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Multiple Cyclic AMP Receptors are Linked to Adenylyl Cyclase in *Dictyostelium*

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cAMP receptor 1 and G-protein α-subunit 2 null cell lines (*car1*$^-$ and *g*α*2*$^-$) were examined to assess the roles that these two proteins play in cAMP stimulated adenylyl cyclase activation in *Dictyostelium*. In intact wild-type cells, cAMP stimulation elicited a rapid activation of adenylyl cyclase that peaked in 1–2 min and subsided within 5 min; in *g*α*2*$^-$ cells, this activation did not occur; in *car1*$^-$ cells an activation occurred but it rose and subsided more slowly. cAMP also induced a persistent activation of adenylyl cyclase in growth stage cells that contain only low levels of cAMP receptor 1 (cAR1). In lysates of untreated wild-type, *car1*$^-$, or *g*α*2*$^-$ cells, guanosine 5′-O-′(3-thiotriphosphate) (GTPγS) produced a similar 20-fold increase in adenylyl cyclase activity. Brief treatment of intact cells with cAMP reduced this activity by 75% in control and *g*α*2*$^-$ cells but by only 8% in the *car1*$^-$ cells. These observations suggest several conclusions regarding the cAMP signal transduction system. 1) cAR1 and another cAMP receptor are linked to activation of adenylyl cyclase in intact cells. Both excitation signals require Gα2. 2) cAR1 is required for normal adaptation of adenylyl cyclase. The adaptation reaction caused by cAR1 is not mediated via Gα2. 3) Neither cAR1 nor Gα2 is required for GTPγS-stimulation of adenylyl cyclase in cell lysates. The adenylyl cyclase is directly coupled to an as yet unidentified G-protein.

INTRODUCTION

Dictyostelium discoideum uses a G-protein linked signal transduction system to guide the initial stage of its developmental program, the spontaneous aggregation of thousands of isolated amoebae to form single multicellular organisms (Devreotes, 1989). The known components of this system are a seven transmembrane domain surface cyclic AMP (cAMP)[1] receptor (cAR1), a G-protein α-subunit (Gα2), and a membrane bound aggregation stage adenylyl cyclase (ACA) (Kesbeke *et al.*, 1988; Klein *et al.*, 1988; Kumagai *et al.*, 1989; Pitt *et al.*, 1992). ACA is topologically homologous to mammalian adenylyl cyclases, containing 12 putative transmembrane domains and two homologous cytosolic domains. Three additional cAMP receptors, cAR2, cAR3, cAR4, and seven additional G-protein α-subunits also have been shown to be expressed transiently at specific times in the developmental program (Hadwiger and Firtel, 1991; Saxe *et al.*, 1991; Wu and Devreotes, 1991; Pupillo, Pitt, Vogelmaier, and Devreotes, unpublished data). A single β-subunit is constitutively expressed throughout growth and development (Pupillo *et al.*, 1988).

There are several well-studied examples for activation and inactivation of receptor-dependent GTP-sensitive adenylyl cyclase systems (Gilman, 1987). In avian erythrocytes and mammalian brain, reconstitution of β-adrenergic receptor, G_s, and adenylyl cyclase is sufficient for activation (Gilman, 1987). The occupied receptor catalyses the exchange of GTP for GDP causing the dissociation of the α_s- and $\beta\gamma$-subunits. The activated α_s-subunit then stimulates the adenylyl cyclase. Occupancy of the receptor also leads to its phosphorylation by a specific receptor kinase. A regulatory protein, β-arrestin, binds to the phosphorylated receptor, blocking

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[1] Abbreviations used: ACA, adenylyl cyclase, aggregation; cAMP, cyclic AMP; cAR, cyclic AMP receptor; DB, development buffer; DTT, dithiothreitol; Gα2, G-protein α-subunit; GTPγS, guanosine 5′-O-′(3-thiotriphosphate); LB, lysis buffer; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

further interaction with G_s (Benovic *et al.*, 1988; Dohlman *et al.*, 1991). Recent observations, however, suggest that other activation and inactivation mechanisms may exist. For instance, excitation of G_i or G_q related pathways can lead to stimulation of a G_s-linked adenylyl cyclase by releasing $\beta\gamma$-subunits that stimulate the type II enzyme (Tang and Gilman, 1991; Federman *et al.*, 1992).

