From Drought Sensing to Developmental Control: Evolution of Cyclic AMP Signaling in Social Amoebas

Allyson V. Ritchie,[1] *Saskia van Es,*[2] *Celine Fouquet,*[3] *and Pauline Schaap*

College of Life Sciences, University of Dundee, Dundee, Angus, UK

Amoebas and other protists commonly encyst when faced with environmental stress. Although little is known of the signaling pathways that mediate encystation, the analogous process of spore formation in dictyostelid social amoebas is better understood. In *Dictyostelium discoideum*, secreted cyclic AMP (cAMP) mediates the aggregation of starving amoebas and induces the differentiation of prespore cells. Intracellular cAMP acting on cAMP-dependent protein kinase (PKA) triggers the maturation of spores and prevents their germination under the prevalent conditions of high osmolality in the spore head. The osmolyte-activated adenylate cyclase, ACG, produces cAMP for prespore differentiation and inhibition of spore germination. To retrace the origin of ACG function, we investigated *ACG* gene conservation and function in species that span the dictyostelid phylogeny. *ACG* genes, osmolyte-activated ACG activity, and osmoregulation of spore germination were detected in species that represent the 4 major groups of Dictyostelia. Unlike the derived species *D. discoideum*, many basal Dictyostelia have retained the ancestral mechanism of encystation from solitary amoebas. In these species and in solitary amoebas, encystation is independently triggered by starvation or by high osmolality. Osmolyte-induced encystation was accompanied by an increase in cAMP and prevented by inhibition of PKA, indicating that ACG and PKA activation mediate this response. We propose that high osmolality signals drought in soil amoebas and that developmental cAMP signaling in the Dictyostelia has evolved from this stress response.

Introduction

Unicellular organisms are the most abundant life forms in terms of numbers and total biomass. They have occasionally given rise to the more familiar multicellular forms of animals and plants. One of the most intriguing questions in biology is how such transitions occurred. Multicellular life is typically dependent on highly orchestrated signaling between cells, whereas unicellular organisms respond mainly to environmental signals. A major aspect of the evolution of multicellularity is therefore how the sensory systems of unicellular organisms were elaborated to allow for more sophisticated signaling between cells. The social amoebas or Dictyostelia are eminently suited to study this problem because they can still alternate between unicellular and multicellular lifestyles.

Dictyostelid amoebas feed as single cells on soil bacteria. However, when starved, thousands of amoebas aggregate and differentiate to form fruiting structures with an exquisitely proportioned pattern of stalk cells and spores. In the model organism *Dictyostelium discoideum*, the signal molecule cyclic AMP (cAMP) has multiple roles as secreted chemoattractant and differentiation inducer and as intracellular messenger for other external stimuli (Anjard et al. 1998; Aubry and Firtel 1999; Meima and Schaap 1999). cAMP is produced by 3 adenylate cyclases ACA, ACB, and ACG. *ACA* is highly expressed during aggregation and provides cAMP for chemotactic aggregation (Pitt et al. 1992). *ACB* is expressed in prestalk cells (Alvarez-Curto et al. 2007) and is essential for maturation of stalk cells and spores (Soderbom et al. 1999). ACG is present in prespore and spore cells and produces cAMP for the induction of prespore differentiation (Pitt et al. 1992; Alvarez-Curto et al. 2007). ACG also regulates spore germination in the fruiting body. Here spores are kept dormant by ambient high osmolality, which acts on the intramolecular osmosensor of ACG to activate cAMP synthesis (Van Es et al. 1996; Saran and Schaap 2004).

To provide a framework for evolutionary studies, a molecular phylogeny based on small subunit (SSU) ribosomal RNA (rRNA) and α-tubulin sequence data was recently constructed for all known species of Dictyostelia (Schaap et al. 2006). The phylogeny shows subdivision of species into 4 major groups, which were tentatively called the parvisporids (group 1), after their small spores, the heterostelids (group 2), which consist of the 2 former genera acytostelids and polyphondylids, the rhizostelids (group 3), which contain a cluster of crampon-based species, and the dictyostelids (group 4). *Dictyostelium discoideum* belongs to the evolutionary most derived group 4, whereas group 1 is closest to the root of the tree. Species in groups 1–3 commonly form small clustered fruiting bodies, which are often branched, whereas group 4 species form robust, solitary, and unbranched fruiting bodies. In groups 1–3, the stalk cells form by dedifferentiation of prespore cells, whereas in group 4, they differentiate from a specialized cell type, the prestalk cell (Schaap et al. 1985). Group 4 species also stand out by using cAMP as chemoattractant for aggregation, with other compounds are being used in groups 1–3. Additionally, group 4 species have lost the ability to encyst individually. This survival strategy of their ancestors, the solitary amoebozoans (Baldauf et al. 2000) is retained by many species in groups 1–3 (Schaap et al. 2006).

We retraced the evolutionary history of extracellular cAMP signaling in the Dictyostelia by examining the presence of cell surface cAMP receptors in species from all 4 taxon groups. This work showed that the role of extracellular cAMP in coordinating aggregation of group 4 species is evolutionary derived from a role in coordinating fruiting body morphogenesis in all Dictyostelia (Alvarez-Curto et al. 2005). The use of extracellular cAMP is thus far unique

[1] Present address: Department of Haematology, University of Cambridge, Cambridge, UK.

[2] Present address: Earth and Life Sciences, Free University of Amsterdam, Amsterdam, The Netherlands.

[3] Present address: LPPA UMR7152 Collège de France, Paris, France.

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E-mail: p.schaap@dundee.ac.uk.

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to the Dictyostelia. However, intracellular cAMP is a very common signal molecule in all domains of life. Best known as intermediate for hormone action in humans, it also mediates cellular responses to a wide range of external stimuli in prokaryotes, protests, and fungi. This is reflected by the presence of its synthesizing enzymes, the adenylate cyclases, in all groups of pro- and eukaryotes (Linder and Schultz 2003; Schaap 2005). It is therefore likely that the use of extracellular cAMP is derived from an intracellular role of cAMP.

In this work, we explore the evolutionary history of intracellular cAMP signaling in the Dictyostelia by retracing the conservation and function of the adenylate cyclase ACG. In contrast to ACA, which produces cAMP for extracellular use, and ACB, which is expressed in prestalk cells that are only present in group 4, ACG is expressed in the prespore and spore cells that are common to all Dictyostelia. Here it triggers spore differentiation and controls germination, respectively. In this study, we present evidence that these roles were derived from similar functions in encystation and excystation of single-celled amoebas.

Materials and Methods

Materials, Strains, and Cell Culture

8-Bromo-cAMP (8Br-cAMP), N6,2′-O-dibutyryl-cAMP (DB-cAMP), and 8-(4-chlorophenylthio)-N6-phenyl-cAMP (8C-6P-cAMP) were obtained from Biolog (Bremen, Germany), [2,8-^{3}H]cAMP was obtained from Amersham (Little Chalfont, UK), dithiothreitol (DTT), 3-isobutyl-1-methylxanthine (IBMX), Calcofluor, and G418 were obtained from Sigma (St Louis, MO) and Complete protease inhibitors from Roche (Basel, Switzerland).

