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Characterization of Two Unusual Guanylyl Cyclases from Dictyostelium*

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Guanylyl cyclase A (GCA) and soluble guanylyl cyclase (sGC) encode GCs in Dictyostelium and have a topology similar to 12-transmembrane and soluble adenylyl cyclase, respectively. We demonstrate that all detectable GC activity is lost in a cell line in which both genes have been inactivated. Cell lines with one gene inactivated were used to characterize the other guanylyl cyclase (i.e. GCA in sgc− null cells and sGC in gca− null cells). Despite the different topologies, the enzymes have many properties in common. In vivo, extracellular cAMP activates both enzymes via a G-protein-coupled receptor. In vitro, both enzymes are activated by GTPγS (Kₐ = 11 and 8 μM for GCA and sGC, respectively). The addition of GTPγS leads to a 1.5-fold increase of Vₘₐₓ and a 3.5-fold increase of the affinity for GTP. Ca²⁺ inhibits both GCA and sGC with Kᵢ of about 50 and 200 nM, respectively. Other biochemical properties are very different; GCA is expressed mainly during growth and multicellular development, whereas sGC is expressed mainly during cell aggregation. Folic acid and cAMP activate GCA maximally about 2.5-fold, whereas sGC is activated about 8-fold. Osmotic stress strongly stimulates sGC but has no effect on GCA activity. Finally, GCA is exclusively membrane-bound and is active mainly with Mg²⁺, whereas sGC is predominantly soluble and more active with Mn²⁺.

cAMP and cGMP are important signaling molecules. In prokaryotes, cAMP regulates gene expression. Cyanobacteria contain high levels of cGMP relative to other bacteria, but their function as intracellular signaling molecules is not well understood (1). In eukaryotes, cAMP and cGMP regulate enzyme activities, channel activity, and gene expression, mainly via cAMP- and cGMP-dependent protein kinase (2, 3). A large and complex family of adenylyl cyclase (AC)³ and guanylyl cyclase (GC) is responsible for the synthesis of cAMP and cGMP (4, 5).

The crystal structure of mammalian AC (6, 7) suggests that the core of the enzyme consist of two cyclase domains that are associated in an antiparallel manner. In metazoan, four cyclase subgroups are recognized (4, 5, 8): 1) the 12-transmembrane adenylyl cyclase is composed of two different cyclase domains and is regulated by G-proteins; 2) The single-transmembrane guanylyl cyclase contains one cyclase domain and functions as a homodimer, and GC activity is stimulated by extracellular peptides; 3) The nitric oxide-sensitive soluble guanylyl cyclase is a complex of two different proteins with one cyclase domain each; 4) The recently discovered soluble adenylyl cyclase (sAC) from rat and human possesses two cyclase domains, which share the highest degree of identity with bacterial adenylyl cyclases (9).

In the social amoeba Dictyostelium, cGMP is implicated as one of the second messengers for chemotaxis (10), although its precise role is not known. On the other hand, cAMP can act as both first and second messenger (11, 12). As first messenger, cAMP induces chemotaxis, cAMP signal relay, and gene expression. Dictyostelium possesses three AC and two GC genes. ACA is similar to mammalian 12-transmembrane AC and controls cAMP signaling during cell aggregation. ACG is an AC with the topology of a membrane-bound GC containing one cyclase domain and one transmembrane region (13); the enzyme produces cAMP, which regulates spore germination. ACR, encoded by the acrA gene, is most similar to cyanobacterial AC and controls spore maturation (14–16). Recently we identified two Dictyostelium GC gene, gcaA and sgcA, encoding GCA (17) and sGC (18), respectively. The deduced amino acid sequences and predicted topologies suggest that GCA and sGC are more related to animal AC than to animal GC enzymes. GCA has the topology of 12-transmembrane AC in which the two cyclase domains appear to be functionally swapped. Thus, the second cyclase domain of mammalian AC, which provides most catalytic interactions with ATP, is similar to the first domain of GCA interacting with GTP. GC enzymes with this topology have also been found in Paramecium and Plasmodium (19, 20), but unlike these proteins have a P-type ATPase at their N terminus. The second Dictyostelium guanylyl cyclase, sGC, contains two cyclase domains and two long (~1000 amino acids) N- and C-terminal regions. The cyclase domains and the C-terminal region of sGC shows a high degree of identity with the corresponding segments of human soluble AC. Interestingly, homologs of sAC are present in bacteria and rat but are absent from the completely sequenced genomes of Drosophila, Caenorhabditis elegans, Arabidopsis, and yeast. Phylogenetic analysis places the Dictyostelium sGC as the evolutionary intermediate between the bacterial and vertebrate sequences (18, 21).