We recently have reported the construction of cAR1 and Gα2 null mutants (*car1*$^-$ and *gα2*$^-$) of *Dictyostelium* through homologous recombination (Kumagai *et al.*, 1991; Sun and Devreotes, 1991). Both of these genes are required for development. In the current study, we used these mutants to analyze the mechanism of cAMP signal transduction. This genetic approach has led to several surprising observations. First, although cAR1 contributes to stimulation of adenylyl cyclase, activation can occur in its absence, suggesting that another cAMP receptor is also linked to the enzyme. Second, the mechanisms for receptor-mediated adenylyl cyclase activation and adaptation appear to be different than those proposed by the standard models.

MATERIALS AND METHODS

Cell Growth and Development Conditions

Cells were grown in HL5 medium with G418 or thymidine supplements as required. For development, the cells were washed in development buffer (DB), resuspended at 2×10^7/ml in DB, and shaken at 100 rpm for 4.5 h. cAMP was added (to a final concentration of 50 nM) at 6-min intervals. To interrupt the natural cAMP oscillations and obtain cells with basal adenylyl cyclase activity, the developed cultures were diluted to 2×10^6/ml in DB and shaken rapidly (~200 rpm) for ≥30 min at 22°C. Cells were washed once in DB without calcium at 0°C, resuspended at 8×10^7/ml, and held at 0°C.

cAMP Stimulation of Adenylyl Cyclase

Cells with basal adenylyl cyclase activity were shaken in beakers either on ice or at room temperature. Dithiothreitol (DTT) (to inhibit phosphodiesterase) and cAMP were added sequentially to final concentrations of 10 mM and 10 μM, respectively. DTT alone does not elicit a response. At times after cAMP stimulation, cells were removed and mixed 1:1 (vol/vol) with 2× lysis buffer (LB: 20 mM tris(hydroxymethyl)aminomethane [Tris], pH 8, 2 mM $MgSO_4$). The mixture was lysed by rapid push through a 5-μm pore size membrane. Two hundred microliters of this lysate was immediately added to the adenylyl cyclase assay as described below.

Guanosine 5′-O′-(3-thiotriphosphate) (GTPγS) Stimulation

Cells were mixed 1:1 (vol/vol) in 2× LB ± 80 μM GTPγS (final 40 μM) ± 2 μM cAMP (final 1 μM) and lysed by rapid push through a 5-μM pore size nucleopore membrane. The lysates were incubated at 4°C for 2 min (to allow GTPγS activation) and then 200 μl was added to the adenylyl cyclase assay.

Adenylyl Cyclase Assay

Two hundred microliters of cell lysates prepared as described above were added to 20 μl of 10× reaction mix consisting of 0.1 M Tris, pH 8, 3 mM ATP, 5 mM cAMP, 0.1 M DTT, and ~30 nM α^{32}P-ATP (440 Ci/mmol). The reaction continued for 1 min in a room temperature water bath, after which it was stopped and cAMP purified (Solomon, 1979).

Total cAMP Synthesis Assay

Total cAMP produced during persistent stimulation with the cAMP analogue 2′ deoxy cAMP was determined by the method described by Van Haastert (1984).

Membrane Preparation and Western Blotting

Membranes were prepared as described in Klein *et al.* (1988). The membrane pellet was resuspended at 5×10^7 cell equivalents/ml in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer, and 2×10^6 cell equivalents were electrophoresed on a 10% SDS polyacrylamide gel. The membrane proteins were transferred to nitrocellulose and probed with antiserum directed against cAR1.

Genotypes

The genotypes of the cell lines used are as follows. KAX-3, axenic wild-type; HC-6, axenic parent; HPS400, thymidine auxotroph of AX-3; JS8, car1::neo in HC-6; JS12, "random" integrant in HC-6 (derived from same transformation as JS8); JS14, car1::neo in KAX-3; MP2, gα2::THY1 in HPS400; JM1, gα2::THY1 (retransformed with cAR1/ACT15 expression vector); aca$^-$/ACA_{act15}, aca::URA (retransformed with ACA/ACT15 expression vector).