Dictyostelium discoideum NC4, *Dictyostelium rosarium* M45, *Dictyostelium mucoroides* S28b, *Dictyostelium minutum* 71-2, *Polysphondylium pallidum* TNS-C-98, and *Dictyostelium fasciculatum* SH3 were grown with *Klebsiella aerogenes* on lactose peptone (LP) agar (Raper 1984). *Dictyostelium discoideum aca-* and *aca-/DdisACG* cells (Pitt et al. 1992) were grown in HL5 medium, supplemented with 20 μg/ml G418 for *aca-/DdisACG*. *Polysphondylium pallidum* strains TNS-C-98 and WS320 were also grown in axenic A-medium (Sussman 1963), which for strain WS320, transformed with A15::PKA-Rm (Funamoto et al. 2003) was supplemented with 200 μg/ml G418. A control cell line for the latter strain was generated by transforming WS320 with pDV-CYFP (Meima et al. 2007), followed by selection at 200 μg/ml G418. Lower G418 concentrations are not lethal for untransformed *Ppal* cells.

Gene Identification and Expression

Identification

A pilot library, prepared by cloning *Eco*RI-digested *Dmin* genomic DNA (gDNA) into phage λgt11, was screened at low stringency with a *DdisACG* DNA probe yielding one positive plaque. Its phage DNA contained a 1,954-bp insert with an incomplete open reading frame (ORF) that was 54% identical to *DdisACG*. The insert was used to screen an λZapII library of sheared *Dmin* gDNA (Alvarez-Curto et al. 2005). Three positive plaques were identified, and their pBluescript phagemids were isolated by in vitro excision. Two phagemids carried identical 2.4-kb inserts and the third an overlapping 4.8-kb insert, which was sequenced to 4-fold coverage by primer walking. Apart from a complete 2,241-bp ACG-like ORF, interrupted by a single 60-bp intron, the insert contained 2 partial ORFs, which proved to have highest identity to genes *DdisMtrpS* and *DdisDDB0233813* (http://dictybase.org/) that flank *DdisACG*.

Degenerate oligonucleotide primers, 5′-TCTTCTTC-GCCGACATCGYNGGNTTYAC-3′ and 5′-TCCCATG-CCGTACCANGTYTTCAT-3′, were designed to match amino acid sequences FFLDIAGF and MKTWYLMG that are conserved between *DdisACG* and *DminACG*. These primers were used to amplify *ACG* sequences by touchdown polymerase chain reaction (PCR) (Alvarez-Curto et al. 2005) from *Ppal*, *Dfas*, and *Dmuc* gDNAs. The PCR products were subcloned in the pGEM-T Easy vector (Promega, Madison, WI), and their sequence was determined from at least 3 independent clones.

Expression

Initial experiments using *DminACG* gDNA did not yield protein expression in *Ddis*, an observation that was also made with the intron containing *DminPdsA* gene (Schaap P, unpublished data). An intronless fusion of the constitutive *Ddis* actin15 promoter, *DminACG*, and yellow fluorescent protein (YFP) was prepared by amplifying segments 1–285 (A) of the *DminACG*-coding region with primer pair 5′-CCCGGATCCAAAAAATCTTCATGTT-CAAGTATTAAGAATTCAAC-3′ and 5′-CCGCTTAA-GATTAATTGAATTTGTTTCTAATGTTGTAC-3′ and segments 346–2,241 (B) with primer pair 5′-CCC-CTTAAGGCATTATATTATACAATTGGTCAATATG-3′ and 5′-CCCTCTAGATGTGGTATCGTTATTTTCTTGG-TTATTAAGTTGATC-3′. After digestion of segment A with *Bam*HI and *Bfr*I and segment B with *Bfr*I and *Xba*I, the 2 segments were cloned into *Bam*HI- and *Xba*I-digested pDV-CYFP (Meima et al. 2007) by 3-point ligation. The construct and empty vector (mock) were introduced into aca- cells by electroporation. Transformants were selected in HL5 medium with 20 μg/ml G418.

cAMP and Adenylate Cyclase Assays

cAMP Accumulation during Encystation

Polysphondylium pallidum cells, diluted to 10^6 cells/ml, were shaken at 200 revolutions per minute (rpm) in A-medium in the presence and absence of 0.25 M sorbitol. At 4-h intervals, 10^8 cells were pelleted by centrifugation. Cell pellets were mixed with 100 μl 3.5% (v/v) perchloric acid and vortexed for 15 min with 100 μl of glass beads to break cyst walls. Lysates were neutralized by addition of 50 μl of 50% saturated $KHCO_3$ and 250 μl of cAMP assay buffer (4 mM ethylenediaminetetraacetic acid [EDTA] in 150 mM K-phosphate, pH 7.5). Samples were centrifuged for 5 min at 3,000 × g, and cAMP was assayed in 30 μl of the supernatant fraction by isotope dilution assay, using purified beef muscle protein kinase regulatory subunit (PKA-R) as

cAMP-binding protein and [2,8-^{3}H]cAMP as competitor (Alvarez-Curto et al. 2007). Pellets were dissolved in 1 ml of 0.1 M NaOH and assayed for total protein content.

ACG Activity in Intact Cells

Exponentially growing *Ddis* cells were harvested and resuspended to 3×10^7 cells/ml in 10 mM phosphate buffer, pH 6.5. Cells were shaken at 150 rpm and 22 °C with 5 mM of the phosphodiesterase inhibitor DTT and osmolytes for variable time periods. The reaction was terminated by addition of an equal volume of 3.5% (v/v) perchloric acid. Lysates were neutralized, and cAMP was assayed as described above.

In Vitro Trap Assay for ACG Activity

Vegetative *Ppal* cells were resuspended to 10^8 cells/ml in lysis buffer (10 mM Tris, pH 8 with 1 tablet Complete protease inhibitors per 50 ml) and incubated for 15 min at 22 °C with variable osmolyte concentrations. Cells were rapidly lysed through nuclepore filters (pore size 3 μm), and aliquots of 10 μl cell lysate were added on ice to 5 μl of variables at 4× the desired final concentration and 5 μl of assay mix (4 mM adenosine triphosphate [ATP], 8 mM $MnCl_2$, 0.8 mM IBMX, and 40 mM DTT in lysis buffer). Reactions were started by transferring the samples to a 22 °C water bath and were terminated by adding 10 μl of 0.4 M EDTA, pH 8.0, followed by boiling for 1 min. cAMP was assayed directly in the boiled lysate.

Spore Germination, Excystation, and Encystation Assays

Spore Germination

For bacterially grown strains, spores were harvested from 2-day-old fruiting bodies and plated on LP agar with *K. aerogenes* at 50 spores/plate. After 5–8 days, the number of amoeba colonies per plate was counted. For strains that can grow axenically, spores were diluted into axenic medium at 3×10^5 spores/ml and incubated for 24 h at 22 °C. Suspensions were then mixed with 0.1 volumes of 0.01% Calcofluor, a fluorescent dye that stains the cellulose in spore and cyst walls, and examined by microscopy. Total cell number was counted under phase contrast illumination and stained elliptical spores under UV light using filter cube A on a Leica DM LB2 microscope. Excystation of *P. pallidum* cysts was measured using the same method.

