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Gα3 Regulates the cAMP Signaling System in *Dictyostelium*

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The *Dictyostelium discoideum* developmental program is initiated by starvation and its progress depends on G-protein–regulated transmembrane signaling. Disruption of the *Dictyostelium* G-protein α-subunit *Gα3* (*gα3*$^-$) blocks development unless the mutant is starved in the presence of artificial cAMP pulses. The function of Gα3 was investigated by examining the expression of several components of the cAMP transmembrane signaling system in the *gα3*$^-$ mutant. cAMP receptor 1 protein, cyclic nucleotide phosphodiesterase, phosphodiesterase inhibitor, and aggregation-stage adenylyl cyclase mRNA expression were absent or greatly reduced when cells were starved without exogenously applied pulses of cAMP. However, cAMP receptor 1 protein and aggregation-stage adenylyl cyclase mRNA expression were restored by starving the *gα3*$^-$ cells in the presence of exogenous cAMP pulses. Adenylyl cyclase activity was also reduced in *gα3*$^-$ cells starved without exogenous cAMP pulses compared with similarly treated wild-type cells but was elevated to a level twofold greater than wild-type cells in *gα3*$^-$ cells starved in the presence of exogenous cAMP pulses. These results suggest that Gα3 is essential in early development because it controls the expression of components of the transmembrane signaling system.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* initiates a developmental program as a self-protective mechanism when nutrients are depleted. Starvation induces the aggregation of individual amoebae into an initial multicellular structure, a mound. The mound undergoes further morphogenesis to form a slug, then finally a fruiting body consisting of two major differentiated cell types, stalk cells and spores. Stalk cells function to hold the spore head containing about 10^5 spores above the ground. Within 24 h of the onset of starvation, a terminally differentiated organism that can survive for long periods without food is produced from a culture of identical amoebae (Devreotes, 1994).

Much is known about the mechanics of aggregation, the first stage of development, at both the biochemical and the molecular level (Devreotes, 1983, 1994). Aggregation is accomplished by the chemotaxis of individual amoebae toward extracellular cAMP produced by the starving cells. A series of cyclic AMP (cAMP) concentration gradients are generated through G-protein–regulated activation of adenylyl cyclase so that cAMP is synthesized and secreted in bursts at approximately 6-min intervals. Between the bursts, a secreted form of cAMP phosphodiesterase (PDE)[1] degrades the ligand to prepare the system for a subsequent stimulus. The activity of the secreted PDE, and consequently extracellular cAMP, is further regulated by a small secreted inhibitor protein (phosphodiesterase inhibitor, PDI). Development is dependent on coordinated regula-

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[1] Abbreviations used: ACA, adenylyl cyclase of aggregation; cAR, cyclic AMP receptor; CMF, conditioned medium factor; DB, development buffer; DTT, dithiothreitol; PDE, phosphodiesterase; PDI, phosphodiesterase inhibitor; PKA, protein kinase A; PSF, prestarvation factor; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing Tween 20.

tion of this mechanism because targeted disruptions of the cAMP receptor 1 (*cAR1*), the G-protein subunits $G\alpha 2$ and $G\beta$, and mutations in *PDE* block development (Faure *et al.*, 1989; Kumagai *et al.*, 1991; Sun and Devreotes, 1991; Lilly *et al.*, 1993).

In addition to coordinating aggregation, cAMP pulses are required for the expression of many developmentally regulated genes (Schaap and Van Driel, 1985; Mann and Firtel, 1989; Firtel, 1995). Some genes of the cAMP signaling system, such as aggregation-stage adenylyl cyclase (*ACA*), are induced to a low level by starvation and others, such as *cAR1* and *PDE*, can also be induced before nutrient deprivation by the density-sensing agent prestarvation factor (PSF; Rathi and Clarke, 1992). In either case, as cAMP pulses are established, much higher expression is induced to create the signaling system necessary to coordinate aggregation (Mann and Firtel, 1989; Firtel, 1995). Later in development, expression of cell type-specific genes such as *SP60* and *ECM A*, which are dependent on the *Dictyostelium* transcription factor G-box factor, require continuous high levels (millimolar) of cAMP (Schnitzler *et al.*, 1994). cAMP pulses during aggregation confer competency on the cells to respond to higher concentrations of continuous cAMP for the induction of cell type-specific gene expression later in development (Schaap and Van Driel, 1985; Schnitzler *et al.*, 1994; Firtel, 1995).

Stimulation of the transmembrane cARs by secreted cAMP may be the only cellular requirement for cAMP at early stages of development. This hypothesis is supported by the observation that exogenously applied pulses of cAMP rescue aggregation in a range of mutants with blocks in the production of cAMP. Without this treatment, these mutants fail to aggregate. Mutants that display this phenotype include strains lacking ACA, cAR1, or Gα2 (aca^-, $car1^-$, and $g\alpha 2^-$, respectively; Pitt *et al.*, 1993; Soede *et al.*, 1994; Schnitzler *et al.*, 1995). Presumably cAMP signaling through cARs links extracellular cAMP to proteins that regulate gene expression.

We have recently reported the sequence, developmental regulation, and targeted disruption of a *Dictyostelium* G-protein α-subunit, Gα3 (Brandon *et al.*, 1997). The $g\alpha 3^-$ mutant cannot aggregate, but its developmental defect can be partially rescued by cAMP pulses so that it proceeds to the tipped mound stage of development. In this work, we investigate the role of Gα3 by analyzing the expression and activities of components of the cAMP-stimulated transmembrane signaling system in the $g\alpha 3^-$ mutant. Our results suggest that Gα3 functions in the earliest stages of development to regulate the expression of genes that are required for cAMP synthesis. Gα3 may be a link between cARs and the gene regulation machinery.

