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 $\beta\gamma$ 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 $\beta\gamma$-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 $\beta\gamma$-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 $\beta\gamma$ complexes (Tang and Gilman, 1991; Logothetis et al., 1987). The phospholipases share a region, the pleckstrin homology (PH)[1] 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 $\beta\gamma$-subunit complexes (Musacchio et al., 1993). This hypothesis is supported by the recent discovery that fusion proteins containing various PH domains bind to dissociated $\beta\gamma$-subunits (Touhara et al., 1994). No adenylyl cyclases have been found to contain PH domains; the mechanisms by which $\beta\gamma$-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 10^5 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, cAR1 (Klein et al., 1988). cAMP binding to cAR1 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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1. *Abbreviations used in this paper*: ACA, adenylyl cyclase; cAR1, cell surface cAMP receptor; CRAC, cytosolic regulator of ACA; PH, pleckstrin homology domain; PKA, protein kinase A; REMI, restriction enzyme-mediated integration; YAC, yeast artificial chromosome.

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Recent evidence suggests that, like mammalian adenylyl cyclases, ACA is stimulated by the $\beta\gamma$-subunit complex released from G2 (Pupillo et al., 1992; Kesbeke 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 NH_2-terminal sequences of *dagA* and CRAC coincide, and expression of *dagA* in human tissue culture cells yields cytosol containing active CRAC protein.

Materials and Methods

Culture Conditions, Strains, and Stains

Cells were grown in HL5 medium and developed on filters as described by Sussman (1987). Strain HL330, a derivative of strain AX4, carries a deletion of the *pyr5-6* gene (Kuspa and Loomis, 1992). HL328, also a derivative of strain AX4, carries a mutation in *pyr5-6*, making the cells dependent on uracil for growth. Strain TL50 was derived from HL328 by transformation with a *cotB::lacZ* fusion construct, where expression of β-galactosidase is under the control of the prespore *cotB* promoter (Fosnaugh and Loomis, 1993). Strain TL51 was derived from HL328 by transformation with a *ecmA::lacZ* fusion construct, where expression of β-galactosidase is under the control of the prestalk *ecmA* promoter (Jermyn and Williams, 1991). Strain TL43 was derived from HL328, where expression of β-galactosidase is under the control of the actin 15 promoter (Cohen et al., 1986). Strains AK516, AK501, and AK512 are *dagA*$^-$ derivatives of TL43, TL50, and TL51, respectively, generated by homologous recombination with ClaI-digested pl20Cla. They were stained for β-galactosidase activity with X-Gal as described previously (Shaulsky and Loomis, 1993). When developing in mixtures with wild-type cells, a counterstain, 0.02% eosin Y, was used to stain all cells pink.

Insertional Mutagenesis

Insertional mutagenesis was performed by REMI of the plasmid DIV2, using the restriction enzymes BamHI or Sau3AI, as described previously (Kuspa and Loomis, 1992, 1993). REMI transformants were spread with *Klebsiella aerogenes* on SM plates such that clonal populations grew and developed in isolated plaques (Sussman, 1987). Mutant strains were subcultured in HL-5 medium and stored at –70°C (Sussman, 1987).

Molecular Cloning

Genomic clones carrying the *dagA* locus were recovered from several *dagA*$^-$ mutants after digestion of genomic DNA with ClaI or BglII, religation of plasmids, and ampicillin selection in *Escherichia coli* (Kuspa and Loomis, 1992). cDNAs were identified in a λgt11 cDNA bank prepared from RNA isolated at 2–4 h of development (Klein et al., 1988). cDNA clones of *dagA* inserted into the EcoRI site of Bluescript, and were recognized by hybridization with the ClaI/BamH1 fragment from pl20Cla after hexamer labeling (Maniatis et al., 1982). The largest insert (2.3 kb) was subcloned downstream of the actin 15 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 pl20Bgl was isolated, labeled with α[^{32}P]dCTP (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 ApaI and BglI (Kuspa et al., 1992). The probe for the linked gene, *pkeB*, was kindly provided by Joe Dynes and Richard Firtel (Center for Molecular Genetics, UCSD, La Jolla, CA). Sequence analysis of the *pkeB* clone suggests that it encodes a protein kinase.

Molecular Analyses

RNA was prepared from strains AK108 (*dagA*$^-$), RI-6d (*actinl5::dagA* over-expression construct in a *dagA*$^+$ background), RI-7b (*actinl5::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 p70.4 (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 cAR1 (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 sulphate as described by Klein et al. (1988); each sample analyzed 2×10^6 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 10^8/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 α[^{32}P]ATP.

Reconstitution of GTPγS 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-nM 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 GTPγS 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.

