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GTPγS Regulation of a 12-Transmembrane Guanylyl Cyclase Is Retained after Mutation to an Adenylyl Cyclase*

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DdGCA is a *Dictyostelium* guanylyl cyclase with a topology typical for mammalian adenylyl cyclases containing 12 transmembrane-spanning regions and two cyclase domain. In *Dictyostelium* cells heterotrimeric G-proteins are essential for guanylyl cyclase activation by extracellular cAMP. In lysates, guanylyl cyclase activity is strongly stimulated by guanosine 5′-3-*O*-(thio)triphosphate (GTPγS), which is also a substrate of the enzyme. DdGCA was converted to an adenylyl cyclase by introducing three point mutations. Expression of the obtained DdGCA^{kqd} in adenylyl cyclase-defective cells restored the phenotype of the mutant. GTPγS stimulated the adenylyl cyclase activity of DdGCA^{kqd} with properties similar to those of the wild-type enzyme (decrease of K_m and increase of V_{max}), demonstrating that GTPγS stimulation is independent of substrate specificity. Furthermore, GTPγS activation of DdGCA^{kqd} is retained in several null mutants of Gα and Gβ proteins, indicating that GTPγS activation is not mediated by a heterotrimeric G-protein but possibly by a monomeric G-protein.

Adenylyl cyclases (ACs)[1] and guanylyl cyclases (GCs) have a high degree of amino acid sequence identity and are expected to have a similar structure of their catalytic sites (1–3). Despite these similarities, the mechanism of activation is essentially different for ACs and GCs. Mammalian 12-transmembrane adenylyl cyclases are regulated by G-protein-coupled receptors (GPCRs). Ligand binding to the GPCR causes intracellular dissociation of the heterotrimeric G-protein into its Gα and Gβγ subunits. These subunits can activate (Gα$_s$, Gβγ) or inhibit (Gα$_i$, Gβγ) AC (4–6). In cell lysates GTPγS, a GTP analogue that cannot be hydrolyzed by Gα, is often used as indication for G-protein regulation (7–9). Mammalian GCs can be separated into two groups, soluble and membrane-bound enzymes (10, 11). The soluble GCs form a heterodimer and are activated by NO, which binds to a heme group that is associated to the dimer (12). Membrane bound GCs have an extracellular domain, which often functions as a receptor, and ligands have been identified for a number of these GCs.

Recently, several unusual GCs have been cloned in lower eukaryotes. We have characterized a GC in *Dictyostelium*, DdGCA, that has the topology of mammalian ACs, consisting of two stretches each with six membrane-spanning regions and two cyclase domains (13). In *Paramecium*, *Tetrahymena,* and *Plasmodium,* GCs with the same topology have been found, but at the N terminus these proteins contain a P-type ATPase, providing an additional 10 membrane-spanning region (14, 15). The regulation of GC in these last three organisms is essentially unknown. Although G-protein regulation has been considered for the *Paramecium* and *Tetrahymena* GCs, no 40-kDa heterotrimeric G-protein family members have been found in these organisms (16).

In *Dictyostelium*, activation of GC *in vivo* is known to be dependent on GPCRs and on heterotrimeric G-proteins. cAMP and folic acid are two well known extracellular ligands that induce a transient rise in cGMP. Disruption of the single Gβ gene abandons the cGMP response to both stimuli (17), whereas disruption of Gα$_2$ or Gα$_4$ inhibits the cGMP response to cAMP and folic acid, respectively (18–24). In cell lysates of wild-type cells, GTPγS stimulates AC and GC activity. However, in lysates of the *gβ$^-$* cell line, this activation is lost for AC but not for GC (17). Thus, the GTPγS activation of ACA depends on heterotrimeric G-proteins. The mechanism by which GTPγS regulates GC activity is still unclear, especially because GTPγS is known to be a substrate of GC from *Dictyostelium* and several other organisms (25, 26). It has been speculated that GTPγS-mediated stimulation of cGMP formation is a substrate effect (17, 26).

To study the mechanism by which GTPγS regulates GC activity, we converted DdGCA to an adenylyl cyclase. The results demonstrate that a protein with three point mutations, DdGCA^{kqd}, shows strong AC activity and can rescue the phenotypic defects of the adenylyl cyclase null mutant, *aca$^-$*. Next we analyzed the effect of GTPγS on DdGCA^{kqd} and show that GTPγS stimulates AC activity; this indicates that the GTPγS effect is independent of substrate specificity and not acting on the catalytic site. Finally, by expressing DdGCA^{kqd} in several Gα and Gβ deletion mutants, we have demonstrated that a heterotrimeric G-protein does not mediate GTPγS activation of GC. The role of monomeric G-proteins in GC regulation is discussed.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—The following strains were used in this study: wild-type strain AX3, *gβ$^-$* strain LW6 (27), *gα2$^-$* strain JH104 (20), *gα4$^-$*(28), *aca$^-$* (29), *gca$^-$* (13), and *gα2$^-$gα4$^-$* (see below). Cells were grown in HG5 medium supplemented with 10 μg/ml blasticidin S or 10 μg/ml neomycin based upon the selection marker present. To study morphogenesis, cells were plated on non-nutrient agar at different densities (2×10^6, 4×10^5, and 8×10^4 cells/cm^2) and incubated at 22 °C to develop. Cells were transfected with DNA by electroporation as described (30).

