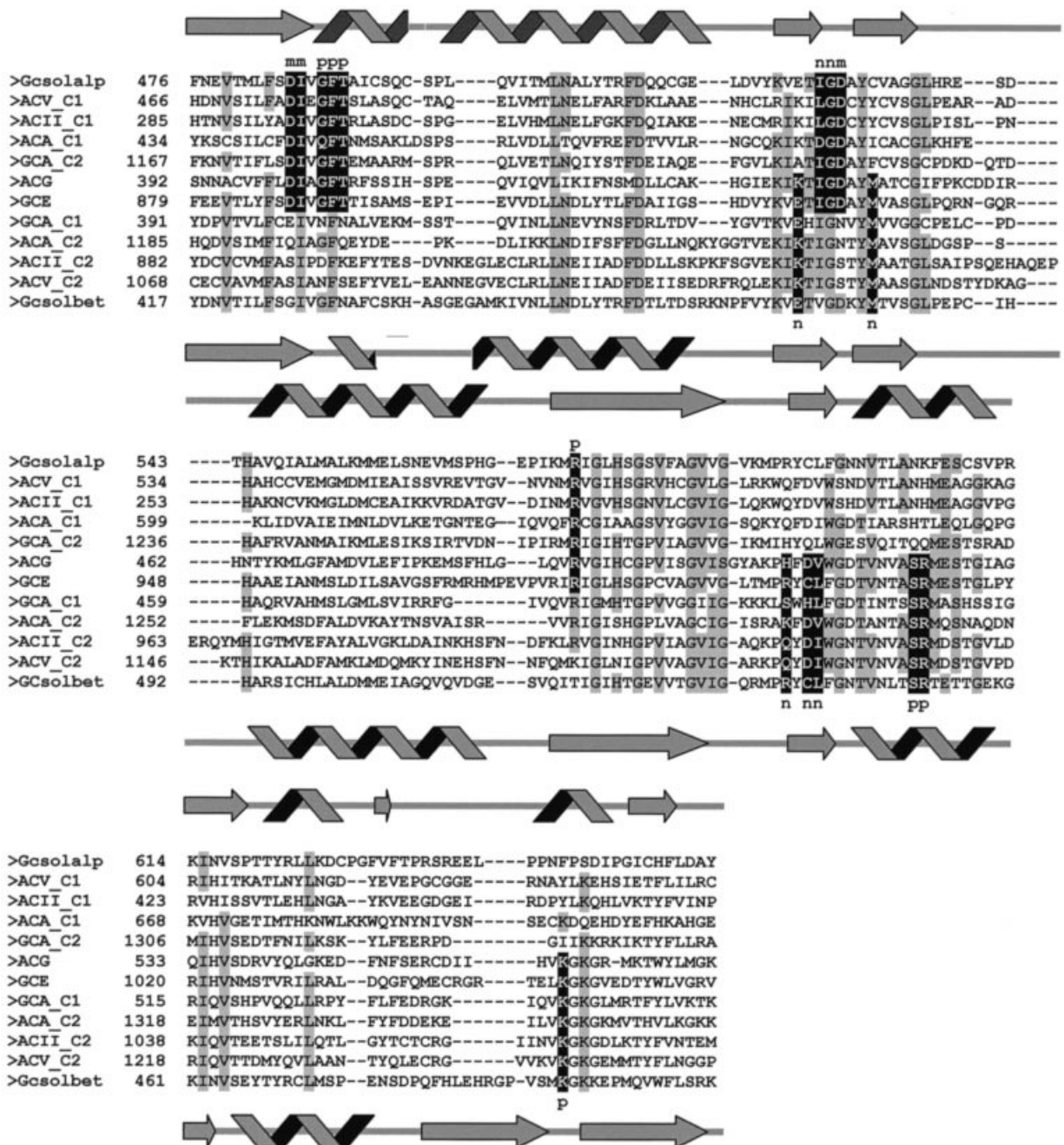


Figure 3 Amino acid sequence alignment of several cyclase homology domains

Function of specific amino acids as deduced from the structure and modelling of mammalian adenylate cyclase are shown as white-on-black with markings above or below the column: m, amino acids interacting with Mg^{2+} ; p, amino acids that interact with ribose or pyrophosphate; n, amino acids interacting with GTP or ATP [21,35,36,43]. Other conserved amino acids are shown with a grey background. Gcsolalp and Gcsolbet correspond to the α and β subunits of rat soluble guanylate cyclase, ACV and ACII correspond to mammalian adenylate cyclases type V and II. ACA and ACG correspond to *Dictyostelium* adenylate cyclases A and G. GCE corresponds to mammalian membrane-bound guanylate cyclase type E. The arrows indicate β sheets, and the helical lines α helices. In ACA_C1, a region, consisting mainly of asparagine residues (amino acids 497 to 596), has been deleted in this alignment.

