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An adenylyl cyclase that functions during late development of *Dictyostelium*

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SUMMARY

A variety of extracellular signals lead to the accumulation of cAMP which can act as a second message within cells by activating protein kinase A (PKA). Expression of many of the essential developmental genes in *Dictyostelium discoideum* are known to depend on PKA activity. Cells in which the receptor-coupled adenylyl cyclase gene, *acaA*, is genetically inactivated grow well but are unable to develop. Surprisingly, *acaA*$^-$ mutant cells can be rescued by developing them in mixtures with wild-type cells, suggesting that another adenylyl cyclase is present in developing cells that can provide the internal cAMP necessary to activate PKA. However, the only other known adenylyl cyclase gene in *Dictyostelium*, *acgA*, is only expressed during germination of spores and plays no role in the formation of fruiting bodies. By screening morphological mutants generated by Restriction Enzyme Mediated Integration (REMI) we discovered a novel adenylyl cyclase gene, *acrA*, that is expressed at low levels in growing cells and at more than 25-fold higher levels during development. Growth and development up to the slug stage are unaffected in *acrA*$^-$ mutant strains but the cells make almost no viable spores and produce unnaturally long stalks. Adenylyl cyclase activity increases during aggregation, plateaus during the slug stage and then increases considerably during terminal differentiation. The increase in activity following aggregation fails to occur in *acrA*$^-$ cells. As long as ACA is fully active, ACR is not required until culmination but then plays a critical role in sporulation and construction of the stalk.

Key words: Adenylyl cyclase, *Dictyostelium discoideum*, Sporulation

INTRODUCTION

cAMP is used as an intercellular chemotactic signal during aggregation and as a second messenger throughout development of *Dictyostelium* (Konijn et al., 1968; Bonner et al., 1969; Bonner, 1970; Kay, 1989; Schaap et al., 1993; Reymond et al., 1995). Two distinct genes have been characterized that encode the enzyme, adenylyl cyclase, responsible for the synthesis of cAMP (Pitt et al., 1992). One of them, *acaA*, encodes a protein with 12 transmembrane domains that is expressed shortly after the initiation of development while the other, *acgA*, is only expressed during germination of spores. Analyses of the phenotypes of null mutants have shown that *acaA* is essential for chemotactic aggregation while *acgA* is essential for the maintenance of dormancy when spores are dispersed into an environment of high osmolarity (van Es et al., 1996). Somewhat surprisingly, it was found that cells lacking the aggregation adenylyl cyclase, ACA, would form spores if they developed in chimeric mixtures with wild-type cells and that addition of extracellular cAMP could restore post-aggregative gene expression and morphogenesis (Pitt et al., 1993). While extracellular cAMP is known to activate ACA by binding to the G-protein coupled cAMP receptor CAR1 (Sun and Devreotes, 1991), this pathway could not be functioning in the *acaA*$^-$ null cells. Ligand binding to CAR1 also elicits several G-protein independent responses such as induction of prespore-specific genes (Jin et al., 1998a,b). There is direct evidence that gene expression in prespore cells as well as their encapsulation during terminal differentiation is mediated by the cAMP-dependent protein kinase PKA (Hopper et al., 1995; Kay, 1989; Richardson and Loomis, 1992; Loomis, 1998; Anjard et al., 1998). However, until recently the source of cAMP needed to activate PKA in *acaA*$^-$ null cells has not been apparent. The first indication that there might be another adenylyl cyclase came from the demonstration of low levels of adenylyl cyclase activity in vegetative cells of a double mutant in which both *acaA* and *acgA* were inactivated (Kim et al., 1998). Unlike the activity measured during aggregation, this vegetative activity which they named ACB was found to be maximally active in the presence of magnesium and was not stimulated by addition of GTPγS (Kim et al., 1998).

A near-saturation screen for morphological mutants in *Dictyostelium* recently turned up a gene that affects terminal differentiation and encodes a large protein with a domain in the carboxy-terminal half that shows significant similarity to cyanobacterial adenylyl cyclases and the recently characterized cytosolic adenylyl cyclase that is preferentially expressed in mammalian testis (Buck et al., 1999). The predicted product of the gene also has domains closer to the N terminus that resemble those in the signal transducing hybrid histidine kinases (Loomis et al., 1998). However, it is missing the

conserved H motif where the histidine phosphate is found and so it cannot encode a true histidine kinase. Nevertheless, well conserved motifs surrounding the phosphorelay aspartate are present raising the possibility that this gene product may interact and be regulated by one or more of the histidine kinases that regulate various aspects of terminal differentiation in *Dictyostelium* (Loomis et al., 1998; Wang et al., 1996; Zinda and Singleton, 1998). Although the consequences to disruption of the gene seemed to be restricted to terminal differentiation, it might be responsible for the vegetative activity, ACB, as well. Most adenylyl cyclases have two active sites, C1 and C2, present in one or more subunits (Tucker et al., 1998; Buck et al., 1999) but the predicted product of the disrupted gene has only a single active site. Since it is likely to encode for a subunit of adenylyl cyclase and carries a potential response regulator domain we named the gene *acrA*.

