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Structurally Distinct and Stage-Specific Adenylyl Cyclase Genes Play Different Roles in Dictyostelium Development

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Summary

We have isolated two adenylyl cyclase genes, designated *ACA* and *ACG*, from Dictyostelium. The proposed structure for ACA resembles that proposed for mammalian adenylyl cyclases: two large hydrophilic domains and two sets of six transmembrane spans. ACG has a novel structure, reminiscent of the membrane-bound guanylyl cyclases. An *aca*⁻ mutant, created by gene disruption, has little detectable adenylyl cyclase activity and fails to aggregate, demonstrating that cAMP is required for cell-cell communication. cAMP is not required for motility, chemotaxis, growth, and cell division, which are unaffected. Constitutive expression in *aca*⁻ cells of either *ACA* or *ACG*, which is normally expressed only during germination, restores aggregation and the ability to complete the developmental program. *ACA* expression restores receptor and guanine nucleotide–regulated adenylyl cyclase activity, while activity in cells expressing ACG is insensitive to these regulators. Although they lack ACA, which has a transporter-like structure, the cells expressing *ACG* secrete cAMP constitutively.

Introduction

Cyclic AMP coordinates the early stages of the developmental program during which Dictyostelium aggregates into a multicellular organism. Triggered by nutrient depletion, this developmental program culminates in the formation of a fruiting body consisting of two distinct cell types that result from differentiation of pluripotent amoebae. Durable spores rest atop a stalk of nonviable, vacuolated cells. Extracellular cAMP, secreted by cells at maturing aggregation centers in an oscillatory fashion, initially serves to organize the amoebae during aggregation (Devreotes, 1982). Surrounding cells respond both by chemotaxis and by secreting more cAMP so that they relay the signal to cells further away. The cAMP oscillations are necessary for gene expression as well as aggregation; supplying constant levels of cAMP exogenously blocks the developmental program at its outset. Adenylyl cyclase activity and cAMP levels, low during growth, increase when aggregation begins (Klein, 1976). Within the resulting multicellular organism, cAMP continues to influence development. cAMP and its hydrolysis product adenosine act as morphogens and pattern organizers to influence differentiation of individual cells into either stalk cells or spore cells (Devreotes, 1989; Schaap, 1986).

During aggregation, the extracellular cAMP functions analogously to hormones and other signals whose effects are mediated by G protein–linked signal transduction pathways (Gilman, 1987). Stimulation of cell surface cAMP receptors (cAR1) (Klein et al., 1988) activates a heterotrimeric G protein, whose α subunit has been designated $G_\alpha 2$ (Kumagai et al., 1989; Pupillo et al., 1989), to elicit the chemotactic response and cAMP synthesis by a membrane-bound adenylyl cyclase activity. The gene products for both *cAR1* and $G_\alpha 2$ share significant topological or sequence homology with their counterparts in mammalian G protein–linked pathways. Both cAR1 and $G_\alpha 2$ are necessary to elicit the chemotactic response and for early gene expression, as was demonstrated by targeted gene disruption of the *cAR1* and $G_\alpha 2$ genes (Kumagai et al., 1991; Sun and Devreotes, 1991). These *car1*⁻ and $g_\alpha 2^-$ cells fail to respond to cAMP stimuli, fail to aggregate, and are blocked in the developmental program.

Two lines of evidence suggest that $G_\alpha 2$ does not activate adenylyl cyclase directly, however. Guanine nucleotides stimulate adenylyl cyclase in $g_\alpha 2^-$ lysates (Kesbeke et al., 1988), suggesting that, although $G_\alpha 2$ is necessary for transducing the cAMP signal from cAR1 to adenylyl cyclase, a different guanine nucleotide–binding protein directly couples to adenylyl cyclase. Recent observations suggest that mechanisms of indirect activation of adenylyl cyclase also occur in mammalian systems (Felder et al., 1989; Tang and Gilman, 1991).

Other lower eukaryotes also utilize cAMP, but activate adenylyl cyclase through a signal transduction pathway different from the G protein motifs found in mammals and in Dictyostelium. In Saccharomyces cerevisiae, for example, *RAS* gene products rather than a heterotrimeric G protein activate adenylyl cyclase (Kataoka et al., 1985). The ligand and receptor that activate RAS have not yet been identified. The disparities between the activation of adenylyl cyclase in the mammalian and yeast signal transduction pathways are reflected in differences within the structures of the adenylyl cyclases themselves.

cDNAs for several adenylyl cyclases from higher eukaryotes recently isolated predict integral membrane proteins of about 1100 aa that span the membrane 12 times and contain two homologous cytoplasmic domains; one, designated C_1, lies between the sixth and seventh transmembrane segments, and a second, C_2, is at the carboxyl terminus. Within each of these cytoplasmic domains is a region of homology (the C_{1a} and C_{2a} regions) that is also found in the catalytic domain of S. cerevisiae adenylyl cyclase (Krupinski et al., 1989). Outside of these domains, the yeast and the mammalian adenylyl cyclases share little homology; the yeast enzyme has a distinct repetitive sequence motif, no transmembrane spans, and only the one catalytic domain. Since adenylyl cyclase in aggregating Dictyostelium cells has been shown to be activated by a

G protein (Theibert and Devreotes, 1986) that does not appear to be a *ras* gene product (Reymond et al., 1986), we predicted that the aggregation adenylyl cyclase would more closely resemble mammalian adenylyl cyclase.

Results

Two Structurally Diverse Adenylyl Cyclases

We designed degenerate primers based on the C_2 domains (Gao and Gilman, 1991) of Type I and Type III adenylyl cyclases, and *rutabaga*, an adenylyl cyclase involved in learning and memory in Drosophila (Bakalyar and Reed, 1990; Krupinski et al., 1989; Levin et al., 1992), for use in polymerase chain reaction (PCR) to amplify part of a C_2 domain from a Dictyostelium adenylyl cyclase. The resulting product was used to screen genomic libraries from which we isolated the gene. We designated this gene *ACG*, since it is expressed during germination (see below).

Analysis of the nucleotide sequence from the *ACG* genomic fragments predicts an open reading frame of 2574 nt encoding an 858 aa protein, after the removal of two introns (Figure 1A). Dictyostelium introns are small, rare, and readily identifiable by sequence because of their almost exclusive AT composition, as well as by consensus splice sites (Kimmel and Firtel, 1982). Their size and location were confirmed by PCR analysis of mRNA (Perkin-Elmer Cetus, 1990). The 3′ end of the coding region is unusually AT rich; in the last 375 nt, there are only 41 G or C residues. Sixty of the carboxy-terminal 125 codons are AAT, encoding asparagine, including one stretch of 14 contiguous asparagines. Asparagine-rich sequences appear in other Dictyostelium proteins, such as several members of the cAMP receptor family (R. Johnson, C. Saxe, A. Kimmel, and P. D., unpublished data), although the functions of these repeats are not yet known. Repetitive elements contained within open reading frames are found in other organisms, such as the *opa* repeat in Drosophila (Wharton et al., 1985), yet their function has also not been identified.

The absence of *ACG* mRNA in aggregating cells (see below) prompted us to renew our search for the adenylyl cyclase involved in cell–cell signaling. We used a fragment of *ACG* containing the portion that encodes its C_2-like domain to screen at low stringency a cDNA library made from aggregating cells. Five overlapping clones were initially isolated, all of which appeared to be derived from the same mRNA as judged from sequence and cross-hybridization analysis. Since none contained a full-length message, the largest was used to screen both the genomic and cDNA libraries to obtain overlapping fragments that constituted the entire gene. This gene was labeled *ACA* to denote that it is an adenylyl cyclase expressed during aggregation (see below).

The *ACA* nucleotide sequence predicts a 4221 nt open reading frame encoding a 1407 aa protein (Figure 1B). There are three introns, as demonstrated by comparing sequence from the cDNA and the genomic DNA isolates, each of which has consensus splice sites and the typical AT-rich base composition (Kimmel and Firtel, 1982). Analysis of the cDNA clones identifies three additional regions of AT-rich sequence within the coding region. These encode for asparagine-rich amino acid sequences similar to the one noted in ACG.