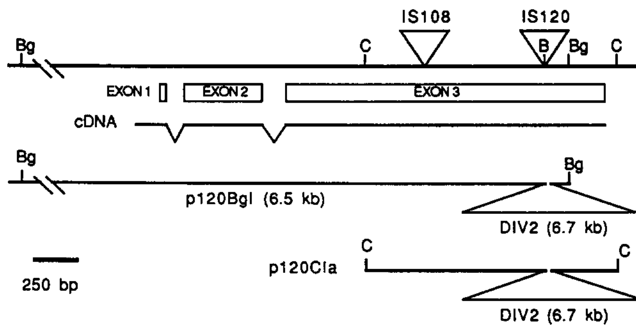

Figure 1. Restriction map of the *dagA* locus. Insertions at IS108 and IS120 (*triangles*) occurred in Sau3AI and BamHI sites, respectively. Other restriction sites are BglII (*Bg*) and ClaI (*C*). A cDNA clone, pRHI31, is shown beneath the predicted exons 1-3 of the *dagA* gene. Two genomic clones (p120Bgl and p120Cla) are shown below the cDNA clone.

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, p120Bgl, 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 generated in a Sau3AI REMI mutagenesis (Kuspa and Loomis, 1992) revealed that a distinct insertion event at IS108 had occured 0.6 kb away from IS120 in this strain.

To prove that the insertions into IS108 and IS120 are responsible for the inability to aggregate, wild-type cells were transformed with plasmids from the 10 independent strains to recreate the mutated genomic structure by homologous recombination in the regions flanking the insertions. Plasmids from each of the mutant strains (Fig. 1) gave rise to transformants where the locus was disrupted, and these strains were found to be unable to aggregate under developmental conditions, confirming that the locus, which we named *dagA*, is essential for aggregation.

Several cDNA clones were isolated by probing a library prepared from RNA isolated early in development with the insert in p120Bgl. The longest cDNA was 2.3 kb and spanned both of the insertion sites of the *dagA* mutant strains. Both cDNAs and appropriate genomic fragments that had been rescued from AK108 and AK120 were sequenced (Fig. 2 *A*). Several of the cDNA clones started exactly 360 bp upstream of the single long open reading frame and may include the 5′ end of the transcript. Comparison of the cDNA sequence to the genomic sequence revealed two short introns near the 5′ end (Figs. 1 and 2 *A*). The splice junctions have the consensus sequences, except that exon 3 starts with a T rather than a G. The introns are short and highly skewed to high A and T, as is common for *Dictyostelium* introns (Kimmel and Firtel, 1982). The open reading frame in the cDNA sequence corresponds to the longest open reading frame in the genomic sequence and encodes a predicted protein of 698 amino acids with a molecular mass of 78.5 kD (Fig. 2 *A*). Northern blots probed with a *dagA* cDNA reveal a 3.2-kbp mRNA of low abundance in RNA from wild-type cells; *dagA*$^-$ cells contain a barely detectable level of a larger mRNA (data not shown; see also Fig. 7).

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 *Drosophila 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

A

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-360  ACCTTTTTTT TTTAATTTTT TTTTTCCACT TTTAAAAAAG GTAATATTTA GTTTTTTTTT
      TTTTTTTACT TTATAAAATT TTTTTTTTTT TTTTATTTAT TTATTAATAA TAAATAACAC
      AAAAAATTGT ACATATACCA AAAAAAAAAA AAAAAAAAAG AAAGAAAGAA AGAAAGAAAT
      ATTTTATTTG TAAATTAAAG CATTACATTT GATTGTATTT TTTTTTTTTT CTCTTCAACT
      ATAAATATTA ATCAATTTTA AAAAAAATAC AAATAGACGC GCACACCCAC ATACTCTTTT
      TTAAGTAAAT AAATAAAATA TATATATATA AATATATATA TTTTTTAAAA TTAAAAAAA

   1  ATG GGG AAA ACA GAG AGA AAG AAA GAG CTT TTA   GTAAGTACAA  AAATAATAAA  AAAAAAAAAT
   1  Met gly lys thr glu arg lys lys glu leu leu   .............intron 1............

  64  ACATACACAT  GCATTTATGT ATTAACATTT ATTTTTTTTT ATTTTTTTTT TTTTATATAA TAG        GAA TTA
  12  ............................intron 1..................................        glu leu

 133  TTT GAA TAT GAA AAA ATT AAA GGA GAT GTA TCA TAT TCA TCA ATA ATG AAG AAA GCA GGT GGA AAT
  14  phe glu tyr glu lys ile lys gly asp val ser tyr ser ser ile met lys lys ala gly gly asn

 199  GGT AAA GGA TTT TTA GAT CGT TAT TTT GCA CTT CAT AGA AAC TAT ATA TTA TAT TAT AAA CTT GGT
  36  gly lys gly phe leu asp arg tyr phe ala leu his arg asn tyr ile leu tyr tyr lys leu gly