To better understand the role of cGMP and GC enzymes in Dictyostelium, we have characterized GCA and sGC. First we show that all GC activity is lost in a cell line in which both genes are inactivated, suggesting that GCA and sGC represent all GC activity in Dictyostelium. Subsequently, GCA was characterized in a cell line with a deletion of sGC, and sGC was characterized in a strain without GCA.
Characterization of Dictyostelium Guanylyl Cyclases

MATERIALS AND METHODS

Strains and Culture Conditions—AX3 (wild type), gca− null cells (17), sgc− null cells (18), and gca−/sgc− double null cells (see below) were grown in HG5 medium. When grown with selection, HG5 medium was supplemented with 10 μg/ml blasticidin S. To select for the survival of the uracil auxotroph DH1 cells by the pyr5/6 cassette (see below), cells were grown in uracil-deficient FM medium (22) (ICN). Cells were starved for up to 6 h by shaking in 10 mM phosphate buffer, pH 6.5, at a density of 10⁶ cells/ml.

Gene Inactivation—The gca−/sgc− double knock-out cell lines were obtained in the uracil auxotroph strain DH1 using the pyr5/6 gene (23) and the bar gene (24) as selection markers. The pyr5/6 cassette contains the coding region of the Pyr5/6 protein (23), which was amplified by PCR and cloned into a vector between the actin-15 promoter and the actin-8 terminator. To make the double knock-out strain, first gSC was inactivated in DH1 using the knock-out construct of sGC (see Ref. 18) in which the DNA segment encoding the Bar selection marker was replaced by the pyr5/6 selection cassette, yielding psCycKOpyr. A linear fragment with the sGC flanks and the pyr5/6 selection cassette was obtained by two rounds of PCR. Homologous integration of this fragment in DH1 resulted in the replacement of the region coding for amino acids 1192–1224 of sGC for the pyr5/6 selection cassette. Subsequently, two independent sgc− knock-out clones were used to disrupt the GCA gene with the Bar-containing construct as described previously (17). The disruption of both genes was confirmed by PCR as well as by Northern analysis. Two independent clones were used in this study.

Guanylyl Cyclase Assays—AX3, gca− null, and sgc− null cells were harvested and starved for 1 or 5 h in phosphate buffer. Unless mentioned otherwise, the procedure for determining GC activity was as follows. Cells were washed and resuspended in ice-cold lysis buffer (1.5 mM EGTA and 2.5 mM sucrose in 10 mM Tris, pH 8.0) and lysed through nucleopore filters (pore size, 3 μm) in the absence or presence of 100 μM GTP·S·P. For separation into soluble and particulate fractions, 0.5-ml aliquots of filter lysates were centrifuged for 1.5 min at 14,000 × g, and the pellets were resuspended in 0.5 ml of lysis buffer (particulate fraction). Lysates and fractions were incubated at 22 °C with 0.5 mM GTP in the presence of 10 mM dithiothreitol, 2 mM MgCl₂ or MgOAc, or 0.75 mM EGTA, 250 mM sucrose, and 15 mM Tris, pH 8.0. Reactions were terminated after 30 and 60 s by the addition of an equal volume of 3.5% perchloric acid. After neutralization, cGMP levels were measured by radioimmunoassay (25). The data shown are generally the means and standard error of the mean from three independent experiments with lysis in triplicates.

cGMP Response to Folic Acid, cAMP, and Osmotic Stress—Cells were starved for 1 h to measure the cGMP response to folic acid or osmotic stress. After starvation, the response to cAMP or GTP·S·P was measured. Upon return to normal buffer, cells were washed, resuspended in phosphate buffer at 10⁵ cells/ml, and stimulated with 1 μM folic acid, 300 mM glucose, or 0.1 μM GTP·S·P. The reactions were terminated with perchloric acid, and cGMP levels were measured by radioimmunoassay.

