Developmental regulation and evolution of cAMP signalling in Dictyostelium

Alvarez-Curto, E.

Chapter One

Adenylyl cyclase G triggers prespore differentiation in *Dictyostelium* slugs

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cAMP produced by adenylyl cylase G triggers prespore differentiation in *Dictyostelium* slugs
Elisa Alvarez-Curto, Shweta Saran, Marcel Meima, Jenny Zoebel, Claire Scott and Pauline Schaap

Abstract

Encystation and sporulation are crucial developmental transitions for solitary and social amoebas, respectively. While little is known of encystation, sporulation requires both extra- and intracellular cAMP. After aggregation, extracellular cAMP binding to surface receptors and intracellular cAMP binding to cAMP dependent protein kinase (PKA), act together to induce prespore differentiation. Later, a second episode of PKA activation triggers spore maturation. Adenylyl cyclase B (ACB) produces cAMP for maturation, but the cAMP source for prespore induction is unknown. I show in this chapter that adenylyl cyclase G (ACG) protein is upregulated in prespore tissue after aggregation. *acg* null mutants show reduced prespore differentiation, which becomes very severe when ACB is also deleted. ACB is normally expressed in prestalk cells, but is upregulated in the prespore region of *acg null* structures. These data show that ACG induces prespore differentiation in wild-type cells, with ACB capable of partially taking over this function in its absence.

Introduction

Encystation and sporulation are common life cycle transitions that allow protists, fungi and lower plants to survive nutrient depletion and other forms of stress. Little is known about the signalling pathways that control encystation, which in the case of pathogenic protists is of significant medical importance. For instance for *Entamoeba histolytica*, which causes the second most lethal parasite borne disease, amebiasis, the cyst is the infective stage of the disease (Stanley and Samuel, 2003). Infections with *Acanthamoeba castellani*, that causes keratitis and amoebic encephalitis, are difficult to treat because the amoebas differentiate into highly resistant cysts inside host tissues (Lloyd et al., 2001; Marciano-Cabral and Cabral, 2003; McClellan et al., 2002). Mainly due to lack of genetic tools to investigate this process, little is known of the signalling pathways that control encystation.

Social amoebas respond to nutrient stress by either encysting individually or by aggregating to form fruiting structures, where most of the cells differentiate into spores. A small proportion of cells altruistically build a stalk to support the spore mass and to aid in their dispersal. Particularly the species *D.discoideum* has excellent genetic tractability, and the pathways that control sporulation have been extensively studied. Here, sporulation involves a first phase, prespore differentiation that occurs shortly after aggregation. In this stage the cells synthesize spore-coat components in prespore vesicles, but remain otherwise amoeboid. Prespore differentiation is triggered by extracellular cAMP acting on cAMP receptors (cARs), and intracellular cAMP acting on PKA (Schaap and Van Driel, 1985; Hopper et al., 1993). The second phase, spore maturation, occurs after the stalk is formed and this process is triggered solely by a high level of PKA activity (Mann et al., 1994). Spore maturation involves relatively minor changes in gene expression, but is accompanied by major physiological changes: prespore vesicles fuse with the plasma membrane, laying down the first layers of the spore coat and releasing precursors for synthesis of the outer layers (West and Erdos, 1990).

PKA activation during spore maturation requires the activity of the adenylyl cyclase ACB, encoded by *AcrA*, which is maximally expressed during culmination and fruiting body stages (Kim et al., 1998; Meima and Schaap, 1999; Soderbom et al., 1999). In addition, the process requires inactivation of the intracellular cAMP phosphodiesterase, RegA. This unusual enzyme harbours a response regulator domain, which is the target of a phosphorelay system that is regulated by sensor histidine kinases/phosphatases (Shaulsky et al., 1996; Shaulsky et al., 1998; Thomason et al., 1998; Thomason et al., 1999). A peptide released by stalk cells, SDF-2, activates the sensor histidine phosphatase *DhkA*, causing dephosphorylation and hence inactivation of RegA. This in turn causes cAMP accumulation and the activation of PKA (Anjard and Loomis, 2005; Wang et al., 1999). PKA remains important in the spore stage, where it controls spore dormancy. The ambient high osmolality in the spore head keeps the spores dormant, and this effect is mediated by the adenylyl cyclase ACG, which harbours an intramolecular osmosensor (Saran and Schaap, 2004; Van Es et al., 1996; Virdy et al., 1999).

The requirements of ACB and ACG for PKA activation in spore maturation and dormancy

are well documented. However, it is not clear which enzyme produces the extracellular cAMP that triggers prespore differentiation. The third *Dictyostelium* adenylyl cyclase, ACA, is mainly active during aggregation and disappears from the prespore region once slugs start to form (Pitt et al., 1992; Verkerke-van Wijk et al., 2001). Null mutants in ACB/*AcrA* show normal prespore gene expression (Soderbom et al., 1999) and ACG mRNA was only detectable in spores (Pitt et al., 1992). However, biochemical analysis of adenylyl cyclase activities in *aca-* slugs demonstrated the presence of an adenylyl cyclase activity, which similar to ACG preferred Mn^{2+}-ATP over Mg^{2+}-ATP as a substrate. Since the reverse is true for ACB, this suggested that ACG could be expressed in slugs (Meima and Schaap, 1999).