The deduced amino acid sequence of ACA contains two hydrophilic domains (Kyte and Doolittle, 1982) that exhibit homology with a single domain in ACG. The amino-terminal regions of these domains are also homologous

A

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MKKTFVKILS KSYVEGYPVG FFIGLIILAI FGSMVCIFSF MHYSEEENSN
IQMDLERSSK QIIHNIQMNA MYLLSSIDTL KALYYVNPNF DRNDFNVFLN  100
TTLKNSEFQY LFWIKKINNN DRNCFEEKFS KEIKDTFQIY SFDENTNSIH
VAKNKSSYFP ILHAFPDINK DIIGLDINST DYMNETIKKS IFNKKPTVHL  200
NKKVLLSKRN IDILIVSPII VTKTLESTNE TMEDMSHISS GLFLMEKNVQ
ASRIEVENGN DFTIFLSTTN GEIVYQENYL NFKTLYQVHE SGLFEDRLKY  300
ESSLKIADCV LKLWIFTTEE YENNSKTYLP LLVSIISAVI LVLLITYSVD
QRKQKSLIAK IMREKNNLIN KILPLEVSVK LENGEDVVAE RSNNACVFFL  400
DIAGFTRFSS IHSPEQVIQV LIKIFNSMDL LCAKHGIEKI KTIGDAYMAT
CGIFPKCDDI RHNTYKMLGP AMDVLEFIPK EMSFHLGLQV RVGIHCGPVI  500
SGVISGYAKP HFDVWGDTVN VASRMESTGI AGQIHVSDRV YQLGKEDFNF
SERCDIIHVK GKGRMKTWYL MGKKSSDFSL KKDFSRSRVQ PSLFNRKQSH  600
VHCIYPEFPS GLQALNIENN LNNTDAGCEN CSKILKKTYA YSPDHSTSNY
YYHGDDNSPP PPSLNSNDLI DGSEYHDDPF PSDSNVGYHD TSKDIKEDEN  700
EQNETLLFNQ EQLKKKQIEN IQRDLSLNDS IEAIKILNNN NNNNINDNNI
NNTNLNNNNN DININNSDNV NNYENNNNFS DKIENNDGDN NNINDNNYKS  800
TNENNIKSKT LFKDSKSLIN DIKMAKENCD NNDDNNNNNN NNNNNNNNDE
NVESKKNK
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B

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MASSSPMFND HAIARSKYAL NSVLQQTNEL HDGNGGGGYT PSSPHLGGVS
LNKSQNQPYT QYNNGGGGGG GGGGHINPMH LNLNSITNNH NNHHNHHPNT  100
LSTPHNNNHN NNNHSTSHHP HSNSVANGGH LSQSITQQRG GLADLANAVI
NRKNRSDSVQ TKMKPTDSAS NIESWAKVEK FSSSIFDSEK SKKSNIFQKY  200
TLRLKNSYEK GYLHQHYNSQ IMLLRITNLI GIVAVSYGFT KEAIFMLIAI
RILCFNLFAF SIFLSFLRNR ELYKKLFHPL FLFSFTTFFI TILLEYKTTT  300
TTLILFLYVV IFCCLYALGC LLFIWMVMCN LMNAICFIIF IFLESTLDRN
NLISFVIYIL TMFLVGASHL YVLEKFRKES FIAEKKLIKE SNILKNEKEK  400
SSKLLNNILP DFIIENIVYD FEKRDIVIPE PEEYKSCSIL CFDIVQFTNM
SAKLDSPSRL VDLLTQVFRE FDTVVLRNGC QKIKTDGDAY ICACGLKSKK  500
KAKKQMPNSK STPLLQSTSS TSVNNIDLDK DNKDNNNNNN NNKNSNNNFK
NKNNIINNNN NSNSNNTTNN NSNNNNITTS GNDDDDEEIE DSELEHPEKL  600
IDVAIEIMNL DVLKETGNTE GIQVQFRCGI AAGSVYGGVI GSQKYQFDIW
GDTIARSHTL EQLGQPGKVH VGETIMTHKN WLKKWQYNYN IVSNSECKDQ  700
EHDYEFHKAH GECITSYFVD WKDDYREKKK KDLSCDFSIN KVLNAETIES
KSNNNNYNNN NYNNNNYNNN YNNNNLNNNS NNNNNEYGSS SSSSSVLGEA  800
VTEQIDCNNT NPPLQHKKSQ SILTNNENDI VSPSLTSNSP ILDTTVNNNN
NNNNTNNNNK NQNNIYGNNN NNEEDFKIKS KSNSSFEIEM SNIKKPKSRF  900
IDRVMGILHH VKISNDKIDK EIIQIDEDFV KVTKLRKYFY FFENLTTEKF
FHKYVIINNV VETKFFLVIG LILHLMFYLD DHIMDSAPYF NSNVIYLVMG 1000
IAFLVYIGLS FTRIFRTPLV YQIAFFILLC AFGVCTVLEL IRFQNPLARS
SLTRVCATLF YLNVFHSLNF LSVLFLNLFI FSFFIICSIL ISPTLTNHLY 1100
ETDYIGFVIV LLIQICSSYG MKLAMRKAWV VNCKINFKTI SVNKEKDKFN
FLLKSIFPQS ALTKLRDMID TPNIETKGIV YVQPHQDVSI MFIQIAGFQE 1200
YDEPKDLIKK LNDIFSFFDG LLNQKYGGTV EKIKTIGNTY MAVSGLDGSP
SFLEKMSDFA LDVKAYTNSV AISRVVRIGI SHGPLVAGCI GISRAKFDVW 1300
GDTANTASRM QSNAQDNEIM VTHSVYERLN KLFYFDDEKE ILVKGKGKMV
THVLKGKKDL EQTNKWFTKP PEVWEVNATP AGIASPLSGT LLGEIGSFTT 1400
PRFHLSS
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Figure 1. Deduced Amino Acid Sequences of ACG and ACA

(A) Deduced amino acid sequence of ACG. (B) Deduced amino acid sequence of ACA.

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ACA-C1a  ....NEKEKS SKLLNNILPD .......FII ENIVYDFEKR DIVIPEPEEY
ACA-C2a  ..KEKDKFN. ..FLLKSIFP QSAL.TKLRD MIDTPNIETK GIVYVQP..H
ACG-C    ITYSVDQRKQ KSLIAKIMRE KNNLINKILP LEVSVKLENG EDVVAER..S
I-C1a    LRLEDENEKQ ERLLMSLLPR NVAMEMKEDF LKPPERIFHK ..IYIQR..H
I-C2a    DDMEKVKLDN KRILFNLLPA HVAQ.HFL.. MSNPRNMD.. ..LYYQS..Y
Consen.        K K    LL   LP    A   K          E     VY Q

ACA-C1a  KSCSILCFDI VQFTNMSAKL D...SPS RLVDLLTQVFREF DTVVLRN...
ACA-C2a  QDVSIMFIQI AGFQEY.... ..DE.PK DLIKKLNDIFSFF DGLLNQKYGG
ACG-C    NNACVFFLDI AGFTRF.... SIHSSPE QVIQVLIKIFNSM DLLCAKH...
I-C1a    DNVSILFADI VGFTGL.... AQCTSAQ ELVKLLNELFGKF DELATENHCR
I-C2a    SQVGVMFASI PNFNDFYIEL DNNMGGV ECLRLLNEIIADF DELMDKDFYK
Consen.    VSI F DI  GFT          S    L  LL  IF  F D L

