Regulation and Function of Gα Protein Subunits in Dictyostelium

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Summary

We have examined the developmental regulation and function of two G_α protein subunits, $G_\alpha 1$ and $G_\alpha 2$, from Dictyostelium. $G_\alpha 1$ is expressed in vegetative cells through aggregate stages while $G_\alpha 2$ is inducible by cAMP pulses and preferentially expressed in aggregation. Our results suggest that $G_\alpha 2$ encodes the G_α protein subunit associated with the cAMP receptor and mediates all known receptor-activated intracellular signal transduction processes, including chemotaxis and gene regulation. $G_\alpha 1$ appears to function in both the cell cycle and development. Overexpression of $G_\alpha 1$ results in large, multinucleated cells that develop abnormally. The central role that these G_α proteins play in signal transduction processes and in controlling Dictyostelium development is discussed.

Introduction

Upon starvation, Dictyostelium initiates a multicellular developmental program. After several hours, the cells begin to aggregate to form a loose mound of $\sim 10^5$ cells. The aggregate then proceeds through a series of morphological changes that lead to the formation of a fruiting body containing spores and stalk cells. The aggregation processes are mediated chemotactically by cAMP interacting with cell-surface receptors that activate two intracellular signal transduction processes (Klein et al., 1985; Devreotes et al., 1987; Janssens and van Haastert, 1987; Vaughan and Devreotes, 1988; Mann et al., 1988; Newell et al., 1988). These pathways are activated by two kinetic classes (fast and slow dissociating) of cAMP receptors. The faster-dissociating class activates a cAMP relay system in which cAMP causes the activation of adenylate cyclase with the resultant synthesis and secretion of cAMP into the extracellular medium. The secreted cAMP interacts with receptors on adjacent cells, thereby relaying the signal outward from the aggregation centers. The more slowly dissociating class of receptors mediates chemotaxis and activation of guanylate cyclase and is involved in the regulation of a number of classes of developmentally regulated genes. The receptor-mediated responses

adapt rapidly, and thus a continued presence of cAMP produces only an initial, transient activation of both adenylate and guanylate cyclase. In vivo, a cell-surface and membrane cAMP phosphodiesterase destroys the extracellular cAMP, and receptors return to an active state within ~ 5 min, whereupon a new cAMP pulse is initiated. Thus, a pulsatile cAMP signal that oscillates every 6–7 min is established that acts as the chemotactic signal for aggregation and the formation of a multicellular organism.

Previous studies have suggested the cAMP receptor transmits signals to these two divergent pathways via G (guanine nucleotide binding) proteins (Theibert and Devreotes, 1986; van Haastert et al., 1986, 1987). A cAMP receptor that is preferentially expressed during aggregation has been cloned. Sequence analysis of the clone suggests that the protein has seven transmembrane domains, characteristic of receptors that are known to be linked to G proteins in other systems (Klein et al., 1987). The linkage of the receptor to G proteins is supported by biochemical observations of mutants that affect each of these two pathways. Mutants within the FrigidA complementation group do not carry out chemotaxis to extracellular cAMP, fail to aggregate, and lack the activation of guanylate and adenylate cyclase by cAMP in vivo. In addition, exogenously applied pulses of cAMP do not stimulate expression of developmentally regulated genes. Inhibition of cAMP binding by GTP, a standard indicator of G protein–linked receptors, is nearly absent in several FrigidA alleles. However, wild-type levels of GTP-stimulated adenylate cyclase activity can be demonstrated in vitro even in the most severe allele. These results suggest that FrigidA mutants are defective in a G protein that interacts directly with the receptor which activates guanylate cyclase, leading to chemotaxis and gene regulation, and that some product of this pathway is needed to activate adenylate cyclase in vivo but not in vitro (Coukell et al., 1983; Kesbeke et al., 1988; Mann et al., 1988; Snaar-Jagalska et al., 1988b).

A second group of mutants, Synag, fail to aggregate but can carry out chemotaxis in external cAMP gradients. Exogenously applied pulses of cAMP induce expression of developmentally regulated genes. Synag mutants express cAMP receptors; guanylate cyclase activity is stimulated by cAMP; and GTP inhibits cAMP binding in isolated membranes. However, Synag mutants fail to produce cAMP in response to cAMP stiumli and do not show endogenous cAMP oscillations. In vitro, Synag mutants are defective in GTP stimulation of adenylate cyclase activity and appear to lack a soluble protein component found in wild-type cells. These data suggest that a defect in the signal transduction pathway is responsible for the aggregation-minus phenotype (Theibert and Devreotes, 1986; Theibert et al., 1986; van Haastert et al., 1987; Snaar-Jagalska, 1988; Mann et al., 1988).

Several classes of genes are differentially regulated during the preaggregation/aggregation stages. The expression of a major class of genes that is induced prior to aggregation and is maximally expressed during the

aggregation stage appears to require the periodic activation of the cell-surface receptor. This class includes proteins that function during aggregation, such as contact sites A (CsA or gp80), serine esterase D2, and the cAMP receptor (Gerisch et al., 1975; Klein et al., 1985; Mann and Firtel, 1987; Klein et al., 1988). Another class is induced very rapidly upon starvation and then repressed during aggregation in response to pulsatile cAMP application (Mann and Firtel, 1987). High, continuous levels of cAMP result in a continuous expression of the "pulse-repressed" genes and only a transient, low level of expression of the "pulse-induced" genes. Experiments with pharmacologic agents, cAMP analogs, and mutant strains suggest that the repression of the "pulse-repressed" genes and the activation of the "pulse-induced" are mediated by the "chemotactic" signal transduction pathway described above. There are a number of other classes of genes, including prestalk and prespore genes, that are either repressed or induced by cAMP, apparently acting through the cell-surface receptor (see Mann et al., 1987; Datta et al., 1987; Williams, 1988).

In a separate report, we describe the cloning of cDNAs encoding two different G_α protein subunits ($G_\alpha 1$ and $G_\alpha 2$) that show strong amino acid sequence identity to G_α protein subunits from other eukaryotes (Pupillo et al., submitted). We also show that $G_\alpha 1$ and $G_\alpha 2$ cDNA probes are complementary to multiple mRNAs differentially expressed in development. In this paper, we examine the regulation of expression of the two G_α proteins and the function of the two G_α proteins in controlling cellular processes in Dictyostelium.

