CRAC, a Cytosolic Protein Containing a Pleckstrin Homology Domain, Is Required for Receptor and G Protein–mediated Activation of Adenylyl Cyclase in Dictyostelium

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Abstract. Adenylyl cyclase in Dictyostelium, as in higher eukaryotes, is activated through G protein–coupled receptors. Insertional mutagenesis into a gene designated dagA resulted in cells that cannot activate adenylyl cyclase, but have otherwise normal responses to exogenous cAMP. Neither cAMP treatment of intact cells nor GTPγS treatment of lysates stimulates adenylyl cyclase activity in dagA mutants. A cytosolic protein that activates adenylyl cyclase, CRAC, has been previously identified. We trace the signaling defect in dagA cells to the absence of CRAC, and we demonstrate that dagA is the structural gene for CRAC. The 3.2-kb dagA mRNA encodes a predicted 78.5-kD product containing a pleckstrin homology domain, in agreement with the postulated interaction of CRAC with activated G proteins. Although dagA expression is tightly developmentally regulated, the cDNA restores normal development when constitutively expressed in transformed mutant cells. In addition, the megabase region surrounding the dagA locus was mapped.

We hypothesize that CRAC acts to connect free G protein βγ subunits to adenylyl cyclase activation. If so, it may be the first member of an important class of coupling proteins.

Responses to light, odorants, chemoattractants, and many hormones and neurotransmitters are mediated by G protein–coupled receptors. When excited, these receptors activate heterotrimeric G proteins, catalyzing the exchange of GTP for GDP on the α-subunit and the dissociation of the α from the βγ-subunit complex. Both of these components can stimulate or inhibit effectors including adenylyl cyclases, phosphodiesterases, phospholipases, and ion channels (Gilman, 1987).

Increasing evidence has highlighted the role of βγ-subunits in directly regulating effectors, rather than in merely modulating α-subunit activity (Birnbaumer, 1992). Several particular adenylyl cyclase subtypes, ion channels, and phospholipases are activated by free βγ complexes (Tang and Gilman, 1991; Logothetis et al., 1987). The phospholipases share a region, the pleckstrin homology (PH) domain, which is also found in a variety of other signal transduction proteins (Parker et al., 1994). It has been suggested that the PH domains are sites of interaction with βγ-subunit complexes (Musacchio et al., 1993). This hypothesis is supported by the recent discovery that fusion proteins containing various PH domains bind to dissociated βγ-subunits (Touhara et al., 1994). No adenylyl cyclases have been found to contain PH domains; the mechanisms by which βγ-subunits control adenylyl cyclase activity are as yet unknown.

Dictyostelium cells feed and grow singly, but on starvation, they aggregate to form a millimeter-sized organism containing up to 10⁶ cells. This process is controlled by a complex G protein–linked signal transduction system that is highly homologous to those used by higher eukaryotes (reviewed in Devreotes, 1989, 1994). Central cells secrete pulses of cAMP at 6-minute intervals; the surrounding cells advance chemotactically towards the center and reinforce (or "relay") the signal by secreting additional cAMP. cAMP pulses also induce specific changes in gene expression in the aggregating cells. All three processes, chemotaxis, relay, and control of gene expression, are mediated by a cell surface cAMP receptor, cARI (Klein et al., 1988). cAMP binding to cARI activates the G protein G2, which leads to an elevation of intracellular cGMP levels and other responses required for chemotaxis. Activation of G2 also leads to an increase in the activity of the adenylyl cyclase (ACA), which generates the signal relay.

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Recent evidence suggests that, like mammalian adenylyl cyclases, ACA is stimulated by the βγ-subunit complex released from G2 (Pupillo et al., 1992; Keskebe et al., 1988; Lilly et al., 1993). Genetic analysis indicates that there are several components in the pathway that connects G2 to ACA activation. One such protein has been designated CRAC, for cytosolic regulator of adenylyl cyclase (Lilly and Devreotes, 1994). CRAC was originally identified as an activity that is lacking in a mutant strain, synag7; these cells are unable to activate adenylyl cyclase and, therefore, they cannot aggregate or develop. ACA activity in synag7 lysates may be reconstituted by adding cytosol from wild-type cells (Theibert and Devreotes, 1986). This reconstitution has been used as an assay to partially purify CRAC and to identify it as an 88-kD protein (Lilly and Devreotes, 1994).

