Developmental regulation and evolution of cAMP signalling in Dictyostelium

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To Phil, always

A Mamá, siempre

Contents

General Introduction

Dictyostelium discoideum: a model for cell and developmental biology

The social amoeba *Dictyostelium discoideum* is a popular system to study different aspects of cell and developmental biology such as cell motility, cell signalling and differentiation. In addition to this, *Dictyostelium* is a very attractive system to study the origins of multicellularity. The Dictyostelids are members of the Mycetozoa, which consist of three different groups: the syncytial slime moulds or Myxogastrids (e.g. *Physarum polycephalum*), which exist as single-celled spores and amoebas and as multinucleate syncytia, the Protostelids, which form a spore with an acellular stalk from a single cell and the Dictyostelids or social amoebas that aggregate to form multicellular structures consisting of up to 100.000 cells. The use of molecular data to reconstruct species phylogenies has shown that the three groups are all members of the Amoebazoa, a major group of protists, that is a sister clade to the animals and fungi (Baldauf, 2003). The Amoebazoa is a diverse group that comprise a great variety of solitary amoebas, amoeba-flagellates and amitochondrial pelobionts. The Dictyostelids are however the only organism in the group that shows true multicellularity.

Dictyostelium development might seem at first glance very simple when compared with that of higher Metazoans. However, all the events leading to and taking place during the transition from unicellular to multicellular organism show that this organism provides an multifaceted challenge to understand fundamental mechanisms of development and one of the many strategies by which multicellularity has been achieved. The transition to multicellularity is a critical step in eukaryotic evolution and it has generated an enormous morphological and behavioural diversity among species, including the diversity found within the Dictyostelids. Social amoebas aggregate to form a bigger organism (the fruiting body) in response to nutrient stress. The formation of multicellular structures by aggregation has occurred several times during evolution in unrelated amoeba species, the acrasid slime molds and also in prokaryotes of the genus *Myxococcus* (Dao et al., 2000). The architecture of the fruiting body in the myxococci varies between different species, as is also the case for Dictyostelids. We find very simple, basic structures in *M.xanthus* consisting of a ball of cells differentiated into spores, and quite elaborated fruiting bodies in *C.crocatus* with tree-like acellular stalks that support spores at its ends.

Multicellularity offers several advantages such as division of labour between differentiated cells, as well as increased size and protection against predators in the soil. Nematodes feed on *Dictyostelium* amoebas but they are not able to penetrate the slug once it is formed, hence preventing the *Dictyostelium* cells from being eaten. The fruiting body itself provides an easier way of propagating spores as they are elevated from the substratum and they can be more easily carried by small arthropods or other soil dwelling organisms (Kessin, 2001). However, differentiation and specialization to achieve multicellularity comes at a price. There is a necessity for novel genes, pathways and regulatory elements, and it might also mean that part of the population will have to be sacrificed. In the case of *Dictyostelium* about 20% of cells that form the aggregate altruistically die to form the cellulose stalk that supports the spore mass ensuring the spores are elevated from the substrate (Hudson et al., 2002). As a consequence, if cells with different genotypes coexist within the same aggregate, which is to be expected in the natural environment, the genetic information of the cells forming the stalk will be lost.

Development in all systems is based on the formation of a complex structure such as an embryo (or a fruiting body) from a much simpler one such as an egg (or an amoeba). In the biology of the Dictyostelids we find some features that remind us of characteristics found in other Metazoans. *Dictyostelium* has vegetative cells that are highly motile like some animal cells (e.g. leukocytes), the cells in the stalk are highly vacuolated and with cellulose as we find in plants and finally, the spores are in some aspects quite fungal-like (Kessin, 2001). Although the fundamental principles of development that we find in higher eukaryotes and Dictyostelids are comparable, plants, animals and Dictyostelids are physiologically and morphologically very different and this must be taken into account when extrapolating findings.

In animal development the zygote divides clonally up to a specific point in which cells responding to certain signals will choose their fate. After this, the cells will keep dividing giving rise to the several differenciated structures. Therefore differential growth and death of cell types, accompanies development throughout (Slack, 2001). Cell and tissue movements are a requirement for *Dictyostelium* and embryonic animal development but these are highly reduced in plant development, where pattern formation occurs mainly by oriented cell division and cellular differentiation. In *Dictyostelium* however, we find that growth by means of cell division is separated from development. *Dictyostelium* development is based on the aggregation of a genetically heterogeneous population, albeit belonging to the same species, of vegetative cells that will differenciate rather than on cell division. Multicellular development also requires of regional specification that will establish optimal ratios of the newly formed specialized cell types and define the final pattern throughout the newly formed structure.

Dictyostelium discoideum life cycle

Dictyostelium amoebas are free-living cells that feed on bacteria present in the soil and divide for as long as food is available. Upon starvation, *Dictyostelium* cells stop dividing and enter the developmental program that will lead to the formation of a multicellular fruiting body (Figure 1). *Dictyostelium* cells secrete quorum-sensing factors such as PSF (pre-starvation factor) and CMF (conditioned-medium factor) (Clarke and Gomer, 1995). These factors combined with starvation conditions help establish whether the optimal cell density for multicellular development has been reached and trigger induction of early gene expression. The expression of the some early genes is fast and transient whilst expression of genes that encode proteins involved in cAMP signalling is sustained. This causes cells to secrete nanomolar pulses of cAMP to the extracellular medium. cAMP acts as a chemoattractant and will summon cells to the aggregate. At the same time every cAMP pulse that is produced elicits a new cAMP pulse in neighbouring cells. This relay response causes the pulses to propagate as waves through the entire cell population leading to the formation of aggregates of up to 100.000 amoebas. The cAMP pulses also further accelerate the expression of cAMP signalling genes and other genes that are involved in aggregation. Once aggregates have formed, a tip develops at the centre and elongates giving rise to a finger-shaped structure called the slug. When the slug topples over it starts to migrate guided by signals as light and temperature. Cell differentiation takes place in parallel to morphogenesis and two main cell types arise in newly formed *D.discoideum* slugs: prestalk cells (precursors of the stalk cells) and prespore cells (precursors of the spores). In the slug the two cell populations are arranged along an anterior-posterior axis in which the prestalk cells are in the most anterior third of the slug, leaving the prespore cells to occupy the posterior part. Intermixed between the prespore cells there is a third cell type that has prestalk-like properties known as anterior-like cells (ALC). Fruiting body formation or culmination takes place through complex morphogenetic movements and a complete rearrangement of the slug cells. The *Dictyostelium* fruiting body consists of a cellulose-rich stalk tube that is filled with highly vacuolated dead prestalk cells and a spore head that contains the spores embedded in a mucous matrix. The proportion of spore cells to stalk cells in the fruiting body is roughly 3:1, so the majority of cells differentiate into spores. The mass of spores is lifted during culmination to the top of the stalk tube by an unknown mechanism, which probably involves active movement of the prespore cells and expansion of cells within the stalk tube. When favourable conditions such as the right humidity, temperature or the presence of food are met, spores germinate into new vegetative amoebas closing the cycle.

In addition to cAMP, PSF and CSF, a number of signalling molecules that regulate other developmental transitions have been identified. DIF (Differentiation Induction Factor) regulates the expression of a subclass of prestalk genes (Thompson and Kay, 2000). The catabolite ammonia inhibits spore and stalk maturation during slug migration (Gee et al., 1994; Hopper et al., 1993). The small peptides SDF1 and SDF2 (Spore Differentiation Factor 1 and 2) control culmination and spore maturation respectively (Anjard et al., 1998; Anjard and Loomis, 2005). Adenosine, an end product of cAMP degradation, is proposed to act as long-

range inhibitor of tip formation and as an inhibitor of prespore differentiation in the prestalk region (Newell, 1982; Newell and Ross, 1982; Schaap and Wang, 1986).

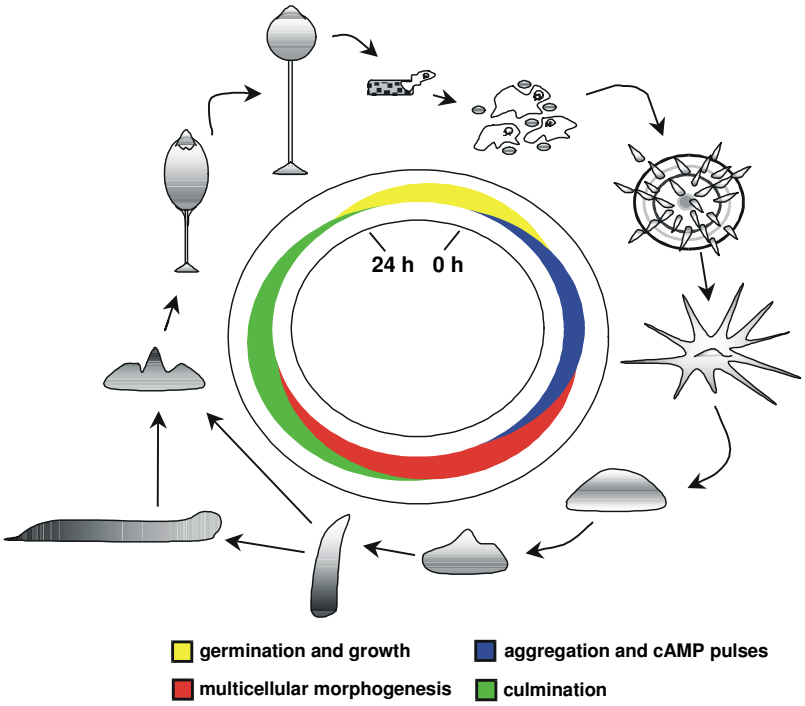

Figure 1. Life cycle of *Dictyostelium discoideum*

cAMP signalling during development

cAMP has multiple roles in *Dictyostelium* discoideum development. It acts as an extracellular signal controlling chemotaxis, expression of aggregative genes and prespore genes. cAMP also functions as in intracellular signal controlling initiation of development, spore and stalk maturation and spore germination (Figure 2) (Saran *et al.,* 2002). It is therefore of great importance to understand how cAMP is synthesized, detected and degraded and how these processes are regulated in the different stages of development.

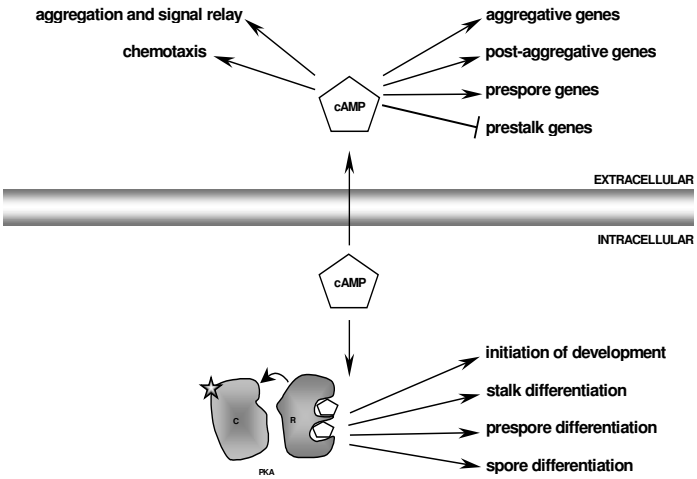

Figure 2. Roles of extracellular and intracellular cAMP

cAMP synthesis

There are three adenylyl cyclases producing cAMP during *Dictyostelium* development. The extracellular cAMP required for aggregation is produced by ACA. The *acaA* gene was identified by PCR through its homology with the catalytic domain of the *Drosophila* and mammalian adenylyl cyclases (Pitt et al., 1992). ACA consists of 12 transmembrane domains and two cyclase catalytic domains. The two catalytic domains represent half-sites of the catalytic core and ACA requires dimerization of these sites to be active. The mechanism of activation of ACA is complex, involving many molecular components (Figure 3). Binding of cAMP to the surface cAMP receptor cAR1, causes the heterotrimeric G-protein to dissociate releasing the Gα2 and βγ-subunits. This triggers the activation of the phosphoinositol specific kinase PI3-kinase, resulting in an increased production of phosphatydyl-inositol 3-phosphate, PIP3. Newly formed PIP3 acts as anchoring place binding to the pleckstrin homology domain of the cytosolic regulator of adenylyl cyclase, CRAC (Dormann et al., 2002), causing its translocation to the membrane and consequently promoting ACA activation. In addition to G-proteins and receptors, full activation of ACA requires of a number of other proteins such as ERK2 (Segall et al., 1995), Rip3 (Lee et al., 1999), RasC (Kae et al., 2004), Aimless and Pianissimo (Chen et al., 1997; Insall et al., 1996). Expression of *acaA* is highest during aggregation and remains present at lower levels in later stages. *acaA* deletion blocks aggregation but this defect can be restored by the addition of exogenous extracellular cAMP (Pitt et al., 1993).

Oscillatory cAMP signalling controls major aspects of *Dictyostelium* development. ACA activity is regulated by positive and negative feedback loops, which causes developing cells to produce and secrete cAMP in a spontaneous manner at regular intervals (Figure 3). The positive loop is caused by the self-stimulatory effect of extracellular cAMP produced by ACA acting on the cAMP receptor cAR1. However, persistent stimulation with cAMP causes desensitisation of the cells also known as adaptation. This process represents a negative feedback loop in the regulation of ACA (Martiel and Goldbeter, 1987; Tang and Othmer, 1994). Neither of the processes leading to excitation or adaptation of ACA are fully understood. Regulators of G-protein signalling (RGS), ligand-induced phosphorylation of the surface receptor, and the action of the intracellular cAMP-stimulated phosphodiesterase *PdeE* have been suggested to participate at different levels in the adaptation process (Devreotes, 1994; Manahan et al., 2004; Meima et al., 2003).

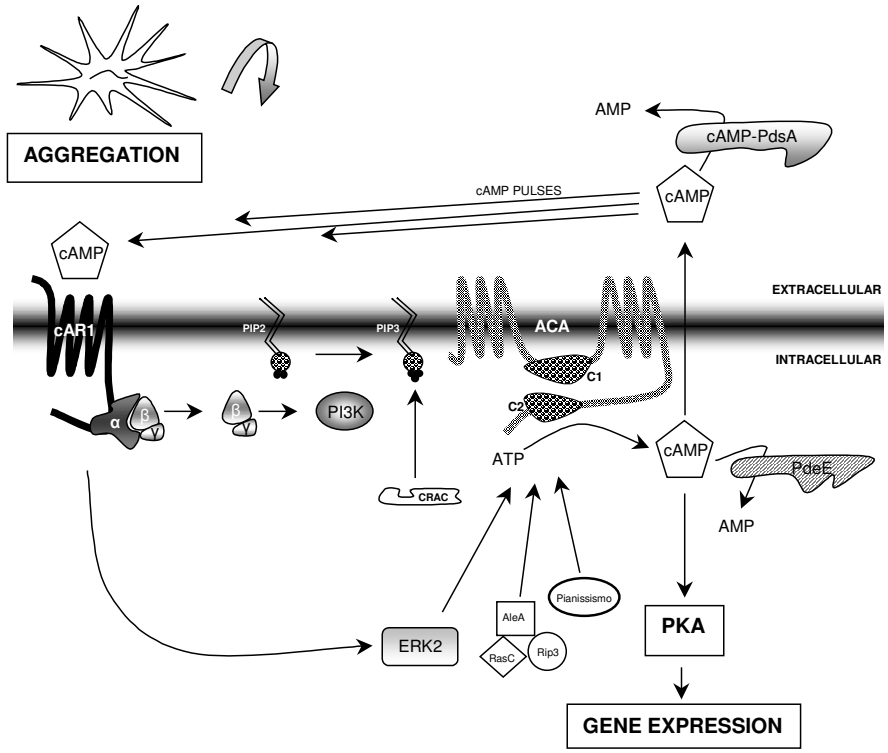

Figure 3. Signalling pathways controlling the aggregation specific cyclase ACA. ACA: adenylyl cyclase A; cAR1: cAMP-specific receptor 1; α, β ,γ: G-protein subunits; PIP2 and PIP,3: phosphatydylinositol phosphate 2 and 3; C1, 2: catalytic domain 1 and 2; PI3K: phospho-inositol specific kinase 3; CRAC: cytosolic regulator of adenylyl cyclase; AleA: Aimless; Pia: Pianissimo; Rip3: Ras interacting protein 3; PKA: protein kinase A; PdsA: extracellular phosphodiesterase; PdeE: intracellular phosphodiesterase.

AcgA, originally described as a germination specific enzyme, was the first adenylyl cyclase identified in the screening that yielded *AcaA*. Structurally ACG is reminiscent of membrane-bound guanylyl cyclases (Pitt et al., 1992). ACG has a single catalytic domain, a transmembrane domain and an extracellular domain. The extracellular domain shares homology with the CHASE-type domains (CHASE=Cyclases/Histidine Kinases Associated Sensory Extracellular). This type of domain is found in bacteria and lower eukaryotes and in receptor-like proteins of plants like the cytokine receptor *Cre1* (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). ACG activity is stimulated by high osmolarity (Figure 4), and although the mechanism of activation of ACG is not yet resolved, heterologous expression of the protein in yeast has established that the osmosensor is intrinsic to the ACG protein (Saran and Schaap, 2004). ACG is present in cells as a homodimer as demonstrated using engineered mutant ACG proteins lacking the catalytic domain that act as dominant-negative inhibitors (Saran and Schaap, 2004). Although the development of *acgA* null mutants is not significantly altered, germination of the spores under high osmotic conditions is not inhibited (Van Es et al., 1996). Spores are suspended in the spore head in a droplet of fluid that contains ammonium phosphate at concentrations higher than 100 mM (Cotter et al., 1999). This high osmolarity keeps ACG active, elevating the levels of cAMP inside the spore (Van Es et al., 1996; Virdy et al., 1999). Consequently, PKA remains active and spore germination is inhibited.

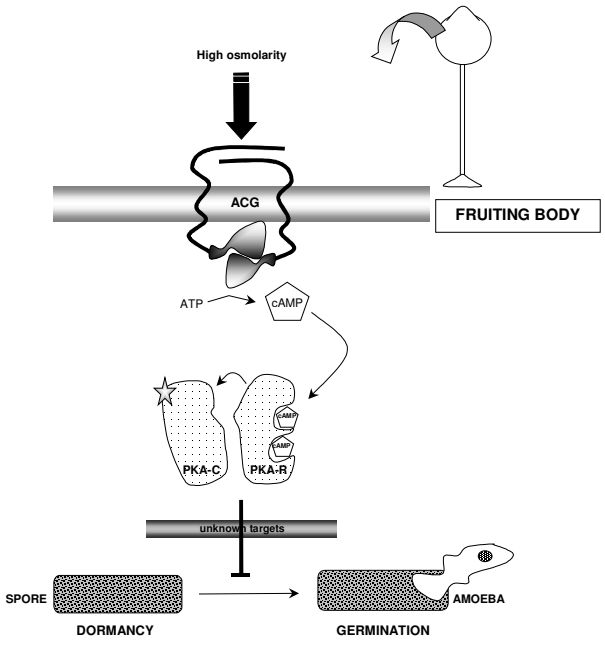

Figure 4. ACG is an osmosensor and controls spore dormancy in *Dictyostelium*

Lastly, the third *Dictyostelium* adenylyl cyclase (*AcrA*) encodes a protein (ACB/AcrA) that harbours a single cyclase domain, a response regulator (RR), most commonly involved in phosphorelay pathways, and a histidine kinase homology domain (HK) (Figure 5). This histidine kinase domain is predicted to be inactive as it lacks the histidine residue essential for phosphorylation. ACB/AcrA shares greatest homology with the CyaC adenylyl cyclases of the cyanobacterias *Spirulina platensis* and *Anabaena spirulensis* (Soderbom et al., 1999). ACB/AcrA sequence information also suggests that the enzyme has two putative transmembrane domains separated by an extracellular region. Maximal ACB activity is found associated to the particulate fraction in *vitro* assays, which supports that the protein is associated to the membrane (Meima and Schaap, 1999). ACB differs biochemically and in its developmental regulation from the other two cyclases ACA and ACG. ACB activity shows preference for Mg^{+2}/ATP than Mn^{+2}/ATP, as is the case for ACA and ACG. Furthermore, ACB

does not seem to be activated by any known external stimuli such as cAMP, DIF, GTPγS, ammonia or bicarbonate (Kim et al., 1998, Meima and Schaap, unpublished results). Mutants of *acrA* show severe defects in terminal differentiation. They aggregate normally but they produce only about 10% of viable spores. Their fruiting bodies show abnormally long stalks and glassy spore heads due to the reduced number of spores (Soderbom et al., 1999).

cAMP hydrolysis

To maintain dynamic signalling, the degradation of cAMP by cyclic nucleotide phosphodiesterases is as important as its synthesis. Phosphodiesterases are generally classified into three classes, Class I to III. The main variations found between classes are in the sequence of their catalytic domains and within classes in cyclic nucleotide specificity, presence of additional domains and cellular localization.

Dictyostelium has several cAMP and cGMP specific phosphodiesterases that regulate both extracellular and intracellular cyclic nucleotide levels. The cAMP-specific phosphodiesterase *RegA* (Shaulsky et al., 1996; Thomason et al., 1998) and the cGMP-specific *PDE3* (Kuwayama et al., 2001) belong to the class I phosphodiesterases, whereas the extracellular *PdsA* (Lacombe et al., 1986) is related to the fungal class II of phosphodiesterases.

Hydrolysis of extracellular cAMP

PdsA can be secreted or bound to the extracellular face of the membrane (Gerisch and Malchow, 1976; Malchow et al., 1972). *PdsA* hydrolyses both cAMP and cGMP (Lacombe et al., 1986) but shows the highest affinity for cAMP. The catalytic domain is similar to the low-affinity yeast phosphodiesterase PDE1 (Nikawa et al., 1987). *PdsA* is under a tight developmental regulation and three different promoters control its expression. The vegetative promoter directs expression during growth, the aggregative promoter during aggregation and the late promoter after mound formation. The different promoters are also expressed in different cell types, with the late *PdsA* promoter being most active in prestalk cells and the aggregative promoter in anterior-like and rear-guard cells (Weening et al., 2003). A soluble glycoprotein inhibitor (PDI) that is secreted by cells regulates PdsA activity (Franke and Kessin, 1981).

PdsA knockout mutants are defective in aggregation. This defect can be rescued by expression of the gene under its aggregation promoter, but development is arrested at the mound stage (Darmon et al., 1978; Sucgang et al., 1997). Only expression under the late promoter is able to drive development into slug and fruiting body formation. Constitutive overexpression of the gene causes accelerated aggregation, but development beyond the mound stage is blocked (Faure et al., 1988). These data demonstrate the essential role of PdsA in the regulation of dynamic cAMP signalling during the entire course of development.

Hydrolysis of intracellular cAMP

Hydrolysis of intracellular cAMP occurs by the action of two different phosphodiesterases. RegA is a cAMP-specific phosphodiesterase that harbours a prokaryote-type response regulator in addition to a mammalian-type phosphodiesterase domain. *RegA* was first identified from a REMI sporogenous mutant that showed accelerated development (Shaulsky et al., 1996; Thomason et al., 1998). Response regulators are found in two-component phosphorelay systems and act as targets for phosphoryl groups that are passed through a relay cascade initiated by sensor histidine kinases (Figure 5).

Two sensor histidine kinases have been proposed to control RegA activity. *DhkC* functions as an ammonia sensor in slug cells. Ammonia is a well-known inhibitor of culmination and *DhkC* mutants show unnaturally prolonged slug migration (Singleton et al., 1998). A phosphoryl group donated by the auto-phosphorylated histidine in *DhkC* is transferred to *RegA* through the action of the intermediate phospho-donor protein RdeA. This phosphorylation causes a 20-fold increase of the RegA phosphodiesterase activity (Thomason et al., 1999).

The resulting hydrolysis of cAMP and consequent inactivation of PKA activation inhibits the transition from slug migration to culmination.

The second sensor histidine kinase, *DhkA*, acts as a histidine phosphatase when activated by its ligand SDF-2, causing dephosphorylation of RegA and therefore inactivation (Anjard and Loomis, 2005; Wang et al., 1999). SDF-2 is a small peptide that is produced by stalk cells and triggers the maturation of spores (Anjard *et al.,* 1998). Inactivation of *RegA* causes an elevation on cAMP levels and cAMP in turn activates PKA inducing terminal differenttiation.

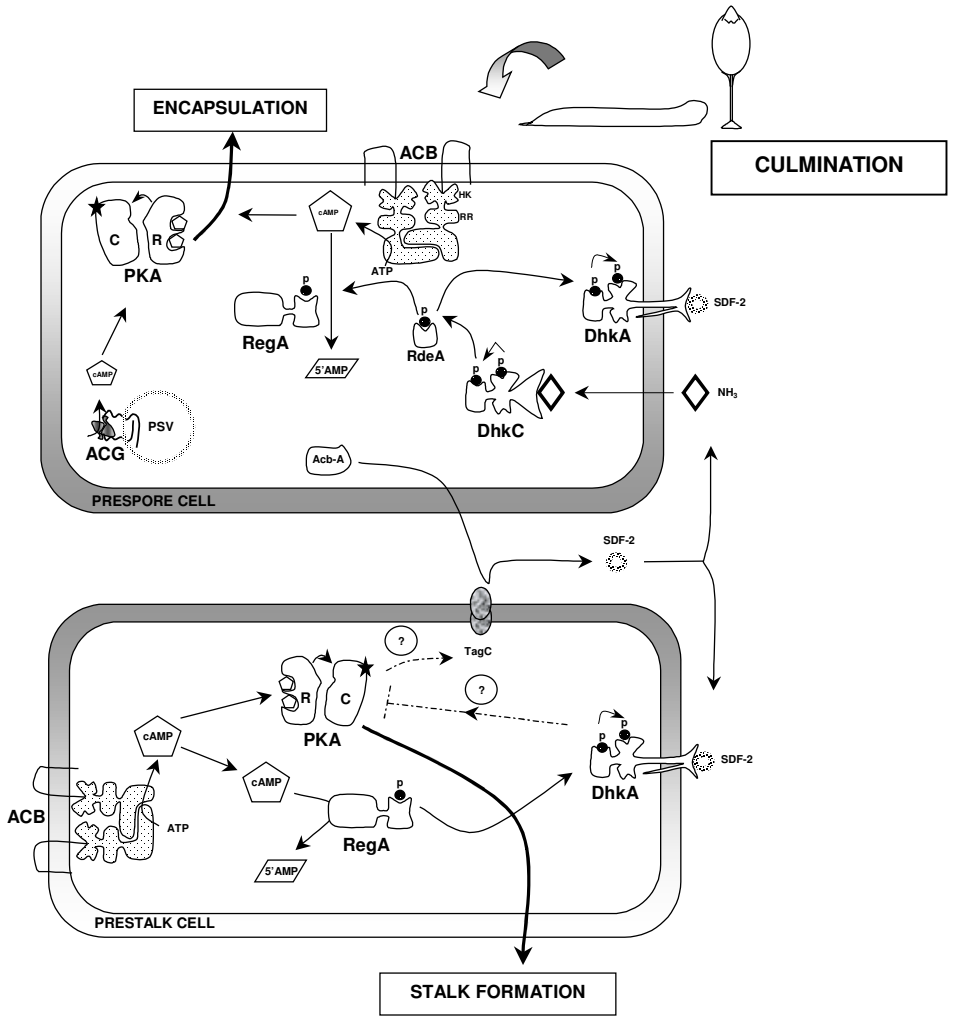

Figure 5. Regulation of post-aggregative development by cAMP, ACB/AcrA and RegA
ACG and ACB: adenylyl cyclases G and B; HK: histidine kinase domain; RR: response regulator domain; PKA: protein kinase A; C and R: catalytic and regulatory PKA subunits; RegA: intracellular phosphodiesterase; RdeA: phospho-donor protein; DhKA and C: hybrid histidine kinases A and C; SDF-2: spore differentiation factor 2; AcbA: SDF-2 precursor protein; TagC: serin protease C; PSV: prespore vesicle; p: phosphoryl group.

The other intracellular cAMP phosphodiesterase, *PdeE*, is an unusual protein that harbours two cyclic nucleotide-binding domains similar to those found in the regulatory subunit of the bovine PKA and a metallo-β-lactamase domain (Meima et al., 2003). PdeE is structurally similar to PdeD, a cGMP specific phosphodiesterase, and work on *PdeD* has shown that the metallo-β-lactamase domain is responsible for the cyclic nucleotide phosphodiesterase activity, while the cyclic nucleotide binding domains act as allosteric activators of the PDE activity (Meima et al., 2002). PdeE is mostly active during aggregation. Development of *PdeE* null mutants is not significantly altered and they show a modest increase in cAMP relay response. Overexpression of the *PdeE* gene blocks aggregation, but development is

restored when overexpressing cells are developed in synergy with wild type. The timing of expression as well as the elevated cAMP relay response suggests a role for *PdeE* during aggregation, possibly controlling the adaptation process.

cAMP detection

Extracellular cAMP

Extracellular cAMP controls both chemotaxis and gene expression in different developmental stages by acting on cell surface cAMP receptors. Four homologous cAMP receptors with different cAMP affinities are expressed at different stages of development (Johnson et al., 1993; Klein et al., 1988; Louis et al., 1994; Saxe III et al., 1993). These cAMP receptors (*cAR1 to cAR4*) belong to the class E of G-protein coupled seven transmembrane receptors (GPCRs). The four cARs differ in their affinity for cAMP in a manner that correlates with their timing of expression. The receptor with the highest cAMP affinity (*car1*) is expressed before and during aggregation (Johnson et al., 1992a; Johnson et al., 1992b). *cAR3* is also a high affinity receptor and is expressed a few hours after *cAR1*, while the low affinity receptors *cAR2* and *cAR4* are expressed during post-aggregative stages in slugs and fruiting bodies

cAR activation of several target enzymes, such as the two guanylyl cyclases sGC and GCA, PI3-kinase, ACA and phospholipase C is mediated by heterotrimeric G-proteins (Aubry and Firtel, 1999). Expression of the several Gα subunits that form the heterotrimeric G-proteins is also developmentally regulated. In *Dictyostelium* there are more than twelve different α-subunits that are transiently expressed at different times of development (Eichinger et al., 2005). Of these α-subunits only Gα2 seems to be essential for development. On the other hand there is a unique β-subunit expressed at a constant rate throughout development. Disruption of the gene encoding the β-subunit results in failure to aggregate (Wu et al., 1995).

Some of the cAR-mediated pathways are independent of G-proteins such as the induction of Ca^{+2} influx, ERK2 activation, STATa translocation to the nucleus and prespore gene expression (Araki et al., 1998; Jin et al., 1998; Maeda et al., 1996; Milne et al., 1995; Wu et al., 1995). The immediate targets for the receptors in these responses are still unknown.

Intracellular cAMP

Similarly to other organisms, in *Dictyostelium* cAMP is also used as an intracellular second messenger that activates cAMP-dependent protein kinase (PKA). *Dictyostelium* PKA is a heterodimer consisting of one regulatory subunit (PKA-R) and one catalytic subunit (PKA-C) (Mann et al., 1992). The homologous vertebrate enzyme consists of two PKA-C and two PKA-R subunits. In *Dictyostelium*, activation of PKA leads to the dissociation of the PKA-C-R complex upon binding of two cAMP molecules to the regulatory subunit. PKA activity is therefore mainly regulated by intracellular cAMP. However, the protein and mRNA of both PKA-R and PKA-C subunits differentially accumulate during the first 12 hours of development, which indicates the presence of additional regulation at the transcriptional and translational levels. PKA is not essential for vegetative growth but it is involved in almost every other aspect of *Dictyostelium* development from aggregation to terminal differentiation (Mann et al., 1992; Simon et al., 1992).

Null mutants in PKA-C do not aggregate by themselves, but can aggregate in synergy with wild type. They show normal induction of aggregation specific genes in response to cAMP pulses, which suggests the presence of a second PKA-C protein (Mann et al., 1992, Meima and Schaap, unpublished results). On the other hand, overexpression of PKA-C leads to rapid development and to a sporogenous phenotype (Anjard et al., 1992). In addition to this, altering the levels of expression of the regulatory subunit modulates PKA activity. A version of the regulatory subunit containing mutated cAMP binding sites (PKA-Rm) acts as dominant negative inhibitor of PKA and its overexpression blocks the relay response and early gene induction (Schulkes and Schaap, 1995).