Differentiation of spores and stalks cells must be coordinated in both time and space during fruiting body formation to avoid premature encapsulation that would leave the spores at the base of the stalk. Several peptide signals that stimulate prespore cells to encapsulation are released by prestalk cells during culmination (Anjard et al., 1997, 1998). The response to one of the these peptides, SDF-2, has been shown to be mediated by the receptor histidine kinase DhkA and to ultimately lead to the activation of PKA (Wang et al., 1999; Anjard et al., 1998). Mutant strains in which *dhkA* is disrupted form long thin stalks and very few spores (Wang et al., 1996). It is of considerable interest that the recently isolated *acrA*$^-$ strain shares these phenotypic properties.

The signal transduction pathway from DhkA to PKA activation appears to be mediated by inhibition of the cytoplasmic cAMP phosphodiesterase, RegA, such that cAMP can accumulate to higher levels. The gene encoding this phosphodiesterase, *regA*, was isolated in a suppressor screen for strains able to encapsulate in the absence of prestalk signaling (Shaulsky et al., 1996). It was subsequently shown to encode a cAMP-specific phosphodiesterase (Shaulsky et al., 1998; Thomason et al., 1998). Null mutations in *regA* not only suppress the block to encapsulation in *dhkA*$^-$ cells but also result in constitutive release of SDF-2 (Wang et al., 1999; Anjard et al., 1998). While inhibition of RegA could certainly account for the accumulation of cAMP seen during culmination if ACA were still maximally active (Abe and Yanagisawa, 1983), the levels of ACA during terminal differentiation may be considerably less than those during the aggregation stage since *acaA* mRNA decreases markedly after 6 hours of development (Pitt et al., 1992). The newly recognized adenylyl cyclase gene, *acrA*, continues to be expressed until culmination and appears to be responsible for many late functions.

MATERIALS AND METHODS

Chemicals

Guanosine 5′-O-(3-thiotriphosphate) (GTPγS) and phenyl-methyl-sulfonyl fluoride (PMSF) were purchased from Boehringer Mannheim, Indianapolis, Ind. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), pepstatin A and benzamidine were purchased from Sigma, St. Louis, MO. Biotrak Cyclic AMP [^{3}H] assay system was purchased from Amersham Pharmacia Biotech, Arlington Heights, Ill. Blasticidin S was purchased from ICN Pharmaceuticals Inc., Irvine, CA. Nucleopore Track-Etch Membrane was from Corning Corp., Corning, NY.

Cells and culture

All strains were grown in HL5 medium and induced to synchronously develop by depositing them at 5×10^7 on nitrocellulose filters supported on buffer-saturated pads (Sussman, 1987). Strain AK299 (*dhkA*$^-$) and strain TL123 (*dhkA*$^-$ *dhkB*$^-$) have been previously described (Wang et al., 1996, 1999). Strain TL135 has a deletion in *dhkC* (Singleton et al., 1998) and was the kind gift from Charles Singleton.

Transformation and development

Strain AX-4 was used as the host for a *Dpn*II REMI mutagenesis with the pBSR1 vector (Kuspa and Loomis, 1992; Shaulsky et al., 1995). Cells were transformed by electroporation and transferred to tissue culture plates with HL5 medium. Blasticidin S resistant transformants were selected by adding 5 μg/ml blasticidin S a day after electroporation and selecting for one week (Adachi et al., 1994). Morphological mutants were recognized by the structures formed within plaques generated by the transformed cells. Strain DG1100 was selected because it forms tall, thin fruiting bodies with clear sori.

Filter development and slug migration on agar plates were carried out as described by Shaulsky and Loomis (1993). Chimeric development was accomplished by mixing vegetative cells from two different strains in a 1:1 ratio and depositing them on nitrocellulose filters.

Spore viability assay

Mid-culminants were collected from nitrocellulose filters, dissociated and washed in 20 mM phosphate buffer pH 6.2 containing 20 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. Cells were distributed in 24 wells plates at 10^5 cells/cm^2 with 0.5 ml buffer in each well. At the indicated times 25 μl of 10% Triton X-100 was added to a well and the contents collected. Detergent resistant spores were then pelleted, washed and spread on SM agar plates together with bacteria. After 4 days incubation, the number of plaques observed on duplicate plates gave an indication of the number of viable spores.

DNA analyses

Regions flanking the plasmid insertion site were isolated by plasmid rescue (Kuspa and Loomis, 1992). Genomic DNA from strain DG1100 was digested with either *Nde*I, *Bgl*II or *Hin*dIII, ligated and electroporated into *E. coli* SURE cells (Stratagene, Inc.). Plasmid DNA was isolated from the transformants and sequenced on an ABI 377 automated sequencing machine (Perkin-Elmer, Inc.). A 7922 bp sequence including the *acrA* gene has been deposited in GenBank (Accession no. AF153362).

The disruption of *acrA* was recapitulated by homologous recombination (Kuspa and Loomis, 1992) with linearized *Hin*dIII plasmid where 4.3 kb of the gene flanks the insertion site.

Northern blots

Northern blots were performed as described by Shaulsky and Loomis (1993). Probes for *acrA* mRNA were generated by amplifying a 743 bp region covering the adenylyl cyclase catalytic domain (5225 to 5968 bp in the sequence deposited in GenBank). *csaA* (Noegel et al., 1986), *ecmA* (Jermyn et al., 1987), *cotB* (Fosnaugh and Loomis, 1989), *spiA* (Richardson and Loomis, 1992) probes were made from appropriate clones. RNA was isolated from 10^8 developed cells using the Trizol Reagent (Gibco BRL). 20 μg of each sample was run on a 0.8% RNA gel and northern blotted.