Double Knock-out Strain gα2$^-$/gα4—To make a double null of Gα2 and Gα4, *gα4$^-$* cells were transformed with a Gα2 knock-out construct obtained as follows. A PCR fragment, obtained by using primers *ga2f1* (5′- AGCCTC CGCGGG CGCGCA AGAAAT GGGTAT TTGTGC ATC-3′) and *ga2r1* (5′-CTAGCT AGCCAT ACCACT AGTACC AGAATA TA-

[1] The abbreviations used are: AC, adenylyl cyclase; GC, guanylyl cyclase; GPCR, G-protein-coupled receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DdGCA, *Dictyostelium discoideum* guanylyl cyclase A; PCR, polymerase chain reaction.

AACC AGC-3′) on genomic *Dictyostelium* DNA, was cloned into the pCR2.1 vector (Invitrogen). A *Bsr* cassette (31) was cloned in the *Hin*dII site of the obtained plasmid. The resulting Gα2 knock-out construct was digested with *Eco*RI, and the fragment containing the *Bsr* cassette with the Gα2 flanks was isolated and used as template in a PCR reaction with primers *ga2f1* and *ga2r1*. In this way sufficient knock-out fragment was obtained devoid of any residual circular plasmid that could cause a high background of random insertion in the transformation of *Dictyostelium* cells. Transformation by electroporation and selection of clones was done as described previously (13).

Mutagenesis of DdGCA—All mutations were made using PCR (32). Before creating the mutant cyclases, two silent mutations were introduced in pGEM7GCA (13) at nucleotide positions 1365 (A to T) and 2049 (T to C), creating unique *Pst*I and *Hin*dIII restriction sites, respectively, that flanked the coding region of the first cyclase domain. The plasmid obtained was used as the template for PCR mutagenesis to introduce three point mutations, E440K, S502Q, and H504D, which have been confirmed by sequencing. The obtained PCR products were cloned in the *Pst*I and *Hin*dIII sites of full-length DdGCA, which were subsequently cloned in the expression plasmids MB12N (*bsr* selection) and MB12Neo (G418 selection) (33) to create DdGCA^{kqd}. The sequence of the plasmid is similar to that of A^{15}-Δ5′-A$_5$-GCA (13) with the exception of the introduced restriction sites and point mutations. DdGCA^{kqd}(*bsr*) was transformed to *aca*$^-$ cells, and DdGCA^{kqd}(*bsr*) or DdGCA^{kqd}(*neo*) to the other *Dictyostelium* strains depending on the selection markers available for the specific strain.

Guanylyl and Adenylyl Cyclase Assays—GC assays were performed as described previously (34). In short, cells (10^8 cells/ml) in lysis buffer (40 mM Hepes/NaOH, 6 mM MgSO$_4$, and 6 mM EGTA, pH 7.5) with or without GTPγS (0.1 mM) were lysed by forced filtration through a Nucleopore filter (pore size, 3 μm). The reactions were started at 30 s after lysis by the addition of an equal amount of assay mixture (10 mM dithiothreitol and 1 mM GTP). Reactions were terminated at the indicated time points by the addition of an equal amount of 3.5% (v/v) perchloric acid; the time zero sample was taken immediately after cell lysis. The AC assay was performed similar to the GC assay, except that the assay mixture contained 1 mM ATP instead of GTP. The cGMP or cAMP content was measured using an isotope dilution assay as described previously (34).

In Vivo Responses—To measure responses to cAMP, cells were starved for 5 h and resuspended at 10^8 cells/ml in 10 mM phosphate buffer. Cells were stimulated with 10 μM 2′-deoxy-cAMP, and reactions were terminated by the addition of an equal volume of 3.5% perchloric acid. cAMP and cGMP levels were measured in the neutralized lysates.