 265  AAA TCA TCA CTC AAA CCA GAC GAT AAA CAA GAA CCA CAA GGT TAT ATA AAT TTA ATG GAT TGT AAT
  58  lys ser ser leu lys pro asp asp lys gln glu pro gln gly tyr ile asn leu met asp cys asn

 331  CCA GAT GAC ACC AAA GAG ATC GCA CCC TTA ATG TTT CAA ATC TCT CAT AAA CAT AGA ACA TAC ATT
  80  pro asp asp thr lys glu ile ala pro leu met phe gln ile ser his lys his arg thr tyr ile

 397  GTA AAA GCA AAA GAT GAG AGT TCA ATG AAA CAA TTC TTA ACT TTA CTC ATC GCA AGA ATT AGA TCA
 102  val lys ala lys asp glu ser ser met lys gln phe leu thr leu leu ile ala arg ile arg ser

 463  CTT GAA AAA ATT GAT ATT GAT AAA CTT GGT TGT ACA GTT GTA GTT TTA ACT AAA GTT AAA AAG TTT
 124  leu glu lys ile asp ile asp lys leu gly cys thr val val val leu thr lys val lys lys phe

 529  AGA GAA GTT TTA ACA AAT CCA T  GTATGTATTA  CAAATATATT  TATATTATTT  ATTAATATAT  ATATATAT
 146  arg glu val leu thr asn pro l  .......................intron 2.........................

 599  TT  ATATATTATT  TATTAATATA  TATATATATA  TATTTATAAC  TAATATATTT  TTTTAAAAAT  AG    TA ATT
 153  ..................................intron 2.....................................    eu ile

 668  TTA CCA GAT AGA GTT TCA CCA GAA ATG GCA GAA GAA TGG GTA AAA CAA ATG AAA AAT TAT AAT GCA
 155  leu pro asp arg val ser pro glu met ala glu glu trp val lys gln met lys asn tyr asn ala

 734  TCA TTT AAT CTT GCA GAT CCA TTT ATT AAA CAA GTT GAA CAA ATT TCA GAA TTT TGT AGA GGT GAA
 177  ser phe asn leu ala asp pro phe ile lys gln val glu gln ile ser glu phe cys arg gly glu

 800  GTT AAA GAG TAT ATT GAT TGG TTT GGT GGA CCA GAG GGA CCA CGT TTA GCA ATG ATT CGT TGT GAA
 199  val lys glu tyr ile asp trp phe gly gly pro glu gly pro arg leu ala met ile arg cys glu

 866  GAG ACT GTA TTG TCA AAT TGG GTT GAG TAT ATT AAT AAG ACG AGT TCA GAG ATT ACC ACC TAT CAA
 221  glu thr val leu ser asn trp val glu tyr ile asn lys thr ser ser glu ile thr thr tyr gln

 932  GAT AAT AGA TTC TTT AGA GAG GAT TTC AAA GAT ATT GCA GTT CAT CTT AAG AAT ATG ACC ACC TTT
 243  asp asn arg phe phe arg glu asp phe lys asp ile ala val his leu lys asn met thr thr phe

 998  ATC GAT TGT TAC AAT GAC TAT ATG ATT CAT TGT AGA AAG TAT AAT AAT AAT AAA CCA AAT ACG AAA
 265  ile asp cys tyr asn asp tyr met ile his cys arg lys tyr asn asn asn lys pro asn thr lys

1064  TTC TTG GAA GAG AAA CAA ACA TTT AAA GAA TAC ATT GAG AAA TTC ATT CCA AAA GTT GCC TCT TGT
 287  phe leu glu glu lys gln thr phe lys glu tyr ile glu lys phe ile pro lys val ala ser cys

1130  AAT GAC GTT AGT TTA AAT CAG TTC TAT GAC AGA TCG CTC ATT CAA AGC TCT GAT GGT ATT GTA ACT
 309  asn asp val ser leu asn gln phe tyr asp arg ser leu ile gln ser ser asp gly ile val thr

1196  ATA AAT ACA AGT GGT ATA AAG AAA ACA TTG ATT AAT CAA TCC AAT ATC ATT TCA ATC ACC TCC ACA
 331  ile asn thr ser gly ile lys lys thr leu ile asn gln ser asn ile ile ser ile thr ser thr

1262  ACC ACC ACC ACC ACC ACA ACC ACC ACT ACA ACC TGT TCA ATG CCA AAT ATG TCA AAT TTA ATT CAT
 353  thr thr thr thr thr thr thr thr thr thr thr cys ser met pro asn met ser asn leu ile his

1328  TCA TTA GAT CAT ACT AAT CTT AAT ATT ATT GAT TTA AAT CAT TCA AAA TCA CAA CAA CAA TTA CAT
 375  ser leu asp his thr asn leu asn ile ile asp leu asn his ser lys ser gln gln gln leu his