Adenylyl cyclase assay

At each time point about 10^8 cells were collected, washed in 10 mM sodium/potassium phosphate buffer, pH 6.5 and resuspended in 200

μl of lysis buffer (250 mM sucrose in 10 mM Tris buffer, pH 8.0). Cells were lysed by passage through 3 μm pore size nucleopore membranes and the extracts kept on ice. 35 μl of extract was mixed with 17.5 μl of 0.8 mM IBMX to inhibit cAMP phosphodiesterases (Kim et al., 1998). After adding either $MgCl_2$ or $MnCl_2$, samples were placed in a 22°C waterbath and the reaction started by adding 17.5 μl of a solution of 2 mM ATP and 40 mM DTT in 2× lysis buffer. 20 μl aliquots were withdrawn at various times and added to 10 μl of 0.2 M EDTA (pH 8.0) before heating them at 100°C for 1 minute. cAMP levels were then determined using the Biotrak Cyclic AMP [^{3}H] assay system. Protein was determined by the Bradford procedure using the Bio-Rad Protein Assay. In certain experiments GTPγS was added to the cells before lysing them and the extracts were incubated on ice for 8 minutes prior to initiating the reaction in the presence of 10 mM $MgCl_2$.

The assay was found to be linear with extract and time over the course of the reaction (10 minutes).

Cell fractionation

Cells were dissociated from culminants after 22 hours of development, washed and suspended in lysis buffer containing the protease inhibitors PMSF (1 mM), pepstatin A (1 μg/ml), and benzamidine (1 mM). Extracts were prepared by passage through nucleopore membranes and centrifuged at 16,000 ***g*** for 30 seconds to remove unbroken cells before centrifuging the supernatant at 100,000 ***g*** for 10 minutes at 4°C. The supernatant was collected and the pellet was dissolved in lysis buffer and immediately assayed for adenylyl cyclase activity.

RESULTS

Isolation and characterization of strain DG1100

Populations of *D. discoideum* were mutagenized using Restriction Enzyme Mediated Integration (REMI) to facilitate random plasmid insertions into the genome (Kuspa and Loomis, 1992). Transformants were plated clonally on bacterial lawns and the developmental potential of each clone was determined by visual screening of the structures formed within the plaques. One of the transformed strains, DG1100, formed fruiting bodies with long, thin stalks and and glassy colorless sori (Fig. 1). This is a fairly rare phenotype but is seen in strains lacking the receptor histidine kinase DhkA (Wang et al., 1996, 1999). Some spores can be found during early culmination but upon dissociation in buffer they appear to germinate almost immediately ($T_{\frac{1}{2}}$ ~30 minutes). Under similar conditions wild-type spores remain encapsulated for over 6 hours. Thus, the *acrA*$^-$ mutant has a defect in sporulation that can account for the glassy appearance of its sori, a phenotype similar to that of strains lacking the histidine kinases DhkA or DhkB (Wang et al., 1996; Zinda and Singleton, 1998).

Cells of strain DG1100 grew in axenic media at the same rate as the parental strain, AX4, and proceeded to develop normally up to culmination when washed free of nutrients and deposited on buffer-soaked filters. However, as soon as stalk tubes were formed in strain DG1100, they could be seen to be less rigid than those of wild-type strains and the prespore masses retained their elongated slug shapes as they rose up the stalks. When fruiting bodies were collected after either 24 or 48 hours of development, dissociated and washed in 0.1% NP40, less than 1% of the initial cells were found to be viable. Wild type spores are completely resistant to such detergent treatment.

To determine whether or not impaired sporulation is a cell autonomous defect we mixed equal numbers of cells from strains AX4 and DG1100 and allowed them to develop together. Fruiting bodies were collected after 24, 36, or 48 hours of development and the spores plated clonally after detergent treatment. Visual inspection of the morphological structures formed within each plaque showed that less than 1% of the spores were derived from strain DG1100. Thus the mutation in this strain results in a cell autonomous phenotype that is not rescued by the presence of wild-type cells.

The mutated gene (*acrA*) was isolated by plasmid rescue into *E. coli* and used to recreate the insertion mutation in other host strains by homologous recombination. Disruption of *acrA* in wild-type cells produced strains that had the same phenotype as strain DG1100. This rules out the possibility of extraneous mutations accounting for the culmination defects in the original mutant.

Characterization of acrA

We have cloned and sequenced over 8 kb surrounding the plasmid insertion site in *acrA* and found an open reading frame (ORF) that encodes a large protein of 243 kDa (Fig. 2). A single intron is found near the 5′ end (open triangle). There are several strongly hydrophobic domains in the N-terminal region of the predicted product, ACR, followed by an ATP binding motif of the type found in histidine kinases such as DhkA; however, the H motif where a histidine is autocatalytically