RESULTS

cAMP Stimulates Adenylyl Cyclase Activity in *car1*$^-$ Cells

Previous experiments have shown that cell lysis causes a rapid loss of receptor-mediated activation of adenylyl cyclase. However, the state of activation of the enzyme during cAMP stimulation of intact cells can be assessed by rapid lysis followed by brief assay (Roos *et al.*, 1977). Such an "activation-trap" experiment is illustrated in Figure 1. Cells were allowed to develop, prepared in the basal state, and then stimulated with cAMP. At time

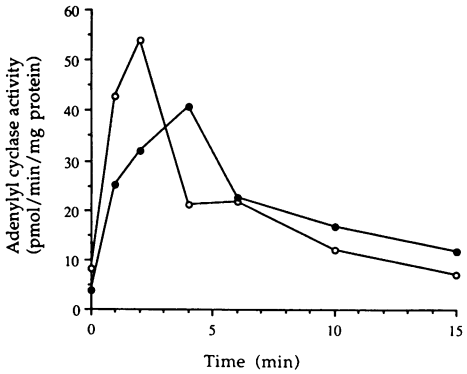

Figure 1. "Activation trap" assay of adenylyl cyclase activation. Whole cells were stimulated with 10 μM cAMP for the indicated times, rapidly lysed, and adenylyl cyclase activity was measured as described in METHODS. Wild-type (HC6-vector) cells (—— ○ ——); *car1*$^-$ cells (—— ● ——).

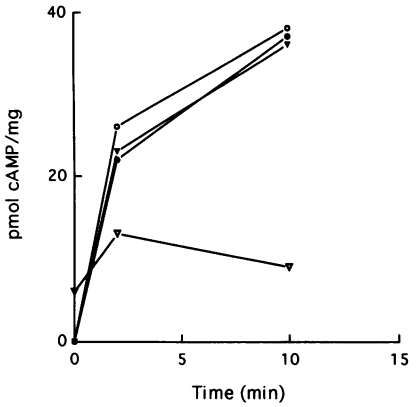

Figure 2. cAMP-stimulated increases in total cAMP levels. Wild-type (JS12) and *car1*⁻ cells (JS8) were prepared in a basal state. At the indicated times before and after addition of 10 μM 2′-dcAMP plus 10 mM DTT, aliquots were removed and assayed for cAMP as described in METHODS. Composite of two independent experiments. JS12 (○, ●); JS8 (▽, ▼).

points after stimulation, an aliquot of cells was removed from the suspension, lysed, and immediately incubated with adenylyl cyclase reaction mix for 60 s at 22°C.

In the wild-type cells (AX-3), adenylyl cyclase activity increased immediately on cAMP addition, peaked at 1–2 min, and then returned toward the prestimulus level within 5–10 min (Figure 1). The return to the prestimulus state of activation occurs in the continued presence of 10 μM cAMP and reflects the adaptation of the system. The properties of this adaptative behavior have been characterized extensively and are discussed in greater detail below (Devreotes, 1989).

Surprisingly, the *car1*⁻ cells (JS14) also display cAMP-stimulated adenylyl cyclase activity. The sharp peak typically observed at 1–2 min in wild-type cells, however, does not occur. Instead, activity in the *car1*⁻ cells peaks at 3–5 min and subsides slowly (Figure 1). Similar results were obtained in an independent cAR1⁻ cell line (JS8) created in a different parent (HC-6). Thus, the absence of cAR1 affects the time course of the activation but cAR1 is not essential for the response, suggesting that another cAMP receptor can activate the enzyme.

To further document capacity of *car1*⁻ cells to respond, we monitored total (the sum of internal and secreted) cAMP during persistent stimulation of intact *car1*⁻ cells. As shown in Figure 2, on addition of the stimulus, total cAMP levels rose rapidly and then plateaued between 2 and 10 min. In an average of four independent determinations of the amount of cAMP accumulated within 10 min of addition of the stimulus, the *car1*⁻ (JS8) cells produced 99 ± 73% (mean ± SD) as much as the wild-type parental cells (HC-6). Thus, total cAMP levels reflected the elevated adenylyl cyclase activities observed in the activation trap assays (Figure 1).

