The G Protein β Subunit Is Essential for Multiple Responses to Chemoattractants in *Dictyostelium*

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Abstract. Increasing evidence suggests that the βγ-subunit dimers of heterotrimeric G proteins play a pivotal role in transducing extracellular signals. The recent construction of Gβ null mutants ($g\beta^-$) in *Dictyostelium* provides a unique opportunity to study the role of βγ dimers in signaling processes mediated by chemoattractant receptors. We have shown previously that $g\beta^-$ cells fail to aggregate; in this study, we report the detailed characterization of these cells. The $g\beta^-$ cells display normal motility but do not move towards chemoattractants. The typical GTP-regulated high affinity chemoattractant-binding sites are lost in $g\beta^-$ cells and membranes. The $g\beta^-$ cells do not display chemoattractant-stimulated adenylyl cyclase or guanylyl cyclase activity. These results show that in vivo Gβ links chemoattractant receptors to effectors and is therefore essential in many chemoattractant-mediated processes. In addition, we find that Gβ is required for GTPγS stimulation of adenylyl cyclase activity, suggesting that the βγ-dimer activates the enzyme directly. Interestingly, the $g\beta^-$ cells grow at the same rate as wild-type cells in axenic medium but grow more slowly on bacterial lawns and, therefore, may be defective in phagocytosis.

Heterotrimeric guanine nucleotide–binding proteins (G proteins), composed of α, β, and γ subunits, play a central role in coupling surface receptors to intracellular effectors. When occupied by agonists, the activated receptors catalyze the exchange of GDP for GTP on the α subunit of the G protein with the concomitant dissociation of the α subunit from the βγ subunit dimer. In mammals, about twenty α subunits, four β subunits, and seven γ subunits have been identified (Simon et al., 1991; Birnbaumer, 1992; Clapham and Neer, 1993). These subunits can presumably combine with each other to form a large variety of heterotrimers.

βγ-subunit dimers initially were viewed only as attenuators of α activity, but recently they have come into the limelight as playing an active role in transmitting signals. They can directly activate certain effectors such as adenylyl cyclase, phospholipase C (PLC)[1], and ion channels (for reviews, see Clapham and Neer, 1993; Birnbaumer, 1992) and they may specify receptor interaction (Kleuss et al., 1992). For example, βγ subunits stimulate mammalian adenylyl cyclase subtypes II and IV in vitro (Tang and Gilman, 1991). Expression of the type II adenylyl cyclase in COS cells renders it sensitive to stimulation by α-adrenergic ligands presumably via release of βγ from Gi (Federman et al., 1992). In addition, it has been shown that βγ subunits play a role in activation of certain isoforms of PLC in chemotactic cells such as leukocytes (Camps et al., 1992; Katz et al., 1992). The first genetic evidence that the βγ dimer plays an active role in signaling came from studies in yeast where it transmits the mating signal to the MAP kinase pathway; mutants lacking the α subunit are constitutively active.

The G protein–linked signal transduction strategy plays an essential role in the developmental program of *Dictyostelium* (Devreotes, 1994; Firtel, 1991; Wu et al., 1993). In this program, individual amoebae aggregate to form multicellular structures which undergo morphogenesis and differentiation. This spontaneous process is organized by extracellular cAMP that binds to surface cAMP receptors (cARs), which in turn evoke numerous physiological responses. Genes encoding four cARs (Klein et al., 1988; Saxe et al., 1991*a,b*), eight G protein α subunits (Pupillo et al., 1989; Hadwiger et al., 1991; Wu and Devreotes, 1991; Wu et al., 1994; Pupillo, M., and P. N. Devreotes, manuscript in preparation) and one β subunit (Lilly et al., 1993), two adenylyl cyclases (Pitt et al., 1993), and one phospholipase C_δ (PLC) (Drayer et al., 1993) have been identified. Genetic analyses indicate that many of these components serve critical functions in the signaling processes and in controlling development. For example, coupling of cAR1

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1. *Abbreviations used in this paper:* cAR, cAMP receptors; CRAC, cytosolic regulator of adenylyl cyclase; $g\beta^-$, Gβ null mutants; PH domain, pleckstrin homology domain; PLC, phospholipase C.

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to G2 is essential for early development (Kumagai et al., 1989, 1991) while G4 is required for late development and folate chemotaxis (Hadwiger and Firtel, 1992; Hadwiger et al., 1994).

The recent construction of Gβ null mutants ($g\beta^-$) provides a unique opportunity to study the role of βγ dimers in chemotaxis and cell–cell signaling, to determine whether there are receptor-mediated, G protein–independent responses, and to carry out a genetic analysis of βγ function. The Gβ gene is expressed constantly during growth and development. It is highly homologous to the mammalian β subunits, and low stringency hybridization studies indicate that there are no close homologues in *Dictyostelium* (Lilly et al., 1993). We have shown that $g\beta^-$ cells fail to aggregate and differentiate. In this study, we report the detailed characterization of $g\beta^-$ cells. Not surprisingly, we find that the Gβ is required to couple cAR1 to G2 and is therefore essential in many agonist-mediated processes. In addition, we find that Gβ is required for GTPγS stimulation of adenylyl cyclase activity, suggesting that the βγ dimer activates ACA directly. We speculate that βγ subunits may directly activate a variety of effectors.