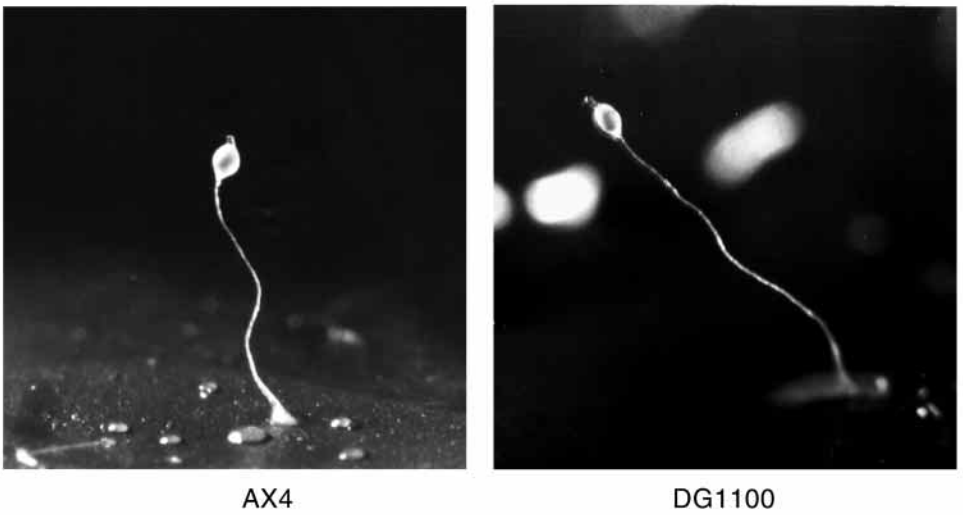

Fig. 1. Terminal Structures. Wild type cells of strain AX4 and *acrA*$^-$ mutant cells of strain DG1100 were developed on buffer saturated filters and representative fruiting bodies were photographed. Many of the fruiting bodies formed by cells of strain DG1100 toppled over before they could be photographed due to their abnormally thin stalks. DG1100 fruiting bodies have translucent, glassy sori.

phosphorylated in true histidine kinases is missing in ACR (Fig. 2B). While the N motif of histidine kinases is well conserved in ACR and indicates that these domains are derived from a common ancestoral gene, critical amino acids in the G1 and F boxes have drifted in ACR bringing in to question whether it is still an ATP binding protein. The D motif of the response regulator domain in which an aspartate accepts a phosphate in phosphorelay systems appears to have been well conserved as is the K motif found a little further along the ACR protein (Fig. 2C). The overall sequence similarity of this domain with other response regulators is consistent with it being phosphorylated by two component systems.

A 190 amino acid region in the carboxy-terminal half of ACR shows significant similarity to adenylyl cyclases (Fig. 2D). ACR is most closely related to the adenylyl cyclases of the photosynthetic bacteria *Spirulina platensis* and *Anabena spirulensis* but is also clearly related to the *Dictyostelium* adenylyl cyclase ACG that is expressed in germinating spores. The *cyaC* genes of both *Spirulina* and *Anabena* encode proteins that have similar structures to that of ACR. They all have an N-terminal domain related to hybrid histidine kinases as well as an adenylyl cyclase domain (Katayama and Ohmori, 1997; Kasahara et al., 1997). It appears that a gene encoding such a composite protein evolved before the separation of blue-

Fig. 2. Structure and predicted product of *acrA*. (A) A single intron interrupts the two exons that are indicated by boxes. Two hydrophobic domains follow the intron (filled boxes). The regions encoding the pseudo-histidine kinase domain, the response regulator domain and the adenylyl cyclase domain are indicated as shaded boxes. The site of plasmid insertion in strain DG1100 occurred between the kinase and response regulator domains and is indicated by a triangle. The ClustalW program was used to align the sequences of the three domains. (B) The kinase domain of ACR was aligned with similar regions in the *Dictyostelium* histidine kinase DhkA and the CyaC gene products of *Spirulina platensis* and *Anabena spirulensis*. There is no significant similarity in ACR to the conserved H motif surrounding the phosphohistidine of DhkA. The conserved N, G1, F and G2 domains of histidine kinase can be recognized in ACR but are less conserved than in other members of this family. (C) The D and K motif conserved in response regulator domains of DhkA and CycA are carried in ACR. (D) The ATP binding and catalytic domains of ACR were aligned with those of the germination adenylyl cyclase of *Dictyostelium* ACG as well as those in the CyaC products of the blue-green bacteria *Spirulina platensis* and *Anabena spirulensis*. Highest similarity was found to the CyaA adenylyl cyclase of *Anabena spirulensis* which does not have histidine kinase or response regulator domains. The 8 kb genomic sequence encompassing *acrA* has been deposited in GenBank (Accession no. AF153362).

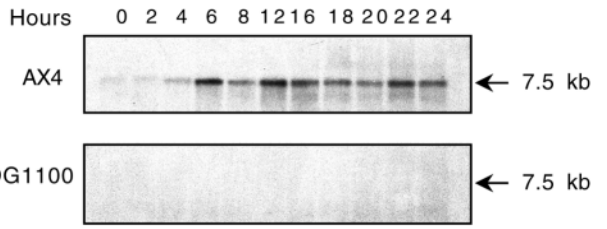

Fig. 3. Accumulation of *acrA* mRNA during development. The various times at which cells were collected after the initiation of development are indicated above the lanes (hours). RNA was prepared and electrophoretically separated on agarose gels before being blotted to nitrocellulose. Northern blots prepared from cells of stain AX4 (*acrA*$^+$) and DG1100 (*acrA*$^-$) were probed for the 7.5 kb *acrA* mRNA and exposed to film for the same period of time.

green bacteria and eukaryotes. Comparison of the aligned sequences of multiple adenylyl cyclases and guanylyl cyclases shows that the determinitive amino acids that distinguish between ATP and GTP as substrates predict that ACR is an adenylyl cyclase (Tucker et al., 1998).