ACA-C1a  GCQKIKTDGD AYICACGLKS KKKAKKQMPN SKSTPLLQST --poly N--
ACA-C2a  TVEKIKTIGN TYMAVSGLDG SP..... ..... .....
ACG-C    GIEKIKTIGD AYMATCGIFP KC..... ..... .....
I-C1a    ...RIKILGD CYYCVSGLTQ PK..... ..... .....
I-C2a    DLEKIKTIGS TYMAAVGLAP TA..... ..... .....
Consen.    EKIKTIGD  YMA   GL

ACA-C1a  TSGNDDDDEE IEDSELEHFE KLIDVAIEIM NLDVLKETGN TEGIQVQFRC
ACA-C2a  .......... ......SFLE KMSDFALDV. .KAYTNSVAI SR....VVRI
ACG-C    .......DDI RHNTY..... KMLGFAMDV. .LEFIPK.EM SFHLGLQVRV
I-C1a    .......TDH AHCCV..... ...EMGLDM. .IDTITSVAE ATEVDLNMRV
I-C2a    .......GTK AKKCISSHLS TLADFAIEM. .FDVLDEINY QSYNDFVLRV
Consen.                        K  DFA D     D                RV

ACA-C1a  GIAAGSVYGG V.GSIQK YQFDIWGDTIARS HTLEQLGQPG KVHVGETIMT
ACA-C2a  GISHGPLVAG C.GIISR AKFDVWGDTANTA SRMQSNAQDN EIMVTHSVY.
ACG-C    GIHCGPVISG VSGYIAK PHFDVWGDTVNVA SRMESTGIAG QIHVSDRVY.
I-C1a    GLHTGRVLCG V.GLLRK WQYDVWSNDVTLA NVMEAAGLPG KVHITKTTL.
I-C2a    GINVGPVVAG V.GAIRR PQYDIWGNTVNVA SRMDSTGVQG RIQVTEEVH.
Consen.  GI  GPV  G V G I K  QFDVWGDTVN A SRMES G  G  IHVT  V

