Developmental regulation and evolution of cAMP signalling in Dictyostelium

Alvarez-Curto, E.

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).

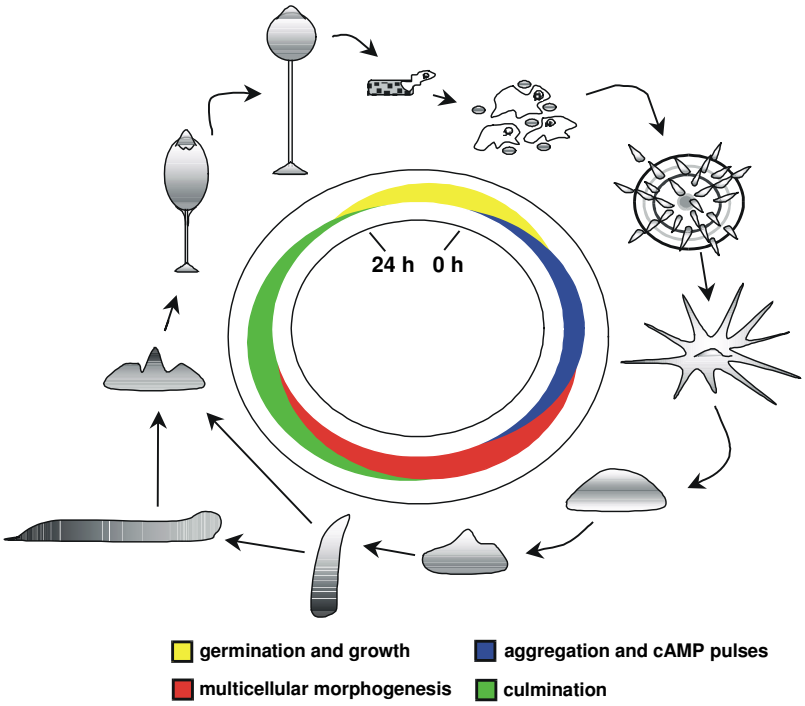

Figure 1. Life cycle of *Dictyostelium discoideum*

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.

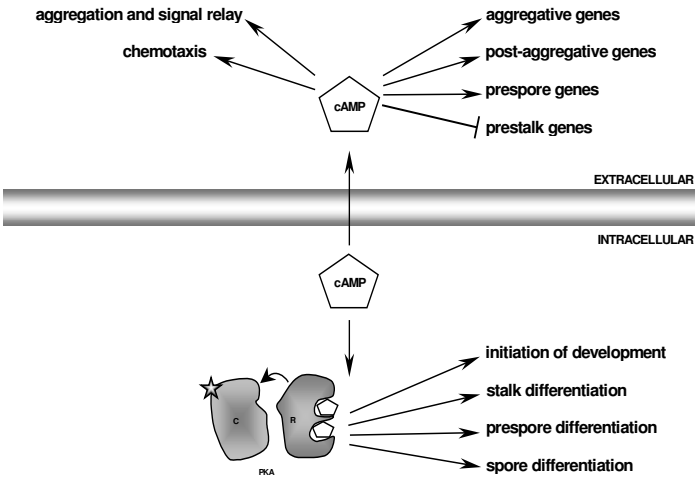

Figure 2. Roles of extracellular and intracellular cAMP

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).