In this work I analyse the pattern of *ACG* transcription and translation more closely by studies with ACG promoter-reporter gene fusions and an ACG specific antibody. Our data indicate that *ACG* is transcribed at low levels throughout development, while ACG protein is markedly upregulated after aggregation in the prespore regions of slugs. Analysis of single and double null mutants in ACG and ACB indicates that ACG is essential for prespore differentiation, but that its function is partially redundant with ACB. This work complements parallel studies where we show that ACG is deeply conserved in amoebazoan evolution and regulates encystation and excystation in analogy to its roles in spore formation and germination.

Materials and methods

Cell culture and development

D.discoideum cells were grown in standard axenic medium, which was supplemented with antibiotics as indicated. To induce multicellular development cells were harvested from exponentially growing cultures, washed twice in PB (10 mM Na/K-phosphate buffer pH 6.5) and incubated at 22°C on PB agar (1.5% agar in PB).
To induce competence for prespore gene induction, cells were starved on PB agar for 16 hours at 6°C and 2 hours at 22°C until aggregation territories had formed. Cells were then resuspended to 2 x 10^6 cells/ml in PB and shaken at 150 rpm and 22°C in the presence and absence of cAMP.

Gene constructs and transformation

Fusion constructs of the ACG promoter were made with the *LacZ* (gal) reporter gene and with a modified LacZ, called ile-gal. In ile-gal, *LacZ* is modified by N-terminal addition of the ubiquitin gene and replacement of the *LacZ* start codon with an isoleucine codon. The ubiquitin moiety is cleaved off during translation, leaving β-galactosidase with an exposed isoleucin, which decreases protein stability to a half-life of 30 minutes (Detterbeck et al., 1994). For both constructs, 2855 bp of *ACG* DNA sequence, comprising 2810 bp of the complete 5' intergenic region and 45 bp of coding sequence, were amplified from vector pGACG (Pitt et al., 1992) using primers ACGpr5' and ACGpr3' (Table 1), which harbour XbaI and BglII sites respectively. After digestion with XbaI and BglII, the amplified product was cloned into XbaI/BglII digested pDdGal-17 (Harwood and Drury, 1990) to create ACG::gal, and used to replace the XbaI/BglII *psA* promoter fragment from vector *PsA*-ile-gal (Detterbeck et al., 1994) to generate ACG::ile-gal. The vectors were introduced into AX3 cells and *acrA-* mutants by electroporation and transformants were selected for growth at 100 µg/ml G418 (Sigma) for ACG::gal and at 200 µg/ml for the ACG::ile-gal constructs.

Gene fusions of the *AcrA* promoter with labile ile-gal and stable ala-gal (Detterbeck et al., 1994) were made by amplification of the 819 bp *AcrA* 5'intergenic region from AX2 genomic DNA with primers AcrApr5' and AcrApr3', containing XbaI and BglII restriction sites (Table 1). The amplifed product was inserted into both the ile-gal and ala-gal vector as described above to create *AcrA*-ile-gal and *AcrA*-ala-gal. Both vectors were introduced into AX2 and *acg-* cells. Transformants were selected for growth at 100 µg/ml G418.

To prepare an *ACG* gene disruption construct, two DNA fragments of the *acgA* gene comprising nucleotides 29-922 and 1761-2184 were amplified by PCR from vector pGACG

(Pitt et al., 1992), using oligonucleotides AcgKO1-4 (Table 1) that add a 5'-BamHI and 3'-KpnI site to the first fragment and a 5'-XbaI and 3'-BamHI site to the second fragment. These fragments were cloned sequentially into BamHI/KpnI digested and XbaI/BamHI digested pBsrΔBam (Sutoh, 1993). The construct was linearized with BamHI, which yielded the pBsrΔBam plasmid flanked by 894 bp and 423 bp of 5' and 3' *AcgA* sequence respectively, and introduced into wild-type AX2 cells. Transformed cells were selected for growth at 5 µg/ml blasticidin and selected clones were screened for homologous recombination by two separate PCR reactions and analysis of Southern blots of genomic digests.

Histochemical and spectrophotometric β-galactosidase assays

For visualization of β-galactosidase activity in developing structures, cells were distributed at 10^7 cells/cm^2 on nitrocellulose filters supported by PB agar and incubated at 22°C. Structures were fixed in 0.25% glutaraldehyde, containing 2% Tween-20 and stained with X-gal as described previously (Dingermann et al., 1989).

For spectrophotometric measurement of β-galactosidase activity, cells were lysed by three rounds of freeze-thawing. 100 µl aliquots of lysate were incubated at 22°C in microtiterplate wells with 30 µl of 2.5 x Z-buffer and 20 µl of 40 mM chlorophenolred-β-D-galactopyranoside (Schaap et al., 1993). The OD_{574} was measured at regular time intervals using a microtiter plate reader. β-galactosidase activity in ΔOD_{574}/minute was calculated from the time intervals where reaction product accumulated linearly and was standardized on the protein content of the samples. The activity observed in untransformed cells was subtracted as the assay blanc.