Results

Regulation of $G_\alpha 1$ and $G_\alpha 2$

In a separate report we show that both $G_\alpha 1$ and $G_\alpha 2$ probes are complementary to multiple, developmentally regulated mRNAs (Pupillio et al., submitted). The major 1.7 kb and the minor 2.6 kb $G_\alpha 1$ mRNAs are expressed at moderate levels in vegetative cells and increase several-fold in abundance, peaking at the loose aggregate stage. Two minor mRNAs (2.2 kb and 2.3 kb) have different patterns (Pupillo et al., submitted; Figure 1). Both are induced at the aggregation stage and are expressed through culmination. The levels of expression of the 2.2 kb and 2.6 kb transcripts are 5- to 10-fold lower than that of the 1.7 kb RNA. In contrast, all of the $G_\alpha 2$ mRNAs are either not expressed or expressed at very low levels in vegetative cells. The major 2.7 kb $G_\alpha 2$ mRNA is maximally expressed during aggregation. The expression of the minor 2.9 kb mRNA peaks slightly earlier, while the minor 2.3 and 1.9 kb RNAs are maximally expressed during late aggregation or early aggregation stages, respectively, and at a level 5- to 10-fold lower than the 2.7 kb species. The pattern of developmental regulation of $G_\alpha 2$ is very similar to other aggregation stage genes whose expression is regulated by pulses of cAMP interacting with the cell-surface receptor (pulse-induced genes) (Gomer et al., 1985; Gerisch et al, 1985; Mann and Firtel, 1987).
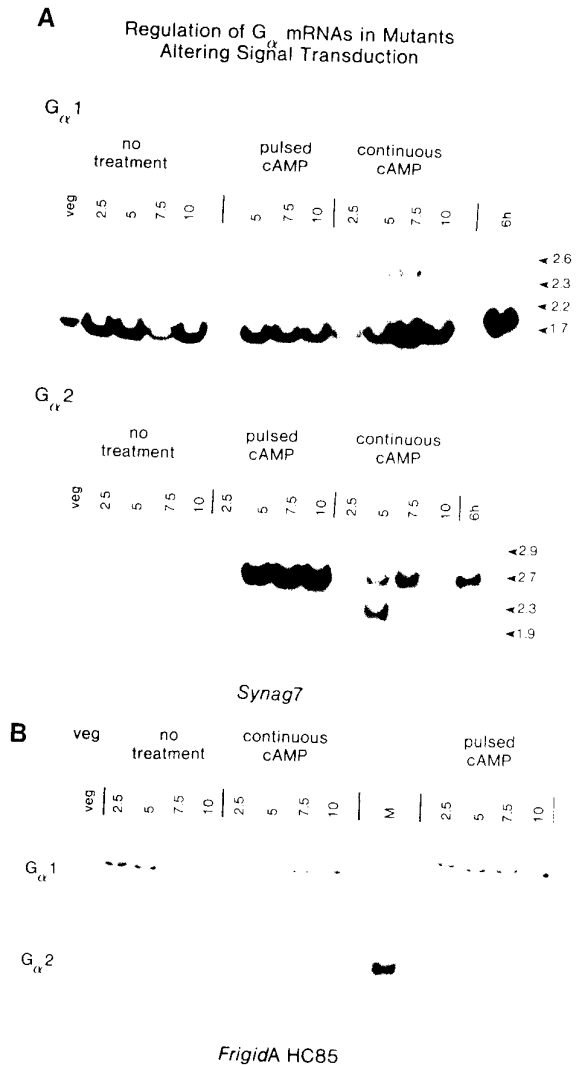
To understand better the mechanisms controlling the

Synag7

FrigidA HC85

Figure 1. Regulation of $G_\alpha 1$ and $G_\alpha 2$ in Mutants with Altered Signal Transduction

(A) Expression in *Synag7* cells. *Synag7* cells were grown in HL-5 axenic medium, washed, and placed in shaking culture at a density of 5×10^6/ml. RNA was isolated from aliquots of cells taken at the times indicated. "No treatment" indicates cells were shaken in buffer without exogenous cAMP. In the "pulsed cAMP" culture, cells were given pulses of cAMP to 25 nM every 6 min. In the "continuous cAMP" culture, cells were given cAMP to 300 μM at the time of starvation and additional cAMP to 100 μM every other hour. These RNAs are the same as those used previously to analyze other pulse-induced genes (Mann et al., 1988). See Mann and Firtel (1987) and Mann et al. (1988) for details. The marker lane 6h contains RNA isolated from wild-type NC-4 cells at 6 hr of development. In that particular experiment, 6 hr was at the beginning of visible streaming and represented the peak point of the expression of $G_\alpha 2$.

(B) Expression in HC85. Expression of $G_\alpha 1$ and $G_\alpha 2$ in HC85 in shaking culture was examined under the same conditions of treatment with cAMP used for *Synag7* cultures in (A). "Veg" represents RNA isolated from 0 hr vegetative cells. The marker lane M is the same RNA used as a marker in A. The arrow points to the $G_\alpha 1$ major 1.7 kb and the $G_\alpha 2$ major 2.7 kb RNAs. Note that during development, $G_\alpha 1$ RNA is overexpressed in this strain relative to wild-type cells. The level of expression of $G_\alpha 1$ mRNA in vegetative cells is similar to that in wild-type cells. In HC85 only the major 1.7 kb $G_\alpha 1$ mRNA is expressed under these conditions.

expression of $G_\alpha1$ and $G_\alpha2$, we have examined the expression of these genes in the mutant Synag7 (See Introduction) in response to exogenous cAMP. Extracts of Synag7 mutants lack a soluble protein necessary for receptor-mediated, GTP-stimulated activation of adenylate cyclase, and can be complemented with extracts from wild-type cells in vitro (Theibert and Devreotes, 1986; Snaar-Jagalska, 1988). Pulse-induced genes are expressed at very low levels in Synag7 cells in the absence of exogenous cAMP but are induced to maximal levels when the cells are pulsed at 6 min intervals with 25 nM cAMP, which mimics the cAMP oscillations observed in vivo in wild-type strains (Mann et al., 1988). Under these conditions, the cAMP receptor molecules oscillate between active and adapted states (Klein et al., 1987).

Figure 1A shows that $G_\alpha2$ is expressed at low levels in vegetatively growing Synag7 cells. When cells are starved in the absence of exogenous cAMP, there is a slight increase in expression of $G_\alpha2$ mRNA during 10 hr in shaking culture. However, pulses of cAMP rapidly induce a high level of expression of the 2.3 and 2.7 kb $G_\alpha2$ RNAs, similar to the pulse-induced genes encoding D2, M3 and CsA (Mann et al., 1988; Mann and Firtel, 1989). The level of the 2.7 kb mRNA is severalfold above that in control NC-4 6 hr cells. Continuous, moderate levels of cAMP result in a small induction of the major 2.7 kb $G_\alpha2$ mRNA species. These results are consistent with results observed with other pulse-induced mRNAs. In addition, there is a high level of expression of the 2.3 kb RNA at 5 hr. In contrast, $G_\alpha1$ shows approximately the same pattern of expression under all three conditions. The results indicate that $G_\alpha2$ expression is responsive to pulses of cAMP.