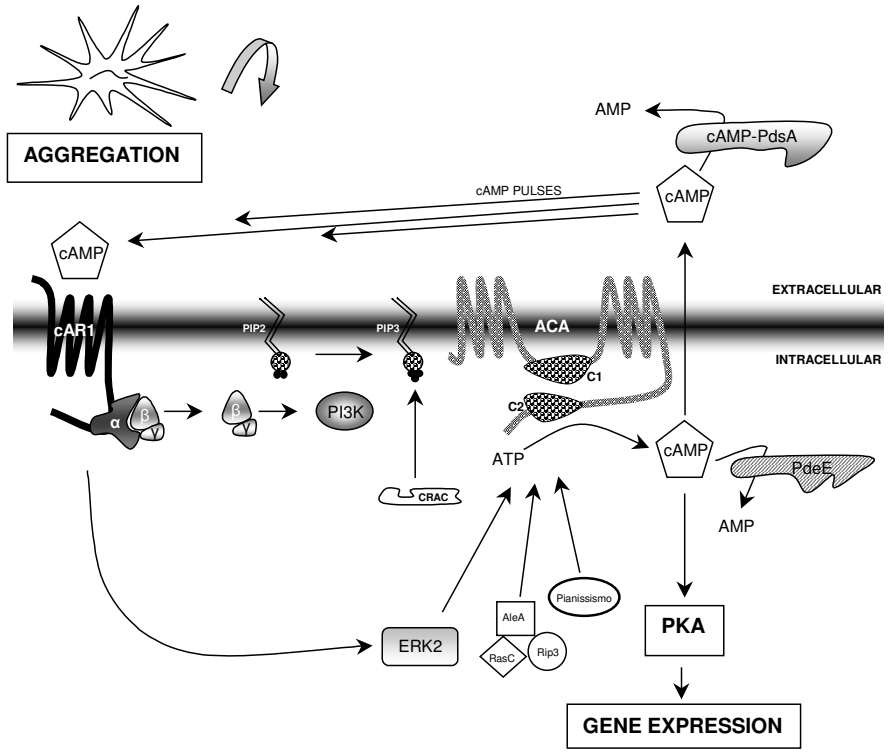

Figure 3. Signalling pathways controlling the aggregation specific cyclase ACA. ACA: adenylyl cyclase A; cAR1: cAMP-specific receptor 1; α, β ,γ: G-protein subunits; PIP2 and PIP,3: phosphatydylinositol phosphate 2 and 3; C1, 2: catalytic domain 1 and 2; PI3K: phospho-inositol specific kinase 3; CRAC: 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.

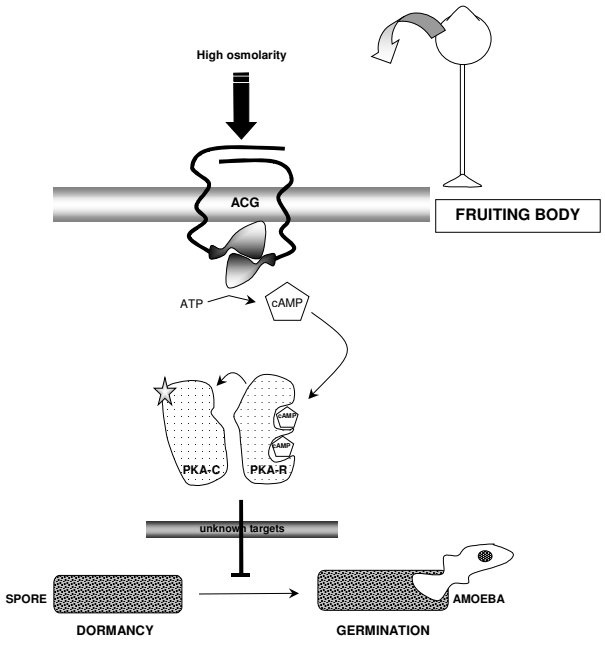

Figure 4. ACG is an osmosensor and controls spore dormancy in *Dictyostelium*

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.