Immunological techniques

For immuno-blotting, samples of 2 x 10^7 cells were pelleted and boiled in 50 µl SDS sample buffer. 50 µg samples of total protein were size-fractionated on 8% SDS-PAA gels and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with a 1:2000 dilution of an αACG peptide antibody (Saran and Schaap, 2004), washed and incubated with 1:2000 diluted horse radish peroxidase-coupled goat-anti-rabbit antibody (Promega, USA). Detection was performed with the Supersignal chemoluminescence kit (Pierce, USA) according to the manufacturer's instructions.

For immuno-cytochemistry, slugs were harvested in 20 mM EDTA in PB and dissociated into single cells by passing through a 23 gauge needle. Cells were placed as 10 µl aliquots of 10^7 cells/ml on 8-well multitest slides, overlayed with agarose (Fukui et al., 1986) and fixed for 10 minutes in ice-cold methanol. Slides were incubated overnight with 1:500 diluted αACG antibody, and with 1:200 diluted FITC-conjugated goat-anti-rabbit IgG (GARFITC) for 1 hr. Subsequently cells were incubated for 1 hr with a 1:500 diluted mouse monoclonal antibody 83.5 (Zhang et al., 1999) and for 1 hr with 1:500 diluted Texas Red-conjugated goat anti-mouse IgG. Spores were harvested from fruiting bodies and stained with αACG antibody and GARFITC.

For whole mount immuno-staining, intact structures were gently floated from an inverted slice of supporting agar to 10 µl PB deposited in the wells of polylysine coated 8-well multitest slides. The fluid was aspirated and the structures were fixed in methanol and incubated with αACG antibody and GARFITC as described above. Preparations were photographed using a Leica TCS SP2 confocal laser-scanning microscope.

To measure the proportion of prespore cells, fully migrating slugs were dissociated into single cells by repeated aspiration in 1% (w/w) cellulase in 2 mM EDTA, pH 6.5. Cells were then fixed in methanol and incubated for 16 hours at 4°C with 1:50 diluted spore antiserum (Takeuchi, 1963) and for 1 hour with 1:200 diluted GARFITC. The samples were counter-stained with 1 µg/ml of 4,6-diaminidino-2-phenylindole (DAPI). Cells were photographed using a Leica DM LB2 fluorescence microscope and total cells (DAPI-stained) and prespore cells (cells with 3 FITC-stained vesicles) were counted.

RNA isolation and analysis

All strains were developed on PB agar until fruiting bodies had formed. Total RNA was extracted from 2 x 10^7 cells at 2 hour intervals, size-fractionated on 1.5% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Nellen *et al.,* 1987). Cells in the culmination stages were vortexed for 5 min with glass beads during RNA extraction. Northern blots were hybridised to a [^{32}P]dATP-labeled *CotB* probe at 65^{o}C, then stripped and re-probed with the constitutively expressed gene 1G7 (Williams et al., 1987).

For induction of prespore gene expression aggregation competent wild-type*, acrA*-, *acg*- and *acrA-/A15::ACGΔcat* cells were shaken for 8 hours in the presence and absence of 300 µM cAMP, added every hour. Total RNA was isolated at 2 hour intervals and all RNA samples were size-fractionated on a single gel and transferred to a single membrane, which was successively probed with [^{32}P]dATP-labeled *CotB*, *PsA* and *1G7* DNA probes.

RNA detection by *in situ* hybridization and RT- PCR

In situ hybridization. Cells were incubated at 10^6 cells/cm^2 on dialysis membrane, supported by PB agar, until the desired developmental stages had been reached. *In situ* hybridization with 200 ng/ml of digoxigenin (DIG) labelled *AcrA* RNA was carried out as previously described (Escalante and Loomis, 1995). An antisense *AcrA* probe was used as a control. To prepare the probes, a 520 bp *AcrA* fragment was amplified from genomic DNA using primers AcrAcat5' and AcrAcat3' (Table 1) and cloned into EcoRI/BamHI digested pBluescript KS+. The *AcrA* fragment was subsequently amplified by PCR using the universal M13-20 and “Reverse” primers. The purified PCR product served as template for synthesis of sense and antisense DIG-labeled *AcrA* RNA probes using the SP6 and T7 RNA polymerases and reagents from a DIG RNA labelling kit (Roche, UK).

RT- PCR. For semi-quantitative detection of ACG mRNA during development, RNA was extracted using an RNAeasy minikit (Qiagen, Crawley, UK) at 2 hours intervals from cells developing on PB agar. RT-PCR reactions were performed on 400 ng total RNA using primers ACGRT5' and ACGRT3' (Table 1) and a One-step RT-PCR kit (Qiagen, Crawley, UK).

Table 1. Oligonucleotides used in this work.