Encystation

Exponentially growing *P. pallidum* cells were resuspended to 3×10^5 cells/ml in A-medium and incubated for 24–48 h at 22 °C with sorbitol as described in the figure captions. The cells were then stained with Calcofluor as described above, and the numbers of amoeboid unstained cells and round fluorescent cysts were counted.

Molecular Phylogeny of Adenylate Cyclases

Alignment

All protein sequences were analyzed by SMART (Schultz et al. 1998) to localize the position of the PFAM 00211 adenylate and guanylate cyclase catalytic domains (Bateman et al. 2004), which was excised and used for global alignment with ClustalW (Chenna et al. 2003) using default parameters. Each sequence was also individually aligned to the RatAC2-C2 domain for which detailed structure–function information is available (Zhang et al. 1997). This alignment was used to edit the global alignment when necessary, to ensure that functionally essential residues were matched up. A few long insertions that were only present in single sequences were removed, as well as a central block of around 30 residues that was not unambiguously alignable. Other unalignable sequences from more diverged proteins were entered as missing data.

Phylogenetic Reconstruction

Bayesian inference was used to assess the phylogenetic relationships between the proteins identified in this work and other adenylate or guanylate cyclases. A total of 152 aligned amino acids from 38 proteins were analyzed with the software package MrBayes-3.1.2 (Ronquist and Huelsenbeck 2003) using a mixed amino acid model with rate variation across sites estimated by a gamma distribution with 4 rate categories and a proportion of invariable sites. The analysis used the default settings of 2 parallel runs, with 3 heated and 1 cold chain each, and was run for 2 million generations. At this point, the average standard deviation of split frequencies had fallen well below 0.01. Posterior probabilities were averaged over the final 75% of trees.

Data Deposition

DNA sequences obtained in the course of this work were submitted to GenBank under accession numbers EF612188–EF612191 for successively *Dmin contig2*, PpalACG, *DfasACG*, and *DmucACG*.

Results

Conservation of *ACG* Genes

To identify ACG genes in basal amoeba species, we first probed genomic libraries of the group 3 species *D. minutum* (*Dmin*) with a *D. discoideum* (*Ddis*) *ACG* DNA probe. One positive clone with a 4.8-kb insert contained the complete *ACG* gene with 2 flanking genes. The latter genes share greatest similarity with 2 genes that flank DdisACG, albeit that in *Ddis* the intergenic region between *ACG* and the 5′ ortholog is interrupted by 2 short intron-rich genes (fig. 1*A*). This indicates synteny between the *Ddis* and *Dmin* chromosomes and orthology between their *ACG* genes. *DminACG* contains one intron at the same position as the first of the 2 *DdisACG* introns. The deduced amino acid sequence of *DminACG* shows the same domain architecture as DdisACG with 2 transmembrane domains and a single cyclase catalytic domain (fig. 1*A*; supplementary fig. S1, Supplementary Material online).

To test whether DminACG was activated by high osmolality, its gene was expressed as a YFP fusion protein

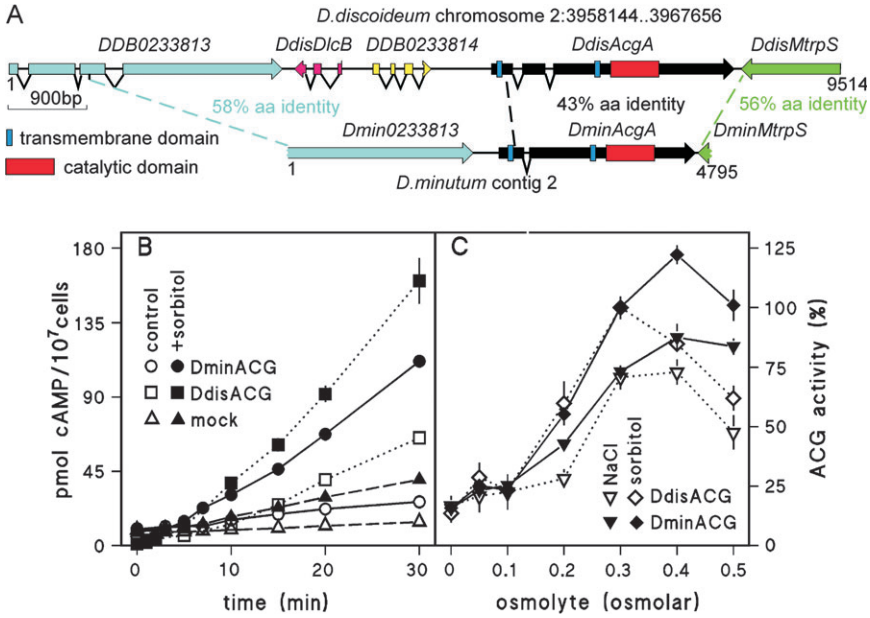

FIG. 1.—Cloning and functional analysis of a *Dictyostelium minutum* ACG. (*A*) *ACG* gene cloning from *D. minutum*. A partial *DminACG*fragment obtained by a low-stringency screen of a *Dmin* gDNA library in λgt11 with a *DdisACG* probe was used to screen a second *Dmin* gDNA library in λZAPII. This yielded 3 overlapping clones of which the largest contained the entire *DminACG* gene and 2 flanking genes, which were orthologous to genes that flank *DdisACG*. The derived amino acid sequence of DminACG showed a similar domain architecture with 2 transmembrane domains (dark blue) and 1 catalytic domain (red) as DdisACG. (*B*/*C*) *DminACG* functional analysis. A *DminACG-YFP* fusion construct was expressed from the constitutive A15 promoter in *Ddis aca*-cells. Vegetative *aca-*/*DminACG*, *aca-*/*DdisACG*, and *aca-*/*YFP* (mock) cells were incubated with 5 mM DTT in the absence (open symbols) or presence (closed symbols) of 0.3 M sorbitol and assayed for cAMP accumulation at the indicated time points. (*B*) *aca-*/*DminACG* and *aca-*/*DdisACG* cells were incubated for 30 min with DTT and NaCl or sorbitol at the indicated osmolarities and assayed for cAMP. Data are expressed as percentage of cAMP accumulation obtained with 0.3 M sorbitol (*C*). Means and standard error of 3 experiments performed in triplicate are presented.

from the constitutive actin15 promoter in *Ddis aca-* cells, which have little basal AC activity (Pitt et al. 1992). It proved to be necessary to remove the single intron from *DminACG* because a genomic *DminACG-YFP* construct was not expressed in *Ddis* (data not shown). Cells transformed with *DdisACG* or with the empty YFP vector were used as positive or negative controls, respectively, in the adenylate cyclase assays. Earlier work showed that the antibiotic G418, which is used for selecting transformed cells, triggers a low level of endogenous *ACG* expression