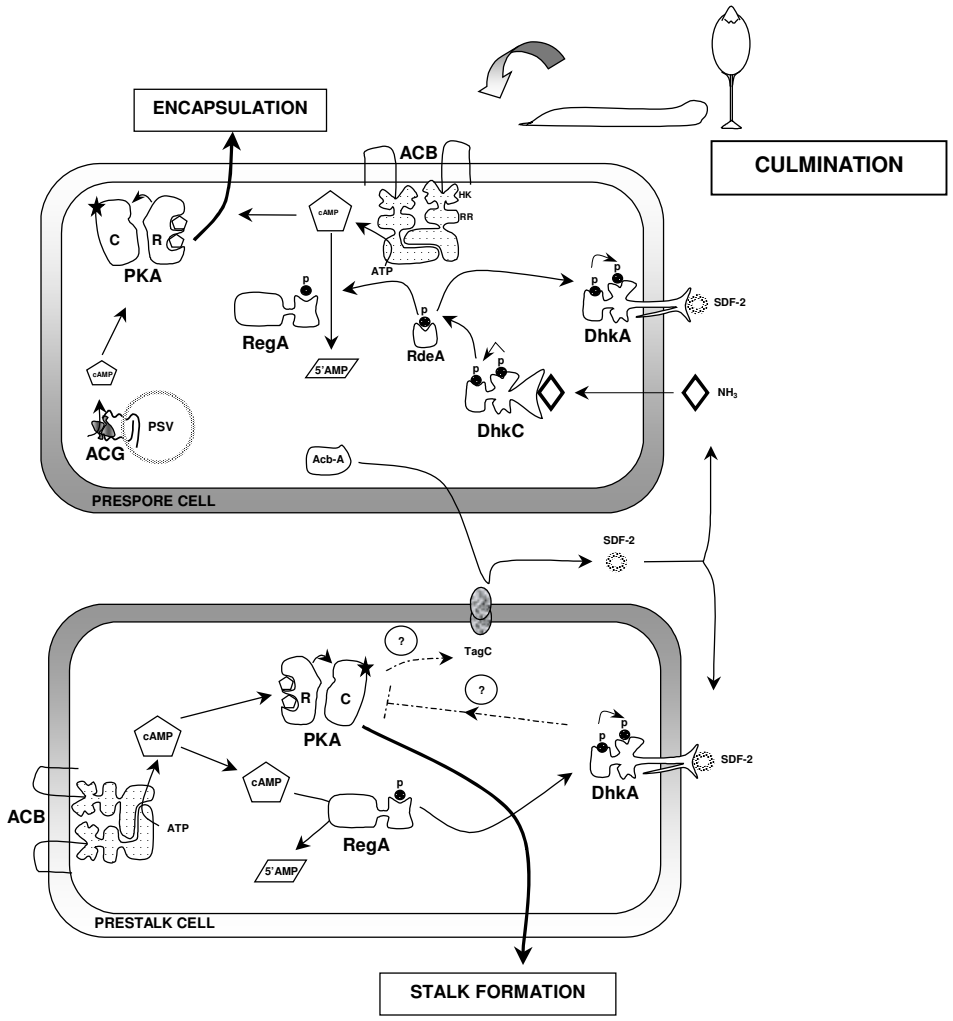

Figure 5. Regulation of post-aggregative development by cAMP, ACB/AcrA and RegA
ACG and ACB: adenylyl cyclases G and B; HK: histidine kinase domain; RR: response regulator domain; PKA: protein kinase A; C and R: catalytic and regulatory PKA subunits; RegA: intracellular phosphodiesterase; RdeA: phospho-donor 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).

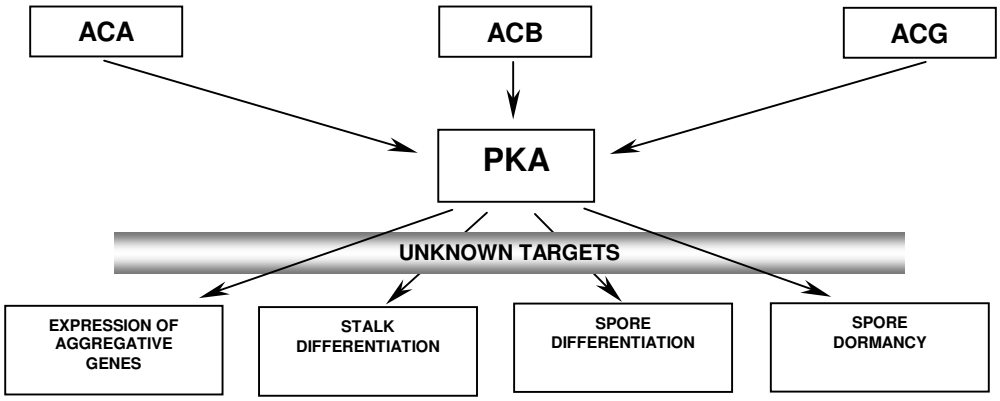

Figure 6. Roles of PKA at different stages of *Dictyostelium* development.