1394  CCA CCA CCA TCA CCA CAT CAT CAG CAT CTT CAT CAT CAA ATA GTA TCA AAT TCA AAA GAT TTC AAT
 397  pro pro pro ser pro his his gln his leu his his gln ile val ser asn ser lys asp phe asn

1460  ATT AGT GTT TCA TCA AAT AAT TTT AAT GAT GGT AAT TCT GAA TTT CCA AAT CTT GAT ATT AAT TGT
 419  ile ser val ser ser asn asn phe asn asp gly asn ser glu phe pro asn leu asp ile asn cys

1526  GAT TTT GAT TTA ACC AGT GCT AGT AAT TTA TCT TCA CCA ATA CTA TCA AGT GAA GTA CCA TCA AAT
 441  asp phe asp leu thr ser ala ser asn leu ser ser pro ile leu ser ser glu val pro ser asn

1592  GTA GTT GAC CCA ATT GGT TCA GGT CAA GGT GGT GGT GGT AGT GGT GGT GGT GGT GTG ACA GCT GTC
 463  val val asp pro ile gly ser gly gln gly gly gly gly ser gly gly gly gly val thr ala val

1658  ACT GAA GAG GCA ATC AAT GAA AAA TGG CAT TTC GAT TGC AAC ACT TCA ATG ATC TTT AAA CCG CCA
 485  thr glu glu ala ile asn glu lys trp his phe asp cys asn thr ser met ile phe lys pro pro

1724  TCT GAA GAT GGT AGA AAT GAG GGT AGT AAC ATG TCA ACT AGT TCA ATC ACA AGT AAA ATG TCA CTT
 507  ser glu asp gly arg asn glu gly ser asn met ser thr ser ser ile thr ser lys met ser leu

1790  TCA TTG AAT GGT GGT TTC GAT ATG AAA TGG GTT TAT CAA TGT GGT TAT TTT AAA AGT AAG AAT ATG
 529  ser leu asn gly gly phe asp met lys trp val tyr gln cys gly tyr phe lys ser lys asn met

1856  GGC TCA ATT TCA TGG AAT GGT AAA CAT TGG TGT TGG TCT CAT CCA AGA ACA AGT TAT AAA ATT AAA
 551  gly ser ile ser trp asn gly lys his trp cys trp ser his pro arg thr ser tyr lys ile lys

1922  TAC ATT TGG GAT CCA ACT AAA CAA TCA TTT TTA AAT ATA CCT TTT AAA TCT AGA GTT GGT GCC ACT
 573  tyr ile trp asp pro thr lys gln ser phe leu asn ile pro phe lys ser arg val gly ala thr

1988  GGT GGT GGT TCT GTC CCT TCC TCA CAA TCT ACC AAT AAT TTA CAA TCT TCA ACA AGT TCA ATG TCA
 595  gly gly gly ser val pro ser ser gln ser thr asn asn leu gln ser ser thr ser ser met ser

2054  TCT TTA TCA TCT TCC TCA ACT TCC ACT ACT AAA AGA TCT CAT CCA ACT ACA CTT TAT CCT GAT TAT
 617  ser leu ser ser ser ser thr ser thr thr lys arg ser his pro thr thr leu tyr pro asp tyr

2120  CAA TTC AAA GAT AAT CTT CTC ACT CCA ATC ATT ATT GAA GGT GGA CAT CAA CCA TCC TTA ACT TTA
 639  gln phe lys asp asn leu leu thr pro ile ile ile glu gly gly his gln pro ser leu thr leu

2186  ATT GAT TCA CCT TTA ACA ATT CCA AAT GCT TGT CTT TTA ACA ATT GCA ATG ACT CAA TAC ATT CAA
 661  ile asp ser pro leu thr ile pro asn ala cys leu leu thr ile ala met thr gln tyr ile gln

2252  GAT GCT TTA ATT CAT CTT TCA CTT GGT CCA AAA GTT TTA TCA AGT AAA TAA  AATAATTGGA
 683  asp ala leu ile his leu ser leu gly pro lys val leu ser ser lys stop

      ATAAAAAAAC ACTTGTAAAT ATCGAT
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B

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                .         .         .         .
 23 VSYSSIMKKAGGNGKGFLDRYFALHRNYILYYKLGKSSLKPD
    |  .:.:|| |: ||::  |||.|: . : |||
    VIKEGYLKKKGS.GKSWKRRYFVLRDAGLSYYKDS.......
    <----1---->      <---2----> <--3--->

       .         .         .         .
 65 DKQEPQGYINLMDCNPDTKEIAPLMFQISHKHRTYIVKAKDES
    |.  |.|.|:| :..  . | :.         |   ||. ||:|
    DEYRPKGLIDLENIQIVEVEDGKKGK.....HCFEIVT.KDGS
       <--------4------->          <-----5---->

       .         .         .         .
108 SMKQFLTLLIARIRSLEKIDIDKLGCTVVVLTKVKKFREVLTN

    .........................................