Restriction enzyme-mediated integration (REMI) of plasmid DNA into the genome has recently been developed as a way to identify and clone genes of Dictyostelium (Kuspa and Loomis, 1992). From a general screen of BamHI REMI transformants, mutants defective in aggregation were selected for further analysis. Nine independent mutants were found to have suffered insertions in a gene that we named dagA. dagA− mutant cells fail to show any evidence of mutual attraction but respond to wild-type cells in chimeric mixtures by coaggregating. Chemotaxis to cAMP appears to be normal, but relay of the cAMP signal by the stimulation of adenylyl cyclase activity is impaired. The defect in dagA− cells was traced to the absence of CRAC. Two results demonstrate that dagA encodes the CRAC protein. The NH2-terminal sequences of dagA and CRAC coincide, and expression of dagA in human tissue culture cells yields cytosol containing active CRAC protein.

Molecular Cloning

Genomic clones carrying the dagA locus were recovered from several dagA− mutants after digestion of genomic DNA with CiaI or BglII, religation of plasmids, and ampicillin selection in Escherichia coli (Kuspa and Loomis, 1992). cDNAs were identified in a Agt1 CDNA bank prepared from RNA isolated at 2–4 h of development (Klein et al., 1988). cDNAs of dagA inserted into the EcoRI site of Bluescript vectors were identified by hybridization with the CiaI/BamHI fragment from p20Cla after hexamer labeling (Maniatis et al., 1982). The largest insert (2.3 kb) was subcloned downstream of the act15 promoter region in pBluescript KS to generate the act15::dagA expression vector.

Clones were sequenced by the dideoxy chain termination method using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) and pertinent primers (Sanger et al., 1977). All sequences were read independently on both strands.

For expression in mammalian tissue culture cells, the dagA cDNA was cloned into pGW1 (British Biotechnology Ltd., Oxford, U.K.), and was transfected into human embryonic kidney 293 cells as described in Levin et al. (1992).

Physical Mapping

The insert in p20Bgl was isolated, labeled with α32PdCTP (Feinberg and Vogelstein, 1983), and hybridized to Southern blots of an arrayed set of yeast artificial chromosome clones carrying large inserts of Dictyostelium DNA (Kuspa et al., 1992). The pertinent yeast artificial chromosome (YAC) clones were positioned on the long-range restriction map generated with rare-site restriction enzyme digests of high molecular weight Dictyostelium genomic DNA surrounding the dagA locus by probing digests prepared with restriction enzymes including Apal and BglII (Kuspa et al., 1992). The probe for the linked gene, pkb, was kindly provided by Joe Dynes and Richard Firtel (Center for Molecular Genetics, UCSD, La Jolla, CA). Sequence analysis of the pkb clone suggests that it encodes a protein kinase.

Molecular Analyses

RNA was prepared from strains AK108 (dagA−), RI-6d (actin15::dagA over-expression construct in a dagA− background), RI-7b (actin15::dagA over-expression construct in a dagA− background), and AX4 at various times of development and used in Northern blot analyses as described previously (Fosnaugh and Loomis, 1991). The hybridization probe specific for cotB was obtained from pG04 (Fosnaugh and Loomis, 1989) and the probe for ecmA was obtained from pDD63 (McRobbie et al., 1988). The dagA probe was a full-length cDNA.

Western analyses were carried out after electrophoresis in 10% SDS polyacrylamide gels by transfer to nitrocellulose and probing with antibodies specific to cARI (Klein et al., 1988) or adenylyl cyclase (the kind gift of Dr. C. Parent, Dept. of Biological Chemistry, Johns Hopkins University). Membranes were prepared with ammonium sulfate as described by Klein et al. (1988); each sample analyzed 2 × 106 cell equivalents.

Biochemical Assays

cGMP was determined in cell lysates prepared as described by Mato et al. (1977) using the scintillation proximity assay (Amersham International Corp., Arlington Heights, IL).