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.

References

1. Anantharaman, V., and Aravind, L. A. (2001). The CHASE domain: a predicted ligand-binding molecule in plant cytokinin receptors and other eukaryotic and bacterial receptors. Trends Biochem Sci 26.
2. Anjard, C., Chang, W. T., Gross, J., and Nellen, W. (1998). Production and activity of spore differentiation factors (SDFs) in Dictyostelium. Development 125, 4067-4075.
3. Anjard, C., and Loomis, W. F. (2005). Peptide signaling during terminal differentiation of Dictyostelium. Proc Natl Acad Sci U S A 102, 7607-7611.
4. Anjard, C., Pinaud, S., Kay, R. R., and Reymond, C. D. (1992). Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of Dictyostelium discoideum. Development 115, 785-790.
5. Araki, T., Gamper, M., Early, A., Fukuzawa, M., Abe, T., Kawata, T., Kim, E., Firtel, R. A., and Williams, J. G. (1998). Developmentally and spatially regulated activation of a Dictyostelium STAT protein by a serpentine receptor. EMBO J 17, 4018-4028.
6. Aubry, L., and Firtel, R. (1999). Integration of signaling networks that regulate Dictyostelium differentiation. Ann Rev Cell Dev Biol 15, 469-517.
7. Baldauf, S. L. (2003). The deep roots of eukaryotes. Science 300, 1703-1706.
8. Chen, M. Y., Long, Y., and Devreotes, P. N. (1997). A novel cytosolic regulator, pianissimo, is required for chemoattractant receptor and g protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in dictyostelium. Genes And Development 11, 3218-3231.
9. Clarke, M., and Gomer, R. H. (1995). PSF and CMF, autocrine factors that regulate gene expression during growth and early development of Dictyostelium. Experientia 51, 1124-1134.
10. Cotter, D. A., Dunbar, A. J., Buconjic, S. D., and Wheldrake, J. F. (1999). Ammonium phosphate in sori of Dictyostelium discoideum promotes spore dormancy through stimulation of the osmosensor ACG. Microbiology-Uk 145, 1891-1901.
11. Dao, D. N., Kessin, R. H., and Ennis, H. L. (2000). Developmental cheating and the evolutionary biology of Dictyostelium and Myxococcus. Microbiology 146, 1505-1512.
12. Darmon, M., Barra, J., and Brachet, P. (1978). The role of phosphodiesterase in aggregation of Dictyostelium discodeum. J Cell Sci 31, 233-243.
13. Devreotes, P. N. (1994). G protein-linked signaling pathways control the developmental program of Dictyostelium. Neuron 12, 235-241.
14. Dormann, D., Weijer, G., Parent, C., Devreotes, P., and Weijer, C. (2002). Visualizing PI3 Kinase-Mediated Cell-Cell Signaling during Dictyostelium Development. Curr Biol 12, 1178.
15. Eichinger, L., Pachebat, J. A., Glockner, G., Rajandream, M. A., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., et al. (2005). The genome of the social amoeba Dictyostelium discoideum. Nature 435, 43-57.
16. Faure, M., Podgorski, G. J., Franke, J., and Kessin, R. H. (1988). Disruption of Dictyostelium discoideum morphogenesis by overproduction of cAMP phosphodiesterase. Proc Natl Acad SciUSA 85, 8076-8080.
17. Franke, J., and Kessin, R. H. (1981). The cyclic nucleotide phosphodiesterase inhibitory protein of Dictyostelium discoideum. Purification and characterization. J Biol Chem 256, 7628-7637.
18. Gee, K., Russell, F., and Gross, J. D. (1994). Ammonia hypersensitivity of slugger mutants of D. discoideum. J Cell Sci 107, 701-708.