We have also examined the expression of $G_\alpha1$ and $G_\alpha2$ in wild-type NC-4 cells in shaking culture in response to cAMP (data not shown). Pulses of cAMP result in a precocious, high level of expression of $G_\alpha2$, similar to that seen for pulse-induced genes. High levels of cAMP result in some delay of $G_\alpha2$ expression and a moderate, but reduced, level of $G_\alpha2$ mRNA accumulation. In contrast, expression of the other pulse-induced genes is almost completely repressed under these conditions (Mann and Firtel, 1987; Mann et al., 1988). These results indicate some differences between the CsA/D2/M3 class of pulse-induced genes and $G_\alpha2$. $G_\alpha1$ expression, as it was in Synag7 cells is unaffected by addition of cAMP (data not shown).

Function of G_a2

Biochemical results have suggested the direct coupling of G proteins with the cell-surface cAMP receptor in a manner similar to other eukaryotic receptor–G protein systems (see Janssens and van Haastert, 1987). The FrigidA complementation group identified by Coukell and co-workers has four known alleles, all of which have an aggregation-minus phenotype (Lo et al., 1987; Coukell et al., 1983). Three of the alleles (fgdA33, 85 and 213) have no detectable (<5%) activation of adenylate or guanylate cyclase in response to cAMP, do not respond chemotactically to cAMP, and do not activate pulse-induced gene expression in response to cAMP pulses (Coukell et al., 1983; Jans-

sens and van Haastert, 1987). Moreover, FrigidA alleles contain defects in membrane-associated cAMP activation of GTP binding and GTPase activity, and GTP-mediated reduction in cAMP binding to the cell-surface receptor. In a fourth FrigidA allele (fgdA112), all of these functions are substantially reduced relative to wild-type cells.

After analyzing the expression of $G_\alpha1$ and $G_\alpha2$ in a number of developmental mutants, we decided to analyze the four FrigidA strains further. The expression of $G_\alpha1$ and $G_\alpha2$ RNA was examined during development and in response to cAMP. The expression of $G_\alpha1$ and $G_\alpha2$ mRNAs in shaking culture in response to cAMP in FrigidA HC85 (the strain with the strongest phenotype) is shown in Figure 1B. As can be seen, the expression of $G_\alpha1$ is greater than in wild-type cells (note the relatively low level of marker at this exposure), while $G_\alpha2$ expression is not detectable, even if the RNA blots are exposed 10 times longer (data not shown). The higher level of $G_\alpha1$ expression is confirmed by G_α subunit protein analysis (see below). FrigidA strains HC33 and HC213 have barely detectable levels of $G_\alpha2$ expression, while $G_\alpha2$ expression in FrigidA strain HC112 is ~20%–30% of wild-type levels (data not shown). This level of expression of $G_\alpha2$ is approximately the same as the relative level of cAMP-stimulated adenylate and guanylate cyclases seen in this strain relative to the parental strain (Coukell et al., 1983; Kesbeke et al., 1988).

One possible explanation for the reduced level or lack of $G_\alpha2$ expression in strains carrying fgdA mutations is a deletion or gene rearrangement associated with the $G_\alpha2$ gene. To examine this, DNA isolated from the four mutant and the two parental strains was used to study the gross structure of the $G_\alpha2$ gene in these strains by DNA blot hybridization (see Figure 2). While the patterns of hybridization seen with $G_\alpha2$ probes to DNA from strains HC33, HC112, HC213, and the two parentals (HC6 and HC91) are indistinguishable, no hybridization to HC85 DNA is detected with a probe that lacked only the N-terminal 60 nucleotides of the coding region and part of the 5' untranslated region (probe A in Figure 2). When probe B, containing the entire coding sequence and 65 nucleotides of the 5' untranslated region, is used, hybridization to HC85 DNA is observed but the hybridization is weak and the bands have altered mobility. The bands produced by EcoRI, EcoRV, and HindIII, enzymes that do not have a recognition site in the cDNA clone, are reduced in size by ~2.2 kb (see HindIII digest for best resolution). HincII, which cuts in the coding region, yields a single band that is ~2.2 kb shorter than the sum of the bands that contain the 5' and 3' portions of the gene. The 1.2 kb DraI band is deleted (see map in Figure 2) and the shorter DraI band, which may also be partially deleted, migrated off this gel. (Note: there are several DraI sites in the 3' untranslated region [unpublished data]). These results indicate that the $G_\alpha2$ gene in HC85 contains a ~2.2 kb deletion removing the majority of the coding sequence. A map of the $G_\alpha2$ genomic region is shown in Figure 2. These data strongly suggest that fgdA encodes $G_\alpha2$ and that mutations in this gene effect all known cAMP receptor–mediated signal transduction processes.
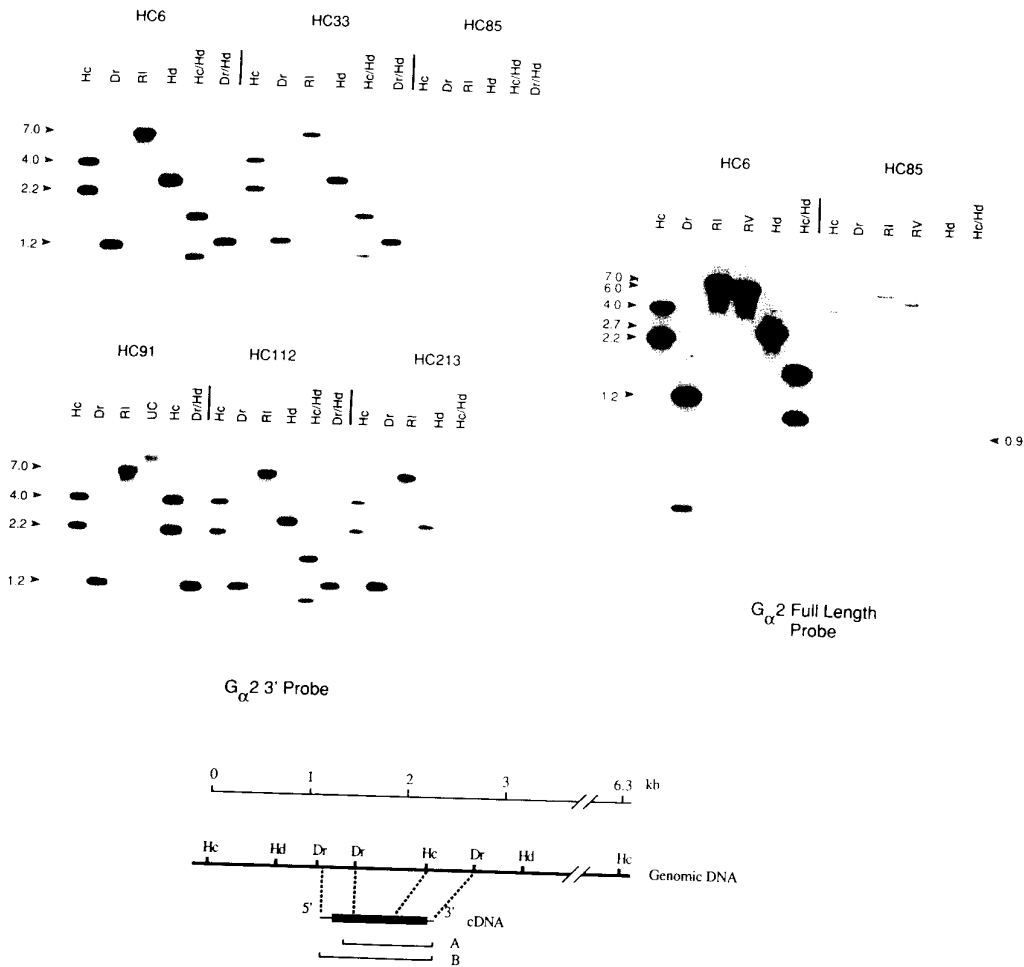
Genomic Mapping of
FrigidA Alleles