Adenylyl cyclase activation was measured as described by Pupillo et al. (1992). Briefly, cells were shaken at 10−5 ml in 16 mM phosphate buffer, pH 6.4, and stimulated with 10 μM cAMP. At various times after cAMP addition, cells in 100-μl suspension were lysed, and the adenylyl cyclase activity of the lysate was assayed using α32PdATP.

Reconstitution of GTP·S stimulation of adenylyl cyclase was performed as described by Lilly and Devreotes (1994). Lysates of wild-type and dagA− mutant cells were prepared after 5 h, and synag7− cells after 10 h of development in suspension with addition of 100 mM cAMP pulses. Cytosol containing CRAC was prepared from cells after 4 h of development. To prepare partially purified CRAC, the supernatant was fractionated on a Sepharose Q Fast Flow column, and the peak fractions further fractionated on a Sepharose S Fast Flow column (Pharmacia, Uppsala, Sweden) (Lilly and Devreotes, 1994). The peak fractions were used to reconstitute GTP·S stimulation of adenylyl cyclase. Human 293 cells were collected in PBS and EDTA 3 d after transfection, snap-frozen, then thawed and sonicated for 1 min in sucrose buffer (10 mM Tris-Cl, pH 8, 0.2 mM EGTA, 200 mM sucrose), and then treated exactly as Dictyostelium lysates.
Results

Isolation of the dagA Gene by REMI Mutagenesis

By visually screening populations of REMI-mutagenized Dictyostelium cells as colonies on bacterial lawns, mutants that were arrested in development before tight mound formation were recovered (Kuspa and Loomis, 1992). After screening, 8,500 BamHI REMI transformants, 24 mutants were isolated. DNA flanking the insertion site in strain AK120 was cloned by digesting AK120 genomic DNA with BglII or ClaI, circularizing the linear fragments by ligation, and transforming E. coli (Kuspa and Loomis, 1992). The genomic fragment in one of the plasmids recovered, pl20Bgl, was used as a probe in Southern blot analysis of the other 23 mutants. Restriction site mapping, along with sequencing the DNA just proximal to the insertion sites, established that the mutations in six independent strains resulted from insertions into the identical BamHI site (named IS120) (Fig. 1). Cells of each of these strains are completely unable to aggregate or develop further. Several additional aggregationless mutant strains were isolated in a series of independent experiments and were shown by sequence comparisons to have also suffered insertions into IS120. In addition, sequencing of a plasmid rescued from mutant strain AK108 revealed that a distinct insertion event at IS108 had occurred 0.6 kb away from IS120. IS108 and IS120 were found to be unable to aggregate, under conditions that initiate development in wild-type cells, they fail to show any signs of aggregation or subsequent morphogenesis. Under these conditions dagA cells become mutually adhesive and can be shown to chemotactically respond to exogenous gradients of cAMP but do not accumulate the cell type–specific mRNAs, cotB or ecmA, that normally appear after aggregation (data not shown).

dagA Encodes a Hydrophilic Protein Containing a PH Domain

The predicted dagA protein product is a generally hydrophilic protein with a high proportion of serine and threonine residues, many of which are potential sites for phosphorylation. No proteins with significant similarity to the dagA product were found in GenBank or EMBL protein databases. However, the region between amino acids 23 and 174 encompasses a PH domain (Fig. 2 B), a motif typically found in proteins that interact with G proteins (Musacchio et al., 1993). PH domains are typically divergent between different proteins, and they show insertions of various lengths between different homologous blocks. In the case of dagA, there is an overall 37% identity with the PH consensus over 94 amino acids, with a 44-amino acid insert between the 5th and 6th blocks (numbered according to Musacchio et al., 1993). To test the significance of this alignment, we used the PH domain consensus of Musacchio et al. (1993) as the query in a BLAST search (Altschul et al., 1990) of the entire PIR, Swissprot, and Genpept databases. This search produced alignments with dagA, as well as several other PH domain–containing proteins. The alignment with dagA was less significant than those with pleckstrin, dynamin, and some but not all rasGAPs, but more significant than those with several canonical PH domain proteins, including Dro sophila SOS, β adrenergic receptor kinases 1 and 2, and phospholipase Cγ.