19. Gerisch, G., and Malchow, D. (1976). Cyclic AMP receptors and the control of cell aggregation in Dictyostelium. Adv Cyclic Nucl Res 7, 49-68.
20. Harwood, A. J., Hopper, N. A., Simon, M.-N., Driscoll, D. M., Veron, M., and Williams, J. G. (1992). Culmination in Dictyostelium is regulated by the cAMP-dependent protein kinase. Cell 69, 615-624.
21. Hopper, N. A., Harwood, A. J., Bouzid, S., Véron, M., and Williams, J. G. (1993). Activation of the prespore and spore cell pathway of Dictyostelium differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. EMBO J 12, 2459-2466.
22. Hopper, N. A., Sanders, G. M., Fosnaugh, K. L., Williams, J. G., and Loomis, W. F. (1995). Protein kinase a is a positive regulator of spore coat gene transcription in Dictyostelium. Differentiation 58, 183-188.
23. Hopper, N. A., and Williams, J. (1994). A role for cAMP-dependent protein kinase in determining the stability of prespore cell differentiation in Dictyostelium. Dev Biol 163, 285-287.
24. Hudson, R. E., Aukema, J. E., Rispe, C., and Roze, D. (2002). Altruism, cheating, and anticheater adaptations in cellular slime molds. American Naturalist 160, 31-43.
25. Insall, R. H., Borleis, J., and Devreotes, P. N. (1996). The aimless RasGEF is required for processing of chemotactic signals through G-protein-coupled receptors in Dictyostelium. Curr Biol 6, 719-729.
26. Jin, T., Soede, R. D. M., Liu, J. C., Kimmel, A. R., Devreotes, P. N., and Schaap, P. (1998). Temperature-sensitive g beta mutants discriminate between g protein-dependent and -independent signaling mediated by serpentine receptors. EMBO Journal 17, 5076-5084.
27. Johnson, R. L., Gundersen, R., Hereld, D., Pitt, G. S., Tugendreich, S., Saxe, C. L., Kimmel, A. R., and Devreotes, P. N. (1992a). G-protein-linked signaling pathways mediate development in Dictyostelium. Cold Spring Harbor SympQuantitBiol LVII, 169-176.
28. Johnson, R. L., Van Haastert, P. J. M., Kimmel, A. R., Saxe III, C. L., Jastorff, B., and Devreotes, P. N. (1992b). The cyclic nucleotide specificity of three cAMP receptors in Dictyostelium. J Biol Chem 267, 4600-4607.
29. Johnson, R. L., Saxe III, C. L., Gollop, R., Kimmel, A. R., and Devreotes, P. N. (1993). Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of Dictyostelium development. Genes Dev 7, 273-282.
30. Kae, H., Lim, C. J., Spiegelman, G. B., and Weeks, G. (2004). Chemoattractant-induced Ras activation during Dictyostelium aggregation. EMBO Rep 5, 602-606.
31. Kessin, R. H. (2001). Dictyostelium: Evolution, cell biology and the development of multicellularity (Cambridge, UK, Cambridge University Press).
32. Kim, H. J., Chang, W. T., Meima, M., Gross, J. D., and Schaap, P. (1998). A novel adenylyl cyclase detected in rapidly developing mutants of Dictyostelium. J Biol Chem 273, 30859-30862.
33. Klein, P. S., Sun, T. J., SaxeIII, C. L., Kimmel, A. R., Johnson, R. L., and Devreotes, P. N. (1988). A chemoattractant receptor controls development in Dictyostelium discoideum. Science 241, 1467-1472.
34. Kuwayama, H., Snippe, H., Derks, M., Roelofs, J., and Van Haastert, P. J. M. (2001). Identification and characterization of DdPDE3, a cGMP-selective phosphodiesterase from Dictyostelium. Biochem J 353, 635-644.