Materials and Methods

Cell Growth and Development

Cells were grown either in HL5 media or on SM nutrient agar plates in association with *K. aerogenes* at 22°C as described (Watts and Ashworth, 1970). JH10 (a thymidine auxotrophic mutant, Hadwiger and Firtel, 1992) and DH1 (a uracil auxotrophic mutant, Caterina et al., 1994) cells were supplemented with 100 μg/ml thymidine and 20 μg/ml uracil, respectively. To score for developmental phenotypes and determination of plaque sizes, cells were plated for individual clones (~40–50 cells per 10-cm SM/Ka plate) and incubated for 5–7 d at room temperature. Development in shaking culture was carried out by pulsing cells with 75 nM cAMP at 6-min intervals as described (Devreotes et al., 1987).

Construction of $g\beta^-$ Cells

We reported previously the construction of $g\beta^-$ cells (LW5) using JH10 parent, a thymidine auxotrophic cell line (Lilly et al., 1993). We noted that JH10 cells do not always have robust development, especially during the later stages of development. Therefore, an independent $g\beta^-$ cell line was created using DH1, a uracil-deficient cell line which exhibits growth and development characteristics more similar to wild type. The Gβ gene was disrupted via homologous recombination, in the same way as previously described except that a *URA* marker was used. The $g\beta^-$ cell line created in this host (LW6) will subsequently be referred to as $g\beta^-$. For most of the experiments described, both LW5 and LW6 were tested.

Plasmid Construction and Other Recombinant DNA Techniques

Plasmids were constructed using standard cloning techniques (Maniatis et al., 1982). For construction of Gβ expression vectors, full-length Gβ cDNA was cloned into the extrachromosomal vector pJK1 (Pitt et al., 1992) or the integrating vector B18 (Johnson et al., 1991). In both vectors, the inserted gene was driven by an actin promoter and terminator. Other recombinant techniques were carried out as described by Maniatis et al. (1982).

Immunoblot Analysis

Total cellular proteins and ammonium sulfate–extracted membrane proteins (Theibert et al., 1984) were analyzed by immunoblot according to the standard procedures using ^{125}I-labeled protein A (Towbin et al., 1979) or an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL) as described by the manufacturer.

Chemotaxis

Small population assays were used to measure chemotaxis to cAMP, folic acid, bacteria, and urine as described (Konijn, 1970; Devreotes et al., 1987). Cells were resuspended in PB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 6.1) at a density of 5×10^6 cells/ml. Small droplets (0.1 μl) were deposited on the surface of 1% washed agar, giving a final radius of 0.3 μm. After 30 min (vegetative cells) or 6 h (starved cells), test solutions (0.1 μl) were deposited close to the small populations of amoebae. The distribution of the amoebae within at least 20 small droplets was observed at 5–10-min intervals. The response was scored positive if at least twice as many amoebae were pressed against the edge closest to the test solution as to the opposite edge. Additionally, the agar-cutting assay was also used to measure chemotaxis to cAMP and folic acid (Kuwayama et al., 1993). Cells were inoculated in the center of a SM/3 plate on lawn of *E. coli*. After a few days of incubation at 21°C, a 4-mm colony was formed, which contained both vegetative and aggregative amoebae. A 0.5 × 2-mm agar block aligned radially through the edge of the colony was excised and placed upside down on the surface of a 1% purified agar plate containing 100 μM folic acid or 1 μM cAMP. Due to secretion of folic acid deaminase and cAMP phosphodiesterase, folic acid and cAMP are degraded under the agar block giving rise to gradients of folic acid and cAMP. After 1 h of incubation, the dispersion of mutant cells was compared with that of control cells. The response was scored positive if cells were dispersed at least twice as far from the agar block as on control agar containing no folic acid or cAMP.

^{3}H-cAMP Binding and Scatchard Analysis

Cells developed for 6 h were washed and resuspended to 10^8/ml in PB. ^{3}H-cAMP binding in 3 M ammonium sulfate was carried out essentially as described (Van Haastert, 1985; Johnson et al., 1991) in the presence of 10 mM DTT. For ^{3}H-cAMP binding on intact cells in PB, the silicone oil spin assay was used (Van Haastert, 1984). ^{3}H-cAMP binding to membranes in the presence or absence of 0.1 mM GTP was carried out as described (Caterina et al., 1994; Van Haastert, 1984) except that the membranes were resuspended in 10^8 cell equivalents/ml. Each binding assay was done in triplicate. Scatchard plots were generated and the data analyzed by LIGAND (Munson and Rodbard, 1980). Nonspecific binding was presubtracted and set to zero in the program. No other initial conditions were fixed.

Guanylyl Cyclase Assays and Adenylyl Cyclase Assays

The amount of cGMP produced upon stimulation by 1 μM cAMP (developed cells) or 10 μM folic acid (vegetative cells) were determined by isotope-dilution assay (Kuwayama et al., 1993). In vitro guanylyl cyclase assays were performed as described by Kuwayama et al. (1993). The in vivo and in vitro adenylyl cyclase assays were carried out as described (Pupillo et al., 1992).