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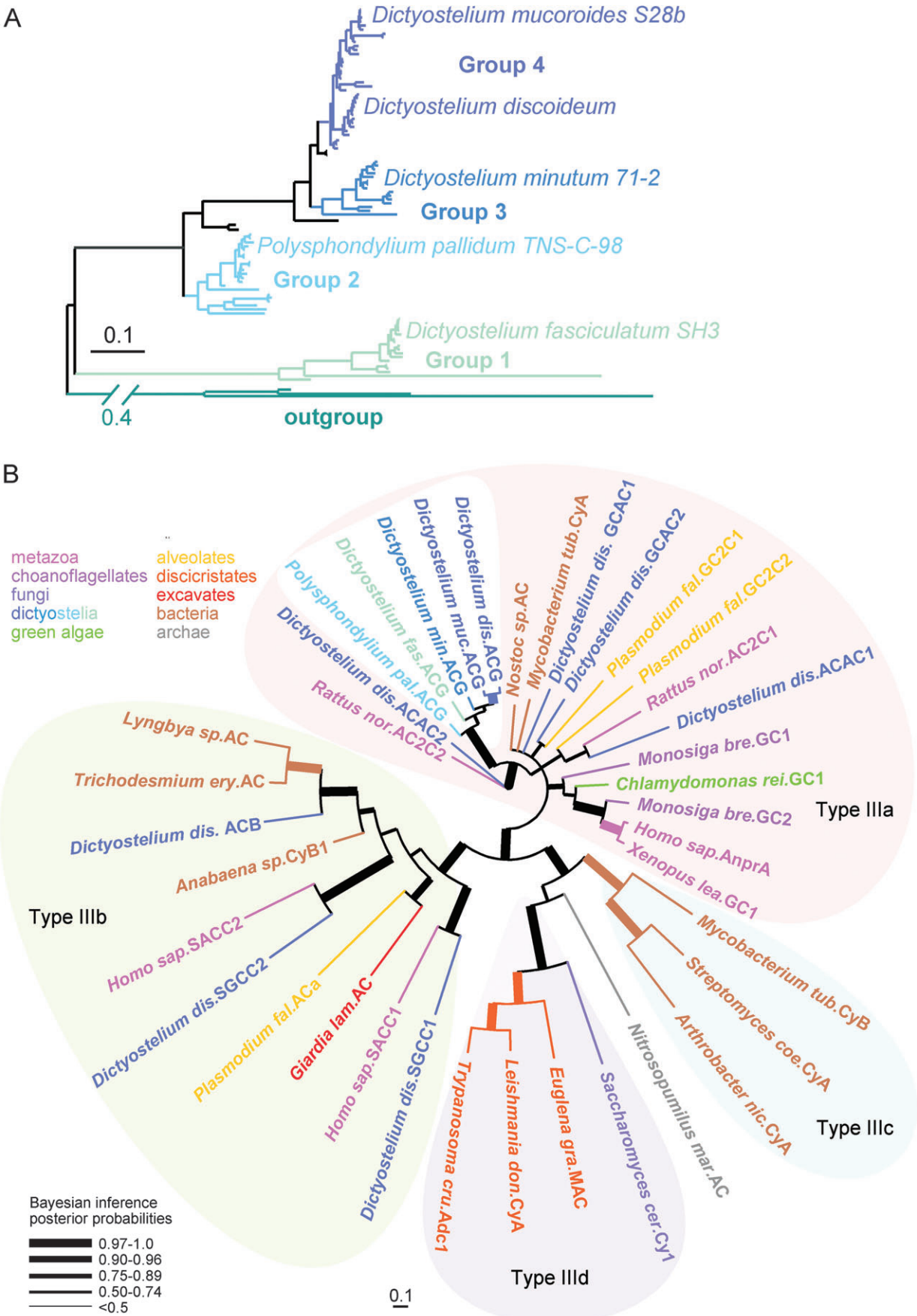
FIG. 2.—Identification of ACG sequences in Dictyostelid species. (*A*) Species selection. Schematic representation of the SSU rRNA phylogeny of the Dictyostelia showing the subdivision of all species into 4 major groups with solitary amoebas as outgroup (redrawn from fig. 1 in Schaap et al. [2006]). The positions of *Dictyostelium discoideum* (*Ddis*) and the group representative test species *Dictyostelium mucoroides* (*Dmuc*), *Dictyostelium minutum* (*Dmin*), *Polysphondylium pallidum* (*Ppal*), and *Dictyostelium fasciculatum* (*Dfas*) are indicated. (*B*) ACGs and their phylogenetic position. Gene fragments were amplified from *Dmuc*, *Ppal*, and *Dfas* genomic DNAs using ACG-specific primers and sequenced. The derived ACG amino acid sequences were aligned with cyclase domains as defined by the PFAM 00211 adenylate and guanylate cyclase catalytic domain profile (Bateman et al. 2004) of all Dicytostelid adenylate and guanylate cyclases and their most closely related outgroup sequences as selected by Blast search of GenBank. The set also contains sequences representative of cyclase subtypes IIIa–d (Linder and Schultz 2003) and sequences obtained by BLAST search within taxonomic divisions, such as bacteria, archae, and the 8 divisions of eukaryotes (Baldauf 2003). This set is not comprehensive, but representative of all subtypes within specific divisions. No sequences were found in nondictyostelid amoebozoans, cercozoa, or heterokonts. Note that several cyclases have 2 catalytic domains, denoted C1 and C2. The alignment, shown in supplementary figure S2 (Supplementary Material online), was used to assess phylogenetic relationships between the cyclases by Bayesian inference (Ronquist and Huelsenbeck 2003) as indicated in the Materials and Methods. Approximate posterior probabilities of nodes are indicated by line thickness, with exact values shown in supplementary figure S3A (Supplementary Material online). An alternative phylogeny, derived by maximum likelihood, shows the same overall topology, with minor variations in the nodes with low statistical support (supplementary fig. S3B, Supplementary Material online). The taxonomic position of the source organisms is indicated by the color code of branches and sequence names, as outlined top left in panel (*B*). GenBank accession numbers of all sequences: *Rattus nor(vegicus)*AC2: P26769; *Dictyostelium dis(coideum)*ACG: Q03101; *Dictyostelium muc(oroides)*ACG: ABU89566; *Dictyostelium min(utum)*ACG: ABU89562; *Polysphondylium pal(lidum)*ACG: ABU89564; *Dictyostelium fas(ciculatum)*ACG: ABU89565; *Dictyostelium dis*.ACA: Q03100; *Dictyostelium dis*.GCA: Q553Y7; *Homo sap(iens)*AnprA: P16066; *Xenopus lae(vis)*GC1: BAA83786; *Monosiga bre(vicollis)*GC1: XP_001748626; *Monosiga bre*.GC2: XP_001748448; *Plasmodium fal(ciparum)*GC2: XP_001350316; *Chlamydomonas rei(nhardtii)*GC1: Q5YLC2; *Mycobacterium tub(erculosis)*CyA: EAY59895; *Nostoc sp(ecies PCC 7120)*AC: NP_484705; *Dictyostelium dis*.ACB: Q55F68; *Trichodesmium ery(thraeum)*AC: YP_722386; *Lyngbya sp(ecies PCC 8106)*AC: ZP_01621902; *Anabaena sp(ecies)*CyB1: BAA13998; *Homo sap*.SAC: AAF65931; *Dictyostelium dis*.SCG: AAK92097; *Plasmodium fal*.ACa: AAO64441; *Giardia lam(blia)*AC: XP_001704657; *Streptomyces coe(licolor)*CyA: P40135; *Arthrobacter nic(otianae)*CyA: P27580; *Mycobacterium tub*.CyB: NP_215780: *Saccharomyces cer(evisiae)*Cy1: YJL005W; *Leishmania don(ovani)*CyA: Q27675; *Euglena gra(cilis)*MAC: BAD20741; *Trypanosoma cru(zi)*Adc1: CAA09919; and *Nitrosopumilus mar(itimus)*AC: YP_001583100.