              .         .
151 PLILPDRVSPEMAEEWVKQMKNYN 174
    .||| :    |.|  ||||  .::.
    SLIL.QAESEEEREEWVAALRRAI
     <---------6----------->
```

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 cDNA is indicated in bold type; regions of pleckstrin homology are underlined. (*B*) Comparison of the pleckstrin homology domains of CRAC (*upper sequence*) with the consensus sequence (*lower sequence*). The domains, shown below the consensus sequence, are numbered to correspond with those in Musacchio et al. (1993).

6 min to mimic normal signaling. In the absence of added cAMP, *dagA* cells accumulated less than wild-type levels of both ACA and cAR1 (see Fig. 3). When cells were repeatedly stimulated with cAMP, however, both *dagA* and wild-type cells accumulated similar levels of ACA and cAR1 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^{-4} to 10^{-8} M (data not shown). Furthermore, mutant cells responded to a cAMP stimulus, in a similar fashion to wild-type cells, by a rapid increase in cGMP (Fig. 4). Thus, most of the machinery for responding to cAMP signals is present in *dagA*$^-$ mutant cells, but they are nonetheless unable to aggregate in pure populations.

When *dagA*$^-$ mutant cells marked with a constitutively expressed *actin15*::*lacZ* reporter construct were mixed with wild-type cells, the marked cells were found to stream to aggregation centers (Fig. 5 *A*). However, mutant cells were relegated to the edges and left behind when wild type cells culminated to form fruiting bodies (Fig. 5, *B–D*). In *dagA*$^-$ mutant cells that carried either prespore-specific *cotB*::*lacZ*, or prestalk-specific *ecmA*::*lacZ* reporter constructs (see Materials and Methods), no cells were found to express either reporter gene, whether they were developed in pure populations or in mixtures with wild-type cells. This could either imply that *dagA*$^-$ cells are unable to form spores, or that they fail to receive the signals required for spore differentiation as a result of being excluded from the aggregate.

When detergent-washed spores were plated in association with bacteria, a few clones showed the *dagA*$^-$ phenotype from the mixtures in which the ratio of *dagA*$^-$ cells to wild-type cells was 1:10, 1:1, or 10:1. From a total of 6,000 plaques scored for developmental phenotype, 106 were found to be derived from mutant spores. The fact that a few of the mutant spores were viable demonstrates that all the genes necessary for spore formation are present but seldom used in the

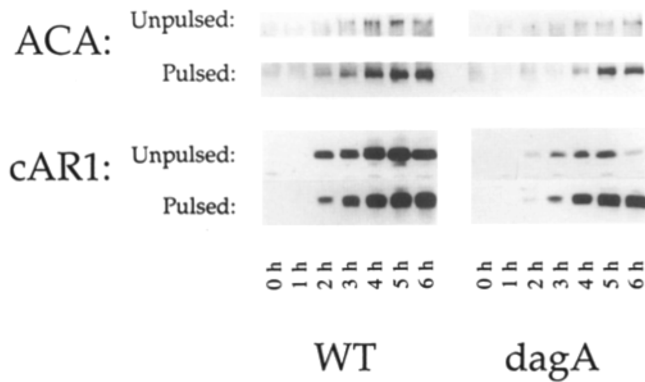