PKA activity is also required for both prespore and prestalk differentiation. Expression of PKA-Rm under prespore or prestalk promoters inhibits the expression of prespore and prestalk genes respectively (Harwood et al., 1992; Hopper et al., 1993; Hopper et al., 1995; Hopper and Williams, 1994; Zhukovskaya et al., 1996). Terminal spore and stalk maturation are dependent on PKA as described previously. Once the spores are formed, high osmolarity in the spore head activates adenylyl cyclase G to produce cAMP. Here PKA activation by cAMP inhibits the germination of spores (Saran and Schaap, 2004; Van Es et al., 1996). This mechanism ensures that the spores do not germinate while still in the fruiting body or under adverse conditions.

PKA activity is found in both the cytosol and in the nucleus, but despite being so ubiquitous and having such prominent effects during development the direct targets for PKA phosphorylation are still unknown (Figure 6).

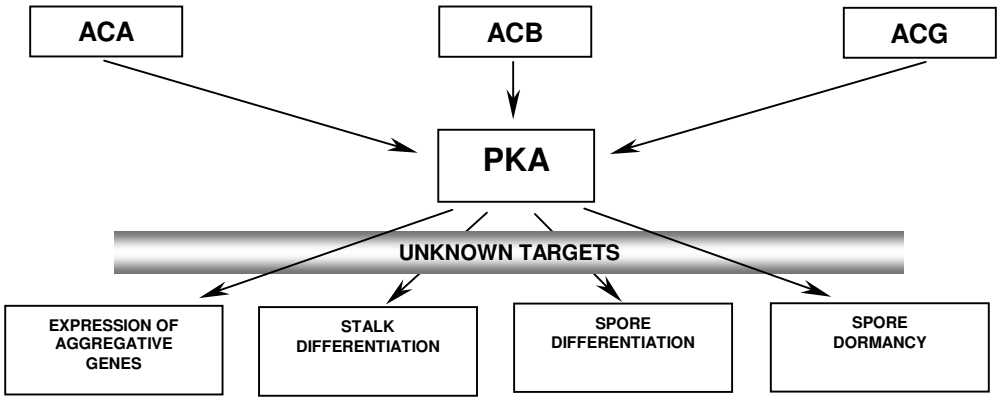

Figure 6. Roles of PKA at different stages of *Dictyostelium* development.

Regulation of pattern formation in *Dictyostelium*

One of the most dramatic aspects of *D.discoideum* development is the differentiation of an initially homogeneous population of amoebas into prespore and prestalk cells, whose proportions and spatial patterning are carefully regulated. Early work showed that prespore differentiation in the rear of the slug requires the sustained presence of extracellular cAMP (Schaap and Van Driel, 1985, Schaap and Wang, 1986), but until now it has been unclear which of the three *Dictyostelium* adenylyl cyclases produces the cAMP required for this purpose. DIF was previously proposed to trigger the differentiation of prestalk and stalk cells, but recent studies with DIF-deficient mutants indicate that DIF is not required for the stalk cell differentiation and its absence only affects a small subpopulation of prestalk genes. Major challenges therefore still exist to establish how the prespore/prestalk pattern is generated.

Prespore cell differentiation is triggered once aggregation has taken place by the combined action of extracellular cAMP binding to surface receptors and intracellular cAMP binding to PKA. However, neither the developmental regulation of the three *Dictyostelium* adenylyl cyclases nor the phenotypes of null mutants in their respective genes provides any clue how this might occur. ACA is expressed in all cells during aggregation, but during slug formation expression is lost from all cells except those at the anterior tip. *AcaA* null mutants cannot aggregate, but development can be restored to some extent by overexpression of PKA or by prolonged treatment with extracellular cAMP. *ACG* mRNA was only found in spores and *acgA* null mutants were reported to form normal fruiting bodies. ACB is the most likely to produce the cAMP that triggers prespore differentiation, since it is most active in the slug and early culmination stage. However, although null mutants in ACB display a late defect in spore maturation, they express prespore genes normally.

The failure to identify the source of cAMP for prespore differentiation may have several causes: i. There might be another yet unidentified cyclase. This possibility we consider unlikely because the fully sequenced *Dictyostelium* genome contains no other genes with the highly conserved nucleotidyl cyclase domain than the adenylyl cyclases ACA, ACB and ACG and the guanylyl cyclases sGC and GCA. ii. The three adenylyl cyclases are functionally redundant. This is a more likely scenario that could particularly complicate interpretation if the three cyclases negatively regulate each other's expression. A lesion in one of the cyclases would then automatically lead to upregu-lation of expression of the three others. Such a mechanism is already indicated by ACA expression, which is down-regulated by cAMP in slug cells (Verkerke-van Wijk et al., 2001).

Aims of this thesis

In this thesis I will concentrate in identifying the specific roles of the three adenylyl cyclases and cAMP in cell differentiation, pattern formation and particularly prespore gene induction. The first chapter describes a series of studies of the spatio-temporal expression pattern of *ACG* and *ACB* in *Dictyostelium* and the manner in which each adenylyl cyclase influences the expression of the others as well as the induction of prespore cells and the maturation of spores. This work shows an unexpected role for *ACG* in the induction of prespore differentiation.

Due to the functional redundancy that seems to be present between the adenylyl cyclases I performed a search for specific inhibitors for any of the three adenylyl cyclases and this is described in Chapter Two. Such inhibitors can then be used to study the effects on development of acute inhibition of a specific adenylyl cyclase, without compensation by up-regulation of the other enzymes. This work has lead to the identification of two enzyme specific inhibitors.

In Chapter Three I have explored the role of adenosine in morphogenesis by studying the effects of gene disruption of an adenosine kinase that converts extracellular adenosine into 5'AMP, thus regulating the extracellular levels of the molecule.

In Chapters Four and Five I have investigated cAMP signalling from an evolutionary perspective. In the course of this work I have contributed to the construction of the first molecular phylogeny of the Dictyostelids, which is described in Chapter Four. In Chapter Five I present a novel approach to dissect cAMP signalling pathways by reconstructing their evolutionary history. Using this approach I have studied how deeply each cAMP-signalling pathway is conserved in order to identify the ancestral core functions for cAMP signalling. I have then tried to reconstruct how each pathway was elaborated and modified during evolution and how these innovations in signalling are correlated with the appearance of novel morphologies and behaviours.

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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
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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 counterstained 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).

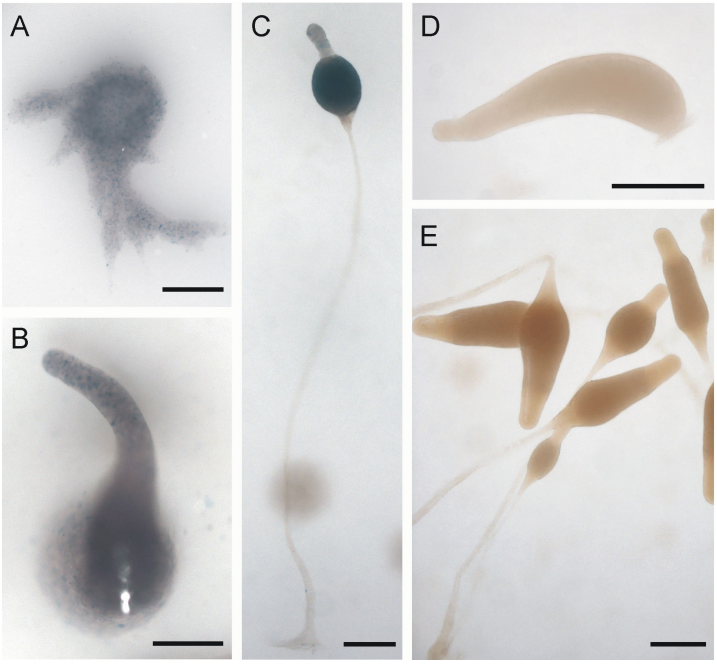


Figure 1. ACG promoter activity in developing structures
(A-C) *D. discoideum* wild-type cells were transformed with vector ACG::gal, which contains a gene fusion of the ACG promoter and the β-galactosidase reporter gene. Cells were starved on nitrocellulose filters supported by PB agar and developing 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.

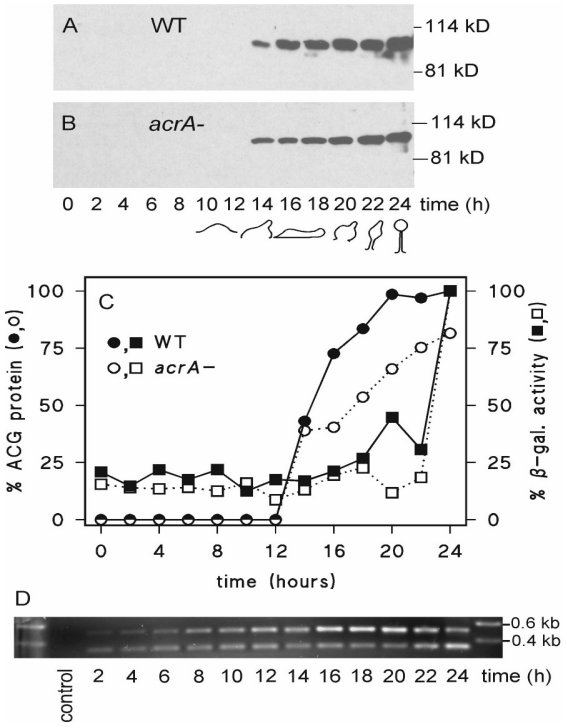


Figure 2. Developmental regulation of ACG transcription and ACG protein accumulation
(A,B). *Dictyostelium* wild-type (WT) cells (A), the *acrA* mutant (B) and both cell lines transformed with the ACG::ile-gal construct were incubated for 24 hours on PB agar. Every 2 hours, WT and *acrA*- cells were 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.

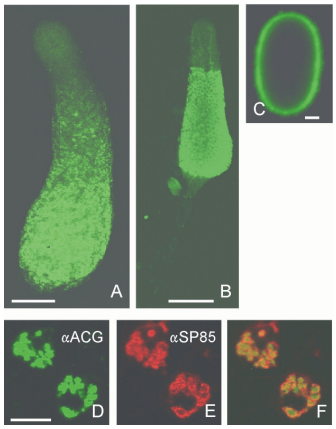


Figure 3. ACG protein in intact structures and cells
(A-C) Intact wild-type slugs (A), fruiting bodies (B), spores (C) werefixed in methanol and stained with ACG antibody and FITC conjugated goat-anti-rabbit IgG (GARFITC). (D-F). Slugs were dissociated into single cells, which were first stained with 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.

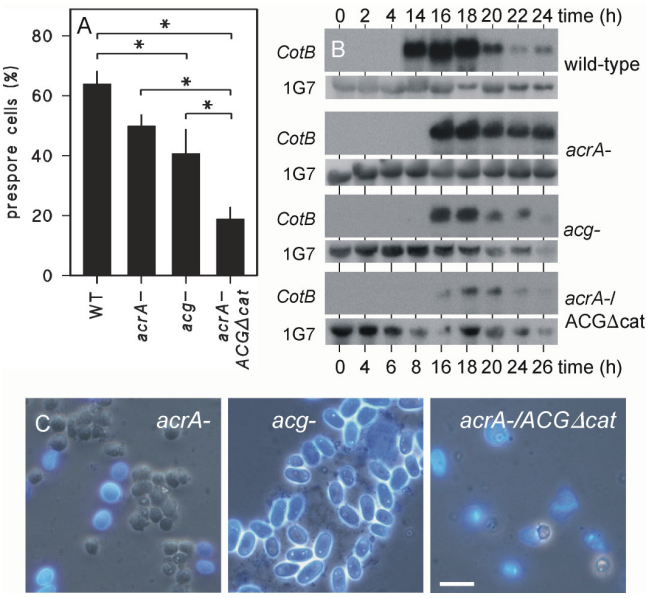
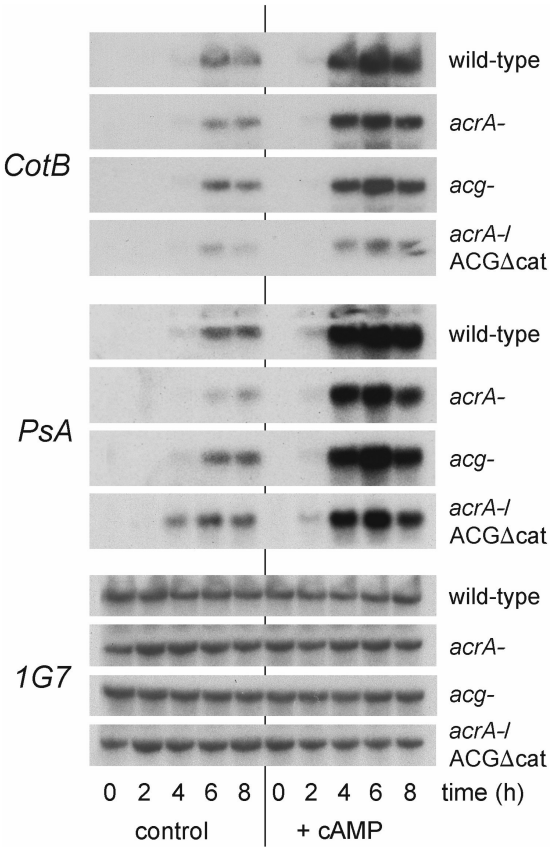


Figure 4. Prespore and spore differentiation in adenylyl cyclase mutants
(A) Slugs of wild-type, *acrA*-, *acg*- and *acrA-/A15::ACGΔcat* cells that had migrated for 2-3 hours were dissociated and stained with a spore-specific antiserum and GARFITC. Cell nuclei were counterstained with DAPI. The percentage 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.

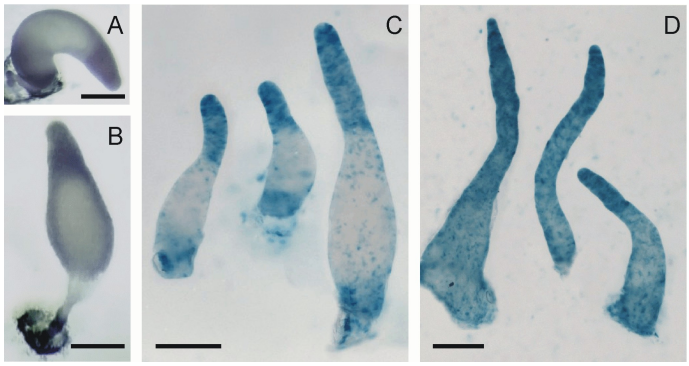


Figure 6. *AcrA* expression in wild type and *acg-* cells
(A,B) *AcrA* expression detected by *in situ* hybridization. Wild-type cells were starved on dialysis membrane supported by PB agar. *AcrA* mRNA was visualized in migrating slugs (A) and mid-culminants (B) by in situ hybridisation to a DIG-labeled *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.

Acknowledgments
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Chapter Two

Pharmacological profiling of the *Dictyostelium* adenylyl cyclases ACA, ACB, and ACG

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Pharmacological profiling of the *Dictyostelium* adenylyl cyclases ACA, ACB and ACG
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Abstract

Intracellular and secreted cAMP play key roles in controlling cell movement and regulation of numerous genes throughout development of the social amoeba *Dictyostelium discoideum.* cAMP is produced by three structurally distinct adenylyl cyclases, ACA, ACG and ACB which have distinctive but overlapping patterns of expression and, as concluded from gene disruption studies, seemingly overlapping functions. In addition to gene disruption, acute pharmacological abrogation of protein activity can be a powerful tool to identify its role in the biology of the organism. I have analysed the effects of a range of compounds on the activity of ACA, ACB and ACG to identify enzyme-specific modulators. Caffeine, which was previously used to specifically block ACA function, also inhibited cAMP accumulation by ACB and ACG. 2'3'-O-methyl isopropylidene adenosine (IPA) specifically inhibits ACA when measured in intact cells, without affecting ACB or ACG. All three enzymes are inhibited by the P-site inhibitor 2'5'dideoxyadenosine (DDA) when assayed in cell lysates, but not in intact cells. Tyrphostin A25 and SQ22536 proved to be effective and specific inhibitors for ACG and ACA respectively. Both compounds acted directly on enzyme activity assayed in cell lysates, but only SQ22536 was also a specific inhibitor when added to intact cells.

Introduction

The evolution of social amoebas or Dictyostelids was accompanied by extensive elaboration of cAMP signalling pathways (1). In *Dictyostelium discoideum*, cAMP acts as a classical second messenger for external stimuli. In this role, cAMP controls the initiation of multicellular development, the maturation of stalk and spore cells and the germination of spores. cAMP is also secreted in a highly regulated manner. As an extracellular signal, it coordinates the aggregation of starving cells and the directional movement of cells in multicellular structures. In addition, extracellular cAMP acts as a trigger for gene regulation at different stages of development (2,3).

D.discoideum has three structurally distinct adenylyl cyclases (ACs), ACA, ACB and ACG, for synthesis of cAMP. ACA produces cAMP for cell aggregation. It is structurally similar to the mammalian adenylyl cyclases with two different catalytic domains that are interspersed by two sets of six transmembrane helices (4). Similar to mammalian adenylyl cyclases, ACA is activated by a serpentine receptor, in this case the cAMP receptor cAR1 that interacts with a heterotrimeric G-protein, G2. However, in contrast to mammalian ACs (5), the G-protein does not interact directly with ACA. Instead the G2 βγ-subunit activates a phospholipid inositol kinase that generates plasma membrane binding sites for the Cytosolic Regulator of ACA, CRAC, which activates ACA upon recruitment to the plasma membrane (6).

ACG has an extracellular sensor domain, one or two transmembrane helices, and a single intracellular catalytic domain. ACG is an osmosensor that controls the germination of spores (7,8), and has an overlapping role with ACA and ACB in triggering prespore differentiation (See Chapter One). The catalytic domain of ACB (9), encoded by the *AcrA* gene (10), is homologous to that of the bicarbonate regulated bacterial adenylyl cyclases (11). Similar to the CyaC adenylyl cyclases from the cyanobacteria *Anabena spirulensis* and *Spirulina platensis*, ACB also harbours a response regulator domain and a histidine kinase domain. ACB is required for the maturation of spores (10).

Information on the role of each of the enzymes in particular aspects of the developmental programme has been derived from studies with null mutants in their respective genes (4,7,10). However, this approach precludes the demonstration of late developmental roles for those enzymes that are essential for an early stage of development. Moreover, the results shown in Chapter One indicate that the *Dictyostelium* adenylyl cyclases negatively regulate each other's expression. As a consequence, gene disruption in each of the genes will lead to overexpression of the others and partial or full restoration of the function of the abrogated gene . The use of enzyme-specific inhibitors with acute effects circumvents such problems.

Similar to the mammalian adenylyl cyclases, the *D.discoideum* enzyme ACG is active as a dimer, potentially creating two binding sites for ATP binding and catalysis (8). For ACA, ran-

dom mutagenesis studies have identified amino acids that are either essential for catalysis or for regulation by upstream components in the signalling pathway (12,13). However, apart from these data no structural information on enzyme regulation is available. In addition to studies of the protein crystal structure, the elucidation of the catalytic mechanism of the mammalian adenylyl cyclases has benefited greatly from pharmacological interference with enzyme activity. Notably the use of some ribose-modified adenosine analogs (known as P-site inhibitors) and of the AC activator forskolin, have contributed considerably to the understanding of how ATP interacts with the catalytic site and how the catalytically active dimer is formed (14,15).

In *Dictyostelium*, ACA is not activated by forskolin, but its activity in intact cells is inhibited by caffeine and by ribose-modified adenosine analogs (16-18). However, it is not clear whether the target for the analogs is ACA itself or the cAMP receptor that activates ACA (19,20). Neither these compounds nor any of the drugs known to directly modulate the activity of the mammalian adenylyl cyclases have yet been tested on ACG or ACB.

In this work we perform a systematic investigation of the effects of caffeine, ribose-modified adenosine analogs and other known regulators of mammalian adenylyl cyclases on the activities of ACA, ACB and ACG. This study identifies two enzyme-specific inhibitors for the *Dictyostelium* adenylyl cyclases and indicates that the effects of caffeine on adenylyl cyclase inhibition are mediated by two different targets.

Material and methods

Materials, cell lines and cell cultures

Guanosine 5'-O-(3-thiotriphosphate) (GTPγS), 2'-deoxyadenosine 3',5'-monophosphate (DcAMP), 2',3'-O-isopropylideneadenosine (IPA), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), sodium pyrophosphate (PPi) and G418 were from Sigma (St.Louis, USA). 2',5'-dideoxyadenosine (DDA), α-cyano-(3,4,5-trihydroxy)cinnamonitrile (tyrphostin A25), 9-(tetrahydro-2'-furyl)adenine (SQ22536), cis-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine, HCl (MDL-12,330A), 2-Amino-7-(furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80) were from Calbiochem (San Diego, USA). [2,8-^{3}H]cAMP was from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). *Naja messambica* snake venom was from SA venom suppliers (Louis Trichardt, South Africa)

Wild type NC4 cells, aca*-/A15::ACG* (4) and *aca-/rdeA*- (9) mutants were grown in standard axenic medium, which was supplemented with 20 µg/ml G418 for *aca-/A15::ACG* cells. A *pdeE-/regA*- double null mutant was created by transforming *pdeE*- cells (21) with the *pRegA*KO construct (22). Null mutants were selected from blasticidin resistant transformed clones by two PCR reactions and Southern analysis of genomic digests. While *pdeE*- cells develop normally (21), the *pdeE-/regA*- cells displayed the rapidly developing phenotype of *regA*- mutants (22). aca-/A15::ACG, *aca-/rdeA*- and *pdeE-/regA*- cells were harvested during exponential growth, washed once with 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB) and resuspended in either PB or lysis buffer (2 mM $MgCl_2$ and 250 mM sucrose in 10 mM Tris, pH 8.0) to 10^8 cells/ml. Wild-type cells were plated on PB agar (1.5% agar in PB) at 2.5 x 10^6 cells/cm^2, starved for 6-8 hr at 22^{o}C until aggregation territories were formed, and subsequently collected and resuspended in PB or lysis buffer to 10^8 cells/ml.

Adenylyl cyclase assays in intact cells

Cells were resuspended in PB and exposed to either 5 mM DTT (ACB, ACG) or stimulated with 5 µM DcAMP in 5 mM DTT (ACA) in a total volume of 30 µl in microtiterplate wells under gentle agitation. After variable time periods, the reaction was terminated by addition of 30 µl of 3.5% (v/v) perchloric acid. Lysates were neutralized by addition of 15 µl of 50% saturated $KHCO_3$ and 75 µl cAMP assay buffer (4 mM EDTA in 150 mM K-phosphate, pH 7.5). Microtiterplates were centrifuged for 5 min at 3000 x g to precipitate protein and perchlorate. cAMP was assayed in 30 µl of the supernatant fraction by isotope dilution assay, using purified PKA

regulatory subunit (PKA-R) from beef muscle as cAMP-binding protein (23) and [2,8-^{3}H]cAMP as competitor. Since several of the compounds used in this work to alter adenylyl cyclase activity have some structural similarity to cAMP, and could potentially compete with [2,8-^{3}H]cAMP for binding to PKA-R, we compared and show t=0 time points for each assay without and with the highest concentration of the compound. No significant interference of any of the compounds with the cAMP assay could be detected.

Adenylyl cyclase assays in cell lysates

Cells were resuspended in ice-cold lysis buffer and lysed through nuclepore filters (pore size, 3 µM), conditionally in the presence of 30 µM GTPγS for NC4 cells. Aliquots of 10 µl cell lysate were added to 5 µl of variables at 4x the desired final concentration and 5 µl of assay mix (2 mM ATP, 0.8 mM IBMX and 40 mM DTT in lysis buffer), which was supplemented with 8 mM $MnCl_2$ for ACG and 38 mM $MgCl_2$ for ACB assays. After 5 min of incubation on ice, reactions were started by transferring the samples to a 22°C water bath. Reactions were terminated by adding 10 µl of 0.4 M EDTA, pH 8.0, followed by boiling for 1 min (23). cAMP was assayed directly in the boiled lysate. For all assays the cAMP levels were standardized on the protein content of the lysate or cell suspension.

cAMP phosphodiesterase assay

Cells were resuspended to 10^8 cells/ml in 10 mM DTT in PB, and incubated for 30 min with 10^{-7} M [2,8-^{3}H]cAMP, caffeine and IBMX as indicated in a total volume of 20 µl. Reactions were stopped by boiling, and the reaction product was converted into [2,8-^{3}H]adenosine by incubation for 30 min with 10 µg *Naja messambica* snake venom (which contains 5'nucleotidase). [2,8-^{3}H]adenosine was separated from [2,8-^{3}H]cAMP by adsorption of the latter to Dowex anion exchange resin and measured by scintillation counting.

Results

Effects of caffeine on ACA, ACB and ACG

The modified purine caffeine acts as an antagonist for adenosine A1 and A2A receptors in human brain (24), and also inhibits some mammalian cAMP phosphodiesterases (PDEs) (25). In *D.discoideum,* caffeine is commonly used to inhibit ligand-induced ACA activation (17). Its mode of action is not clear. It was suggested that caffeine could act by increasing cytosolic Ca^{2+} levels (17), but later studies showed that ACA activity was not inhibited by Ca^{2+} (26). To study whether caffeine is a specific inhibitor of ACA, we compared its effect on the activities of ACA, ACB and ACG in intact cells.

To measure each enzyme separately, we chose the following conditions: ACA was measured in wild-type NC4 cells that were starved for 6 hours to induce maximal expression of *ACA*. Cells were stimulated with the cAR agonist DcAMP in the presence of dithiothreitol (DTT) that acts here as an inhibitor of the extracellular phosphodiesterase PdsA (27). In *Dictyostelium,* cAMP is rapidly secreted after synthesis, and its extracellular accumulation can therefore be readily measured when PdsA is inhibited.

ACB is closely associated with the intracellular cAMP phosphodiesterase RegA. It appears to be constitutively active, but its activity can only be measured when RegA or the RegA activator RdeA is absent (9). ACB shows significant activity in vegetative *aca-/rdeA-* cells, which is not obscured by the presence of ACA. ACG is maximally expressed in spores, which are virtually inaccessible for measurement of adenylyl cyclase activity. This enzyme was therefore measured in vegetative *aca-* cells that express ACG from the constitutive A15 promoter (*aca-/A15::ACG*) (4). Although ACB is also present in vegetative cells, its activity (0.83 pmol/min.mg protein) is negligable compared to that of ACG (38 pmol/min.mg protein). cAMP production by both ACB or ACG can be measured during exposure of intact cells to DTT. Figure 1A shows that caffeine inhibits ACB, ACG and DcAMP stimulated ACA activity

equally effectively with an IC_{50} (effective concentration that produces halfmaximal inhibition) of 0.2-0.4 mM. Inhibition is complete at 10 mM, except for ACG where the higher caffeine concentrations become less effective. We next measured whether caffeine inhibited the three enzymes directly by testing its effect on the conversion of ATP into cAMP in cell lysates. Figure 1B shows that under these conditions caffeine does not inhibit ACB or ACG activity, nor the basal activity of ACA and even slightly stimulates the three enzymes at 3-10 mM. However, caffeine does inhibit GTPγS induced activation of ACA. These data indicate that neither ACA, ACB nor ACG are direct targets for the inhibitory effects of caffeine. They further more suggest that there are at least two different caffeine targets that mediate either its effect on GTPγS stimulation of ACA in lysates, or on cAMP production by at least two of the three ACs in intact cells.

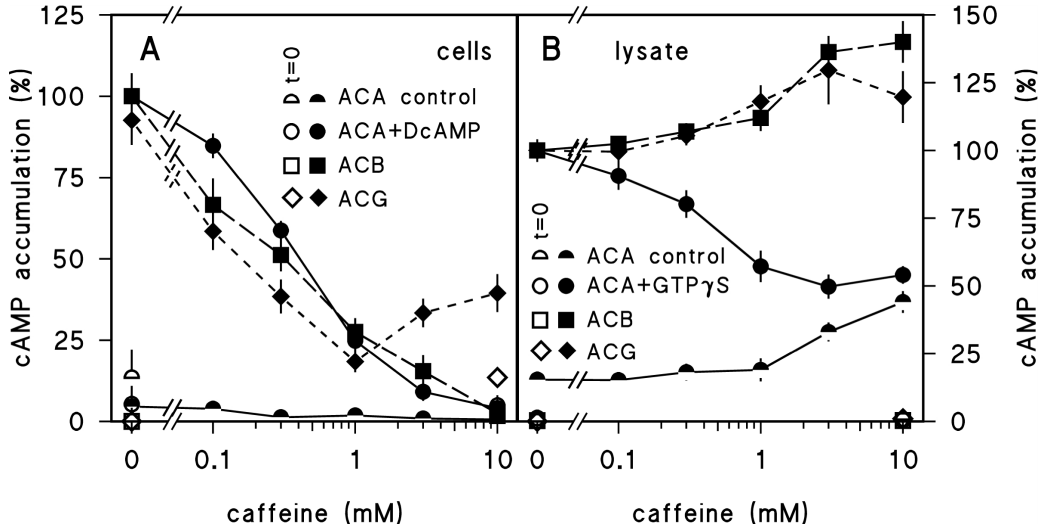

Figure 1. Effects of caffeine on ACA, ACB and ACG activity in intact cells and lysates
(A) *Intact cells.* For assay of ACA mediated cAMP accumulation, 6 hour starved NC4 cells were incubated for 0 and 3 min with 5 mM DTT in the presence and absence of 5 µM DcAMP. For assay of ACG, vegetative *aca-/A15::ACG* cells were incubated for 0 and 5 min with 5 mM DTT and for assay of ACB, vegetative *aca-/rdeA-* cells were incubated for 0 and 30 min with 5 mM DTT. Caffeine was present during incubation at the indicated concentrations. Reactions were terminated by addition of perchloric acid to 1.75% and cAMP was assayed in the neutralized cell lysates. Data are expressed as percentage of cAMP accumulation measured at the maximal incubation time for each enzyme in the absence of caffeine, and for ACA in the presence of DcAMP. This was 43.3±15.5, 24.6±5.1 and 190±53 pmoles cAMP/mg protein for ACA+DcAMP, ACB and ACG respectively.
(B) *Cell lysates.* Six hour starved NC4 cells were filter-lysed in the presence and absence 30 µM GTPγS and incubated for 0 and 5 min with 0.5 mM ATP, 2 mM $MgCl_2$ and 10 mM DTT for assay of ACA. Vegetative *aca-/A15::ACG* cells were lysed and incubated for 0 and 5 min with 0.5 mM ATP, 2 mM $MnCl_2$ and 10 mM DTT for assay of ACG. Vegetative *aca-/rdeA-* cell lysates were assayed for 0 and 30 min with 0.5 mM ATP, 10 mM $MgCl_2$ and 10 mM DTT for assay of ACB. Caffeine was present during incubation at the indicated concentrations. Reactions were terminated by addition of EDTA to 0.13 M and boiling, and cAMP was assayed in the boiled lysate. Data are expressed as percentage of cAMP accumulation measured at the maximal incubation time for each enzyme in the absence of caffeine, and for ACA in the presence of GTPγS. This was 34.0±6.0, 27.1±4.5 and 193±51 pmoles cAMP/mg protein for ACA+GTPγS, ACB and ACG respectively. Means and s.e.m. of at least three experiments performed in triplicate are presented.

A global effect of caffeine on cAMP accumulation could occur if caffeine inhibited cAMP secretion, in which case cAMP would be degraded by intracellular cAMP-PDEs, or if caffeine strongly stimulated a cAMP-PDE. Figure 2A shows that when ACB activity is measured in cells and medium separately, caffeine does not increase the amount of cAMP associated with the cell fraction. The small amount of cAMP that is produced in the presence of caffeine is fully secreted. Therefore caffeine is unlikely to inhibit cAMP secretion. *D.discoideum* has two intracellular cAMP-PDEs, *RegA* (22) and *PdeE* (21), and two cell surface associated enzymes, *PdsA* and *PDE4* (28). PdsA is in our assays inhibited by DTT, while RegA requires RdeA for activity (9) and should not be active in the *aca-/rdeA-* mutants that are used to assay ACB. PDE4 can be inhibited by the common PDE inhibitor IBMX (28). To test whether caffeine inhibition of cAMP accumulation is due to activation any of the four cAMP-PDEs, we measured its effect on cAMP accumulation by ACB in *PdeE-/RegA-* double null mutants with DTT to

inhibit PdsA and IBMX to inhibit PDE4. Figure 2B shows that in the absence of IBMX, cAMP accumulation is effectively inhibited by caffeine, which rules out PdeE, RegA and PdsA as caffeine targets. However in the presence of IBMX, caffeine inhibition is reduced. This suggests that PDE4 could be activated by caffeine. To test this directly we measured the effect of caffeine on ^{3}H-cAMP hydrolysis by intact cells, with DTT added to inhibit PdsA. Figure 2C shows that the IBMX-sensitive PDE activity, which is most likely PDE4, is inhibited instead of stimulated by caffeine. This indicates that caffeine does not inhibit cAMP accumulation by activating PDE4 either. Inhibition by IBMX of the effects of caffeine on cAMP accumulation as observed in figure 2B is perhaps due to true antagonism. IBMX (3-isobutyl-1-methylxanthine) and caffeine (1,3,7-trimethylxanthine) are similar in structure and may both bind to the target of caffeine, which, for the time being, remains obscure.