35. Lacombe, M.-L., Podgorski, G. J., Franke, J., and Kessin, R. H. (1986). Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum. J Biol Chem 261, 16811-16817.
36. Lee, S., Parent, C. A., Insall, R., and Firtel, R. A. (1999). A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate signal relay in Dictyostelium. Molecular Biology of the Cell 10, 2829-2845.
37. Louis, J. M., Ginsburg, G. T., and Kimmel, A. R. (1994). The cAMP receptor CAR4 regulates axial patterning and cellular differentiation during late development of Dictyostelium. Genes Dev 8, 2086-2096.
38. Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996). Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in Dictyostelium. J Biol Chem 271, 3351-3354.
39. Malchow, D., Nagele, B., Schwartz, H., and Gerisch, G. (1972). Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of Dictyostelium discoideum. Eur J Biochem 28, 136-142.
40. Manahan, C. L., Iglesias, P. A., Long, Y., and Devreotes, P. N. (2004). Chemoattractant signaling in dictyostelium discoideum. Annu Rev Cell Dev Biol 20, 223-253.
41. Mann, S. K. O., Yonemoto, W. M., Taylor, S. S., and Firtel, R. A. (1992). DdPK3, which plays essential roles during Dictyostelium development, encodes the catalytic subunit of cAMP-dependent protein kinase. Proc Natl Acad Sci USA 89, 10701-10705.
42. Martiel, J.-L., and Goldbeter, A. (1987). A model based on receptor desensitization for cyclic AMP signaling in Dictyostelium cells. Biophys J 52, 807-828.
43. Meima, M. E., Biondi, R. M., and Schaap, P. (2002). Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants. Mol Biol Cell 13, 3870-3877.
44. Meima, M. E., and Schaap, P. (1999). Fingerprinting of adenylyl cyclase activities during Dictyostelium development indicates a dominant role for adenylyl cyclase B in terminal differentiation. Dev Biol 212, 182-190.
45. Meima, M. E., Weening, K. E., and Schaap, P. (2003). Characterization of a cAMP-stimulated cAMP phosphodiesterase in Dictyostelium discoideum. J Biol Chem 278, 14356-14362.
46. Milne, J. L. S., Wu, L., Caterina, M. J., and Devreotes, P. N. (1995). Seven helix cAMP receptors stimulate Ca2+ entry in the absence of functional G proteins in Dictyostelium. J Biol Chem 270, 5926-5931.
47. Mougel, C., and Zhulin, I. B. (2001). CHASE: an extracellular sensing domain common to transmembrane receptors from prokaryotes, lower eukaryotes and plants. Trends Biochem Sci 26, 582-584.
48. Newell, P. C. (1982). Cell surface binding of adenosine to Dictyostelium and inhibition of pulsatile signalling. FEMS Microbiol Lett 13, 417-421.
49. Newell, P. C., and Ross, F. M. (1982). Inhibition by adenosine of aggregation centre initiation and cyclic AMP binding in Dictyostelium. J Gen Microbiol 128, 2715-2724.
50. Nikawa, J., Sass, P., and Wigler, M. (1987). Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of Saccharomyces cerevisiae. Mol Cell Biol 7, 3629-3636.
51. Pitt, G. S., Brandt, R., Lin, K. C., Devreotes, P. N., and Schaap, P. (1993). Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenylyl cyclase (ACA). Genes Dev 7, 2172-2180.
52. Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., and Devreotes, P. N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development. Cell 69, 305-315.
53. Saran, S., Meima, M. E., Alvarez-Curto, E., Weening, K. E., Rozen, D. E. and Schaap, P. (2002) cAMP signaling in Dictyostelium : Complexity of cAMP synthesis, degradation and detection. J. Muscle Res. Cell Motil. 23, 793-802
54. Saran, S., and Schaap, P. (2004). Adenylyl cyclase G is activated by an intramolecular osmosensor. Mol Biol Cell 15, 1479-1486.
55. Saxe III, C. L., Ginsburg, G. T., Louis, J. M., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1993). CAR2, a prestalk cAMP receptor required for normal tip formation and late development of Dictyostelium discoideum. Genes Dev 7, 262-272.
56. Schaap, P., and Van Driel, R. (1985). Induction of post-aggregative differentiation in Dictyostelium discoideum by cAMP. Evidence of involvement of the cell surface cAMP receptor. Exp. Cell Res. 159, 388-398.
57. Schaap, P., and Wang, M. (1986). Interactions between adenosine and oscillatory cAMP signaling regulate size and pattern in Dictyostelium. Cell 45, 137-144.
58. Schulkes, C., and Schaap, P. (1995). cAMP-dependent protein kinase activity is essential for preaggegative gene expression in Dictyostelium. FEBS Lett 368, 381-384.
59. Segall, J. E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R. A., and Loomis, W. F. (1995). A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in Dictyostelium. J Cell Biol 128, 405-413.
60. Shaulsky, G., Escalante, R., and Loomis, W. F. (1996). Developmental signal transduction pathways uncovered by genetic suppressors. Proc Natl Acad Sci USA 93, 15260-15265.
61. Simon, M.-N., Pelegrini, O., Veron, M., and Kay, R. R. (1992). Mutation of protein kinase A causes heterochronic development of Dictyostelium. Nature 356, 171-172.
62. Singleton, C. K., Zinda, M. J., Mykytka, B., and Yang, P. (1998). The histidine kinase dhkC regulates the choice between migrating slugs and terminal differentiation in Dictyostelium discoideum. Dev Biol 203, 345-357.
63. Slack, J. (2001). Essential Developmental Biology, Blackwell Science Ltd).
64. Soderbom, F., Anjard, C., Iranfar, N., Fuller, D., and Loomis, W. F. (1999). An adenylyl cyclase that functions during late development of Dictyostelium. Development 126, 5463-5471.
65. Sucgang, R., Weijer, C. J., Siegert, F., Franke, J., and Kessin, R. H. (1997). Null mutations of the dictyostelium cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates. Dev Biol 192, 181-192.
66. Tang, Y., and Othmer, H. G. (1994). A G protein-based model of adaptation in Dictyostelium discoideum. Math Biosciences 120, 25-76.
67. Thomason, P. A., Traynor, D., Cavet, G., Chang, W.-T., Harwood, A. J., and Kay, R. R. (1998). An intersection of the cAMP/PKA and two-component signal transduction systems in Dictyostelium. EMBO J 17, 2838-2845.
68. Thomason, P. A., Traynor, D., Stock, J. B., and Kay, R. R. (1999). The RdeA-RegA system, a eukaryotic phospho-relay controlling cAMP breakdown. J Biol Chem 274, 27379-27384.
69. Thompson, C. R., and Kay, R. R. (2000). The Role of DIF-1 Signaling in Dictyostelium Development. Mol Cell 6, 1509-1514.
70. Van Es, S., Virdy, K. J., Pitt, G. S., Meima, M., Sands, T. W., Devreotes, P. N., Cotter, D. A., and Schaap, P. (1996). Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores. J Biol Chem 271, 23623-23625.