MATERIALS AND METHODS

Growth and Development Conditions

Dictyostelium cells were grown in HL5 medium to densities between 5 and 10×10^6 cells/ml with thymidine (100 μg/ml) supplements added as needed (Podgorski and Deering, 1984; Sussman, 1987).

Development was initiated by harvesting cells, washing them one time in development buffer (DB: 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4), and resuspending them to 2×10^7 cells/ml in DB. The cultures were shaken at 22°C for 4.5 to 8 h with or without externally applied pulses of 50 nM cAMP delivered every 6 min.

Development was observed by placing the cells on agar starvation plates. A 1-ml suspension of 5×10^6 cells was placed on 1.5% agar dissolved in DB on 35 mm in diameter Petri dishes. The starvation plates were incubated at 22°C for up to 96 h. The HPS400 cells completed development in 24–27 h.

Adenylyl Cyclase Assay

Cells were developed for 4.5 to 5 h in the presence or absence of pulses of 50 nM cAMP as described above. To interrupt the natural cAMP pulses and obtain cells with basal adenylyl cyclase activity, the developed cells were diluted to 2×10^6 cells/ml in DB and shaken rapidly (~200 rpm) for at least 30 min at 22°C. The cells were washed once in DB without calcium at 0°C, resuspended in ice-cold DB without calcium at 8×10^7 cells/ml, and held at 0°C if needed. Before cAMP stimulation, the cells were gently shaken in beakers on ice for 10 min to recover from centrifugation. Dithiothreitol (DTT; to inhibit phosphodiesterase) and cAMP were added sequentially to final concentrations of 10 mM and 10 μM, respectively. A prestimulus sample was removed from the culture prior to cAMP addition. At various times after cAMP stimulation, cells were removed and mixed 1:1 (vol/vol) with 2× lysis buffer (20 mM Tris, pH 8, 2 mM $MgSO_4$). The diluted cells were lysed by rapidly pushing through a double 5-μm pore-size membrane. Two hundred microliters of the whole cell lysate were immediately added to 20 μl of a reaction mixture consisting of 0.1 M Tris, pH 8, 3 mM ATP, 5 mM cAMP, 0.1 M DTT, and approximately 30 nM [α-^{32}P]ATP (440 Ci/mmol). The reaction was incubated for 1 min in a room-temperature water bath and stopped with the addition of 100 μl of stop solution (45 mM ATP, 1.3 mM cAMP, 2% SDS). The radiolabeled cAMP was purified by sequential Dowex/alumina chromatography as described by Solomon (1979).

cAMP-stimulated Phosphorylation of cAR1

Cells were starved by shaking gently for 5 h and then rapidly for 30 min to interrupt the cAMP oscillations and washed in ice-cold DB as described above. The cells were resuspended in ice-cold DB to 1×10^8 cells/ml and gently shaken in a beaker on ice for 10 min to recover from centrifugation. DTT was added to 10 mM and a prestimulus sample was removed from the culture for membrane preparation. cAR1 kinase activity was stimulated by the addition of 10 μM cAMP and samples were removed for membrane preparation at 1- or 2-min intervals for up to 10 min after cAMP stimulation.

Preparation of a Membrane-enriched Cell Fraction

Approximately $8–10 \times 10^6$ cells in a volume of 0.1 ml were rapidly diluted in 0.9 ml of ice-cold saturated ammonium sulfate, vortexed vigorously, and centrifuged at $6000 \times g$ for 6 min at 4°C. The supernatant was gently aspirated. The cell pellet was resuspended in 0.2 ml of ice-cold receptor assay buffer containing protease inhibitors (described in Theibert *et al.*, 1984) to lyse the cells. A membrane-enriched cell fraction was collected by centrifugation at $16,000 \times g$ for 10 min at 4°C. The final pellet was resuspended in sample buffer (10% glycerol, 5% DTT, 3% SDS, 62.5 mM Tris, pH 6.8, 2% bromophenol blue) at 5×10^7 cell equivalents/ml. Approxi-

mately 2 × 10^7 cell equivalents were loaded per lane on a 10% SDS-PAGE gel.

Protein Blotting and Detection

Proteins present in the membrane-enriched cell fraction were transferred in 24 mM Tris base, 192 mM glycine, and 25% methanol to methanol-soaked Immobilon P membranes by using a semidry Transblotter (Bio-Rad Laboratories, Hercules, CA). Electrophoretic transfer was carried out at 15 V for 30 min.

Specific proteins were detected using enhanced chemiluminescence according to the manufacturer's specifications (Amersham, Arlington Heights, IL). Briefly, nonspecific protein-binding sites on the Immobilon P membranes were blocked with a solution of Tris-buffered saline (20 mM Tris, pH 7.6, 137 mM NaCl) plus 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk by gently shaking for 1 h at room temperature or overnight at 4°C. The membrane was then soaked in an appropriate dilution of the primary antibody in TBS without Tween 20 but containing 1% nonfat dry milk and then gently shaken for 1 to 2 h at room temperature or overnight at 4°C. The membrane was then washed in TBS-T for 30 min at room temperature with three or four changes of TBS-T. The horseradish peroxidase-labeled second antibody was diluted 1:5000 in TBS containing 1% nonfat dry milk and incubated with the membrane for 1 h at room temperature. The final washes were carried out in TBS-T for 1 h at room temperature with changes of the buffer every 10–15 min. Equal amounts of the detection reagents provided by the manufacturer were mixed and immediately incubated with the membrane for 1 min. The detection reagents were discarded and the membrane exposed to film for 2 to 30 min.