(Figure 4b). One subgroup is formed by all membrane-bound guanylate cyclases; a second group consists of soluble guanylate cyclases. The other two groups contain the first and the second cyclase homology domains, C_1 and C_2 , of the twelve-transmembrane-segment adenylate cyclases. It appears that both cyclase homology domains of DdGCA group with the

adenylate cyclases, but they branch closely to the guanylate cyclases. Furthermore, the first cyclase homology domain C_1 of DdGCA groups with the C_2 domains of all other adenylate cyclases, whereas C_2 of DdGCA groups with C_1 of the adenylate cyclases. This phylogenetic analysis confirms the observations made in the alignment.

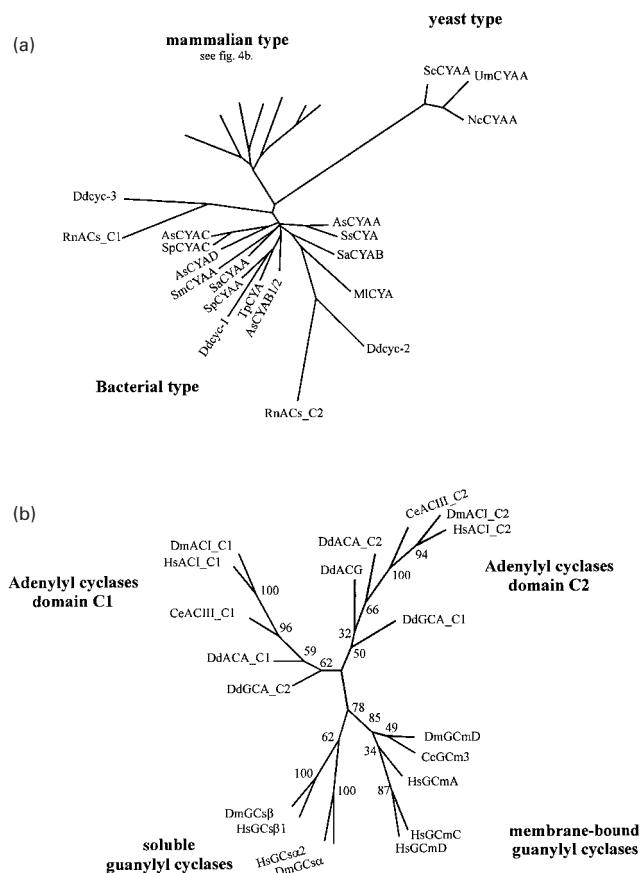


Figure 4 Phylogenetic analysis using the cyclase homology domains of several adenylyl and guanylate cyclases