ACA-C1a  HKNWLKKWQY NYNIVSNSEC KDQEHDYEFH KAHGECITSY FVDWK.....
ACA-C2a  ..ERLNKLFY .F........ .DDEKEIL.V KGKGKMVTHV LKG K.....
ACG-C    ..Q.LGKEDF NF........ .SERCDIIHV KGKGRMKTWY LMG K.....
I-C1a    ..ACLN.... .......... ....GDYEVE PGHGHERNSF LKTHNIETFF
I-C2a    ..RLLRRGSY RF........ .VCRGKVS.V KGKGEMLTYF LEG RTDGN.
Consen.     L K  Y  F            D   V KGKG M T   L   K
```

Figure 2. Homology Among the C Domains of ACA, ACG, and Type I Adenylyl Cyclase

Alignment of the C_{1a} and C_{2a} domains of ACA and Type I (bovine brain) adenylyl cyclase and the C domain of ACG. Positions where at least 3 of the 5 aa are identical are indicated by boldface in the consensus sequence. The poly asparagine–rich region in ACA-C_{1a} has been labeled poly N. The sequences aligned are aa 386–722 for ACA-C_{1a} (the poly N region is 532–609); 1144–1360 for ACA-C_{2a}; 345–573 for ACG; 238–487 for Type I-C_{1a}; and 806–1067 for ACA-C_{2a}. The C_{1a} and C_{2a} domains for Type I (Gao and Gilman, 1991) were extended slightly on either side to demonstrate additional homology.

with the domains C_{1a} and C_{2a} of mammalian adenylyl cyclases (Figure 2; mammalian adenylyl cyclase Type I is shown in the figure). The amino and carboxy hydrophilic domains in ACA have been labeled ACA-C_1 and ACA-C_2, respectively, to reflect the nomenclature for the mammalian adenylyl cyclase family (Gao and Gilman, 1991). The single domain in ACG has been labeled ACG-C. Relevant pairwise comparisons (% similarity) are 52% ACA-C_{1a} vs. I-C_{1a}; 51% ACA-C2a vs. I-C_{2a}; 61% ACA-C_{1a} vs. ACG; 58% ACA-C_{2a} vs. ACG; 53% ACG vs. I-C_{1a}; 60% ACG vs. I-C_{2a}. One of the asparagine-rich sequences in ACA interrupts the C_{1a} domain in a region that is least conserved among the C domains of all adenylyl cyclases.

The topology of the two putative adenylyl cyclases differed. Hydropathy analysis reveals that ACA contains two hydrophobic regions, one amino terminal to ACA-C_1 and the other between ACA-C_1 and ACA-C_2. Each hydrophobic region contains candidates for six closely spaced transmembrane spans. The predicted topology of ACA in the membrane, therefore, resembles that predicted for the family of mammalian adenylyl cyclases (Figure 3). In contrast, ACG has only two candidates for transmembrane spans. A single putative transmembrane span (denoted as "II" in Figure 1A and Figure 3) is shown at the amino-terminal side of the catalytic domain. A second segment near the amino terminus likely represents a signal sequence for insertion into the endoplasmic reticulum (Pugsley, 1989).

We propose that ACG's putative catalytic domain is in the cytoplasm, leaving a large extracellular domain connected by the transmembrane span to the catalytic domain. This model is reminiscent of the structure proposed for the family of membrane-bound guanylyl cyclases. Members of this family of transmembrane receptors, such as the egg peptide receptor on sea urchin sperm, the mammalian atrial natriuretic peptide receptor, and the heat-stable enterotoxin receptor (Chinkers et al., 1989; Schulz et al., 1990; Singh et al., 1988), have an intracellular domain that shares homology with the cytoplasmic domains in the adenylyl cyclase family (Krupinski et al., 1989). ACG is 34% identical to the atrial natriuretic peptide receptor in this domain.

ACG Is an Adenylyl and Not a Guanylyl Cyclase

To demonstrate that the *ACG* gene encodes an adenylyl cyclase, Dictyostelium cells were transformed with an expression vector in which *ACG* is under transcriptional control of the Dictyostelium actin 15 promoter (Cohen et al., 1986; Dynes and Firtel, 1989). This promoter directs *ACG* expression during the growth stage of the life cycle, when endogenous adenylyl cyclase activity is very low. Lysates of these cells (*ACG* cells) had a basal adenylyl cyclase activity of 31.2 pmol/min per mg, 53-fold greater than lysates of control cells transformed with the vector only (0.6 pmol/min per mg). Adenylyl cyclase activity in *ACG* cells increased in the presence of Mn^{2+} (Ross and Gilman, 1980) to 307 pmol/min per mg, 10-fold over the basal activity, while activity in control cell lysates remained the same (1.3 pmol/min per mg).

Because ACG has a similar structure to and shares significant homology with the membrane-bound family of guanylyl cyclases, we also examined whether ACG is a guanylyl cyclase. *ACG* cell lysates had similar guanylyl cyclase activity to wild-type control cells, 5.9 pmol/min per mg and 6.4 pmol/min per mg, respectively. Further, adenylyl cyclase activity was not inhibited by 1 mM GTP, which is more than 3-fold higher than ACG's K_M for ATP (data not shown), demonstrating that *ACG* does not encode a guanylyl cyclase.

Stage-Specific Expression of *ACA* and *ACG*

To determine whether *ACA* could encode the adenylyl cyclase that controls aggregation, we examined whether its expression correlated with the induction of adenylyl cyclase activity during aggregation. Northern blot analysis demonstrates a 5.8 kb mRNA that first appears at three hours of development and peaks at 6 hr after starvation (Figure 4A). This time course parallels adenylyl cyclase activity during aggregation (Klein, 1976). *ACA* mRNA decreases after aggregation, and is induced again in the later stages of development. This pattern was observed in several independent experiments. ACA may therefore also synthesize the cAMP that influences differentiation

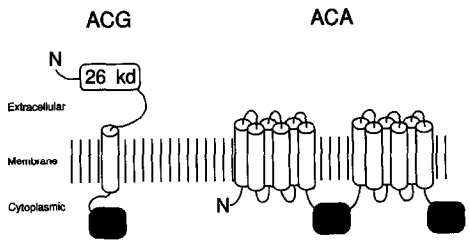

Figure 3. Models of ACA and ACG

The vertical bars represent the plasma membrane; "N" represents the respective amino termini; the boxed and shaded C_1, C_2, and C represent the respective C_1 and C_2 regions in ACA and the C region in ACG.

and morphogenesis of cells within the resulting multicellular structure (Schaap, 1986).

In striking contrast, *ACG* mRNA is expressed as a 3.5 kb transcript only at the culmination of the developmental program—after fruiting bodies have formed—and during germination (Figure 4B). Growing amoebae and cells in the starvation-induced developmental stage of the life cycle do not express *ACG*. Fruiting bodies contain about 10^5 dormant spores that germinate when nutrients again become available to release a single amoeba from each spore to begin the life cycle anew. During germination, the level of *ACG* mRNA increases. It peaks during the first 1.5 hr of germination and then decays, so that by 3 hr, when germination is complete, mRNA is no longer present (data not shown). Because *ACG* mRNA is absent in the amoebae that emerge from the spores at germination, any protein in these amoebae, which grow and divide indefinitely while nutrients are plentiful, would be soon diluted to less than one copy per cell. This implies that ACG functions specifically during germination.

ACA Is the Adenylyl Cyclase That Controls Aggregation

We examined whether ACA is the adenylyl cyclase that coordinates aggregation by creating an *aca*$^-$ mutant by gene targeting (Figure 5A). The result was unambiguous. The *aca*$^-$ cells failed to aggregate and remained as a homogeneous monolayer indefinitely (>100 hr), while the wild-type strain began to aggregate within 3 hr of starvation and formed fruiting bodies by 30 hr (Figures 5B and 5C). Nine independent uracil protrophic clones were examined by Southern blot analysis and phenotype. Eight of these were disrupted at the *ACA* locus and revealed a pattern identical to that shown for the mutant in Figure 5A. All of these eight failed to aggregate when starved on agar plates. Southern blot analysis of the ninth clone demonstrated that the *ACA* locus remained intact. This clone aggregated and completed the developmental program, identical to wild-type. To further confirm these results, an independent *aca*$^-$ cell line was created in a thymidine auxotroph (Podgorski and Deering, 1984) by disrupting the *ACA* locus with the *Thy1* gene as a selectable marker (Dynes and Firtel, 1989). These cells also failed to aggregate when deprived of nutrients (data not shown). The disruption of the *ACA* locus thus correlates with the inability to aggregate, suggesting that *ACA* is the adenylyl cyclase gene that controls aggregation.