RNA Preparation and Analysis

Aliquots of 2 × 10^7 cells were removed every 2 h from HPS400 and $g\alpha3^-$ cultures developing in shaking culture as described in Wu *et al.* (1995a). Some cultures were treated with 50 nM cAMP added every 6 min to mimic endogenous pulses of cAMP. RNA was extracted using the method of Chomczynski and Sacchi (1987). Five micrograms of RNA from each developmental time point were treated with glyoxyl and electrophoresed on a 1% agarose gel. Ethidium bromide staining of rRNAs in the gel prior to transfer provided evidence for even loading and integrity of the RNAs. RNAs were transferred to a charged nylon membrane in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). After overnight blotting, the gels were restained with ethidium bromide to examine the extent of RNA transfer. The RNA blot was hybridized overnight at 65°C with radiolabeled PDE and PDI in a solution of 1 M NaCl, 10% polyethylene glycol (M_r 8000), 1% SDS, and 100 μg/ml boiled herring sperm DNA. The blots were washed at the hybridization temperature using standard techniques (Sambrook *et al.*, 1989).

RESULTS

cAR1 Is Aberrantly Regulated in the gα3⁻ Mutant

The expression of cAR1 protein was examined in the $g\alpha3^-$ mutant and its $G\alpha3^+$ parent, HPS400. Cells were developed in the presence or absence of 50 nM cAMP pulses for up to 8 h (see MATERIALS AND METHODS). Aliquots of cells were removed at 1-h intervals for preparation of the membrane-enriched cell fraction. cAR1 was detected by a specific polyclonal antibody. In the absence of exogenous cAMP pulses, cAR1 could be detected in the HPS400 cells at 4 h of development. Its expression increased as development progressed through at least 8 h (Figure 1). In the $g\alpha3^-$ mutant, cAR1 expression was not detected in the absence of exogenous cAMP pulses even with an exposure 10 times longer than that used for HPS400. This experiment was repeated five times with similar results. In the experiment shown in Figure 1, cAR1 appeared 1 h later in HPS400 cells developed in the presence of exogenous cAMP pulses. This difference in timing, however, is not significant and is due to experimental variation. When the $g\alpha3^-$ mutant was treated with exogenous cAMP pulses, cAR1 expression was completely rescued, a result obtained in nine independent experiments. In most cases, cAR1 expression in the $g\alpha3^-$ mutant developed with exogenous cAMP pulses was greater than that in HPS400 cells developed under the same conditions. These results demonstrate that cAR1 expression is dependent on Gα3, but this requirement is bypassed if cells are treated with cAMP pulses.

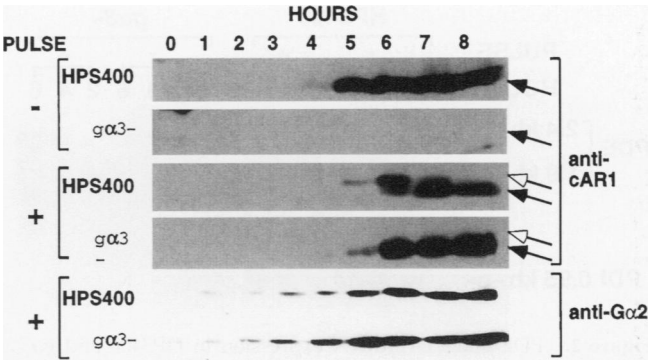

Figure 1. cAR1 and Gα2 protein expression in HPS400 and $g\alpha3^-$ cells. A membrane-enriched cell fraction was prepared every hour for 8 h from HPS400 and $g\alpha3^-$ cells developing with (+) or without (−) exogenous cAMP pulses. The protein blots were probed with anti-cAR1 or anti-Gα2 polyclonal antibodies. The blot prepared from $g\alpha3^-$ cells developed without exogenous cAMP pulses was exposed 10 times longer than that of the HPS400 cells. The two bands of cAR1 protein seen in lanes 6 and 7 of the blot prepared from HPS400 cells developed with exogenous cAMP pulses are the phosphorylated (open arrowhead) and nonphosphorylated (closed arrowhead) forms of cAR1. Only the nonphosphorylated form is seen in the blot prepared from $g\alpha3^-$.

Phosphorylation of cAR1 is required for the ligand-induced reduction of receptor affinity that is a hallmark of G-protein–coupled receptors (Caterina *et al.*, 1995). The phosphorylated form of cAR1 is visible as the slower mobility form in HPS400 cells developed with exogenous cAMP pulses (Figure 1, open arrowhead). Phosphorylated cAR1 was not visible in the membrane-enriched cell fraction prepared from the $g\alpha3^-$ mutant. To determine whether cAR1 kinase was altered in the $g\alpha3^-$ mutant, we assayed cAR1 kinase activity in cells starving for 8 h with exogenous cAMP pulses (see MATERIALS AND METHODS). Under these conditions, the phosphorylated form of cAR1 was detected in the $g\alpha3^-$ mutant at the same level and