An amino acid alignment of 34 different cyclase homology domains (as shown in Figure 3) was used to create the tree. (a) Analysis of all sequences, including the bacterial class III enzymes, using ProtDis and Fitch (PHYLP 3.5). (b) Within the group containing the DdGCA sequences a more refined analysis was performed. Sequences were aligned using the program CLUSTAL W, version 1.6 [27]. Phylogenetic analyses using the maximum parsimony (MP) method were performed with the PHYLP program package, version 3.572 from Felsenstein J (1993) PHYLP (Phylogeny Inference Package). Support values were established by bootstrapping with 100 replicates. Maximum likelihood (ML) analyses were performed with the PUZZLE program, version 4 [47]. The consensus trees constructed with the maximum-likelihood and the maximum-parsimony methods exhibited the same topology. Sequences used: AsCYAA (*Anabaena* sp.: BAA13997), SsCYA (*Synechocystis* sp.: BAA16969), SpCYAC (*Spirulina platensis*: BAA22997), AsCYAC (*Anabaena* sp.: BAA14000), AsCYAB2 (*Anabaena* sp.: BAA13999), AsCYAB1 (*Anabaena* sp.: BAA13998), SpCYAA (*Sp. platensis*: BAA22996), RnACs (AAD04035), SaCYAA (*Stigmatella aurantiaca*: P40137), TPCYA (*Treponema pallidum*: AAC65466), SaCYAB (*St. aurantiaca*: CAA11548), AsCYAD (*Anabaena* sp.: BAA14001), SmCYAA (*Sinorhizobium melioli*: P19485), MICYA (*Mycobacterium leprae*: CAA18817), ScCYA (*Saccharomyces cerevisiae*: P08678), NcCYA (*Neurospora crassa*: Q01631), UmCYA (*Ustilago maydis*: P49606), *cyc-2* and *cyc-3* can be assembled using several raw sequence data from the *Dictyostelium* genomic sequencing project, starting from JC2d13b12.r (*cyc-2/3*). *cyc-1* was assembled from IIBP1D1703 and later cloned by others and named *acrA* (AAD50121). (b) DmGCSα (*Drosophila melanogaster*: AAA87940), HsGCSα2 (*Homo sapiens*: CAA90393), DmGCSβ (*Dr. melanogaster*: AAA87941), HsGCSβ1 (*H. sapiens*: AAB94877), HsGCMc (*H. sapiens*: AAB19934), HsGCMd (*H. sapiens*: JH0717), DmGCMd (*Dr. melanogaster*: CAA51318), HsGCMa (*H. sapiens*: g4505435), CeGCM3 (*Caenorhabditis elegans*: Q09435), DdACA (*D. discoideum*: Q03100), DdACG (*D. discoideum*: Q03101), DdGCA (*D. discoideum*: CAB42641), CeACIII (*C. elegans*: CAA84795), DmACI (*Dr. melanogaster*: P32870), HsACI (*H. sapiens*: Q08828).

Function of DdGCA in *Dictyostelium*

To study the function of the DdGCA guanylate cyclase in *Dictyostelium*, we performed a Northern-blot analysis to discover at which developmental stages DdGCA is transcribed. The