Figure 3. Adenylyl cyclase and cAMP receptor proteins in wild type and *dagA*⁻ mutant cells. Washed cells were shaken in phosphate buffer. At 6-min intervals, cAMP pulses (100 nM) were given to some of the cells. Samples were taken hourly, separated by SDS-PAGE, transfered to nitrocellulose, and stained with antibodies to adenylyl cyclase or the cAMP receptor.

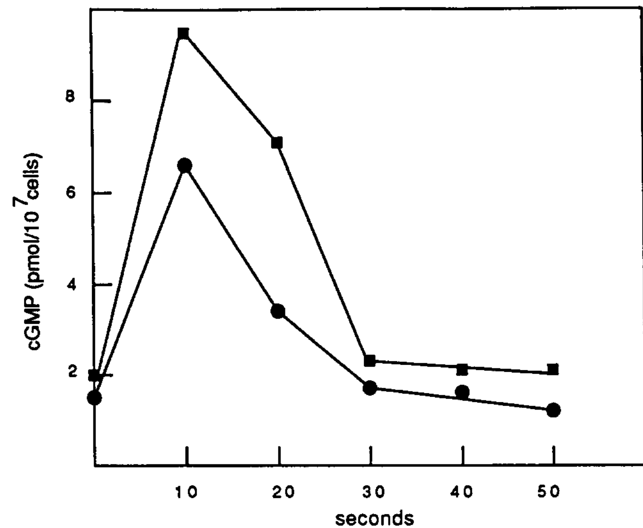

Figure 4. The cGMP response of *dagA* cells. The increase in intracellular cGMP concentration was measured after a 10-nM pulse of cAMP in wild-type cells (*circles*) and *dagA*⁻ mutant cells (*squares*) that had developed in suspension for 8 h.

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 GTPγS, which activates and dissociates trimeric G proteins (Theibert and Devreotes, 1986). While GTPγS 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

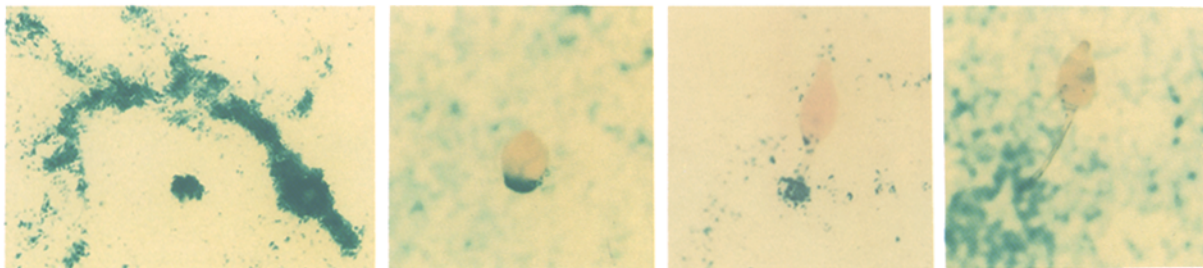

Figure 5. Coaggregation and sorting out of *dagA*⁻ and wild-type cells. Cells of strain AK516 (*dagA*⁻, *actinl5::lacZ*) were mixed with an equal number of wild-type AX4 cells and developed on nitrocellulose filters. Mutant cells were stained blue with X-gal and unstained cells were visualized by staining pink with eosin Y. (*A*) Streams entering an aggregate. (*B*) A tipped aggregate. (*C*) An early culminant. (*D*) A mature fruiting body.

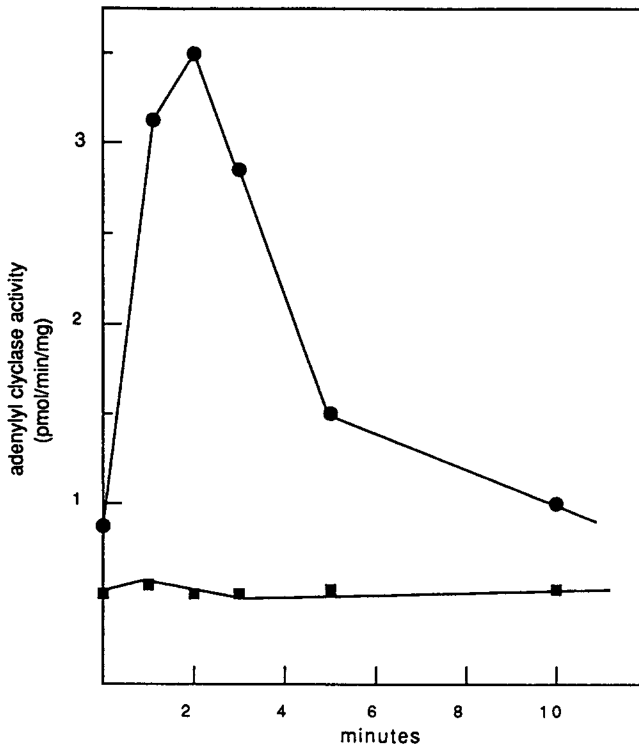

Figure 6. Lack of cAMP signal relay in *dagA*⁻ cells. Wild-type (*circles*) and *dagA*⁻ (*squares*) cells were allowed to develop in phosphate buffer for 6 h and pulsed with 100 nM cAMP at 6-min intervals during the last 5 h. Developed cells were washed and then stimulated with 10 μM cAMP. Samples were 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

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 $MnSO_4$ (Pupillo et al., 1992). After 2 min of activation, adenylyl cyclase activity was assayed as described in Materials and Methods.

Table II. Reglation of Adenylyl Cyclase in dagA⁻ Cells

Lysates	Cytosol	Adenylyl cyclase stimulation (-fold)
synag7	None	1.0
	synag7	1.0
	dagA⁻	1.2
	AX3 (wild-type)	6.5
	Partially purified CRAC	6.4
dagA⁻	None	1.0
	synag7	0.9
	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.

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

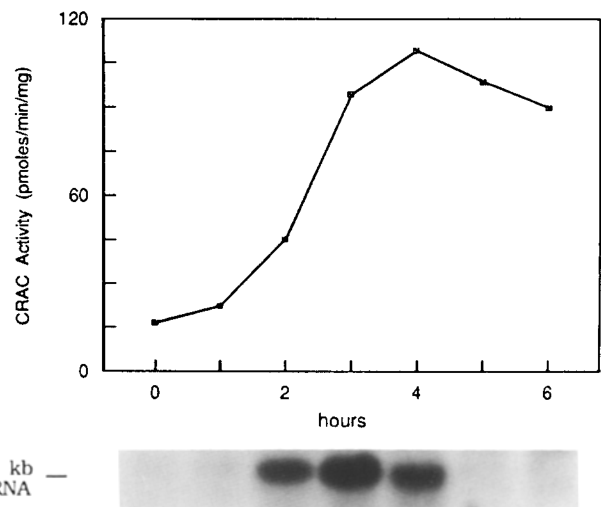

Figure 7. Expression of *dagA* RNA and CRAC activity during normal development. Wild-type cells were allowed to develop in phosphate buffer and were pulsed with 100 nM cAMP at 6-min intervals. At 1-h intervals, cells were harvested and CRAC activity was assayed (*top*), and RNA was extracted, blotted, and probed with a *dagA* cDNA (*bottom*). The 3.2-kbp mRNA shown was the only band detected under these conditions.