Developmental Defects of dagA Mutants

When dagA cells are deposited on moist filters under conditions that initiate development in wild-type cells, they fail to show any signs of aggregation or subsequent morphogenesis. Under these conditions dagA cells become mutually adhesive and can be shown to chemotactically respond to exogenous gradients of cAMP but do not accumulate the cell type–specific mRNAs, cotB or ecmA, that normally appear after aggregation (data not shown).

To test whether dagA was required for normal gene expression, mutant and wild-type cells were allowed to develop in suspension, with or without addition of 100 nM cAMP every
Figure 2. The dagA sequence. (A) The predicted amino acid sequence is given in the standard three-letter code directly beneath the nucleotide sequence derived from genomic DNA clones. The positions of the two short introns were determined by comparing the cDNA sequence to the genomic sequence. The start of the eDNA sequence to the genomic sequence. The start of the positions of the two short introns were determined by comparing the consensus sequence with the consensus sequence.

6 min to mimic normal signaling. In the absence of added cAMP, dagA cells accumulated less than wild-type levels of both ACA and cARI (see Fig. 3). When cells were repeatedly stimulated with cAMP, however, both dagA and wild-type cells accumulated similar levels of ACA and cARI proteins. This suggests that the dagA cells are defective in the production of cAMP signals, but they can respond normally.

After 5 h of stimulation with cAMP, cells were tested for chemotaxis and cAMP-induced cGMP synthesis. dagA- mutant and wild-type cells were equally responsive to doses of cAMP over the range of 10^-5 to 10^-8 M (data not shown). Furthermore, mutant cells responded to a cAMP stimulus, while wild-type cells did not. This suggests that the dagA cells are defective in the production of cAMP signals, but they can respond normally.

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Adenylyl cyclase and cAMP receptor proteins in wild type and dagA- mutant cells. Washed cells were shaken in phosphate buffer. At 6-min intervals, cAMP pulses (100 nM) were given to some of the cells. Samples were taken hourly, separated by SDS-PAGE, transferred to nitrocellulose, and stained with antibodies to adenylyl cyclase or the cAMP receptor.

dagA- cells. This is reminiscent of the behavior seen in aca- cells, which cannot develop alone, but are able to develop all the way to spore formation in an appropriate environment (Pitt et al., 1993).

cAMP Synthesis in dagA- Cells

Although dagA- cells can respond normally to cAMP in both chemotaxis and early gene expression, their ability to produce additional cAMP is defective. When wild-type cells are stimulated with cAMP, adenylyl cyclase is activated and the cells produce additional extracellular cAMP, thus relaying the chemotactic signal (Roos and Gerisch, 1976). When dagA- cells were treated with 100 nM cAMP in a standard ACA activation assay, adenylyl cyclase activity did not increase above background (Fig. 6).

ACA activity can be assayed in vitro in cell-free lysates or in partially purified cell membranes (Loomis et al., 1978; van Haastert et al., 1987; Devreotes et al., 1987). The activity in membrane preparations is greatly stimulated by addition of the nonhydrolyzable GTP analogue GTPTS, which activates and dissociates trimeric G proteins (Theibert and Devreotes, 1986). While GTPyS stimulated adenylyl cyclase activity about eightfold in lysates prepared from wild-type cells, it failed to significantly stimulate the activity in lysates prepared from dagA- cells (Table I). In the presence of Mn++ ions, which directly stimulate ACA activity, lysates of dagA- cells had as much ACA activity as did extracts of wild-type cells, confirming that the defect in dagA- cells does not affect the catalytic activity of adenylyl cyclase (Table I).

dagA- Cells Lack CRAC Activity

Since ACA in dagA- cells could not be stimulated in vivo by cAMP nor in vitro by GTPγS, a phenotype similar to that seen in the synag7 mutant (Theibert and Devreotes, 1986), we examined their CRAC activity. As shown in Table II, dagA- cells contain no measurable CRAC activity. Cytosol prepared from either dagA- or synag7 cells fails to restore the adenylyl cyclase response to GTPγS in synag7 lysates. Similarly, adenylyl cyclase was not activated in dagA- membranes when cytosol from synag7 cells was added along with GTPγS. ACA activity could be reconstituted in dagA- membranes by adding cytosol from wild-type cells (Table I).
Lack of cAMP signal relay in dagA- cells. Wild-type (circles) and dagA- (squares) cells were allowed to develop in phosphate buffer for 6 h and pulsed with 100 nM cAMP at 6-min intervals during the last 5 h. Developed cells were washed and then stimulated with 10 μM cAMP. Samples were taken at various times after stimulation, and adenylyl cyclase activity was assayed by the method of Roos and Gerisch (1976).