RESULTS

Regulation of DdGCA Activity by GTPγS—The guanylyl cyclase activity of DdGCA is strongly dependent on GTPγS (13). To analyze the catalytic activity in more detail. DdGCA was overexpressed in AX3 cells, and guanylyl cyclase activity was determined at different concentrations of GTP or GTPγS (see Fig. 1*A*). The Lineweaver-Burk plot shows that the K_m for GTP and GTPγS are 340 and 200 μM, respectively, with a similar V_{max} of 75 pmol/min/mg for both substrates. Thus, it seems that GTPγS is a better substrate than GTP; however, GTPγS is also a potent activator of GC activity in *Dictyostelium* (26). GC activity was measured at different GTP concentrations in the presence of 50 μM GTPγS. Without GTP no cGMP formation was detected at this concentration of GTPγS. The results reveal that GTPγS leads to a reduction of the K_m for GTP from 340 to 88 μM and to an increase of the V_{max} with a factor of 1.5 (Fig. 1*A*). The K_a of GTPγS stimulation is about 5 μM as determined by measuring GC activity with 500 μM GTP at increasing concentrations of GTPγS (Fig. 1*B*).

These measurements have two intrinsic caveats. First, GTPγS is an activator as well as a substrate of the enzyme, making it difficult to distinguish substrate-related effects from non-catalytic activation. Second, although the measurements were done in cell lysates from DdGCA-overexpressing cells, these cells still express another GC (13). We may circumvent both problems upon mutation of DdGCA to an adenylyl cyclase and expression in *aca*$^-$ cells, because GTPγS will no longer be
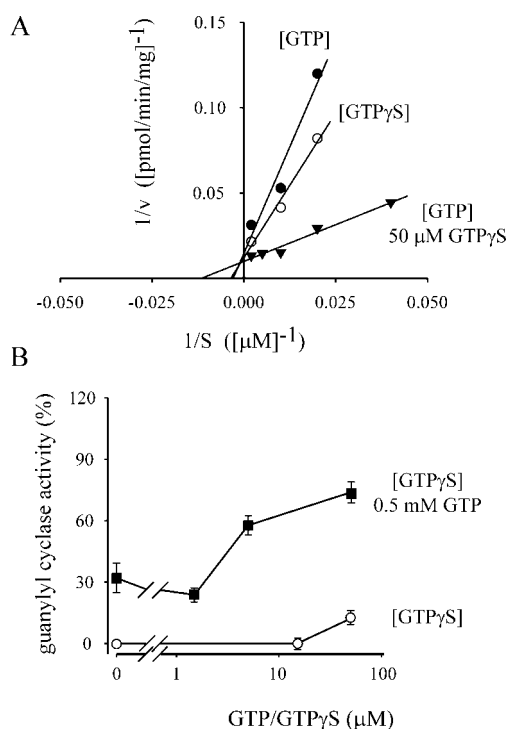

FIG. 1. **Lineweaver-Burk plots of guanylyl cyclase activity in cell lysates of AX3 cells overexpressing DdGCA.** *A*, guanylyl cyclase activity (*v*) was measured in the presence of 3 mM EGTA, 3 mM Mg^{2+}, 10 mM dithiothreitol, 40 mM Hepes (pH 7.0), and increasing substrate concentrations of GTP (●), GTPγS (○), or GTP in the presence of 50 μM GTPγS (▲). *B*, guanylyl cyclase activity was measured with (■) or without (○) 500 μM GTP in combination with increasing concentrations of the activator GTPγS; the results show that 10 μM GTPγS is not a substrate but stimulates cGMP formation from GTP. Experiments were performed in triplicate.

a substrate and *aca*$^-$ cells do no contain AC activity under our assay conditions.

Change of Substrate Specificity—DdGCA has a typical mammalian AC topology consisting of two clusters each with six transmembrane-spanning regions and two cyclase domains (Fig. 2*A*). The cyclase domains show homology to the domains of eukaryotic ACs and GCs. Sequence analysis clearly show that the C1 domain of DdGCA is functionally equivalent to the C2 domains of mammalian ACs, and the C2 domain of DdGCA corresponds to the mammalian C1 domains (13). The crystal structure of a homodimer of the rat AC type II C2 domain and a heterodimer of canine AC type V C1 and rat AC type II C2 domains together with modeling studies gave a good indication of which amino acids are involved in substrate binding and catalysis (1–3). Based on this information and on alignments of several AC and GC sequences (Fig. 2*B*), we might be able to predict which amino acids in DdGCA are involved in determining substrate specificity.