RESULTS

Topology and Catalytic Pockets of GCA and sGC—Dictyostelium contains two genes that encode guanylyl cyclase enzymes, GCA and sGC. Both enzymes contain two catalytic domains, which is essentially the only feature that these enzymes have in common (Fig. 1A). GCA has the topology of a membrane-bound mammalian adenylyl cyclase with two stretches of six transmembrane-spanning segments. In contrast, sGC has the topology of soluble mammalian adenylyl cyclase, including a homologous long C-terminal segment. Based on the three-dimensional structure of mammalian adenylyl cyclase, the two cyclase domains are expected to form an antiparallel dimer with potentially two catalytic pockets, α and β. In mammalian adenylyl cyclase, ATP is bound in the β pocket; the α pocket contains many amino acids that could prevent binding and catalysis of ATP (6, 7). Instrumental in this respect is the transition state mimicking Arg₁⁰₂⁹ from the C2 domain of mammalian adenylyl cyclase. The guanidino acids that cover the potential binding pockets of sGC indicate that GTP is bound in the β pocket of sGC (Fig. 1, B and C). In contrast, in GCA the catalytic arginine is provided by the C1 domain, and consequently GTP is predicted to be bound and hydrolyzed in the α pocket.

The three-dimensional structure of mammalian adenylyl cyclase with bound ATP analogs in the catalytic site suggests that two aspartates (Asp₁⁰⁰⁶ and Asp₁⁰⁴⁰) interact via two metal ions with the triphosphate moiety of ATP (35). These two metal-binding aspartates are conserved in GCA and sGC (Fig. 1, B and C). The γ-phosphate of ATP interacts with two positively charged amino acids (Arg₉⁴⁸ and Lys₁⁰₆⁰⁵ in ACII), of which one (Arg₉⁴⁸) forms a salt bridge to Glu₁⁵¹⁸. These amino acids are conserved in GCA and sGC as well, except for Lys₁⁰₆⁰⁵, which is a histidine in sGC (His₁¹⁴⁹⁷). Although this histidine may still interact with Pγ, it can no longer interact with the conserved glutamate (Glu₁¹⁸⁶⁵) unless it is protonated.

The purine moiety is bound to AC in a cleft that contains many hydrophobic amino acids. These hydrophobic amino acids are conserved in GCA and sGC, except for a lysine in sGC (Lys₁³³₄). Interestingly, sAC, the mammalian homolog of sGC, also contains a lysine at this position. The substrate specificity of cyclases is determined predominantly by a lysine for adenylyl cyclases (Lys₉³⁸ in ACII), which is a glutamate in guanylyl cyclases (Glu₉₂⁵ in GCE). Both GCA and sGC comply with this general observation, as they do not possess the positively charged lysine but contain the negatively charged glutamate in GCA and aspartate in sGC. In mammalian GCE it has been modeled that the O-6 of the guanidine moiety forms a weak hydrogen bond to the side chain thiol of Cys₁⁰⁰⁰. This hydrogen bond may still be possible with the histidine at this position in GCA (His₅⁰⁴) but not with the alanine in sGC (Ala₁³⁵⁷); perhaps in sGC the O-6 group forms a hydrogen bond with the backbone amide of Val₁³⁵⁸. Glu₉₂⁵ of GCE has been modeled to interact with Arg₉⁹⁸. At the position of this arginine GCA contains a histidine (His₁₂⁸⁴) that may fulfill this function. In contrast, in sGC this amino acid is replaced by a glutamate (Glu₁₁⁷⁰), which cannot provide the interaction with the corresponding aspartate; perhaps Glu₁₁⁷⁰ interacts with the NIH of guanine in sGC.

The noncatalytic pocket of GCA as well as that of sGC contain many hydrophobic amino acids and lack the amino acids that bind the metal ion and the phosphates of GTP. In this respect they have the properties of the noncatalytic sites of many mammalian adenylyl and soluble guanylyl cyclases. Forskolin, which binds in the noncatalytic site of mammalian membrane adenylyl cyclase, does not affect GCA or sGC activity (Ref. 17 and data not shown).