ACGpr5'	5'-CACTCTAGAGGCGGCGATGTCACCAAAG
ACGpr3'	5'-TGGAGATCTTTCAACATATGATTTAGATAG
AcrApr5'	5'-GCTCTAGATGATCTTGAATTTTGTTGATTTTCC
AcrBpr3'	5'-GGGAGATCTATCTAATTTTGAACAATTATTAC
AcgKO1	5'-CGGGATCCCTAAATCATATGTTGAAGGATATCC
AcgKO2	5'-AGGTACCCCCACTTGATATATGACTCATATC
AcgKO3	5'-CGCCTCTAGAATGGAGTCTACGGG
AcgKO4	5'-CGGGATCCGGTGGTGGTGGAGAATTATCAT
AcrAcat5'	5'-CCTAGAATTCAACCACTGAGAAAATGTTGG
AcrAcat3'	5'- TTACGGATCCCGTTCACCATCGAT

Results

ACG transcription during *Dictyostelium* development

Low levels of ACG mRNA were previously only detected in spores (Pitt et al., 1992). This does not preclude expression at an earlier stage, because 2-3 fold lower levels would go undetected in Northern blots. To obtain more information on the spatio-temporal pattern of ACG transcription, we fused 2.8 kb of 5' flanking sequence of the ACG gene to the β-galactosidase (gal) reporter gene. The parent strain AX3 was transformed with the *ACG::gal* construct and developing structures were stained with X-gal for β-galactosidase activity. Surprisingly, β-galactosidase activity was already present in aggregating cells and newly formed slugs, although activity was most pronounced in the spore head of fruiting bodies (Fig. 1A-C).

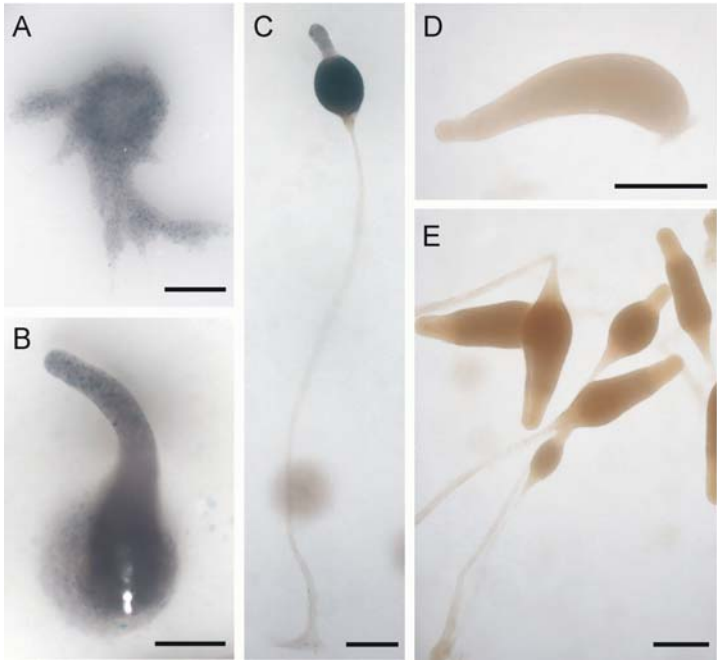

Figure 1. ACG promoter activity in developing structures
(A-C) *D. discoideum* wild-type cells were transformed with vector ACG::gal, which contains a gene fusion of the ACG promoter and the β-galactosidase reporter gene. Cells were starved on nitrocellulose filters supported by PB agar and developing structures were fixed and stained with X-gal. A) aggregate, B) standing slug C) mature fruiting body. (D, E) *D. discoideum* wild-type cells were transformed with vector ACG::ile-gal, where ile-gal encodes a labile form of β-galactosidase with a 30 minute half-life. Slugs (D) and mid-culminants (E) were stained with X-gal. Bar length is 100 µm.

Because the β-galactosidase protein is stable, it will progressively accumulate in cells, even if gene transcription is low. To investigate whether this caused the discrepancy between the *ACG::gal* and earlier mRNA data, we made a second gene fusion of the ACG promoter with ile-gal that encodes a labile β-galactosidase protein (Detterbeck et al., 1994). In cells transformed with this construct, β-galactosidase activity was barely detectable in slugs (Fig. 1D), but did become visible in the prespore region of mid-culminants (Fig. 1E). This indicates that ACG promoter activity must be low during early development and only increases significantly at the onset of fruiting body formation.

ACG protein levels during development

Next, I measured the developmental regulation of ACG protein expression by immunoblotting using an ACG peptide antibody that was raised and tested for specificity previously (Saran and Schaap, 2004). Figure 2A shows that in wild-type cells, ACG protein levels rapidly increased during tip formation to reach a plateau in migrating slugs. Fruiting body formation was accompanied by a further modest increase in ACG protein levels. This expression pattern is more consistent with the expression of prespore genes than that of spore genes. To test this further we measured ACG expression in an ACB/*acrA* null mutant (Kim et al., 1998; Soderbom et al., 1999). ACB is essential for the expression spore genes, but not for the differentiation of prespore cells. However, also in the *acrA*- mutant, ACG protein accumulated rapidly during tip formation, with only a minor increase in mature fruiting bodies (Fig. 2B).

The profiles of ACG protein accumulation measured here, and *ACG* mRNA measured earlier (Pitt et al., 1992) are quite different, with mRNA only being detected in the spore stage. We used the more sensitive reporter gene assay to determine the developmental profile of *ACG* promoter activity in wild-type and *acrA*- cells, both transformed with the ACG::ile-gal constructs. Figure 2C shows that consistent with the earlier data, *ACG* promoter activity shows a dramatic increase during fruiting body formation. However, there is low but detectable activity during the entire course of development. This explains why stable β-galactosidase protein could accumulate in early development (Fig. 1A, B). Both *ACG* promoter activity and ACG protein synthesis were normal in *acrA*$^-$ cells. This indicates that unlike other spore genes (Soderbom et al., 1999), the expression of *ACG* is not dependent on ACB activity.