In ACs, three amino acids seem to be most important for ATP recognition: a lysine (Lys-938 in ACII), a glutamine (Gln-1016 in ACII), and an aspartate (Asp-1018 in ACII). These three amino acids are highly conserved among all ACs in higher eukaryotes. Their counterparts in GCs, a glutamate (Glu-928 in GCE), an arginine (Arg-998 in GCE), and a cysteine (Cys-1000 in GCE), are also strongly conserved among GCs. In DdGCA, the glutamate (Glu-440 in DdGCA) is conserved as well, but the other amino acids are unique, being a serine (Ser-502 in DdGCA) and a histidine (His-504 in DdGCA). To study the involvement of these amino acids in substrate specificity, we mutated all three amino acids to their counterparts in AC, creating DdGCA^{kqd}.
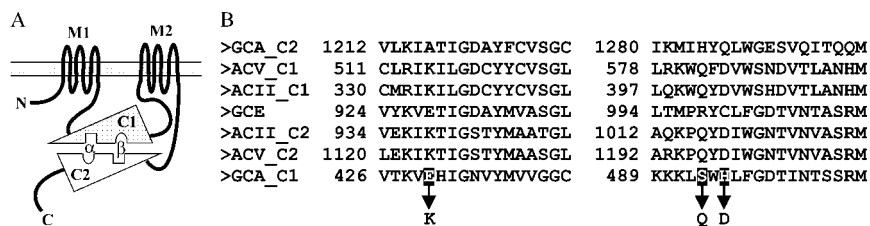
FIG. 2. **Schematic and amino acid alignment of the cyclase domains of DdGCA.** *A*, the putative topology of *Dictyostelium discoideum* guanylyl cyclase, DdGCA. Two hydrophobic stretches, each consisting of six transmembrane-spanning regions, are intersected and followed by two cyclase homology domains, C_1 and C_2, respectively. There are two potential catalytic sites, α and β. In mammalian adenylyl cyclases, ATP is converted in the β site, and the α site has evolved into a forskolin-binding site. In contrast, sequence alignment (*panel B*) indicates that in *Dictyostelium* DdGCA, GTP is hydrolyzed in the α catalytic site, whereas the β site is presumably inactive. *B*, the alignment shows the region of cyclase domains containing the amino acids involved in substrate specificity. *Black arrows* indicate amino acids that have been mutated to make DdGCA^{kqd}.

Adenylyl Cyclase Activity of DdGCA^{kqd} and Rescue of aca$^-$ Cells—Disruption of the *DdACA* gene leads to an aggregation defective phenotype of the *aca$^-$* cells (29). As demonstrated in Fig. 3, expression of DdGCA^{kqd} in *aca$^-$* cells resulted in the formation of small aggregates that developed into fruiting bodies with spores. Expression of DdGCA did not result in any kind of rescue. AC activity in lysates from in *aca$^-$* cells is very low under our assay conditions (~2 pmol/min/mg protein). Expression of DdGCA in *aca$^-$* cells did not yield AC activity, whereas expression of DdGCA^{kqd} resulted in enhanced AC activity (25 pmol/min/mg protein; Fig. 4*A*). GC activity levels as well as basal cGMP levels are significantly higher upon expression of DdGCA in *aca$^-$* or wild-type cells (Fig. 4*B* and Ref. 13). Expression of DdGCA^{kqd} in *aca$^-$* cells did not result in enhanced GC activity or a higher basal cGMP level (Fig. 4*B*). Thus, by making three point mutations DdGCA was successfully converted to an adenylyl cyclase.

Regulation of DdGCA^{kqd} by Ca^{2+} in Vitro and cAMP in Vivo—To investigate the effect of known regulators of GC activity, the inhibition of DdGCA^{kqd} by calcium *in vitro* (35) and stimulation by cAMP *in vivo* was tested. As shown in Fig. 5, AC activity of *aca$^-$* cells overexpressing DdGCA^{kqd} is strongly inhibited by similar Ca^{2+} concentrations as GC activity of wild-type AX3 cells. This finding suggests that both DdGCA^{kqd} and DdGCA are regulated by a protein that strongly binds calcium, like *e.g.* guanylyl cyclase activating protein (GCAP) modulating GC activity in the retina (36, 37).

Dictyostelium cells show a transient rise in cGMP levels, peaking at 10–15 s after stimulation with cAMP. Overexpression of DdGCA^{kqd} in *aca$^-$* cells may result in a similar response, but now a transient rise in cAMP should occur. This cell line has the important advantage that the simultaneous detection of cGMP and cAMP levels provide information on the activation of endogenous GC enzymes and overexpressed DdGCA^{kqd}, respectively. Cells were stimulated with 2′-deoxy-cAMP, an analogue that stimulates the receptor but does not interfere with cAMP determinations. As shown in Fig. 6, cGMP levels increase about 8-fold to a maximum at 10 s after stimulation. In addition, cAMP levels produced by DdGCA^{kqd} increase about 4-fold; the response is biphasic with enhanced cAMP levels after prolonged stimulation. This difference in cGMP and cAMP response could be because of the phosphodiesterases that degrade cGMP and cAMP, respectively, which have very different regulatory properties in *Dictyostelium* (38–44). These results of Fig. 6 demonstrate that DdGCA^{kqd} is activated by extracellular cAMP, probably via a cAR1 receptor dependent pathway.