Inactivation of GCA and sGC—To investigate whether GCA and sGC encode all guanylyl cyclases in Dictyostelium, these two genes were inactivated separately and in combination. Wild-type cells contain high levels of Mg₂⁺-dependent guanylyl cyclase activity (Fig. 2). Guanylyl cyclase activity in sgc− cells is reduced to about 30% of the activity of wild-type cells. In gca− cells, the reduction of guanylyl cyclase activity is much smaller, to about 65% of that in wild-type cells. The double null mutant sgc−/gca− does not contain detectable guanylyl cyclase activity either with Mg₂⁺/GTP (Fig. 2) or with MgOAc/GTP as substrate (data not shown). The sensitivity of the assay implies that the guanylyl cyclase activity in the double null cells is maximally 1% of the activity in wild-type cells. Basal cGMP levels are significantly reduced in sgc− cells and slightly reduced in gca− cells. Again, the double null cell line does not contain cGMP above the detection limit (Fig. 2). Additional experiments at different developmental stages and measurements of intracellular cGMP levels in vivo after cAMP stimulation all failed to detect significant levels of guanylyl cyclase or cGMP in the double null cells (data not shown). The guanylyl cyclase double null cells can aggregate and form fruiting bodies; these cells show reduced chemotaxis with approximately the same mild defects as sgc− cells (data not shown).
The results indicate that GCA and sGC account for all guanylyl cyclase activity in Dictyostelium. Consequently, this implies that sgc cells can be used to characterize GCA without background of any other guanylyl cyclase, and conversely, gca cells can be used to characterize sGC.

**Mg$$^{2+}$$ and Mn$$^{2+}$$ Dependence of GCA, and sGC and Inhibition by Ca$$^{2+}$$**—Adenylyl and guanylyl cyclases require bivalent cations to support enzyme activity. In Fig. 3 dose-response curves are shown for gca and sgc cells at different concentrations of Mg$$^{2+}$$ and Mn$$^{2+}$$. The activity of sGC (gca-cells) is about 5-fold higher with Mn$$^{2+}$/GTP than with Mg$$^{2+}$/GTP, with maximal activity at 1–2 mM for both cations (Fig. 3A). Surprisingly, GCA (sgc-cells) is predominantly active with Mg$$^{2+}$/GTP, whereas Mn$$^{2+}$/GTP support only about 30% of the Mg$$^{2+}$-dependent activity (Fig. 3B). As the intracellular concentration of Mn$$^{2+}$$ is only 10 $$\mu$$M and Mg$$^{2+}$$ reaches a concentration of 3 mM (26), the...
physiological substrate of both enzymes is probably Mg$^{2+}$/GTP. Ca$^{2+}$ ions are known to inhibit Mg$^{2+}$-dependent guanylyl cyclase in Dictyostelium (27–29). Fig. 4 reveals that both GCA and sGC are sensitive to Ca$^{2+}$ inhibition. The Ca$^{2+}$ dose dependence suggests that GCA is slightly more sensitive to Ca$^{2+}$ inhibition than sGC, showing half-maximal inhibition at about 50 nM for GCA and at about 200 nM for sGC. The Mn$^{2+}$-dependent activity of sGC (18) or GCA (data not shown) is not inhibited by 10 μM Ca$^{2+}$.