II). Furthermore, partially purified CRAC from wild-type cytosol also restored ACA activity. Lysates of synag7 cells carrying the actl5::dagA expression vector showed more than 15-fold stimulation of adenylyl cyclase upon addition of GTPγS (data not shown); moreover, they contained high levels of CRAC activity (Lilly and Devreotes, 1994). These observations demonstrate that dagA is required for the production of CRAC activity.

Control of CRAC and dagA Expression during Development

Fig. 7 shows the levels of CRAC activity and dagA mRNA in identical cells during the first 6 h of development. CRAC activity has been detected in both growing and developed cells (Lilly and Devreotes, 1994). As shown in Fig. 7, however, the level of activity is regulated during development, increasing eightfold during the first 4 h of starvation, and then slowly decreasing thereafter. The amount of the 3.2-kbp mRNA shows a complementary pattern of expression; a barely detectable level in vegetative cells is followed by a rise during the first 3 h of starvation, and then a sharp decline between 4 and 5 h. Levels of CRAC peak earlier in development than other proteins involved in cAMP signaling (for example, cAR1; Klein et al., 1988), which may implicate CRAC in the decision to start cell-to-cell signaling early in development. The level of CRAC activity corresponds with

Table I. Adenylyl Cyclase Activity in Cell Lysates

| Cell lysate | Addition | Adenylyl cyclase activity* (pmol/min/mg) |
|-------------|----------|----------------------------------------|
| Wild-type   | None     | 1.5                                    |
|             | GTPγS    | 12.4                                   |
|             | Mn**     | 12.6                                   |
| dagA-       | None     | 1.9                                    |
|             | GTPγS    | 2.5                                    |
|             | Mn**     | 14.0                                   |

Cells that had developed for 5 h in suspension with pulses of cAMP were lysed in the presence or absence of either 40 μM GTPγS and 1 μM cAMP or 5 mM MnSO4 (Pupillo et al., 1992). After 2 min of activation, adenylyl cyclase activity was assayed as described in Materials and Methods.

Table II. Regulation of Adenylyl Cyclase in dagA- Cells

| Lysates         | Cytosol | Adenylyl cyclase stimulation (-fold) |
|-----------------|---------|-------------------------------------|
| synag7          | None    | 1.0                                 |
| synag7          | synag7  | 1.0                                 |
| synag7          | dagA-   | 1.2                                 |
| AX3 (wild-type) |         | 6.5                                 |
| Partially purified CRAC |   | 6.4                                 |
| AX3 (wild-type) |         | 9.6                                 |
| Partially purified CRAC |   | 7.4                                 |

Supernatants from the indicated cell types were mixed with activated lysates from strain synag7 or dagA- cells and incubated on ice for 8 min. Cells were developed in suspension with the addition of 100-nM pulses of cAMP every 6 min. Adenylyl cyclase activity was then assayed for 1.5 min. Fold stimulation was determined by dividing the activity obtained with each addition by that from adding buffer alone. The mean activity of unstimulated lysates was 15.9 pmol/min per mg.
the level of dagA mRNA after a short lag, which suggests that
the gene encodes the CRAC protein.

dagA Encodes the CRAC Protein

CRAC has been partially purified from wild-type cells, using
its ability to reconstitute GTPyS-stimulated ACA activity in
synag7 lysates as an assay. When wild-type and synag7 super-

The dagA sequence is from Fig. 2A; the CRAC sequence is from Lilly and Devreotes (1994). Asterisks mark tentative assignments, with the most likely residue shown on the top line; crosses mark clear assignments where the indicated residue was recovered at a lower than expected abundance.