The effect of GTPγS on the AC Activity of DdGCA^{kqd}—To study the effect GTPγS on the activity of GCA, we used *aca$^-$* cells expressing DdGCA^{kqd}. As shown in Table I, AC activity is hardly detectable without GTPγS, whereas in the presence of GTPγS substantial production of cAMP is observed. The kinetic

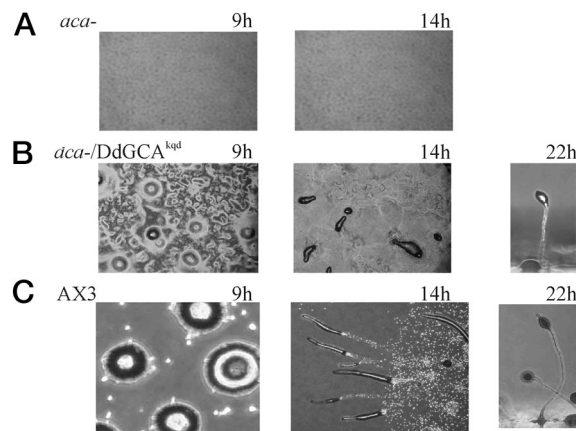FIG. 3. **Rescue of aca$^-$ cells by overexpressing DdGCA^{kqd}.** To study phenotypes, vegetatively growing cells were plated on agar at a density of 2×10^6 cell/cm^2. *A*, *aca$^-$* cells, which have a disruption of the adenylyl cyclase ACA (29), do not form aggregates or fruiting bodies. *B*, *aca$^-$* cells overexpressing DdGCA^{kqd}, form small aggregates and fruiting bodies. *C*, wild-type AX3 cells.
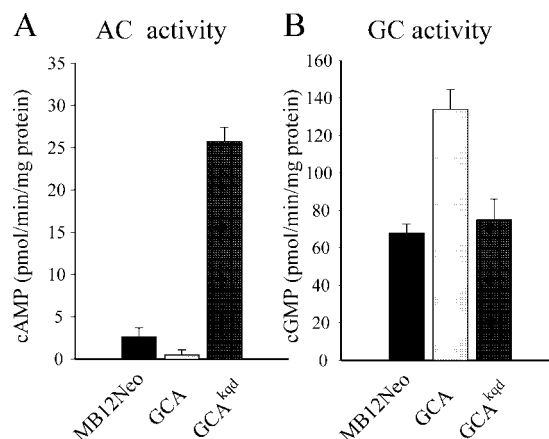

FIG. 4. **Adenylyl and guanylyl cyclase activity of aca$^-$ cells overexpressing DdGCA^{kqd}.** Adenylyl cyclase (*A*) and guanylyl cyclase (*B*) activities were measured in cell lysates of *aca$^-$* cells expressing MB12Neo (empty plasmid), DdGCA, or DdGCA^{kqd}. Activities were determined in the presence of 50 μM GTPγS and 500 μM ATP or GTP.

constants of DdGCA^{kqd} were determined from activity measurements at different substrate concentrations (Fig. 7), demonstrating that GTPγS regulates AC activity of DdGCA^{kqd} by reducing the K_m from 270 to 141 μM ATP and by increasing the V_{max} from 17 to 38 pmol/min/mg. These kinetic data for the AC activity of DdGCA^{kqd} are essentially identical to the effect of GTPγS on the kinetic data for the GC activity of DdGCA (Fig. 1), suggesting that the mechanism of stimulation by GTPγS did not change dramatically upon mutation of DdGCA.

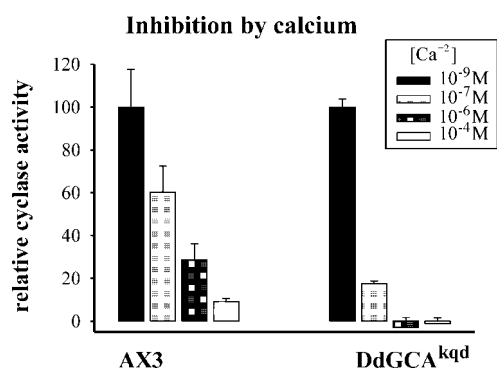The activation of *Dictyostelium* GCs was studied *in vitro* and

Inhibition by calcium

FIG. 5. **Calcium inhibition of guanylyl cyclase activity in AX3 and adenylyl cyclase activity in DdGCA^{kqd} expressed in *aca⁻* cells.** Cells were lysed in the presence of 50 μM GTPγS and different concentrations of free calcium as achieved by a calcium/EGTA buffer. Activities at 10^{-9} free calcium were set at 100%.
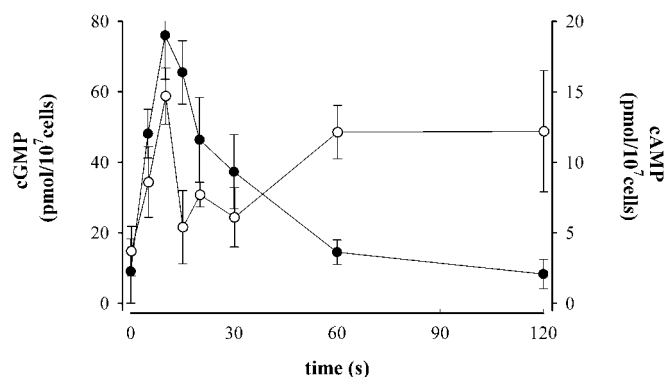