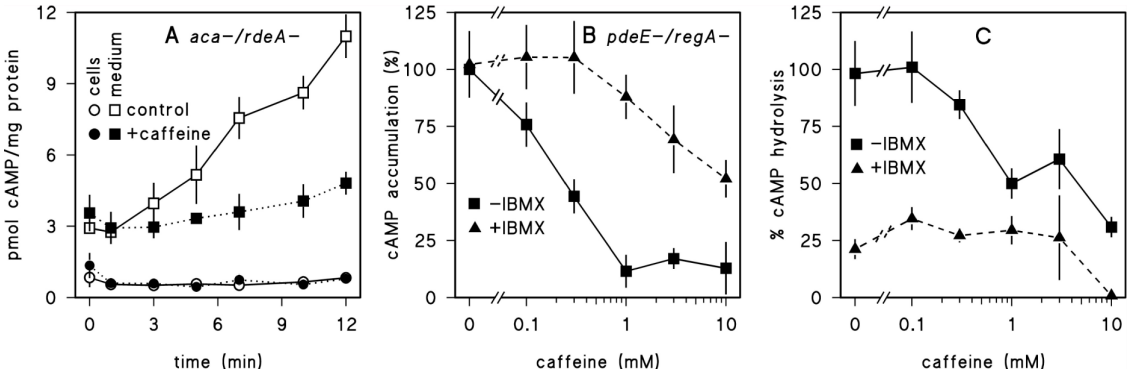

Figure 2. Effects of caffeine on cAMP secretion and cAMP-PDE activity.
(A) *cAMP secretion.* Vegetative *aca-/rdeA-* cells were incubated at 10^{8} cells/ml with 5 mM DTT in PB in the presence and absence of 2 mM caffeine. At the indicated time periods 50 µl aliquots of cell suspension were centrifuged for 5 s at 10.000 *g*. Supernatant (medium) and pellet (cells) fractions were rapidly separated and boiled for 30s. cAMP was assayed in both fractions. (B) *cAMP production in PDE null mutants.* Vegetative *pdeE-/regA-* cells were incubated for 30 min with 10 mM DTT in PB in the presence and absence of 3 mM IBMX and the indicated caffeine concentrations and assayed for cAMP. Data are expressed as percentage of cAMP accumulation in the absence of IBMX and caffeine. (C) *PDE activity.* Vegetative wild-type cells were incubated with 10 mM DTT and 10^{-7} M ^{3}H-cAMP in the presence and absence of IBMX and caffeine as indicated. After 30 min the samples were assayed for ^{3}H-5'AMP production. Data are presented as percentage of ^{3}H-cAMP hydrolysis obtained in the absence of IBMX and caffeine. Means and s.e.m. of two experiments performed in duplicate for (A) and triplicate for (B,C) are presented.

Effects of P-site inhibitors on AC activity in intact cells and cell lysates

In *Dictyostelium*, most if not all cAMP-induced responses that are mediated by the cAMP receptor cAR1, including the activation of ACA, are inhibited by adenosine (18,20,29,30). Adenosine analogs with modifications in the purine moiety are generally less effective than adenosine. Ribose-modified adenosine analogs are more effective and this is particularly the case for 2'3'-O-isopropylidene adenosine (IPA) (18,20,30-32). These effects were attributed to inhibition by adenosine of cAMP binding to cAR1, which shows a similar adenosine analog specificity (20,29).

Many mammalian adenylyl cyclases are directly inhibited by adenosine. Also here modification of the purine moiety reduces efficacy, while some ribose modified analogs, such as 2'-deoxyadenosine (DA) and particularly 2'5'-dideoxyadenosine (DDA) and 2'5'dideoxy-3'AMP are more active than adenosine (33). Due to its dependence on an intact purine moiety, this type of adenylyl cyclase inhibition is known as P-site inhibition (34). Co-crystallization of the C1 and C2 catalytic domains of mammalian AC with P-site inhibitors and pyrophosphate (PPi) showed that the complex occupied the ATP binding pocket of the enzyme, mimicking the enzyme-product complex in the transition state. Apart from this direct interaction, adenosine also has both stimulatory and inhibitory indirect effects on mammalian adenylyl cyclases that are mediated by a large class of G-protein coupled adenosine receptors (35). These receptors require an intact ribose-moiety and were traditionally called R-sites (34). However, this class of adenosine receptors has never been detected in *Dictyostelium*.

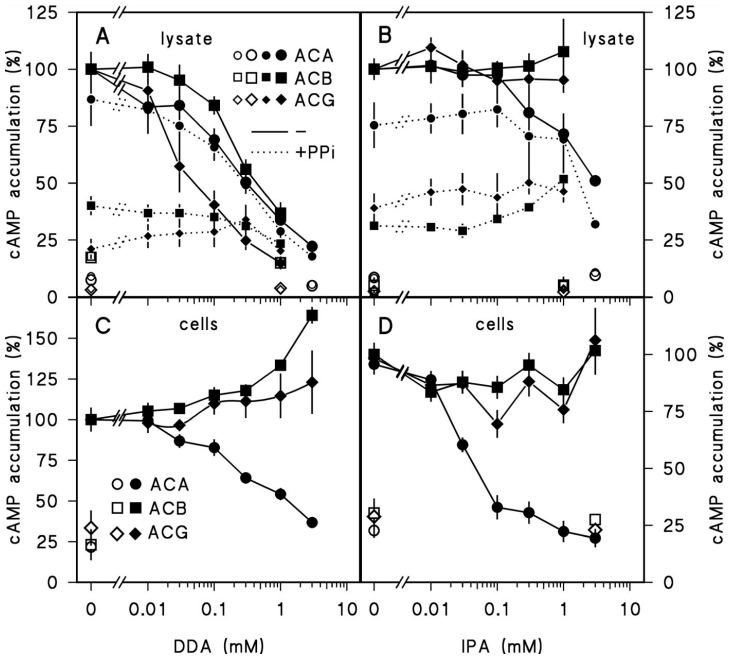

Figure 3. Effects of IPA and DDA on adenylyl cyclase activities
(A, B) *Cell lysates.* For assay of ACB, ACG or ACA activity, *aca-/rdeA-, aca-/A15::ACG* or NC4 cells were lysed without or with 30 µM GTPγS (NC4). Lysates were incubated in the presence (small symbols, dotted lines) and absence (large symbols, solid lines) of 1 mM Ppi and the indicated concentrations of IPA or DDA. Accumulated cAMP levels were determined after 0 (open symbols), 5 (closed symbols - ACA, ACG) or 30 minutes (closed symbols, ACB). C,D. *Intact cells. aca-/rdeA-, aca-/A15::ACG* and NC4 cells were incubated with DTT or DcAMP/DTT (NC4) in the presence of the indicated concentrations of DDA and IPA. Accumulated cAMP levels were determined after 0 (open symbols), 3 (circles, ACA), 5 (diamonds, ACG) or 30 minutes (squares, ACB). Data are standardized on cAMP levels obtained in the absence of DDA, IPA or PPi. Means and s.e.m of two to three experiments performed in triplicate are presented.

In *Dictyostelium*, ACA inhibition by DA and DDA was previously reported (19), but effects of P-site inhibitors on ACB or ACG were never investigated. It is also not known whether IPA inhibits *Dictyostelium* ACs directly. We therefore tested the effect of both DDA and IPA on the three *Dictyostelium* ACs. PPi was added conditionally at 1 mM. Figure 3A shows that DDA inhibits ACG activity in cell lysates most effectively (IC_{50}~75 µM), and inhibits ACA and ACB at 3-5 fold higher concentrations. In crude lysates the inhibitory effects of DDA are not dependent on added PPi, although the compound itself markedly inhibits both ACG and ACB activity. IPA has no effect on ACG and ACB and only slightly inhibits ACA activation in lysates (Fig. 3B). When the three enzymes are measured in intact cells, the effects of DDA and IPA are quite different. Neither of the two analogs inhibits ACG or ACB activity (Figs. 3C,D). IPA strongly inhibits DcAMP-induced ACA activation (IC_{50}~30 µM), while at least 15-fold higher concentrations of DDA are required to inhibit this response. These data indicate that the effects of IPA on ACA activation in intact cells are due to inhibition of cAMP binding to cAR1 as previously proposed. ACG, ACB and ACA are inhibited by the “classical” P-site inhibitor DDA when assayed in lysates, but DDA is apparently not sufficiently membrane-permeable to inhibit the enzymes when added to intact cells.

Searching for compounds that specificaly inhibit ACA, ACB or ACG

To identify inhibitors that act specifically on ACA, ACB or ACG, we tested a range of compounds that inhibit adenylyl cyclases in other organisms, such as NKY80 (36), MDL-12,330A (37) and SQ22536 (38). In addition, we tested tyrphostin A25, a compound that was initially identified as a tyrosine kinase inhibitor, but also proved to inhibit a variety of mammalian guanylyl- and adenylyl cyclases (39). NKY80 and tyrphostin A25 both act directly at the adenylyl cyclase catalytic core (36,39). We first tested the effects of the four inhibitors on ACA, ACB and ACG activity measured in cell lysates. Figure 4A shows that NKY80 does not inhibit ACG and has only a partial inhibitory effect on ACA and ACB. On the other hand, MDL-12,330A inhibits all three enzymes with IC_{50}'s that vary between 0.3 and 0.9 mM (Fig. 4B, Table 1). Tyrphostin A25 did not alter ACA or ACB activity (Fig. 4C), but was an effective ACG inhibitor with an IC_{50} of ~16 µM in cell lysates. However when added to intact cells, ACG inhibition required a 40-fold higher concentration. These concentrations also start to inhibit ACA and ACB, suggesting that this inhibition is due to a pleiotropic effect (Fig. 4E). SQ22536 effectively inhibited both GTPγS stimulated ACA activity in cell lysates and DcAMP stimulated ACA activity in intact cells (Figs. 4D,F). Remarkably, SQ22536 stimulated ACG activity

strongly and ACB activity weakly, both when assayed in lysates and intact cells. To conclude, both tyrphostin A25 and SQ22536 are specific inhibitors for ACG and ACA, respectively, when assayed in lysates. However, only SQ22536 can be used to inhibit ACA specifically in intact cells.

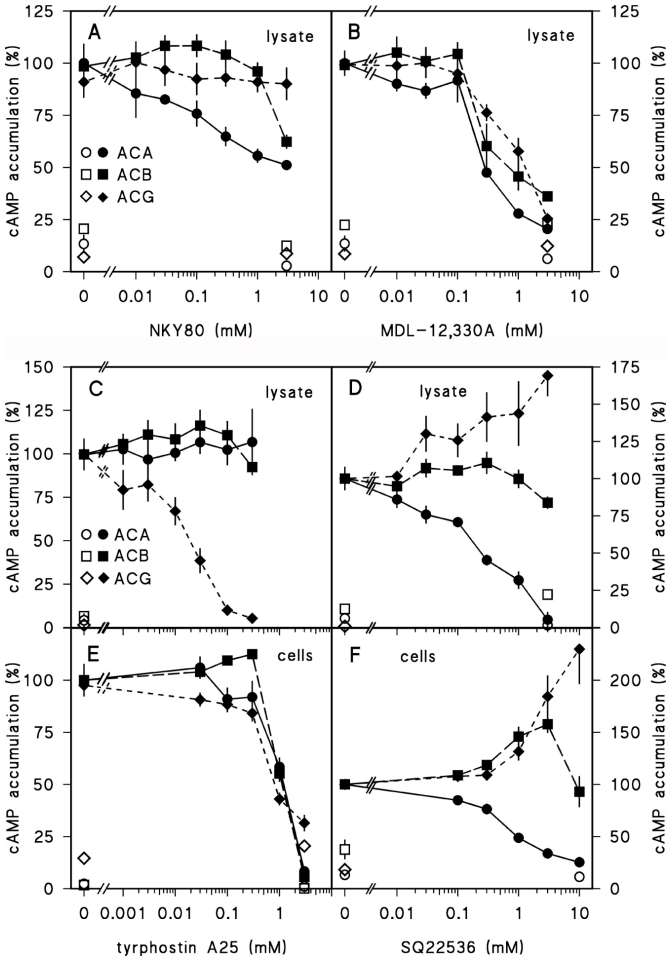

Figure 4. Effects of NKY80, MDL-12,330A, tyrphostin A25 and SQ22536 on ACA, ACB and ACG activities
(A-D) *Cell lysates.* For assay of ACB, ACG or ACA activity, *aca-/rdeA-, aca-/A15::ACG* or NC4 cells were lysed without or with 30 µM GTPγS (NC4) and incubated with the indicated concentrations of NKY80 (A), MDL-12,330A (B), tyrphostin A25 (C) or SQ22536 (D). Accumulated cAMP levels were determined after 0 (open symbols), 5 (closed symbols - ACA, ACG) or 30 minutes (closed symbols, ACB). Data were standardized on control cAMP levels. (E,F) *Intact cells. aca-/rdeA-*, *aca-/A15::ACG* or NC4 cells were incubated with DTT and/or DcAMP/DTT in the presence of the indicated concentrations of tyrphostin A25 or SQ22536. Accumulated cAMP levels were determined after 0 (open symbols), 3 (ACA), 5 (ACG) or 30 min (ACB). Data were standardized on levels obtained in the absence of inhibitor, and in case of ACA in the presence of DcAMP. Means and s.e.m of two to three experiments performed in triplicate are presented.

Discussion

This work was initiated to identify specific inhibitors for the three *Dictyostelium* adenylyl cyclases in order to identify and study specific roles of each of the enzymes during the life cycle of the organism. We first studied the effects of caffeine, which has long been used to study specific roles of ACA in cell aggregation and development (17,40-42). Our experiments showed that in intact cells caffeine inhibited ACB and ACG as efficiently as ACA (Fig. 1). The effects of caffeine on the three enzymes must be indirect, since it does not directly inhibit their basal activities when measured in cell lysates. In case of ACA, it does prevent activation of the enzyme by GTPγS, which activates the G-protein G2, that is part of the signal transduction cascade that normally activates ACA (2,3). However, neither ACB nor ACG are activated by G-proteins (4,9). It therefore appears that there is one target for caffeine that is at or downstream from the G-protein G2, and another that acts globally to prevent cAMP accumulation by either ACB or ACG and possibly also ACA.

We explored the possibility that caffeine could inhibit cAMP accumulation by either activating a cAMP-PDE activity or by inhibiting cAMP secretion, causing cAMP to be degraded intracellularly. However, no effects of caffeine on cAMP secretion were observed (Fig. 2A), and caffeine still inhibited cAMP accumulation when three out of the four cAMP-PDEs were inactivated (Fig. 2B).

Table 1. Pharmacological profiles of the three *Dictyostelium* ACs

ND: not determined; NA: not applicable: 0: no effect; -: inhibition; --: strong inhibition; +: stimulation; ++: strong stimulation; EC_{50}: effective concentration that yields half-maximal stimulation; *Cm*: concentration that yields maximal effect. IC_{50} values were determined by non-linear curve fitting of the data presented in figures 1-4 to $y = 100\text{-}[I] \times 100/(IC_{50}+[I])$, where y denotes % cAMP accumulation, with *t=0* cAMP levels subtracted, and [I] the inhibitor concentration. IC_{50} values are presented as means ± 95% confidence interval. The goodness-of-fit (r^2) is indicated between brackets.

	ACA (6h starvation)		ACB (vegetative)		ACG (vegetative)	
	Cells	Lysates	Cells	Lysates	Cells	Lysates
GTPγS	NA		NA		NA	
Mg^{+2}	NA		NA		NA	
Mn^{+2}	NA		NA		NA	
Caffeine	-- IC_{50}:361±113 µM (0.99)(17)	-* IC_{50}:825±114 µM (0.99)	-- IC_{50}:300±107 µM (0.99)	+ EC_{50}: 1-2 mM	-- IC_{50}:207±312 µM (0.79)	+ EC_{50}: 1-2 mM
IPA	-- IC_{50}:31±13 µM (0.98)	0/- IC_{50}:2.18±0.65 mM (0.96)	0	0	0	0
DDA	- IC_{50}:465±217 µM (0.97)	- IC_{50}: 274±153 µM (0.98)	+ EC_{50}:> 1 mM	- IC_{50}: 336±82 µM (0.99)	0/+	-- IC_{50}: 76±25 µM (0.99)
PP_i	NA	0/-	NA	-	NA	-
NKY80	ND	0/- IC_{50}:1.31±1.36 mM (0.78)	ND	0/- IC_{50}:5.78±4.97 mM (0.89)	ND	0
MDL-12,330A	ND	- IC_{50}: 329±174 µM (0.95)	ND	- IC_{50}: 497±383 µM (0.93)	ND	- IC_{50}: 885±258 µM (0.99)
Tyrphostin A25	0/- 1119±879 µM (0.94)	0	0/- 1336±715 µM (0.89)	0	0/- IC_{50}: 602±336 µM (0.95)	-- IC_{50}:16.6 ± 7.4 µM (0.97)
SQ22536	- IC_{50}: 812±280 µM (0.99)	-* IC_{50}: 247±134 µM (0.96)	+	0	++	+

* GTPγS-stimulated activity

The fourth cAMP-PDE was inhibited rather than activated by caffeine (Fig. 2C), in agreement with the known effect of caffeine on mammalian cAMP-PDEs (25). The observation that caffeine still inhibits cAMP accumulation in the *pdeE-/regA*- mutant that lacks intracellular cAMP-PDEs, confirms that it does not act on cAMP secretion, since in this mutant cAMP should then accumulate intracellularly.

We next analyzed the potential of ribose-modified adenosine analogs as specific AC inhibitors. 2'5'dideoxyadenosine (DDA) is a characteristic P-site inhibitor for mammalian adenylyl cyclases that acts directly at the catalytic core of the enzymes (15). It also inhibited the three *Dictyostelium* adenylyl cyclases, and particularly ACG, when measured in cell lysates indicating that also here it may interact directly with the catalytic region of the enzymes. However, DDA did not inhibit ACB nor ACG activity when added to intact cells, which implies that there is insufficient uptake of the compound. There was an inhibitory effect of DDA on ligand-induced ACA activation, but this, as discussed below, could be due to interference with ligand binding. 2'3'-O-isopropylideneadenosine (IPA) is another ribose-modified adenosine analog that is very effective in inhibiting cAR1-mediated responses in *Dictyostelium* (30-32). However, it has no reported merits as P-site inhibitor for mammalian adenylyl

cyclases and had no effects on ACB and ACG, and only modest effects on ACA activity measured in cell lysates (Fig 3C). IPA was however a very effective inhibitor of cAR1 mediated ACA activation in intact cells (Fig. 3D). Other ribose modified cAMP analogs, such as 2'-chloroadenosine and 2'O-methyladenosine also inhibit cAMP induced ACA activation in intact cells (18). These compounds as well as IPA and 2'deoxyadenosine are also effective inhibitors of cAMP binding to cAR1 (20). This strongly suggests that all inhibitory effects of ribose-modified analogs on cAMP-induced responses in intact cells are due to inhibition of cAMP binding to cAR1.

Lastly we tested four known inhibitors of adenylyl cyclases in other organisms for effects on ACA, ACB and ACG. One compound, NKY80, was not effective, while another, MDL-12,330A, was effective, but not specific for either of the three enzymes. The third compound tyrphostinA25 proved to be a specific and effective inhibitor of ACG in lysates. However, in intact cells it required much higher concentrations to have an effect, which was then no longer specific for ACG. The fourth inhibitor SQ22536, was a specific ACA inhibitor, also acting both in lysates and in intact cells. SQ22536 is highly lipophilic and may prove to be a useful agent for investigation of specific roles of ACA in *Dictyostelium* chemotaxis and development.

This work added a large number of traits that distinguish the adenylyl cyclases from each other. The novel traits are summarized with previously reported distinguishing features in Table 1. They will prove useful for enzyme identification in specific cell types or during specific stages in development of *Dictyostelium discoideum* or to recognize similar activities in other organisms.

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Chapter Three

Characterization of an adenosine kinase in *Dictyostelium discoideum*

Abstract

cAMP plays a pivotal role in *Dictyostelium* development controlling morphogenesis, cell movement and cell differentiation. The degradation of extracellular cAMP by the action of the extracellular phosphodiesterases produces 5'AMP that is subsequently degraded to adenosine by the activity of the 5'nucleotidase enzyme. Adenosine is known to block several cAMP-induced responses by inhibiting the binding of cAMP to surface cAMP receptors. Adenosine is also proposed to suppress secondary tip formation and to regulate pattern formation in the slug stage. This work describes the identification and characterization of the *Dictyostelium* adenosine kinase gene, *DdADK*. *DdADK* phosphorylates adenosine to form 5'AMP and is present both inside and outside the cells. The deduced DdADK protein sequence shares homology with adenosine kinases of higher eukaryotes and with microbial ribokinases. *DdADK* is expressed during the entire course of development, but expression decreases about three-fold during tip and slug formation. Up to culmination *DdADK* is expressed in all cells, but in fruiting bodies expression is confined to the upper and lower cup regions. *DdADK* null mutants (*adk*$^-$) develop normally but show a two-fold increase in slug size, which agrees with a putative role of adenosine in tip suppression.

Abbreviations*: ADK: adenosine kinase; Ado: adenosine; 5'AMP: adenosine 5'-monophosphate; cAMP: adenosine 3',5'-monophosphate; 5'NT: 5'nucleotidase; ALP: alkaline phosphatase; AMD: adenosine monophosphate deaminase; AC: adenylyl cyclase; PDE: phosphodiesterase; SpcAMPs: adenosine 3',5'-monophosphorothioate Sp-isomer.*

Introduction

Dynamic cAMP signalling regulates key aspects of multicellular development in the social amoeba *Dictyostelium discoideum*. Upon starvation, some cells start to secrete pulses of the chemoattractant cAMP, which are relayed by neighbouring cells. This results in the propagation of cAMP waves through the cell population and aggregation of cells towards the source of the pulses. Mound-shaped aggregates are formed which give rise to one or several tips that elongate into finger-shaped slugs. Each tip is an autonomous oscillator that attracts and controls cells within the aggregate. The size of the resulting slug is determined by the potency of the tip to suppress competing oscillators, a property known as tip dominance (Dormann and Weijer, 2001; Schaap, 1986; Schaap and Wang, 1984). During slug formation cells differentiate into prespore and prestalk cells with prespore cells at the posterior and prestalk cells in the anterior part of the slug. This pattern is established as follows: in the slug posterior there is a constant inter-conversion of prespore cells into anterior-like cells that is proposed to be caused by the secretion of a prespore inhibitor, DIF, by the prespore cells (Kay and Thompson, 2001). The anterior-like cells move forwards by chemotaxis towards cAMP and establish the prestalk region. In addition, adenosine has been put forward as the inhibitor of prespore differentiation in the prestalk region (Schaap and Wang, 1986).

Adenosine acts as a cAMP antagonist. It inhibits cAMP-induced responses such as cAMP binding to the specific receptors, cARs, therefore blocking cAMP relay (Theibert and Devreotes, 1984), the formation of competing tips and prespore differentiation (Schaap and Wang, 1986; Weijer and Durston, 1985). *Dictyostelium* has two cell surface adenosine-binding sites, the β-receptor (K_d 350 μM) and the α-receptor (K_d 0.8 μM) (Newell, 1982; Van Haastert and Kien, 1983), as opposed to higher organisms that present up to four surface adenosine receptors (Fredholm et al., 2001) and intracellular adenosine binding sites (Londos and Wolff, 1977). The cell surface and intracellular adenosine binding sites show different affinities for adenosine analogues. The cell surface receptors do not tolerate modification of the ribose moiety, and are therefore called R-sites, while the intracellular binding sites does not tolerate modification of the purine moiety and are called P-sites. Recent studies have indicated that the P-site resides in the catalytic core of mammalian adenylyl cyclases (Fredholm et al., 2001; Johnson et al., 1989; Londos and Wolff, 1977). In *Dictyostelium*, the inhibition of cAMP binding

to cAR1 shows P-site specificity, as do the inhibition of all cAMP induced responses (Alvarez-Curto et al., 2006; Theibert and Devreotes, 1984; Van Lookeren Campagne et al., 1986).

In *Dictyostelium* adenosine is produced mainly by the action of the extracellular cAMP phosphodiesterase (*PdsA*) and 5'nucleotidase (*5'nt*) respectively (Fig. 1), but several other enzymes are involved in its metabolism. In this work I present the identification of an adenosine kinase gene (*DdADK*) that encodes the enzyme responsible for the conversion of adenosine into 5'AMP. I have investigated the biochemical activity of the ADK enzyme and consequences of loss of the *ADK* gene for *Dictyostelium* development.

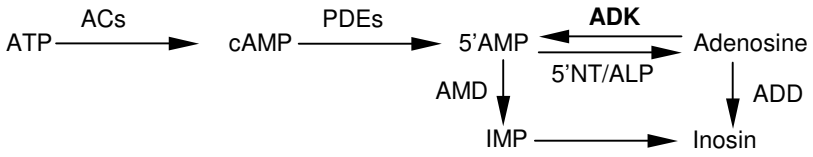


Figure 1. cAMP and adenosine metabolism in *Dictyostelium*
ADK: adenosine kinase; Ado: adenosine; 5'AMP: adenosine 5'-monophosphate; cAMP: adenosine 3', 5'-monophosphate; 5'NT: 5' nucleotidase; ALP: alkaline phosphatase; AMD: adenosine monophosphate deaminase; ADD: adenosine deaminase; ACs: adenylyl cyclases; PDEs: phosphodiesterases.

Material and methods

Cell culture and development conditions

D.discoideum NC4 cells were grown in association with *Klebsiella aerogenes* on SM agar plates. AX2 cells were grown in HL5 axenic medium. After transformation the medium was supplemented with either 5 µg/ml blasticidin (ICN, USA) for knockout selection or with 20-200 µg/ml G418 (Sigma, USA) for cells transformed with the *PsA-LacZ* construct. 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 10 mM Na/K phosphate buffer pH 6.5), unless otherwise stated.

Identification and reconstruction of the gene

The *Dictyostelium* genome and the Japanese cDNA projects (http://genome.imb-jena.de/*Dictyostelium*; http://dictycdb.biol.tsukuba.ac.jp/) were screened for sequences sharing homology to the sequence of the rat adenosine kinase. A putative *Dictyostelium* ADK (*DdADK*) consensus sequence was assembled using 7 cDNA sequences obtained from the Japanese database in combination with other 5 genomic DNA hits. This yielded a 1280 nucleotides long sequence plus 1000 bp 3' UTR. The deduced coding sequence contains a single ~250 bp long intron starting at nucleotide +135, as recognized by the conserved intron boundary sequence GT/AG. RT-PCR was performed to confirm the intron using primers P1 and P4 (Table 1) and the One-step RT-PCR kit (Quiagen, Crawley, UK) on 250 ng of NC4 total RNA. The deduced consensus DNA fragment has been confirmed by the finished *Dictyostelium* genome project as the gene DDB230174 (http:// dictybase.org). Translation of the *DdADK* gene predicts a soluble, 340 amino acids long protein. Functional domains of the protein were predicted using the SMART program (http://smart.embl-heidelberg.de). Conservation of the functional domains was analyzed through multiple sequence alignments with the CLUSTALW program (http://ebi.ac.uk/clustalw/).

Gene constructs, PCR screening and cell transformations

To generate a gene disruption construct, two fragments of 460 bp and 440 bp respectively of the *DdADK* gene were amplified by standard PCR using Taq DNA polymerase (Promega, USA) on wild type AX2 genomic DNA. The oligonucleotides used were P1 and P2 that add a 5'-BamHI and 3'-EcoRI site to the 460 bp fragment and oligonucleotides P3 and P4,

that add a 5'-XbaI and a 3'-BamHI site to the 440 bp fragment (See Table 1 for primers sequences and Figure 5 for details). Both fragments were subsequently cloned in tandem into the BamHI/EcoRI and XbaI/BamHI sites of the pUC118BsrΔBamHI plasmid that harbours the blasticidin selection marker (Sutoh, 1993). The resulting construct was made linear by digesting it with BamHI, prior to transformation into *Dictyostelium* cells (Knecht et al., 1990). Homologous recombination of the linear construct with specific flanking sequences of the *ADK* gene will cause the integration of the entire plasmid, including the resistance cassette. Knockout clones were selected for growth with 5 µg/ml blasticidin. Genomic DNA was isolated from transformed clones and screened for homologous recombination or random integration by two separate PCR reactions using primers P6 and P5 or P6 and P4 respectively (Table 1, Fig. 5). Two knockout clones (KO1 and KO2) and one random integrant (RI) were selected for further analysis For analysis of prespore gene expression all three clones were transformed by electroporation (Knecht et al., 1990) with a fusion construct of the *PsA* prespore gene promoter and the LacZ reporter gene, *PsA-LacZ* (gift from Jeff Williams, University of Dundee, UK).

RNA isolation, Northern blotting and *in situ* hybridisations

To study the developmental regulation of *DdADK* expression, NC4 cells were incubated for 24 h on PB agar. Total RNA was isolated from 2 x 10^7 cells at 2 hours intervals. RNA was size fractionated on 1.5% agarose gels containing 2.2 M formaldehyde (Nellen et al., 1987) and transferred to a nylon membrane. As a probe for Northern Blot hybridisation a 460 bp *DdADK* DNA fragment was amplified by PCR using primers P3 and P4 (Table 1), radiolabelled with [^{32}P]-dATP (Amersham, UK) and hybridised as previously described (Sambrook et al., 1989).

For *in situ* hybridisation, 10^6 cells were spread on 1-cm^2 pieces of dialysis membrane, supported by PB agar and incubated at 22°C. After the desired developmental stages had been reached, membranes were transferred to a parafilm coated wet-box, where *in situ* hybridisation was carried out as previously described (Escalante and Loomis, 1995) using 250 ng of DIG-labelled RNA probes. For construction of the *DdADK*-RNA probe a 460 bp DNA fragment was amplified by PCR using primers P3 and P4 (Table 1) yielding a fragment with flanking XbaI/BamHI sites. This fragment was cloned into the XbaI and BamHI sites of pBluescript KS+ and subsequently used as template for the *DdADK* RNA probe using the T3 and T7 RNA polymerases and DIG-RNA labelling kit following the manufactures instructions (Roche, UK). Similarly, a 700 bp BamHI/HindIII fragment of the prespore specific gene *cotB* was cloned into compatible sites of the multiple cloning site of the Bluescript SK- vector (Strategene, California, USA) (gift from Nick Oswald, University of Dundee, UK) and used to create the *CotB*-DIG-labelled probe.

Determination of number of cells per slug and analysis of slug size

Cells were harvested from exponentially growing cultures and resuspended in PB buffer at 2 x 10^8 cells/ml. Droplets of 10 µl of cell suspension were deposited on 1.5% water agar. Cells were incubated at 22°C until slugs have formed. The droplet area was then photographed and the number of slugs per droplet was counted. The number of slugs per 10^6 cells and cells per slug was calculated from at least five droplets.