Figure 8. Alignment of the dagA sequence with the NH2-terminal sequence of CRAC. The dagA sequence is from Fig. 2A; the CRAC sequence is from Lilly and Devreotes (1994). Asterisks mark tentative assignments, with the most likely residue shown on the top line; crosses mark clear assignments where the indicated residue was recovered at a lower than expected abundance.

| Cytosol | Adenylyl Cyclase Activity (pmol/mg/min) |
|---------|----------------------------------------|
| None    | 10.1                                   |
| AX3     | 144.5                                  |
| 293 cells (vector control) | 12.1 |
| 293 cells + dagA (expt. 1) | 80.1 |
| 293 cells + dagA (expt. 2) | 87.4 |

Figure 9. Expression of dagA in human 293 tissue culture cells. (A) Western blot of whole cells transfected with empty vector (lane V) and two different transfections with dagA under the control of a cytomegalovirus promoter (lanes 1 and 2). One confluent 8-cm plate was harvested, and one tenth of the cells were dissolved in sample buffer, separated on a 7.5% SDS gel, blotted onto poly-
vinyldifluoride, then probed with anti-CRAC antibody as described in Lilly et al. (1994). (B) Adenylyl cyclase activity in supernatants from transfected cells. Supernatants from the indicated cell types were mixed with activated lysates from synag7 cells and incubated on ice for 8 min. Adenylyl cyclase activity was then assayed for 1.5 min.

Physical Mapping and Characterization of the dagA Gene

The insert in pl20Bgl was used as a hybridization probe to physically map the dagA gene to an arrayed set of YAC clones (Kuspa et al., 1992). The probe recognized 3 of the 1,016 YACs that carry large inserts of Dicyostelium DNA. The dagA YACs were digested with Apal and BglII, restriction enzymes that cut rarely in the Dicyostelium genome, and the fragments separated by pulsed-field electrophoresis before probing with the individual arms of the YAC cloning vector (Kuspa et al., 1992). The YACs in this contig could then be positioned relative to the Apal and BglII sites. Further probing of the fragments with the insert in pl20Bgl positioned the dagA gene to within 20 kb (Fig. 10).

Figure 10. Megabase genomic map of the region surrounding the dagA locus. YAC clones recognized by probes from the dagA and pkeB genes were positioned on the genomic map relative to rare restriction sites as described previously (Kuspa et al., 1992). Smal; A, Apal; B, BglII; N, NarI.
A megabase map around the \textit{dagA} locus was constructed by digesting high molecular weight genomic DNA with combinations of rare cutting restriction enzymes and resolving the large fragments by pulsed-field electrophoresis before probing with the insert in pl20Bgl (Kuspa et al., 1992). The \textit{dagA} gene was found to lie between a Smal site and an ApaI site closely flanked by a BglII site (Fig. 10). The genomic map confirmed the arrangement of YACs in the \textit{dagA} contig. The megabase map surrounding the \textit{dagA} locus was found to be congruent with the map surrounding \textit{pkeB}, a gene encoding a protein kinase, for which we had already established a contig of three YACs (Kuspa, A., and W. I. Loomis, unpublished observation). Linkage of these genes was directly demonstrated by digesting high molecular weight DNA from wild-type AX4 cells and mutant AK120 cells with BglII and showing that both the \textit{dagA} probe and the \textit{pkeB} probe recognized an 850-kb fragment from wild type DNA and a smaller (780 kb) fragment from DNA of the mutant strain. The insertion of DIV2 \textasciitilde 70 kb from the BglII site in strain AK120 introduces a new BglII site that generates this restriction fragment length polymorphism.

Finer scale mapping was carried out by digesting genomic DNA with combinations of the frequent-cutting restriction enzymes, BglIII and ClaI, electrophoretically separating the fragments and probing with the insert in pl20Bgl. The resulting map was used to position plasmids pl08Bgl, pl08Cla, pl120Bgl, and pl120Cla before sequencing (see Fig. 1). Such physical mapping establishes that \textit{dagA} is a unique gene and defines the surrounding genomic structure.