Expression of acrA

Northern analyses of RNA taken at various stages of development using a probe from *acrA* have shown that a 7.5 kb mRNA is present at low levels in growing cells, accumulates to high levels after 4 hours of development, and then remains high throughout the remainder of development (Fig. 3A). Similar studies on RNA collected from cells of strain DG1100 (*acrA*$^-$) have shown that this mRNA is missing at all stages of development (Fig. 3B). Thus, insertion of the plasmid into *acrA*$^-$ appears to have generated a null mutation.

Adenylyl cyclase activity

The specific activity of adenylyl cyclase was determined in crude extracts of cells taken throughout development (Fig. 4). Assays were carried out in the presence of either magnesium or manganese. The activity measured in the presence of magnesium was very low for the first 4 hours of development in wild-type cells and then increased throughout the remainder of development. When adenylyl cyclase activity was measured in the presence of manganese no significant activity could be detected until 6 hours. It then increased to reach a peak at 10 hours. The activity during aggregation was higher when assayed in the presence of manganese than in the presence of magnesium as had been previously observed (Loomis et al., 1978); however, at later stages of development the activity in wild-type cells was higher when assayed in the presence of magnesium.

The late activity measured in the presence of magnesium was almost completely missing in the mutant *acrA*$^-$ cells (Fig. 4). Moreover, no activity could be detected in the mutant *acrA*$^-$ cells assayed in the presence of either magnesium or manganese until 6 hours of development. When measured in the presence of manganese, adenylyl cyclase activity increased to a peak at 10 hours of development in *acrA*$^-$ cells and declined slowly thereafter (Fig. 4). When measured in the presence of magnesium, adenylyl cyclase activity paralleled the manganese assayed activity but was lower at all stages of development. Thus, the late magnesium assayed activity seen in wild-type cells is dependent on the *acrA* gene. The low level of activity seen during the first few hours of development also appears to be dependent on *acrA* since it could not be detected in *acrA*$^-$ mutant cells and was preferentially active in wild-type extracts when assayed in the presence of magnesium.

We determined the characteristics of the adenylyl cyclase activity isolated from cells that had developed for 22 hours and were in the process of forming fruiting bodies. Unlike the aggregation stage adenylyl cyclase, ACA, and the germination cyclase, ACG, that are activated 2 to 3 fold by manganese (Loomis et al., 1978; Pitt et al., 1992), the late activity is preferentially active when assayed with magnesium (Table 1). Likewise, lysis in the presence of 100 μM GTPγS strongly stimulates the activity of ACA (Pitt et al., 1992), but has no effect on the late adenylyl cyclase activity (Table 1). Thus, besides having different patterns of expression, *acaA* and *acrA* are responsible for enzymes with significantly different characteristics.

The primary sequence of ACR includes two strongly hydrophobic domains near the N terminus suggesting that this

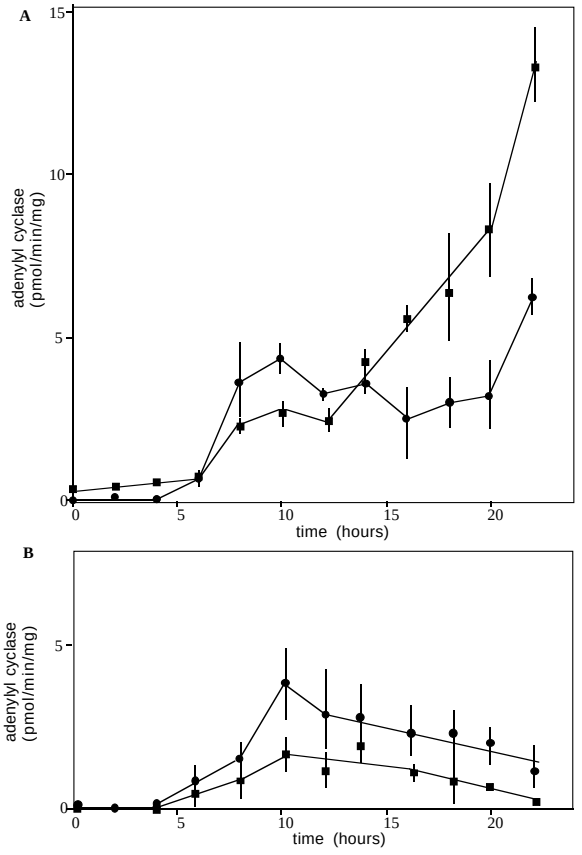

Fig. 4. Adenylyl cyclase activity throughout development. (A) Wild-type cells of strain AX4 and (B) *acrA*$^-$ mutant cells of strain DG1100 were developed on filters and collected at various times following the initiation of development. Extracts were assayed for adenylyl cyclase activity in the presence of magnesium (filled squares) or manganese (filled circles). The activity seen during the aggregation stage (T = 6-12 hours) is preferentially active in the presence of manganese as expected for the product of the aggregation gene, *acaA*. Average of duplicate samples in 3 or more separate experiments. Error bars are shown except where the error fell within the point.