To assess whether *ACG* mRNA is synthesized throughout development, we used RT-PCR to amplify an *ACG* cDNA fragment that spans the two (spliced out) introns of *ACG* from RNA isolated during development. Figure 2D shows that the RNA derived product with a predicted size of 333 bp was amplified from all developmental stages, but most strongly from fruiting bodies. A 532 bp band that is expected for a genomic DNA derived product was also amplified. These data confirm that *ACG* is transcribed throughout development.

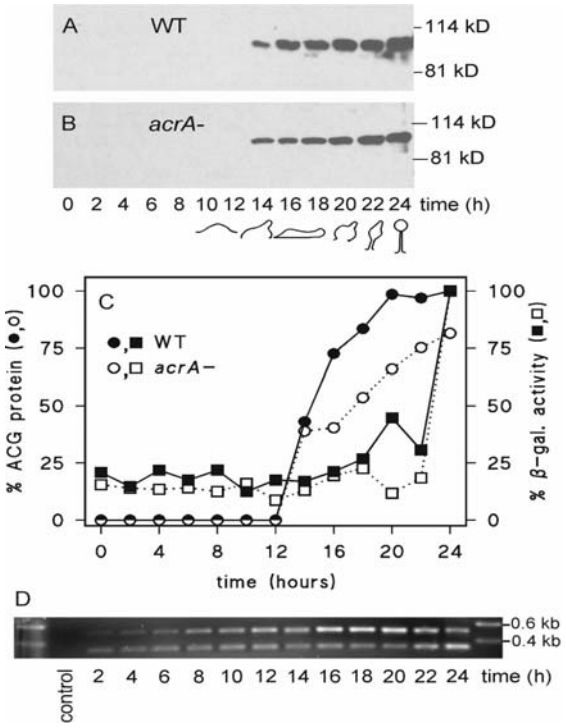

Figure 2. Developmental regulation of ACG transcription and ACG protein accumulation
(A,B). *Dictyostelium* wild-type (WT) cells (A), the *acrA* mutant (B) and both cell lines transformed with the ACG::ile-gal construct were incubated for 24 hours on PB agar. Every 2 hours, WT and *acrA*- cells were harvested, lysed in SDS-PAGE sample buffer and immunoblotted with αACG antibody. The αACG antibody reacts to a single band around 98 kD, the predicted size of ACG. C. The ACG-ile-gal transformed cells were lysed and assayed for β-galactosidase activity using a spectrophotometric assay (■, □). The data are expressed as percentage of β-galactosidase activity measured at 24 hours in wild-type cells. The means of two experiments assayed in triplicate are presented. This panel also shows data obtained from a densitometric scan of the ACG bands in the immunoblots in panels A, B (●, ○). The optical density values of the scan are expressed as percentage of the value obtained for WT at 24 hours. D. Total RNA was extracted from developing WT cells at 2-hour intervals and subjected to RT-PCR for 25, 30 and 35 cycles using primers that yield a product that spans the two introns in the ACG gene. Product was first detectable after 30 cycles (shown here) from both cDNA (lower band) and contaminating gDNA (upper band) amplification. The control reaction lacked RNA.

Localization of ACG in cells and tissues

To gain insight in the role of ACG in slugs, we first visualized the pattern of ACG protein expression. Figure 3A shows that in newly formed slugs ACG protein was exclusively localized at the posterior prespore region. In mid-culminants, ACG protein was highly expressed throughout the prespore region, while being absent from the stalk, prestalk and lower cup regions (Fig. 3B). In spores, ACG was localized at the cell periphery as would be expected for a transmembrane osmosensor (Fig. 3C). However, in slugs αACG staining was distributed in a punctuated fashion over the cells, reminiscent of the distribution of prespore vesicles. To test this we double stained slug cells with αACG antibody (Fig. 3D) and with an αSP85 antibody (Fig. 3E) (Zhang et al., 1999). SP85 is a spore coat protein that is associated with prespore vesicles (Zhang et al., 1998). The superimposed image (Fig. 3F) shows that ACG and SP85 are colocalized in the same cellular compartments, which are most likely the prespore vesicles.

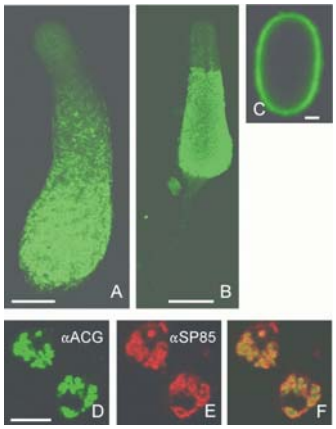

Figure 3. ACG protein in intact structures and cells
(A-C) Intact wild-type slugs (A), fruiting bodies (B), spores (C) werefixed in methanol and stained with ACG antibody and FITC conjugated goat-anti-rabbit IgG (GARFITC). (D-F). Slugs were dissociated into single cells, which were first stained with αACG antibody and GARFITC, and subsequently with mouse monoclonal antibody mAb83.5 that was raised against the spore coat protein SP85 (Zhang et al., 1999) and Texas Red-conjugated goat-anti-mouse IgG. Intact structures and cells were photographed using a Leica TCS SP2 confocal laser-scanning microscope, using 596 nm excitation and 620 nm emission for Texas Red, and 495 nm excitation and 520 nm emission for FITC. In panel F, images D and E are superimposed to show colocalization of ACG and SP85.
Bar lengths in A,B: 100 µm, C: 1 µm and D: 10 µm.