71. Verkerke-van Wijk, I., Fukuzawa, M., Devreotes, P. N., and Schaap, P. (2001). Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression. Dev Biol 234, 151-160.
72. Virdy, K. J., Sands, T. W., Kopko, S. H., van Es, S., Meima, M., Schaap, P., and Cotter, D. A. (1999). High cAMP in spores of Dictyostelium discoideum: association with spore dormancy and inhibition of germination. Microbiology 145, 1883-1890.
73. Wang, N., Soderbom, F., Anjard, C., Shaulsky, G., and Loomis, W. F. (1999). SDF-2 induction of terminal differentiation in Dictyostelium discoideum is mediated by the membrane-spanning sensor kinase DhkA. Mol Cell Biol 19, 4750-4756.
74. Weening, K. E., Wijk, I. V., Thompson, C. R., Kessin, R. H., Podgorski, G. J., and Schaap, P. (2003). Contrasting activities of the aggregative and late PdsA promoters in Dictyostelium development. Dev Biol 255, 373-382.
75. Wu, L., Valkema, R., Van Haastert, P. J. M., and Devreotes, P. N. (1995). The G protein b subunit is essential for multiple responses to chemoattractants in Dictyostelium. J Cell Biol 129, 1667-1675.
76. Zhukovskaya, N., Early, A., Kawata, T., Abe, T., and Williams, J. (1996). cAMP-dependent protein kinase is required for the expression of a gene specifically expressed in Dictyostelium prestalk cells. Dev Biol 179, 27-40.