Adenosine kinase assay

Adenosine kinase activity was determined by measuring the conversion of ^{3}H-adenosine (Perkin Elmer Life Sicences, Boston, USA) into ^{3}H-5'AMP in the presence of ATP and Mg^{+2}. 50 µl of either cleared cell lysate or secreted cell fraction samples were incubated in a final volume of 100 µl with 10 µl assay mix (1 mM ATP, 1 mM $MgSO_4$, 10 mM adenosine, 10^{-5} M ^{3}H-adenosine in 20 mM Tris pH 8.0). Samples were incubated for 20 min at 23°C. Reactions were stopped by addition of 30 µl 0.1 M EDTA and boiling of the plate for 1 minute. The negatively charged 5'AMP was separated from adenosine by shaking 100 µl of sample for

2 min with a 100 µl slurry of 1:1 diluted AG1x8-Dowex anion exchange resin (Biorad). The resin was precipitated by centrifugation for 2 min at 663 x g and the supernatant discarded. The resin was washed twice with 1 ml of 10% methanol by shaking tubes for 30 seconds and centri-fugation at 663 x g for 1 minute. To elute the bound ^{3}H-5'AMP, samples were shaken for 5 min with 0.5 ml of 2 M NaCl. Samples were centrifuged at 663 x g for 2 min and the supernatant was recovered, mixed with scintillation cocktail and measured by liquid scintillation counting.

Assay for secreted and cell associated adenosine kinase activity in slugs

Cells were incubated on PB agar at 7 x 10^6 cells/cm^2 at 22^{o}C until slugs were formed. Slugs were harvested, washed and dissociated by vigorous pipetting in 20 mM Tris pH 8.0 (TB). The cell density was adjusted to 10^7cells/ml, and 2 ml of cell suspension were shaken for 1 h at 150 rpm and 22^{o}C. Samples were centrifuged 2 min at 1125 x g. The supernatant was separated from the pellet and placed on ice; the pellet was then resuspended in 2 ml TB, and lysed through Nucleopore filters (pore size, 3 µM). Both supernatant and cell lysate were subsequently assayed for adenosine kinase activity.Protein levels of all cell lysates and extracts were measured using the Bradford assay and used to standardize adenosine kinase activities.

Developmental time course of accumulated and newly produced adenosine kinase activity

10^8 cells were plated on 2 cm^2 nitrocellulose filters placed on 2.5 cm^2 pieces of Whatman paper drenched in 5 mM PB pH 6.5, on PB agar plates. Plates were incubated in a wet box at 22^{o}C over a 24 h period. Every two hours a filter sandwich was transferred to 2 ml of ice-cold TB and vortexed for 15 seconds. The liquid fraction was transferred to a fresh tube and the remaining filters were washed once more with 2 ml TB. The combined washes were centrifuged for 2 min at 720 x g. The supernatant ("accumulated" fraction) was transferred to a fresh tube and kept on ice until assayed for adenosine kinase activity. The pellet containing the developing cells was washed once more and finally resuspended in 1.2 ml TB. One ml was shaken for 20 min at 150 rpm and 22^{o}C and subsequently centrifuged for 2 min at 720 x g. The supernatant fraction was then recovered as the freshly "produced" fraction and assayed alongside with the "accumulated" fraction for adenosine kinase activity.

Prespore induction assay

Cells transformed with the *PsA-LacZ* construct were starved on PB agar until loose mounds had formed. Cells were subsequently harvested and resuspended to 3x10^6 cell/ml in PB. Aliquots of 90 µl of cell suspension were incubated in a total volume of 100 µl with 30 µM SpcAMPS (Biolog, Germany) and variable adenosine (Sigma, USA) concentrations (0, 10^{-5} M, 3x10^{-5} M, 10^{-4} M, 3x10^{-4} M, 10^{-3} M, 3x10^{-3} M). Cell suspensions were intermittently (10s on/1s off) shaken for 8 hours at 800 min^{-1} rpm and 22^{o}C. Cells were lysed for spectrophotometric measurement of β-galactosidase activity by two freeze-thawing cycles whilst vigorously shaking (Schaap et al., 1993). 30 µl of 2.5x Z-buffer (150 mM Na_2HPO_4, 100 mM NaH_2PO_4, 25 mM KCl, 25 mM $MgSO_4$, 5 mM $MgCl_2$ and 1% 2-β-mercaptoethanol, pH 7.0) and 10 µl of ortho-nitrophenyl-β-galactoside (ONPG) (Sigma, USA) were added to the cell lysates and the OD_{450} was measured at regular time intervals using a microtiter plate reader.

Table 1: Oligonucleotides used in this work

P1	TCATGGATTCCACTCACTAAAATGTCAAACATTAAAATTCTTTGCGC
P2	CAGTGAATTCGTGAACAGCAGAGTCTGGTGAAACGG
P3	TGACTCTAGACCATTCCTCTACGGTTTAGCCGC
P4	GACCGGATTCGGCTGGGACGGTGGCACCATTTTGTC
P5	CACAAGATCCCTCAGGTAGAGC
P6	GATTTGATGGGATTAATTAATTTGTAATC
P7	GTGGTGCTGCACAAAATACATCACGTC

Results

Dictyostelium adenosine kinase gene sequence and protein analysis

The sequence for the *Dictyostelium* adenosine kinase gene was obtained by reiterated query of single sequence runs of the ongoing *Dictyostelium* Japanese cDNA and genomic sequencing projects using the rat adenosine kinase sequence. Initial hit sequences were assembled into a final consensus sequence for the *Dictyostelium* adenosine kinase homologue gene, *DdADK*. The recently finished *Dictyostelium* genome has shown that the deduced sequence corresponds to the gene *DDB230174*. The adenosine kinase *DdADK* gene is positioned on chromosome 4 and shares 37% amino acid sequence identity with the human and rat adenosine kinases. The *DdADK* DNA coding region is 1280 nucleotides long and carries a single intron (Fig. 2A).

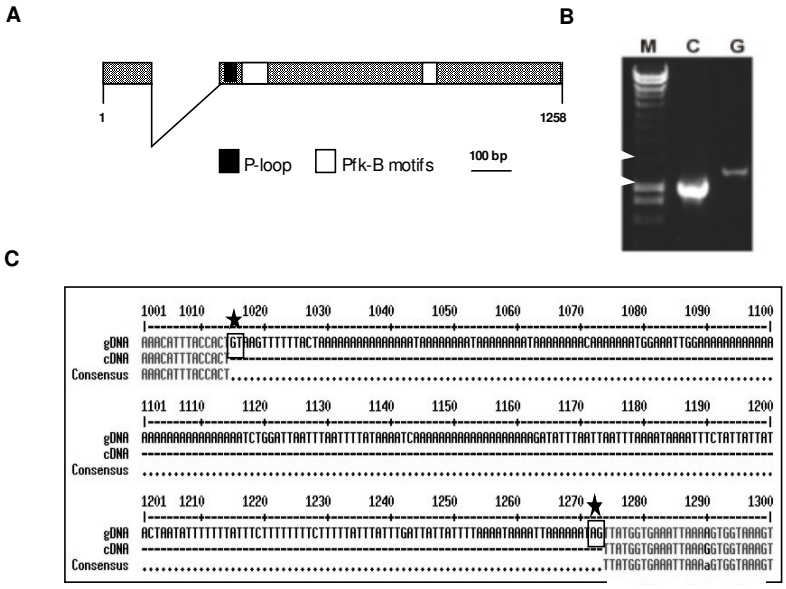


Figure 2. Structure of the *Dictyostelium ADK* gene
(A) Schematic representation of the *DdADK* gene. Solid boxes represent the two exons (134 and 888 bp long respectively), separated by a single 258 bp intron. The conserved domains ATP binding P-loop (solid box) and sugar kinase (PfkB family) homologous motifs (white boxes) are shown (See text for details). (B) Amplification of *DdADK* gene by RT-PCR was performed on AX2 cells RNA at 4 hours of development, using primers P1 and P4 (Table 1), yielding a 1 kb long cDNA. The same set of primers was used in a PCR reaction on AX2 genomic DNA yielding the 1.25 kb expected genomic band. Both PCR and RT-PCR products were run alongside for size comparison on 1% agarose gel stained with ethidium bromide. The size difference between the cDNA and the genomic bands confirms the existence of a ~250 bp intron. As a reference, arrowheads mark the position of 1.5 kb and 0.8 kb bands on the DNA marker (M = 1kb molecular marker; c = RT-PCR cDNA; g = PCR genomic DNA). (C) Alignment of sequences from the genomic PCR and the RT-PCR products further proved the position of the 258 bp intron and conserved intron boundary (GT/AG). The intron boundary is boxed and marked with a star. This is the result of one sequence run using primers P1 and P4 (Table 1).

Amplification of the gene by RT-PCR using RNA from NC4 cells confirms the presence of a 258 bp intron, yielding the expected 1023 bp cDNA fragment. A second PCR reaction on wild type genomic DNA using the same set of primers performed as control yielded the expected 1280 bp (Fig.2B). Sequencing of the RT-PCR product provided further evidence of the presence of the intron starting at nucleotide number 135 and revealed the presence of the conserved intron boundary represented by the nucleotide sequence GT/AG (Fig. 2C)

The deduced protein sequence is 340 amino acids long and topology analysis performed with the SMART program suggest that it has no putative transmembrane domains and is therefore a soluble protein (Fig. 3A). DdADK protein is homologous to the rat and human adenosine kinases and they all share homology to conserved domains found in sugar kinases of yeast, plants and lower eukaryotes (Spychala et al., 1996). Alignment of the predicted protein sequence with some of these homologous sequences highlights the principal motifs found in other adenosine kinases, fructokinases and microbial ribokinases (Fig. 3B). The conserved N-terminal P-loop that is essential for ATP/GTP binding in most kinases is also conserved in all aligned enzymes. However, in *Dictyostelium* ADK there is a transition from a

lysine to an arginine in a critical position. Despite this substitution the protein binds ATP in all cases that it has been investigated (Sinha et al., 1999). There are other two conserved motifs likely to be involved in binding to the sugar moiety of the adenosine molecule (Fig. 3B) that share homology to the microbial sugar kinases belonging to the family of phosphofructo-kinases B.

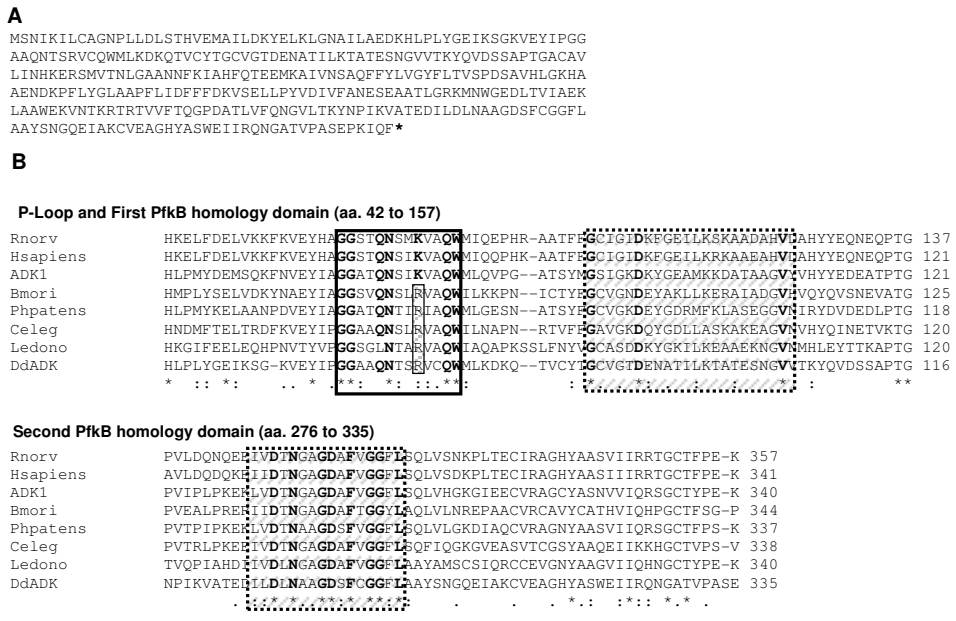


Figure 3. DdADK predicted protein and conserved domains
(A) Amino acid sequence of DdADK predicted protein. (B) Alignment of DdADK with homologous protein sequences from rat (Rnorv; NP_037027.2), human (Hsapiens; P55263), yeast (ADK1; P47143), the invertebrates silkworm (Bmori; ABD36182.1) and C.elegants (Celeg; NP_502104.1), the moss P.pathens (Phpatens; CAA75628.1) and the protozoan L.donovani (Ledon; AAC80288). The solid line box indicates the position of the ATP-binding P-loop. Conserved critical amino acids are shown in bold and the K to R amino acid substitution is highlighted with a dashed box. The broken line boxes show the two conserved motifs found in other microbial phospho-fructo kinases belonging to the PfkB family, with conserved amino acids in bold.

Developmental regulation of *DdADK*

To investigate when *DdADK* is expressed during development, total RNA of NC4 cells was isolated at two-hour intervals during a 28 hours time course. A single *DdADK* mRNA is detected by Northern blot showing a bimodal pattern of expression. Expression is high in growing cells and during the first and final 8 hours of development, but significantly lower from 10 to 18 hours (Fig. 4A).

Earlier pilot experiments showed the presence of both a cell-associated and a secreted adenosine kinase activity (P. Michielsen and P. Schaap, unpublished results). To assess whether the developmental regulation of both activities is similar to that of the *DdADK* mRNA, we measured cell-associated (accumulated) and secreted (produced) ADK activity during the entire course of development. The developmental time course presented in Figure 4B shows that cell-associated and secreted activities are co-regulated and about equal throughout. Both activities reach their maximal peaks with about four hours of delay compared to the timing of mRNA synthesis. There is a peak at 8-12 hours followed by a decrease in activity from 16 to 22 hours and a second sharper peak during late culmination. The delay in ADK activity could be due to additional translational regulation of the protein. However, although in both experiments wild type cells were used, the Northern blot was performed using NC4 cells and the biochemical analysis was done on the axenic line AX2. It is possible that the development of both cell strains may not be completely synchronous.

The spatial pattern of expression of *DdADK* was analysed by *in situ* hybridisation on AX2 developing structures. *DdADK* mRNA does not have a specific pattern during earlier stages, showing low and uniform expression up to the slug stage (Fig. 4C1). However, a clear

pattern appears during culmination showing very strong and specific expression of the gene in the upper and lower cups of culminants (Fig. 4C2).

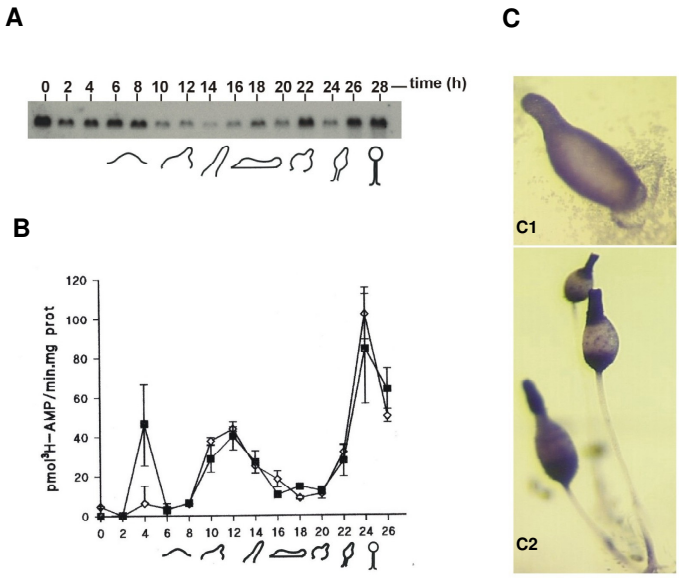


Figure 4. Developmental regulation and pattern of adenosine kinase
(A) Wild type NC4 cells were let to develop until fruiting bodies had formed on non-nutrient agar. Total RNA was extracted every two hours, size fractionated, blotted and hybridised with a [^{32}P]-dATP-labelled *DdADK* DNA probe. (B) AX2 cells were incubated on nitrocellulose filters supported on PB-drenched Whatman paper on PB agar. Cells and Whatman paper were collected every two hours and the levels of both produced and accumulated 5'-AMP were determined as measurement of adenosine kinase activity. Means and SE of one experiment performed in triplicate are shown. All data were standardized on protein content of the extracellular suspension. Closed symbols: produced activity; Open symbols: accumulated activity. (See methods for details).
(C) AX2 cells were let to develop on 1 cm^2 dialysis membranes supported by PB agar. *DdADK* pattern was visualized by *in situ* hybridisation in fingers (C1) and late culminants (C2) using a specific *DdADK* DIG-labelled-RNA probe.

Disruption of the *DdADK* gene

To understand the role of ADK in *Dictyostelium* development the *DdADK* gene was inactivated by homologous recombination and insertion of the blasticidin resistance cassette, in the wild type strain AX2 (Fig. 5A). Screening for positive clones was performed using at least two independent PCR reactions as shown in Figure 5B, and Southern blotting (not shown). Two independent knockout clones (KO1 and KO2) and one random integrant clone (RI) obtained from the same transformation were selected and used for further phenotypic analysis.

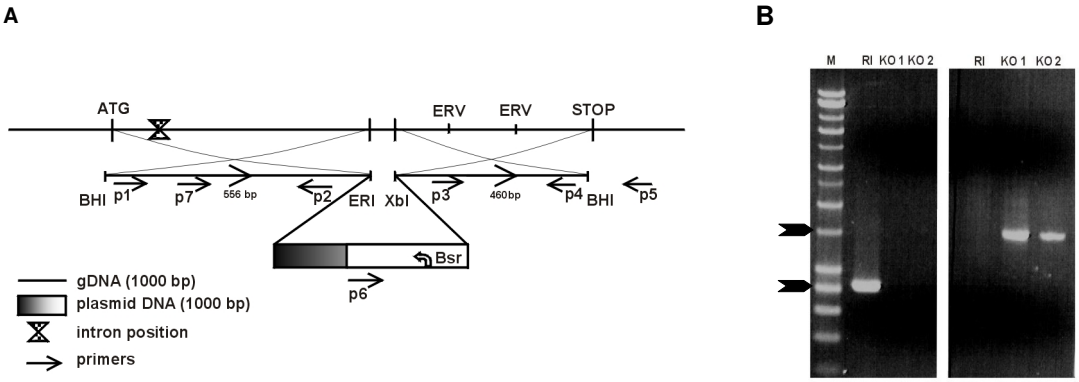
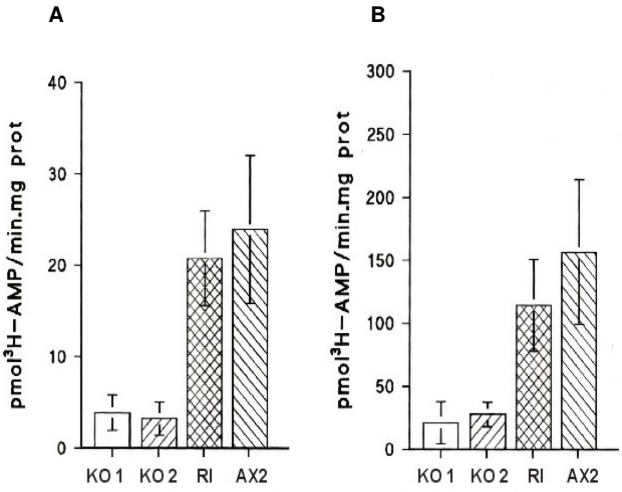


Figure 5. Gene disruption and screening of knockout clones
(A) Schematic representation of the *DdADK* gene knockout construct. The blasticidine resistance cassette (Bsr) is inserted into the gene by homologous recombination. Primers used to make the knockout construct and later PCR screening of knockout clones are represented by P1 to P7 (See Table 1 for sequences). Restriction sites are marked as: BHI: BamHI, ERI: EcoRI, XbI: XbaI, ERV: EcoRV. (B) At least two separate PCR reactions were performed in the screening of knockout clones. PCR1 was performed using primers P7 and P4 yielding a 0.8 kb fragment corresponding to a wild type band, which is present in the random integrant clone, RI. PCR2 shows the band obtained with primers P5 and P6 that is only present when specific disruption of the gene has taken place. This band is present in the knockout clones, KO1 and KO2, and absent in the RI clone. Arrowheads mark the positions of 1.5 and 0.8 kb on DNA marker.

Analysis of the ADK null (*adk*$^-$) mutant phenotype.

ADK controls the levels of nucleotides and could potentially interfere with basic cellular metabolism. However, the KO strains do not show any growth defects (data not shown). The null mutant also displayed normal aggregation and development of slugs and fruiting bodies. Any adenosine kinase activity should be lost in this cell line and Figure 6 shows that in slugs both the cell-associated and secreted adenosine kinase activities are about four-fold reduced in the two null-mutant clones. This demonstrates that most of the *Dictyostelium* adenosine kinase activity is due to DdADK.

Figure 6. Absence of adenosine kinase activity on *Ddadk* knockouts
Knockout clones KO1 and KO2, a control random integrant (RI) and AX2 cells were let to develop into slugs on non-nutrient agar. Disaggregated slug cells were resuspended in 20 mM Tris at a density of 3 x 10^7cells/ml and shaken at 150 rpm for 1h at 22°C. Cells were then collected by centrifugation and separated from the supernatant. The remaining cells were resuspended in fresh 20 mM Tris and lysed through Nucleopore filters. Both supernatant and lysate dilutions were assayed in triplicate in the presence of saturating concentrations of ATP/Mg, and 10^{-6} M ^{3}H-adenosine for 20 min at 22°C. Final adenosine kinase activity was calculated by measuring the levels of newly formed ^{3}H-5'AMP after being separated from ^{3}H-adenosine by anion exchange chromatography. Extracellular (A) and cell associated (B) adenosine kinase activities are shown as means and SEM of at least three experiments performed in triplicate. All assays were standardised on total protein content.



Extracellular levels of adenosine have been reported to modulate slug size and cell type proportions (Wang and Schaap, 1985). Slug size is largely determined by the number of tips appearing on aggregates with each tip directing the formation of a single slug. Every tip produces cAMP pulses autonomously and secretes a compound that inhibits formation of competing oscillators. Adenosine has been put forward as a likely candidate to play this inhibiting role. Since DdADK is active also extracellularly it could be potentially controlling extracellular levels of adenosine and therefore slug size. We measured the number of slugs that were formed from a known number of cells placed at close proximity in a small droplet. Figure 7A shows that *adk*$^-$ cells formed less than half the number of slugs from 10^6 cells as the control line and these slugs were consequently more than two times larger. From this experiment we can conclude that adenosine plays a role in controlling slug size, although the levels of extracellular adenosine should be measured to validate this affirmation.

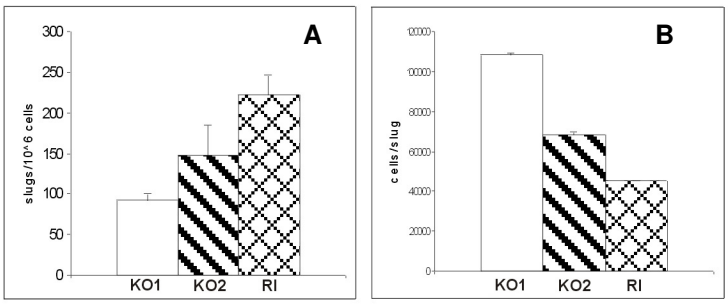


Figure 7. Calculation of slug size and number of cells per slug
Droplets of cell suspensions of the *adk*$^-$ KO1 and KO2, and of one random integrant used as control were deposited on water agar and allowed to develop at 22°C. Once slugs were formed, they were photographed and counted. The number of slugs per 10^6 cells (A) and number of cells per slug are shown (B).

Adenosine has also been proposed to be an inhibitor of prespore differentiation in the prestalk region of slugs (Schaap and Wang, 1986). However, millimolar concentrations of adenosine are required to have a significant effect and it is questionable whether these concentrations can be reached by degradation cAMP. Yet it is possible that active phosphorylation of adenosine by ADK obscures the actual local adenosine concentration. If adenosine

has a role in prespore differentiation, inhibition of prespore induction in the *adk*- cells will be hypersensitive to adenosine. Additionally we will expect the knockout to show a reduction in size of the prespore region of the slug. To test for adenosine hypersensitivity, *adk*- and RI cells were transformed with a β-galactosidase reporter construct under the prespore specific *PsA* promoter (*PsA-LacZ)*. This yielded the cell lines KO1/*PsA-LacZ* and RI/*PsA-LacZ.* Aggregation competent cells of the transformed strains were then incubated with 30 µM SpcAMPs and increasing concentrations of adenosine during a period of 8 hours. Figure 8A shows that PsA promoter activity was induced by SpcAMPs and inhibited by all concentrations of adenosine with equal efficiency in both cell lines.

The pattern of prespore gene expression was visualized by *in situ* hybridisation in *adk*- and random integrant clones using the prespore gene *CotB* as a probe (Fig. 8B). No significant differences in the proportional size of the prespore region were found between the knockouts and control cell lines. This suggests that loss of adenosine kinase does not have a major effect on prespore expression in slugs.

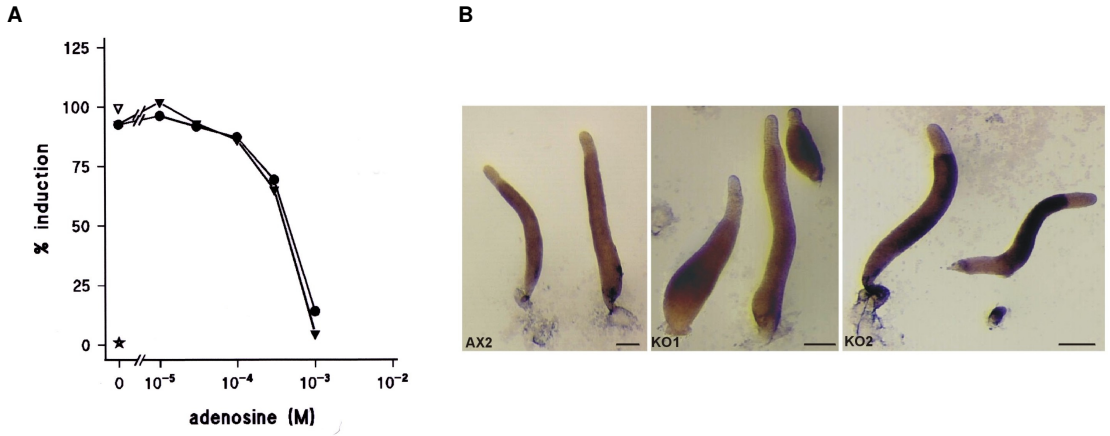


Figure 8. Inhibition of prespore induction by adenosine and prespore pattern in *DdADK* knockouts
(A) Knockout clones and the control random integrant were transformed with the prespore specific construct *PsA-LacZ.* Transformed lines were let to develop to loose mounds, harvested, and resuspended to $3x10^6$ c/ml. Cell suspensions were incubated in the presence of 30 µM SpcAMPs and increasing concentrations of adenosine during 8 hours. 30 µM SpcAMPs alone was used as a positive control. Prespore induction was measured by spectrophotometric methods following the activity of the reporter *PsA-LacZ* on cell lysates using ONPG as a substrate. (Star: no treatment; Open triangle: 30 µM SpcAMPs; Closed triangle: RI; Closed circle: *adk*-). (B) *In situ* hybridisation to a prespore specific DIG-labelled-cotB riboprobe was performed on slugs of *adk*-, RI and wild type strains.

Discussion

We have identified the gene for an adenosine kinase (*DdADK*) by screening the *Dictyostelium* genomic and Japanese cDNA databases. *DdADK* is highly conserved and it is homologous to the human and yeast adenosine kinases and to enzymes of the phospho-fructo kinase B (PfkB) family. PfkB proteins are involved in pathways of carbohydrate metabolism such as glycolysis, and share with adenosine kinases the motifs for ATP binding and pentose recognition. All adenosine kinases analysed are closer to this sugar kinases than to other nucleoside kinases such as uridine or thymidine kinases. This might suggest that a sugar kinase might have been recruited at some point during evolution to synthesise nucleotides as a different function. Analysis of the predicted topology of the protein suggests that *DdADK* does not have any signalling peptide for subcellular localization or transmembrane domains, so it is likely to be a soluble protein and I have shown that cell-associated and extracellular adenosine kinase activities are present at significant levels throughout development. However, the mechanism of secretion is yet unknown.

DdADK is developmentally regulated, with two peaks of maximal expression, one during the first hours of development and the second one after 18-20 hours. On the other hand, the maximal secreted and cell-associated kinase activities are found between 10 and 12 hours and between 22 and 24 hours, showing a shift of four to six hours in relation to the gene

transcription. This might be due to translational regulation of the protein or enzyme regulation. A similar discrepancy between mRNA levels and levels of enzymatic expression has been observed in the human adenosine kinase. The human ADK is widely distributed among tissues. However, there are cases such as the brain in which the high levels of ADK activity detected do not correlate to the low levels of mRNA found in this tissue (Spychala et al., 1996).

DdADK is specifically expressed at the upper and lower cups in culminants. This pattern of expression is related to that of *5'NT* and the alkaline phosphatase that also degrades 5'AMP to adenosine, *ALP*. *5'NT* is specifically expressed at the lower cup and stalk in late culminants whereas the *ALP* is found in the boundary between prespore and prestalk cells in slugs and its expression becomes specific of the upper and lower cups in culminants. These results point at the prespore/prestalk boundary in the slug and later in culminants, as important signalling centres from which cell differentiation and movement might be controlled.

The *DdADK* knockouts show normal growth and development, but form significantly larger slugs when compared to wild type, indicating that fewer tips are formed in each aggregation field. Null mutants in 5' nucleotidase, the enzyme that produces adenosine, show an opposite phenotype with multi-tipped aggregates and consequently much smaller slugs (Rutherford et al., 2003). Taken together, these results support the hypothesis that adenosine mediates tip dominance and thereby controls slug size (Newell and Ross, 1982; Schaap and Wang, 1986).

Treatment of aggregation competent cells with concentrations of adenosine in the millimolar range inhibits the expression of the prespore specific genes *psA* and *cotC* (Verkerke-VanWijk et al., 1998), which suggests that extracellular adenosine plays an important role controlling prespore induction. The lack of the kinase activity in the knockouts would potentially lead to an increase in the levels of intra- and more importantly, extracellular adenosine. The consequences of this in terms of prespore expression in slugs are that the total levels of prespore expression would be greatly reduced in the knockouts, showing a smaller prespore area. Furthermore, less adenosine would be required to inhibit induction of prespore genes. The data presented here cannot support these predictions. I have not observed any significant reduction or alteration on prespore pattern in slugs as seen by *in situ* hybridisation. Inhibition of prespore gene induction is also not altered in the *adk* null strain. This does not exclude the possibility of alternative pathways that might take over controlling prespore expression in the *adk* null strain and that would require further investigation.

As a final conclusion, these results tend to support a role for adenosine in the regulation of slug size but I have found no evidence to support a role for extracellular adenosine in the establishment of prespore/prestalk pattern.

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Chapter Four

Molecular phylogeny and evolution of morphology in the social amoebas

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Molecular phylogeny and evolution of morphology in the social amoebas
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Abstract

The social amoebas (Dictyostelia) display conditional multicellularity in a broad variety of forms. Apart from the genome sequence of *Dictyostelium discoideum*, there is little molecular data from the rest of the group. This Chapter introduces the first dictyostelid molecular phylogeny using two parallel datasets - small subunit ribosomal RNA and α-tubulin. The phylogeny shows a molecular depth for Dictyostelia similar to animals, and indicates that their taxonomy requires complete revision. A mapping of well-documented characters onto the phylogeny identifies increased size and cell-type specialization of fruiting structures, but not architectural complexity, as trends in dictyostelid evolution. This major taxon is now uniquely well suited to identify molecular changes underlying phenotypic innovation.

Introduction

Animals and plants display an enormous variety of forms, but their underlying genetic diversity is relatively small compared to the immense genetic diversity of the microbial world. Eukaryotic microbes include a broad range of morphologically or physiologically distinct unicellular life forms, with multiple independent and novel inventions of multicellularity. One of the most intriguing and challenging problems in biology is to understand how the latter has happened, and why this particular evolutionary stratagem has repeatedly arisen.

The social amoebas or Dictyostelia (*1*) are a striking group of organisms that hover on the borderline between uni- and multicellularity. Their life cycle consists of a unicellular feeding stage that can take three directions when cells are starved. i) Individual cells may encyst to form microcysts (*2*). ii) Cells fuse and two nuclei combine to form a zygote that subsequently cannibalizes neighboring cells and then encapsulates to form a macrocyst. iii) Numerous cells may aggregate and differentiate to form a multicellular fruiting body consisting of spore and stalk cells. Microcysts represent the ancient survival strategy of the solitary amoebas from which the social amoebas arose (*3*). Apart from the cannibalistic attribute of the dictyostelid macrocyst, zygotic cyst formation is also a common survival strategy for a diversity of eukaryotes from green algae to dinoflagellates to fungi.