Results

$g\beta^-$ Cells Are Aggregation-Deficient and Form Small Plaques on Bacterial Lawns

We have previously reported that $g\beta^-$ cells do not aggregate and differentiate (Lilly et al., 1993). They remain as a smooth monolayer of cells on either starvation agar plates or on nutrient agar plates in association with bacterial lawns. The relative growth rate of $g\beta^-$ cells was measured in shaking culture in axenic medium or on bacterial lawns by determining cell numbers for 2–3 d after inoculating with $g\beta^-$ or wild-type cells. The $g\beta^-$ cells grew at the same rate as wild-type cells in axenic medium (doubling time ~12 h), but grew about two times more slowly than wild-type cells on bacterial lawns (doubling times ~4.5 h and ~9 h, respectively). As an apparent correlation, the sizes of the $g\beta^-$ clonal plaques formed in the lawns were smaller than those of wild type (Fig. 1). All of the growth and de-

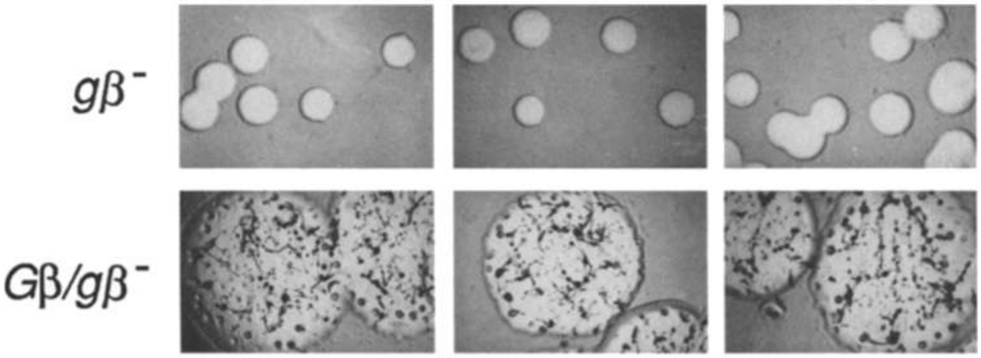

Figure 1. Phenotype of $g\beta^-$ cells and $g\beta^-$ cells rescued by Gβ cDNA on SM/Ka. The cells were mixed with *K. aerogenes* and plated on SM nutrient agar plates for clonal plaques. The photograph was taken 1 week after the plating.

velopmental defects of the $g\beta^-$ cells were completely reversed by transformation with the Gβ cDNA in a variety of expression plasmids where the inserted gene was driven by a constitutively expressed actin-15 promoter (Fig. 1). Therefore, all of the defects are specifically caused by the absence of the Gβ gene.

The aggregation defect in the $g\beta^-$ mutants is strongly cell autonomous. A class of aggregation mutants, designated as "*synag*," failed to differentiate in isolation, but can form spores when developed in a chimeric mixture with wild-type cells. Synergy is an indication of the ability of these mutants to sense and respond to the signals provided by the wild-type cells. The $g\beta^-$ cells completely failed to synergize with the wild-type cells; examination of over 1,000 spores from 50:50 mixtures revealed no $g\beta^-$ spores. Observation of the chimeric mixtures indicated that the $g\beta^-$ cells did not coaggregate with the wild-type cells. These observations suggest that the $g\beta^-$ cells cannot respond appropriately to extracellular cAMP stimuli.

To examine whether the absence of Gβ causes the lack of expression of other essential components which in turn lead to the defective aggregation observed in the $g\beta^-$ cells, the expression of a number of early genes was examined. Fig. 2 shows an immunoblot analysis of cAR1, Gα2, and ACA, isolated from vegetative or pulse-developed cells. All of these proteins were induced appropriately and there was only a slight difference in the levels between $g\beta^-$ and wild-type cells, suggesting that the phenotypic defects in $g\beta^-$ cells are not pleiotropic effects.

It has been noted, however, that the level of cAR1 in $g\beta^-$ cells could vary greatly depending on the growth conditions and the age of $g\beta^-$ cells. Specifically, when cultures were maintained in axenic medium in petri dishes for 3–4 wk, the level of cAR1 appearing within 6 h after starvation was significantly diminished. The level of cAR1 expression was also severely affected if cells were grown for more than a week in shaken axenic medium. Therefore, for all the experiments reported here, a fresh culture of $g\beta^-$ cells was always used. However, even when cAR1 was constitutively expressed in the $g\beta^-$ cells, it did not detectably alter the phenotypes described here.

$g\beta^-$ *Cells Are Generally Nonchemotactic*

Aggregation-stage wild-type cells display strong chemotactic responses to cAMP, while vegetative-stage cells carry out chemotaxis to folic acid as well as to unidentified components in bacterial extracts and urine. Genetic analyses have shown that G protein α subunits are required for chemotactic responses to cAMP and folic acid. Gα2 is essential for cAMP chemotaxis mediated by either cAR1 or cAR3. However, chemotaxis to other chemoattractants such as folic acid remains intact in $g\alpha 2^-$ cells (Coukell et al., 1983; Kumagai et al., 1991). Conversely, Gα4 is not required for chemotaxis to cAMP but is essential for chemotaxis to folic acid (Hadwiger et al., 1994).

Chemotaxis to a variety of chemoattractants was examined in wild-type and $g\beta^-$ cells. These responses are typically measured by the small population assay (Konijin, 1970; Devreotes et al., 1987) and agar-cutting assay (Kuwayama et al., 1993). We tested cAMP in the range from 10^{-10} to 10^{-3} M, folic acid in the range from 10^{-8} to 10^{-3} M, and a wide range of dilutions of the other chemoattractants. In multiple trials using these assays, the $g\beta^-$ cells never displayed a detectable chemotaxis response to any of these chemoattractants. In these tests, the motility of the $g\beta^-$ cells appeared to be similar to that of wild-type cells. However, unlike wild-type cells, the $g\beta^-$ cells did not extend pseudopods towards cAMP released from micropi-