Moreover, ACA accounts for all measurable adenylyl cyclase activity. We assayed the *aca*$^-$ and parental cells under a variety of conditions. After 5 hr of starvation, when adenylyl cyclase activity in wild-type cells is near its peak, lysates of *aca*$^-$ cells have little detectable adenylyl cyclase activity even with the addition of Mn^{2+} or guanine nucleotides (Table 1). Lysates of the parental cells, in contrast, have adenylyl cyclase activity that is stimulated by both Mn^{2+} and guanine nucleotides.

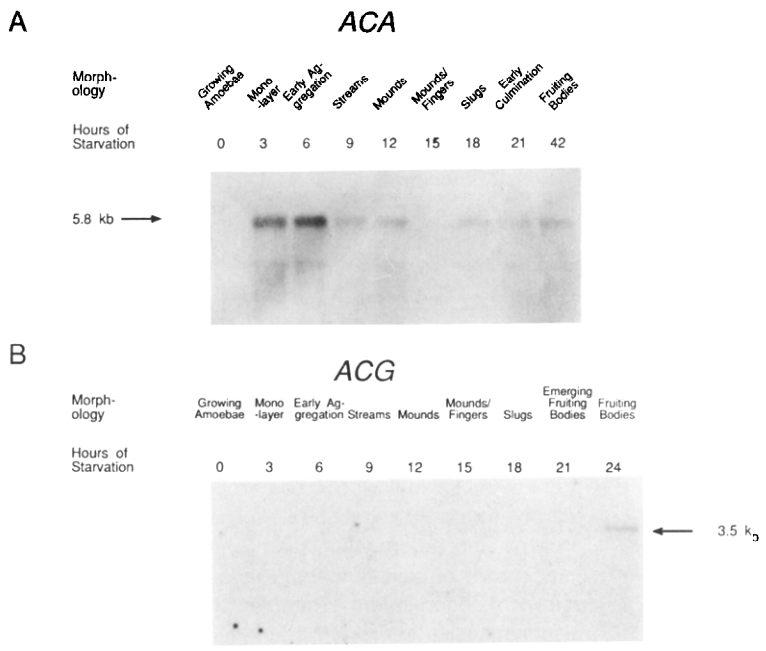

Figure 4. The Developmentally Regulated Expression of *ACA* and *ACG*

RNA was prepared from either growing cells or cells that had been starved on agar plates for the times indicated.

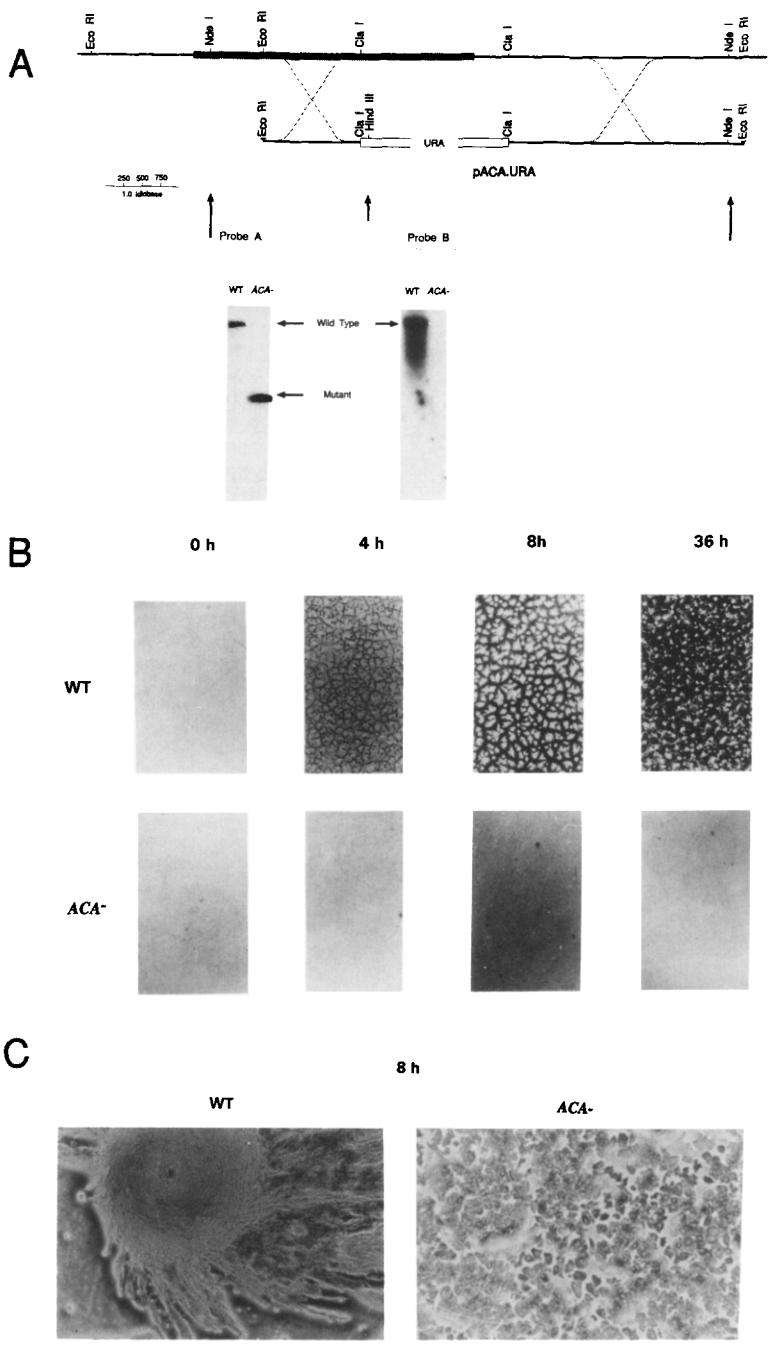

Figure 5. Disruption of the *ACA* Locus by Gene Targeting Results in the Loss of the Capability to Aggregate

(A) Southern blot analysis of genomic DNA from wild type and cells that had been transformed with a genomic fragment of *ACA* in which an internal ClaI fragment had been replaced with the Dictyostelium UMP Synthase gene (pACA.URA). The stippled box represents the *ACA* coding region. DNA was digested with NdeI and HindIII. In the parental cell line (WT), Probe A detects a ~6 kb NdeI fragment. Gene targeting introduces a HindIII site (within the UMP Synthase gene) into the *aca*⁻ mutants so that Probe A detects a 1.3 kb fragment. The filter was stripped and then probed with Probe B to confirm that the predicted double crossover event had occured in the *aca*⁻ cells. Probe B detects the same ~6 kb NdeI fragment in the parental cell line, but does not hybridize to DNA from the *aca*⁻ cell line, confirming that the ClaI fragment had been lost from the genome.

(B) Developmental phenotype of *aca*⁻ cells. Wild-type and *aca*⁻ cells were placed on starvation plates and photographs were taken at the time indicated. Each photograph shows the middle one-third of a 100 cm^2 plate.

(C) Higher magnification showing aggregate or individual cells at the 8 hr stage.

The defect in the *aca*⁻ cells appeared to be very specifc. The growth of the *aca*⁻ cells in shaking culture, when nutrients are plentiful, was indistinguishable from the parental cells. Time-lapse video analysis revealed that motility was qualitatively unaffected. Further, in tests of chemotaxis in gradients of extracellular cAMP, the *aca*⁻ cells performed as did wild type (data not shown).

ACA and *ACG* Rescue the *aca*⁻ Phenotype

Expression of *ACA* in *aca*⁻ cells restored the ability to aggregate, further demonstrating that the mutation was specifc. Similar results were obtained whether we used an *ACA* genomic fragment in an extrachromosomal vector (*aca*⁻/*ACA* cells) or the *ACA* genomic fragment cloned into an expression vector downstream from the actin 15 promoter, which directs high levels of expression during growth and development (*aca*⁻/$ACA_{act\text{-}15}$ cells) (Cohen et al., 1986). Both of these cell lines regained the ability to aggregate when placed on nutrient-poor agar. Within 8 hr, cells had aggregated into mounds, and by 30 hr, fruiting bodies were evident.

As shown in Table 1, at the 5 hr stage of development, *aca*⁻/*ACA* cell lysates had similar levels of basal and unregulated (Mn^{2+}-dependent) adenylyl cyclase activity com-

Table 1. Cells Overexpressing *ACA* Have Adenylyl Cyclase Activity

Cell Line	Adenylyl Cyclase Activity (pmol·min^{-1}·mg^{-1})		
	Mg^{2+}	Mn^{2+}	GTPγS
WT 5 hr starvation	3.2	8.4	70
aca$^-$ 5 hr starvation	0.1	0.7	0.2
aca$^-$/*ACA* 5 hr starvation	3.9	7.6	54
aca$^-$/*ACA*$_{act\text{-}15}$ growing cells	26	96	63
aca$^-$/*ACA*$_{act\text{-}15}$ 5 hr starvation	80	190	290

Adenylyl cyclase assays were performed as described (Theibert and Devreotes, 1986) in the presence or absence of 5 mM $MnSO_4$. GTPγS stimulation was determined in the presence of 40 μM GTPγS and 1 μM cAMP in the lysate.

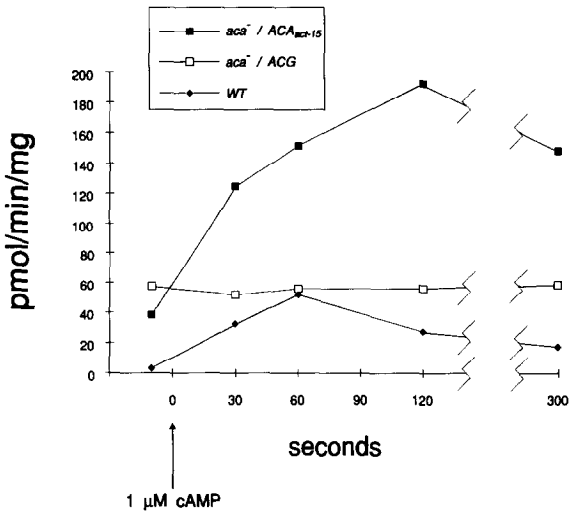

Figure 6. ACA but Not ACG Couples to cAR1

Adenylyl cyclase assays were performed on 5 hr starved Ax-3, *aca*$^-$/*ACG*, and *aca*$^-$/*ACA* cells stimulated with cAMP. Cells were removed at the times indicated, lysed, and assayed for one minute for adenylyl cyclase activity. The first point of every curve represents a sample that was assayed before the cAMP stimulus was applied.

pared with wild-type cells. While growing wild-type cells have almost undetectable adenylyl cyclase activity, the growing *aca*$^-$/*ACA*$_{act\text{-}15}$ cells had high levels of basal and Mn^{2+}-dependent adenylyl cyclase activity (8-fold and 12-fold higher, respectively, than aggregation-competent wild-type cells), reflecting the high expression from the actin 15 promoter. When these cells were starved for 5 hr, basal and unregulated adenylyl cyclase activity increased slightly. The GTPγS-stimulated activity increased almost 5-fold, however, suggesting that the G protein that activates ACA is developmentally regulated.

The capacity of the *aca*$^-$ cells to aggregate could be partially restored by constitutive expression of *ACG*. The *aca*$^-$/*ACG*$_{act\text{-}15}$ cells aggregated into small mounds (compared with wild type) within 6 hr after they were placed on a nonnutrient agar surface. Development continued within some of the mounds, which gave rise to small fruiting bodies. The spores in these fruiting bodies successfully germinated in rich media (data not shown). The appearance of mounds suggests that the cells were capable of chemotaxis and likely responded to cAMP through cAR1. It did not appear that the cells produced pulsatile waves of cAMP emanating from aggregation centers, however, since the territories were less than 3 mm.

To investigate whether ACA and ACG were coupled to cAR1, lysates of *aca*$^-$/*ACA*$_{act\text{-}15}$ and *aca*$^-$/*ACG*$_{act\text{-}15}$ cells were assayed for adenylyl cyclase activity after a 1 mM cAMP stimulus in vivo (Roos and Gerisch, 1976), as shown in Figure 6. Lysates of *aca*$^-$/*ACA*$_{act\text{-}15}$ cells made at 30 s, 1 min, 2 min, and 5 min after the cAMP stimulus had higher levels of adenylyl cyclase activity than untreated cells. Adenylyl cyclase activity peaked near 2 min after cAMP stimulation, about 5-fold higher than in untreated cells, before beginning to decay. In wild-type cells subjected to the same stimulus, adenylyl cyclase activity increased about 16-fold in response to a cAMP stimulus, and the peak was closer to 1 min. In contrast, adenylyl cyclase activity in *aca*$^-$/*ACG*$_{act\text{-}15}$ cells was insensitive to cAMP stimulation.

Because ACG was not regulated by cAR1, we examined whether ACG was sensitive to guanine nucleotide regulation. The high adenylyl cyclase activity in *ACG* cell lysates (56.7 pmol/min per mg) was not altered by GTPγS addition (53.2 pmol/min per mg). Since GTPγS regulation may be due to a factor that is present in aggregation-competent cells, but not in growing cells, we examined adenylyl cyclase activity in lysates of *aca*$^-$/*ACG*$_{act\text{-}15}$ cells starved for 4 hr, the time when GTPγS stimulates ACA maximally (Theibert and Devreotes, 1986) and the time when multiple G proteins are expressed (Wu and Devreotes, 1991). Again, ACG was insensitive to the addition of GTPγS (15.0 pmol/min per mg basal activity; 12.6 pmol/min per mg with GTPγS addition). This suggests that this novel form of adenylyl cyclase may not be subject to regulation by a G protein.