Thus, it is the process of fruiting body formation in social amoebas that has long fascinated developmental biologists. This has been best described for the model organism *Dictyostelium discoideum*. Here the aggregate of up to 100,000 cells first encases itself in a skin-like matrix, and then transforms into a macroscopic finger-shaped structure, the “slug”. This slug possesses specialized head and body regions whose roles in the final fruiting structure are now set. The head region senses environmental stimuli such as temperature and light, and directs migration of the slug towards the soil’s outer surface, where spores will be most readily dispersed. Migration ends with the slug “standing up” to form the fruiting body or sorocarp. The cells in the head region first deposit a cellulosic stalk tube; they then move into this tube, swell up, deposit more cellulose and die. The remaining “body” cells then crawl up this newly formed stalk and encapsulate to form spores. Thus the Dictyostelia display distinct characteristics of true multicellularity, such as cell-cell signalling, cellular specialization, body axes formation, coherent cell movement and altruism.

Traditionally, classification of the social amoebas has been based on their most striking trait, fruiting body morphology. Based on this, three genera were proposed: *Dictyostelium*, with unbranched or laterally branched fruiting bodies, *Polysphondylium,* whose fruiting bodies consist of repetitive whorls of regularly spaced side branches, and *Acytostelium*, which, unlike the other genera, forms acellular fruiting body stalks (*2*).

Despite the widespread use of *D. discoideum* as a model organism (*4,5*), the Dictyostelia as a whole are very poorly characterized in molecular terms, with nearly all currently available data being from a single species. Nonetheless, the social amoebas provide us with a unique opportunity to understand the evolution of multicellularity (*6-8*). A primary and essential prerequisite for this is an accurate and complete understanding of the true phylogeny of the group. Only then can we meaningfully correlate the history of phenotypic change with the history of genetic change and begin to infer the molecular genetic modifications that led to the

generation and diversification of multicellular life. This work describes the first reconstruction of the tree of life of most known social amoeba species, and traces the acquisition of morphological and functional complexity during their evolution.

Material, methods and supporting material

Cell culture and genomic DNA extraction

All species were cultured on LP agar (1). When necessary, fruiting body formation of the more delicate species was facilitated by placing about 20 pellets of activated charcoal in the petri dish lid. To isolate genomic DNA, cells were harvested and washed three times with 10 mM K-phosphate buffer pH 6.5. Around 3 x 10^7 cells were lysed in 1 ml HMN (10 mM NaCl, 30 mM Mg-acetate, 10% (w/v) sucrose and 0.5% (v/v) Nonidet P-40 in 30 mM HEPES, pH 7.5), and nuclei were pelleted by centrifugation for 5 minutes at 6500 rpm. Pellets were resuspended in 50 µl HMN and 150 µl of resuspension solution from the GenElute Mammalian Genomic DNA Kit (Sigma, St.Louis, USA). Further DNA isolation was performed according to the kit's protocol.

PCR amplification, cloning and DNA sequencing

PCR and sequencing of SSU rDNA. A ~2000 base pair (bp) fragment of SSU rDNA was amplified by PCR (2) using primers A (forward) and B (reverse) as previously described (*3*) with 50°C annealing. Following amplification, PCR products were separated on 1% agarose gels, and appropriate sized bands excised and extracted from the gel, using the QIAquick gel extraction kit (Qiagen, Crawley, UK). Purified DNA was then cloned into the pGEM-T easy T-tailed vector (Promega, Southampton, UK) and transformed into DH5α competent cells (Invitrogen, Paisley, UK). A minimum of eight positive colonies were screened by PCR using flanking primer sequences (Sp6 and T7) to confirm the presence of inserts and to screen for possible multiple products (4). For sequencing, DNA was first amplified by PCR using the T7 and SP6 primers, precipitated with polyethylene glycol and processed further as described (3). Two clones for each PCR product were sequenced completely on complementary strands using the T7 or SP6 primers and one walking primer (forward primer D542-F: 5'-ACAATTG-GAGGGCAAGTCTG-3'; reverse primer D1340R: 5'-TCGAGGTCTCGTCCGTTATC-3'). To control for a possible multiple of SSU rRNA genes in *P. violaceum*, 50-150 PCR product clones for each of three independent isolates were screened by restriction fragment polymorphism (RFLP) analysis (3). Sequencing was performed using ABI PRISM BigDye Terminator v3.0 (Applied Biosystems) on a 3730 DNA Analyzer at the Oxford Sequencing Facility (http://polaris.bioch.ox.ac.uk/dnaseq/). Sequences were initially analyzed using Chromas version 2.23 (Technelysium Pty ltd) and then assembled into contigs using BioEdit version 5.0.9 (5).

PCR and sequencing of α-tubulin (tubA). Amplification, cloning and sequencing of *tubA* followed the same protocol as above except for using the primers atF3 and atR3 (*3*). Some *tubA* PCR reactions also required annealing at 40°C, and others required a second round of amplification using 1µl of the original reaction as substrate in order to obtain sufficient material for cloning. A minimum of 12 positive clones from each PCR product was screened by reamplification and size fractionation, which always yielded only identical sized clones from any given DNA. Two clones were then sequenced completely on complementary strands. Since *tubA* reactions utilized degenerate PCR primers, some reactions yielded multiple products of the correct or larger size. In all cases, these additional products were also cloned, screened and completely sequenced. However, in no case were multiple *tubA* genes detected.

Multiple sequence alignment

Alignment of SSU rDNA sequences consisted of five steps. 1) Based on a rough preliminary phylogeny, sequences were assigned to one of four major groups. 2) Sequences for

each group and for the amoebozoan outgroup were each aligned separately in ClustalX (*6*) using default parameters. 3) Each alignment was imported separately into the alignment editor Bioedit (5) where minor modifications were made by eye to minimize hypothetical insertion-deletion events, and 70% consensus sequences were calculated (also 50% and 60% sequences for amoebas). 4) The resulting consensus sequences were imported into PAUP* (7) and aligned to each other by eye along with individual sequences not obviously belonging to any group (*D. polycarpum, D. polycephalum, P. violaceum, D. laterosorum*). 5) Finally, the five separate alignments were imported into the same file. These were aligned to each other following their respective consensus sequences in the master consensus alignment, with minor final adjustments made by eye.

Only unambiguously aligned regions of the SSU rDNA alignment were used for phylogenetic analyses. These were defined as regions bordered by a consensus sequence entry for all Groups and with no internal insertions or deletions greater than 1 nucleotide in length (1674 sites). In order to resolve the highly similar Group 4 sequences, separate analyses were conducted including additional sites only alignable within the group (1861 total sites). The position of the Dictyostelia root was tested using only sites alignable among all 4 groups plus the amoebas (1374 sites). Very little length variation is seen in α-tubulin across all eukaryotes (8). Therefore, tubA nucleotide sequences were aligned manually in Bioedit (5) and then automatically translated into amino acids following intron removal. All analyses were conducted at the amino acid level, and all sites were included.

Phylogenetic analysis

Bayesian inference. Bayesian inference was used to calculate posterior probability of clades (biPP) utilizing the program MrBayes (version 3.1 or 3.1.1) (9). Final analyses consisted of two sets of four chains each (one cold and three heated) run for 1-10 million generations with trees saved every 10 generations and parameters sampled every 100. All analyses were run at least until a split frequency of <0.01 between the two run sets was reached. Posterior probabilities were averaged over the final 75% of trees (25% burn in), which was well passed convergence (log probability plateau, usually reached within <10,000 generations) for all analyses. Bayesian analyses of SSU rRNA sequences utilized the general time reversible model with a proportion of sites designated invariant, and rate variation among sites modelled after a gamma distribution divided into six categories (GTR+I+G) (10), with all variable parameters estimated by the program based on BioNJ starting trees. Amino acid sequences of α-tubulin were analyzed using a mixed amino acid model with sites again weighted according to a six-category gamma distribution and relevant parameters estimated by the program as above.

Maximum likelihood. Maximum likelihood was used to calculate bootstrap support values (mlBP) utilizing the program PHYML (11) over the web (http://atgc.lirmm.fr/phyml /) or on a PC. A total of 500 (web-based analyses) or 1000-10,000 bootstrap replicates were conducted using the GTR+I+G model for SSU rRNA and the WAG+I+G model (12) for amino acids. All variable parameters were estimated using the program from BioNJ starting trees.

Phylogenetic controls. All sets of analyses were also conducted with sequential and combined exclusion of relevant disproportionately long-branched sequences (overall tree and root analyses – *D. multistipes, A. ellipticum, D. polycarpum, Planoprotostelium, Thecamoeba*; Group 4 analyses - *D. septentrionalis*). From these results it was determined that neither topology nor support values were substantially affected by long-branches (13). That is, there were no changes in the significantly resolved portions of the topology (clades with mlBP>70% and/or biPP>0.90), and no substantial change in support values (mlBP +/- 5%, biPP +/- 0.01).

Combined sequence analyses. Combined analyses utilized concatenated alignments of SSU rRNA with either α-tubulin deduced amino acid sequences (tubA-AA) or first and second codon position nucleotides (tubA-NT). Differential weighting of the α-tubulin matrix was also performed in an attempt to compensate for the roughly 2 or 4 fold difference in the number of phylogenetically informative sites between SSU rRNA versus tubA-NT and tubA-AA datasets (564, 273 and 161 parsimony informative sites, respectively). Weighting was

accomplished by double, triple or quadruple entry of the tubA (NT or AA) matrix. Bayesian analyses utilized separate models for each data type (partition), with separate GTR+I+G models for SSU rRNA and tubA-NT, and a mixed amino acid model tubA-AA.

Maximum likelihood (PHYML) and paralinear distance (LogDet) analyses were performed at the nucleotide level only (SSU rRNA + tubA-NT), as mixed nucleotide and amino acid model implementations are not currently available for either of these methods. PHYML analyses were performed as described above. LogDet analyses were performed under the minimum evolution model with PAUP 4b10 (7) and a fraction of 0.34 sites designated as invariant as estimated by the program based on an initial neighborjoining tree calculated under the Kimura 2-parameter model (14). Analyses consisted of 1000 bootstrap replicates with trees derived by neighborjoining.

Results

Molecular phylogeny of Dictyostelia

Nearly complete small subunit ribosomal RNA (SSU rRNA) gene sequences were determined from over 100 isolates of Dictyostelia, including nearly every described species currently in culture worldwide. Phylogenetic analyses of these data identify four major subdivisions of the group, here numbered 1 to 4 (Fig. 1). Group 1 consists of a morphologically diverse set of *Dictyostelium* species. Group 2 is a mixture of species with representatives of all three traditional genera including all pale coloured species of *Polysphondylium*, at least two species of *Dictyostelium*, and all species of *Acytostelium*. Group 3 is again a diverse set of purely *Dictyostelium* species, also including the single cannibalistic species, *D. caveatum*. The largest group is Group 4, which consists almost entirely of *Dictyostelium* species possibly also including a clade of two violet colored species from two separate traditional genera, *P. violaceum* and *D. laterosorum.* With the exception of the latter, Group 4 is a fairly homogeneous set of large robust species including the model organism *D. discoideum* and the cosmopolitan species, *D. mucoroides*, which appears to be polyphyletic (9t).

These four SSU rRNA (rDNA) groupings are confirmed by α-tubulin phylogeny (Fig. 2) with two exceptions: i) *A. ellipticum* is only weakly placed with Group 2 in the α-tubulin tree (Fig. 2), and in fact appears as a poorly supported independent branch with maximum likelihood (not shown, 27t). ii) The *D. laterosorum* plus *P. violaceum* clade is grouped together with *D. polycephalum* as the sister group to a weakly supported Group 3 plus 4 clade (0.64 Bayesian inference posterior probability (biPP), 51% Maximum likelihood bootstrap (mlBP)), rather than as the exclusive sister lineage to Group 4 as in the SSU rRNA tree (Fig. 1). Weak resolution of the *A. ellipticum* branch and several other deep branches in the α-tubulin tree may be due to the much smaller size of this molecule versus SSU rRNA (322 and 1655 variable sites, respectively). Resolution of the position of the *D. laterosorum* plus *P. violaceum* clade may be further complicated by the accelerated evolution of α-tubulin sequences in Group 4 (Fig. 2) (*10*).

One taxon in particular, *D. polycarpum*, appears as a strongly supported solo branch in the SSU rRNA tree (Fig. 1) and the combined SSU rRNA plus α-tubulin nucleotide trees (not shown). This suggests that this delicate species with bunched fruiting bodies may represent an ancient dictyostelid lineage. This would also suggest that this species may, in fact, be a collection of morphologically similar cryptic species as suggested by the considerable difference in SSU rRNA sequence between the two separate isolates of *D. polycarpum* examined (Fig. 1). This could also be the case for *D. polycephalum*, as mentioned above, another delicate species with bunched fruiting bodies that is not definitively placed with any group by either gene.

One of the most striking features of the dictyostelid molecular phylogeny is the split of the genus *Polysphondylium*. The violet coloured *P. violaceum* is unequivocally grouped together with *D. laterosorum*, which lie together at the base of Group 4 (Fig. 1) or Groups 3 plus 4 (Fig. 2). Meanwhile the pale coloured Polysphondylids are all found nested within Group 2 (Fig. 1 and Fig. 2). With regard to Group 2, the phylogenetic position of *A. ellipticum* is particularly

intriguing. Both the SSU rRNA and α-tubulin trees place this taxon on a separate branch from the other *Acytostelium*s (Figs. 1 and 2), as does elongation factor 1α (EF-1α) phylogeny (B. Elgie, P. Schaap and S. Baldauf, unpublished results). This placement is also unequivocally supported in all combined SSU rRNA plus α-tubulin trees (not shown, *27*). This would be very interesting if true, because it would mean that the pale *Polysphondylium*s evolved from an *Acytostelid* ancestor. Thus the polysphondylids would not only have invented a relatively complex morphology from a morphologically simple ancestor, but they would also have reinvented the cellular stalk.

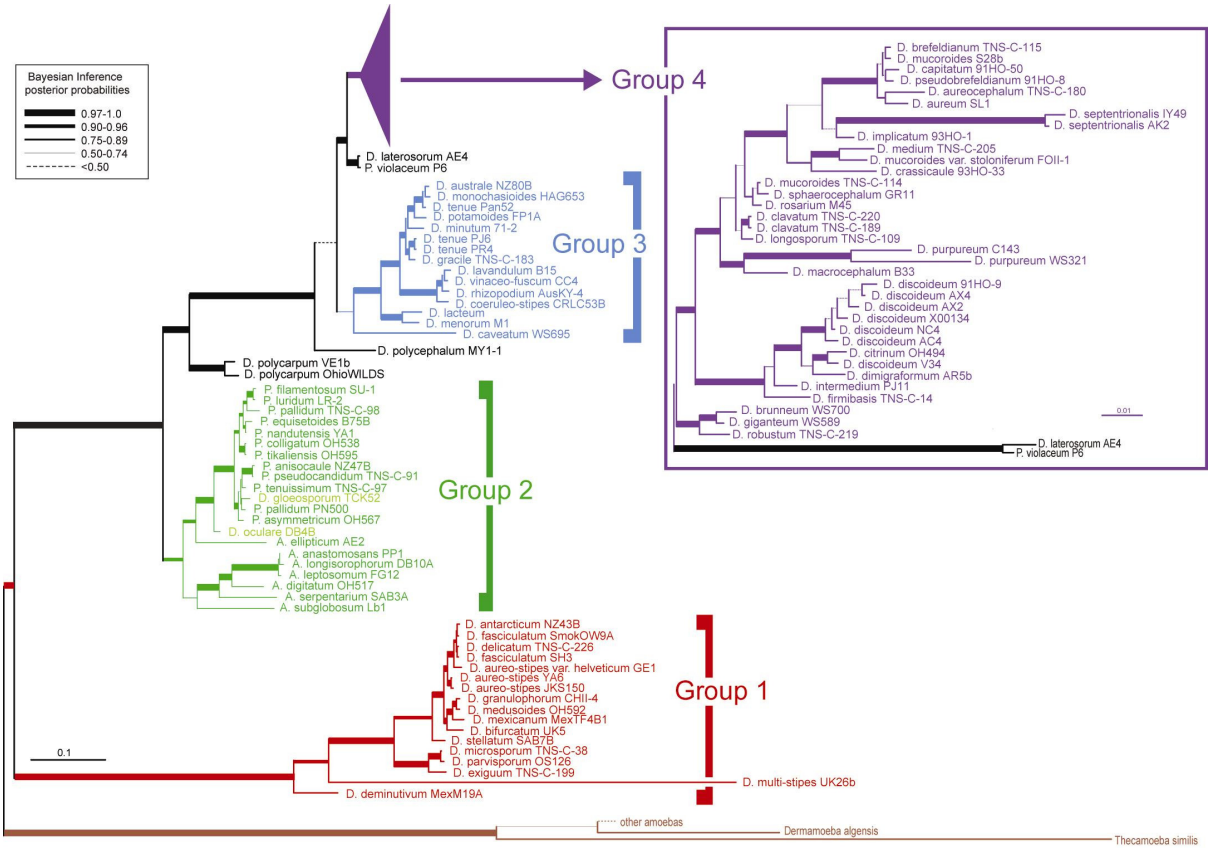

Figure 1. A universal phylogeny of the Dictyostelia based on SSU rRNA sequences
The tree shown was derived by Bayesian inference from 1655 well-aligned positions of SSU rRNA sequences. Four major taxa (Groups 1-4) are indicated by separate colours and to the right of the figure beside brackets (*Dictyostelium* species within Group 2 are indicated in lighter green). The tree includes nearly all known and described species of *Dictyostelium* (*D.*), *Polysphondylium* (*P.*) and *Acytostelium* (*A.*). Bayesian inference posterior probabilities (biPP) are roughly indicated by line width (key at the upper left of the figure). The tree is rooted based on separate analyses of 1355 positions alignable with sequences from closely related lobosan amoebas (*23, 24*). Branches lengths for the latter were scaled up to compensate for the smaller number of sites, based on the length of the first two internal branches (*27*).

The SSU rRNA phylogeny strongly supports Group 1 as the deepest major divergence in Dictyostelia (Fig. 1), as do analyses of combined SSU rRNA and α-tubulin nucleotide sequences (*27*). Although this root is not recovered in the α-tubulin amino acid phylogeny, which tentatively places Group 2 as the first major split (Fig. 2). These data only weakly resolve most of the deep branches in the tree (Fig. 2). Identifying the root of a tree is one of the most difficult problems in phylogenetic reconstruction, due to the antiquity of these events and the often-large divergences involved (*11*). Therefore, the position of the root of the dictyostelid tree still requires confirmation by broad sampling of additional genes and, eventually, combined multi-sequence analyses (*12,13*).

Initial inspection of the first taxonomically comprehensive molecular data set from Dictyostelia (Fig. 1 and Fig. 2) indicates that, at least in molecular terms, Dictyostelia is a very

deep and complex taxon. In fact, the dictyostelid SSU rRNA tree possesses a molecular depth roughly equivalent to that of a broad sampling of animals and substantially larger than that of a broad sampling of fungi.

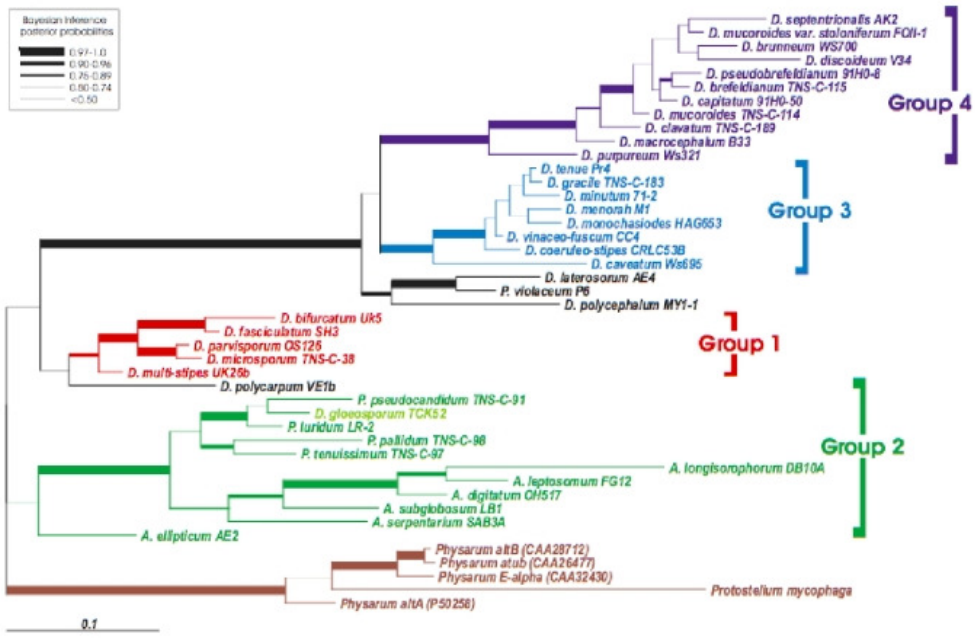

Figure 2. Phylogeny of the Dictyostelia based on α-tubulin amino acid sequences
The tree shown was derived from 322 universally aligned α-tubulin amino acid positions using Bayesian inference with a mixture of amino acid models and a six-category gamma correction. The tree includes a broad representation of all four major groups identified in the comprehensive SSU rRNA phylogeny. Colours and brackets indicate major groups with labels as in Figure 1. All biPP values are roughly indicated by line width according to the key shown in the upper left. Branch lengths are drawn to scale as indicated by the scale bar at the lower left (*27*).

Evolution of morphology in the Dictyostelia

Social amoeba species show striking differences in the size and branching patterns of their fruiting bodies and the presence/absence and shape of support structures. They may also vary in spore characteristics, cell aggregation patterns, slug migration characteristics and presence/absence of alternative life cycles, such as the microcyst and macrocyst. To understand how these traits might have evolved, we mapped all well-documented dictyostelid traits onto the molecular phylogeny, summarising a >50 year literature (Fig. 3). Some of the most significant traits are summarized below.

Spores and cysts. Few of the traditionally noted morphological characters show any clear trend across the tree, although a number show interesting within group trends (Fig. 3). The most globally consistent character appears to be spore shape (Fig. 3, column 2). Spores can be either round (globose) or oblong, in the latter case often with granules at their poles. Groups 1 and 3 are characterised by having oblong spores with tightly grouped (consolidated) granules. In Group 2, the granules have become loosely grouped (unconsolidated), while polar granules are lost entirely in Group 4. Group 1 is further characterised by having markedly smaller spores than the other taxa (Fig. 3, column 1).

Species that form sexual macrocysts are present in all four groups (Fig. 3, column 4). Although homothallic macrocysts are not uncommon, many species require cells of opposite mating type for sex (*2*). The number of species that show sexual activity is therefore probably underestimated, because the proper mates were never brought together in the laboratory.

Many species in Groups 1, 2, and 3 individually encapsulate to form microcysts, but these have not been observed for Group 4 species (Fig. 3, column 3). The number of microcyst forming spe-cies is probably also underestimated, since not every species may have been subjected to conditions that trigger this alternative survival strategy.

Aggregation and slug migration. To aggregate, cells can either come together as individuals, creating mound-shaped aggregates, or line up with each other to form inflowing streams. Stream formation is the most common mode of aggregation, with only a few species in Groups 1 and 3 aggregating as individuals (Fig. 3, column 6). The chemoattractant (acrasin) that is used for aggregation has been identified for only a few species (Fig. 3 column 5). For all investigated Group 4 species, including *D. discoideum*, the acrasin is cAMP. In Group 3, the acrasin has been identified for only three species, all of which differ: *D. minutum* uses folic acid, *D. lacteum* a pterin analog and *D. caveatum* the modified dipeptide glorin (Fig. 3, column 5). The latter may be fairly widespread, since it is used as acrasin in Groups 2 and 3 and in the *P. violaceum* and *D. laterosorum* clade.

Fruiting body formation is often preceded by a period of slug migration, where slugs can either migrate freely or lay down a stalk while migrating. Slug migration occurs in all four taxon groups, but is most common among Group 4 species (Fig. 3, column 7). A small cluster of Group 4 species and a single non-Group 4 species, *D. polycephalum,* however, are the only ones to show free migration, suggesting that this character has evolved twice independently.

Size and shape of fruiting bodies. A primary determinant of fruiting body (sorocarp) size is the number of cells that can be collected into one aggregate. However, most of the sorocarp size and shape variation depends on the extent and manner of subsequent aggregate subdivision (Fig. 3, columns 9-14). These characteristics are controlled by so-called organizing centers or “tips”, the first of which appears as a small protrusion on top of a newly formed aggregate. Additional tips can appear during streaming aggregation, breaking up streams into smaller aggregates. This gives rise to a gregarious sorocarp habit. Alternatively, multiple tips may appear on completed aggregates, giving rise to a clustered or coremiform (bunched) habit.

The rising cell masses (sorogens) that form the sorocarps can subdivide even further, forming secondary spore heads. This can occur either by new tips arising along the main sorogen axis, yielding lateral branches, or by groups of cells detaching themselves from the rear of the sorogen. The latter abstricted masses can differentiate directly into spores to yield sessile sori (spore heads), as in *D. rosarium* (*16*), or form new tips giving rise to whorls of branches that are either irregular as in *D. tenue* (*17*) or evenly spaced as in the polysphondylids (18t).

Species in Groups 1-3 usually display a clustered or gregarious sorocarp habit, while Group 4 species mainly form solitary fruiting bodies (Fig. 3, column 9). Additionally, branched forms are much more common in Groups 1-3 than in Group 4. However, both lateral and whorled-type branching are found in most groups (Fig. 3, column 10). Not surprisingly, there is an inverse relationship between a tendency for aggregates and sorogens to subdivide and the size of stalk and sorus. Thus, the Group 4 species with their consolidated aggregates also have the largest sori and the thickest and longest stalks (Fig. 3, columns 11-13).

Other characteristics, such as the presence of support structures formed from stalk-like cells such as basal disks, triangular supporters or crampons, also appear to be markedly correlated with large fruiting body size, particularly in Groups 3 and 4 (Fig. 3, column 14). Group 4 especially appears to be the culmination of an evolutionary trend towards larger-sized units of stalk and spore-head. This trend was accompanied by increased cell-type specialization resulting in the formation of robust stalk support structures.

Conclusions

We present the first molecular phylogeny of the social amoebas based on two independent markers. The use of two structurally and functionally unrelated genes, in this case SSU rRNA and α-tubulin, is critical to control for gene-specific artefacts to which all single gene trees are vulnerable (B. Elgie, P. Schaap and S. Baldauf, unpublished results).

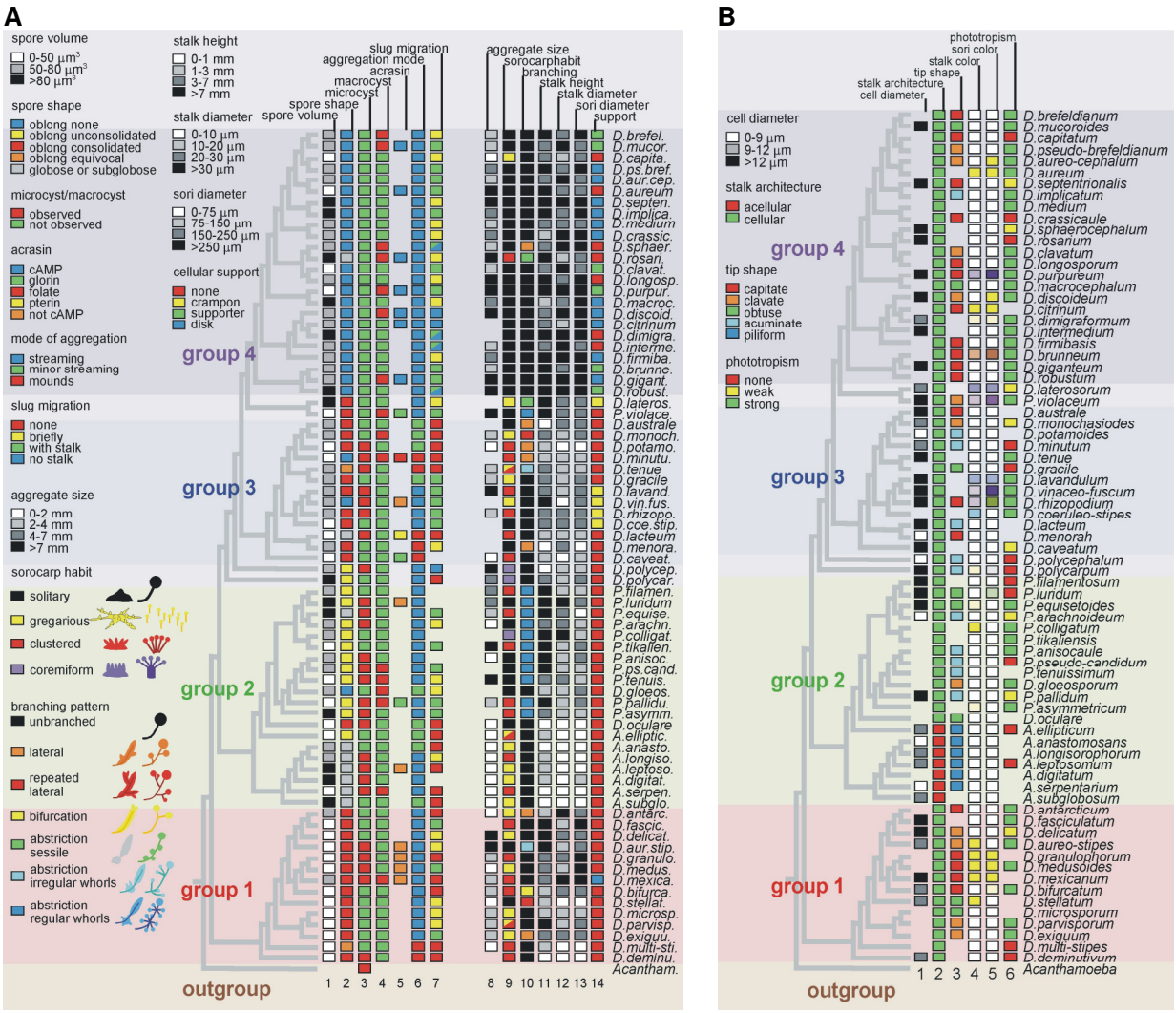

Figure 3. Trait mapping of dictyostelid characters (A) Consistently documented characters were retrieved from primary species descriptions (Table 1) and from *Dictyostelium* monographs (*2,24*). Character states were numerically coded and mapped to the dictyostelid SSU rRNA phylogeny (Fig. 1) using the MacClade 4 software package (*25*). For comprehensive presentation the most informative characters are combinatorially presented on a single tree with the numerical code converted into colour code for qualitative traits and into greyscale for quantitative traits. The code key for the character states is shown on the left side of the figure and in Table 2 (see main text for details). (B) An additional six characters were mapped onto the dictyostelid SSU rRNA phylogeny. Cell size, though inconsistently documented, shows no strong group-specific trend (column 1). A very restricted trait is the acellular stalk, which is found only in a subset of Group 2 species, the acytostelids (column 2). The tips of acellular stalks are thinly pointed (piliform) (column 3). Pointy (acuminate) or blunt (obtuse) tips also mark the cellular stalks of other group 2 species and of some group 3 species. Group 1 and Group 4 species usually have extended (capitate or clavate) stalk tips. Another character that is of importance for taxonomy is the color of the spores and stalk. This is represented here as an approximation of the observed color. Species with similarly coloured stalks and spore heads are often related, but no colour is specific for any of the four major groups (columns 4 and 5). *D. discoideum* slugs show phototaxis and thermotaxis, but these properties have rarely been investigated for other species. However, a related character is phototropism, the tendency of fruiting structures to veer towards the light (*1*). This trait appears to be common to all four groups, but not to all species within the groups (column 6).

In addition, congruent support from independent data is the strongest form of proof in evolutionary study (*19*). SSU rRNA was chosen for the primary phylogeny because it is a powerful and highly tractable phylogenetic marker, backed by the single most taxonomically broad molecular database available (*20*). A taxonomically broad sampling of α-tubulin sequences was then determined to test all major features of the SSU rRNA tree.