Figure 2. Genomic Mapping of DNA from FrigidA Strains

DNA blot hybridization of DNA isolated from four FrigidA strains, HC33 and HC85 and their parental strain HC6, plus HC112 and HC213 and their parental strain HC91, digested with restriction endonucleases, size fractionated on agarose gels, and blotted to nylon membranes. The membranes were hybridized to the appropriate nick-translated probe, washed, and placed on X-ray film. The blots at left show hybridization to $G_\alpha 2$ 3' probe A (see map of the $G_\alpha 2$ genome shown at the bottom). In the blot on the right, DNA from the parental strain HC6 and FrigidA strain HC85 was probed with the $G_\alpha 2$ full-length probe B. The bottom shows a crude restriction map of the $G_\alpha 2$ region determined from the genomic DNA blots and from isolated genomic clones. Note that additional DraI restriction sites are found in the genomic DNA outside of the region shown, but have not been mapped and are not relevant to the results presented here since they are outside the region that hybridized to either of our probes. There is an increase in the size of the genomic DNA fragment relative to the cDNA between the middle DraI site and the middle HincII site due to an intron in the $G_\alpha 2$ gene (unpublished data). The numbers on the sides of the blots indicate DNA fragment lengths in kb. Lanes are each labeled according to the restriction enzymes used to digest the DNA samples. Abbreviations: Hc, HincII; Dr, DraI; RI, EcoRI; Hd, HindIII; Dr/Hd, double digest of DraI plus HindIII; Hc/Hd, double digest of HincII and HindIII; Uc, undigested DNA; Rv, EcoRV. See Experimental Procedures for additional details.

Identification of G_α Protein Subunits

We have used anti-peptide antibodies specific for $G_\alpha 1$ and $G_\alpha 2$ to identify the proteins encoded by the $G_\alpha 1$ and $G_\alpha 2$ genes. Two antibodies were generated against unique $G_\alpha 1$ and $G_\alpha 2$ N-terminal sequences. A third antibody was elicited using a highly conserved sequence in G_α subunits that has been identified as part of the GTP binding site (see Experimental Procedures). On immunoblots of Dictyostelium membrane proteins, the affinity-purified G_α common antibody recognizes two proteins, one 38 kd and one 40 kd. When affinity-purified $G_\alpha 1$-specific antibodies were used, the $G_\alpha 1$ antibody detects a band with an apparent size of 38 kd, while the $G_\alpha 2$ anti-peptide antibody labels a band of approximately 40 kd
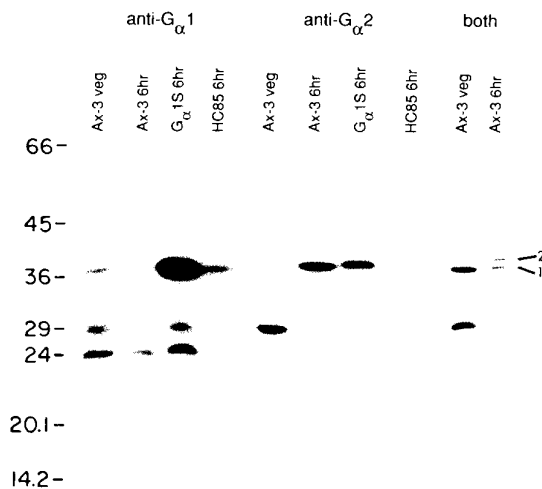
Figure 3. Immunoblot of Dictyostelium Lysate Pellets with Affinity-Purified Anti-Peptide Antibodies to $G_\alpha 1$ and $G_\alpha 2$

Dictyostelium cells were lysed, and washed 12,000× g pellets were subjected to SDS–polyacrylamide gel electrophoresis and then transferred to nitrocellulose for immunoblotting. Each sample is the equivalent of 3 × 10^6 cells. The left four lanes were probed with anti-$G_\alpha 1$ antibodies and contain samples from the wild-type axenic strain Ax-3 from growing cells (veg), Ax-3 cells developed for 6 hr, an overexpressing $G_\alpha 1$ strain developed for 6 hr, and fgdA strain HC85 cells developed for 6 hr. The next four lanes contain an aliquot of the same samples probed with anti-$G_\alpha 2$ antibody. The right two lanes contain samples from Ax-3 vegetative and 6 hr cells probed with a mixture of the two antibodies. Immunoblots were probed with ^{125}I-protein (see Experimental Procedures). Lines point to the $G_\alpha 1$ and $G_\alpha 2$ proteins (labeled "1" and "2", respectively).

(see Figure 3). Both the $G_\alpha 1$ and $G_\alpha 2$ antibodies also label, presumably nonspecifically, additional bands of roughly 24 kd and 30 kd; the G_α common antibody labels only the 38 kd and 40 kd bands.

The identity of the $G_\alpha 1$ and $G_\alpha 2$ proteins was confirmed using the developmental pattern of expression of $G_\alpha 1$ and $G_\alpha 2$ mRNA in wild-type cells, the mutant strain HC85, and transformed wild-type strains that overexpress $G_\alpha 1$ or $G_\alpha 2$ (see below). The results of the immunoblot are shown in Figure 3. The $G_\alpha 1$ 38 kd band is seen in both vegetative and developed wild-type cells and in developed HC85. Only the 38 kd band is increased in the cells that overexpress $G_\alpha 1$ (see below). In HC85 the 38 kd band is more intense relative to wild-type cells, as expected from the higher level of $G_\alpha 1$ mRNA in these cells. The pattern for $G_\alpha 2$ is quite different. The 40 kd $G_\alpha 2$ band is predominant in developed wild-type cells as well as the cells that overexpress $G_\alpha 1$. In vegetative wild-type cells $G_\alpha 2$ is minimally expressed, and in strain HC85 it is not detected at all. In addition, overexpression of $G_\alpha 2$ mRNA using a similar vector system as described below for $G_\alpha 1$ results in a more intense 40 kd band (data not shown). The G_α common antibody labels only the lower, 38 kd band in HC85. In developed cells it labels both the 38 kd and 40 kd bands (data not shown). The additional bands stained by the two G_α species–specific anti-peptide antibodies

do not show this same pattern of expression and are not labeled with the affinity-purified G_α common antibody. Their identify is unknown at this time, but we do not believe that they are G_α protein subunits.