ACA Is Not Necessary for cAMP Transport

Aggregation of the *aca*$^-$/*ACG*$_{act\text{-}15}$ cells, presumably in response to extracellular cAMP, suggested that *aca*$^-$ cells were able to secrete cAMP. We exploited the constitutive adenylyl cyclase activity of ACG to measure cAMP secretion in *aca*$^-$/*ACG*$_{act\text{-}15}$ cells. Cells were pre-equilibrated with ^{3}H-adenosine, and extracellular cAMP was measured after blocking extracellular phosphodiesterase activity with dithiothreitol (DTT) (Kessin et al., 1979). As shown in Figure 7, extracellular cAMP accumulated linearly with time, while adenylyl cyclase activity was unchanged. Similar results were obtained when the extracellular phosphodiesterase activity was blocked with 100 μM cAMP (data not shown). ACG, which has only a single transmembrane span, is topologically distinct from ACA and the mammalian adenylyl cyclases. Thus, although it is possible that ACA may be involved in cAMP secretion in wild-type cells,

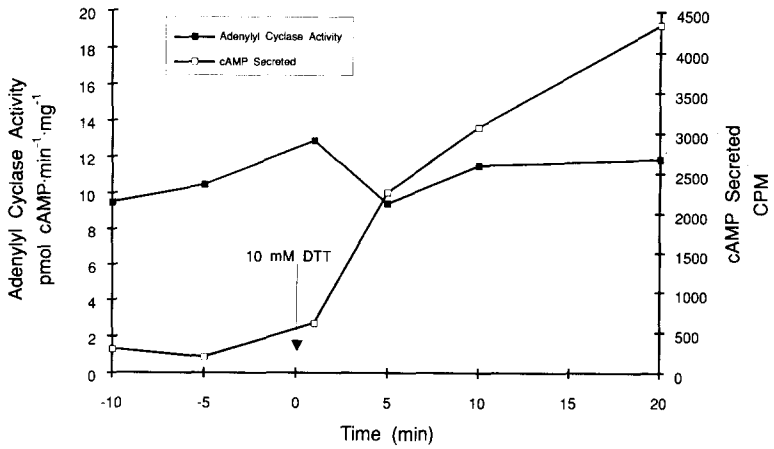

Figure 7. *aca*⁻ Cells Can Secrete cAMP
aca⁻/*ACG*$_{act\text{-}15}$ cells were pre-equilibrated with ^{3}H-adenosine. The supernatant of samples taken at the times indicated, either before or after the addition of 10 mM DTT to inhibit extracellular phosphodiesterase, was assayed for ^{3}H-cAMP.

it is not required. The rapid secretion of cAMP from *aca*⁻/*ACG*$_{act\text{-}15}$ cells demonstrates the existence of an independent pathway for cAMP secretion.

Discussion

Stimulation or inhibition of adenylyl cyclase in response to extracellular signals is part of the repertoire of cellular regulation in diverse organisms. Activation of adenylyl cyclase and the consequent synthesis of cAMP as an intracellular second messenger results, for example, from stimulation of β-adrenergic receptors by β-agonists in mammalian heart cells, while inhibition of adenylyl cyclase is mediated by α-agonists acting through α-adrenergic receptors (Dowd et al., 1989).

Dictyostelium contains two topologically distinct adenylyl cyclases: a twelve transmembrane–span form (ACA), topologically equivalent to the adenylyl cyclases identified in higher metazoans; and a novel single transmembrane–span form (ACG). The two genes are expressed at specific developmental stages and subject to different modes of regulation. ACA is expressed during aggregation and is the adenylyl cyclase activated in response to stimulation of cAR1, a G protein–linked receptor. *ACG* is expressed only during germination, and it is insensitive to guanine nucleotides.

Among adenylyl cyclases studied in eukaryotic protozoa, ACA is unique in its membrane topology and its regulation by guanine nucleotides. Neither the S. cerevisiae nor Schizosaccharomyces pombe adenylyl cyclases (Kataoka et al., 1985; Young et al., 1989), for example, share ACA's topology. While S. cerevisiae adenylyl cyclase activity is regulated by a *ras* gene product, and S. pombe adenylyl cyclase is insensitive to guanine nucleotide regulation, ACA is regulated by a surface receptor through a guanine nucleotide binding protein which does not appear to be the *ras* gene product. The appearance of a form of enzyme that is similar to that in mammals in one of the earliest multicellular organisms may signify an evolutionary branch point in the function of adenylyl cyclases and cAMP. The accessible genetics and biochemistry of Dictyostelium may make it a convenient vehicle for the study of mammalian adenylyl cyclases.

Our identification and studies of ACG demonstrate a novel form of eukaryotic adenylyl cyclase. In our model of ACG, a single transmembrane span connects a large extracellular domain, which may serve to bind ligands, to the internal catalytic domain. ACG's extracellular domain may be involved in recognition of an extracellular signal present during germination, when ACG is expressed, which leads to activation or inhibition of cAMP synthesis by the cytoplasmic domain. This suggests that ACG has the receptor component, signal transduction apparatus, and catalytic component within the same molecule. This model is analogous to the atrial natriuretic peptide receptor in mammals and to the chemotactic egg peptide receptor in sea urchin sperm, which are guanylyl cyclases whose activities are regulated by binding of their respective ligands to extracellular domain (Chinkers et al., 1989; Schulz et al., 1990; Singh et al., 1988). It should be noted that while there may be an evolutionary relationship, ACG is clearly an adenylyl cyclase and not a guanylyl cyclase. We are currently searching for a signal that activates or inhibits ACG activity and are investigating the function of ACG during germination in an *acg*⁻ cell line created by gene targeting. A search of the NBRF Protein Bank fails to identify any proteins with homology to ACG's putative extracellular domain.

ACA and the other examples of the twelve transmembrane–span form of adenylyl cyclase represent only one part of a multicomponent signal transduction pathway. As part of a G protein–linked signal transduction pathway, these adenylyl cyclases are indirectly coupled to a transmembrane domain receptor and may be just one of several intracellular effectors activated by the receptor. A single receptor has been shown to interact with several G proteins, and individual G proteins may activate several effectors (Freissmuth et al., 1989). Indeed, activation of ACA is only one of many cAMP-stimulated responses during aggregation in Dictyostelium.

Where the entire signal transduction apparatus (receptor, transducer, and effector) is combined in one molecule, such as in the family of membrane-bound guanylyl cyclases and possibly ACG, the extracellular signals are more specific in their intracellular effects, regulating the alteration in the concentration of only one second messen-

ger molecule. It is conceivable that there may be tissue-specific or developmental stage–specific requirements for the regulation of cAMP as the sole intracellular second messenger in response to a particular stimulus in higher eukaryotes. The presence of both forms of adenylyl cyclase in Dictyostelium suggests that metazoa may express both this novel form as well as the twelve transmembrane form.

A family of genes from the Trypanosoma equiperdum and brucei species, homologous to *ACG* in sequence and structure, has recently been identified (Alexandre et al., 1990; Ross et al., 1991). Like ACG, the members of this family appear to have a single transmembrane span separating an extracellular domain from a cytoplasmic domain that shares homology with the catalytic domains of the adenylyl and guanylyl cyclase families. While expression of a partial open reading frame from a T. equiperdum gene in S. cerevisiae results in an increase in adenylyl cyclase activity, it has not yet been demonstrated whether the T. brucei genes encode adenylyl or guanylyl cyclases. The functions of these enzymes in the Trypanosome life cycle have not yet been identified. All of the mammalian adenylyl cyclases for which the genes have been cloned have the twelve transmembrane domain structure and are regulated by G proteins, but an adenylyl cyclase activity insensitive to guanine nucleotide regulation has been identified in mammalian sperm (Garbers and Kopf, 1980). This enzyme may be structurally homologous to ACG.