Thus, phylogenetic analyses of two independent molecular markers, SSU rRNA and α-tubulin, consistently subdivide nearly all dictyostelid species into four major groups with strong support from one or both molecules. Both trees also strongly support a Group 3 plus 4 supergroup, and both SSU rRNA (Fig. 1), and combined SSU rRNA plus weighted α-tubulin nucleotide sequences (*27*) place Group 1 as the earliest branching lineage in the tree. However, the

latter remains to be confirmed with additional data, as α-tubulin lacks adequate phylogenetic signal to resolve the deepest branches in the tree (Fig. 2) and combined analyses of only these two very disparate genes can only provide weak additional support (*27*). Although more limited sampling of EF1α data also confirms several of the major trends in the SSU rRNA and α-tubulin trees, interpretation of the EF1α data is complicated by multiple gene duplications within Dictyostelia (B. Elgie, P. Schaap and S. Baldauf, unpublished results).

None of the four major groups identified in the new molecular phylogeny correspond to traditional dictyostelid classification. The molecular tree is dominated by *Dictyostelium* species, which appear in all four groups. Most surprisingly, *Polysphondylium*s are found in two separate locations, within Group 2 and at the base of Group 4. This split of purple and pale polysphondylid species is supported by phylogenetic analyses both SSU rRNA (Fig. 1) and α-tubulin (Fig. 2), and by a cladistic analysis of morphological traits (*21*). While the *Acytostelium*s probably all reside in Group 2, this group also includes *Dictyostelium* and *Polysphondylium* species. Therefore, none of the four molecularly defined dictyostelid groups correspond to traditional genera, and none of the traditional genera, with the possible exception of *Acytostelium*, are even monophyletic. This indicates that fruiting body morphology is a very plastic trait in Dictyostelia and is apparently of little use as a taxonomic determinant. This is even more evident from the scattered distribution of similar branching morphologies over the four taxon groups (Fig. 3, columns 9-10). For instance, the rosary-type, coremiform and laterally branched morphologies appear respectively two, three and seven times independently across the tree.

The strongest evolutionary trend in dictyostelid fruiting body morphology appears to be related to size. Within each group the more derived species tend to have larger fruiting bodies, but this is most clearly shown when comparing entire groups. Whereas the species in Groups 1, 2 and 3 generally split up their aggregates into multiple sorogens, which then subdivide even further to yield branched fruiting bodies, the aggregates of Group 4 species usually give rise to a solitary fruiting body that is only rarely branched. As a result, the Group 4 species have more robust fruiting structures with much larger spore heads than the other groups. These large structures are typically supported at their base by basal discs or triangular supporters that are derived from a third cell-type, the anterior-like cells. In at least one species, *D. discoideum*, this cell-type diverges even further to produce two more structures, the upper- and lower cup that support the spore head. This is an interesting example of the correlation between the size of an organism and its cell-type diversity, which marks the evolution of many multicellular organisms (*22*).

To conclude, we have defined a radically new phylogeny for a major taxonomic group that includes an important model organism, and we have mapped a variety of morphological and behavioral characteristics onto the tree. The phylogeny suggests that Dictyostelia is a molecularly deep and complex group that is in need of major taxonomic revision. This new dictyostelid phylogeny also indicates that many widely studied characteristics of the group are highly plastic and therefore probably controlled by a small number of genes. This means that, using the new phylogeny as a guide, Dictyostelia is now uniquely well suited for unravelling the underlying mechanisms controlling these phenomena and thereby to add important information to our understanding of fundamental aspects of the evolution of eukaryotic cell-cell signalling and multicellularity.

Compilation of data used in the trait analsis represented in Figure 3.

Table 1. Consistently documented characters were retrieved from the original species diagnoses and from secondary publications, such as the *Dictyostelium* monographs by Raper and Hagiwara (1,15). The varieties (states) described for qualitative characters, such as the shapes of spores or the branching patterns of fruiting bodies were numerically coded. The alphabetical code key for each character and the numerical code key for each character state are listed in table 2. For quantitative characters (usually size) a frequency distribution of data for all species was prepared first, and the data range was subdivided into 3-4 intervals in such a manner that each interval contained about the same number of species. Each interval then represents a character state, which was again numerically coded as shown in table 2. Quantitative characters have usually been reported as a range of observed values. For trait mapping this range had to be reduced to a single parameter. For characters such as the size of aggregates, stalks and sori, the upper and intermediate value of the range were first analysed separately. However, this revealed virtually identical trends in character evolution (data not shown). We chose to show the analysis of the upper values here, because this is a better indication of the maximal number of cells that can be incorporated into a single structure (all species will form small structures when few cells are available, but only few will form large structures at high cell densities). For spore and cell sizes, intermediate values were used or average values, when reported. Spore sizes were usually reported as length (L) and width (w) of oblong spores and diameter (d) of globose spores. These values were further reduced to a single parameter by calculating the volume of oblong spores as a cylinder ($L\pi(\frac{1}{2}w)^2$) and that of globose spores as a sphere ($4/3\pi\ (\frac{1}{2}d)^3$).

Species / Character	Character states																		Citation
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
D.brefeldianum	1	0	1	0	?	2	1	1	4	0	3	2	3	2	?	0	0	2	(15, 16)
D.mucoroides	1	0	1	0	0	2	2	1	4	0	2	3	2	2	2	0	2	2	(1, 15, 17)
D.capitatum	0	0	1	1	?	2	1	0	0	0	2	1	2	0	?	0	0	0	(15, 18)
D.pseudo-brefeldia.	1	0	1	1	?	2	2	2	4	0	3	2	3	3	?	0	1	2	(19)
D.aureocephalum	1	0	1	1	?	2	2	1	4	0	2	3	2	3	?	0	1	2	(20)
D.aureum	0	0	1	1	0	2	2	3	4	0	3	2	2	0	?	0	?	2	(1, 21, 22)
D.septentrionalis	2	0	1	1	?	2	1	3	4	0	3	3	3	3	2	0	0	1	(1, 15, 23)
D.implicatum	2	0	1	1	?	2	2	2	4	0	3	2	3	3	?	0	3	2	(15, 24)
D.medium	1	0	1	1	?	2	1	1	4	0	2	2	2	3	?	0	?	2	(25)
D.crassicaule	1	0	1	1	?	2	1	2	4	0	1	3	3	3	?	0	0	0	(15, 24)
D.sphaerocephalum	1	0	1	0	?	2	3	1	4	1	2	3	3	0	2	0	?	1	(1, 26, 27)
D.rosarium	2	5	1	0	0	2	1	3	2	4	3	1	2	0	2	0	?	0	(1, 28)
D.clavatum	0	0	1	1	?	2	2	1	4	0	2	3	2	2	?	0	1	2	(25)
D.longosporum	1	0	1	1	?	2	1	3	4	0	3	2	3	0	?	0	0	2	(29)
D.purpureum	0	0	1	0	0	2	2	3	4	0	3	3	3	2	2	0	0	2	(1, 15, 30)
D.macrocephalum	2	0	1	1	?	2	1	2	4	0	1	2	3	3	?	0	2	2	(15, 31)
D.discoideum	1	0	1	0	0	2	4	3	4	0	2	3	3	3	2	0	1	2	(1, 15, 32)
D.citrinum	1	0	1	1	0	2	4	?	4	0	2	1	3	3	?	0	0	?	(33)
D.dimigraformum	2	0	1	1	?	2	3	?	4	0	3	3	3	0	1	0	?	2	(1, 34)
D.intermedium	1	0	1	1	?	2	3	1	4	0	2	3	2	0	2	0	?	2	(1, 35)
D.firmibasis	1	0	1	1	?	2	1	2	4	0	3	3	3	3	?	0	0	2	(1, 15, 27)
D.brunneum	1	0	1	1	?	2	2	1	4	0	3	2	3	2	?	0	0	2	(1, 36)
D.giganteum	1	0	1	0	0	2	2	3	4	0	3	3	3	3	2	0	0	2	(1, 37-39)
D.robustum	2	0	1	1	?	2	3	3	4	0	3	3	3	4	?	0	0	2	(19)
D.laterosorum	2	2	1	1	?	2	1	?	0	4	3	2	2	1	1	0	?	1	(1, 34)
P.violaceum	0	2	1	0	3	2	2	3	4	6	3	2	2	0	2	0	1	2	(1, 15, 40)

D.australe	1	2	1	1	?	1	0	?	4	1	0	2	2	0	2	0	?	?	(41)
D.monochasioides	1	2	1	0	?	1	2	1	0	2	2	1	2	0	1	0	1	1	(1, 15, 42, 43)
D.potamoides	0	2	0	1	?	1	0	0	2	1	1	0	0	0	0	0	?	?	(44)
D.minutum	0	0	0	0	1	0	0	?	2	1	1	1	1	0	1	0	3	0	(1, 15, 45)
D.tenue	1	3	0	1	?	0	0	1	1	5	2	1	1	0	2	0	?	2	(1, 46)
D.gracile	1	2	1	1	?	1	2	1	0	0	2	1	2	0	?	0	2	0	(29)
D.lavandulum	1	2	0	1	?	2	2	3	2	0	2	1	2	1	2	0	?	2	(1, 15, 47)
D.vinaceo-fuscum	1	0	0	1	4	2	2	?	0	0	3	0	2	1	2	0	?	2	(1, 47, 48)
D.rhizopodium	1	2	0	1	?	2	2	1	2	0	2	2	2	1	2	0	0	2	(1, 15, 47)
D.coeruleo-stipes	0	2	1	1	?	2	2	?	4	0	2	2	2	1	?	0	?	2	(1, 47)
D.lacteum	0	4	0	1	2	0	0	?	2	0	1	1	1	0	2	0	3	?	(1, 49)
D.menorah	1	2	1	1	?	0	1	?	4	1	0	2	0	0	0	0	0	?	(50)
D.caveatum	0	2	1	1	3	0	?	0	2	0	1	0	1	0	2	0	?	1	(1, 51)
D.polycephalum	1	1	0	1	?	2	4	3	3	0	0	1	1	0	0	0	3	0	(1, 15, 52)
D.polycarpum	2	1	1	1	?	2	0	2	3	0	2	1	2	0	2	0	3	0	(1, 53)
P.filamentosum	1	1	1	1	?	2	?	2	2	6	3	1	1	0	2	0	?	0	(1, 53)
P.luridum	2	1	0	1	4	2	?	2	4	6	3	3	2	0	2	0	2	0	(54)
P.equisetoides	2	4	0	1	?	2	2	1	2	6	3	2	0	0	2	0	2	2	(55)
P.arachnoideum	1	1	0	1	?	2	1	0	4	6	2	2	1	0	0	0	3	1	(56)
P.colligatum	1	1	1	1	?	2	?	?	3	6	3	3	1	0	?	0	?	2	(57)
P.tikaliensis	0	1	1	1	?	2	2	3	2	6	3	1	2	0	?	0	?	2	(57)
P.anisocaule	1	1	0	1	?	2	?	0	4	6	3	1	2	0	?	0	3	2	(41)
P.pseudo-candidum	1	1	0	0	?	2	2	?	4	6	3	1	1	0	?	0	3	0	(1, 15, 43, 58)
P.tenuissimum	0	1	0	0	?	2	1	3	4	6	3	2	1	0	?	0	3	?	(1, 15, 58)
D.gloeosporum	1	0	1	0	?	1	1	?	4	0	1	1	2	0	?	0	1	2	(59)
P.pallidum	0	1	0	0	3	2	2	1	2	6	3	1	2	0	2	0	3	1	(1, 15, 21)
P.asymmetricum	2	1	0	1	?	2	2	?	2	6	2	1	1	0	?	0	?	2	(57)
D.oculare	0	2	0	1	?	1	0	0	4	0	0	0	0	0	?	0	?	?	(44)
A.ellipticum	0	1	1	1	?	1	0	0	1	0	1	0	0	0	1	1	4	0	(1, 34)
A.anastomosans	1	4	1	1	?	1	0	0	0	0	0	0	0	0	0	1	?	?	(44)
A.longisorophorum	1	4	0	1	?	2	1	0	2	0	1	0	0	0	1	1	?	?	(44)
A.leptosomum	2	4	0	1	4	2	0	0	0	0	1	0	0	0	1	1	4	0	(1, 48, 60)
A.digitatum	2	4	0	1	?	2	?	0	0	0	1	0	1	0	?	1	4	?	(61)
A.serpentarium	0	4	0	0	?	2	0	0	2	0	0	0	0	0	0	1	?	?	(44)
A.subglobosum	2	4	1	1	?	1	0	0	0	0	0	0	0	0	1	1	?	?	(1, 35, 61)
D.antarcticum	1	2	1	1	?	2	0	0	0	1	1	3	2	0	?	0	?	2	(41)
D.fasciculatum	0	2	1	1	?	2	2	?	2	0	3	1	3	0	2	0	?	2	(1, 53)
D.delicatum	0	2	1	1	?	2	1	3	0	0	3	2	2	0	2	0	1	1	(1, 15, 27)
D.aureo-stipes	0	2	0	1	4	2	1	3	4	5	3	1	2	0	1	0	1	2	(1, 15, 46)
D.granulophorum	0	2	0	1	4	2	0	1	2	0	2	2	3	0	?	0	0	?	(33)
D.medusoides	0	2	0	1	4	2	2	0	0	0	2	1	3	0	?	0	0	2	(33)
D.mexicanum	1	2	0	0	4	2	0	1	2	0	2	3	3	3	2	0	0	?	(1, 22)
D.bifurcatum	0	2	1	1	?	2	0	1	2	3	1	2	2	0	1	0	0	2	(1, 35)
D.stellatum	0	2	1	1	?	2	1	0	4	2	1	0	0	0	1	0	2	?	(44)
D.microsporum	0	2	1	1	?	2	1	1	0	0	1	1	1	0	?	0	2	0	(1, 15, 62)
D.parvisporum	0	2	1	1	?	2	1	1	1	0	3	1	2	0	?	0	1	2	(15, 63)
D.exiguum	0	2	1	1	?	2	2	1	0	1	2	2	2	0	?	0	1	2	(29)
D.multi-stipes	0	3	1	1	?	0	0	0	2	0	1	0	0	0	?	0	?	0	(1, 35)
D.deminutivum	0	2	0	1	?	0	0	?	2	0	0	0	0	0	1	0	?	0	(1, 64)

Table 2. This table lists the letter code for character names and the numerical code for character states as are used in Table 1. The colour code for character states as used in figure 4 is indicated in the last column.

Character name	Code	Character state	Code	Colour code
Spore volume	A	0-50 μm^3	0	white
		50-80 μm^3	1	grey
		>80 μm^3	2	black
		not measured or unknown	?	absent
Spore shape and granule position	B	oblong, no polar granules	0	blue
		oblong, unconsolidated polar granules	1	yellow
		oblong, consolidated polar granules	2	red
		oblong, granule position equivocal	3	orange
		globose or subglobose	4	grey
Microcyst	C	observed	0	red
		not observed	1	green
Macrocyst	D	observed	0	red
		not observed	1	green
Acrasin	E	cAMP	0	blue
		glorin	1	green
		folate	2	red
		pterin	3	yellow
		not cAMP	4	orange
Mode of aggregation	F	mounds	0	red
		minor streaming	1	green
		streaming	2	blue
Slug migration	G	none	0	red
		briefly	1	yellow
		with stalk being formed	2	green
		with and without stalk	3	green/blue
		without stalk formation	4	blue
Aggregate size	H	0-2 mm	0	white
		2-4 mm	1	light grey
		4-7 mm	2	dark grey
		>7 mm	3	black
Sorocarp habit	I	gregarious (loosely grouped)	0	yellow
		gregarious and clustered	1	yellow/red
		clustered (closely grouped)	2	red
		coremiform (bunched)	3	purple
		solitary	4	black
Branching pattern	J	unbranched	0	black
		lateral branches	1	orange
		repeated lateral	2	red
		bifurcation	3	yellow
		abstriction from posterior - sessile	4	green
		abstriction - irregular whorls	5	light blue
		abstriction - regular whorls	6	dark blue
Stalk height	K	0-1 mm	0	white
		1-3 mm	1	light grey
		3-7 mm	2	dark grey
		>7 mm	3	black

Stalk diameter	L	0-10 µm	0	white
		10-20 µm	1	light grey
		20-30 µm	2	dark grey
		>30 µm	3	black
Sori diameter	M	0-75 µm	0	white
		75-150 µm	1	light grey
		150-250 µm	2	dark grey
		> 250 µm	3	black
Cellular support	N	none	0	red
		crampon	1	yellow
		supporter	2	green
		disk	3	blue
Cell diameter	O	0-9 µm	0	white
		9-12 µm	1	grey
		>12 µm	2	black
Stalk shape	P	cellular	0	green
		acellular	1	red
Stalk tip shape	Q	capitate (head-shaped)	0	red
		clavate (club-shaped)	1	orange
		obtuse (blunt)	2	green
		acuminate (pointed)	3	light blue
		piliform (thread-shaped)	4	dark blue
Phototropism	R	none	0	red
		weak	1	yellow
		strong	2	green

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Chapter Five

Evolutionary origins of cAMP signalling in the social amoebas

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Evolutionary origin of cAMP-based chemoattraction in the social amoebae
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Abstract

Phenotypic novelties can arise if integrated developmental pathways are expressed at new developmental stages and are then recruited to serve new functions. I have analysed the origins of a novel developmental trait of Dictyostelid amoebas: the evolution of cAMP as a developmental chemoattractant. I show in this Chapter that cAMP's role to attract starving amoebas arose through recruitment of a pathway that originally evolved to coordinate fruiting body morphogenesis. cAMP receptors (*cARs*) from basal species are only expressed during fruiting body formation, but can fully rescue cAR deletions in derived species. Abrogation of cAR function blocks all development in derived species, but only fruiting body formation in basal species. This indicates that aggregation by cAMP signalling arose through co-option of a pre-existing pathway that originally evolved to coordinate morphogenesis.

Introduction

The origin of species diversity is the story of the origin of novel features. These can arise through the development of entirely new genes (1), or when pathways underlying existing functions are co-opted to perform new ones through altered regulation of the component genes. Novel features of development, which can cause dramatic shifts in species form, are particularly thought to arise by this manner (2-4). However, few data exist to support this common view, and even fewer to document the steps involved at high phylogenetic and biochemical resolution. Here I report on the analysis of the derived origin of a novel, even group-defining, feature of Dictyostelid social amoebas: the origin of cAMP based chemoattraction.

The Dictyostelid amoebas are a diverse group of organisms that display conditional multicellularity with a range of phenotypes (5). In the model system *Dictyostelium discoideum* extracellular cAMP pulses coordinate the aggregation of starving amoebas (6). cAMP has also been implicated as a morphogen that directs subsequent fruiting body formation (7). cAMP is produced by an adenylyl cyclase, ACA, and degraded by an extracellular phosphodiesterase, PdsA (8,9). Together with cAR1 or cAR3, two of the four *D.discoideum* cAMP receptors, these enzymes are essential for oscillatory cAMP signalling (10,11).

Material and methods

Cell lines and culture

D.minutum 71-2, *D.fasciculatum* SH3, *P.pallidum* TNS-C-98 and *D.rosarium* M45 cells were grown in association with *Klebsiella aerogenes* on LP agar (5). *D.discoideum* DH1 cells were grown in HL5 medium (13). For developmental time courses, cells were harvested while in exponential phase and incubated at 22°C and 8 x 10^5 cells/cm^2 on non-nutrient agar (1.5% agar in 10 mM phosphate buffer, pH 6.5).

Gene identification

The degenerate oligonucleotides, 5'-GGTAGTTTCGCATGYTGGYTNTGGAC-3' and 5'-TCACCGAAGTATCGCCACATNTRNGGRTT-3' designed to match amino-acid sequences GSFACWLWT and NPLMWRYFG that are conserved between cARs 1-4 of *D.discoideum* were used to amplify putative cAR genes by touch-down PCR (14) from genomic DNAs of the four test species. The touch-down protocol started with 4 cycles with annealing at 60°C for 30s, 10 cycles with an annealing temperature decrement of 1°C and 20 cycles with annealing at 50°C. The PCR products were subcloned in the pGEM-T Easy vector (Promega, Madison, USA) and their sequence was determined from at least three independent clones. The *DmcAR* PCR product was used to screen a λZapII library of sheared *D.minutum* gDNA, which was custom-made by Stratagene (La Jolla, USA) from *D.minutum 71-2* gDNA provided by us. Three positive plaques, C2, C6 and C10, were identified and their pBluescript phagemids were

isolated by *in vitro* excision according to the manufacturer's instructions. The respective 3.5, 4.7 and 1.25 kb inserts were sequenced to four-fold coverage by primer walking. The sequences could be assembled into a 4873 bp contig, which apart from the *DmcAR*, contained two other ORFs. BLAST searches of the entire Genbank database showed that one partial ORF was most similar to *D.discoideum SpkA* (15), and the other complete ORF to the putative *D.discoideum* protein *DDB0217155* (http://dictybase.org/). The *D.minutum* protein was called DtmA for its only structural feature of Dual TransMembrane helices.

RNA isolation and analysis

Total RNA was isolated from 2 x 10^7 cells, size-fractionated in 1.5 % agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (16). Cells in the culmination stages were vortexed for 5 min with glass beads during RNA extraction to break stalk cells and spores. Membranes were hybridised at 65°C to [^{32}P]dATP-labelled DNA probes and washed at high stringeny according to standard procedures (17). Three microlitres of 0.28- to 6.6-kb RNA markers (Promega, UK) were run on the same gel and stained with ethidium bromide to estimate the size of the *cAR* mRNAs.

Heterologous expression of DmcAR

A 1525 bp fragment was amplified from λZAPII clone C6 using oligonucleotides that will generate BglII restriction sites (5'-CCAGATCTAAAATGGAACAATCACCCGATG-3' and 5'-CCAGATCTCAACCCCAAAACCAACAAC-3'). This fragment includes the complete 1173 bp coding region of DmcAR with 3 bp of 5'UTR and 364 bp of 3'UTR. The BglII digested product was subcloned into the BglII site of vector PJK1 (10), which placed *DmcAR* downstream of the *D.discoideum* actin15 promoter and yielded vector A15:DmcAR. The integrity of the A15:DmcAR fusion was verified by DNA sequencing. The *car1car3* mutant (18) was transformed with either A15:DmcAR or A15:DdcAR1 in PJK1 (19) and selected for growth at 20 µg/ml G418 (Sigma, USA).

cAMP binding assay

To measure cell surface cAMP binding activity, 1.6 x 10^7 cells were incubated for 1 min at 0°C with 1 or 10 nM ^{3}HcAMP (Amersham, Little Chalfont, UK), 5 mM dithiothreitol (Sigma, USA) and variable concentrations of cAMP in a total volume of 100 µl. Cells were separated from unbound ^{3}HcAMP by centrifugation for 10 s at 16,000 x g through a 4:11 mix of AR200:AR20 silicon oil (Wacker-Chemie, Burghausen, Germany). The ^{3}HcAMP associated with the cell pellet was measured by liquid scintillation counting.

Video microscopy and image analysis

An Axiovert 135 microscope (Zeiss; objectives: FLUAR 10´/NA 0.5, Plan NEOFLUAR 10´/NA 0.3) equipped with a cooled CCD camera (Hamamatsu, C4880-82) was used to record wave propagation in aggregating cells and darkfield waves during development (20,21). Images were saved as TIFFs and transferred to a PC for analysis with the Optimas software (MediaCybernetics, version 6.1). Standard image processing techniques like image subtraction were applied to improve the visibility of the optical density waves. To determine wave propagation speed and periodicity time-space-plots were generated and analysed as described previously (20,21).

Phylogenetic analysis

For the cAR protein tree, sequences were aligned with ClustalX (22) using default parameters. Only ungapped regions (or with small gaps in single sequences) and flanked by 70% consensus sites were used. The tree shown was derived by maximum likelihood and

Bayesian Inference analyses on 291 unambiguously aligned amino acid positions. The Bayesian inference utilised MrBayes V3.0 (23) with posterior probabilities (biPP) values estimated from 10^7 chains and discarding a burnin of 1000. Maximum likelihood bootstrap percentage (mlBP) values were determined from 500 replicates using the PROML program from the PHYLIP package (24). Both analyses utilized the JTT model (25) for weighting amino acid substitutions and a gamma correction for rate variation among sites. An α value of 1.39 was used for the gamma distribution in the mlBP analyses, as determined by the program Tree-Puzzle (26). Support values for the *cAR*+*TasA* subtree were determined from a dataset consisting of only these nine sequences, to avoid loss of resolution due to long-branch attraction to the distantly related outgroup sequences. The full dataset of 13 sequences was then used to test the deeper nodes. Four G-protein coupled receptor (GPCR) sequences were used to root the tree as these were shown to be the most conservative (relative to the cAR sequences) based on phylogenetic analyses using a range of cAR-related sequences.

For the small subunit ribosomal RNA (SSU rDNA) tree, complementary DNA sequences were aligned by eye and only unambiguously aligned, ungapped regions were used to construct the tree. Both Bayesian Inference and maximum likelihood analyses utilised the general-time-reversible model with a gamma correction for rate variation among sites and a designated proportion of invariant sites (GTR+I+G). The biPP values were estimated from 10^7 chains with a burnin of 10000 and mlBP values from 100 replicates. All parameters were estimated from the data by the respective phylogenetic programs.

Results

Identification of cAR-like sequences in four Dictyostelid species

As shown in the phylogeny of Chapter Four, the Dictyostelids can be subdivided into 4 major groups. *D.discoideum (Dd)* lies within the most derived Group 4, which is nested within a series of three progressively deeper lineages, the most basal of which is Group 1, the taxon closest to the outgroup of solitary amoebas (27). We selected four taxa, *D.fasciculatum (Df)*, *P.pallidum (Pp)*, *D.minutum (Dm)* and *D.rosarium (Dr)* for study as representatives of Groups 1,2,3 and 4 respectively (Fig. 1A). Similar to *D.discoideum* and other investigated group 4 taxa, *D.rosarium* uses cAMP as attractant. However, none of the other taxa do: *D.minutum* uses folate, *P.pallidum* glorin, and *D.fasciculatum* an unknown compound to aggregate (5, 12).

Degenerate oligonucleotide primers were designed to match amino acid sequences that are conserved between the four homologous *D. discoideum (Dd*) cARs 1–4. These primers were used to amplify cAR-like sequences by touch-down PCR from genomic DNAs of the four test species, *D. fasciculatum*, *P.pallidum*, *D. minutum*, and *D. rosarium.* Single cAR-like sequences were obtained from *D. fasciculatum* (*DfcAR*), *P. pallidum* (*PpcAR*), and *D. minutum* (*DmcAR*) and dual sequences from *D. rosarium* (*DrcARI* and *DrcARII*). The sequences varied in size due to a variable-length intron, present in all sequences except in *DmcAR*. These introns were located at the same conserved position as the single intron in *D. discoideum* cAR1–4. The derived amino acid sequences of the *cAR* genes showed 71–87% identity with DdcAR1 (Fig. 1*A*). *PpcAR* was identical to *TasA*, a putative receptor from *P. pallidum* (28). Phylogenetic analysis showed that *DfcAR, DmcAR, PpcAR, DrcARI*, and *DdcAR1* represent the ancestral cAR receptor lineage from which *cAR2–4* were derived, including *DrcARII*, which is specifically related to *DdcAR2* (Fig. 1*B*). The cAR phylogeny closely mirrors the small subunit RNA phylogeny of the five species (Fig. 1*C*) (27), albeit that in both trees, the nodes that define the relative positions of *P. pallidum* and *D. fasciculatum* are less well resolved than the other nodes.

Developmental regulation of *cAR* expression

We assessed the developmental role of the putative *cARs* by comparing their expression during the life cycles of the five taxa. A *D.discoideum* developmental time course was included for comparison. In the most basal taxa *D.fasciculatum* and *P.pallidum*, a single

cAR mRNA appears after aggregation is completed, and this mRNA remains present until fruiting bodies are formed (Fig. 2).

D.minutum expresses two *cAR* transcripts, a smaller mRNA that occurs during growth and then decreases, and a larger mRNA that appears once tight aggregates have formed. *D.rosarium* (*DrcARI)* also yields two different size transcripts, but here, as for its close relative *DdcAR1* (29, 30), the smaller mRNA species appears just before aggregation, while a larger species appears after aggregation is completed.

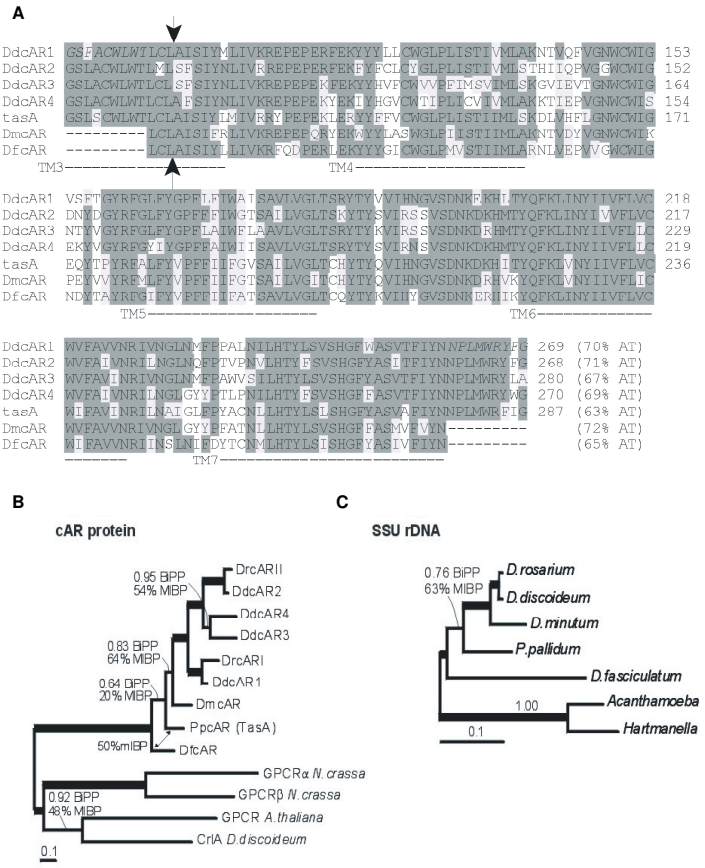

Figure 1. Identifation of cAR-like sequences in four Dictyostelid species. (*A*) Alignment of cAR-like sequences from four test species with the *D. discoideum cARs*. DNA fragments of 543–627 bp were amplified from *D. fasciculatum*, *D.minutum*, *P. pallidum*, and *D.rosarium* genomic DNA by using degenerate oligonucleotides that match conserved sequences in the four *D. discoideum* cARs. After excision of a variable length intron at a conserved position (arrows), the derived amino acid sequences were determined and aligned by using CLUSTAL-X. Amino acid residues that are identical in the majority or at least four of the nine sequences are shaded grey. The conserved regions used for oligonucleotide design are shown for *DdcAR1–4*, for *PpcAR*, which is identical to *TasA* (28), and for *DmcAR*. The positions of the putative transmembrane (TM) domains 3–7 of *DdcAR1* (35) are indicated. GenBank accession nos: A41238 (*DdcAR1*), A46390 (*DdcAR2*), A46391 (*DdcAR3*), A54813 (*DdcAR4*), and AB045712 (*TasA*). (*B*) Phylogenetic analysis of cAR-like sequences. The tree shown was derived by maximum likelihood analysis (mlBP) and Bayesian inference (BiPP) and is drawn to scale, as indicated by the scale bar (0.1 substitutions per site). Thick lines indicate nodes with 1.00 Bayesian inference posterior probabilities and 100% mlBP support. An alternative branching pattern among the two deepest cAR nodes favored by mlBP is indicated by a double-headed arrow. Four putative G protein-coupled receptor sequences were used to root the tree. *N. crassa*. *Neurospora crassa*. GenBank accession nos.: AAM20722 (*AtGPCR*), AAO62367 (*DdcrlA*), EAA35706 (*NcGPCRα*), and EAA28751 (*NcGPCRβ*). (*C*) Molecular phylogeny of Dictyostelids based on small subunit rDNA sequences. The tree shown was derived by using Bayesian inference and maximum likelihood analysis on 1,556 unambiguously aligned nucleotide positions. Sequences from solitary amoebas were used to root the tree.