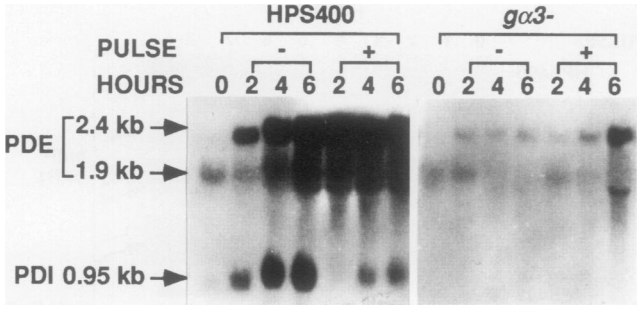

Figure 2. PDE and PDI mRNA expression in HPS400 and $g\alpha3^-$ cells. RNA was prepared from HPS400 and $g\alpha3^-$ cells developing with (+) and without (−) exogenous cAMP pulses at 0, 2, 4, and 6 h. The blots were hybridized simultaneously with radiolabeled PDE and PDI probes. The lowest band in the final lane is due to displacement of degraded PDE mRNA by the 1.8-kb rRNA and does not represent hybridization to the 1.9-kb PDE mRNA.

with the same kinetics as in the HPS400 cells (our unpublished results).

Expression of Gα2 Is Not Altered in the gα3⁻ Mutant

The *Gα2* gene, like cAR1, is required for aggregation and its expression can be induced by cAMP pulses (Kumagai *et al.*, 1991). The expression of Gα2 protein was compared by Western blot in HPS400 and $g\alpha3^-$ mutant cells. In HPS400 cells that received exogenous cAMP pulses, Gα2 expression was detected at 1 h of development and increased through 8 h of development as described previously (Figure 1; Kumagai *et al.*, 1991). The pattern of Gα2 expression in the $g\alpha3^-$ mutant developed with exogenous cAMP pulses was not significantly different from HPS400. When HPS400 and $g\alpha3^-$ cells were developed without cAMP pulses, Gα2 expression in both strains was nearly identical to that shown in Figure 1. Therefore, Gα2 expression is not dependent on Gα3.

PDE Expression Is Reduced in the gα3⁻ Mutant

The *PDE* gene contains three nested promoters that are differentially regulated during development (Faure *et al.*, 1990). During aggregation, two promoters are active, producing a 1.9-kb mRNA and a 2.4-kb mRNA. The expression of the 2.4-kb mRNA is required for aggregation and is induced by cAMP pulses.

In the HPS400 cells, the 1.9-kb and 2.4-kb PDE mRNAs were expressed as described previously (Faure *et al.*, 1990). In the absence of exogenous cAMP pulses, the expression of the 2.4-kb mRNA increased throughout 6 h of development (Figure 2). Treatment with exogenous cAMP pulses induced the 2.4-kb mRNA to maximum levels by 2 h of development. This level was constant throughout the remaining 4 h of the experiment. In the $g\alpha3^-$ mutant developed without exogenous cAMP pulses, both the 1.9-kb and the 2.4-kb PDE mRNAs were expressed, but at significantly lower levels than those of HPS400 (Figure 2). The 2.4-kb PDE mRNA in the $g\alpha3^-$ mutant was induced after 6 h of development with exogenous cAMP pulses, although the amount was significantly lower than that of identically treated HPS400. The 1.9-kb PDE mRNA was not induced under any of the conditions tested. Therefore, normal expression of PDE is dependent on Gα3 and it is possible to partially bypass this requirement by treatment with exogenous pulses of cAMP.

PDI Is Not Expressed in the gα3⁻ Mutant

The *PDI* gene is expressed coordinately with the *PDE* to regulate levels of extracellular cAMP. Like the *PDE*, cAMP regulates *PDI* gene expression, though in this case cAMP represses rather than induces transcription (Franke *et al.*, 1991). We examined the effect of the *Gα3* mutation on *PDI* gene regulation.

In the HPS400 cells that were developed without exogenous cAMP pulses, a 0.95-kb PDI mRNA was expressed as described previously (Figure 2; Franke *et al.*, 1991). As expected, exogenous cAMP pulses inhibited accumulation of PDI mRNA. In the $g\alpha3^-$ mutant, PDI mRNA expression was not detected under any developmental condition. Therefore, Gα3 is required for *PDI* gene expression.

Adenylyl Cyclase mRNA Levels Are Reduced in the gα3⁻ Mutant

cAR1 is linked through Gα2 to the activation of ACA (Kumagai *et al.* 1991; Pitt *et al.*, 1993). We examined the effects of loss of Gα3 activity on the developmental induction of ACA mRNA.

Cells were developed for 6 h in the presence or absence of exogenous cAMP pulses, and RNA was isolated at 0, 2, 4, and 6 h of development and then analyzed for ACA mRNA as described in MATERIALS AND METHODS. In the HPS400 cells developed without exogenous cAMP pulses, a 5.8-kb ACA mRNA was detected at 2 h of development and its expression increased through 6 h of development (Figure 3). Developing the cells with exogenous cAMP pulses induced maximal ACA expression earlier in development and increased ACA mRNA levels. In the $g\alpha3^-$ mutant that did not receive exogenous cAMP pulses, ACA expression was dramatically reduced. ACA mRNA was not detected at 0, 2, or 4 h and only weak expression occurred at 6 h (Figure 3). Developing the $g\alpha3^-$ mutant with exogenous cAMP pulses increased ACA mRNA expression so that at 4 and 6 h, ACA mRNA levels approached, but were still less than, those of HPS400. These results demonstrate that *ACA* gene expression is dependent on Gα3 and, as is

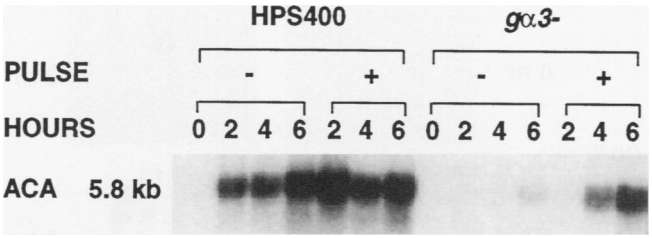

Figure 3. ACA mRNA expression in HPS400 and $g\alpha3^-$ cells. RNA was prepared from cells developed and treated with exogenous cAMP pulses as described in Figure 2 and used to prepare a Northern blot that was hybridized with a radiolabeled ACA probe.

true for cAR1 and PDE, exogenous cAMP pulses partially restore the expression of ACA mRNA in the absence of Gα3.