Function of $G_\alpha 1$

We have investigated the function of $G_\alpha 1$ protein by overexpression of the cDNA using the actin 15 (Act15) promoter (see Figure 5A). RNA blot experiments indicate that there is a high level of Act15–$G_\alpha 1$ mRNA present (~10- to 20-fold greater than endogenous $G_\alpha 1$ mRNA), and a ~20-fold excess of $G_\alpha 1$ protein is observed on Western blots (see Figure 3). We examined the growth properties and developmental pattern of these cells and observed two phenotypes. When $G_\alpha 1$-overexpressing cells are grown in contact with plastic on Petri dishes, the majority of the cells are substantially larger than either untransformed cells or cells transformed with a control construct. When the nuclei are stained with Hoechst dye 33342, we observe that the majority of the control transformed cells have a single nucleus, a small fraction have two nuclei, and very few of the cells have three or four nuclei (See Figures 4B). By contrast, in the cells overexpressing $G_\alpha 1$ only ~25%–30% of the cells have one nuclei, ~20%–25% have two nuclei, and ~45%–50% have more than two nuclei, with some cells having 10 or more nuclei (Figure 4C). When these cells are plated for development, we observe the phenotypes shown in Figure 5A. The majority of the cells do not aggregate, and the cells that do aggregate produce a small and abnormal fruiting body.

When $G_\alpha 1$-overexpressing cells are grown in shaking culture, they are indistinguishable from wild-type cells in size and number of nuclei. It is possible that shaking shears the multinucleated cells. When these cells are plated for development, most aggregate. However, they produce an abnormal fruiting body. The stalks of the $G_\alpha 1$ overexpressers are 2–3 times longer than control fruiting bodies (see Figure 5B). Each aggregate produces one sorocarp (mass of spores), but in >90% of the fruiting bodies the spore head appears to have slipped down the stalk. Frequently, two or three sorocarps are arranged in linear sequence along the stalk. This phenotype may be secondary to a misproportioning of the fraction of stalk and spore cells. The results of these experiments suggest that $G_\alpha 1$ has a function in both growth and development, as might be expected from the developmental pattern of $G_\alpha 1$ mRNA expression.

Discussion

Development of Dictyostelium is a highly regulated process. Many aspects of early and mid-development are controlled by signal transduction processes mediated by the cell-surface cAMP receptor and G proteins. These events control aggregation and differential gene expression during preaggregation (interphase) and aggregation and in prestalk and prespore cells (Klein et al., 1985; Gomer et al., 1985; Datta et al., 1987; Mann et al., 1987; Williams, 1988). Moreover, extracellular cAMP appears to be involved in controlling the patterning of the migrating
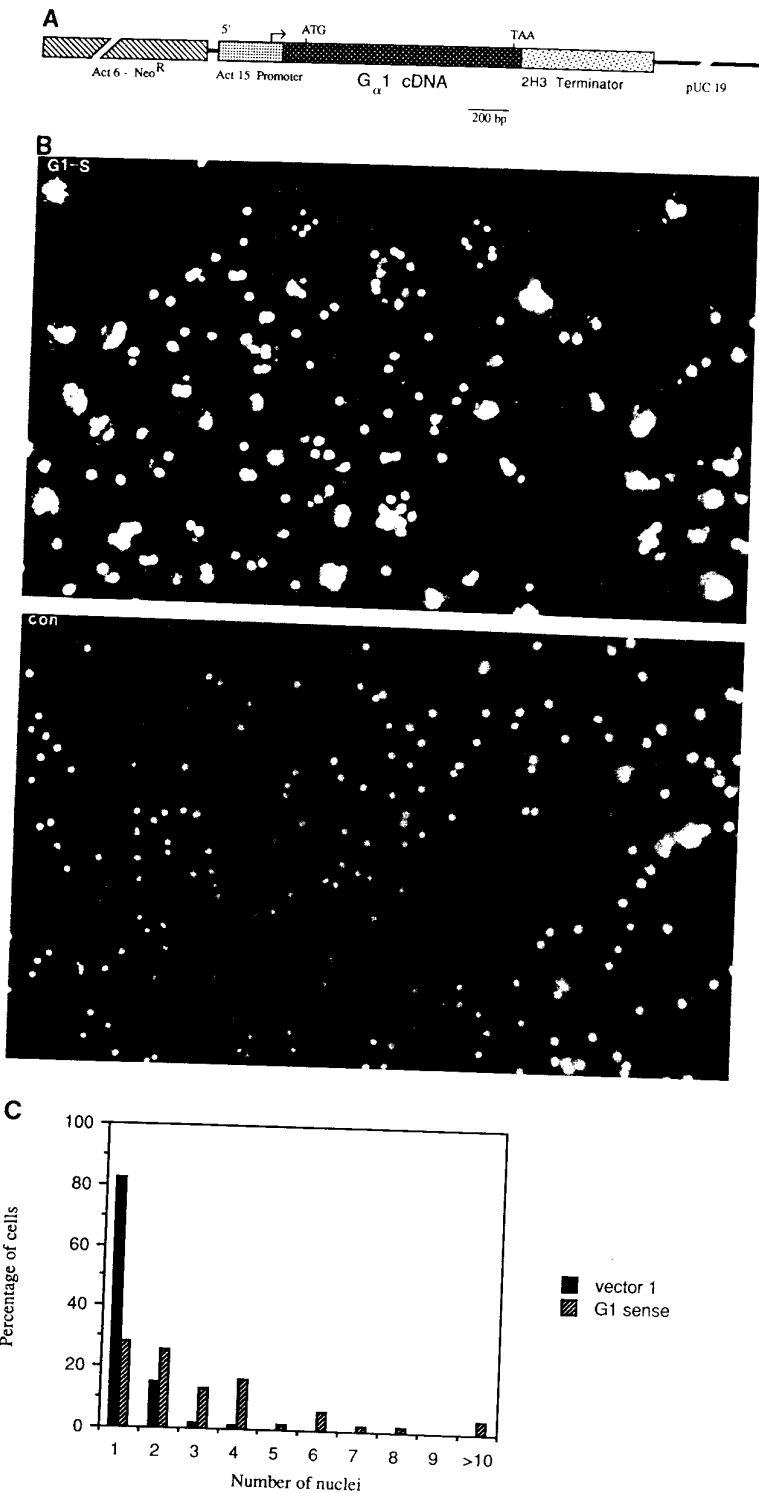
A