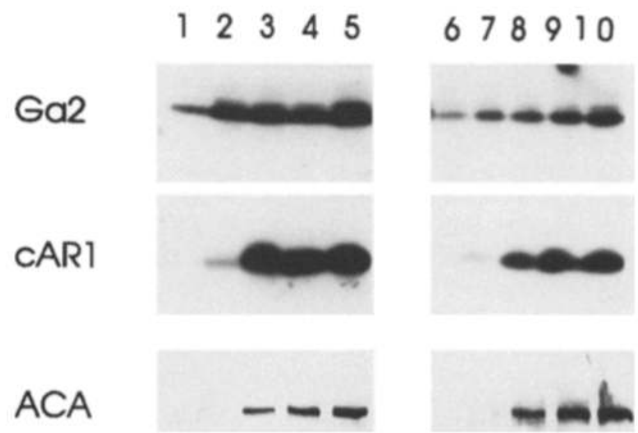

Figure 2. Western blot of cAR1, Gα2, and ACA in wild-type and $g\beta^-$ cells. Cells were either grown in HL-5 medium or developed in DB with pulses of 75 nM cAMP at 6-min intervals for various times. Membrane proteins were extracted by ammonium sulfate, run on SDS-PAGE, and subjected to immunoblot. Lanes *1–5*, proteins isolated from wild-type cells; *6–10*, from $g\beta^-$ cells. The length of the development is: 0 h (lanes *1* and *6*); 3 h (lanes *2* and *7*); 4 h (lanes *3* and *8*); 5 h (lanes *4* and *9*); and 6.5 h (lanes *5* and *10*).

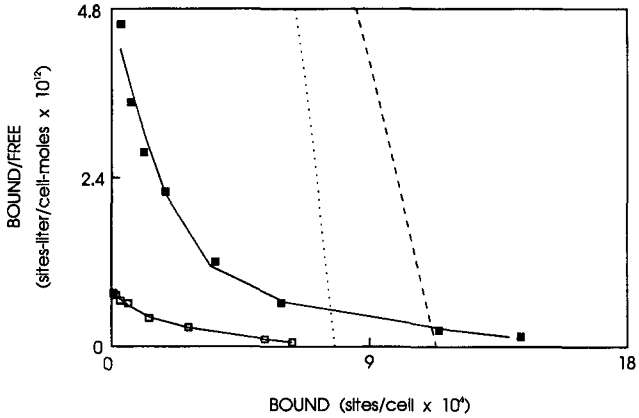

Figure 3. Scatchard analysis of ^{3}H-cAMP binding to intact cells in ammonium sulfate (*in dashed lines*, -----, wild type; ·····, gβ^{-}) and phosphate buffer (*solid squares*, wild type; *open squares*, *gβ*$^{-}$). Receptor affinity was determined by the binding of ^{3}H-cAMP to cells in ammonium sulfate or phosphate buffer in the presence of increasing amounts of cAMP. The data shown are means of a single experiment with triplicate determinations. Two other independent experiments were done and the similar results were obtained.

pets (Ecke, M., personal communication). These results suggest that the Gβ subunit is required specifically for appropriate transduction of signals from the receptors for all of the chemoattractants tested.

gβ^{-} *Cells and Membranes Do Not Display GTP-regulated High Affinity cAMP-Binding Sites*

The affinity and number of cAMP-binding sites were determined for *gβ*$^{-}$ cells and wild-type cells in parallel. ^{3}H-cAMP binding to intact cells under physiological conditions and in the presence of ammonium sulfate was determined and the data analyzed by Scatchard plots. Ammonium sulfate uniformly increases the affinity of all of the cAMP-binding sites to ~5 nM and thereby facilitates the detection of the total number of binding sites (Van Haastert, 1985). The *gβ*$^{-}$ cells display essentially wild-type binding characteristics under this condition; a single binding component was detected for both wild-type and *gβ*$^{-}$ cells (Fig. 3, Table I). The binding affinities for the wild-type and *gβ*$^{-}$ cells are 5.7 nM and 2.9 nM, respectively. Consistent with the immunoblot results, these data show that these *gβ*$^{-}$ cells express nearly wild-type levels of cAR1.

When cAMP binding to intact cells was measured under physiological conditions (10 mM phosphate buffer), the Scatchard plot of the wild-type cells was curvilinear and could be approximated by assuming two affinities of 5.4 nM and 268 nM, respectively. For the *gβ*$^{-}$ cells, the overall affinity of the receptors was lower. The lower affinity binding component in *gβ*$^{-}$ cells was similar to that of wild-type cells (compare kD of 254 nM for *gβ*$^{-}$ and 268 nM for wild-type cells). The highest affinity sites were lacking and a small fraction of the binding (~11% of the total binding sites) displayed an intermediate affinity (16 nM) (Fig. 3, Table I). These results indicate that Gβ is required for the cAR1 on intact cells to display the appropriate high affinity–binding sites under physiological conditions.

To determine whether the altered binding properties of *gβ*$^{-}$ cells are due to the lack of interaction between cAR1 and a G protein, the effects of GTP on the cAMP binding to isolated membranes were assessed. In wild-type membranes, the presence of 100 μM GTP greatly inhibits the binding to 20 nM cAMP (Kesbeke et al., 1988). The GTP effects are attributed primarily to G2 since in *gα2*$^{-}$ cells, they are substantially mitigated (only ~10% vs 70–80% inhibition). Membranes of wild-type and *gβ*$^{-}$ cells were prepared and binding at 20 nM ^{3}H cAMP measured in the presence and absence of 100 μM GTP. In wild-type membranes, addition of the nucleotide reduced cAMP binding by 80%, whereas in the membranes of *gβ*$^{-}$ cells, GTP had little effect (less than 5% inhibition). These data suggest that a Gβ subunit is essential for maintaining the appropriate coupling between cAR1 and a G protein, presumably G2.