A
Dictyostelium mucoroides S28b
Group 4
Dictyostelium discoideum
Dictyostelium minutum 71-2
Group 3
Polysphondylium pallidum TNS-C-98
Group 2
0.1
Dictyostelium fasciculatum SH3
Group 1
0.4
outgroup
B
metazoa
choanoflagellates
fungi
dictyostelia
green algae
alveolates
discicristates
excavates
bacteria
archae
Dictyostelium dis.ACG
Dictyostelium muc.ACG
Dictyostelium min.ACG
Dictyostelium fas.ACG
Polysphondylium pal.ACG
Dictyostelium dis.ACAC2
Rattus nor.AC2C2
Nostoc sp.AC
Mycobacterium tub.CyA
Dictyostelium dis. GCAC1
Dictyostelium dis.GCAC2
Plasmodium fal.GC2C1
Plasmodium fal.GC2C2
Rattus nor.AC2C1
Dictyostelium dis.ACAC1
Monosiga bre.GC1
Chlamydomonas rei.GC1
Monosiga bre.GC2
Homo sap.AnprA
Xenopus lea.GC1
Type IIIa
Lyngbya sp.AC
Trichodesmium ery.AC
Dictyostelium dis. ACB
Anabaena sp.CyB1
Type IIIb
Homo sap.SACC2
Dictyostelium dis.SGCC2
Plasmodium fal.ACa
Giardia lam.AC
Homo sap.SACC1
Dictyostelium dis.SGCC1
Mycobacterium tub.CyB
Streptomyces coe.CyA
Arthrobacter nic.CyA
Type IIIc
Nitrosopumilus mar.AC
Saccharomyces cer.Cy1
Euglena gra.MAC
Leishmania don.CyA
Trypanosoma cru.Adc1
Type IIId
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0.90-0.96
0.75-0.89
0.50-0.74
<0.5
0.1

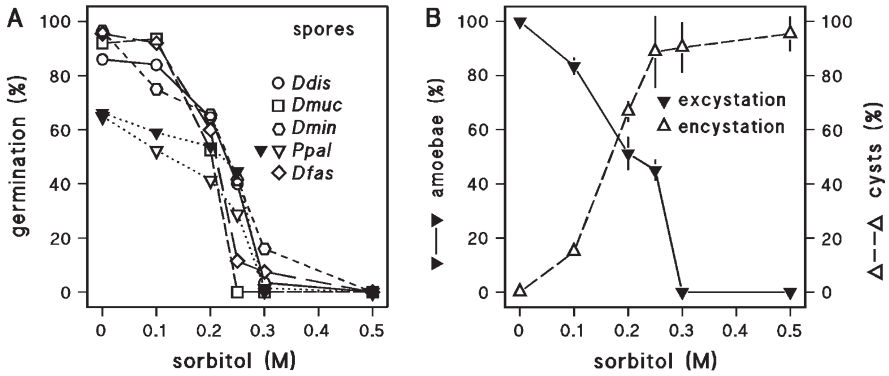

FIG. 3.—Osmoregulation of sporulation and encystation. (*A*) Spore germination. Spores of group-representative species were plated with *Klebsiella aerogenes* on LP agar with the indicated sorbitol concentrations. After 5–8 days, the number of amoeba colonies per plate was counted. Data are expressed as percentage of spores plated. Means of 4 plates are presented. *Ppal* spores were also allowed to germinate in axenic growth medium (▼). After 24 h, cells were stained with Calcofluor, and the number of total cells and stained elliptical spores was counted. The means of 2 experiments, measuring 200 cells per sample each, are presented. (*B*) Excystation and encystation. For excystation, *Ppal* cysts were diluted to 3×10^5 cells/ml in growth medium with the indicated sorbitol concentrations and incubated for 24 h at 22 °C. For encystation, growing *Ppal* cells were similarly incubated in growth medium. After 24 h, the cells were stained with Calcofluor, and the percentage of unstained amoebas or peripherally stained round cysts to total cells was determined. Means and standard deviation of 2 experiments are presented.

in *Ddis* cells (Schulkes et al. 1995). This is evident by the presence of some osmolyte-stimulated cAMP production in the cells transformed with the empty YFP vector (fig. 1*B*). However, osmolyte-stimulated cAMP synthesis is strongly increased when cells are transformed with *DminACG* or *DdisACG*. For both DminACG and DdisACG, sorbitol activates slightly better than NaCl. The osmolyte dose dependency of DdisACG and DminACG for both solutes is almost the same (fig. 1*C*), indicating that DminACG is a fully functional osmosensor.

Is ACG common to all Dictyostelia? To answer this question, we selected 3 more species: *D. mucoroides* (*Dmuc*), *P. pallidum* (*Ppal*) and *D. fasciculatum* (*Dfas*) to represent groups 4, 2, and 1, respectively, of the dictyostelid phylogeny, which is schematically represented in figure 2*A*. We designed degenerate oligonucleotide primers, complementary to conserved regions between *DdisACG* and *DminACG* and used the primers to amplify putative *ACG* genes from genomic DNAs of the test species. This yielded products with the expected size of 0.53 kb. Their derived amino acid sequences showed extensive sequence identity with DdisACG and DminACG (supplementary fig. S2, Supplementary Material online).

All eukaryote adenylate and guanylate cyclases contain 1 or 2 deeply conserved catalytic domains, which are also found in the class III prokaryote adenylate cyclases. Prokaryotes additionally have 4 unrelated classes of adenylate cyclases (Linder and Schultz 2003). The class III domain can be subdivided into 4 subtypes, a–d, of which type a is more dominant in metazoans and type b in prokaryotes. Although traditionally the metazoan adenylate and guanylate cyclases have been most thoroughly studied, several protist cyclase genes are currently emerging from genome sequencing projects. To analyze genetic relationships between the ACGs and prokaryote, protist, and metazoan cyclases, we aligned representative cyclase domains from major taxonomic divisions with the 5 ACG cyclase domains, the cyclase domains of the other Ddis adenylate and guanylate cyclases ACA, ACB, GCA, and sGC and representative type IIIa–d domains (supplementary fig. S2, Supplementary Material online).

Bayesian inference (Ronquist and Huelsenbeck 2003) was used to assess the relatedness of the aligned sequences. Figure 2*B* shows that the 5 ACGs are tightly grouped together in the cluster of type IIIa enzymes, which also contains metazoan, choanoflagellate, alveolate, algal, and prokaryote enzymes. Both catalytic domains of DdisACA and GCA are also members of this cluster, but it is unresolved whether the ACGs are more related to these enzymes than to some of the metazoan or prokaryote cyclases.

As previously reported, DdisACB is most similar to type IIIb prokaryote cyclases, both with respect to protein sequence and its array of functional domains. This suggested that the gene was most likely acquired by horizontal gene transfer (Soderbom et al. 1999). The IIIb cluster also contains DdisSGC, human SAC, and an excavate and alveolate AC, which are all less related to the prokaryote ACs than ACB. The IIIc cluster contains only prokaryote cyclases and is probably ancestral to cluster IIId, which contains the single type of fungal AC, the discicristate ACs, and perhaps an archaean AC.