As is the case for the more basal Dictyostelids, the post-aggregative mRNA remains present until development is completed. In case of *DrcAR1* the smaller mRNA species also persists. Because both hybridization and washing of the Northern blots were performed at high stringency, the additional bands are unlikely to result from nonspecific hybridization to other cAR genes. Expression of two mRNA species from a single gene was previously demonstrated for *DdcAR1* (30). The *cAR* mRNAs varied between 1.4 and 2.1 kb in size; however, even the smallest 1.4-kb mRNA of *D. minutum* is large enough to accommodate the complete

1.16-kb *DmcAR* coding region. We could not detect any mRNA hybridizing to the *DrcARII* probe, which indicates that this gene is expressed only at very low levels, if at all. To conclude, it appears that in the course of Dictyostelid evolution, the expression of a single *cAR1*-type mRNA during culmination became supplemented with expression of a second mRNA from the same gene during pre-aggregative development.

Functional Analysis of the D. minutum cAR

The expression of *cAR1* during *D. discoideum and D. rosarium* aggregation is fully concordant with the fact that these species use cAMP to aggregate. However, the expression of a *cAR1*-like gene during *D. minutum* aggregation is enigmatic in view of the fact that *D.minutum* cells use folate and not cAMP for aggregation (31).

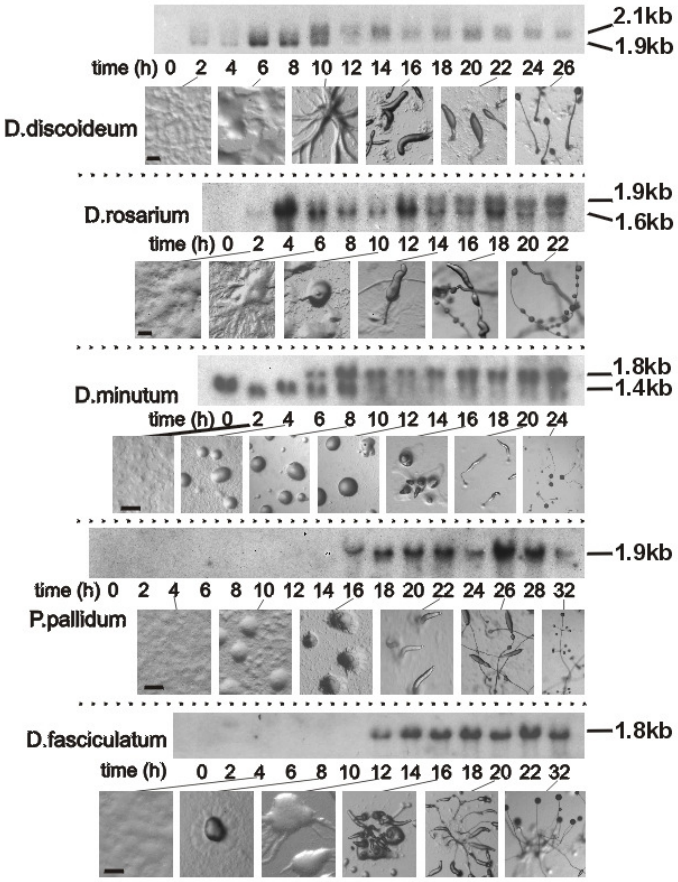

Figure 2. Developmental regulation of *cAR* gene expression Cells of the indicated five species were incubated on nonnutrient agar until fruiting bodies had formed. Total RNA was extracted at 2 hours intervals, and the progression of development was photographed. Northern blots were probed at 65°C with [^{32}P]dATP-labeled *DdcAR1* cDNA or with the [^{32}P]dATP-labeled *DrcARI*, *DmcAR*, PpcAR, and *DfcAR* PCR products, respectively, and washed at high stringency. Bar, 200 µm.

It is therefore particularly important to establish for this species that its cAR-like gene encodes a functional cAR. To do so, we cloned the full-length *DmcAR* gene from a *D. minutum* genomic DNA library and expressed it in the *D. discoideum car1car3* mutant for assay of cAMP-binding activity and functional complementation. The library screen yielded three overlapping clones, which could be assembled into a 4,873-bp contig (Fig. 3*A*). In addition to the complete 1.16-kb *DmcAR* coding sequence, this contig also contained a complete second gene, which was named *DmDtmA*, and a gene fragment, which was named *DmSpkA*. BLAST searches of the entire GenBank protein database with these sequences identified the *D. discoideum* genes *DdSpkA* and *DDB02170155* as their most related orthologues. The *D. discoideum* genes occupy the same position relative to *DdcAR1* as their *D. minutum* orthologues to *DmcAR*. The flanking genes of *DdcAR2*, *DdcAR3*, and *DdcAR4* bear no similarity to the DmcAR flanking genes. This indicates that *DmcAR* is a true orthologue of

DdcAR1, and that there is at least partial synteny between the *D. discoideum* and *D. minutum* genomes.

To determine whether the putative cARs encode functional cAMP receptors, we cloned the full-length DmcAR gene from a genomic library (Fig. 3A) and used it to complement the *D.discoideum car1car3* mutant (18). This mutant lacks high affinity receptors due to lesions in both its *cAR1* and *cAR3* genes and can consequently neither aggregate nor form fruiting bodies. We fused the DmcAR coding sequence to the constitutive *D.discoideum* A15 promoter and transformed *car1car3* with the A15:DmcAR gene fusion. *car1car3* transformed with A15:DdcAR1 was used as a control. Figure 3B shows that cells transformed with A15:DmcAR or A15:DdcAR1 bound significant amounts of ^{3}HcAMP, whereas the host *car1car3* strain bound none at all. Competition curves and Scatchard plots of ^{3}HcAMP binding (Fig. 3C) show that DdcAR1 and DmcAR give rise to both high (K_D~30 nM) and low (K_D~1 µM) affinity binding sites as reported for cAR1 in wild-type *D.discoideum* cells (32).

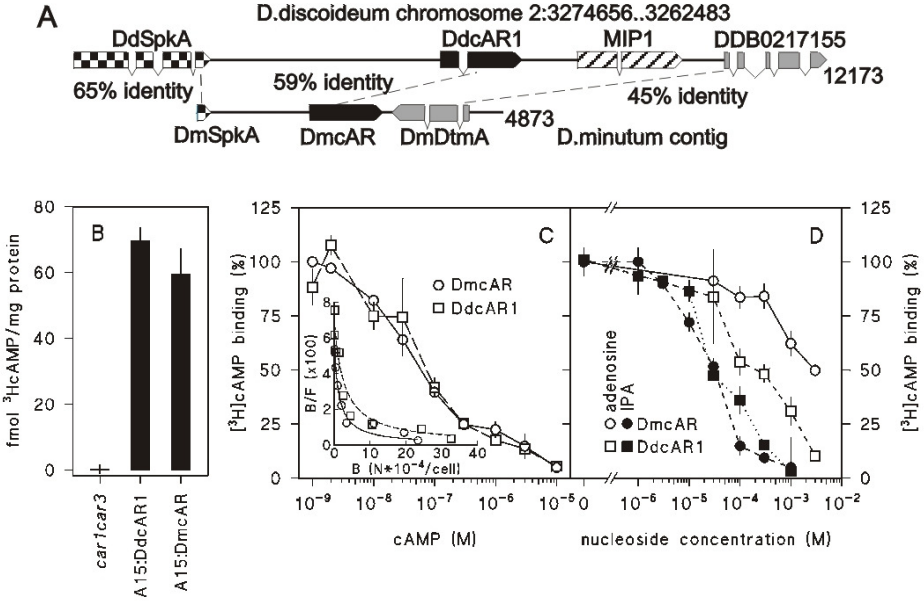

Figure 3. Cloning and cAMP-binding properties of DmcAR (*A*) Cloning of DmcAR. Screening of a *D. minutum* genomic DNA library with the *DmcAR*PCR product yielded a 4.87-kb contig of three clones. This contig contains *DmcAR* and two flanking genes, which we denote *DmSpkA* and *DmDtmA*. These genes are most similar to the *D. discoideum* genes *SpkA* and *DDB0217155*, respectively, which occupy the same positions relative to *DdcAR1* on chromosome 2 (39). The percentages of amino acid identity between the orthologous genes are indicated. (*B*) cAMP binding. *car1car3* cells, transformed with either *A15:DmcAR*, *A15:DdcAR1*, or no construct, were incubated with 10 nM [^{3}H]cAMP and assayed for cell-surface-associated [^{3}H]cAMP-binding activity. (*C*) Competition curve for cAMP. *A15:DmcAR*- or *A15:DdcAR1*-transformed *car1car3* cells were incubated with 1 nM [^{3}H]cAMP and the indicated concentrations of cAMP and assayed for [^{3}H]cAMP binding to the cell surface. The data are presented as percentage of [^{3}H]cAMP binding in the absence of cAMP and as a *Scatchard* plot (38) (*Inset*). B, bound; F, free cAMP; N, number of molecules. (*D*) Inhibition of [^{3}H]cAMP binding by adenosine and 2_3_isopropylidene adenosine (IPA).The transformed cell lines were incubated with10 nM [^{3}H]cAMP and the indicated concentrations of adenosine and IPA and assayed for [^{3}H]cAMP binding to the cell surface. The data are presented as percentage of [^{3}H]cAMP binding in the absence of nucleosides. All data represent the means and SEM of two experiments performed in triplicate.

The binding of cAMP to DdcAR1, but not to any of the other *D. discoideum* cARs, is inhibited by adenosine and more potently by the adenosine analog 2_3_isopropylidene adenosine (IPA) (19,33). We investigated whether this was also the case for the DmcAR. Fig. 3*D* shows that both adenosine and IPA inhibit ^{3}HcAMP binding to DmcAR, although inhibition by adenosine occurs less effectively for DmcAR than for DdcAR1. In conclusion, these data show that the cAMP-binding properties of DmcAR are much more similar to those of DdcAR1 than to any of the other *D. discoideum* cARs. This confirms the genetic evidence that *DmcAR* is a *DdcAR1* orthologue.

Binding of cAMP is not the sole function of cAR1, as full functionality requires DmcAR to interact with other components of the cAMP signalling system. We assessed this by compa-

ring development in the A15:DmcAR cells to the *car1car3* mutant and its parent DH1. Figure 4A shows that transformation with A15:DmcAR fully restored aggregation and fruiting body formation in *car1car3*. During aggregation of *D.discoideum* cells, cAMP pulses are propagated in complex spiral wave patterns (20,21,26,34). To investigate whether DmcAR can mediate similar complex behaviour, we tracked the optical density waves that are diagnostic for pulsatile cAMP signalling during aggregation of *car1car3*/A15:DmcAR cells. The time-lapse movie presented in figure 4B shows spiral waves propagating from an aggregation center into a field of cells, which causes the cells to move towards the center. This indicates that DmcAR fully supports pulsatile cAMP signalling in *D.discoideum*, and thus couples to downstream components of the cAMP signalling machinery. Together with the biochemical data presented above, we therefore conclude that DmcAR is a functional high affinity cAMP receptor.

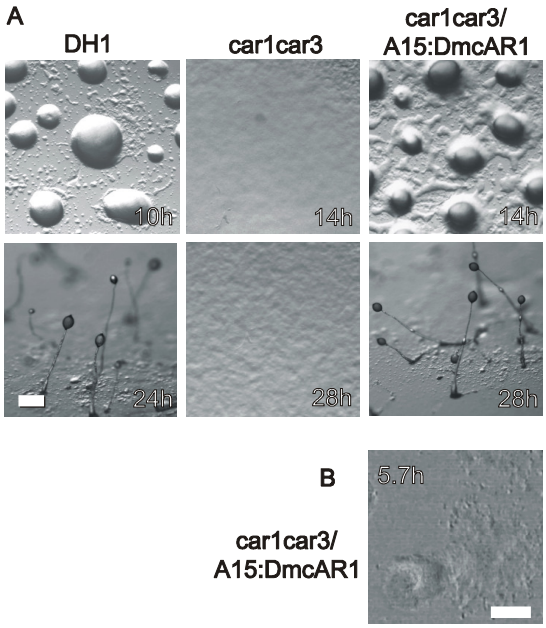

Figure 4. Complementation of *D.discoideum car1car3* by *DmcAR* (*A*) Restoration of development. The *D.discoideum car1car3* mutant, its parent strain DH1, and *car1car3* transformed with *A15:DmcAR* were incubated on non-nutrient agar at 22°C and photographed at 2-h intervals. Bar, 100 µm. (*B*) Oscillatory signaling. *car1car3_A15:DmcAR* cells were incubated for 5h at 4 x 10^5 cells per cm^2 on agar and subsequently tracked during 50 min at 10-s intervals by time-lapse video-microscopy under phase-contrast illumination. Optical density waves were enhanced by image subtraction (30). The 256th video frame is shown. Bar, 100 µm.

The Role of cARs in Basal Dictyostelid Species

Similar to all investigated group 4 taxa, *D.rosarium* uses cAMP as chemoattractant, but this is not the case for *D.fasciculatum*, *P.pallidum* and *D.minutum* (5,12). What, then, is the function of cARs in these taxa? *cAR1*-mediated signalling adapts to sustained stimulation with cAMP or its non-degradable analog SpcAMPS; this feature enables cAR1 function to be pharmacologically abrogated by exposure to excess ligand (35,36). Consistent with the known role of cAMP during *D. discoideum* aggregation, development on SpcAMPS inhibits aggregation of *D.discoideum* cells, mimicking the phenotype of *car1car3* cells. SpcAMPS can thus be used to specifically determine which aspects of development (aggregation, fruiting body formation, or both) are regulated by cAMP signalling.

Figure 5 shows that the development of all species is curtailed by SpcAMPS, although the manner in which this occurs differed among taxa. The most basal species, *D .fasciculatum*, aggregated normally with inflowing streams of cells when developing on agar containing SpcAMPS. However, while control aggregates rapidly developed into several robust upright culminants, the aggregates on SpcAMPS agar remained spread out and formed only small and aberrant structures. Similarly, in neither *P.pallidum* nor *D.minutum* was the aggregation process itself affected by SpcAMPS. In *D.minutum*, the aggregates failed to form tips, and thereafter dispersed. *P.pallidum* showed an interesting dose-dependency for the effects of SpcAMPS. At 10 µM, fruiting bodies were formed, but these had lost the whorls of side-branches that characterise this taxon. This phenotype was also reported for the *PpcAR* (*TasA*) null mutant (28). At 300 µM, tip formation on mounds was delayed and fruiting structures became very abnormal. In both *D.discoideum* and *D.rosarium*, SpcAMPS blocked the

aggregation process, consistent with the fact that both species use cAMP to aggregate. These experiments show that the basal species, *D.fasciculatum, P.pallidum and D.minutum* only require dynamic cAMP signalling during fruiting body formation. This is in contrast to the more derived species *D.discoideum* and *D.rosarium,* which also use cAMP for aggregation.

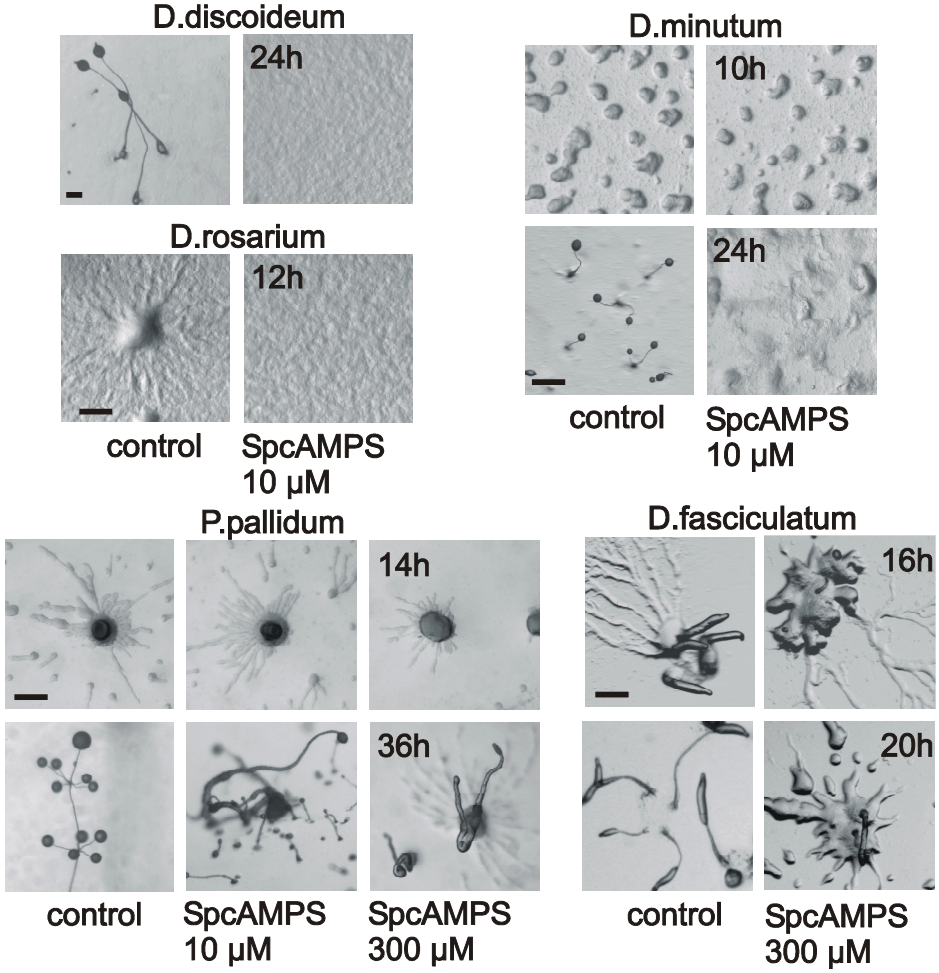

Figure 5. Effects of SpcAMPS on *Dictyostelid* development Cells from the indicated species were distributed at 2 x 10^5 cells per cm^2 on nonnutrient agar (control) or agar with 10 or 300 µM SpcAMPS and incubated at 22°C. The progression of development was photographed at 2-h intervals. Bar, 200 µm.

Conclusions

We identified *DdcAR1* orthologs in species belonging to three taxa, *D.fasciculatum, P.pallidum and D.minutum* that do not use cAMP to aggregate. The cAMP binding properties of the *DmcAR* are similar to those of *DdcAR1*, and *DmcAR* fully restores oscillatory signalling and development in a *D.discoideum car1car3* mutant. *D.minutum, P.pallidum* and *D.fasciculatum* each represent earlier branches off the main line of descent leading to *D.discoideum* (Fig. 1A) (20). In contrast to *D.discoideum,* two of these species express *cAR1* only during fruiting body formation (Fig. 1C). In all three cases only fruiting body formation, and not aggregation, is disrupted when cAR function is blocked (Fig.5) (21). This strongly suggests that coordination of fruiting body morphogenesis is the ancestral function of extracellular cAMP signalling, while its role in *D.discoideum* and *D.rosarium* aggregation is evolutionary derived.

The spiral waves of cell movement that are triggered by cAMP oscillations in a field of starving *D.discoideum* cells are one of the most striking examples of self-organization in biology. We now show that they also represent a stunning example of a derived evolutionary novelty. How might this novel feature have come about? The promoter structure of cAMP sig-

nalling genes in *D. discoideum* suggests a mechanistic explanation for this alteration. The extracellular phosphodiesterase *DdPdsA* gene has three separate promoters for expression during growth, aggregation and fruiting body (late) morphogenesis respectively. The late promoter is proximal to the coding sequence, followed by the growth-specific promoter and finally the aggregation promoter (37). *DdcAR1* has two separate promoters; the late promoter, proximal to the coding sequence, is for expression during fruiting body formation, whereas the aggregation promoter is distal to the late promoter (30). We hypothesize that the proximal promoters direct the ancestral function of the cAMP signalling genes in fruiting body morphogenesis, while the distal promoters were acquired later to accommodate the derived roles of cAMP in early development.

Pathway co-option through the acquisition of novel promoter elements is not the entire story, however, as evidenced by the intermediate species *D. minutum*, which shows altered cAR gene expression but lacks aggregation to cAMP. We show that *DmcAR* encodes a fully functional cAMP receptor, which suggests that aggregation to cAMP has not been lost in *D. minutum*. Rather, we propose that aggregation to cAMP has simply not been fully gained, either because this species lacks other components of the signalling pathway, or because the recruited pathway is not completely coupled to the downstream factors required for activity. The cAMP signalling system in Dictyostelids is composed of at least three major parts: cAMP production by adenylyl cyclases, reception by cARs, and degradation by specific phosphodiesterases. Here just one part of this apparatus is considered, but future work will seek to elucidate the route taken towards this evolutionary novelty by considering each component independently and then together. Through this approach, we hope to begin to understand the origin of new traits via gene recruitment.

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Genbank accession numbers: DdcAR2: A46390; DdcAR3: A46391; DdcAR4: A54813; DrcARI: AY839643; DrcARII: AY839644; DmcAR: AY518271; DfcAR: AY518272; PpTasA: AB045712; AtGPCR: AAM20722; DdcrlA: AAO62367; NcGPCRα: EAA35706 and NcGPCRβ: EAA28751.

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Discussion

Discussion

Regulation of prespore differentiation in *Dictyostelium* slugs

During *Dictyostelium* development cAMP is used as a signalling molecule for cell aggregation and cell differentiation. The combined action of extracellular and intracellular cAMP on cAMP receptors and PKA respectively, promotes prespore differentiation (Mehdy and Firtel, 1985; Schaap and Wang, 1986; Wang and Schaap, 1985; Wang et al., 1986). The cyclases ACA, ACB, and ACG, show different regulatory properties but overlapping timings of expression (Figure 1), making it particularly difficult to establish the direct source of cAMP required for induction of prespore genes during the slug stage.

ACG is responsible for triggering prespore differentiation in slugs (Chapter One). ACG is expressed specifically in prespore cells in the slug most posterior region, and it localizes specifically to the prespore vesicles that carry the components that will form the several layers of the spore coat (West and Erdos, 1990).

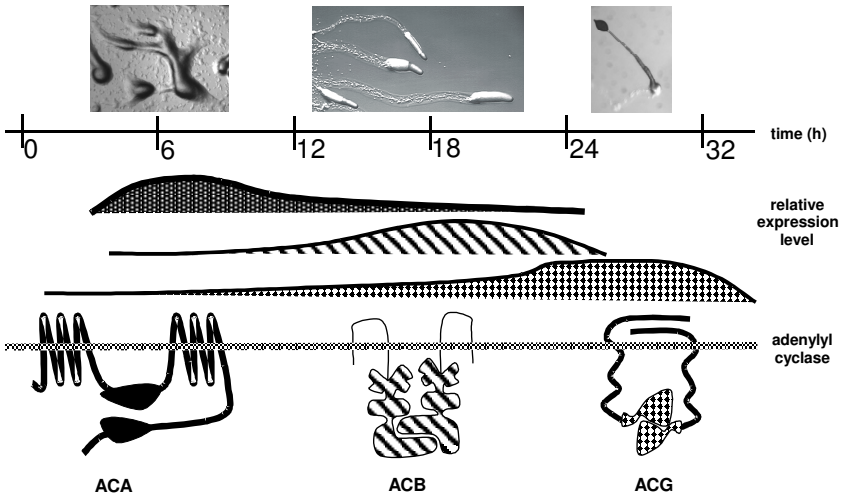

Figure 1. Timing and levels of expression of ACA, ACB and ACG during development

ACG translation is up-regulated after aggregation in the absence of the corresponding increase in transcription. This suggests that there are factors controlling ACG translation that might be responding to intra- or extracellular signals, although these signals are not known. Pilot experiments showed that micromolar concentrations of cAMP and low concentrations of DIF have an inductive effect on ACG protein levels (Alvarez-Curto, unpublished). These results are in consonance with previous findings from Oohata and co-workers, where they showed that very low levels (~0.1 nM) of DIF promote prespore differentiation in submerged conditions (Oohata, 1995).

ACB shows a prestalk specific pattern in slugs; however, when ACG is absent ACB expression extends to the entire prespore area. This would explain why *acgA*$^-$ cells show only a partial defect on prespore differentiation and how the *acgA*$^-$ mutant is able to produce encapsulated spores. Only when both *ACG* and *ACB* are lacking, there is an almost total loss of prespore gene expression and a dramatic defect on spore formation (Chapter One).

Prespore cells encapsulate into spores during culmination and it has been suggested that signals coming from prestalk cells contribute to complete terminal spore differentiation (Anjard et al., 1998b). Spore maturation is triggered by the peptide SDF2 (spore differentiation factor 2) that is produced by prestalk cells. The precursor molecule of SDF2 (AcbA) is secreted by prespore cells (Anjard and Loomis, 2006; Anjard and Loomis, 2005) but it is processed by the serine protease TagC that is found in prestalk cells (Anjard et al., 1998a). TagC exposure to the intercellular space seems to be controlled through a cascade involving GABA (γ-aminobutyric acid) and PKA, though the intermediaries are still unknown (Anjard and Loomis, 2006; Anjard et al., 1998b). The pattern of ACB described in Chapter One supports

that the cAMP required for the activation of PKA in this particular cascade is synthesized by ACB. However, the role of ACB in spore maturation might be more indirect than initially thought, as there is more than one pathway leading to SDF2 release and spore maturation (Anjard and Loomis, 2006). Other questions about the regulation of ACB remain yet unanswered. A detailed structure-function analysis of ACB dissecting the function of individual domains of the protein would shed some light into the enzyme's regulation (ACB has a response regulator and a putatively inactive histidine kinase domain). Although ACB is not regulated by neither of the histidine kinases *DhkA*, *DhkB* or *DhkC* (Soderbom et al., 1999) there are at least 14 sensor histidine kinase homologous genes in *Dictyostelium*. These hybrid histidine kinases are responsive to many effectors and signalling molecules such as adenine-based cytokinins like discadenine, which are also involved in spore maturation and germination (Anjard and Loomis, unpublished). ACB's response regulator domain could be phosphorylated as part of a different phosphorelay cascade that has not yet been characterized, triggering terminal differentiation. ACB shares homology with the adenylyl cyclase *CyaC* of the cyanobacteria *Spirulina platensis* and this cyclase requires phosphorylation in a receiver domain for full activation (Kasahara and Ohmori, 1999). Phosphorylation of RegA in a similar response regulator domain causes a dramatic increase in the enzyme activity (Thomason et al., 1999) so it is plausible that ACB is under similar regulation. In addition to this, ACB putative extracellular domain might act as a sensor for extracellular signals. Furthermore there might be other alternative and redundant pathways controlling terminal spore differentiation.

cAMP produced by ACB is difficult to detect unless the intracellular phosphodiesterase RegA is inactivated (Kim et al., 1998; Meima and Schaap, 1999). RegA controls PKA activation by regulating intracellular cAMP levels (Thomason et al., 1998; Thomason et al., 1999). This phosphodiesterase is prestalk specific as shown by *in situ* hybridisation (Shaulsky et al., 1996; Tsujioka et al., 2001). So at least two of the components that control the cAMP levels in the cell that subsequently regulate PKA activity, RegA and ACB, co-localize to the same cell type in the slug. It would be interesting to find out about the sub-cellular localization of these proteins to see if they are physically in close proximity or together forming a complex. In mammalian cells it is known that PKA can form complexes with phosphodiesterase enzymes. These complexes are formed through the action of scaffolding proteins known as A-kinase anchoring proteins or AKAPs (Michel and Scott, 2002). In *Dictyostelium* however, no sequence-based homologue of these proteins has been found but there might be a functional homologue present involved in the compartmentalization of cAMP signalling components. The topologies of RegA and ACB are compatible with the idea of these enzymes being part of a complex or of a micro-domain for cAMP signalling within prestalk cells.

In summary, these findings indicate that neither ACB nor ACG are solely responsible for prespore gene induction and that as long as there is a source of cAMP, prespore differentiation can take place. ACG and ACB play combinatorial and redundant roles in prespore and spore differentiation respectively. In later stages of development ACG acts as an osmosensor maintaining spore dormancy (Figure 2) (Saran and Schaap, 2004; Van Es et al., 1996). This functional redundancy between the two cyclases seems to ensure that prespore and ultimately spore differentiation takes place so that survival is guaranteed. Work carried out in our laboratory shows that both ACG gene and function are deeply conserved within the social amoebas. The homologous *ACG* genes found in other *Dictyostelium* species are involved in controlling an alternative survival structure, cyst formation (Ritchie A.V. and Schaap P., in preparation).

Pharmacological profiling of the adenylyl cyclases

The three *Dictyostelium* cyclases differ structurally and in their activation mechanisms (Kim et al., 1998; Meima and Schaap, 1999; Pitt et al., 1992; Van Es et al., 1996). In Chapter Two the effects of several compounds that were selected based on the characteristics of each of the enzymes or their mammalian homologues were studied.

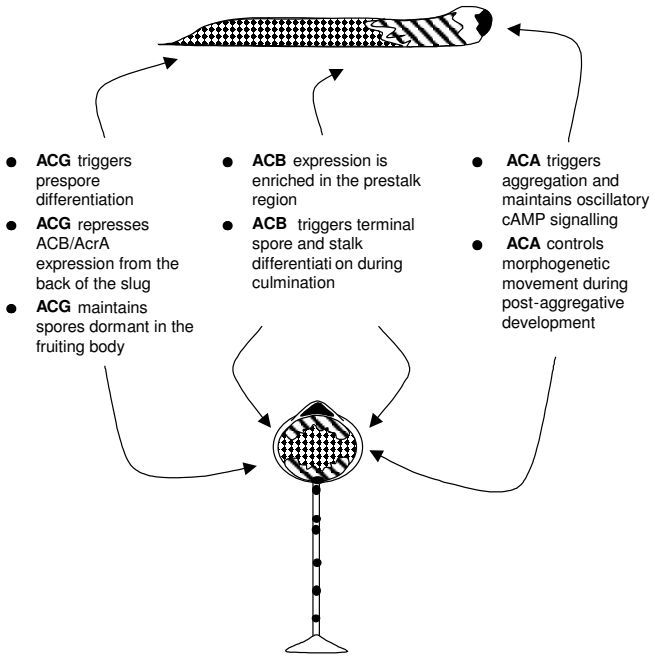

Figure 2. Roles of ACA, ACB and ACG during the slug and fruiting body stages

This pharmacological analysis has produced at least two specific inhibitors for ACG and ACA, the tyrosine kinase inhibitor tyrphostin A25 and the compound SQ22536 respectively. Tyrphostins are a group of recognized tyrosine kinase inhibitors that have an inhibitory effect on guanylyl and adenylyl cyclases (Jaleel et al., 2004). The results presented in Chapter Two show that tyrphostin A25 efficiently inhibits ACG *in vitro* with an IC_{50}= 16.6±7.4 μM. *Dictyostelium* ACG is structurally similar to mammalian membrane associated guanylyl cyclases (Pitt et al., 1992), such as GC-C, so we could speculate that inhibition by tyrphostin A25 in these two types of proteins occurs by similar mechanisms. The furyl-adenine derivate SQ22636, an inhibitor of human adenylyl cyclases (Harris et al., 1979) effectively inhibits ACA activity in intact cells and GTPγS-stimulated activity in cell lysates.

Adenosine and its analogs are effective interfering with *Dictyostelium* extracellular cAMP signalling (Newell, 1982). A selection of P-site inhibitors (adenosine analogues that have an intact purine moiety) was also tested in *Dictyostelium*. The ribose-modified compound DDA (2'5'-dideoxyadenosine) inhibits most efficiently ACG in cell lysates. A second ribose-modified compound, IPA (O-isopropylideneadenosine), is known to be an effective inhibitor of cAMP binding to *cAR1* (Soede et al., 1996; Theibert and Devreotes, 1984; Van Lookeren Campagne et al., 1986; Verkerke-VanWijk et al., 1998), which suggests that most of the effect that we see of ribose-modified analogues is due to interference with cAMP binding to its receptor. IPA robustly inhibits cAMP-induced ACA activation, but has no effect on any of the other two cyclases *in vivo*.

Despite having access to single and some double adenylyl cyclase mutants, until now it has been difficult to determine which of the enzymes produces cAMP at each particular time during development and the functional redundancy of the cyclases added complexity of the problem. Therefore having these pharmacological tools to acutely abrogate specifically one of the cyclases at the time is essential to analyse specific enzyme activity and to uncover new roles for cAMP in *Dictyostelium*. Tyrphostin A25 and SQ22536 can be readily used to dissect pathways in different cell types where more than one of the cyclases in being expressed or when creating a knockout is not viable. Tyrphostin A25 has been already successfully used in our laboratory to characterize an ACG-like activity found in *P.pallidum* and in the solitary amoeba *Acanthamoeba castellani* (Ritchie et al., in preparation). On the other hand, SQ22536 can be used to inhibit ACA in chemotaxis and cell movement studies without the limitations of using knockout cell lines.