To examine whether the high or low affinity binding sites were affected in these experiments, Scatchard analyses were performed on membranes in the presence or absence of GTP (Fig. 4, Table II). As indicated in Table II, wild-type membranes displayed two binding sites with affinities of 4.8 nM and 467 nM, respectively, in the absence of GTP. Approximately 11% of the sites displayed the higher affinity. When GTP was added to the binding reaction, the high affinity binding component was completely lost and the affinity of the remaining site was 450 nM. In the membranes of *gβ*$^{-}$ cells, only the low affinity binding component (kD = 404 nM) was detected even in the absence of GTP; the addition of GTP had no effect (kD = 378 nM). These results suggest that the Gβ subunit is required for cAR1 to display its GTP-sensitive high affinity binding component.

gβ^{-} *Cells Do Not Display cAMP- or Folic Acid–stimulated cGMP Accumulation*

Chemoattractants elicit a rapid increase in intracellular cGMP levels which is correlated with the redistribution of myosin heavy chain from the cytosol to the cell cortex (for review see Van Haastert and Devreotes, 1993). In our ex-

Table I. cAMP Binding in Phosphate Buffer and Ammonium Sulfate

	Ammonium sulfate		Phosphate buffer			
			kD		Sites/cell (×10^{3})	
Cell	kD	Sites/cell (×10^{3})	Site 1	Site 2	Site 1	Site 2
	nM		*nM*			
WT	5.7 ± 0.4	112 ± 5.6	5.4 ± 1.4	268 ± 80	23 ± 5.1	144 ± 13
gβ^{-}	2.9 ± 0.5	78 ± 7.0	16 ± 3.8	254 ± 46	8.6 ± 2.6	67 ± 2.0

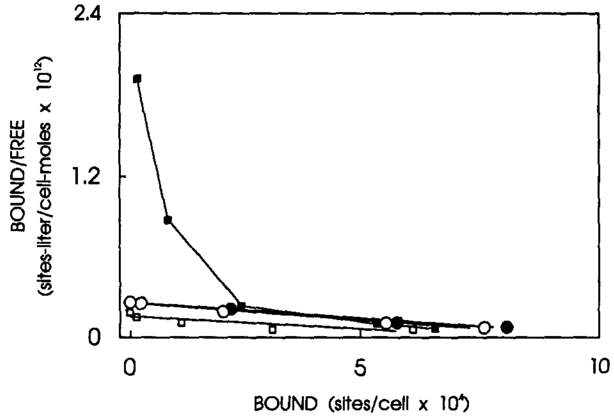

Figure 4. Scatchard analysis of ^{3}H-cAMP binding to membranes in the presence or absence of GTP. Membranes were prepared by filter-lysis and the binding was carried out in the presence of increasing amount of cAMP with or without 100 μM GTP. *Squares,* wild-type cells; *circles, gβ⁻* cells. *Solid symbols,* in the absence of GTP; *open symbols,* in the presence of GTP. The data shown are means of two independent experiments with triplicate determinations.

periments, when vegetative cells were stimulated with 10 μM folic acid, the cGMP levels in wild-type cells typically increased about twofold within 10 s of simulation and then declined to prestimulated levels within 60 s. Under the same conditions, however, there was no detectable change in the cGMP levels in the *gβ⁻* cells (data not shown).

When aggregation competent cells were stimulated with 0.1–1 μM cAMP, wild-type cells displayed a rapid accumulation of cGMP. As shown in Fig. 5 *A*, the intracellular cGMP level peaked (~3–5-fold induction) around 10 s after stimulation and then gradually declined. In contrast, no response was observed in the *gβ⁻* cells under the same conditions. We also noted that the basal level of cGMP in the *gβ⁻* cells is generally lower than that in wild-type cells. These results demonstrate that Gβ is required for cGMP production stimulated by both cAMP and folic acid.

It has been previously reported that the guanylyl cyclase activity was stimulated about threefold by GTPγS in wild-type cell lysates (Janssens et al., 1989). As shown in Fig. 5 *B*, a similar level of stimulation was observed in *gβ⁻* cells. These cells also seem to have an elevated basal level activity. In addition, 1 μM Ca^{++} greatly inhibits the guanylyl cyclase activity in both wild-type and *gβ⁻* cells.