The eukaryotes most likely inherited all cyclase subtypes from prokaryotes and acquired some cyclase genes later by horizontal gene transfer. Subsequently, there appears to have been lineage-specific loss of some subtypes and extensive expansion of others. This makes it very difficult to retrace the origin of any eukaryote cyclase.

Apart from the dictyostelid enzymes, no amoebozoan cyclase genes have yet been identified, although osmolyte-stimulated adenylate cyclase activity has been measured in *Acanthamoeba palestinensis* (Chlapowski and Butcher 1986). The dictyostelid ACGs are therefore likely to be more deeply conserved within the amoebozoans.

Conservation of ACG Function

DdisACG mediates inhibition of spore germination by ambient high osmolality in the fruiting body, a response

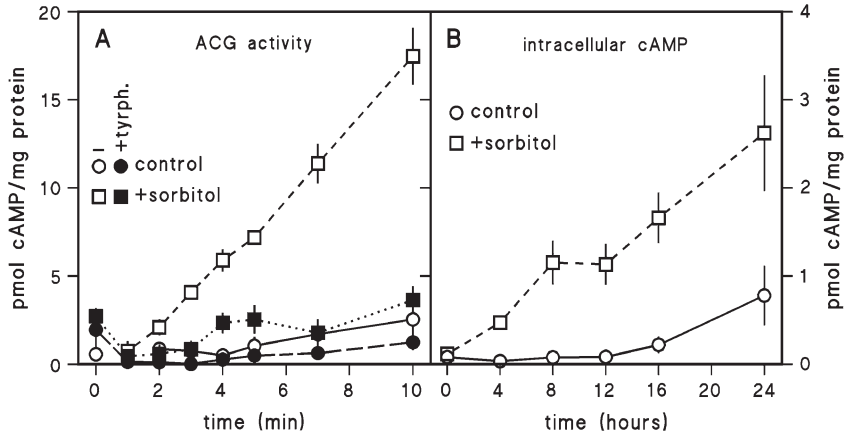

FIG. 4.—Osmoregulation of cAMP production in *Polysphondylium pallidum*. (*A*) AC activity. *Ppal* amoebas were incubated for 15 min in the presence (□, ■) and absence (○, ●) of 0.25 M sorbitol, rapidly lysed through nuclepore filters and incubated with ATP/Mn^{2+} and cAMP phosphodiesterase inhibitors in the absence (open symbols) and presence (closed symbols) of 300 μM tyrphostin A25. At the indicated time points, lysates were assayed for cAMP. Data were standardized on the protein content of the cell lysates. Means and standard error of 2 experiments performed in triplicate are presented. (*B*) cAMP accumulation during encystation. *Ppal* amoebas were shaken in growth medium in the presence (□) and absence (○) of 0.25 M sorbitol. At 4-h intervals, 10^8 cells were pelleted by centrifugation and lysed by addition of perchloric acid. Total cAMP and protein levels were determined in the neutralized cell lysates. Means and standard error of 3 experiments performed in triplicate are presented.

that is lost in *acg* null mutants (Van Es et al. 1996; Cotter et al. 1999). To assess whether this role of ACG is universal, we measured the effect of high osmolality on spore germination in *Dmuc*, *Dmin*, *Ppal*, and *Dfas*. Figure 3*A* shows that the inhibitory effect of sorbitol on spore germination occurs at the same concentrations as its stimulatory effect on ACG activation (fig. 1*C*). This makes it very likely that ACG mediates inhibition of spore germination in the 4 test species.

The deeply conserved role of ACG in spore germination suggests that this role may have been derived from the more ancestral process of cyst germination or excystation. Cyst formation is the universal survival strategy for solitary amoebas and still occurs when more basal dictyostelid species starve under conditions that are unfavorable for aggregation. Indeed, for many basal species, such as *D. deminutivum* and the Acytostelids, encystation appears to be the preferred mode of survival, and they can be very difficult to bring to fruiting body formation (Raper 1984). On the other hand, the species in the most derived group 4, such as *Ddis*, have lost this alternative mode of development (Schaap et al. 2006). The round cysts differ from the usually elliptical spores in being less dehydrated and in having a 2-layered instead of 3-layered cell wall (Hohl et al. 1970).

We chose the group 2 species *Ppal* to test whether ACG regulates excystation. *Ppal* can be grown in axenic medium and encysts after the culture has reached stationary phase. Excystation occurs when cysts are diluted into fresh medium. Figure 3*B* shows that excystation is inhibited by the same osmolyte concentrations as spore germination. Encystation of starving cells is known to be promoted by high osmolality (Toama and Raper 1967). However, we noticed that high osmolality is an independent trigger and induces encystation while the cells are still feeding (fig. 3*B*). Encystation occurs at the same osmolyte concentrations as inhibition of spore and cyst germination, suggesting that all 3 responses are mediated by the same osmosensor, that is, ACG.

The Role of ACG in Encystation

To confirm that PpalACG mediates osmolyte-induced encystation, we first measured whether PpalACG was present before aggregation. Pilot experiments indicated that unaggregated *Ppal* cells do not secrete cAMP, necessitating an in vitro trap assay to measure a putative ACG (Pupillo et al. 1992; Van Es et al. 1996). An AC activity was detected in *Ppal* cell lysates, which was strongly stimulated by preincubation with osmolyte (fig. 4*A*). This activity was inhibited by tyrphostin A25, a compound that specifically inhibits DdisACG but not ACA or ACB. This confirms that *Ppal* cells show ACG activity before aggregation. Tyrphostin A25 only inhibits ACG when applied to cell lysates and cannot be used to block ACG function in intact cells (Alvarez-Curto et al. 2007).

Second, we measured whether high osmolality increases cellular cAMP levels prior to encystation. Figure 4*B* shows that cAMP levels are low in growing *Ppal* cells but increase dramatically when cells are exposed to high osmolality and consequently encyst. Both experiments strongly suggest critical involvement of ACG in osmolyte-induced encystation.

PKA Involvement in Encystation

The effect of ACG on *Ddis* spore germination is mediated by cAMP-dependent PKA (Van Es et al. 1996). To investigate whether PKA also mediates encystation, we first tested whether direct PKA activation can bypass the effect of high osmolality. PKA can be directly activated in living cells by lipophilic analogues of cAMP, which, in contrast to cAMP itself, can penetrate the plasma membrane. We incubated actively feeding *Ppal* cells with 3 different membrane-permeant cAMP analogues that activate PKA effectively. Figure 5*A* shows that all 3 cAMP analogues trigger encystation in the growing cells in the absence of high osmolality. The potency of the analogues is correlated