Exogenous cAMP Pulses Are Required for Adenylyl Cyclase Activity in the $g\alpha3^-$ Mutant

The weak developmental induction of the cAMP induced *cAR1*, *ACA*, and *PDE* genes in the $g\alpha3^-$ mutant suggested that this mutant cannot produce cAMP pulses. We examined this possibility by measuring the cAMP-stimulated adenylyl cyclase activity of the $g\alpha3^-$ mutant cells.

Cells were starved for 5 h with or without exogenous cAMP pulses and used for measurement of adenylyl cyclase activity as described in MATERIALS AND METHODS. Adenylyl cyclase activity is reported as the average fold stimulation (±SD) over basal activity. Basal activity was determined from the adenylyl cyclase activity of unstimulated cells. Figure 4A was compiled from three separate assays for each strain developed without exogenous cAMP pulses. In the HPS400 cells, the basal adenylyl cyclase activity was 0.3 ± 0.06 pmol per min per mg. Adenylyl cyclase activity increased within 30 s of cAMP stimulation and the maximum activity of (5.9 ± 3.0)-fold above basal was reached between 4 and 4.5 min after stimulation. The activity began to decrease due to adaptation 5.5 min after stimulation and remained above prestimulus levels at the end of measurement, 9.5 min after stimulation. In the $g\alpha3^-$ mutant, there was a similar basal level of adenylyl cyclase activity of 0.2 ± 0.06 pmol per min per mg (Figure 4A). A weak increase in adenylyl cyclase activity was detected 1 min after cAMP stimulation. Maximum adenylyl cyclase activity of (2.8 ± 0.03)-fold above basal was achieved at 3 min after stimulation. In contrast to what is seen in HPS400, the adenylyl cyclase activity in the $g\alpha3^-$ mutant remained at the elevated level through the end of the assay. Although the patterns of adenylyl cyclase stimulation we observed in HPS400 and $g\alpha3^-$ cells developed without exogenous cAMP pulses were

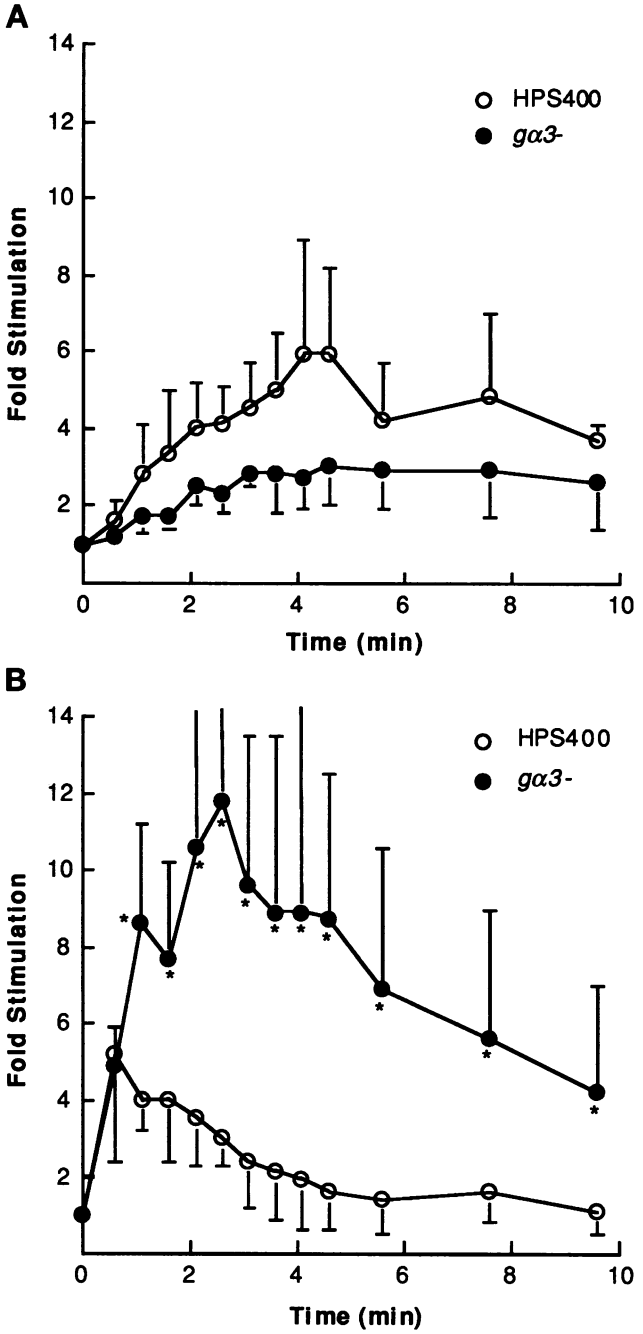

Figure 4. Adenylyl cyclase activity. HPS400 and $g\alpha3^-$ cells were developed for 5.5 h without (A) or with (B) exogenous cAMP pulses. Adenylyl cyclase activity was measured at the indicated times after stimulating the cells with 10 μM cAMP (see MATERIALS AND METHODS). Activity is reported as fold stimulation over basal activity, which was determined as pmoles of cAMP synthesized per minute per mg of total cellular protein from cells just prior to cAMP stimulation. Error bars show the SD between three (A) and five (B) experiments. Asterisks indicate statistically significant differences at a 0.05 confidence level based on a paired *t* test.

consistent, the differences between strains are not statistically significant. In the absence of exogenous

cAMP pulses, the average peak adenylyl cyclase activity of the $g\alpha3^-$ mutant was about 50% of the HPS400 cells and did not adapt.