In an independent series of experiments, we had observed that the aggregation stage adenylyl cyclase (ACA), when overexpressed in growth stage cells, was responsive to cAMP stimulation. Growth stage cells generally express only low levels of cAR1. Yet, as shown in Figure 3, when these cells were stimulated with cAMP and assayed by the activation trap method, a large prolonged activation of the adenylyl cyclase ensued. In these cells, little cAR1 is detectable by immunoblot (inset), again suggesting that another cAMP receptor that must be expressed during the growth stage can mediate the activation of the enzyme.

cAR1 is not Required for GTP-Stimulated Adenylyl Cyclase Activity in Cell Lysates

Although it has been difficult to demonstrate receptor-mediated activation of adenylyl cyclase in broken cells, GTP and GTPγS activation of the enzyme is robust (Theibert and Devreotes, 1986; Snaar-Jagalska and Van Haastert, 1988). We used the *car1*⁻ cells to test whether cAR1 was required for GTP and GTPγS to be effective. The GTPγS activation of adenylyl cyclase is shown for wild-type and mutant cells in Table 1. In each case, the cell lines were repeatedly stimulated with cAMP until the wild-type had reached the aggregation stage. The cells were then prepared in the basal state and lysed in the presence or absence of GTPγS (see METHODS). The *car1*⁻ (JS8) cells displayed GTPγS-stimulated adenylyl cyclase activity comparable with control cells (77 ± 13%), suggesting that cAR1 is not required for maximal activation of the enzyme. We also tested the *gα2*⁻ cells (MP2) in the same experiment. Consistent with previous observations on the *Frd A* mutants (Kesbeke *et al.*, 1988), the *gα2*⁻ cells (MP2) displayed wild-type levels of GTPγS-stimulated adenylyl cyclase activity (98 ± 62%). Thus, both the *car1*⁻ and *gα2*⁻ cell lines must express a G-protein that confers the guanine nucleotide

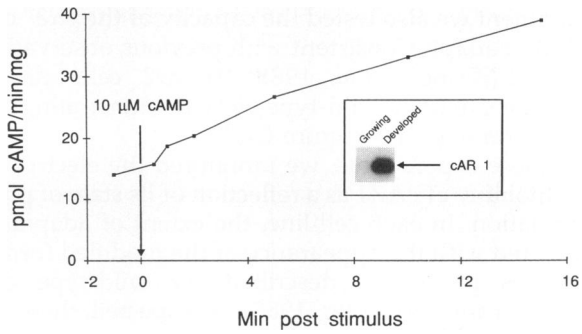

Figure 3. Activation trap assay in undifferentiated cells. Wild-type cells, overexpressing ACA in the growth stage (Pitt *et al.*, 1992), were stimulated with 10 μM cAMP for the indicated times, rapidly lysed, and adenylyl cyclase activity was measured as described in METHODS. Inset, immunoblot of cAR1 in growth stage (t = 0) and 5-h stage (t = 5) cells.

Table 1. GTPγS-stimulated adenylyl cyclase activity

	Percent of maximum	
	Basal	Stimulated
HC6-vector	4 ± 3	100
cAR1⁻ (JS8)	1 ± 2	77 ± 13
HPS400	4 ± 1	100
Gα2⁻ (MP2)	7 ± 6	98 ± 62

Cells were lysed in the presence or absence of 40 μM GTPγS and 1 μM cAMP. Adenylyl cyclase activity was measured after 2 min as described in METHODS. Results are presented as percent of maximum activity (activity of control cells, HC6-vector or HPS400, in the presence of 40 μM GTPγS and 1 μM cAMP). Maximum activity for HC6-vector cells was 178 ± 91 pmol · min^{-1} · mg $protein^{-1}$ (n = 3) and for HPS400 cells was 126 ± 48 pmol · min^{-1} · mg $protein^{-1}$ (n = 4).

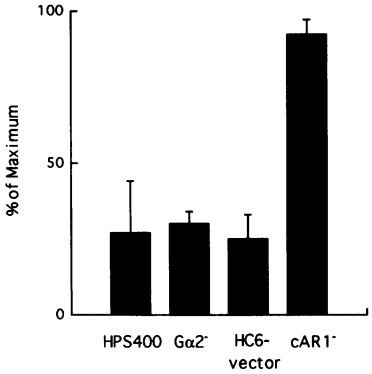

Figure 4. Adaptation of GTPγS-stimulated adenylyl cyclase activity. Whole cells were either stimulated with 10 μM cAMP for 5 min at room temperature to adapt the adenylyl cyclase or held at 0°C to maintain basal activity. The cells were then lysed in the presence or absence of 40 μM GTPγS and 1 μM cAMP as described in METHODS. Maximum activity was obtained from unadapted cells stimulated with 40 μM GTPγS and 1 μM cAMP.

sensitivity to the adenylyl cyclase, and this G-protein is effectively activated in the absence of cAR1.