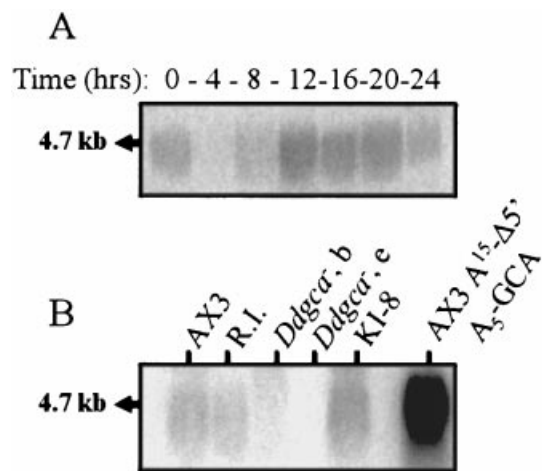


Figure 5 mRNA expression of DdGCA

Total RNA was isolated and blotted on to Nytran filter. The blot was probed using the first cyclase-homology domain of the *DdGCA* gene (nt 1430–2090). The 4.7 kb band was the only band detected; equal loading was confirmed by ethidium bromide staining (results not shown). (A) RNA was isolated from AX3 cells (wild-type), at several stages of development, that had been starved on non-nutrient agar for the times indicated above the panel. (B) Analysis of mRNA isolated from different strains during growth. AX3, wild-type control cells; R.I., random integrant obtained in the transfection experiment yielding the *ddgca*⁻ disruptants; *DdGCA*⁻, cells in which the DdGCA ORF has been disrupted (confirmed by Southern blots; not shown), *ddgca*⁻,b and *ddgca*⁻,e are clones isolated from two transfections with different constructs, creating disruptions at different locations of the ORF; KI-8, cell line with very low guanylate cyclase activity; AX3 A¹⁵-Δ5'-A₅'-GCA, an AX3 cell containing a *Dictyostelium* expression plasmid with the DdGCA ORF behind a constitutive actin-15 promoter.

experiments revealed an mRNA of 4.7 kb, which was developmentally regulated (Figure 5A). Levels were high in vegetative cells, decreased approx. 3-fold during the aggregation stage, and then increased again to twice the level of mRNA in vegetative cells during the later stages of development (mound, finger and slug). This may suggest that DdGCA has a function in *Dictyostelium* during vegetative growth and late development, and not so much during cell aggregation. We would not expect this if DdGCA were the only guanylate cyclase in *Dictyostelium*.

For a further investigation of the function of DdGCA, a knock-out cell line was made. Using two different constructs, several knock-out clones were identified by Southern- and Northern-blot analyses. Two clones, obtained with different knock-out DNA constructs, were used for further analysis (*ddgca*⁻ cells). A Northern blot indicated the absence of detectable DdGCA mRNA in both clones (Figure 5B). Figure 5(B) also shows a normal size and amount of DdGCA mRNA in mutant KI8, which exhibits very little guanylate cyclase activity [8]. Phenotypic analysis of the two different *ddgca*⁻ clones showed that these cells were still able to aggregate and develop normally, forming mounds, fingers, slugs and fruiting bodies composed of stalk and spores (Figure 6). Fruiting bodies were indistinguishable in shape and timing of formation from those of the wild-type, but culminants often had an irregular appearance. Further analysis, including phototaxis/thermotaxis of slugs, chemotaxis of cells to cAMP and survival after osmotic stress, also did not reveal any differences compared with wild-type cells.

We also determined residual guanylate cyclase activity in *ddgca*⁻ cells. This was performed in vegetative and in cells starved for 5 h. In either condition, significant cGMP production could be measured in *ddgca*⁻ cells and the guanylate cyclase activity in *ddgca*⁻ cells was not significantly different from that of AX3 cells

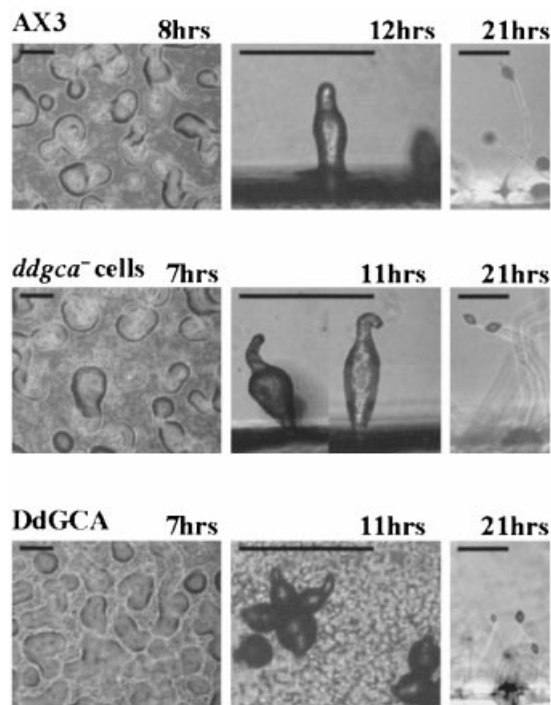


Figure 6 Phenotypes of *ddgca*⁻ and DdGCA overexpressor during development

Cells were starved on non-nutrient agar at a density of about 10^6 cells/cm². Cell aggregation at 7–8 h was not different among the strains (left panels). The culminants of *ddgca*⁻ cells at 11–12 h were often crumby (middle panels) and in the DdGCA overexpressor they often had multiple tips (bottom middle panel). Fruiting bodies of *ddgca*⁻ at 21 h appeared normal (right panels) but were smaller in the DdGCA overexpressor (bottom right panel).