Developing cells in the presence of exogenous cAMP pulses changed the kinetics of cAMP-stimulated adenylyl cyclase activity. Figure 4B was compiled from five separate assays for each strain developed with exogenous cAMP pulses and the data are reported as described above. In the HPS400 cells, basal adenylyl cyclase activity was 0.4 ± 0.2 pmol per min per mg. The maximum activity of (5.2 ± 2.8)-fold over basal was reached earlier in the presence of cAMP pulses, between 0.5 and 1.5 min after cAMP stimulation. The activity began to adapt 2 min after stimulation and slowly reached prestimulus levels by 9.5 min after stimulation. This result compared with the adenylyl cyclase activity in HPS400 cells developed without exogenous cAMP pulses demonstrates that prior cAMP pulsing leads to a more rapid adenylyl cyclase response. We speculate that this is due to the ability of exogenous cAMP pulses to entrain and synchronize the cAMP signaling system so that most of the cells synthesize and secrete cAMP at the same time (Kimmel, 1987).

Developing the $g\alpha3^-$ mutant in the presence of exogenous cAMP pulses dramatically changed the magnitude of cAMP-stimulated adenylyl cyclase activity in this strain. The prestimulus activity of 0.2 ± 0.2 pmol per min per mg and the peak in activity at 2.5 min after cAMP stimulation was similar to that of nonpulsed $g\alpha3^-$ cells (Figure 4, compare A to B). However, the maximum adenylyl cyclase activity of (11.8 ± 5.0)-fold over basal was twice that of the maximum activity of the HPS400 cells under the same conditions. From 1.5 min after cAMP stimulation through the remainder of the assay, the adenylyl cyclase activity of the $g\alpha3^-$ mutant was greater than that of HPS400 cells and this difference was statistically significant (Figure 4B). Unlike the $g\alpha3^-$ cells developed without exogenous cAMP pulses, the adenylyl cyclase activity began to adapt at 3 min after stimulation but did not reach the prestimulus level by 9.5 min after stimulation. Therefore, cAMP pulses elevate cAMP-stimulated adenylyl cyclase activity twofold higher than the parent strain and rescue adaptation in the $g\alpha3^-$ mutant.

Developmental Rescue Depends on the Length of Exposure to Exogenous cAMP Pulses

We previously found that aggregation could be restored in the $g\alpha3^-$ mutant treated with exogenous cAMP pulses (Brandon *et al.*, 1997). To determine the relationship between developmental rescue and exposure to exogenous cAMP pulses, $g\alpha3^-$ and HPS400 cells were removed at 0, 3, 5, and 8 h from cultures developing with or without exogenous cAMP pulses. The aliquots were placed on agar starvation plates to observe the degree of developmental rescue. The results are shown in Figure 5. The development of HPS400 was not dependent on the length of time the cells received pulses. At all time points, these cells completed development after being transferred to agar starvation plates. In contrast, development of the $g\alpha3^-$ mutant was a function of the length of exposure to exogenous cAMP pulses. In $g\alpha3^-$ mutants that received no exogenous cAMP pulses, most cells did not aggregate and a small number formed a few loose mounds. Cells that received 3 h of exogenous cAMP pulses displayed some ability to aggregate and formed a larger number of loose mounds. In cells that received 5 h of exogenous cAMP pulses, a few of the loose mounds progressed to the tipped mound stage. Finally, in $g\alpha3^-$ cells that received 8 h of exogenous cAMP pulses, most of the cells participated in aggregation, many tipped mounds were formed, and some tipped mounds progressed to early culmination. $g\alpha3^-$ cells that were developed for 8 h without exogenous cAMP pulses before being placed on agar starvation plates displayed a phenotype identical to the $g\alpha3^-$ cells that were placed on agar starvation plates at 0 h of development. These results support our observation that the $g\alpha3^-$ mutant lacks the ability to produce endogenous cAMP pulses during early development but can respond to artifically supplied cAMP pulses. Providing the $g\alpha3^-$ mutant with cAMP pulses bypasses the defect in production and drives the cells to nearly complete development.