Table 1. Characteristics of adenylyl cyclases

	Aggregation adenyly cyclase ACA	Culmination adenylyl cyclase* ACR
Ratio of activity in presence of manganese or magnesium (5 mM)	2.5‡,§	0.34
Ratio of activity in presence or absence of 100 μM GTPγS	22§	0.80
Specific activity in cytosol (pmol/min/mg)	1.8¶	2.65
Specific activity in pellet (pmol/min/mg)	188¶	15.05

*Average of two or more separate determinations.
‡Loomis et al., 1978.
§Pitt et al., 1992.
¶Khachatrian et al., 1987.

enzyme might be membrane associated. When cell lysates were prepared by passage through a nucleopore filter and centrifuged at 100,000 ***g***, the activity was preferentially recovered in the pellet (Table 1). Thus, ACR appears to be associated with the particulate fraction as has been shown for ACA (Khachatrian et al., 1987).

It appears that ACR is responsible for the low level of adenylyl cyclase activity seen during the first few hours of development (Kim et al., 1998) and that accumulation of ACA is responsible for the major increase in activity seen between 6 and 12 hours of development as the cells aggregate. Thereafter, accumulation of ACR accounts for an increasing proportion of the total activity such that by culmination it is responsible for most of the measured activity.

Expression of developmental genes

To further define the role of ACR we determined the developmental kinetics and levels of several mRNAs that are expressed at different stages of development. Northern blots were prepared from RNA extracted at various times from synchronously developing wild-type and *acrA*$^-$ mutant cells and probes made from the *csaA*, *ecmA*, *cotB* and *spiA* genes. *csaA* is expressed during aggregation and encodes the cell adhesion molecule, gp80 (Noegel et al., 1986; Wong and Siu, 1986). *ecmA* is expressed in prestalk cells starting at the mound cells and is not expressed in prespore cells; it encodes a large extracellular matrix protein, ST430 (Ceccarelli et al., 1987; Williams et al., 1987; McRobbie et al., 1988). *cotB* is expressed in prespore cells starting at the mound cells and is not expressed in prestalk cells; it encodes the spore coat protein SP70 (Fosnaugh and Loomis, 1989, 1993). *spiA* is expressed in prespore cells as they encapsulate and encodes an inner spore coat protein (Richardson and Loomis, 1992; Richardson et al., 1994). *csaA*, *ecmA* and *cotB* were all expressed in *acrA*$^-$ cells at the expected stages; however, *spiA* was not expressed in the mutant cells (Fig. 5). It appears that differentiation through the early stages of development occurs normally in *acrA*$^-$ cells but that spore differentiation is incomplete in the absence of the late adenylyl cyclase.

Spores in suspension

Up to 25% of the *acrA*$^-$ cells isolated during culmination appeared to be encapsulating into phase bright ellipsoid spores but less than 1% of the total cells showed the detergent resistance of wild-type spores (data not shown). When cells were collected from sori at 22 hours of development and incubated in buffer, only 0.5% were viable after detergent treatment. After being suspended in buffer the number of detergent resistant viable spores increased about 3 fold during the first hour but precipitously fell during the next hour until less than 10^{-5} of the initial number of cells were still detergent resistant (Fig. 6). Wild-type spores remain completely detergent resistant over this time period (data not shown). It appears that mutant prespore cells embark on terminal differentiation but fail to form fully differentiated spores. The few spores that are made rapidly loose viability. While the lack

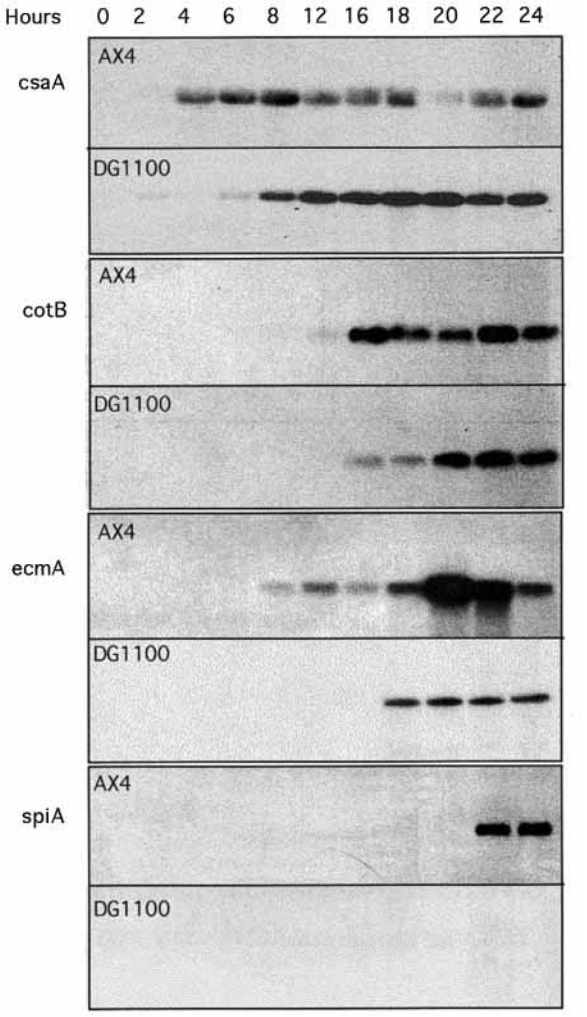

Fig. 5. Expression of developmental genes. Northern blots prepared throughout development of strains AX4 and DG1100 are marked. They were sequentially probed and analyzed for mRNA encoded by *csaA*, *cotB*, *ecmA* and *spiA*. Blots from the two strains were exposed for the same amount of time in each case. Longer exposure of the DG1100 blot probed with *spiA* failed to show any signal.