```
dagA sequence          M  G  K  T  E  R  K  K  E  L  L
CRAC NH2 terminus         X (P)* T  E  R (K)+(K)+(E)*
                            (K)               (S)
                                              (T)
```

Figure 8. Alignment of the *dagA* sequence with the NH_2-terminal sequence of CRAC. The *dagA* sequence is from Fig. 2 *A*; 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.

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 GTPγS-stimulated ACA activity in *synag7* lysates as an assay. When wild-type and *synag7* supernatants were purified in parallel, an 88-kD SDS-PAGE band from wild-type supernatant was absent in the mutant (Lilly and Devreotes, 1994). A short NH_2-terminal sequence obtained from this band was too short to allow cloning of the CRAC gene. This sequence fragment matches the derived protein sequence from *dagA*, starting one amino acid from the NH_2 terminus (Fig. 8). The alignment also implies that the CRAC protein is modified to remove the NH_2-terminal methionine residue.

To demonstrate unambiguously that *dagA* is the structural gene for CRAC, we transiently expressed the cDNA in human embryonic kidney 293 cells under the control of a cytomegalovirus promoter. Western blots probed with a polyclonal antibody against SDS-PAGE–purified CRAC (Lilly and Devreotes, 1994) show the appearance of an 88-kD band corresponding to CRAC in *dagA*-transfected cells, but not in vector control cells (Fig. 9 *A*). This clearly demonstrates that the *dagA* gene encodes CRAC. Cytosol was prepared from control and *dagA*-transfected cells and added to *synag7* lysates. As shown in Fig. 9 *B*, the expressed *dagA* was able to restore GTPγS stimulation of ACA activity in *synag7* lysates, which suggests that no *Dictyostelium*-specific modifications or additional subunits are necessary to produce functional CRAC activity.

Constitutive Expression of *dagA* Restores Development

To show that the cDNA sequence is sufficient for *dagA* function, it was placed under the control of the *actinl5* promoter on a neomycin resistance plasmid (see Materials and Methods). This construct was used to transform *dagA*$^-$ and wild-type cells by selecting cells that carry tandem arrays of the plasmid (Nellen et al., 1984). High levels of *dagA* mRNA were observed in transformants with both *dagA*$^-$ and wild-type backgrounds at 0, 8, and 18 h of development by probing Northerns with the 2.3-kb cDNA (data not shown). Expression of the *dagA* gene in the *dagA*$^-$ mutant transformants restored normal development, and high levels of CRAC activity were found in the cytosol of transformants. Moreover, wild-type cells that overexpress *dagA* appear to develop normally.

A

V 1 2

97.4
68
43

B

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 polyvinyldifluoride, 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 p120Bgl 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 *Dictyostelium* DNA. The *dagA* YACs were digested with ApaI and BglI, restriction enzymes that cut rarely in the *Dictyostelium* 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 ApaI and BglI sites. Further probing of the fragments with the insert in p120Bgl positioned the *dagA* gene to within 20 kb (Fig. 10).

Sma A B N A Sma IS120 A B N B
pkeB
dagA
3283
3667
3831
3423
3476
3565
100 kb

Figure 10. Megabase genomic map of the region surrounding the *dagA* locus. YAC clones recognized by probes from the *dagA* and *pke*B genes were positioned on the genomic map relative to rare restriction sites as described previously (Kuspa et al., 1992). *Sma*, SmaI; *A*, ApaI; *B*, BglI; *N*, NarI.

A megabase map around the *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 *dagA* gene was found to lie between a SmaI site and an ApaI site closely flanked by a BglI site (Fig. 10). The genomic map confirmed the arrangement of YACs in the *dagA* contig. The megabase map surrounding the *dagA* locus was found to be congruent with the map surrounding *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 BglI and showing that both the *dagA* probe and the *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 ~70 kb from the BglI site in strain AK120 introduces a new BglI 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, BglII and ClaI, electrophoretically separating the fragments and probing with the insert in pl20Bgl. The resulting map was used to position plasmids pl08Bgl, pl08-Cla, pl20Bgl, and pl20Cla before sequencing (see Fig. 1). Such physical mapping establishes that *dagA* is a unique gene and defines the surrounding genomic structure.

Discussion