Characterization of a novel *Dictyostelium* adenosine kinase

The metabolism of extracellular cAMP and particularly of its by-products is complex and yet not well understood. Extracellular adenosine is mainly produced in *Dictyostelium* through degradation of extracellular cAMP by the phosphodiesterase *PdsA* and 5' nucleotidase. Adenosine has been put forward as an essential molecule in the regulation of tip dominance, slug size and prespore/prestalk pattern organization (Newell and Ross, 1982; Schaap and Wang, 1986; Wang and Schaap, 1985).

In Chapter Three, I describe the identification of a novel adenosine kinase gene (*DdADK*). DdADK protein is homologous to mammalian and yeast adenosine kinases but it also shares homology to enzymes of the sugar kinase family, rather than to other nucleoside kinases. *DdADK* is predicted to be a soluble protein, and its activity is found at significant levels both intra- and extracellularly. *DdADK* is developmentally regulated and in culminants it localizes specifically to the upper and lower cups.

DdADK knockout mutants form slugs that are about two times bigger than those of the wild type. These results support a role for the enzyme adenosine kinase in controlling slug size and tip dominance therefore supporting a potential role of adenosine in tip dominance. However, I have not been able to find any prominent role for *DdADK* in prespore differenttiation and/or in the establishment of prespore/prestalk pattern during slug formation.

Evolution of morphological change and cAMP signalling in the social amoebas

Unravelling how modifications in genes that control developmental programs have lead to the generation of phenotypic variation is a challenge for modern evolutionary biology. *Dictyostelium* can choose from three developmental strategies to form three types of resistant structures: relatively simple single-celled cysts, macrocysts if they follow their sexual program, or multicellular fruiting bodies. The complexity of the developmental program and the requirements of signalling pathways will be different depending on the strategy followed (Figure 3).

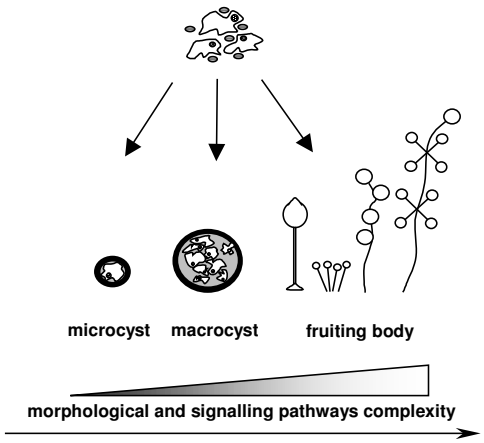

Figure 3. Survival strategies found in the Dictyostelids

Social amoebas provide researchers with an exceptional opportunity to study the molecular basis of phenotypic variation and also to trace the evolutionary origins of multicellularity. All the known Dictyostelid species can form multicellular fruiting bodies when faced with starvation, yet their morphology can be very diverse. However, in all cases fruiting body ontogeny relies on cell signalling, cell movement and cell specialization processes.

The first requisite to understand the origins of morphological complexity of the group and the processes that lie beneath it is to have a true phylogeny of all *Dictyostelium* species. The phylogeny presented in Chapter Four is the first molecular-based phylogeny of all known *Dictyostelium* species. This phylogeny provides the basic framework for any logical evolutionary and comparative analysis. Once the phylogenetic relationships between species are established it will be possible to trace and meaningfully interpret when and how morphological variations have taken place.

Originally, the taxonomy of social amoebas was based on fruiting body characteristics. This new phylogeny highlights the flaws derived from the classification of species based only on phenotypic features. The new molecular tree does not match the traditional taxonomical division into three genera: *Dictyostelium, Polysphondylium* and *Acytostelium* (Raper, 1984),

but reclassifies the species into four distinct groups with *D.discoideum* in the most derived group.

A set of morphological traits plotted onto the phylogenetic tree shows novel and unexpected trends in the evolution of morphological diversity. There is a tendency to form larger fruiting bodies and spores in the more derived, or evolutionary younger species; these species also use cAMP during aggregation. The architecture of a larger fruiting body requires more cell specialization, with a tendency to show different types of supporting structures. However the direct causality between the use of cAMP signalling and increased fruiting body size has yet to be determined in molecular terms. Other traits such as branching or aggregation patterns seem to have evolved independently several times. This suggests that specific architectures cannot be under the control of great numbers of genes and therefore the underlying genetic and molecular mechanisms would be easy to reveal.

To completely unveil the history of morphological change it is necessary to assess the levels of conservation and divergence of signalling genes responsible for those morphological variations. Based on the phylogeny I have tried to infer how ancestral characters might have derived into novel functions such as the use of cAMP as chemoattractant. Although the use of cAMP is a hallmark of multicellular development in the model *D.discoideum* not all the other species use cAMP as chemoattractant. Diverse molecules such as folate (*D.minutum*) (De Wit and Konijn, 1983) or small peptide compounds such as glorin (Polysphondylids) (Shimomura et al., 1982) are used. However there is evidence of functional cAMP oscillatory signalling during late development in other related species such as *D.minutum* (Schaap, 1985; Schaap et al., 1984). So, in order to establish the molecular evolution of cAMP signalling, a comparative analysis of four *Dictyostelium* species from four different taxa in the phylogeny was carried out (Chapter Five). The cAMP specific receptor *cAR1* was used as a marker for early extracellular oscillatory cAMP signalling.

The results from Chapter Five show that the use of cAMP during aggregation as extracellular chemoattractant is a derived role for the molecule. *cAR1* orthologues were identified in the four species analyzed in Chapter Five (from Group 1 to 4: *D. fasciculatum, P.pallidum, D.minutum* and *D.rosarium*). Functional analysis of the receptor shows that the biochemical role of this signalling component has not changed through evolution. However, analysis of the developmental regulation of the *cAR1* orthologues suggests that extracellular cAMP signalling was originally originated to control fruiting body morphogenesis and recruited to aggregation stages later on evolution.

The acquisition of multicellular development and chemotaxis towards cAMP requires of multiple components and it is yet not known how all have been gained. One possibility is that there might have been a progression in the incorporation of regulatory *cis* elements, or mutations in existing ones, before the invention of totally new genes. Components required for oscillatory signalling in *D.discoideum* such as the extracellular phosphodiesterase *PdsA* show three promoters that control expression during growth, aggregation and late development (Faure et al., 1990). Also the *D.discoideum* cAMP receptor *cAR1* has an aggregation and late development specific promoters (Louis et al., 1993). In both cases the late promoters are closer to the coding region whereas the aggregation specific are more distal to it. This might suggest that the distal regions of those promoters were acquired later on evolution to be able to sustain oscillatory cAMP signalling at earlier stages. This is an example of recruitment of an existing signalling pathway to a different stage of development, where it performs a novel function.

Future prospects

Certain aspects of *ACG* regulation, such as the transcription *vs.* translation differences, still need to be resolved. It is possible that ACG protein is highly unstable during earlier stages and there is a higher rate of protein degradation due to more active targeted ubiquitination. There might also be translational suppression mechanisms that down-regulates protein expression until slug formation.

ACG appears to be deeply conserved. An ACG-like activity has been found to control the process of encystations in the solitary amoeba *Acanthamoeba castellani* (Ritchie et al., in preparation) and *ACG* homologous sequences have been found in basal species like *D.fasciculatum* and *P.pallidum*. The latter species can form microcysts as alternative survival structures and changes in the environment osmotic conditions control the encystation and excystation processes in this organism. This reinforces the conserved role of *ACG* in controlling spore and cyst formation under nutritional or fluid stress and its role in spore dormancy will be derived from this. It would be interesting to find out if these homologues act mechanistically similarly to *D.discoideum ACG*.

In future, using the phylogeny as a guide it would be easier to establish possible causal relations between the appearance of novel morphological traits observed and changes in the regulation or activity of developmental signalling genes. In this thesis the conservation of only one gene related to extracellular cAMP signalling has been analyzed (*cAR1*), but homologous studies can be carried out with other genes of this signalling cascade such as *PdsA* or *ACA*. At the same time, a systematic study and mapping into the phylogeny of other morphological characteristics might highlight new evolutionary trends. The engineering of genetically tractable species from the different taxon groups would also prove very useful to carry out functional analyses and help to establish relations between genetic and phenotypic change. The evolutionary studies presented in this thesis highlight a correlation between early cAMP signalling and larger fruiting bodies. The presence of a pre-pattern and different cell types in earlier stages such as the slug also seems to be related to the use of cAMP. It would be interesting to find out when cAMP started having a prominent role controlling not just cell movement and morphogenesis, but post-aggregative gene expression to control cell differenttiation. Immunochemical staining of slugs of an array of species with a prespore specific antibody would show in which species this pre-pattern appeared first through evolution. Similarly studies into the origins of other signalling molecules such as DIF and SDFs should be initiated.

Now that the *Dictyostelium* genome is completed (Eichinger et al., 2005) sequencing the genomes of at least one representative of each of the four groups that form the phylogeny is on the way. This would be very a powerful tool for comparative functional analysis of other signalling genes. At the same time useful information about the conserved domains could be extrapolated to what is known of homologous genes in other systems. These studies are important for the general scientific community as they highlight the viability of *Dictyostelium* as a model system in evolutionary developmental biology to study the origins of cell signalling pathways found in higher eukaryotes.

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Samenvatting

Summary for the non-biologist

Samenvatting voor niet-biologen

Ontwikkelings biologie

Ontwikkelings biologen bestuderen de processen die leiden tot de vorming van een complex, meercellig organisme vanuit een simpeler vorm zoals een embryo of een bevruchte eicel. Een verscheidenheid aan organismen zoals kikkers (*Xenopus*), fruit vliegen (*Drosophila*) en amoebes (*Dictyostelium)* wordt gebruikt als model systemen ter bestudering van deze ontwikkelings processen.

Dictyostelium discoideum behoort tot de groep van sociale amoebes. Dit zijn solitaire eencelligen, die wanneer de voedselbron raakt uitgeput samen stromen en een meercellige structuur vormen. Hoewel mensen, amoebes, vliegen en kikkers geen fysieke gelijkenis vertonen, zijn er vanuit een moleculair gezichtspunt (dus qua hormonen, receptoren, enzymen etc.), veel overeenkomsten in signaal overdracht routes die belangrijke cellulaire en ontwikkelings processen gebruiken (zoals bijv. de ontwikkeling van een embryo). Alle levende organismen ontvangen verschillende soorten stimuli waarop een respons wordt gegegeven (bijvoorbeeld, een toename in het bloed suikergehalte verhoogt de afgifte van een specifiek hormoon: insuline. De juiste respons op een signaal vergt detectie en verwerking door het juiste cel type of orgaan en hiervoor is een efficiente cel communicatie cruciaal. Ontwikkelings biologen onderzoeken dus ook hoe cellen via de juiste boodschappen en antwoorden met elkaar communiceren en organiseren. In hogere organismen is de werking van deze signaal systemen uitermate complex en moeilijk te ontrafelen. Daarom kan de bestudering van signaal overdracht, celdifferentiatie en patroonvorming van een simpele levensvorm als de sociale amoebe helpen inzicht te verschaffen in hoe deze processen in complexere organismen werken.

Dictyostelium discoideum levens cyclus en ontwikkelings programma

Ons leefmilieu verandert continu en daarom is flexibele aanpassing aan nieuwe condities noodzakelijk om te kunnen overleven. *Dictyostelium* cellen leven gewoonlijk in de grond en voeden zich met bacterien en andere micro-organismen. Het is doorgaans een rijke leefomgeving die echter aan grote veranderingen in temperatuur, zuurgraad (pH) en vochtigheid onderhevig kan zijn en waar van tijd tot tijd voedsel op kan raken. Via evolutie hebben *Dictyostelium* amoebes mechanismen ontwikkeld om deze veranderingen aan te kunnen en te overleven als er tijdelijk geen voedsel is. Cellen stromen dan samen tot meercellige aggregaten die zich ontwikkelen tot vruchtlichamen, bestaande uit een steel met daarop een sporen hoofdje. Een vruchtlichaam wordt gevormd uit zo'n 100,000 cellen die aanvankelijk gelijke eigenschappen hebben maar zich uiteindelijk differentieren (=ontwikkelen) in steel- of sporecellen afhankelijk van de signaal moleculen waaraan ze blootgesteld worden.

Ongeveer 20% van de cellen diffentieren zich tot dode steel cellen en 80% tot sporen, die levensvatbaar zijn en ontkiemen als de condities (voedsel, temperatuur, vochtigheid) er gunstig voor zijn. De dode steel tilt de sporen van de grond zodat ze goed kunnen verspreiden. Sporen zijn resistent tegen extreme condities en ontkiemen tot de nieuwe generatie amoebes. De levenscyclus van *Dictyostelium* (de transformatie van eencellige, solitaire amoebe via meercellige tussenstadia tot een vruchtlichaam bestaande uit een steel met sporen hoofd) voltooit zich onder normale omstandigheden in 24 uren. Een belangrijk tussenstadium na celaggregatie is de vorming van een zgn. slak. De slak is een cylinder vormige, bewegende structuur en heeft een "kop tot staart" ontwerp. De kop of pre-steel regio, bestaat uit de voorste 20% en de staart of pre-spore regio bestaat uit de achterste 80% van de cellen. Onder invloed van licht en temperatuur zoekt de slak een gunstige plaats om zich op te richten en het vruchtlichaam te vormen. Het is gedurende deze fase dat de "lot's bepaling" tot spore of steel cel vast komt te liggen.

Het voornaamste signaal molecuul dat de meeste stappen in de progressie van *Dictyostelium's* levens cyclus reguleert heet cyclisch AMP (of cAMP). Amoebes kunnen cAMP produceren en uitscheiden. Tijdens aggregatie detecteren amoebes dit uitgescheiden,

extracellulaire cAMP met specifieke receptoren en worden zo tot elkaar aangetrokken. Dit proces van het bewegen naar een chemische verbinding, heet chemotaxis en cAMP noemen we de “chemo-attractant”. Amoebes die honger krijgen door het opraken van voedsel beginnen cAMP uit te scheiden. Dit cAMP stimuleert buur cellen tot productie en uitscheiding van cAMP, hetgeen leidt tot het doorgeven van het cAMP signaal en het samen stromen van cellen tot aggregaten. Zo start de meercellige ontwikkeling en de sturende rol van extracellulair cAMP is uniek voor *Dictyostelium.*

De eiwitten of enzymen die cAMP produceren heten adenylyl cyclases en *Dictyostelium discoideum* heeft er drie: ACA, ACB and ACG. Het geproduceerde cAMP heeft vele intracellulaire functies maar kan ook uitgescheiden worden om extracellulaire functies te vervullen. De juiste temporele (tijd) en spatiele (plaats) regulering van cAMP concentraties is vereist voor correcte cel differentiatie en uiteindelijke overleving.

Een aantal complexe processen vinden plaats gedurende de levens cyclus en de grote meerderheid hiervan wordt gereguleerd door cAMP. Er zijn morfogenetische bewegingen, wat betekent dat groepen cellen zich mobiliseren om bepaalde structuren zoals de slak en later het vruchtlichaam (bestaande uit de steel en het sporenhoofd) te vormen. In de slak worden cel bewegingen bepaald door de tip (het voorste puntje van de kop) van waaruit cAMP pulsen worden verzonden, die voor de orientatie van de cellen zorgen. Ook cel differentiatie vindt nu plaats. Dit betekent dat cellen pre-spore of pre-steel cel karakteristieken verkrijgen en zich uiteindelijk eigenschappen verwerven die nodig zijn om of spore- of steelcel te worden. Van de voor tot achterkant van de slak, vormt zich een “kop tot staart” as. Langs deze “kop tot staart” as, vind je de pre-steel cellen het dichtst bij de kop (anterior) en de pre-spore cellen bevinden zich in de staart (posterior). Het ontwikkelen en verkrijgen van deze patroonvorming in *Dictyostelium* wordt gereguleerd door de differentiele expressie van de verschillende componenten van de cAMP signaaltransductie machinerie.

Het doel en de conclusies van dit proefschrift

Aangezien cAMP het meest belangrijke signaal molecuul in de ontwikkeling van *Dictyostelium* is, wilde ik onderzoeken waar en wanneer het geproduceerd wordt en hoe de productie ervan gereguleerd wordt. Omdat verschillende *Dictyostelium* soorten homologe ontwikkelings programma’s hebben, heb ik ook onderzoek naar evolutionaire verwantschap tussen de verschillende soorten gedaan, door het vergelijken van de genetische profielen (DNA code) van enkele belangrijke componenten van de cAMP signaal machinerie.

De adenylyl cyclases produceren cAMP op verschillende tijden en in verschillende regios van de slak, hetgeen van belang is voor correcte cel differentiatie (hoofdstuk 1). ACA reguleert de aggregatie van *Dictyostelium* cellen en komt tot expressie in het voorste puntje, de tip van de slak. Van hieruit wordt het geproduceerde cAMP pulsgewijs naar de achterliggende cellen in de slak uitgezonden en wordt de beweging van de (cellen van de) slak bepaald. In hoofdstuk 1 heb ik ook aangetoond dat ACB specifiek tot expressie komt in de pre-steel regio van de slak, in het voorste deel het dichtst bij de tip. ACB reguleert voornamelijk processen in de late ontwikkeling; zoals de oprichting van de slak tot een vruchtlichaam en de maturatie (=rijping) van de sporen en steel cellen. ACB, in combinatie met andere componenten van de signaal machinerie, zorgt voor correcte spore vorming en inkapseling van de pre-spore cellen. ACG komt ook tot expressie in sporen en kan geactiveerd worden door hoge osmolariteit (dat is een hoge concentratie van geladen verbindingen) van het vocht in het sporenhoofd. De hoofd functie van ACG is het voorkomen van ontkiemen onder ongunstige condities. Ik heb ook gevonden dat ACG veel eerder in het slakken stadium aanwezig is (hoofdstuk 1). Tijdens dit stadium is het aanwezig in het achterste deel van de slak, waar het cAMP produceert, dat de pre-spore differentiatie in gang zet. Samenvattend, in de slak komen de cyclases tot expressie in de tip (ACA), de pre-steel regio (ACB) en de pre-spore regio (ACG). In hoofdstuk 1 toon ik ook aan dat functies van de adenylyl cyclases deels overlappend zijn (de afwezigheid van een bepaald cyclase kan gecompenseerd worden door een ander aanwezig cyclase). Dit maakt het moeilijk om de individuele rol van een specifiek cyclase te bepalen op momenten dat ook andere cyclases aanwezig zijn.

Via genetische manipulatie m.b.v. moleculair biologische technieken kunnen specifieke genen uitgeschakeld worden (het maken van gen knock-outs) en kan zo de functie van een bepaald eiwit of enzym worden geanalyseerd. Als bijvoorbeeld het ACG-gen wordt uitgeschakeld, dan is het verwachte fenotype (het zichtbare resulterende defect) dat van sporen die ontkiemen onder condities van hoge osmolariteit. Soms is de uitschakeling van genen onmogelijk (i.g.v. dodelijke consequentie) of niet practisch (als bijv. de ontwikkeling in een heel vroeg stadium wordt geblokkeerd). Een farmacologische aanpak kan hier een uitweg bieden. Synthetische verbindingen die receptoren of enzymen kunnen remmen of activeren kunnen op een zelf gekozen moment worden ingezet. In hoofdstuk 2, onderzoek ik mogelijke remmers van de verschillende adenylyl cyclases en identificeer ik tenminste 2 specifieke remmers voor ACA en ACG.

Als cAMP wordt afgebroken, wordt o.a. adenosine gevormd. Dit afbraak product, is zelf een signaal molecuul en kan tot significante concentraties toenemen. Adenosine kan interfereren met de werking van cAMP in aggregatie en pre-spore differentiatie. In hoofdstuk 3, bestudeer ik de rol van het enzym adenosine kinase, dat adenosine fosforyleert en het daardoor dus in concentratie af doet nemen. De adenosine concentratie blijkt invloed op de grootte van de slak maar niet op pre-spore differentiatie te hebben.

Dictyostelium discoideum is een soort van de *Dictyosteliden* die we als model organisme voor differentiatie en patroonvorming gebruiken. Er zijn echter vele andere soorten beschreven die grote overeenkomsten vertonen en allemaal aggregeren bij uithongering en vruchtlichamen vormen als overlevings strategie. De vorm en grootte van deze vruchtlichamen kunnen behoorlijk verschillen. Sommige soorten hebben zelfs meer dan een sporenhoofd, zoals *Dictyostelium, Polysphondylium*.

In hoofdstuk 4, hebben we de eerste fylogenetische stamboom geconstrueerd op basis van moleculaire verwantschap. Een fylogenetische stamboom geeft inzicht in de evolutionaire ontwikkeling van soorten en vertelt dus welke soorten en families verwanter en/of ouder zijn dan anderen. De gepresenteerde fylogenetische stamboom is gebaseerd op de vergelijking van DNA profielen (van specifieke genen) van meer dan 80 *Dictyostelium* soorten. Gebruikmakend van de stamboom kunnen we concluderen dat de soorten van de *Dictyosteliden* die verder geevolueerd zijn, een groter vruchtlichaam hebben ontwikkeld dan de “oudere” soorten.

Deze stamboom laat ook zien wanneer bepaalde karakteristieken voor het eerst in de evolutie voorkomen; zoals bijvoorbeeld het gebruik van cAMP als chemo-attractant (hoofdstuk 5).

Summary for the non-biologist

Developmental biology

Developmental biologists study the processes that lead to the formation of a complex multicellular organism starting from a much simpler form such as an embryo or an egg. A variety of organisms like frogs (*Xenopus*), fruit flies (*Drosophila*) and social amoebas like *Dictyostelium discoideum* are used as model systems to study these processes. Although humans and amoebas, or flies and frogs, do not physically resemble each other, the mechanisms controlling important developmental processes are very similar between them from a molecular point of view. Therefore, the study of these processes in simpler systems (like the social amoebas) can help us understand how they work in more complex ones.

Developmental biologists also investigate how cells communicate the right message to each other. All living organisms are exposed to different types of stimuli, to which they react or give a response to. For example, the increase of sugar in the blood stream causes the release of a particular type of hormone. The correct response comes after the signal has been processed by the right cell type or organ. *Dictyostelium discoideum* responds to certain changes on the environmental conditions such as lack of nutrients congregating to form a multicellular structure. Therefore, efficient cell communication is paramount for the system to work.

The components of the machinery involved in cell communication and development act through what we know as *signalling pathways*. The main parts of any signalling pathway are: proteins or *enzymes*, which will carry out the reactions, and *signalling molecules* that will pass on the message or the instructions of what needs to be done and where.

Dictyostelium discoideum life cycle and developmental program

Organisms must find a way of adapting to a constant changing environment to ensure surviving. The social amoeba *Dictyostelium discoideum* is found normally in the soil and although this is a rich environment where to live it is also a place that is subject to great changes in temperature, pH, humidity, and where food might not always be available. Through evolution the social amoebas have developed the mechanisms to adapt to those changes and to ensure survival.

Dictyostelium cells normally feed on bacteria and other soil-dwelling micro-organisms but when their food source is depleted they are able to aggregate and form a fruiting body. More than 100.000 cells participate to form a single fruiting body that consists of a mass of spores on top of a cellulose stalk made of dead cells. *Dictyostelium* amoebas that are initially all the same *differentiate* (change their properties) in response to the signalling molecules that they produce and get exposed to during development. During *Dictyostelium* life cycle about 80% of the cells become spores, whereas the other 20% will differentiate into dead stalk cells. The dead stalk is used to lift the spores off the ground facilitating dispersal of the spores by other soil organisms. The spores containing dormant amoebas inside are resistant structures that can withstand harsh environmental conditions. They germinate to a new generation when favourable conditions are met, such as right humidity or replenishment of food. Under normal conditions this life cycle takes about 24 hours to complete. An important intermediate stage of the life cycle is the formation of a so-called slug. The slug is originated from cells in the *aggregate* and is a cylindrical shaped, motile structure organized to the equivalent of a “head to tail” plan. The slug is able to move and it migrates through the soil until it finds the right place to culminate (erect to form a fruiting body). The slug is attracted to light (it is phototactic) and guided by temperature too (it is thermotactic). It is during slug migration that cells determine their fate (*differentiate*) to become either a spore or a stalk cell.

Upon starvation, during the process of aggregation and later on during slug migration *Dictyostelium* amoebas produce and secrete a molecule called cyclic AMP or cAMP. This is the main signalling molecule that controls most of the developmental decisions throughout *Dictyostelium* life cycle. The cAMP secreted initially by a small group of cells accumulates in the extracellular medium and is detected by neighbouring cells attracting them to the

aggregate. This process of moving towards a chemical compound is called chemotaxis (chemo=chemical, taxis=movement) and cAMP acts in this instance as *chemoattractant*. Once a few amoebas start secreting it, the rest will move towards them to form an aggregate at the same time that they relay the signal (*or pass on the message*). This is how multicellular development is triggered and its dependence on extracellular cAMP is a unique feature of *Dictyostelium* development.

The proteins or *enzymes* that produce cAMP are called adenylyl cyclases and we find three in *Dictyostelium discoideum*: ACA, ACB and ACG. The cAMP produced by these enzymes plays different roles depending on whether is secreted by the cells and found extracellularly (outside the cells), or kept inside the cells then remaining intracellular. The regulation of cAMP production in time and space is essential for correct cell differentiation and survival.

The aims and conclusions of this thesis

cAMP seems to be the most prevalent molecule in *Dictyostelium* development therefore I have focus my attention to the regulation of cAMP production during particular developmental stages. A number of complex processes take place during the slug stage of which a big majority are controlled by cAMP. There are morphogenetic movements, which means that groups of cells move to build up a particular form or structure such as the slug and later on the fruiting body. These movements are mainly controlled from the tip of the slug through the emission of pulses of cAMP that will orient the cells. Cell differentiation also occurs during this stage. This means that cells acquire different characteristics becoming either prespore (later spores) or prestalk (later stalk) cells. From the tip of the slug to the rear a “head to tail” axis is established. Along this “head to tail” axis the prestalk cells are closest to the tip occupying one third of the structure and the prespore cells are in the posterior most part, occupying the other two thirds of the slug. *Dictyostelium* has developed mechanisms to achieve and maintain this pattern through the differential expression of the different components of the cAMP signalling machinery.

The production of cAMP by the adenylyl cyclases (ACA, ACB and ACG) at different times and in different places of the slug is involved in controlling cell differentiation (Chapter One). ACA controls early development and aggregation and it is only found at the tip of the slug. From here it emits pulses of cAMP regulating slug movement. The pattern of expression of adenylyl cyclase B was not known but I have shown in Chapter One that ACB is specifically expressed in the prestalk region of the slug, (anterior part closest to the tip). ACB mainly controls events during late development such as the entry into culmination, which is the transition from slug to fruiting body. In combination with other components ACB also contributes to the proper encapsulation of prespore cells into spores and the final formation of the stalk. ACG is expressed in the spores where it is activated by the high osmolarity (high concentration of solutes) found in the spore head fluid. The main function of ACG here is to stop spores from germinating under these adverse conditions. I have discovered that ACG is also present during the slug stage (Chapter One). During this stage ACG is present at the rear most part of the slug. From here it produces cAMP that is responsible for triggering prespore differentiation, a novel role for this enzyme. In summary the cyclases are expressed at the tip (ACA), the prestalk region (ACB) and the prespore region (ACG). This pattern of expression will directly reflect on the regulation of prespore and prestalk differentiation. In Chapter One I also show that the functions of the adenylyl cyclases are partially redundant, so one cyclase can overcome the absence of any of the other three. For example when ACG is not present the expression of ACB changes taking over the role of ACG in the prespore region. This makes it difficult to differentiate between adenylyl cyclase activities at the stages in which more than one is expressed.

The creation of specific gene knockouts by disrupting a particular gene using molecular biology techniques can be useful to analyse the function of the protein encoded by that gene. For example if a gene is involved in maintaining spores dormant under high osmotic conditions, like ACG, and we disrupt this gene, the resulting predicted phenotype of the mutant

(the physical appearance or resulting defect) would be spores that cannot remain dormant and that will germinate under high osmolarity. However, knocking out a gene is not always possible or practical as the gene of interest might be lethal or block development completely. A pharmacological approach can then be used instead using synthetic compounds that inhibit specifically the activity of a particular enzyme as an alternative to gene disruption. The advantage of this approach is that the drug can be used at the time or dose that we are interested in. Chapter Two shows such a pharmacological study performed to find potential inhibitors of the adenylyl cyclases. These studies have yielded compounds that inhibit ACA (SQ22536) and ACG (Tyrphostin A25) specifically.

cAMP degradation originates by-products that can also act as signalling molecules. Adenosine, produced as a result of cAMP degradation, is such a molecule. Because adenosine is quite similar to cAMP and it accumulates significantly during development, it can interfere with cAMP functions such as aggregation or prespore differentiation. In Chapter Three I show a study on an enzyme called adenosine kinase that phosphorylates adenosine (which is a type of chemical modification) potentially reducing the overall adenosine concentration. In this Chapter I show that the lack of adenosine kinase activity can have an effect determining slug size but not in prespore differentiation.

Dictyostelium discoideum is the species that we use in the laboratory as a model organism but it is only one of many described species. *D. discoideum* belongs to a bigger group called Amoebozoa, found as a sister branch to Animals and Fungi on the tree of life. Therefore *Dictyostelium* is in a very interesting evolutionary position to study the origins of multicellularity. All *Dictyostelia* species have in common that they form multicellular fruiting bodies originated from unicellular amoebas. However the shape and sizes of these fruiting bodies can vary greatly and whereas some species have only one spore head (*D. discoideum*), others have many along regular whorls (*Polysphondylids*). How these different phenotypes have evolved and what are the molecular mechanisms that control fruiting body formation on the other species has been one of the interesting questions I have asked in the last two chapters of this thesis. To analyse this we need to know about phylogeny, about the relationships between all the *Dictyostelium* species. Phylogenies are normally represented as trees, similar to a genealogical tree, and they show the sequence of events in the evolution of the species. Looking at the phylogeny one can determine relations between different species. We have built first molecular-based phylogenetic tree using DNA sequence information from more than 80 *Dictyostelium* species. Furthermore, mapping and following species characteristics through the tree highlights interesting evolutionary traits such as that during the Dictyostelids evolution the size of the fruiting body tended to increase in the most evolved species (“younger”) and this is directly linked with their use of cAMP during aggregation.

I have used this phylogeny to track down when certain traits appeared for the first time during evolution, such as the use of cAMP as chemoattractant (Chapter Five). In this Chapter I show that whereas the younger species like *D. discoideum* use cAMP as chemoattractant during aggregation at the onset of development, most “ancient” species such as *D. fasciculatum* or *D. minutum* use cAMP to control later events such as fruiting body morphogenesis, which represents the ancient function of the molecule.

Curriculum Vitae

Elisa Alvarez Curto was born on the 29th of August 1975 in Valladolid, Spain. She moved south to Málaga with her mother in 1987. Here she finished her Secondary Education in 1992, receiving her pre-university high school certificate at the Instituto Nacional de Bachillerato El Palo. She studied Biological Sciences at the University of Málaga, graduating as MSc (*Licenciada*) in 1998 with specialization in Microbiology and Biochemistry. She worked as a graduate in the laboratory of Dr. Jose Angel Aguirre, in the Human Physiology Department of the School of Medicine, University of Málaga, on the expression of dopaminergic receptors from July to December 1998. In January 1999 she moved to Leiden, The Netherlands, to carry out her PhD research project in the Institute of Molecular Plant Sciences. Here she will work under the supervision of Professor Pauline Schaap and funded by the Dutch Science Foundation (NWO). She finished her PhD work in the University of Dundee, Scotland, where Professor Schaap laboratory relocated to from September 1999. She was also Research Assistant to Professor Schaap in this University between 2003 and 2006. She now lives in Glasgow, Scotland, and is married to Dr. Philip McKenna. She currently works in the Molecular Pharmacology group of Professor Graeme Milligan at the University of Glasgow.

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Dankword (Acknowledgments)

I am ultimately responsible and to blame for what it is written here, but most of it wouldn't exist hadn't it been for the many people that have accompany me through this process.

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And now, for something completely different…