(108 ± 10 and 100 ± 6 at zero time, and 168 ± 24 and 183 ± 13 after 5 h starvation respectively). We also tested cGMP responses to known stimuli in *ddgca*⁻ cells. The knock-out cell line still responded to cAMP, folic acid and osmotic shock (0.3 M glucose) with a level of cGMP production that was similar to that of control cells (results not shown).

DISCUSSION

The determination of an X-ray structure, modelling studies and mutagenesis experiments have revealed the general mechanism of action of adenylate and guanylate cyclases [25,30]. The catalytic core of the enzymes consist of two cyclase homology domains which are arranged approximately as two halves of a hemisphere in an antiparallel fashion. The catalytic reaction occurs at the interface of the two domains at positions relatively far from the centre, resulting in two potential catalytic sites α and β (see Figure 1). Membrane-bound guanylate cyclase and mammalian adenylate cyclase form an intermolecular and intramolecular heterodimer respectively. In the homodimer, both cyclase domains are identical and two catalytic centres are present. Similarly, the heterodimer also has two potential catalytic sites. However, structural data, modelling and biochemical experiments suggest that only one site is a catalytic site and the other site is inactive [31–35].

The ATP or GTP molecule binds in a catalytic site by interactions with amino acids from both domains. In the homodimer, all interacting amino acids are present in both the α and

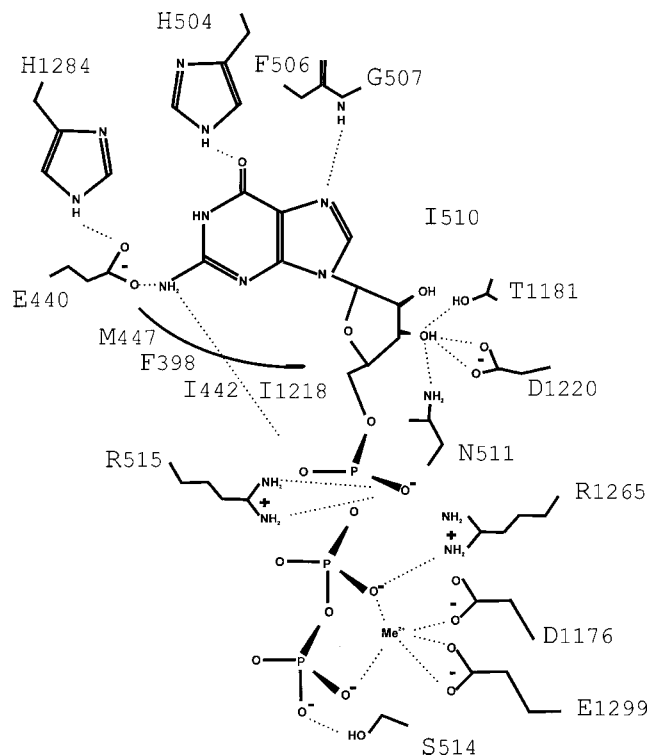


Figure 7 Amino acids of DdGCA possibly involved in GTP binding

The Figure is based on the structure of adenylate cyclase, and on modelling and mutagenesis studies of mammalian adenylate and guanylate cyclases [25,30,48,49]. Most amino acids involved in the binding of GTP are conserved between retGC and DdGCA; therefore we assume similar positioning of the amino acids. These positions were not further modelled using molecular dynamics. The amino acids binding the ribose ring and Mg²⁺ triphosphate are identical in retGC and DdGCA; also, several amino acids interacting with the guanine ring are conserved. Two major differences between retGC and DdGCA are the two histidine residues in DdGCA (H504 and H1284), which, in retGC, are a cysteine and an arginine residue respectively. The arginine in retGC is mainly involved in positioning the glutamic acid residue, the counterpart of which in DdGCA is E440. We have repositioned the glutamic acid residue, E440, based upon mutational studies by Sunahara et al. [48], who showed that the glutamic acid interacts with the 2-NH₂-group and not with 1-NH, as originally proposed by Lui et al. [30].

