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Developmental regulation and evolution of cAMP signalling in *Dictyostelium*

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Discussion

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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).

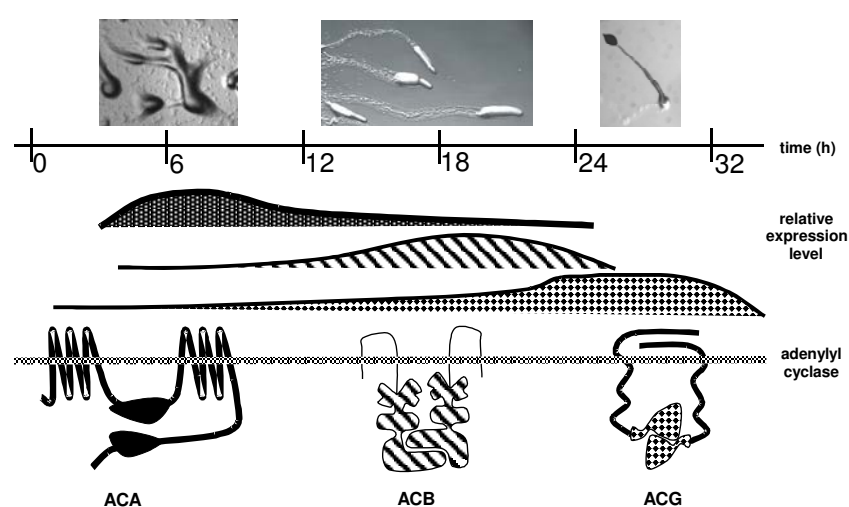


Figure 1. Timing and levels of expression of ACA, ACB and ACG during development

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concentrations of cAMP and low concentrations of DIF have an inductive effect on ACG protein levels (Alvarez-Curto, unpublished).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.

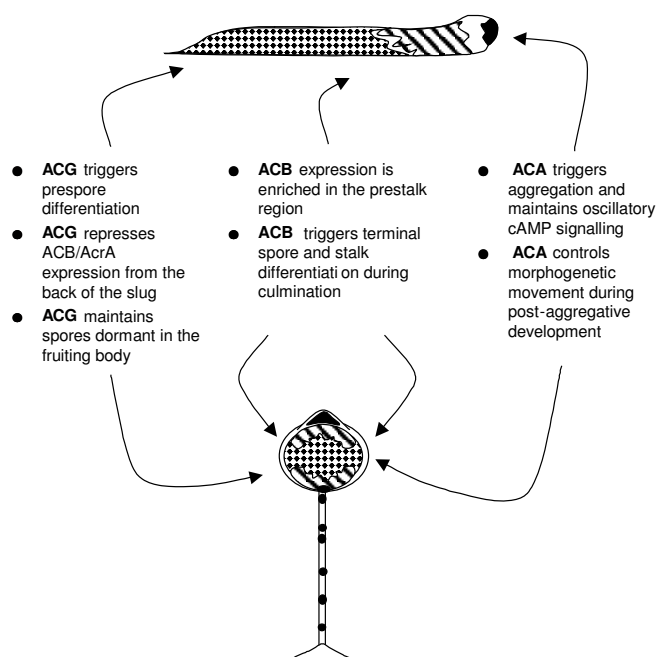


Figure 2. Roles of ACA, ACB and ACG during the slug and fruiting body stages

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 $IC_{50} = 16.6 \pm 7.4 \mu M$. *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 GTP γ S-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 is 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 castellanii* (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 differentiation 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).

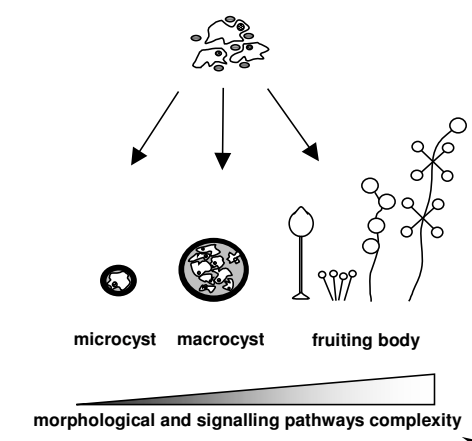


Figure 3. Survival strategies found in the Dictyostelids

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 encystation in the solitary amoeba *Acanthamoeba castellanii* (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 differentiation. 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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