Rapid Adaptation does not Occur in car1⁻ Cells

In wild-type cells, adenylyl cyclase activity subsides after several minutes in the continued presence of cAMP (Dinauer *et al.*, 1980) (Figure 1). As previously reported, this adaptation process is reflected in a reduced capacity of GTPγS to activate the enzyme in vitro (Theibert and Devreotes, 1986). Because, as illustrated in the previous section, cAR1 is not required for GTPγS activation of the enzyme, this assay may be used to assess the role of cAR1 in adaptation. Cells were incubated in the presence or absence of a cAMP stimulus for 5 min and then lysed in the presence of GTPγS. Figure 4 shows the effects of these treatments on *car1⁻* (JS8) and control cells. The control cell lines adapt strongly to cAMP stimulation as shown by an ~75% reduction in GTPγS-stimulated adenylyl cyclase activity. In contrast, GTPγS-dependent adenylyl cyclase activity in the lysates of the *car1⁻* cells is only reduced by 8%, demonstrating that cAR1 is required for normal adaptation. In the same experiment we also tested the capacity of the *gα2⁻* cells (MP2) to adapt. Consistent with previous observations on *Frd A* (Kesbeke *et al.*, 1988), the *gα2⁻* cells adapt to the same extent as wild-type cells, demonstrating that adaptation does not require Gα2.

In these experiments, we monitored the electrophoretic mobility of cAR1 as a reflection of its state of phosphorylation. In each cell line, the extent of adaptation correlated with the appearance of the modified form of cAR1 as previously described for wild-type cells (Vaughan and Devreotes, 1988). As expected, there was no cAR1 band present in the *car1⁻* cells.

cAR1-Dependent and cAR1-Independent Responses Require Gα2

The observations in Table 1 and Figure 4 above verify previous conclusions from *Frd A* cells, that both GTP stimulation and cAR1-induced adaptation of adenylyl cyclase are independent of Gα2 (Kesbeke *et al.*, 1988). It also has been reported previously that in intact *Frd A* cells a cAMP stimulus does not trigger activation of adenylyl cyclase (Kesbeke *et al.*, 1988). Consistently, we found that in the *gα2⁻* cells, there is no cAMP-stimulated adenylyl cyclase activity. However, this failure to respond might be due to deficient expression of cAR1 because the number of cAMP binding sites on either *gα2⁻* or *Frd A* cells is <25% that of the wild-type parental cells (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1991). To rule out this possibility, we examined *gα2⁻* cells transformed with a plasmid overexpressing cAR1 (JM1 cells; Milne, personal communication). This experiment also tests whether excess cAR1 can suppress the defect in *gα2⁻* cells. As shown in Figure 5, the JM1 cells display no stimulation of adenylyl cyclase activity above the prestimulus levels. The protein blots (inset) show that the

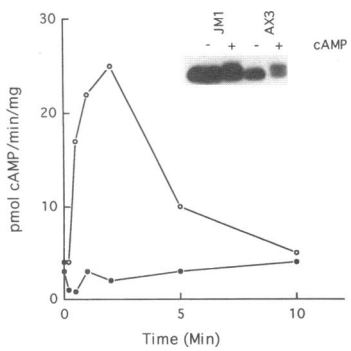

Figure 5. *Gα2⁻* cells containing excess cAR1 fail to activate ACA. Experiments were carried out exactly as for Figure 1. Wild-type (HPS-400) cells (—— ○ ——) and JMI (cAR1 overexpressed in gα2⁻) cells (—— ● ——). Inset, immunoblot of cAR1 in WT and JM1 cells.

JM1 cells contain a large excess of cAR1 protein relative to wild-type AX3 cells. Therefore, in the presence or absence of large amounts of cAR1, there is no cAMP stimulation of adenylyl cyclase without Gα2.

DISCUSSION

A schematic pathway summarizing the observations reported here is illustrated in Figure 6. Surprisingly, cAR1 does not appear to be essential for adenylyl cyclase activation in vivo. Activation of adenylyl cyclase is vigorous in *car1*⁻ cells, although it peaks late and adapts slowly (Figure 1). This indicates that another cAMP receptor mediates adenylyl cyclase activation in the *car1*⁻ cells. It has been noted previously that persistent cAMP stimulation triggers a kinetically biphasic activation of the adenylyl cyclase (Roos *et al.*, 1977; Dinauer *et al.*, 1980; Devreotes, 1989). It is possible then that cAR1 mediates the first component of the biphasic response and a different receptor triggers the second phase.