the β catalytic site. When GTP binds in one catalytic site of membrane-bound guanylate cyclase, it is likely that the two domains change conformation relative to each other, thereby changing the affinity of the other site for GTP. Thus negative or positive co-operativity could be expected for membrane-bound guanylate cyclase, as has been observed experimentally [36,37]. In the heterodimer, however, the amino acids need to be conserved in only one catalytic centre, and amino acids of the other catalytic centre may mutate to yield an inactive site or a site that binds regulatory compounds, such as forskolin in adenylate cyclase. As a consequence, co-operativity is generally absent in adenylate cyclases. DdGCA encodes a membrane-bound guanylate cyclase with the heterodimer topology of adenylate cyclases. It appears that, in DdGCA, the cyclase domains are transposed relative to the architecture of adenylate cyclases. All amino acids providing binding interactions with the pyrophosphate, the ribose moiety and the Mg²⁺ ion are conserved in the α catalytic centre of DdGCA (Figures 3 and 7). In contrast, the β catalytic centre contains many altered amino acids that would preclude binding of GTP or ATP. The observation that DdGCA follows Michaelis–Menten kinetics is also consistent with the presence of a single one functional GTP

catalytic site (Figure 2). It is unlikely that the non-catalytic β site binds forskolin or YC-1, since DdGCA activity was not altered by this compound in cells overexpressing DdGCA.

Nucleotide binding in DdGCA

In cyclases from higher eukaryotes, there is a very strong conservation of the amino acids determining nucleotide specificity. For guanylate cyclases, it has been proposed that the glutamate residue in the VYKVET motif [Glu-928 in mammalian membrane-bound guanylate cyclase type E (GCE)] is the most important amino acid for specific recognition of the N-2 amino group of GTP. Our data support this notion, since DdGCA, which is evolutionarily distant from all other guanylate cyclases, possesses this glutamate residue (DdGCA E440, see Figure 3). In adenylate cyclases, the counterpart of the glutamate residue is a lysine (ACII K938) which interacts with the N-1 atom of the P-site inhibitor 2'd3'AMP in the X-ray structure. Two other amino acid residues, expected to be important for nucleotide specificity, are an arginine (in the MPRYCL motif, GCE R998) and a cysteine (GCE C1000) in guanylate cyclases, the counterparts of which in adenylate cyclases are glutamine (ACII Q401) and aspartate (ACII D 1018) respectively. Modelling followed by site-directed mutagenesis shows that the arginine of guanylate cyclases is important for proper orientation of the glutamate. In DdGCA, the corresponding amino acid is a histidine (DdGCA H1284), which may fulfil the same function as the arginine residue in other guanylate cyclases. DdGCA also has a histidine residue (DdGCA H504) at the position of the conserved cysteine residue, present in all other guanylate cyclases. The 1-NH-group of this histidine residue may still stabilize the doubly bound oxygen in GTP, which is the proposed function of the cysteine of other guanylate cyclases [30]. In summary, DdGCA has two unusual histidines in the catalytic cleft which recognize the guanine moiety of GTP. To obtain a better idea of the role of the various amino acids involved in purine recognition we are currently studying mutations within this region to investigate the effect on substrate specificity.