Constitutive expression of ACA allowed us to investigate the development of its sensitivity to guanine nucleotides. The time course of the appearance of the factor responsible for this regulation is similar to that of *ACA* gene expression. Since ACA is not regulated by a *ras* gene product (Reymond et al., 1986), we predict that a heterotrimeric G protein confers the guanine nucleotide sensitivity. A recently isolated α subunit gene whose pattern of developmental expression mirrors *ACA* expression may be this G protein (Wu and Devreotes, 1991).

It appears that two G proteins are involved in receptor-mediated activation of ACA in vivo. In cell lysates, cAR1 remains coupled to $G_\alpha 2$, but the cAR1/$G_\alpha 2$ complex is uncoupled from ACA (Theibert and Devreotes, 1986; Van Haastert, 1984). Further, while there is no cAR1-mediated ACA activation in intact $g_\alpha 2^-$ cells, ACA activity remains fully sensitive to guanine nucleotide regulation in $g_\alpha 2^-$ cell lysates.

G proteins that are not directly coupled to the activating receptor also appear to be involved in the activation of adenylyl cyclases in other systems. In human neutrophil membranes, for example, activation of adenylyl cyclase by guanine nucleotides appears to be dependent on a different G protein than that coupled to the fMet-Leu-Phe receptor (Verghese et al., 1985). Similarly, in A9 L cells transfected with the m1 muscarinic receptor, carbachol activates synthesis of cAMP in intact cells but not in cell membranes (Felder et al., 1989). Further, in cells expressing recombinant Type II adenylyl cyclase, cAMP synthesis can be triggered via receptors coupled to G_q, but activated $G\alpha_q$ alone does not stimulate the enzyme.

Recent in vitro studies of several subtypes of mammalian adenylyl cyclases have demonstrated alternative mechanisms of G protein regulation that may explain these observations. While the Type I enzyme can be stimulated by activated $G\alpha_s$, maximal stimulation of Type II requires $G\alpha_s$ and $G\beta\gamma$ (Tang and Gilman, 1991). Thus, in the examples cited, receptor stimulation may result in release of a G protein $\beta\gamma$ complex that interacts with $G\alpha_s$ to activate adenylyl cyclase. Likewise, the requirement for two G proteins in the activation of ACA could be explained if stimulation of the cAR1 results in the release of the $\beta\gamma$ complex from $G_\alpha 2$, which then interacts with the as yet unidentified $G\alpha_s$. It is important to note, however, that ACA is no more homologous to Type II than to Type I.

We have demonstrated that adenylyl cyclase and cAMP are essential for Dictyostelium aggregation. We have also shown, by disrupting the *ACA* locus, that *ACA* is not necessary for cell motility, chemotaxis, cell division, or viability. Preliminary evidence suggests that the mutation in *synag 49*, a cell deficient in aggregation and without detectable adenylyl cyclase activity (Pupillo et al., 1988), is at a locus different from *ACA*; we have been able to complement the aggregation-deficient phenotype in *aca⁻/synag 49* diploid cells (Coukell et al., unpublished data). Since the *aca⁻* cells lack detectable adenylyl cyclase activity, they must be devoid of cAMP. Given the universal presence of adenylyl cyclase in eukaryotic cells, it is intriguing that *aca⁻* cells are unaffected in these cellular processes.

The proposed structure for ACA and the mammalian adenylyl cyclases, two sets of six transmembrane spans and two homologous cytoplasmic domains, is topologically similar to a superfamily of proteins that are involved in the transport of small molecules across the plasma membrane. While there is little homology among the amino acid sequences, the family of facilitative glucose transporters (Gould and Bell, 1990) and the *MDR* (multiple drug resistance) gene products, which excrete cytotoxic drugs (Juranka et al., 1989), are two examples in which the topological similarities are particularly striking. Cyclic AMP secretion is displayed by many cell types, and it has been speculated that adenylyl cyclases may facilitate its transport (Krupinski et al., 1989). Since the *aca⁻/ACG$_{act\text{-}15}$* cells constitutively synthesize and secrete cAMP, however, there must be cAMP transporters that are unrelated to the twelve transmembrane form of adenylyl cyclases. It is possible that ACA and the mammalian adenylyl cyclase might be involved in transport of a different molecule.

As shown in Figure 6, ACA activity is sensitive to external cAMP stimuli. Adenylyl cyclase activity in wild-type and *aca⁻/ACA$_{act\text{-}15}$* cell lysates increases within the first 2 min after a cAMP stimulus and decreases to its pre-stimulus level within 10 min. Thus, ACA is both positively and negatively regulated by extracellular cAMP. This regulation is part of the mechanism that establishes the oscillatory cAMP waves during aggregation.

As shown in Figure 6, adenylyl cyclase activity in *aca⁻/ACG$_{act\text{-}15}$* is insensitive to external cAMP stimuli, yet expression of *ACG* in the *aca⁻* cells is able to rescue the phenotype partially. This suggests that supplying an endogenous source of cAMP is sufficient to restore aggregation and development in *aca⁻* cells. Rescue of the *aca⁻* cells can there-

fore be used to establish activity of an expressed adenylyl cyclase. Dictyostelium signal transduction utilizes components (seven transmembrane-spanning receptors, G proteins, and effectors) that are homologous to their mammalian counterparts. Since mammalian proteins have been successfully expressed in Dictyostelium (Devreotes, 1989), the *aca*⁻ cells might provide a valuable tool for the study of mammalian signal transduction.

Experimental Procedures

Cloning of *ACG* and *ACA*

Protocols were performed as described, unless otherwise noted (Sambrook et al., 1989). PCR (Saiki et al., 1988) was perfomed on 100 ng of genomic DNA using primers RR168 5′-CCNGTNNTNGCNGGNGT-3′ and RR170 5′-GCNACA/GTTNACNGTT/GT-3′, where N is A, C, G, or T. Fifty cycles of PCR were performed with a cycle profile of 94°C for 45 s, 37°C for 1 min, and 55°C for 3 min. A 74 bp product, which was similar in size to a predicted 71 bp product, was excised from the gel, eluted, and subjected to an additional round of PCR. This newly amplified product was subcloned and sequenced. This product corresponds to the codons for aa 498–521 in the ACG sequence. A 43-mer oligonucleotide, RR180, 5′-GTCACCCCAAACATCGAAATGTGGCTTTGCATAACCTGAAATG -3′, corresponding to sequence between the PCR primers, was then used to screen EcoRI-digested Dictyostelium genomic DNA cloned into λZAP (Stratagene), which yielded a 1.7 kb EcoRI fragment, pλ22. This fragment was used to screen a Sau3AI partially digested genomic DNA library cloned into pAT153Δ (gift of R. A. Firtel, University of California, San Diego), from which a 4.3 kb overlapping genomic fragment, pG1D, was isolated. These two genomic fragments overlap by about 400 nt.