Kinetics of GCA and sGC—cGMP formation is activated in vivo by extracellular cAMP via a G-protein-coupled signal transduction pathway. Expression of the G-protein subunits Ga2 and the single Gβ are essential to activate guanylyl cyclase (12). The Mg$^{2+}$-dependent guanylyl cyclase activity is stimulated in vitro by GTPγS (10). We observed that GTPγS has no effect on Mn$^{2+}$-dependent sGC (18) or GCA activity (data not shown). To investigate how GTPγS regulates guanylyl cyclase activity, we determined the $K_m$ and $V_{\text{max}}$ of GCA and sGC for Mg$^{2+}$/GTP in the absence and presence of GTPγS (Fig. 5). The results indicate that both enzymes show Michaelis-Menten kinetics without indications for cooperativity and that GTPγS stimulates enzyme activities by increasing the $V_{\text{max}}$ and reducing the $K_m$ of both enzymes. The $K_m$ of GCA for GTP is 250 ± 50 μM (Fig. 5A). GTPγS activates GCA by reducing the $K_m$ to 66 ± 2 μM GTP. In addition, it induces a moderate increase of the $V_{\text{max}}$ from 6.9 ± 0.7 to 9.8 ± 0.1 pmol/min/mg protein. The effect of GTPγS on sGC is approximately the same (Fig. 5B); it reduces the $K_m$ from 414 ± 110 to 112 ± 10 μM and increases the $V_{\text{max}}$ from 16 ± 3 to 27 ± 1 pmol/min/mg protein. The GTPγS dose dependence of enzyme activation is presented in Fig. 5C. The curves are best fitted by an equation with a Hill coefficient of 1.7 ± 0.4 for both GCA and sGC. Half-maximal activation of GCA is induced by 11 ± 2 μM GTPγS and by 8 ± 2 μM GTPγS for sGC. The data suggest that GCA and sGC are regulated by GTPγS via a similar mechanism.

Developmental Expression of GCA and sGC—Northern blots have suggested that GCA is expressed in wild-type cells mainly during growth and development, whereas sGC is expressed maximally during cell aggregation (17, 18). For quantitation of these Northern blots (Fig. 6A) the expression levels for GCA and sGC during development were normalized for the expression of each gene at 0 h of development. The expression of GCA shows a 70% decline at the onset of cell aggregation (4 h), whereas the expression of sGC increases about 2-fold during cell aggregation. In the multicellular stage, the expression of GCA increases strongly to reach a maximum in the slug stage, which is about 2-fold higher than during growth and 10-fold higher than during aggregation. The expression levels of sGC in the multicellular stages decline to the levels during growth.

GCA activity in vegetative sgca– cells is about 6 pmol/min/mg protein, which decreases significantly to 2.5 pmol/min/mg protein in starved cells (Fig. 6B). The Mn$^{2+}$-dependent activity of sGC in vegetative gca– cells amounts to 11 pmol/min/mg proteins and increases to about 17 pmol/min/mg protein during aggregation. These data indicate that during growth 20–40% of the Mn$^{2+}$-dependent GCA activity is attributed to GCA and 60–80% to sGC. After starvation the total activity increases about 1.5-fold; ~90% is attributable to sGC and ~10% to GCA. The reduction in GCA and increase in sGC activity are consistent with the developmental changes of mRNA expression seen on Northern blots.

Stimulation of GCA and sGC by cAMP, Folic Acid, and Osmotic Stress—Folic acid, cAMP, and osmotic stress induce
the activation of guanylyl cyclase activity in wild-type cells (10). These responses were analyzed in gca- and sgc-null cells to investigate which of the two guanylyl cyclases is activated by these agents (Fig. 7). In sgc- cells (regulation of GCA), the responses are small (folic acid and cAMP) or absent (osmotic stress), indicating that GCA is not or is weakly activated by these agents. In contrast, in gca- cells (sGC regulation), guanylyl cyclase activity is potently activated by folic acid, cAMP, and osmotic stress, indicating that sGC is strongly stimulated by these compounds.

Quantification of these responses suggests that GCA contributes about 55% to the basal cGMP levels of vegetative wild-type cells and about 40% in starved cells. Folic acid stimulation of sgc- cells leads to a 2.2-fold increase of GCA-produced cGMP levels (filled bar in Fig. 7A) and cAMP stimulates cGMP levels about 2.7-fold in these cells (Fig. 7B). Interestingly, GCA is insensitive to osmotic stress, as sgc- cells show no increase of cGMP levels upon stimulation with 0.3 M sucrose (Fig. 7C). In contrast to the weak responses of GCA, sGC is potently stimulated by these agents, because in gca- cells folic acid and cAMP induce a 5.5- and 8-fold increase, respectively, in cGMP levels. The increase of cGMP levels after stimulation of wild-type cells by osmotic stress is totally attributable to sGC, because this response is fully preserved in gca- cells and is absent in sgc- cells.