Phylogeny

At least five cyclases have been identified in *Dictyostelium*: ACA, ACG, ACR, GCA and *cyc2/3*. The adenylate cyclases ACA and ACG clearly belong to the group of adenylate cyclases exemplified by the mammalian enzymes. ACA has the typical topology, with two cyclase homology domains and two sets of six transmembrane spanning regions, whereas ACG probably forms a homodimer, as it contains only one cyclase homology domain. Two *Dictyostelium* proteins group together with the bacterial cyclases: the recently cloned ACR, with a single cyclase homology domain (*cyc1*), and a partial ORF deduced from genomic sequence databases containing two cyclase homology domains (*cyc2* and *cyc3*). The latter protein shows a high degree of identity with the soluble rat adenylate cyclase (RnAC), not only in the cyclase homology domains but also in a stretch of about 250 amino acids located C-terminal from the second cyclase homology domain. Can this new *Dictyostelium* bacterial-type cyclases function as a guanylate cyclase? The deduced sequence of *cyc2/cyc3* revealed that one cyclase domain contained the conserved lysine of adenylate cyclases, whereas the other domain contained an aspartic acid residue at the position of a glutamic acid in all mammalian guanylate cyclases. It should be noted, however, that conservation within the group of bacterial cyclases is not as stringent as within the group of twelve-membrane-segment adenylate cyclases or mammalian-type guanylate cyclases.

The cyclase homology domains of the new guanylate cyclase, DdGCA, rank among the adenylate cyclases, but at the basis of these groups, thereby positioning them also close to the guanylate cyclases. This position may be related to the long evolutionary distance, but it may also indicate that DdGCA unites properties of both guanylate cyclase (substrate recognition) and adenylate cyclase (a G-protein-regulated twelve-transmembrane-segment cyclase). Furthermore, the two cyclase domains are transposed in DdGCA relative to adenylate cyclases. The question arises whether the guanylate cyclase we have cloned is unique for *Dictyostelium* or whether it is a member of a new group of guanylate cyclases. Searching the *Caenorhabditis elegans* and *Drosophila melanogaster* databases did not reveal a potential guanylate cyclase with two cyclase homology domains and twelve transmembrane spanning regions, suggesting that this type of guanylate cyclase is not present in higher eukaryotes. In *Paramecium*, *Tetrahymena* and *Plasmodium*, guanylate cyclases with twelve transmembrane spanning regions and two cyclase homology domains have been identified recently [38,39]. In these guanylate cyclases, as in *Dictyostelium* DdGCA, the cyclase domains are transposed when compared with the mammalian adenylate cyclases, which could indicate that these guanylate cyclases, together with DdGCA, belong to a new group. Unlike DdGCA, these guanylate cyclases are fused to a P-type ATPase with ten transmembrane-spanning-region segments.

DdGCA activity in *Dictyostelium*

The expressed cDNA, with an optimal 5'-untranslated sequence, displays high guanylate cyclase activity (about 300 pmol cGMP/min per 10^7 cells above the level of activity in control cells), and no adenylate cyclase activity was detected (less than 10 pmol cAMP/min per 10^7 cells). Thus our data indicate that DdGCA is at least 30-fold more specific for GTP than for ATP. To obtain high expression, it was important to remove the 5'-untranslated region of the cDNA, possibly because secondary structures or silencing sequences were made with the additional 5'-untranslated sequence derived from the actin-15 promoter, thereby inhibiting translation. Incorporation of a preferred *Dictyostelium* Kozak sequence (AAAAA) further increased expression. A striking difference was observed between the elevated *in vivo* cGMP levels on the one hand and *in vitro* guanylate cyclase activity in the overexpressor strains on the other. cGMP levels were increased about 7-fold in A¹⁵- Δ 5'-GCA and 12-fold in A¹⁵- Δ 5'A₅-GCA relative to control cells, whereas guanylate cyclase activity was increased only about 2.5-fold for both expression constructs. This suggests that guanylate cyclase activity *in vitro* is limited by a regulatory factor which is not limiting *in vivo*. Guanylate cyclase activity is strongly inhibited by calcium ions, suggesting that this guanylate cyclase is activated by a GCAP-like protein [40]. This protein could be limiting *in vitro* but not *in vivo*.