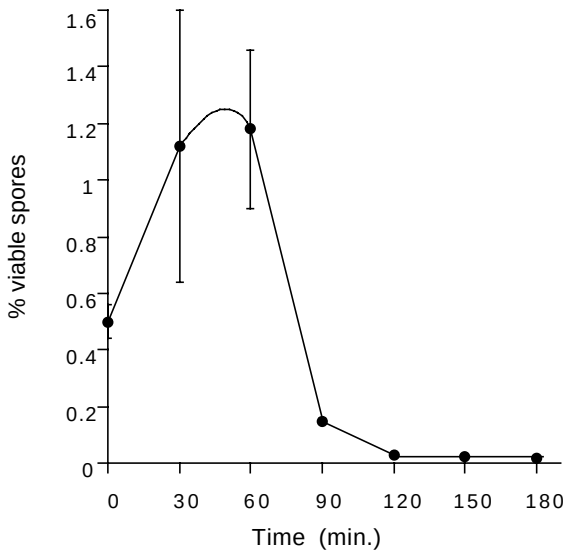

Fig. 6. Unstable spores. Cells of strain DG1100 were collected from fruiting bodies formed after 22 hours of development, dissociated and incubated in multitest wells in buffer. At various times the contents of the wells were collected, treated with 0.5% Triton X-100, washed and spread on plates together with bacteria. The proportion of the initial cells that remained viable was calculated from the number of plaques observed after 4 days. Wild-type spores are completely stable over this time period. Average of 3 separate experiments. Error bars are shown where the error exceeded the data point.

of *spiA* expression is one indication of incomplete spore differentiation, it cannot account for the drastic reduction in spore formation since *spiA*$^-$ cells form viable spores under these conditions (Richardson and Loomis, 1992).

ACR activity in histidine kinase mutant strains

While the region in the N-terminal half of ACR that is homologous to histidine kinases appears degenerate and lacks the essential histidine, the following region that is homologous to response regulators could function in a two-component system to regulate the enzyme activity. Three histidine kinases have been described in *Dictyostelium* that function during development, DhkA, DhkB and DhkC (Wang et al., 1996; Zinda and Singleton, 1998; Singleton et al., 1998). We assayed ACR activity during culmination of mutants lacking one or more of these histidine kinase (Table 2). The levels of activity measured in the presence of magnesium were found to be comparable to those in wild-type cells in *dhkA*$^-$, *dhkA*$^-$ *dhkB*$^-$ and *dhkC*$^-$ cells. In all cases the activity was higher when assayed in the presence of magnesium than when assayed in the presence of manganese, indicating that ACA was responsible for only a minor portion of the adenylyl cyclase activity at this stage of development (Table 2). The results indicate that phosphorelay from these histidine kinases is not essential to activate ACR; however, there is evidence from analyses of the Japanese EST database (Morio et al., 1999) that other histidine kinases are expressed during development. Further studies will be required to see if phosphorylation of the aspartate in the putative response regulatory region affects ACR activity.

Table 2. Adenylyl cyclase in mutant strains

Strain	(genotype)	Hours	Specific activity (pmol/min/mg)* Mg^{2+}	Mn^{2+}
AX4	(*dhkA*$^+$ *dhkB*$^+$ *dhkC*$^+$)	20	8.1	2.9
		22	12.5	3.5
AK299	(*dhkA*$^-$ *dhkB*$^+$ *dhkC*$^+$)	20	9.8	3.8
		22	19.1	6.7
TL134	(*dhkA*$^-$ *dhkB*$^-$ *dhkC*$^+$)	20	11.4	2.5
		22	9.5	2.6
TL135	(*dhkA*$^+$ *dhkB*$^+$ *dhkC*$^-$)	20	9.6	4.8
		22	12.3	3.7

*Assays were carried out in the presence of 10 mM $MgCl_2$ or 10 mM $MnCl_2$. Average of two separate determinations.

DISCUSSION

For some time it has been known that cAMP or its analog, 2′deoxy cAMP which activates the surface receptor, CAR1, but binds weakly to PKA, can induce both prespore and prestalk-specific genes in wild-type cells as well as cells lacking the receptor coupled adenylyl cyclase ACA (Pitt et al., 1993). However, there is good evidence that expression of these post-aggregative genes is dependent on activation of PKA and so should require a source of intracellular cAMP (Simon et al., 1989; Mann et al., 1992, 1997; Harwood et al., 1992a; Hopper et al., 1993a, 1995; Hopper and Williams, 1994). Moreover, *acaA*$^-$ null mutants are able to form spores when developed in chimeric mixtures with wild-type cells (Pitt et al., 1993). Yet, PKA acts in a cell-autonomous manner essential for several aspects of fruiting body formation (Harwood et al., 1993b; Hopper et al., 1993b; Parent and Devreotes, 1996; Firtel, 1996; Loomis, 1998). Up until now it has not been clear how the intracellular cAMP needed to activate PKA during development is synthesized in cells lacking ACA since the germination adenylyl cyclase ACG is not expressed until after fruiting bodies are formed (Pitt et al., 1992). It now appears that ACR may account for the synthesis of intracellular cAMP in *acaA*$^-$ cells since we have found that *acrA* is expressed throughout development and encodes an adenylyl cyclase. Cells that lack ACR due to disruption of the gene are able to aggregate and form normal slugs but cannot complete sporulation and have abnormally long, thin stalks. Thus, in cells that express wild-type *acaA*, ACR is only essential during terminal differentiation.