The role of ACG in *Dictyostelium* slugs

The localization of ACG in the posterior prespore region of the slug suggests that ACG could be required to produce extracellular cAMP that is essential for induction of prespore differentiation (Schaap and Van Driel, 1985; Wang et al., 1988) and/or intracellular cAMP for PKA activation, that is required for expression of a subset of prespore genes (Hopper et al., 1993). Null mutants in ACG were originally described to form fruiting bodies normally, but structures were not studied in great detail (Pitt et al., 1992). We compared prespore and spore differentiation in null mutants for ACG, ACB/AcrA and in a mutant that has neither activity. This mutant was made by expressing ACGΔcat, a dominant negative inhibitor of ACG (Saran and Schaap, 2004) in *acrA*- cells under the constitutive actin15 promoter. A new *acg*- mutant was created because fruiting body formation in the original mutant had deteriorated over time.

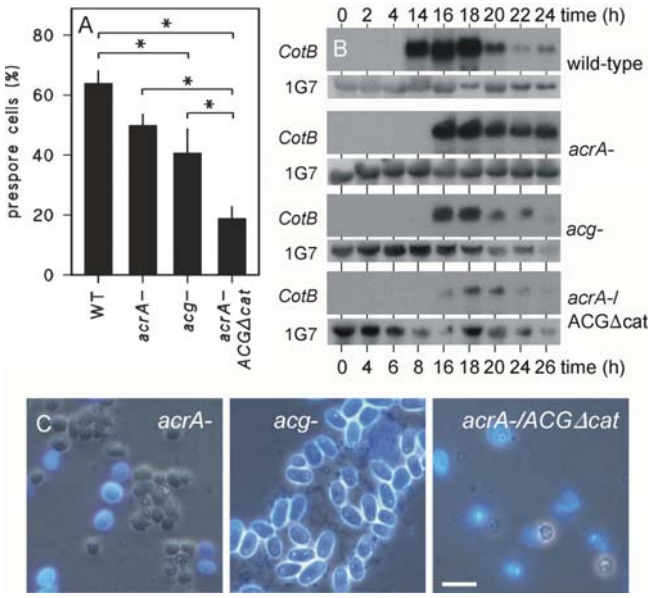

Figure 4. Prespore and spore differentiation in adenylyl cyclase mutants
(A) Slugs of wild-type, *acrA*-, *acg*- and *acrA-/A15::ACGΔcat* cells that had migrated for 2-3 hours were dissociated and stained with a spore-specific antiserum and GARFITC. Cell nuclei were counterstained with DAPI. The percentage of prespore cells (cells with at least 3-4 fluorescent vacuoles) to DAPI stained cells was determined. Means and SE of four experiments are presented. Significant differences (P>0.95) between datasets connected by brackets, as determined by Kruskal-Wallis ANOVA on ranks using SigmaStat software (Systat, San Jose, US), are indicated by asterisks. (B) Wild-type, *acrA*-, *acg*- and *acrA-/A15::ACGΔcat* were developed on PB agar until fruiting bodies had formed. Total RNA was extracted at 2 hour intervals, size-fractionated on 1.5% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Nellen et al., 1987). The four Northern blots were hybridized in the same batch to a [^{32}P]dATP-labeled *CotB* probe at 65°C, then stripped and reprobed with the constitutively expressed gene 1G7 (Williams et al., 1987). (C) 3 day-old fruiting bodies of wild-type, *acrA*-, *acg*- and *acrA-/A15::ACGΔcat* cells were transferred to a slide glass and stained with the cellulose dye Calcofluor at 0.03% (w/v) final concentration. The preparations were photographed under UV by fluorescence microscopy to visualize the Calcofluor stained spores in the presence of a low level of transillumination to obtain a phase contrast image of the remaining amoebas.
Bar length is 10 µm.

To estimate effects of the mutations on prespore differentiation, we measured both the proportion of prespore cells in dissociated slugs and the expression of the prespore gene *CotB* (Fosnaugh and Loomis, 1993; Gomer et al., 1986) during normal development to fruiting bodies. Figure 4A shows that the percentage of prespore to total cells was reduced from 64% to 50% in *acrA*- cells and to 40% in *acg*- cells. The most severe reduction to about 1/4rd of wild-type prespore proportions is observed in the *acrA-/ACGΔcat* cells. The developmental expression of the prespore gene *CotB* showed a similar pattern (Fig. 4B). In *acrA*- cells, *cotB* expression was slightly reduced, in *acg*- cells reduction was more severe and in *acrA-/ACGΔcat* cells *CotB* mRNA was almost gone. All three mutant cell lines still formed fruiting bodies. As previously reported (Soderbom et al., 1999), the spore heads of mature *acrA*- fruiting bodies contained large numbers of amoeboid cells and only few spores (Fig. 4C). In contrast, most cells in the *acg*- spore heads had matured into spore cells. However, in the *acrA-/ACGΔcat* spore heads only a few spores and several empty spore cases were visible. The remaining spores were extremely fragile and often ruptured while being carried over on a slide glass for observation.