There is ample evidence suggesting a role for G-proteins in the regulation of guanylate cyclase activity in *Dictyostelium* cells, but the precise mechanism is unknown. Activation of guanylate cyclase *in vivo* by extracellular cAMP depends on the presence of a G-protein-coupled receptor cAR1, the single G β subunit and a myristoylated G α_2 [1,41-44]. Optimal guanylate cyclase activity *in vitro* requires the presence of both GTP and GTP[S] [10,45], this was also observed for lysates of cells overexpressing DdGCA. The potentiating effect of GTP[S] may suggest that DdGCA is directly regulated by G-proteins. However, this conclusion is premature, because GTP[S] is not only an activator of G-proteins but also a substrate of guanylate cyclases, including DdGCA (J. Roelofs and P. J. M. Van Haastert, unpublished work). To investigate how GTP[S] potentiates guanylate cyclase

activity, we are presently performing reconstitution experiments with DdGCA and G-proteins. In addition, we are mutating DdGCA with the aim of altering its nucleotide specificity and thus transforming it into an adenylate cyclase in which GTP[S] is no longer a substrate.

Function of DdGCA in *Dictyostelium*

Much of the functional information on guanylate cyclase in *Dictyostelium* has been derived from chemically mutated strains. One of these strains, KI8, can not respond chemotactically to any chemoattractant. This strain lacks nearly all guanylate cyclase activity; this was interpreted as either a loss of function in a single guanylate cyclase gene or a loss of function in a hypothetical regulator of guanylate cyclases. The KI8 cells have normal DdGCA mRNA levels and no mutations have been found in the cyclase domains of DdGCA in these cells (J. Roelofs and P. J. M. Van Haastert, unpublished work). Therefore we speculate that KI8 is either mutated in other guanylate cyclases and DdGCA has almost undetectable activity, or KI8 might be defective in a protein that regulates the activity of both DdGCA and the other guanylate cyclase(s). Unfortunately, we have not been able to transform KI8 cells with a plasmid expressing DdGCA; the delicacy of this mutant prevents transfection by any protocol (J. Roelofs and P. J. M. Van Haastert, unpublished work).

Two different *ddgca*⁻ knock-out strains generated in the present study showed no major defects during development. The cells clearly aggregated and formed fruiting bodies with stalks and spores in time range similar to that of wild-type cells. The *ddgca*⁻ cells had guanylate cyclase activity which was not significantly different from wild-type cells during growth or after 5 h of starvation, indicating the presence of another guanylate cyclase(s). The additional guanylate cyclase is membrane bound, dependent on GTP[S] and inhibited by submicromolar Ca²⁺ concentrations. These properties are not different from those of DdGCA, suggesting that the other guanylate cyclase(s) might be similar to DdGCA. However, PCR or screening of the *Dictyostelium* genome database did not reveal other sequences that are likely candidates; at this stage about 80–90% of the genome has been sequenced. Obviously, cloning the other guanylate cyclase(s) is essential to determine the role of DdGCA in chemotaxis.

Besides signalling molecules that act via G-protein coupled receptors, DdGCA is also activated by osmotic stress, which does not require the presence of G-proteins [11,13]. Survival and the cGMP response induced by 0.3 M glucose are not different in *ddgca*⁻ and wild-type cells, implying that DdGCA is not, or is only partially, involved in this response also. Photo/thermotaxis in slugs is another response in which guanylate cyclase could be involved [46]. This would fit nicely with the high mRNA expression of DdGCA in slugs. However, *ddgca*⁻ slugs on a charcoal/agar plate are still able to move towards a light/heat source with good degree of accuracy. In summary, DdGCA is a twelve-transmembrane-segment guanylate cyclase in which the two cyclase homology domains have been transposed relative to mammalian-type adenylate cyclases. DdGCA may be a member of a new class of cyclases which may also include other DdGCAs.

Note added in proof (received 7 February 2001)

We recently obtained a *Dictyostelium* cell line in which the gene encoding the cyclase homology domains *cyc-2* and *cyc-3* (see Figure 4a) has been inactivated. Analysis of this cell line revealed

that this gene encodes a guanylate cyclase (J. Roelofs, M. Miema, P. Schaap and P. J. M. Van Haastert, unpublished work).

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