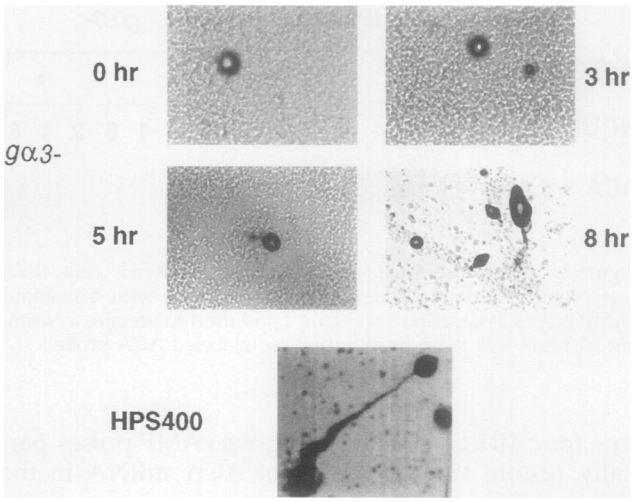

Figure 5. Developmental rescue of $g\alpha3^-$ cells by exogenous cAMP pulses. HPS400 and $g\alpha3^-$ cells were developed with exogenous cAMP pulses. At 0, 3, 5, and 8 h, aliquots were removed from the cultures, placed on agar starvation plates, and incubated at 22°C for 96 h. Photomicrographs of the cells on agar starvation plates were taken at 40× magnification 96 h after starvation was initiated.

DISCUSSION

In this study, we examined the role of the Gα protein subunit Gα3 in regulating gene expression and adenylyl cyclase activity during early *Dictyostelium* development. Gα3 is required for normal expression of the *cAR1*, *ACA*, *PDE*, and *PDI* genes. Not all early development genes, however, are influenced by Gα3, because Gα2 expression is normal in the $g\alpha3^-$ mutant. The requirement for Gα3 can be partially bypassed by supplying exogenous cAMP to the mutant to mimic the normal pulsed secretion of this developmental regulator. cAMP pulses increase ACA mRNA levels nearly to those of the parental strain and cause a twofold increase in ACA activity. cAR1 protein expression is restored in the cAMP-treated mutant and often rises above the level seen in similarly treated wild-type cells. In contrast, cAMP pulses increase PDE expression only slightly and do not restore PDI expression. Our results indicate that Gα3 plays a key role in the induction of several cAMP signaling system genes. The ability of exogenous cAMP pulses to bypass the block imposed by the absence of Gα3 suggests that some other cellular component can partially substitute for Gα3.

Our results on the effects of Gα3 on gene expression can account for the ability of cAMP pulses to rescue aggregation in the $g\alpha3^-$ mutant. Increasing periods of pretreatment with cAMP before placing the cells on agar starvation plates achieved correspondingly more advanced stages of development. Our interpretation of this result is that the activation of gene expression through an alternative signaling pathway is more effective with longer exposure to cAMP pulses. Whether this pathway is used with the Gα3-dependent pathway during normal development is unknown, but its existence suggests the operation of a signal integration and response network controlled by G-protein–mediated second messenger cascades.

The higher level of adenylyl cyclase activity in the $g\alpha3^-$ mutant developed with exogenous cAMP pulses suggests the presence of a negative regulator of adenylyl cyclase activity that requires Gα3 for expression. This putative negative regulator, like the PDI, could not be induced by exogenous cAMP pulses. Alternatively, the higher adenylyl cyclase activity seen in the $g\alpha3^-$ mutant treated with exogenous cAMP pulses may be due to stimulation of the enzyme by excess Gβγ. Although we have no evidence for the existence of excess free Gβγ subunits in the $g\alpha3^-$ mutant, if they are present they would be expected to contribute to adenylyl cyclase activity because the *Dictyostelium* adenylyl cyclase can be activated by Gβγ (Wu *et al.*, 1995b). It is possible that Gα3 itself is a negative regulator of ACA activity, although the evidence for its function as a regulator of gene expression is more compelling.

A model for the function of Gα3 in early development gene expression is presented in Figure 6. We propose that in wild-type cells, Gα3 release from a Gα3/Gβγ heterotrimer is triggered by ligand-mediated activation of a transmembrane receptor (R). The free GTP-bound Gα3 activates an effector (E) that in turn activates two distinct pathways leading to gene expression, one to PDI and another to cAR1 and ACA (Figure 6A). The single arrows connecting Gα3, effector, and gene expression in Figure 6 represent multiple steps within the cell. In the $g\alpha3^-$ mutant, the activated receptor is uncoupled from the effector, blocking the expression of all Gα3-dependent genes (Figure 6B). These include but may not be limited to the genes examined in this study. An alternative pathway can substitute for loss of Gα3 when cells are exposed to exogenous cAMP pulses (Figure 6C). We propose that this pathway is dependent on Gα2 coupled to either or both of the cAMP receptors cAR1 and cAR3. Gα2 is an established regulator of early development and we have shown herein that its expression is not dependent on Gα3. Gα2 may partially substitute for Gα3 in cAMP-stimulated cells by activation of a downstream effector. However, activation by Gα2 is different than that of Gα3 because it induces only the branch of the pathway leading to expression of cAR1 and ACA but not to PDI. Our data do not allow us to predict the contribution of the alternative pathway to normal development.

PDE is not shown in the model because the effect of the Gα3 mutation on its expression is likely to be indirect. Wu *et al.*, (1995a) showed that PDE expression requires cAR1. In our model, Gα3 may influence PDE expression indirectly through the regulation of cAR1, not directly as a regulator of the pathway leading to PDE expression.

Although our results do not allow us to definitively assign a Gα3 effector, striking parallels between the phenotypes of the $g\alpha3^-$ mutant and protein kinase A (PKA) mutants suggest that PKA may function in the Gα3 signaling pathway. PKA activity in *Dictyostelium* has been eliminated by disruption of the catalytic subunit ($PKAcat^-$ mutants) or overexpression of mutant regulatory subunits that lack cAMP-binding sites (RM mutants; Mann and Firtel, 1991; Harwood *et al.*, 1992). In RM mutants, *cAR1*, *PDE*, and *ACA* genes are not expressed (Schulkes and Schaap, 1995). In $PKAcat^-$ mutants, cAR1, PDE, and PDI mRNAs are reduced or absent, but exogenous cAMP pulses partially rescue cAR1 expression (Mann and Firtel, 1991; Wu *et al.*, 1995a). In addition, adenylyl cyclase activity is normal in $PKAcat^-$ mutants developed with exogenous cAMP pulses (Mann and Firtel, 1991). In accordance with a model in which PKA is an effector for Gα3, we have recently determined that PKAcat is expressed in the $g\alpha3^-$ mutant (our unpublished observation).