FIG. 6. ***In vivo* cAMP and cGMP response *aca⁻*/DdGCA^{kqd} cells.** Vegetatively growing *aca⁻* cells overexpressing DdGCA^{kqd} were washed twice in PB, starved in PB at 10^7 cells/ml for 5 h, washed and resuspended to 10^8 cells/ml. These cells were stimulated with 10^{-5} M 2′-deoxy-cAMP and cells were lysed by addition of an equal amount of 3.5% PCA. The levels of cGMP (●) and cAMP(○) were determined in the neutralized lysates.

TABLE I

Adenylyl cyclase activity in cell lines overexpressing DdGCA^{kqd}

Adenylyl cyclase activity was measured in lysates with 0.5 mM ATP in the presence or absence of 50 μM GTPγS. The AC activity was below the detection limit in cells without expression of DdGCA^{kqd} (data not shown).

Cells expressing DdGCA^{kqd}	Adenylyl cyclase activity	
	−GTPγS	+GTPγS
	pmol/min/mg	
aca⁻	−2 ± 1.5	25 ± 1.6
gβ⁻	1.7 ± 1.8	33 ± 2.0
gα2⁻	−3.8 ± 6.5	44 ± 1.8
gα4⁻	0.4 ± 3.0	36 ± 4.0
gα2⁻/gα4⁻	−1.0 ± 2.5	45 ± 0.9

in vivo using several cell lines in which subunits of heterotrimeric G-protein have been deleted. To investigate the role of heterotrimeric G-proteins in the regulation of DdGCA by GTPγS, we expressed DdGCA^{kqd} in the different knock-out cell lines (*gβ*, *gα2⁻*, and *gα4⁻*) and measured the AC activities in lysates plus and minus GTPγS. The endogenous DdACA, which is also stimulated by GTPγS in some of these cells, has not been deleted but is expressed at very low levels in vegetative cell and does not cause interference in the assay (data not shown). *Dictyostelium* cells possess only one Gβ subunit (27), which is essential for a cAMP and cGMP response *in vivo*. Unexpectedly, expressing DdGCA^{kqd} in *gβ⁻* cells results in AC activity that is still strongly activated by GTPγS (Table I).

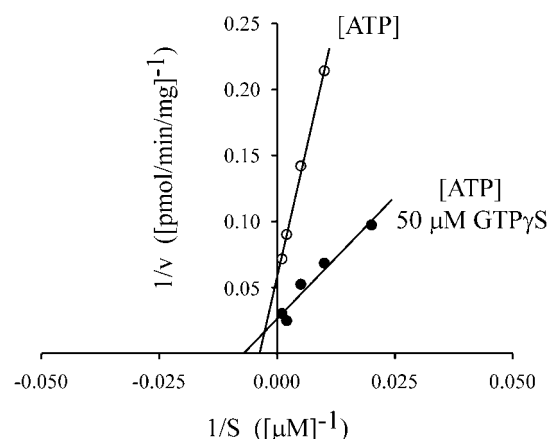At least nine Gα subunits have been described in *Dictyoste-*

FIG. 7. **Lineweaver-Burk plots of adenylyl cyclase activity in lysates from *aca⁻* cells overexpressing DdGCA^{kqd}.** Adenylyl cyclase activities were measured in the presence of 3 mM EGTA, 3 mM Mg^{2+}, 10 mM dithiothreitol, 40 mM Hepes (pH 7.0), and different concentrations of ATP in the presence (●) or absence (○) of 50 μM GTPγS. Experiments were performed in triplicate.

lium (45, 46), but only two of them, Gα2 and Gα4, have been implicated in the activation of AC and GC activity *in vivo* after stimulation of cells with cAMP and folic acid, respectively (18, 19, 21–24). The results shown in Table I indicate that AC activity of DdGCA^{kqd} in lysates from *gα2⁻* or *gα4⁻* cells are still stimulated by GTPγS. It can be argued that *in vitro* GC activity in *gα2⁻* cells is regulated by GTPγS via Gα4 and vice versa. Therefore, a double null cell line was made, in which both Gα2 and Gα4 were deleted. This cell line still shows *in vitro* activation of endogenous GC activity by GTPγS (data not shown). Upon expression of DdGCA^{kqd}, stimulation of AC activity by GTPγS was observed (Table I). The results imply that Gβ, Gα2, and Gα4 are not essential for the GTPγS stimulation of DdGCA^{kqd} in *Dictyostelium* lysates, making the direct involvement of G-proteins unlikely.