It is possible that cAR3, cAR2, or cAR4 mediates this cAR1-independent activation, because they are sequentially expressed at the completion of aggregation (Saxe *et al.*, 1991). However, we can detect no cAR3 or cAR2 on western blots, and photoaffinity labeling does not reveal bands corresponding to the sizes of these three receptors. Furthermore, the cAR1-independent response also can be triggered in growth stage cells (provided that ACA is constitutively expressed) that are less likely to contain these cARs (Figure 3). Because the *car1*⁻ cells display few cAMP binding sites, the novel cAMP receptor might be of low affinity or otherwise undetectable in our standard assay conditions. We are currently investigating the dose dependence and cAMP analogue specificity of the cAR1-independent response. Preliminary results suggest that higher concentrations of cAMP are required in the absence of cAR1. We previously have reported that cAR1 antisense cells do not elevate cAMP levels (Sun *et al.*, 1990). In those early experiments, the antisense cells were allowed to develop on agar surfaces. In the current experiments, cells were shaken and repeatedly stimulated with cAMP at 6-min intervals. The apparent discrepancy in the two experiments might be explained by a failure of the cAR1 antisense cells to express the other cAMP receptor or another component of the system.

Our results indicate that the mechanism of receptor-mediated adenylyl cyclase inactivation can differ from the standard paradigm. We propose that the presence of modified (phosphorylated) cAR1 prevents the in vitro GTPγS activation of adenylyl cyclase (Theibert and Devreotes, 1986). The extent of loss in the capacity of GTPγS to activate the enzyme in vitro is directly correlated with the amount of modified cAR1 present at the moment of cell lysis (Figure 4) (Theibert and Devreotes, 1986). However, cAR1 must not participate in the GTPγS activation of the enzyme in vitro because

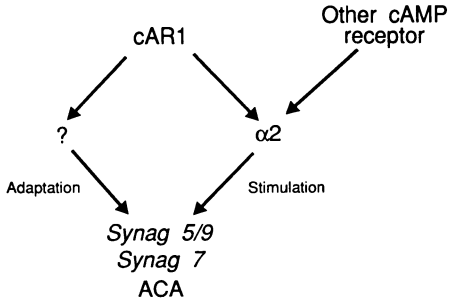

Figure 6. Schematic diagram of signal transduction pathway for receptor-mediated stimulation and adaptation of adenylyl cyclase.

the reaction occurs to its full extent in *car1*⁻ cells (Table 1). To explain then how the effects of in vivo occupancy (and phosphorylation) of cAR1 persist in vitro, we propose that adaptation occurs by modification of a G-protein linked to the adenylyl cyclase or of the enzyme itself. The pathway leading to adaptation appears to be distinct from that for activation because it is independent of Gα2.

Our results also indicate that the mechanism of activation for adenylyl cyclase can differ from the standard paradigm. Gα2 is absolutely required for cAMP stimulation of adenylyl cyclase in intact cells (Figure 5). This implies that the excitation signals from cAR1 as well as that from the novel cAMP receptor are mediated via Gα2. However, when the adenylyl cyclase is activated by GTPγS in vitro, full activation of adenylyl cyclase is achieved in the absence of Gα2, indicating that a component(s) other than Gα2 directly confers GTP sensitivity to adenylyl cyclase. This unusual notion of a "G-protein talking to a G-protein" is not unprecedented. Bourne and co-workers have shown that in cells transfected with type II adenylyl cyclase, activation of G_i or G_q can lead to elevations in cAMP, presumably by release of βγ subunits that, in concert with $G\alpha_s$, stimulate the enzyme (Federman *et al.*, 1992).

In addition to cAR1 and Gα2, at least two other gene products are involved in activation of adenylyl cyclase. Mutations in several complementation groups, represented by the mutants designated *synag 5/9* and *synag 7*, cause a parallel failure in both GTPγS-stimulated adenylyl cyclase in vitro and cAMP receptor-mediated activation in vivo (Theibert and Devreotes, 1986; Vaughan *et al.*, 1987). Both of these gene products appear to lie distal to Gα2 on the pathway because the mutants display wild-type cAMP-mediated chemotaxis and early gene expression that require Gα2. Thus, the products of these genes are candidates for the component(s) that directly confer GTP sensitivity to the enzyme. Current studies are directed at identifying the gene products defective in these mutants and in defining the complementation groups involved in activation.

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