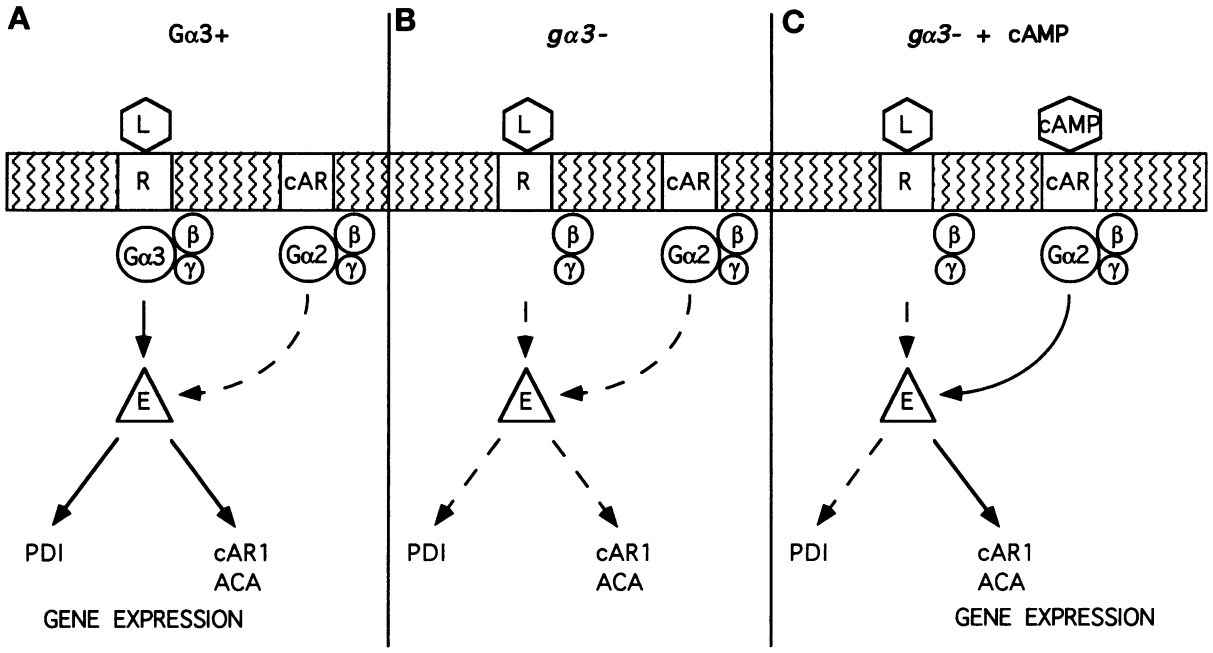

Figure 6. Model for the role of Gα3 in early development gene regulation. (A) Gα3-controlled gene expression in wild-type cells. (B) Absence of gene expression in the $g\alpha3^-$ mutant. (C) Partial rescue of gene expression in the $g\alpha3^-$ mutant developed with exogenous cAMP pulses. Broken lines represent nonfunctioning pathways. L, ligand; R, receptor; E, effector; bar containing zig-zag lines, plasma membrane. The model is described in the DISCUSSION.

cAMP and the receptors cAR1 and/or cAR3 may be coupled to Gα3. Although it appears counterintuitive to propose cAMP as a ligand if it is required to activate synthesis of its own receptor, components of the cAMP-signaling system are subject to dual regulation (Mann and Firtel, 1989). Starvation induces a low level of cAMP-signaling system gene expression, including cAR1, ACA, and the PDE. Later, much higher levels of expression are fueled by a self-reinforcing feedback loop composed of these gene products and secreted cAMP (Mann and Firtel, 1989; Wu *et al.*, 1995a). Consistent with the predictions of this model, we found a low level of ACA mRNA and enzyme activity in the $g\alpha3^-$ mutant (Figures 3 and 4). It is possible that Gα3 is involved in sensing the very low cAMP levels induced by starvation to induce high levels of gene expression necessary to produce the self-reinforcing feedback loop.

Two other known autocrine regulators of early *Dictyostelium* development, PSF and conditioned medium factor (CMF) are not likely to be coupled to Gα3 (Clarke *et al.*, 1988; Jain *et al.*, 1992). Burdine and Clarke (1995) reported that the PSF response was normal in the $g\alpha3^-$ mutant, and in a CMF-antisense cell line, cAR1 and PDE expression are normal, unlike the $g\alpha3^-$ mutant (Yuen *et al.*, 1995).

The results of this study establish a function for Gα3 as a regulator of early development gene expression. Further analysis of the Gα3-signaling pathway will reveal the proteins necessary to transmit a signal from the plasma membrane to the nucleus. The rescue of Gα3-dependent gene expression by exogenous cAMP pulses provides an indication that interconnected G-protein signaling paths control *Dictyostelium* development. Knowledge of the Gα3 signaling pathway may identify the convergence points that are predicted to exist among the multiple G-protein–regulated signaling systems of eukaryotes.

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