These combined data show that loss of ACG is most deleterious for prespore differentiation, while loss of ACB has the strongest effect on spore maturation. However, the two enzymes show considerable functional redundancy and the most severe phenotypes on both prespore and spore differentiation are evident when they are both lost.

cAMP induction of prespore gene expression in adenylyl cyclase mutants

The induction of most prespore genes, such as *CotB*, requires both extracellular cAMP acting on cARs and intracellular cAMP acting on PKA (Hopper et al., 1995; Schaap and Van Driel, 1985). However, the prespore gene *PsA* is less sensitive to ablation of PKA function (Hopper et al., 1993). To examine whether ACG and/or ACB mediate both the intracellular and extracellular functions of cAMP, we measured to what extent *CotB* and *PsA* gene expression were restored by extracellular cAMP in the adenylyl cyclase null mutants. Figure 5 shows that *PsA* gene expression is almost fully restored by extracellular cAMP in both the *acrA*-, *acg*- and *acrA-/ACGΔcat* mutants.

However, *CotB* induction was reduced in the *acrA*- and *acg*- mutant and almost absent in the *acrA-/ACGΔcat* mutant. These results indicate that ACG and ACB have overlapping roles in both cAR and PKA activation.

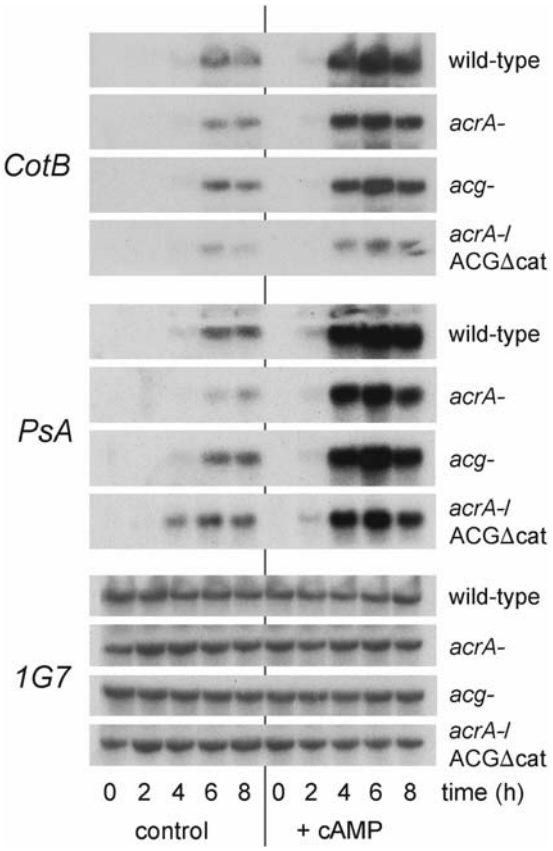

Figure 5. Induction of prespore gene expression in adenylyl cyclase mutants
Aggregation competent wild-type, *acrA*-, *acg*- and *acrA-/A15::ACGΔcat* cells were shaken for 8 hours in the presence and absence of 300 µM cAMP, added every hour. Total RNA was isolated at 2 hour intervals and all RNA samples were size-fractionated on a single gel and transferred to a single membrane, which was successively probed with [^{32}P]dATP-labeled *CotB*, *PsA* and *1G7* DNA probes.

The effect of ACG on the expression of *AcrA*

ACB does not affect the expression of ACG mRNA or protein (Figs. 2B,C), but it is not clear whether ACG affects the expression of ACB/AcrA. We first examined the spatial expression pattern of *AcrA* by *in situ* hybridization (Figs. 6A,B). Surprisingly, *AcrA* is specifically expressed in the prestalk region of slugs and fruiting bodies. To confirm this result and to investigate whether ACG affects the expression pattern of ACB, we prepared a fusion construct of the *AcrA* promoter with the *LacZ* reporter and expressed the construct in wild type and *acg-* cells. Figure 6C shows that in wild-type cells the *AcrA* promoter is almost exclusively active in the prestalk cells. However, in the *acg-* null mutant, *AcrA* promoter activity extends into the entire prespore region (Fig. 6D). This indicates that ACG normally acts to repress *AcrA* promoter activity in prespore cells.