The phenotype of *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 GTPγS to stimulate the enzyme in *dagA*$^-$ lysates. As in *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 *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 GTPγS in vitro. This suggests that CRAC serves to connect G protein dissociation to ACA stimulation. Recent data suggest that the $\beta\gamma$-subunit complex, rather than activated Gα2, 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 $\beta\gamma$-subunits of G proteins (Tang and Gilman, 1991; Federman et al., 1992). We have discovered a PH domain in the NH_2-terminal region of CRAC. This is particularly relevant in the light of recent evidence that PH domains bind to dissociated G protein $\beta\gamma$-subunits (Touhara et al., 1994). It will be important to determine if CRAC binding to $\beta\gamma$-subunits can be biochemically measured. Similarly, it remains to be seen whether CRAC interacts directly with ACA, or is one of several members of the activation pathway.

All other responses to extracellular cAMP and second messenger pathways appear to be normal in *dagA*$^-$ cells, at least early in development. This reinforces the findings of Pitt et al. (1993), who found that cAMP was only required outside the cell for normal development; exogenous cAMP pulses induce normal developmental gene expression, even in ACA deletion mutants. When *dagA*$^-$ mutants are developed in synergy with wild-type cells, a real but very small proportion of the mutant cells produce mature spores. This demonstrates that ACA activation, and therefore presumably intracellular cAMP, is not essential to the formation of a mature spore; however, the efficiency of spore formation is radically reduced in *dagA*$^-$ mutants. This may be explained by the fact that *dagA*$^-$ cells are excluded from the later stages of development (Fig. 5), so they presumably do not experience appropriate inductive stimuli for efficient differentiation. Protein kinase A, the intracellular target of cAMP, is absolutely required for development (Mann and Firtel, 1991). As discussed in Pitt et al. (1993), this indicates either that basal PKA activity is necessary for development, or that some signal other than cAMP is able to stimulate *Dictyostelium* PKA.

9 of 29 BamHI REMI mutants arrested before tight mound formation were found to have integrated the plasmid into the *dagA* gene. This frequency of integration into a single gene might appear to be surprisingly high, considering that integration into any of ~100 genes could have given the same gross phenotype (Loomis, 1978). However, not every gene is expected to carry a BamHI site. The average size of genomic fragments generated by BamHI is 20 kb, while the average size of most genes is 2 kb (Kuspa, A., and W. F. Loomis, unpublished observations). Therefore, there might be only about 10 distinct BamHI sites which would, when disrupted, produce a mutant blocked before tight mound formation. The rate of insertion into the *dagA* locus is somewhat higher than expected but not extremely so. Another gene, *lagC*, was also the target for multiple insertions in this set of BamHI REMI mutants (Insall, R., unpublished results; Dynes et al., 1994) suggesting that most of the BamHI sites in developmental genes have been tagged in the existing set of mutants.

Although the pathway from cell surface receptor to adenylyl cyclase is very similar in *Dictyostelium* and mammalian cells, no functional homologues of CRAC have yet been discovered in any other species. No sequences with a significant homology to CRAC were detected by database searches, and no other cytosolic factor coupling G proteins to adenylyl cyclases has yet been cloned. This might be construed to imply that CRAC is a *Dictyostelium*-specific activity, and that G protein $\beta\gamma$-subunits directly activate types II and IV adenylyl cyclase without the need of coupling factors. Ligand-induced stimulation of mammalian type adenylyl cyclase has been reconstituted using only baculovirus-expressed type II adenylyl cyclase purified $\beta\gamma$-subunits and a constitutively active Gα subunit (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 $\beta\gamma$-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 *Dictyostelium*.

GenBank Accession Number

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

We thank Jeffery E. Segall from Einstein College of Medicine for performing the cAMP chemotaxis assays on six of the original *dagA* mutants, and Carole Parent for helpful discussions about the manuscript. We also thank Mark Floyd for expert technical assistance. This work was supported by National Institutes of Health grants GM23822 (to W. F. Loomis) and GM28007 (to P. N. Devreotes) and a SERC/NATO fellowship to R. Insall.

Received for publication 14 March 1994 and in revised form 17 June 1994.

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