DISCUSSION

Dictyostelium GCA is an unusual GC because it has the topology of an AC and is activated by GTPγS. We have converted DdGCA to an AC, expressed it in *aca⁻* null cells that have no endogenous AC activity, and demonstrated that the obtained AC activity is still stimulated by GTPγS. This clearly demonstrates that GTPγS stimulates DdGCA not by binding in the catalytic site or being a better substrate but via a modulator such as a GTP-binding protein.

Substrate Recognition—For GCs it has been proposed that a glutamate (Glu-928 in GCE, see Fig. 2*B*) is the most important amino acid for specific recognition of GTP as it probably interacts with the N-1 nitrogen of the guanine ring of GTP. This hypothesis has been supported experimentally, because mutant GCs without this glutamate have no GC activity (47, 48). This glutamate is also present in evolutionary distant GCs, such as GCs from *Paramecium*, *Plasmodium* (14, 15), and *Dictyostelium* GCA (13), which most likely have evolved from ACs independently from the traditional GCs. In ACs the counterpart of this glutamate is a lysine (Lys-938 in ACII) that interacts with the N-1 of the P-site inhibitor 2′-deoxy-3′-AMP in the x-ray structure (1). Mutation of the glutamic acid to a lysine (E440K yielding DdGCA^k) caused GC activity to be abandoned but did not yield detectable AC activity (data not shown).

Two other amino acids expected to be important for nucleotide specificity are an arginine (Arg-998 in GCE) and cysteine (Cys-1000 in GCE) in GCs; their counterparts in ACs are a glutamine (Glu-1016 in ACII) and an aspartate (Asp-1018 in

ACII), respectively. The cysteine in GCs (Cys-1000 in GCE) is expected to stabilize the double bound oxygen of GTP (3, 47, 48). The aspartate counterpart in AC has been shown to form a hydrogen bond with the N-6 of 2′-deoxy-3′-AMP and is expected to do the same with ATP. At the position of the very conserved cysteine present in nearly all GCs, DdGCA has a histidine (His-504 in DdGCA), making this a very unusual GC (Fig. 2*B*). We expect the N^1H of histidine to still stabilize the double bound oxygen of GTP. The arginine of GCs (Arg-998 in GCE) is speculated to be important for proper orientation of the glutamate (47). The function of these amino acids in ATP and GTP recognition are supported by mutagenesis studies of mammalian and *Paramecium* cyclases (16, 47, 48).

In addition to mutating the glutamate at position 440 to a lysine, we additionally mutated the serine at position 502 to a glutamine and a histidine at position 504 to an aspartic acid. Expression of the resulting mutant, DdGCA^{kqd}, in *aca*$^-$ yields strong AC activity but no GC activity. This shows that DdGCA is a GC with a similar structure of the catalytic pocket as in mammalian ACs and GCs. Predictions based upon that structure proved to be correct, suggesting that the unusual histidine side chain of DdGCA is actually located in the catalytic cleft as we speculated previously (13). Second, the results imply that the amino acids that determine substrate specificity of DdGCA are located in the C1 domain, unlike mammalian ACs where they are located in the C2 domain, giving experimental proof of the previously observed inverted domain localization within the protein if compared with mammalian ACs.

The mutated DdGCA^{kqd} provides the possibility of studying the regulation of DdGCA in great detail by expressing the mutant DdGCA^{kqd} in *aca*$^-$, which has no background AC activity. GC activity in *Dictyostelium* is inhibited by calcium, probably via a calcium-binding protein like GCAP (26, 35, 49), whereas *Dictyostelium* AC activities are Ca^{2+}-insensitive (50, 51). As shown in Fig. 5, the AC activity of DdGCA^{kqd} is still inhibited by calcium, indicating that in cell lysates the interaction of DdGCA^{kqd} with other proteins is not disrupted by the mutations. Furthermore, cAMP induces a fast 4-fold increase of cAMP levels in *aca*$^-$/DdGCA^{kqd} cells, indicating that the mutated DdGCA is still activated by the G-protein-coupled cAMP receptor. The activation of DdGCA^{kqd} shows the kinetics of activation of DdGCA with a maximum after about 10 s, which is very different from the activation of the adenylyl cyclase ACA, which peaks at about 90 s after stimulation. Despite the differences in activation kinetics of ACA and DdGCA^{kqd}, the expression of DdGCA^{kqd} restores largely the aggregation minus phenotype of *aca*$^-$ cells. This suggests that cAMP production *per se* is sufficient to induce cAMP signaling, as was shown previously by expression of the constitutively active DdACG, an AC normally expressed only during late development in the spores (29).