**Discussion**

The phenotype of \textit{dagA} mutants is caused by an inability to respond to cAMP stimuli by synthesizing and secreting their own cAMP. This failure in receptor-mediated activation of adenylyl cyclase is reflected in the inability of GTP\textsubscript{y}S to stimulate the enzyme in \textit{dagA}\textsuperscript{-} lysates. As in \textit{synag7}, a previously described mutant (Theibert and Devreotes, 1986), this defect can be overcome by addition of cytosol or partially purified CRAC from wild-type cells. In this report, we have shown that the \textit{dagA} gene encodes CRAC, and thereby uncovered a novel component of the signal transduction pathway leading to activation of adenylyl cyclase.

CRAC is required for ACA activation by cAMP in vivo and by GTP\textsubscript{y}S in vitro. This suggests that CRAC serves to connect G protein dissociation to ACA stimulation. Recent data suggest that the \textit{\beta}7-subunit complex, rather than activated \textit{Gz2}, directly stimulates ACA (Pupillo et al., 1992; Wu, L., personal communication). The emergent pathway for the activation of ACA may provide an important precedent because mammalian types II and IV adenylyl cyclase, which are highly expressed in nervous tissue, are activated by the \textit{\beta}7-subunits of G proteins (Tang and Gilman, 1991). However, it is not clear whether the experiment reproduces physiological conditions. One possibility is that CRAC works by facilitating or enhancing the interaction between enzyme and \textit{\beta}7-subunits, in which case high concentrations of these components could partially overcome the need for CRAC. It will be interesting to see if mammalian adenylyl cyclases can be stimulated by cytosolic factors in the same way as ACA from \textit{Dictyostelium}. 

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GenBank Accession Number

The accession number for the sequence reported in this paper is U06228.

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References

Altschul, S., F. W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Brachet, J., P. Barra, M. Darmon, and P. Barrand. 1977. A phosphodiesterase defective mutant of Dictyostelium discoideum. In Development and Differentiation in the Cellular Slime Molds, P. Cappuccinelli and J. M. Ashworth, editors. Elsevier/North-Holland, New York, pp. 125–134.

Birnbaumer 1992. Receptor to effector signalling through G-proteins: roles for β dimers as well as α subunits. Cell. 71:1069–1072.

Cohen, S. M., D. Knecht, H. F. Lodish, and W. F. Loomis. 1986. DNA sequences required for expression of a Dictyostelium actin gene. EMBO (Eur. Mol. Biol. Organ.) J. 5:3361–3366.

Cooley, L., R. Kelley, and A. Spradling. 1988. Insertional mutagenesis of the Dro sophila genome with single P elements. Science (Wash. DC). 239:1121–1128.

Devreotes, P. N. 1989. Dictyostelium discoideum: a model system for cell-cell interactions in development. Science (Wash. DC). 245:1054–1058.

Devreotes, P. N. 1994. G-protein linked signaling pathways control the developmental program of Dictyostelium. Neuron. 12:1–20.

Devreotes, P. N., D. P. Klein, J. Sherryng, and A. Theibert, and P. N. Devreotes. 1987. Transmembrane signalling in Dictyostelium. Methods Cell. Biol. 28:299–331.

Dynes, J. L., A. M. Clark, G. Shaulsky, A. Kuspa, W. F. Loomis, and R. A. Firtel. 1994. Lapg is required for cell-cell interactions that are essential for cell-type differentiation in Dictyostelium. Genes & Dev. 8:948–958.

Federman, A. D., B. Conklin, K. A. Schrader, R. R. Reed, and H. B. Bourne. 1992. Hormonal stimulation of adenylyl cyclase through Gi protein γβ-subunits. Nature (Lond.). 356:159–161.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 136:6–13.

Fosnaugh, K. L., and W. F. Loomis. 1989. Sphore coat genes SP06 and SP70 of Dictyostelium discoideum. Mol. Cell. Biol. 9:5215–5218.

Fosnaugh, K. L., and W. F. Loomis. 1991. Coordinate regulation of the spherule coat genes in Dictyostelium discoideum. Devel. Gen. 12:132–133.

Fosnaugh, K. L., and W. F. Loomis. 1993. Enhancer regions responsible for cell-type differentiation in Dictyostelium. Genes & Dev. 8:948–958.