The gβ⁻ Cells Lack cAMP- and GTPγS-stimulated Adenylyl Cyclase Activity

The central role of Gα2 in the signal transduction via

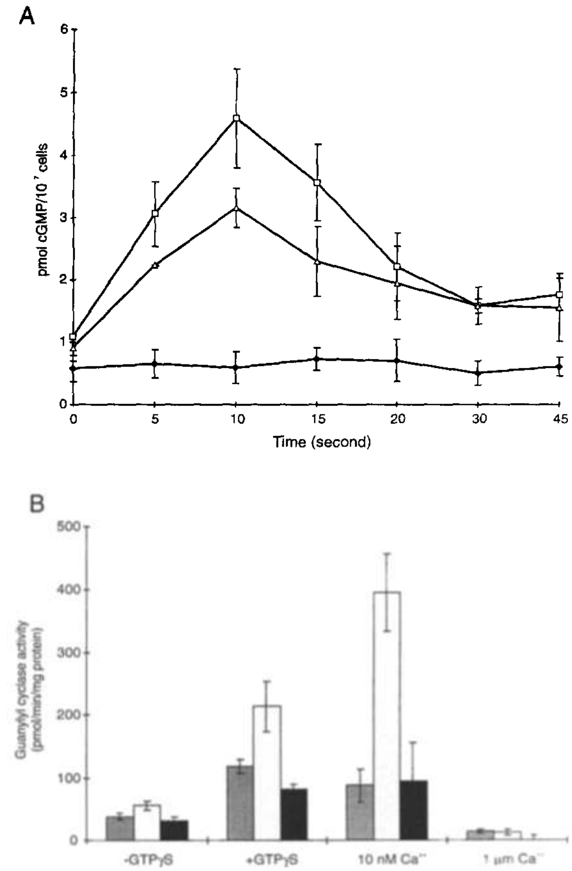

Figure 5. Guanylyl cyclase assay in vivo (*A*) and in vitro (*B*). (*A*) Cells pulsed with cAMP for 5 h were stimulated by 1 μM cAMP and lysed by acid. The cGMP content was determined in the neutralized lysates by isotope-dilution assay. *Triangles, gβ⁻* rescued by Gβ cDNA; *open squares,* an independent wild-type control (a random integrant); *solid symbol, gβ⁻* cells. (*B*) Cells were lysed in the presence or absence of GTPγS and assayed for guanylyl cyclase activity as described. The assay was also performed with different calcium concentrations. *Shaded bars,* wild-type AX2; *open bars, gβ⁻*; *solid bars,* an independent wild-type control (a random integrant). The data shown are means of two independent experiments with triplicate determinations.

cAR1 was demonstrated by its involvement in the activation of adenylyl and guanylyl cyclases, chemotaxis, and in the regulation of the affinity of ^{3}H-cAMP binding. However, these experiments do not indicate whether signals are transmitted via the α or βγ subunits of G2. In fact for adenylyl cyclase, Gα2 does not appear to be the direct ac-

Table II. cAMP Binding to Membranes in the Presence or Absence of GTP

	−GTP				+GTP			
	kD		Sites/cell (×10³)		kD		Sites/cell (×10³)	
Cell	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
	nM					*nM*		
WT	4.8 ± 0.3	467 ± 42	9.9 ± 0.5	80 ± 3.2	–	450 ± 158	–	71 ± 19
gβ⁻	–*	404 ± 24	–	107 ± 4.3	–	378 ± 27	–	98 ± 4.9

*–, analysis by ligand indicated that data was accurately fit by single site and could not be fit to two-site model.

tivator of the enzyme. Although $g\alpha2^-$ cells lack any cAMP-stimulated activation of adenylyl cyclase, lysates of $g\alpha2^-$ cells display an essentially wild-type response to GTPγS stimulation of adenylyl cyclase activity (Kesbeke et al., 1988; Pupillo et al., 1992).

The activation of adenylyl cyclase in vivo and in vitro in wild-type and $g\beta^-$ cells was examined. As shown in Fig. 6 *A*, upon cAMP stimulation, wild-type cells rapidly responded by activating adenylyl cyclase; the activation peaks at 1–2 min and then declines. In contrast, the $g\beta^-$ cells, like the $g\alpha2^-$ cells, did not show any receptor-mediated activation of adenylyl cyclase. As expected, the $g\beta^-$ cells did not produce or secrete cAMP in response to cAMP stimuli (data not shown).

We next examined the direct activation of the adenylyl cyclase in lysates stimulated with GTPγS. As previously reported, in our experiments the presence of GTPγS in lysates of wild-type or $g\alpha2^-$ cells stimulated the adenylyl cyclase activity by ~20-fold. In striking contrast to the observations on $g\alpha2^-$ cells, GTPγS completely failed to activate adenylyl cyclase in the lysates of the $g\beta^-$ cells (Fig. 6 *B*).

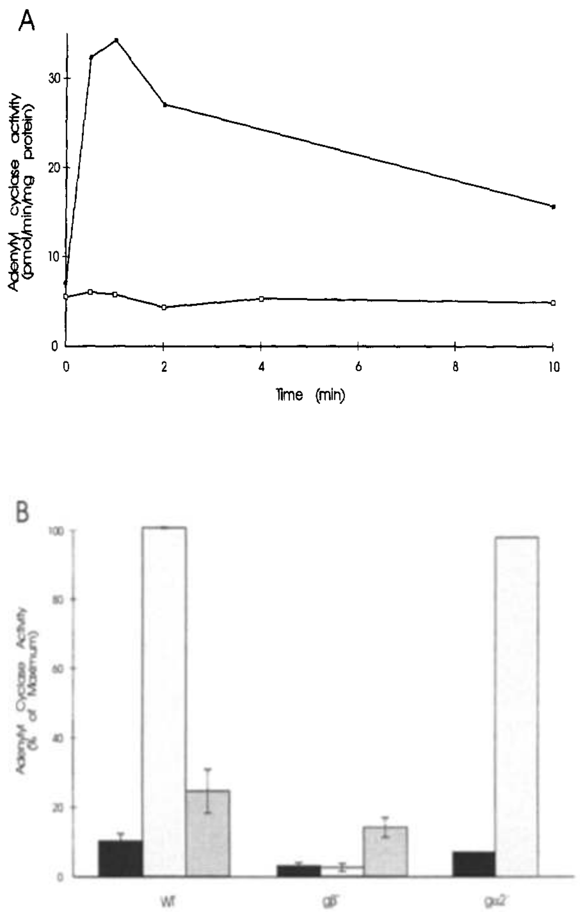

Figure 6. Adenylyl cyclase activity in vivo (*A*) and in vitro (*B*). (*A*) Wild-type (*solid symbol*) or $g\beta^-$ (*open symbol*) cells starved for 6 h were stimulated by cAMP and aliquots of cells were withdrawn to assay for adenylyl cyclase activity. (*B*) Cells were lysed in the presence or absence of GTPγS and adenylyl cyclase activity was measured. *Solid bars*, in the absence of GTPγS; *open bars*, in the presence of GTPγS; *shaded bars*, in the absence of GTPγS but presence of Mn^{++}. The presence of Mn^{++} measures the nonregulated activity. The data shown for *A* are from one experiment with duplicate determinations. An independent experiment was done and similar results were obtained. The data for *B* are means from three independent experiments with duplicate determinations. Values for $g\alpha2^-$ cell lysate were retrieved from Pupillo et al. (1992).