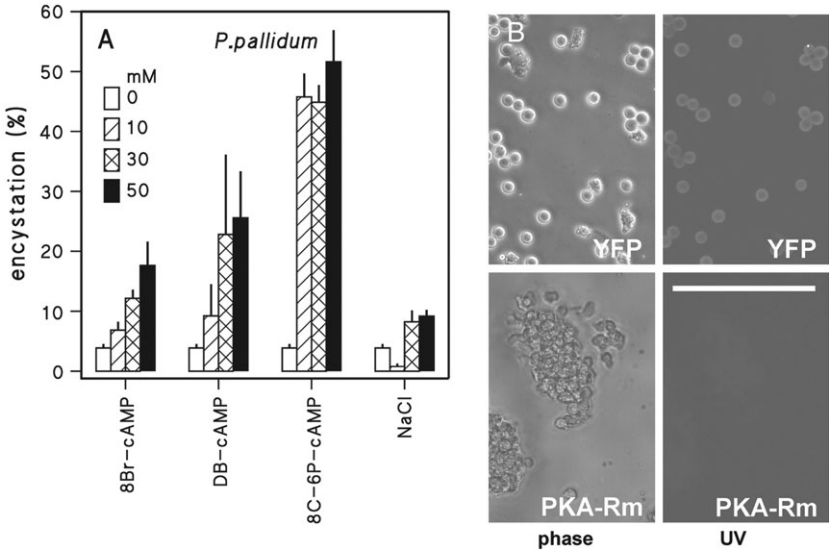

FIG. 5.—PKA involvement in encystation. (*A*) Effects of PKA agonists on encystation. *Ppal* amoebas were incubated at 22 °C in growth medium supplemented with the indicated concentrations of NaCl, 8Br-cAMP, DB-cAMP, or 8C-6P-cAMP. After 24 h, the cells were stained with Calcofluor, and the percentage of stained cysts to total cells was determined. Means and standard error of 3 experiments performed in duplicate are presented. (*B*) Effect of PKA inhibition on encystation. *Ppal* WS320 cells overexpressing YFP (control) or PKA-Rm were incubated for 48 h in growth medium in the presence and absence of 0.25 M sorbitol and stained with Calcofluor. Cells were photographed under phase contrast and UV illumination. Only the sorbitol-treated cells are shown. Bar length is 100 μM.

with their lipophylicity and therefore their ability to pass through the plasma membrane. 8Br-cAMP, DB-cAMP, and 8C-6P-cAMP are, respectively, 2, 20 and 400 times more lipophylic than cAMP (http://www.biolog.de/logkw.html). 8C-6P-cAMP, which is most lipophylic, is the most effective inducer of encystation.

Second, we tested whether PKA activation is essential for osmolyte-induced encystation, using *Ppal* cells that were transformed with a *Ddis* dominant-negative PKA inhibitor, PKA-Rm (Harwood et al. 1992; Funamoto et al. 2003). When transformed in *Ppal*, Ddis PKA-Rm reduces its PKA activity 5-fold. Similar to its effect in *Ddis*, PKA-Rm also blocks normal aggregation and fruiting body formation in *Ppal* (Funamoto et al. 2003). Figure 5*B* shows that the majority of mock transformed *Ppal* cells form round calcofluor positive cysts when exposed to high osmolality. However, the PKA-Rm–transformed *Ppal* cells remain amoeboid and devoid of cellulose after 48 h at high osmolality. The experiments shown in figure 5*A* and *B* indicate that PKA activation is both necessary and sufficient for osmolyte-induced encystation.

Discussion

The ACG Gene Is Functionally Conserved in All Dictyostelia

Multicellular development in the Dictyostelia is a response to nutrient stress. Its purpose is the production of highly resistant viable spores, which are placed on top of a stalk for easy access to dispersal. In the model *D. discoideum*, ACG plays a central role in spore formation. First, it produces extracellular and intracellular cAMP in the posterior region of the slug to activate prespore gene expression. This requires the combined activation of cell surface cAMP receptors and intracellular PKA (Alvarez-Curto et al. 2007). Second, it prevents spores from germinating under the conditions of high osmolality that prevail in the spore head. This effect is mediated solely by PKA (Van Es et al. 1996).

In this work, we show that the *ACG* gene is present in all 4 major groups of Dictyostelia. The cyclase domains of 4 newly identified *ACG* genes are closely related to *DdisACG* but not to the cyclase domains of any of the other *Ddis* adenylate or guanylate cyclases, indicating that they probably evolved independently from these enzymes (fig. 2*B*). The ACG cyclase domains belong to the IIIa subtype, which is found in prokaryotes, several protist phyla, and metazoans. However, there is no clear phylogenetic association of the ACGs with enzymes from either of these groups and their deeper origins therefore remain presently unresolved.

To examine whether the ACGs were also functionally conserved, we expressed a full-length *ACG* gene from the group 3 species *Dmin* in the group 4 species *Ddis*. The DminACG showed the same activation of cAMP synthesis by high osmolality as the DdisACG, indicating that is a fully functional osmosensor (fig. 1*C*). The 4 group representative test species all showed inhibition of spore germination by high osmolality with a concentration dependency that mirrored ACG activation by high osmolality (fig. 3*A*). This strongly suggests that similar to DdisACG, the ACGs of the 4 test species also mediate inhibition of spore germination by high osmolality.

ACG Mediates Drought-Induced Encystation of Basal Dictyostelia

The dormant spores of social amoebas have an analogous role to the dormant cysts of their ancestors, the solitary amoebozoans: survival in response to starvation or other forms of environmental stress. Many dictyostelid species in groups 1–3 have retained encystation as an

alternative survival strategy (Schaap et al. 2006). We found that similar to spore germination, cyst germination is also inhibited by high osmolality. However, unlike spore formation, the formation of cysts is triggered by high osmolality, even when sufficient food is still available (fig. 3*B*). Again these effects of high osmolality occurred at the same concentrations as activation of ACG. A pronounced osmolyte-induced increase in intracellular cAMP levels preceded the final stage of encystment (fig. 4*B*). Together, these data strongly suggest that osmolyte-induced encystation is mediated by ACG.

The target of cAMP produced by ACG is PKA because inhibition of PKA prevented osmolyte-induced encystation and direct activation of PKA with membrane-permeant cAMP analogues induced encystation without the need for high osmolality (fig. 5). Combined, these experiments show that similar to osmolyte-inhibited spore germination, osmolyte-induced encystation was mediated by ACG and PKA.

What is the significance of induction of encystation by high osmolality? We propose that it is most likely a signal for approaching drought, when soil mineral concentrations start to increase. By triggering timely encystation, the demise of soil amoebas by drying out is prevented. Osmolyte-induced encystation was also observed in the solitary amoebozoans *Acanthamoeba castellani* and *Hartmannella rhysodes* (Band 1963; Cordingley et al. 1996), whereas PKA agonists were reported to trigger encystation of *Hartmannella culbertsoni* and *Entamoeba invadens* (Raizada and Murti 1972; Coppi et al. 2002). Evidently, also in these distantly related amoebozoans, high osmolality and PKA activation are associated with encystation. However, up till now, causality between these 2 factors was not suspected.

ACG's Role in Sporulation Is Derived from Its Role in Encystation

The roles of ACG in encystation and spore formation show a striking resemblance. ACG blocks excystation and triggers encystation in individual amoebas. Similarly, ACG blocks spore germination in fruiting bodies and triggers prespore differentiation in the slug stage (Alvarez-Curto et al. 2007). This most likely signifies that the roles of ACG in cell-type specification and spore germination during multicellular *Dictyostelium* development are evolutionary derived from a role as drought sensor triggering encystation in individual amoebas.