Activation of DdGCA by GTPγS—All 12 transmembrane-spanning ACs are regulated by heterotrimeric G-proteins (4–6) in opposition to the soluble or membrane-bound GCs. DdGCA has the topology of G-protein-regulated Acs, and ample evidence suggests its regulation by G-proteins, because activation of GC activity by cAMP *in vivo* requires the presence of the single Gβ and the Gα$_2$ subunit. GC activity in the DdGCA overexpressor strains is stimulated by the presence of GTPγS (Fig. 1), as has been shown previously for GC activity of wild-type strains (26, 52). This activation by GTPγS may suggest regulation of DdGCA by a G-protein, because activation occurs at very low GTPγS concentrations ($K_a \sim 5$ μM). However, GTPγS is a substrate of DdGCA ($K_m = 200$ μM), and binding of GTPγS in the catalytic site might stabilize the enzyme or increase activity for GTP hydrolysis in an unknown manner.

However, in the DdGCA^{kqd} mutant, which does not recognize GTP in the catalytic site, GTPγS still stimulates enzyme activity. The V_{max} or K_m of ATP for DdGCA^{kqd} and of GTP for DdGCA differ by less than 2-fold, indicating that the mechanism of catalysis was not severely affected by the mutations. This could be expected because the mutations are located in the region of the catalytic pocket where the purine is bound, which is relatively far away from the region with the catalytic amino acids. In both wild-type DdGCA and mutant DdGCA^{kqd}, GTPγS results in an increase of V_{max} and reduction of K_m, suggesting the mechanism of activation by GTPγS is not affected.

GTPγS stimulates DdGCA and DdGCA^{kqd} with a K_a of about 5 μM, which is 50-fold lower than the K_m of DdGCA for GTPγS, indicating that the stimulatory effect is mediated by a site with high affinity for GTPγS. In our perception there are two obvious candidates: 1) GTPγS activates heterotrimeric G-proteins, and one of the Gα subunits or the Gβγ subunit activates the enzyme; 2) GTPγS activation of DdGCA is mediated by a monomeric G-protein. Either way means that DdGCA is regulated by a modulator that is also the substrate.

DdGCA Stimulation by Heterotrimeric or Monomeric G-proteins?—In addition to DdGCA, other effectors are known to be activated via the cAR1 receptor *in vivo*, *e.g.* DdACA and DdPLC. In lysates from *gα2*$^-$ cells, GTPγS no longer stimulates phospholipase C activity, indicating the involvement of Gα$_2$ in this activating path (53). On the other hand, ACA stimulation by GTPγS is still present in lysates from *gα2*$^-$ cells but lost in lysates from *gβ*$^-$ cells, suggesting the Gβ subunit mediates activation of ACA (17). For GC activation the mechanism seems more complicated, because *in vivo* experiments show that Gβ is essential in combination with either Gα$_2$ for cAMP stimulation or Gα$_4$ for folic acid stimulation (17, 24, 54). However, *in vitro* neither of the three subunits is necessary to obtain GTPγS activation of endogenous GC activity or AC activity by ectopic expression DdGCA^{kqd}. These data indicate that none of the heterotrimeric G-protein subunits known to be essential for GC activation *in vivo* is a mediator of GTPγS activation *in vitro*. The biochemical properties of GC regulation by GTPγS and other guanine nucleotides have been studied previously (26, 52, 55). GDPβS inhibits GTPγS-mediated activation of GC. GTP has no effect but inhibits when added before GTPγS. These data suggest that the protein mediating GTPγS stimulation of GC rapidly hydrolyses GTP and slowly releases bound GDP; activation is possible only with the non-hydrolyzable GTP analogue. These properties are very different for the regulation of *Dictyostelium* adenylyl cyclase ACA, where both GTP and GTPγS stimulate the enzyme via the heterotrimeric G-protein Gα$_2$βγ (17, 20) but are similar to the regulation of actin polymerization in *Dictyostelium* and leukocytes mediated by the monomeric G-protein Cdc42 (56). The biochemical and genetic data on GC regulation in *Dictyostelium* are consistent with the essential role of heterotrimeric G-protein for cAMP-mediated activation *in vivo*, and monomeric G-protein for GTPγS-mediated activation *in vitro*, which leads to the following model. Extracellular cAMP or folic acid bind to surface receptors thereby activating the heterotrimeric G-proteins Gα$_2$βγ and Gα$_4$βγ, respectively. The activated Gα subunits activate a monomeric GTP-binding protein that stimulates GC activity. Regulation of a monomeric G-protein by a heterotrimeric G-protein might be more common, *e.g.* in the yeast *Saccharomyces cerevisiae* a Gβγ recruits Far1p leading to the activation of Cdc42p (57); other studies in fibroblasts, COS-7 cells, and human airway smooth muscle cells show that G$_i$ mediates the activation of p21^{ras} (58–60).

In *Dictyostelium* an unexpectedly large number of Ras and Rho GTPases have been identified (61, 62). The heterotri-meric-monomeric G-protein model for GC regulation in *Dictyostelium* may help to resolve the pathway and function of cGMP as well as the function of monomeric G-proteins in *Dictyostelium*.

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