Act 6 · Neo^R   5'   ATG   TAA
Act 15 Promoter   G$_\alpha$1 cDNA   2H3 Terminator   pUC 19

200 bp

B

G1-S

con

Figure 4. Overexpression of G$_\alpha$1

(A) Partial map of vector used for overexpression of G$_\alpha$1. All regions of the vector are labeled. Act6-Neo^R encodes G418 resistance, the selectable marker for DNA-mediated transformation isolated from vector B10SX (Nellen et al., 1984; Nellen and Firtel, 1985). Expression of the G$_\alpha$1 cDNA was driven by the Dictyostelium Act15 promoter (Cohen et al., 1986). Fusion was within the 5' untranslated region of Act15 and the 5' untranslated region of the G$_\alpha$1 cDNA. The vector carries the termination region from the 2H3 prespore gene (Crowley et al., 1985). Fragments were cloned into pUC19. The arrow indicates the location of the cap site of the Act15 gene. The start and end of translation of the G$_\alpha$1 coding region are shown. The vector lacking the G$_\alpha$1 cDNA is designated BS18-HindIII and can be used for expressing appropriate constructs. The HindIII site is unique and is the point of insertion of the G$_\alpha$1 cDNA in the 5' untranslated region of Act15 (Kumagai and Firtel, unpublished data).

(B) Photographs of cells overexpressing G$_\alpha$1 (top) and control cells transformed with a vector lacking the G$_\alpha$1 cDNA insert (bottom) grown on plastic slides, fixed, and treated with Hoechst dye 33342 to stain the nuclei. Fluorescent photomicrographs.

(C) Bar graph of the percentage distribution of cells with varying numbers of nuclei. More than 150 randomly selected cells were counted for both the control transformants (solid bars) and for the G$_\alpha$1 sense transformants (hatched bars) from the same experiment as shown in (B).

C

■ vector 1
▨ G1 sense

slug or pseudoplasmodium and the morphogenesis of the fruiting body (see Schaap, 1986). Thus, analysis of the signal transduction processes and the function of G proteins in these processes is essential to understand the regula-

tory mechanisms controlling cellular differentiation in this organism. In this study we have examined the developmental pattern and function of two G$_\alpha$ proteins, G$_\alpha$1 and G$_\alpha$2. In both cases, G$_\alpha$1 and G$_\alpha$2 cDNAs are complemen-
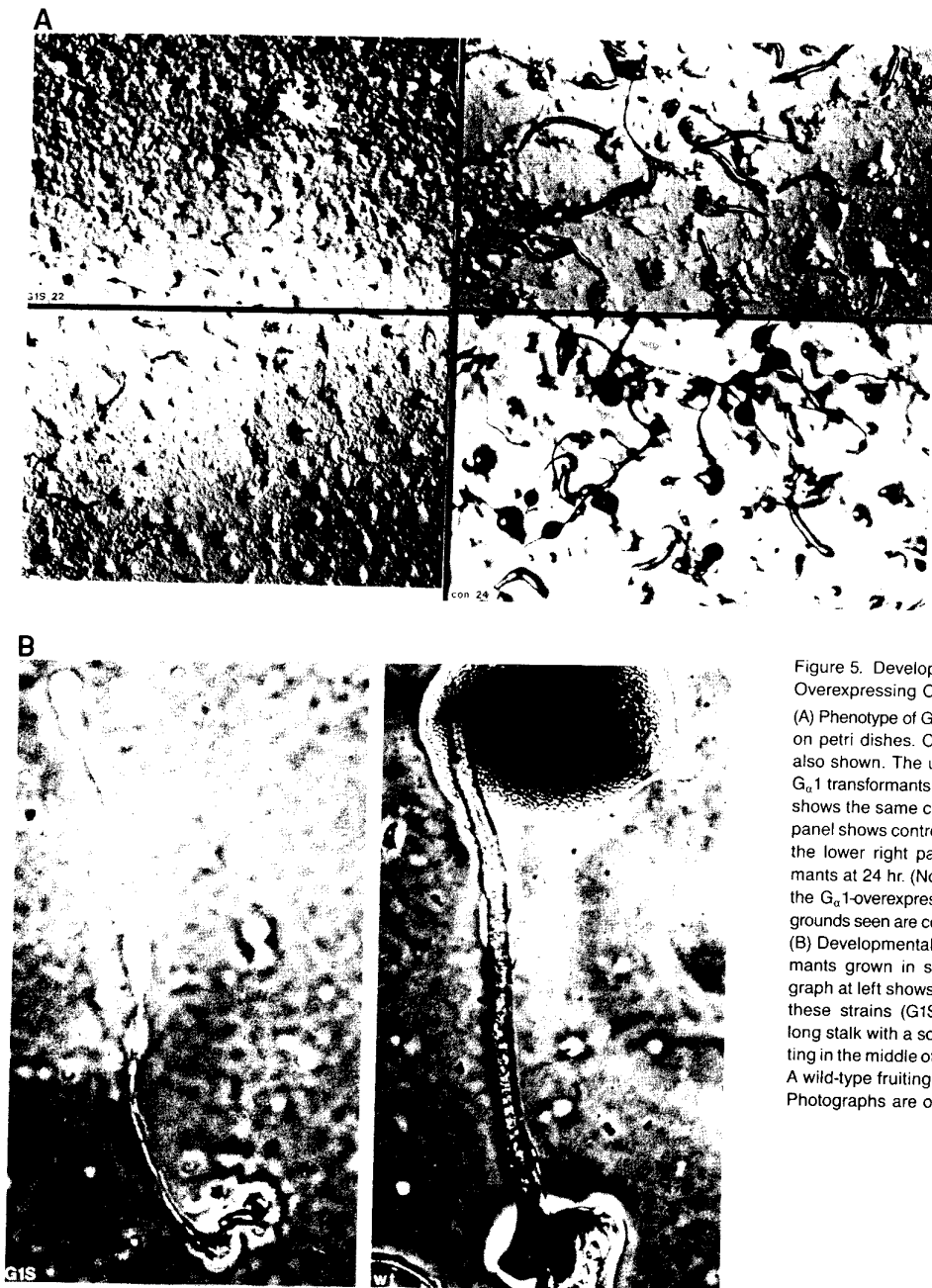
Figure 5. Developmental Phenotype of $G_\alpha 1$-Overexpressing Cells

(A) Phenotype of $G_\alpha 1$-overexpressing cells grown on petri dishes. Control transformed cells are also shown. The upper left-hand panel shows $G_\alpha 1$ transformants at 22 hr. The lower left panel shows the same cells at 30 hr. The upper right panel shows control transformants at 18 hr, and the lower right panel shows control transformants at 24 hr. (Note that in the photograph of the $G_\alpha 1$-overexpressing cells, the rippled backgrounds seen are cells that have not aggregated.)
(B) Developmental phenotype of $G_\alpha 1$ transformants grown in shaking culture. The photograph at left shows fruiting bodies produced in these strains (G1S) containing an extremely long stalk with a sorocarp (mass of spores) sitting in the middle of the stalk rather than on top. A wild-type fruiting body (wt) is shown at right. Photographs are of the same magnification.

tary to multiple mRNAs. Genomic analysis indicates that there are single $G_\alpha 1$ and $G_\alpha 2$ genes. At present the molecular basis for the multiple $G_\alpha 1$ and $G_\alpha 2$ mRNAs is not known.