**DISCUSSION**

*Dictyostelium* contains two genes that encode for unusual GC enzymes, GCA and sGC. Amino acid sequence alignment, phylogenetic studies, and topology suggest that sGC belongs to the small group of soluble AC enzymes present in human, rat, and some bacteria (18, 30). GCA belongs to the large group of prevailing cyclases that harbors nearly all vertebrate ACs and GCs; GCA is most closely related to the family of 12-transmembrane ACs (17). Sequence alignment of cyclase domains and site-directed mutagenesis suggest that one amino acid may be crucial in determining substrate specificity of cyclases, which is a lysine in nearly all adenylyl cyclases and a glutamate in guanylyl cyclase (6, 31–34). In GCA this amino acid is glutamate (Glu440) and in sGC an aspartate (Asp1332). Two other amino acids proposed to be important for determining substrate specificity are an aspartate and a glutamine in adenylyl cyclases, which are an arginine and cysteine at the same positions in guanylyl cyclases. These amino acids are not conserved in GCA and sGC, as they are replaced by two histidines in GCA and by a glutamate and alanine in sGC. Although it is possible to provide a function for these amino acids in substrate recognition (Fig. 1B), it would be interesting to determine the three-dimensional structure of these unusual cyclases. Mutagenesis of GCA has shown that replacing the glutamate and histidine to the corresponding lysine and aspartate converts GCA into a fully active adenylyl cyclase (34). The notion that GCA and especially sGC contain many unusual amino acids at positions that have been shown to provide substrate specificity suggests that conversion of an adenylyl to a guanylyl cyclase can be achieved in different ways.

Regulation of adenylyl and guanylyl cyclases by their natural effectors such as G-proteins or Ca2+ is generally detectable only with Mg2+, whereas Mn2+ uncovers all intrinsic activity (35). This notion also holds for sGC because this enzyme is more active with Mn2+/GTP than with Mg2+/GTP, whereas GTP·S and Ca2+ strongly affect Mg2+-dependent activity but have no effect in the presence of Mn2+. Interestingly, GCA is active predominantly with Mg2+/GTP as substrate, whereas with Mn2+ the activity is reduced at least 3-fold. A trivial but unprecedented explanation for the low Mn2+-dependent GCA...
activity could be that Mn\textsuperscript{2+}-dependent GCA activity remains inhibited by Ca\textsuperscript{2+}. Although lysates contain EGTA to chelate Ca\textsuperscript{2+}, Ca\textsuperscript{2+} will be released during the GC assay because EGTA has a higher affinity for Mn\textsuperscript{2+} than for Ca\textsuperscript{2+} (36). We are not aware of a chelator that binds Ca\textsuperscript{2+} with 10,000-fold higher affinity than Mn\textsuperscript{2+}, which would be needed to test the hypothesis. However, the small Mn\textsuperscript{2+}-dependent GCA activity could not be inhibited by the addition of 10 \textmu M Ca\textsuperscript{2+}, suggesting that GCA either has low intrinsic Mn\textsuperscript{2+}-dependent activity or that the low Mn\textsuperscript{2+}-dependent activity represents a residual Ca\textsuperscript{2+}-insensitive activity.

Despite the different sensitivities of GCA and sGC to bivalent cations, the physiologically relevant cation is probably Mg\textsuperscript{2+} for both sGC and GCA, because the intracellular concentration of Mn\textsuperscript{2+} (\textsim 10 \textmu M) is too low to support activity, whereas the Mg\textsuperscript{2+} concentration (\textsim 3.5 mM) is sufficient (26). It is expected that GCA with 12 hydrophobic segments is found in the particulate fraction of a cell lysate and that sGC without hydrophobic segments resides in the soluble fraction. All Mg\textsuperscript{2+}- and Mn\textsuperscript{2+}-dependent GCA activity was found in the particulate fraction.\footnote{J. Roelofs and P. J. M. Van Haastert, unpublished observations.}

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inactive with Mg\(^{2+}\)/GTP as substrate. Thus, both GCA and sGC are membrane-associated enzymes with the physiologically relevant cation Mg\(^{2+}\).