To be certain that the lack of GTPγS-stimulation of adenylyl cyclase activity in $g\beta^-$ was not due to the absence of the other components in this pathway, we assessed the levels of cAR1, Gα2, and ACA by immunoblot and found that the $g\beta^-$ and wild-type cell lysates contained similar levels of these proteins (also see Fig. 2). Furthermore, the level of adenylyl cyclase activity in the presence of 5 mM Mn^{2+}, which detects relatively unregulated adenylyl cyclase activity, was only slightly lower in the $g\beta^-$ lysates compared to the wild-type lysates (Fig. 6 *B*).

Recently, a cytosolic activator of adenylyl cyclase, designated CRAC (cytosolic regulator of adenylyl cyclase), was identified (Insall et al., 1994). CRAC is essential for appropriate GTPγS stimulation of adenylyl cyclase. Lysates of CRAC null cells ($crac^-$) display only weak activation compared to those from wild-type cells (~50% vs ~20-fold). Addition of purified CRAC to the lysates of $crac^-$ cells restores full GTPγS activation of the enzyme (Lilly and Devreotes, 1994). We determined the amount of CRAC protein by immunoblot and assayed its activity by reconstitution of lysates of $crac^-$ cells; the amount and activity of CRAC are similar in lysates of $g\beta^-$ and wild-type cells (data not shown). These data suggest that the $g\beta^-$ cells contain adequate amounts of functional components in the adenylyl cyclase activation pathway.

Discussion

Dictyostelium contains eight G protein α subunits, one β subunit and as yet unidentified γ subunits. While the α subunits are transiently expressed at specific developmental stages, the single β subunit is constantly expressed and may participate in the formation of heterotrimers with all of the α subunits. If so, the Gβ-null cells ($g\beta^-$) should contain no functional G proteins. Therefore, it was somewhat surprising that we were able to construct these mutants. It suggests that heterotrimeric G proteins are not essential for progress around the cell cycle. Moreover, since the $g\beta^-$ cells grew at the same rate as wild-type cells in axenic medium, these signal transduction components appear not to be required for the diverse physiological processes required for growth. While we did not rigorously quantitate cell motility, our observations of the cells in time lapse videos suggest that Gβ is also not required for this process. The $g\beta^-$ cells do form smaller than wild-type plaques on bacterial lawns. This phenotype is consistent with our observation that the doubling time under this condition is longer than wild type. This defect may be an indication that Gβ plays a role in phagocytosis. We are currently assessing the capacity of the mutant cells to carry out phagocytosis.

Since the $g\beta^-$ cells should contain no functional G proteins, they should be completely unable to receive external signals through G protein–coupled pathways. In fact, we have shown in this report that the Gβ subunit is essential for multiple signaling responses. It has been reported previously that Gα2 is the main regulator of the cAMP-mediated pathways such as chemotaxis, guanylyl and adenylyl cyclase activation (Kumagai et al., 1989, 1991) and Gα4, on the other hand, mediates the folic acid–stimulated responses (Hadwiger et al., 1994). We found that the *gβ*⁻ cells not only lack all the major cAMP-mediated responses as do *gα2*⁻ cells, they also lack all the folic acid–stimulated processes as do *gα4*⁻ cells. Therefore, the *gβ*⁻ cells encompass both *gα2*⁻ and *gα4*⁻ phenotypes. Furthermore, the *gβ*⁻ cells are generally nonchemotactic, unable to sense any chemoattractants, which likely transmit signals via a variety of receptor-G protein units. These results are consistent with the hypothesis that this Gβ subunit interacts with every one of the eight Gα subunits to form functional heterotrimeric G proteins.

The absence of a chemoattractant-induced cGMP response in the *gβ*⁻ cells is most likely a sufficient defect to cause the absence of chemotaxis to any chemoattractants. Kuwayama et al. (1993) have isolated chemotaxis mutants by chemical mutagenesis. One of the isolated mutants (KI-8) has strongly reduced guanylyl cyclase levels, whereas another mutant (KI-10) has normal basal levels but no receptor-mediated guanylyl cyclase activity. In KI-10 mutants, as in wild type, actin is polymerized in response to the stimulus (Liu et al., 1993). The *gβ*⁻ cells fail to extend pseudopods in response to micropipet stimulation, suggesting that the stimulus does not even trigger actin polymerization. The KI-10 chemotaxis mutants also form normal size of plaques on bacterial plates, suggesting that the small plaque phenotype of *gβ*⁻ cells is probably not due to their inability to carry out chemotaxis to folic acid or other compounds secreted by bacteria. It more likely relates to the relatively slower growth rate on bacterial lawns.