The expression of $G_\alpha 2$ is highly developmentally regulated. $G_\alpha 2$ is expressed at very low levels in vegetative NC-4 cells and is maximally expressed during aggregation. The kinetics of $G_\alpha 2$ expression are very similar to those of genes encoding the cAMP receptor, D2, M3 and CsA, proteins that have specific functions during aggrega-

tion and early development (Gomer et al., 1985; Klein et al., 1985; Mann and Firtel, 1987; Gerisch, 1987; Klein et al., 1988; Rubino et al., 1989). All of these genes are regulated by cAMP pulses.

fgdA was identified as a gene required for aggregation. Preliminary analysis by Coukell et al., (1983) showed that strains carrying the strongest FrigidA alleles lacked activation of both adenylate and guanylate cyclases in response to cAMP and that the cells had moderate levels of cAMP receptor. Further biochemical analysis of these mu-

tations (Kesbeke et al., 1988) indicated that *FrigidA* strains (HC213 and HC85) also lack receptor activation of GTP binding and GTPase activity in isolated membranes. In contrast to what is seen in wild-type cells, guanine nucleotides do not affect the affinity of the receptor for cAMP. Moreover, the pulse-induced genes are not induced in strains carrying strong *FrigidA* alleles (Mann et al., 1988). Our analysis indicates that *fgdA* encodes $G_\alpha 2$ and that strain HC85 carries a deletion within the $G_\alpha 2$ gene. Two other strains carrying different alleles have very low levels of $G_\alpha 2$ mRNA, while a fourth (HC112) has moderate levels. Our data combined with biochemical evidence suggest that the strength of the alleles correlates with the level of $G_\alpha 2$ mRNA expression. These results suggest that the $G_\alpha 2$ subunit is directly associated with the cAMP receptor and regulates the activation of the key intracellular responses to cAMP, including chemotaxis, cAMP relay, adenylate and guanylate cyclase activation and the activation of gene expression.

The *FrigidA* mutations are loss-of-function mutations, suggesting that the $G_\alpha 2$ subunit plays a direct role in activation of the effector. Dictyostelium also contains a β subunit that is 77% identical to mammalian β subunits (Lilly and Devreotes, unpublished data). Thus, the D. discoideum system appears similar to most mammalian systems in that it is the α subunit of the G proteins that directly regulates receptor–effector functions; this is in contrast to the yeast pheromone receptor system and the mammalian muscarinic K^+ channel, where the βγ subunit may be the functional entity in directly regulating the downstream effector (Gilman, 1987; Dietzel and Kurjan, 1987; Miyajima et al., 1987; Logothetis et al., 1987; Neer and Clapham, 1988; Levitzki, 1988; Lochrie and Simon, 1988).

It has been postulated that the intracellular signal transduction pathway mediated by the receptor involves the activation of phospholipase C, and that this pathway results in the activation of guanylate cyclase (Janssens and van Haastert, 1987). We propose that $G_\alpha 2$ may be the subunit that activates phospholipase C. We are currently trying to complement *FrigidA* by transformation with $G_\alpha 2$. Successful complementation would provide a powerful system for analysis of this receptor–G protein–effector complex.

Our results and those of others have shown that many of the functions required during aggregation are induced during early development. These include $G_\alpha 2$ and the cAMP receptor, both of which are partially regulated by pulses of cAMP. These observations suggest an autoregulatory control mechanism in which both of these essential components in the signaling system require that system to be functioning for their own maximal expression (Mann et al., 1988; Mann and Firtel, 1989). Other results have shown that a biphasic regulatory pathway controls the expression of the pulse-induced genes, including those encoding $G_\alpha 2$ and receptor. An initial low level of developmentally regulated expression is cAMP-pulse independent, while the second phase of full expression is controlled by cAMP pulses (Mann and Firtel, 1989). This two-phase pathway allows fine-tuning of the developmental response and allows the organism to respond to changes in environmental conditions and cell density.

$G_\alpha 1$ is expressed at a moderate level in vegetative cells and at a high level during aggregation and the loose aggregate stage. By the tight aggregate stage the levels of the two major mRNAs fall, although there is continued expression of additional RNA (Pupillo et al., submitted). While the molecular basis of the multiple $G_\alpha 1$ RNAs is not known, we presume that the predominant mRNA forms encode proteins functional during growth and early development. Some insight has been obtained in our experiments examining the effect of overexpression of $G_\alpha 1$ protein during growth and early development. Of particular interest is the observation that overexpression of $G_\alpha 1$ produces very large cells with multiple nuclei. This is an extremely unusual phenotype and presumably indicates that the protein has an important role in controlling some aspect of cytokinesis during growth. When $G_\alpha 1$-overexpressing cells grown either in shaking culture or attached to plastic are allowed to develop, we observe abnormal phenotypes. The majority of cells grown attached to plastic that are large sized do not aggregate. It is possible the aggregation efficiency may be a function of cell size. Dictyostelium cells lacking myosin heavy chain (Knecht and Loomis, 1988; Wessels et al., 1988) also have large, multinucleated cells that have delayed aggregation. When the $G_\alpha 1$-overexpressing cells are grown in shaking culture, the size and number of nuclei are closer to those in wild-type cells. Many of these cells aggregate, but they produce an abnormal fruiting body that may have an altered prestalk/prespore cell ratio. The biochemical mechanism of these phenotypes is not known, but it is presumably due to competition of excess $G_\alpha 1$ protein subunits for either βγ subunits, the upstream activating protein, or a downstream effector.

Biochemical and physiological experiments have suggested at least three physiological roles for G proteins in controlling various aspects of aggregation in Dictyostelium. From our analysis and previous biochemical examinations, it appears that $G_\alpha 2$ is the G protein coupled to the cAMP receptor, possibly involved in the activation of phospholipase C. A second G_α protein is associated with the activation of adenylate cyclase. Our results presented here and those of Kesbeke et al. (1988) suggest that this is not $G_\alpha 2$, since normal levels of guanine nucleotide activation of adenylate cyclase can be measured in isolated membranes from *FrigidA* cells lacking $G_\alpha 2$. Whether the G protein involved in the activation of adenylate cyclase is of the heterotrimeric class like the G proteins examined here or possibly some other class of G protein is unknown. By analogy with mammalian cells, one would expect it is a member of the heterotrimeric class. A third G protein may be involved in the adaptation response and is sensitive to pertussis toxin (Snaar-Jagalska et al., 1988a), although expression of the ADP-ribosyltransferase gene from Bordetella pertussis does not visibly alter aggregation (Kumagai, Wilkie, Devreotes, and Firtel, unpublished data). Both genes described here lack the pertussis toxin ADP-ribosylation site. An ∼39 kd pertussis toxin substrate

has been observed in Dictyostelium by one laboratory (Khachatrian et al., 1987), although we and P. van Haastert have not observed this (unpublished data; P. van Haastert, personal communication).