An alternative explanation for the homologous roles of ACG in encystation and sporulation would be that ACG's role in sporulation evolved first in the Dictyostelia, whereas the ancestral mechanisms for encystation that were used by solitary amoebas were lost. Encystation was then reinvented in the Dictyostelia, using the same ACG- and PKA-mediated mechanism as was developed for sporulation. This is highly unlikely considering the fact that many, but not all, species in groups 1–3 encyst and none in group 4. Either encystation was reinvented several times independently or reinvented and lost again in a subset of species.

Recent studies also indicate that encystation is ancestral to sporulation and that the accumulation of extracellular cAMP within aggregates informs amoebas to differentiate into spores and not cysts. A *P. pallidum* mutant was created that cannot detect extracellular cAMP due to lesions in its 2 cAMP receptor genes. This mutant forms stunted fruiting bodies because morphogenetic cell movement can no longer be regulated by cAMP (see also Alvarez-Curto et al. 2005), and these fruiting bodies carry cysts instead of spores in the spore head. The mutant is also defective in cAMP induction of prespore gene expression (Kawabe Y, Schaap P, unpublished data). This indicates that encystation is the default strategy that only requires PKA activation, whereas spore formation is a derived strategy that additionally requires cAMP receptor activation. A crucial step in dictyostelid evolution both with respect to the differentiation of spores and the coordination of morphogenesis may therefore have been the ability to secrete and detect cAMP.

This work demonstrates the power of evolutionary reconstruction as an analytical tool. Not only did it uncover a signaling pathway that may mediate a major protist stress response but it also identified the origin of the many roles of cAMP in modern Dictyostelia.

Supplementary Material

Supplementary figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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Literature Cited

Alvarez-Curto E, Rozen DE, Ritchie AV, Fouquet C, Baldauf SL, Schaap P. 2005. Evolutionary origin of cAMP-based chemoattraction in the social amoebae. Proc Natl Acad Sci USA. 102:6385–6390.

Alvarez-Curto E, Saran S, Meima M, Zobel J, Scott C, Schaap P. 2007. cAMP production by adenylyl cyclase G induces prespore differentiation in *Dictyostelium* slugs. Development. 134:959–966.

Alvarez-Curto E, Weening KE, Schaap P. 2007. Pharmacological profiling of the *Dictyostelium* adenylate cyclases ACA, ACB and ACG. Biochem J. 401:309–316.

Anjard C, Zeng CJ, Loomis WF, Nellen W. 1998. Signal transduction pathways leading to spore differentiation in *Dictyostelium discoideum*. Dev Biol. 193:146–155.

Aubry L, Firtel R. 1999. Integration of signaling networks that regulate *Dictyostelium* differentiation. Annu Rev Cell Dev Biol. 15:469–517.

Baldauf SL. 2003. The deep roots of eukaryotes. Science. 300:1703–1706.

Baldauf SL, Roger AJ, Wenk-Siefert I, Doolittle WF. 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science. 290:972–977.

Band RN. 1963. Extrinsic requirements for encystation by soil amoeba, *Hartmannella rhysodes*. J Protozool. 10:101–106.

Bateman A, Coin L, Durbin R, et al. (13 co-authors). 2004. The Pfam protein families database. Nucleic Acids Res. 32:D138–D141.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31:3497–3500.

Chlapowski FJ, Butcher RW. 1986. Activation of adenylate cyclase in *Acanthamoeba palestinensis*. Life Sci. 38:849–859.

Coppi A, Merali S, Eichinger D. 2002. The enteric parasite *Entamoeba* uses an autocrine catecholamine system during differentiation into the infectious cyst stage. J Biol Chem. 277:8083–8090.

Cordingley JS, Wills RA, Villemez CL. 1996. Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. J Cell Biochem. 61:167–171.

Cotter DA, Dunbar AJ, Buconjic SD, Wheldrake JF. 1999. Ammonium phosphate in sori of *Dictyostelium discoideum* promotes spore dormancy through stimulation of the osmosensor ACG. Microbiology. 145:1891–1901.

Funamoto S, Anjard C, Nellen W, Ochiai H. 2003. cAMP-dependent protein kinase regulates *Polysphondylium pallidum* development. Differentiation. 71:51–61.

Harwood AJ, Hopper NA, Simon M-N, Bouzid S, Veron M, Williams JG. 1992. Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. Dev Biol. 149:90–99.

Hohl HR, Miura-Santo LY, Cotter DA. 1970. Ultrastructural changes during formation and germination of microcysts in *Polysphondylium pallidum*, a cellular slime mould. J Cell Sci. 7:285–306.

Linder JU, Schultz JE. 2003. The class III adenylyl cyclases: multi-purpose signalling modules. Cell Signal. 15:1081–1089.

Meima M, Schaap P. 1999. Dictyostelium development—socializing through cAMP. Semin Cell Dev Biol. 10:567–576.

Meima ME, Weening KE, Schaap P. 2007. Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in *Dictyostelium*. Protein Expr Purif. 53:283–288.

Pitt GS, Milona N, Borleis J, Lin KC, Reed RR, Devreotes PN. 1992. Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell. 69:305–315.

Pupillo M, Insall R, Pitt GS, Devreotes PN. 1992. Multiple cyclic AMP receptors are linked to adenylyl cyclase in *Dictyostelium*. Mol Biol Cell. 3:1229–1234.

Raizada MK, Murti CRK. 1972. Transformation of trophic *Hartmannella culbertsoni* into viable cysts by cyclic 3′5′-adenosine monophosphate. J Cell Biol. 52:743–748.

Raper KB. 1984. The dictyostelids. Princeton: Princeton University Press.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

Saran S, Schaap P. 2004. Adenylyl cyclase G is activated by an intramolecular osmosensor. Mol Biol Cell. 15:1479–1486.

Schaap P. 2005. Guanylyl cyclases across the tree of life. Front Biosci. 10:1485–1498.

Schaap P, Pinas JE, Wang M. 1985. Patterns of cell differentiation in several cellular slime mold species. Dev Biol. 111:51–61.

Schaap P, Winckler T, Nelson M, et al. (12 co-authors). 2006. Molecular phylogeny and evolution of morphology in the social amoebas. Science. 314:661–663.

Schulkes CCGM, Verkerke-van Wijk I, Schaap P. 1995. Transformation with vectors harboring the NEOr selection marker induces germination-specific adenylyl cyclase activity in *Dictyostelium* cells. Exp Cell Res. 220:505–508.

Schultz J, Milpetz F, Bork P, Ponting CP. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA. 95:5857–5864.

Soderbom F, Anjard C, Iranfar N, Fuller D, Loomis WF. 1999. An adenylyl cyclase that functions during late development of *Dictyostelium*. Development. 126:5463–5471.

Sussman M. 1963. Growth of the cellular slime mold *Polysphondylium pallidum* in a simple nutrient medium. Science. 139:338.

Toama MA, Raper KB. 1967. Microcysts of the cellular slime mold *Polysphondylium pallidum*. I. Factors influencing microcyst formation. J Bacteriol. 94:1143–1149.

Van Es S, Virdy KJ, Pitt GS, Meima M, Sands TW, Devreotes PN, Cotter DA, Schaap P. 1996. Adenylyl cyclase G, an osmosensor controlling germination of *Dictyostelium* spores. J Biol Chem. 271:23623–23625.

Zhang G, Liu Y, Ruoho AE, Hurley JH. 1997. Structure of the adenylyl cyclase catalytic core. Nature. 386:247–253.

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