Previously it was shown that GTPγS stimulates guanylyl cyclase in vitro (Janssens et al., 1989), possibly via a G protein or a regulatory site on the enzyme. It was also shown that Gα2 is required in vivo for the cAMP-mediated cGMP response, and we have shown in this report that Gβ is required for both cAMP- and folic acid–mediated cGMP responses. However, the GTPγS stimulates guanylyl cyclase in vitro normally in both *gα2*⁻ and *gβ*⁻ cell lysates (Fig. 5 *B* and data not shown). Taken together, these results suggest that transmission of a signal through a heterotrimeric G protein is essential for accumulation of cGMP in response to chemoattractants. However, the effects of GTPγS on the enzyme do not allow us to determine whether the α2 subunit or the βγ dimer is the direct activator of the guanylyl cyclase.

We have used the effects of GTP on agonist binding as a convenient measure of receptor/G protein interactions. It has been shown that Gα2 is required to maintain the majority of the high affinity binding sites in both intact cells and membranes. In *gα2*⁻ membranes, GTP can induce only a slight reduction in high affinity binding. We have found that the *gβ*⁻ cells are completely insensitive to GTP and contain only low affinity sites. This stronger phenotype in *gβ*⁻ compared to *gα2*⁻ cells may indicate a residual activity in the *gα2*⁻ cells of another G protein that weakly couples to cAR1. In any case, the observations again suggest that cAR1 is simply not linked to any G proteins in the *gβ*⁻ cells.

Nevertheless, certain cAR1-mediated responses are retained in the *gβ*⁻ cells. Milne et al. (1995) have shown that the agonist-mediated cAR1 phosphorylation in *gβ*⁻ cells showed a time course and cAMP dose dependence indistinguishable from those of wild-type cells. cAMP-induced loss of ligand binding was also normal. In addition, *gβ*⁻ cells overexpressing cAR1 or cAR3 showed a Ca^{2+} influx response with kinetics, agonist dependence, ion specificity, and sensitivity to depolarization agents that were like those of wild-type cells. In addition, the experiment illustrated in Fig. 2 implies that Gβ is not essential for agonist-mediated enhancement of cAR1 expression. Moreover, Schnitzer et al. (1995) have recently demonstrated that constant levels of cAMP can induce the primary late genes *ras*, CP2, and *lagC* in a Gβ⁻ background. These results further substantiate our initial findings that suggest that responses mediated by G protein–coupled receptors can be independent of the functional heterotrimeric G proteins (Milne et al., 1995).

The α subunit that activates the adenylyl cyclase has been elusive. In *gα2*⁻ cells, agonist activation of the enzyme is essentially absent. However, GTPγS will activate ACA in lysates and membranes from the *gα2*⁻ cells, indicating that Gα2 does not directly confer guanine nucleotide regulation to the enzyme (Kesbeke et al., 1988; Pupillo et al., 1992). Appropriate regulation of ACA is also present in each of the *gα*⁻ cell lines (*gα6*⁻ has not been tested). These observations might be explained if the activation were mediated by the βγ subunit as has been observed for certain subtypes of mammalian adenylyl cyclases (Tang and Gilman, 1991; Federman et al., 1992). As shown in Fig. 7, in intact cells, Gα2 would be required to regulate the transient release of the βγ subunit by cAR1 excitation of G2. In lysates incubated with GTPγS, βγ subunits could be released from any G protein heterotrimer and neither Gα2 nor cAR1 is required. The data presented here strongly support this hypothesis. In the *gβ*⁻ cells, GTPγS completely fails to activate ACA; in fact, even the "basal" activity is slightly lower than that in wild type. We have previously noted that the basal activity was also slightly lowered by GDPβS, suggesting that a low amount of GTP is present in the cell lysates (Theibert and Devreotes, 1986).

An additional component, CRAC, is required to confer guanine nucleotide sensitivity to the adenylyl cyclase. In *crac*⁻ cells, cAMP stimuli do not trigger cAMP synthesis and, in lysates, GTPγS only weakly activates ACA. Interestingly, CRAC contains a pleckstrin homology domain (PH domain) in its NH_2-terminal region. It has been suggested that some PH domains are sites of interaction with βγ-subunit complexes and this hypothesis is supported by the recent discovery that fusion proteins containing various PH domains bind to dissociated βγ subunits (Touhara et al., 1994). We propose that in the system studied here, the agonist or GTPγS activates βγ and the activated βγ creates a binding site for CRAC via its PH domain. CRAC then translocates to the membrane and the CRAC/βγ subunit participates in the activation of ACA. In support of this hypothesis, we have shown that GTPγS treatment of wild-type membranes creates stable binding sites for CRAC, and that these sites cannot be induced in *gβ*⁻ cells (Lilly, P. J., and P. N. Devreotes, in this issue). Experiments are in progress to further investigate this novel mechanism for

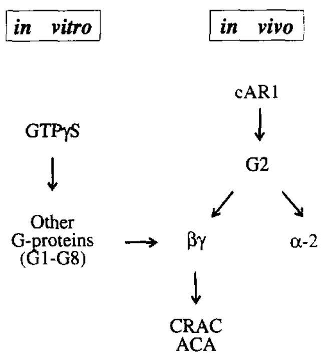

Figure 7. Relationship of multiple components in activation of adenylyl cyclase by βγ subunits. As described in text, both Gα2 and Gβ are required for cAR1-mediated activation of ACA. In lysates stimulated by GTPγS, Gα2 is bypassed by release of βγ complexes from other G-proteins. The cytosolic regulator, CRAC, acts in conjunction with the βγ complex to stimulate ACA.

activation of adenylyl cyclase. Moreover, excited βγ subunits may recruit other cytosolic PH domain–containing effector molecules in Gβγ-mediated signaling.

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