At present we do not know if $G_\alpha 1$ is the G protein associated with adenylate cyclase. However, there are two arguments that suggest this may not be the case. Adenylate cyclase is developmentally regulated, increasing 10–20 times between vegetative cells and aggregation (Janssens and van Haastert, 1987). $G_\alpha 1$ does not show these increases. In addition, we feel that the phenotype observed by overexpressing $G_\alpha 1$ in vegetative cells would be unexpected if $G_\alpha 1$ linked the receptor to adenylate cyclase. Gene disruptions or use of antibodies in in vitro experiments should allow us to determine whether $G_\alpha 1$ is associated with adenylate cyclase.

Our initial molecular analysis has described the developmental pattern of genes for two G_α proteins and has examined potential functions of both of these. Further analysis including gene disruptions, complementation studies, and expression of mutant G_α proteins should further clarify the role each gene plays in regulated cellular processes in Dictyostelium. Additional analysis should identify the presence of other G proteins whose function has been postulated in biochemical and physiological studies. Because of the central role that G proteins play in controlling processes from chemotaxis to gene regulation, further dissection of their functions should give us additional insight into the mechanisms regulating Dictyostelium differentiation.

Experimental Procedures

Growth and Development of Dictyostelium Strains
Cells were grown either axenically in HL-5 medium in shaking culture or on plastic surfaces in petri dishes or in association with Klebsiella aerogenes bacteria on SM plates as previously described (see Mann and Firtel, 1987; Mann et al, 1988). Cells were washed and plated for development either on filters or placed in shaking culture as described in the above references. Conditions of development for the various experiments are given in the figure legends and in the above references.

Molecular Techniques
RNA and DNA were isolated from cells as previously described (Nellen et al., 1987). RNA and DNA blot hybridizations were performed as previously described (Mann and Firtel, 1987; Maniatis et al., 1982). Dictyostelium cells were transformed as described in Nellen et al. (1987). Stable transformants were analyzed.

Production of Anti-Peptide Antibodies
$G_\alpha 1$ and $G_\alpha 2$-Specific Antibodies
Specific anti-peptide antibodies against $G_\alpha 1$ and $G_\alpha 2$ were generated using nonhomologous sequences near the N termini. The $G_\alpha 1$ peptide sequence was a 31-mer starting with the fifth amino acid. The sequence was as follows; H_2N-CGKPELGSPEEIKANQHINSLLKQARS-KLEG-COOH. The $G_\alpha 2$ peptide was a 20-mer starting at residue 4 and had the sequence H_2N-CASSMEGEKTNTDINLSIEK-COOH. The peptides were cross-linked to keyhole limpet hemocyanin, and the conjugate was used to generate antibodies in rabbits. Sera were affinity purified for use on immunoblots as described (Gundersen et al., unpublished).
G_α Common Antibody
Rabbit antiserum was raised against the peptide synthesized by Suzanne Horvath, which is identical to the G_α common peptide used by Mumby et al. (1986). Rabbits were initially immunized with ~1 mg

of keyhole limpet hemocyanin–conjugated peptide in complete Freund's adjuvant. Subsequent boosts used the same amount of conjugated peptide in incomplete Freund's adjuvant. The titer of the resulting serum was estimated by incubating a series of dilutions of the serum with peptide spotted and dried onto nitrocellulose. IgG was purified from whole serum by precipitation with 40% ammonium sulfate. Anti-peptide antibody was then affinity purified from the IgG fraction by passing it over a column of peptide coupled to Affigel-10 (Bio-Rad) and eluting with 3 M KSCN.

Western Blots
Two different procedures were used for immunoblot analysis of $G_\alpha 1$ and $G_\alpha 2$ protein subunits.

Procedure A: Vegetative and developed cell samples were prepared by pushing cells through a 5 μm nucleopore filter into 20 mM Tris–HCl(pH 7.5), 200 mM sucrose, 5 mM EDTA, and a mixture of protease inhibitors at 4°C as previously described (Theibert et al., 1984). The lysates were centrifuged at 12,000× g for 15 min and the pellets were washed and recentrifuged. Washed pellets were resuspended at an equivalent cell density of 8×10^7/ml, mixed 1:1 with hot SDS sample buffer containing 5% β-mercaptoethanol, and boiled for 5 min. Samples of $(3 \times 10^6$ cells) were subjected to SDS–polyacrylamide gel electrophoresis according to the procedures of Laemmli (1970) using 10% gels. Gels were transferred to nitrocellulose (0.45 μm) for 3 hr at 5°C as previously described (Towbin et al., 1979). Blots were stained with Ponceau S (Sigma) to locate molecular weight markers and blocked with 3% BSA (wash buffer). Affinity-purified $G_\alpha 1$- and $G_\alpha 2$-specific antibodies diluted in wash buffer to 1–2 μg/ml were incubated with blots for 1–2 hr and then washed with four changes of wash buffer over 30 min. Bands were visualized by incubating with ^{125}I-protein A (ICN, 2–10 μCi/μg) at 0.1–0.2 μCi/ml in wash buffer for 60 min. Blots were then washed, dried, and autoradiographed.

Procedure B: Membranes were isolated from cells at the appropriate developmental time, and washed with MES-PDF buffer (Mann and Firtel, 1987). Cells were suspended in 20 μM Tris–HCl (pH 7.5), 1 μM EDTA, 1 mM DTT, and the protease inhibitors PMSF, leupeptin, and chymostatin (buffer A). The cells were freeze-thawed until most of the cells were lysed. Crude membranes were isolated by centrifugation in a microcentrifuge at 3500 rpm for 2 min. The pellet was washed four times with buffer A, and the crude membrane fraction was dissolved in SDS sample buffer containing protease inhibitors, run on SDS–polyacrylamide gels (Maniatis et al., 1982), and electroblotted onto nitrocellulose membrane prior to probing with the antibodies.

Proteins were sized by electrophoresis on 12.5% SDS–polyacrylamide gels and electrophoretically transferred to a nitrocellulose filter. The filter was soaked in blocking solution (5% nonfat dry milk in TBST [10 mM Tris–HCl, pH 8.0; 150 mM NaCl; 10.05% Tween-20]) for 30 min and incubated with G_α common antibodies overnight. The filter was washed several times with TBST and incubated with alkaline phosphatase–conjugated anti-rabbit IgG in TBST for 1 hr. After washing with TBST, color development was performed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

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