The adenylyl cyclase catalytic domain of ACR is most similar to that in adenylyl cyclases of the blue-green bacteria, *Spirulina platensis* and *Anabena spirulensis*, but also shows significant similarity to the germination cyclase of *Dictyostelium*, ACG (Fig. 2). Moreover, these cyclases all appear to be homologous to the cytosolic adenylyl cyclase found in mammals (Buck et al., 1999). Like the testis enriched soluble adenylyl cyclase cyclase and ACG, ACR is not stimulated by GTPγS or forskolin (Table 1; and data not shown).

The human adenylyl cyclases carry two active sites, C1 and C2, as does the aggregation cyclase of *Dictyostelium*, ACA (Pitt et al., 1992; Tucker et al., 1998; Buck et al., 1999). In contrast ACR and the cyanobacterial cyclases each carry only a single active site. It has been suggested that such enzymes must

dimerize, either as homodimers or heterodimers (Tucker et al., 1998). It is not yet clear whether the product of *acrA* acts in conjunction with another gene product or is active as a homodimer. Ectopic expression of *acgA* during the early stages of development is able to compensate for the loss of ACA suggesting that ACG functions as a homodimer (Pitt et al., 1992).

Although the response regulator region of ACR indicates that it might accept a phosphate on the conserved aspartate by phosphorelay from a histidine kinase, we found normal levels of adenylyl cyclase activity during culmination of *dhkA*$^-$, *dhkA*$^-$ *dhkB*$^-$ and *dhkC*$^-$ cells. Thus, the histidine kinases encoded by these genes do not appear to play any significant role in regulating ACR. However, the signal transduction pathway from both DhkA and DhkB appears to lead to activation of PKA by increasing the internal cAMP concentrations (Wang et al., 1996, 1999; Zinda and Singleton, 1998). The consequences of null mutations in *dhkA* and *dhkB* can be overcome by adding the cell permeant analog 8-Br-cAMP or genetically inactivating the cytoplasmic phosphodiesterase, RegA (Wang et al., 1996, 1999; Zinda and Singleton, 1998). The phenotypes of *acrA*$^-$ strains may resemble those seen in *dhkA*$^-$ and *dhkB*$^-$ strains because the rate of cAMP synthesis during culmination is insufficient to fully activate PKA even when RegA is inhibited.

The characteristics of the *acrA* dependent activity measured during culmination not only clearly distinguish ACR from the aggregation stage adenylyl cyclase, ACA, but also indicate that the low level of adenylyl cyclase previously seen in vegetative cells of rapid developing strains (Kim et al., 1998) is likely to depend on *acrA*. Consistent with this relationship, we found that the basal adenylyl cyclase activity that we could measure in vegetative cells of wild-type strains was reduced to background levels in *acrA*$^-$ cells. While significant activity is present in growing cells and during the first few hours of development before *acaA* is expressed, this activity does not appear to be essential for either growth or aggregation since neither of these are impaired in *acrA*$^-$ strains. It is only during culmination that we see the dramatic consequences of loss of ACR.

The role of cAMP during development of *Dictyostelium* has been recognized since the pioneering work of Bonner on chemotaxis (Bonner, 1947, 1970; Konijn et al., 1968; Bonner et al., 1969). When the gene encoding the major aggregation stage adenylyl cyclase, *acaA*, was isolated and shown to be essential for chemotactic signaling, it was assumed that it was also responsible for synthesis of cAMP at later stages (Pitt et al., 1992). ACA was found to be activated by the G protein coupled serpentine receptor when it bound extracellular cAMP (Pupillo et al., 1992; Lilly et al., 1993; Parent and Devreotes, 1996). The vegetative adenylyl cyclase activity observed by Kim et al. (1998) in rapid developing strains is not activated by addition of GTPγS and can be distinguished from ACA by its preference for magnesium over manganese (Kim et al., 1998). We find that this activity is missing in *acrA*$^-$ mutants but that there are no significant consequences during early development. Expression of *acrA* increases dramatically at 6 hours of development and remains high throughout the remainder of development. The adenylyl cyclase activity measured at the mound stage seems to be the sum of the ACA and ACR activities. Thereafter, ACA declines to about a third of its peak level while ACR accumulates at least 5 fold and appears to account for much of the cAMP synthesized during culmination. These studies point out that the internal levels of cAMP in this system are determined by a complex balance in the rate of synthesis from multiple adenylyl cyclases and the rate of degradation from one or more phosphodiesterases including RegA. Since cAMP determines the level of PKA activity that is crucial to multiple developmental processes (Firtel, 1996; Loomis, 1998), it would not be surprising to uncover other components that modulate cAMP at the various stages.

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