GCA as well as sGC can be stimulated by the chemottractants folic acid and cAMP, although maximal stimulation of GCA is much weaker (2.5-fold) than maximal stimulation of sGC (8-fold). There is no simple explanation for this difference, because potential regulators such as GTP\(_{\beta}\)S and Ca\(^{2+}\) have nearly the same effect on GCA as on sGC. Perhaps sGC is activated by additional mechanisms, a supposition that is also supported by the observation that osmotic stress activates sGC but not GCA. Translocation of soluble sGC to the membrane or modification of soluble sGC by which it becomes active with Mg\(^{2+}\)/GTP could provide the additional activation of sGC that is not possible for GCA.

The mechanism by which GTP\(_{\beta}\)S stimulates GCA and sGC is not straightforward. Ample evidence indicates that in *vivo* chemotactants stimulate cGMP formation through a G-protein-mediated pathway. No detectable cGMP formation occurs upon stimulation of cells in which the single G\(\beta\) subunit has been deleted, suggesting that chemotactic stimulation of GCA and sGC are both mediated via heterotrimeric G-proteins (37). *In vitro* GTP\(_{\beta}\)S stimulates both GCA and sGC by inducing a 1.5-fold increase of the \(V_{\text{max}}\) of the enzyme and a 3.5-fold reduction of the \(K_{\text{m}}\) for GTP. Also the \(K_{\text{m}}\) and Hill coefficient for the GTP\(_{\beta}\)S dose dependence of GC stimulation are similar for both enzymes, which may suggest that GCA and sGC are regulated by the same GTP-binding protein. The *in vitro* GTP\(_{\beta}\)S activation of sGC and GCA could represent the aforementioned essential requirement of heterotrimeric G-proteins for chemoattractant-mediated stimulation in *vivo*. However, several anomalies have been observed that are not consistent with a heterotrimeric G-protein as the target for GTP before GTP\(_{\beta}\)S stimulation of these enzymes. Third, it has been observed in normal Michaelis-Menten kinetics, suggesting that GTP does

The function of cGMP during chemotaxis and multicellular development is emerging. Previous mutant analysis uncovered several mutants with chemotaxis defects that have an altered cGMP metabolism (see Ref. 10), notably mutant KI-8, which has very low cGMP levels. In comparison with the absolute chemotaxis-null phenotype of KI-8, chemotaxis of *gca* / *sgc* double null cells is relatively normal. It has been demonstrated that cGMP induces the phosphorylation and rearrangement of myosin heavy chain II filaments. Chemotaxis of *mhcII* null cells is disturbed but not absent. The preliminary phenotypic experiments on *gca* / *sgc* double null cells suggest that cGMP may play a role in chemotaxis through myosin filament formation but is not absolutely required for chemotaxis as is the case for myosin heavy chain. Detailed computer-assisted chemotaxis analysis and phosphorylation of the myosin heavy and light chains of *gca* / *sgc* single and double null cells is in progress and should uncover the mechanism by which cGMP regulates chemotaxis.

The identification of two guanylyl cyclases in *Dictyostelium*, GCA and sGC, uncovers unexpected evolutionary traits. GCA has the topology of a 12-transmembrane adenyl cyclase, whereas sGC is the homolog of a soluble adenyl cyclase. No close phylogenetic relationship can be found with mammalian guanylyl cyclase (17, 18), suggesting that the mammalian guanylyl cyclases, GCA and sGC, each developed independently into a guanylyl cyclase. From a biochemical point of view this may not be very surprising, considering the relative ease by which an AC can be converted experimentally to a GC (31–34, 42, 43). However, from an evolutionary perspective, one would expect that AC to GC interconversions would have occurred more frequently. In the family of phosphodiesterases that hydrolyze cAMP and cGMP, a change of substrate specificity during evolution may have been relatively common (44, 45), but it seems also scarce in cAMP- and cGMP-dependent protein kinases. Apparently, the specificity of cyclases and kinases cannot be changed easily because the mechanisms that regulate cyclases and the substrate specificity of kinases have to remain functionally coupled.

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REFERENCES

Characterization of Dictyostelium Guanylyl Cyclases