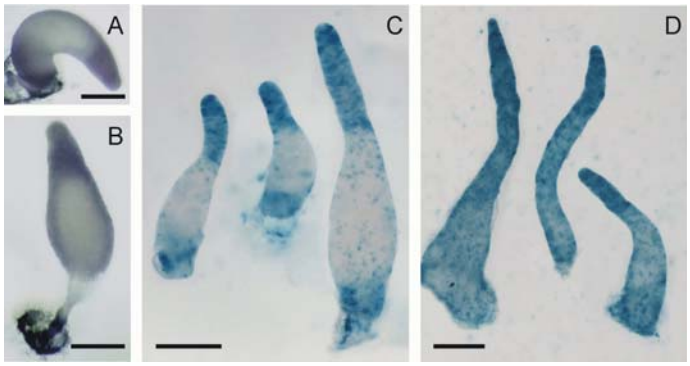

Figure 6. *AcrA* expression in wild type and *acg-* cells
(A,B) *AcrA* expression detected by *in situ* hybridization. Wild-type cells were starved on dialysis membrane supported by PB agar. *AcrA* mRNA was visualized in migrating slugs (A) and mid-culminants (B) by in situ hybridisation to a DIG-labeled *AcrA* RNA-probe. (C,D) Wild-type (C) and *acg-* cells (D) were transformed with vector AcrA::ala-gal, which contains a gene fusion of the *AcrA* promoter and ala-gal, which encodes a stable form of β-galactosidase. Migrating slugs were fixed and stained with X-gal to visualize β-galactosidase activity. AcrA-ile-gal transformed cells yielded the same pattern, but staining intensity was very low (data not shown). Bar length is 100 µm.

Discussion

A low level of *ACG* mRNA was previously only found in spores, and studies of ACG function have up to now concentrated on the spore stage (Pitt et al., 1992; Saran and Schaap, 2004; Van Es et al., 1996). Our present data confirm that *ACG* gene expression is strongly upregulated in maturing fruiting bodies, however there is also significant transcription throughout development. Remarkably, ACG protein is upregulated 12 hours before fruiting bodies are formed in the absence of a corresponding increase in transcription. ACG protein first appears in tipped mounds, to accumulate later in the prespore region of slugs, where it co-localizes with the prespore vesicles. At this location the ACG sensor domain would face the lumen of the vesicle and its catalytic domain would face the cytosol. When prespore vesicles fuse with the plasma membrane in the course of spore maturation, the ACG sensor domain becomes exposed to the cell's exterior.

Significant ACG-like activity (1.7 pmol cAMP/min.10^7 cells) could previously be detected in slug lysates (Meima and Schaap, 1999; and Meima, M. unpublished data), but no osmostimulation of ACG was detectable in intact slug cells. This indicates that the vesicular localization of ACG does not interfere with its enzyme activity, because the catalytic domain would still be exposed to the substrate Mg^{2+}-ATP in the cytosol. However, osmostimulation may either not be possible, or, dependent on the ambient osmolality in the prespore vesicles, the enzyme may always be in the stimulated state. Most of the cAMP that is produced by any of the three *Dictyostelium* adenylyl cyclases is rapidly secreted, suggesting a general non-adenylyl cyclase dependent mechanism for cAMP secretion (Meima and Schaap, 1999; Pitt et al., 1992). This implies that as long as cAMP is produced in the cytosol, it can both act as an

intracellular and extracellular signal by virtue of its constitutive secretion.

Early work showed that extracellular cAMP is both necessary and sufficient for prespore gene induction: micromolar cAMP acting on surface cAMP receptors triggers prespore differentiation (Schaap and Van Driel, 1985), while depletion of extracellular cAMP in slugs causes dedifferentiation of prespore cells (Wang et al., 1988). However, it was less clear how micromolar cAMP concentrations are being produced in slug posteriors. The aggregation-specific adenylyl cyclase ACA is down-regulated in slugs and remains only expressed in the tip (Verkerke-van Wijk et al., 2001). *AcrA* null mutants are defective in spore maturation, but not in prespore differentiation (Soderbom et al., 1999). We show here that prespore differenttiation is significantly reduced in *acg-* cells and has almost disappeared from mutants where both ACG and ACB function is abrogated. Such mutants also do not form any mature spores. These data indicate that ACG and ACB play combinatorial roles in prespore and spore differentiation with ACG predominantly responsible for the former and ACB for the latter response.

Surprisingly *AcrA/ACB* is specifically expressed in prestalk cells, which suggests that its effects on spore maturation may be indirect. In the absence of ACG, *AcrA/ACB* becomes expressed throughout the prespore region, which adequately explains why prespore differentiation is only partially lost in *acg-* cells. The low residual level of prespore gene expression that is still present in slugs where both ACG and ACB function are abrogated could be due to the remaining enzyme ACA.

Expression of the majority of prespore genes not only requires extracellular cAMP acting on cAMP receptors, but also intracellular cAMP acting on PKA (Hopper et al., 1993). We show that ACG produces cAMP for both functions (Figure 5), and it was previously shown to produce cAMP for PKA activation in the spore stage. Here ACG acts as a sensor for the high level of osmolytes in the spore head, which serves to keep the spores dormant (Saran and Schaap, 2004; Van Es et al., 1996; Virdy et al., 1999). Recent work in our laboratory indicates that the ACG gene has been conserved throughout the Dictyostelid phylogeny (Ritchie, A.V. and Schaap, P., in preparation). In addition to spore formation in fruiting bodies, many Dictyostelid species can encyst as single cells, which represent the survival strategy of their ancestors, the solitary amoebas (Raper, 1984). The encystation process is triggered by high osmolality and requires activation of PKA (Ritchie, A.V. and Schaap, P., in preparation). It therefore appears that the role of ACG in prespore differentiation and spore dormancy is derived from a deeply conserved role in encystation.

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