A PCR product corresponding to part of the catalytic domain from ACG was used as a probe to screen at low stringency ~50,000 clones of a λgt11 cDNA library made from aggregation competent Dictyostelium (Klein et al., 1988). This PCR product was generated with primers GP2 (5′-GGACTAGTTGAATGATCGGGTGAGTAAGTCATAAGT-3′) and GP4 (5′-CCTACTTACCATTACTTGTATCAATAATAT-3′). Hybridization conditions were: 50°C in 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 0.1 mg/ml sonicated salmon sperm DNA. The filters were washed at 55°C in 2 × SSC and 0.1% SDS. Five cross-hybridizing inserts were isolated. One of these, pλ5, was used to screen the library of EcoRI-digested genomic DNA cloned into λZAP at high stringency (filters were washed at 65°C in 2 × SSC, 1% SDS). This yielded a 6 kb fragment labeled pGSP2. Since pGSP2 did not contain the entire *ACA* coding region, the 5′ ClaI–EcoRI fragment of pGSP2 was randomly primed and used to screen the library from aggregation-competent cells. Out of 50,000 phage screened, 41 positives were isolated. To determine which of these included additional 5′ sequence, they were screened by PCR using primer ACA1 (5′-GTAAGCATCACCATCAGT-3′) and either λ_{fwd} or λ_{rev} (primers designed to flank the EcoRI cloning site in λgt11). One isolate, labeled λ_{fwd}-ACA1, gave an additional 0.5 kb of 5′ sequence. This fragment was then used to screen the library of EcoRI-digested genomic DNA, from which pGSP8 was isolated. This fragment contained 2 kb of additional 5′ sequence.

Sequence from both *ACG* and *ACA* was determined on both strands using Sequenase (US Biochemical Corp.). For one small stretch of *ACA*, around aa 850, sequence was determined with *Taq* polymerase on an ABI sequencer. The two introns in *ACG* and the three in *ACA* were confirmed either by sequencing RNA–PCR products generated with appropriate primers or by sequencing cDNA isolates. The presence of the AT-rich sequence in the *ACA* and *ACG* coding regions was confirmed by sequencing multiple RNA–PCR products and by sequencing cDNAs. A cDNA library made from RNA isolated from germinating cells, from which we isolated a partial cDNA for *ACG*, in order to confirm its AT-rich sequence and 3′ end, was a gift of H. Ennis (Roche).

Construction of Cell Lines

ACG Cells

A full-length construct of *ACG* was created by inserting a fragment from pλ22 into pG1D at the common XbaI site to create pGACG. The large (about 2 kb) untranslated region at the 5′ end was deleted by cutting a BglII–HindIII fragment from pGACG and inserting this into a plasmid containing a 5′ cDNA, created by RNA–PCR at the BglII site. This leaves less than 100 nt of sequence 5′ to the initiating ATG. This construct was then cloned into the Dictyostelium expression vector pJK1 (J.-Y. Kim, M. Caterina, and P. D., unpublished data), which is a derivative of the Dictyostelium extrachromosomal vector pATANB43 (Dynes and Firtel, 1989) into which the actin 15 promoter and the 2H3 terminator have been inserted. This construct was labeled pGSP1. The pJK1 plasmid bears the neomycin resistance gene that allows the selection of stable transformants. The plasmid pGSP1 or the pJK1 vector alone was electroporated (Howard et al., 1988) into wild-type strain, Ax-3, stable transformants selected, and one, *ACG* cells or vector control cells, was chosen for further study.

aca⁻ Cells

The *ACA* gene was disrupted by gene targeting. A uracil auxotroph RF1 (gift of R. A. Firtel) was transformed (Howard et al., 1988) with a genomic fragment of *ACA*, pGSP2, in which a 2.0 ClaI fragment was replaced by the UMP-synthase gene (pACA.URA) (Kalpaxis et al., 1991). Uracil protrophs were selected, and their genomic DNA was isolated (Nellen et al., 1987) to ascertain whether the *ACA* gene had been disrupted. The DNA was digested with NdeI and HindIII, and Southern blot analysis was performed using randomly primed probes.

aca⁻/*ACA* Cells

A full-length genomic construct of *ACA* (pGSP9) was created by ligating the pGSP8 insert into pGSP7, a derivative of pGSP2 in which 2.5 kb of 3′ untranslated sequence was removed. pGSP9 contains about 1 kb of sequence 5′ to the *ACA* initiating methionine codon. The *ACA* fragment from pGSP9 was then inserted into the Dictyostelium extrachromosomal expression vector pATANB43 (Dynes and Firtel, 1989), and the resulting fragment was labeled pGSP10. The *aca*⁻ cells were transformed with pGSP10, and transformants were selected in HL-5 plus 20 μg/ml G418.

aca⁻/$ACA_{act\text{-}15}$ Cells

The *ACA* fragment from pGSP9 was inserted into the Dictyostelium expression vector pB18 (Johnson et al., 1991) in the sense orientation. The resulting plasmid was labeled pKL2. The *aca*⁻ cells were transformed with pKL2, and transformants were selected in HL-5 plus 20 μg/ml G418.

aca⁻/$ACG_{act\text{-}15}$ Cells

aca⁻/$ACG_{act\text{-}15}$ cells were prepared by transformation of *aca*⁻ cells with pGSP1. The resulting cells were grown in HL-5 supplemented with 20 mg/ml G418.

Adenylyl Cyclase Assays

Adenylyl cyclase assays were performed as described (Theibert and Devreotes, 1986) in the absence or presence of 5 mM $MnSO_4$ on growing or 5 hr starved cells as indicated (Devreotes et al., 1987). GTPγS stimulation was determined in the presence of 40 μM GTPγS and 1 μM cAMP in the lysate. Each assay was determined in triplicate. Experiments were repeated at least three times with similar results, and typical assays are shown.

To examine the effects of cAMP stimulation in vivo, adenylyl cyclase assays were performed on growing or 5 hr starved cells stimulated with cAMP as described (Roos and Gerisch, 1976). In brief, cells shaken on ice were stimulated with 1 μM cAMP and 10 mM DTT. Cells were removed at the times indicated, lysed, and assayed for adenylyl cyclase activity. The first point of every curve in Figure 6 represents a sample that was assayed before the cAMP stimulus was applied. Experiments were repeated at least twice for all three cell lines, and similar results were obtained.

Guanylyl Cyclase Assay

Guanylyl cyclase activity was measured in Dictyostelium whole cell lysates of 1.6 × 10^5 cells and in homogenized rat brain as described (Schultz and Bohme, 1984), except the Dictyostelium reactions were performed at 22°C (as were the Dictyostelium adenylyl cyclase assays). To serve as a positive control, a 45,000 × g supernatant from whole rat brain homogenized in 50 mM Tris (pH 7.5) was used. Each point was assayed in triplicate. This assay was repeated twice with similar results.

Northern Blot Analysis

RNA was prepared as described (Klein et al., 1988) from either growing cells or cells that had been starved on agar plates for the times indicated. Five micrograms of total RNA for each time point was size fractionated on a 1.2% agarose gel and then transferred to a nitrocellulose filter. A partial cDNA isolate (pl4) was randomly primed and used as a probe for *ACA*. A randomly primed 1.2 kb EcoRI fragment that encompasses the 5′ half of the coding region was used to analyze the expression of *ACG*. A randomly primed probe for the G_β subunit (P. Lilly and P. D., unpublished data), which is constitutively expressed during all stages of the life cycle, was used to determine that an equivalent amount of RNA was loaded in each lane.

Secretion of cAMP by *aca*$^-$/*ACG*$_{act\text{-}15}$ Cells

Cells were labeled with (2,8,-^{3}H)-adenine as described (Roos et al., 1975), except cells were labeled at 1 mCi/ml with cells shaking at 1 × 10^8 cells per ml in phosphate buffer for 1 hr. Cells were washed extensively (at least 6 times) and resuspended at 8 × 10^7 cells per ml. Extracellular phosphodiesterase activity was inhibited with 10 mM DTT, and samples were taken at the times indicated. The sample was spun for 15 s at full speed in a microfuge and the supernatant was added to a solution containing cAMP and SDS. ^{3}H-cAMP was quantitated as described (Salomon, 1979). The identity of the ^{3}H-material as cAMP was confirmed by its comigration with standard when chromatographed in 95% ethanol, 1 M ammonium acetate (70:30). This experiment was repeated three times, and three additional times using 100 μM cAMP instead of 10 mM DTT to inhibit extracellular phosphodiesterase, and similar results were obtained.

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