Fosnaugh, K. L., and W. F. Loomis. 1993. Coordinate regulation of the spherule coat genes in Dictyostelium discoideum. Devel. Gen. 12:132–133.

Fosnaugh, K. L., and W. F. Loomis. 1993. Enhancer regions responsible for temporal and cell-type-specific expression of a spherule coat gene in Dictyostelium. Dev. Biol. 157:38–48.

Gilman, A. 1987. G-proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56:615–649.

Greenwald, I. 1985. Two GTP-binding proteins activate extracellular matrix proteins of the slug. Development (Camb.). 104:275–284.

Moeran, D. G., G. B. Benzing, and R. H. Waterston. 1986. Molecular cloning of the muscle gene unc-22 in Caenorhabditis elegans by Tefr transposon tagging. Proc. Natl. Acad. Sci. USA. 83:7906–7910.

Musacchio, A., T. Gibson, R. Firtel, and J. G. Williams. 1988. Functional implications of α and β-subunits of Dictyostelium exchange subunits. Mol. Cell. Biol. 8:1500–1503.

Nelten, W., D. Itaya, A. Saito, S. Mann, R. T. Trow, and A. P. Zampieri. 1997. G-protein linked signal transduction in Dictyostelium discoideum—regulated expression of an actin gene fusion. Mol. Cell. Biol. 4:2890–2898.

Nelten, W., S. Datta, C. Reymond, A. Sivertsen, S. Mann, T. Crowley, and R. A. Firtel. 1987. Molecular biology in Dictyostelium: tools and applications. Methods Cell. Biol. 28:67–100.

Parker, P. J., B. A. Hemmings, and P. Gierschik. 1994. PH domains and phospholipases—a meaningful relationship? Trends Biochem. Sci. 19:54–55.

Pitt, G. S., R. Brandt, K. C. Lin, P. N. Devreotes, and P. Schap. 1993. Extracellular CAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenylyl cyclase (ACA). Genes & Dev. 7:1722–1730.

Pupillo, M., A. Kumagai, G. S. Pitt, R. A. Firtel, and P. N. Devreotes. 1989. Multiple α-subunits of guanine nucleotide-binding proteins in Dictyostelium. Proc. Natl. Acad. Sci. USA. 85:3434–3484.

Pupillo, M., C. Silvan, G. S. Pitt, and R. A. Firtel. 1984. DNA-mediated transformation in Dictyostelium—regulated expression of an actin gene fusion. Mol. Cell. Biol. 4:2890–2898.

Roos, W., and G. Gerisch. 1979. Receptor-mediated adenylyl cyclase activation in Dictyostelium discoideum. FEBS (Fed. Eur. Biochem. Soc.) Lett. 68:170–172.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463–5467.

Shaualsky, G., and W. F. Loomis. 1993. Cell type regulation in response to expression of ricin A in Dictyostelium. Devel. Biol. 160:85–98.

Sun, T. J., and P. N. Devreotes. 1991. Gene targeting of the aggregation stage cAMP receptor cAR1 in Dictyostelium. Genes & Dev. 5:472–582.

Swaisley, G. W., and W. F. Loomis. 1993. Cultivation and synchronous morphogenesis of Dictyostelium under controlled experimental conditions. Methods Cell Biol. 28:9–29.

Tang, W.-j., and A. G. Gilman. 1991. Type-specific regulation of adenylyl cyclase by G-protein βγ-subunits. Science (Wash. DC). 254:1500–1503.

Theibert, A., and P. N. Devreotes. 1986. Surface receptor-mediated activation of adenylyl cyclase in Dictyostelium: regulation by guanine nucleotides in wild type cells and aggregation deficient mutants. J. Biol. Chem. 261:15121–15125.

Touhara, K., J. Inglese, J. Pitcher, G. Shaw, and R. J. Leffkowitz. 1994. Binding of G protein βγ-subunits to pleckstrin homology domains. J. Biol. Chem. 269:10217–10220.

van Haastert, P. J. M., B. E. Snaar-Jagalska, and P. M. W. Janssens. 1987. The regulation of adenylyl cyclase by guanine nucleotides in Dictyostelium discoideum membranes. Eur. J. Biochem. 162:251–258.