Cyclic nucleotide metabolism and function in Dictyostelium discoideum

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Cyclic nucleotide metabolism and function in *Dictyostelium discoideum*

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Voor Suzanne,

voor haar liefde, steun en eindeloos geduld

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Contents

1 General introduction

1.1 Signal transduction

Cells, whether living on their own as the bacteria and protists or in complex multicellular organisms, need to sense environmental signals to survive. These signals can be temperature, pH, light or chemicals, such as food or hormones. Some chemical signals traverse the cell membrane and can directly activate components in the cell, but most signal molecules bind extracellularly to transmembrane receptor proteins. Some receptors have enzymatic activities, such as receptor-kinases, receptor-phosphatases and guanylyl cyclases. Other receptors use intermediates to transduce the signal towards the inside of the cells, such as the G-protein coupled receptors (GPCR) or serpentine receptors. Ligand binding to the receptor results in a conformational change and thereby activation of heterotrimeric guanine-nucleotide binding proteins (G-proteins) at the intracellular side of the receptor. Heterotrimeric G-proteins consist of three subunits. Upon activation, the bound GDP is exchanged for GTP and the protein dissociates into an α- and a $\beta\gamma$-subunit. Both subunits are capable of modulating the activity of intracellular signalling proteins, which in turn produce second messenger molecules that regulate the activity of other signalling proteins. Such signalling cascades provide amplification of the binding of a single ligand molecule and activation of several pathways. In addition to this vertical flow of information, horizontal communication occurs when signalling components of different cascades regulate each other's activity. The resulting network is capable of responding appropriately to subtle changes in the complex environment that the cell faces (for examples see Weng *et al.*, 1999).

The first second messenger ever discovered is involved in the response to glucagon, a hormone which stimulates the degradation of glycogen in liver cells, resulting in the generation of glucose. Earl Sutherland discovered that stimulation with glucagon resulted in the production of a heat-stable compound that could mimic the effect of glucagon in a cell-free system. Sutherland identified this compound as 3',5'-adenosine monophosphate or cyclic AMP (cAMP) and was granted the Nobel prize for his work in 1971 (Sutherland, 1972). Since then, cAMP has been shown to be present in many organisms, ranging from bacteria to mammals and involved in many cellular and physical processes, such as metabolism, pathogenesis, neuronal function (e.g. learning and odorant sensing) and control of heart rate. Likewise, the chemically similar cyclic nucleotide second messenger cGMP was discovered in the 1960s and has since been identified as a regulator of many physiological processes, such as vascular smooth muscle contraction, intestinal fluid and electrolyte homeostasis and retinal phototransduction.

cAMP and cGMP are synthesized by adenylyl cyclases from ATP and guanylyl cyclases from GTP respectively (fig. 1.1). Since termination of the signal is an important feature of signalling systems, the levels of cyclic nucleotides are under strict control of cAMP- and cGMP phosphodiesterases, which hydrolyse the 3' phosphodiesterbond to yield the respective 5'nucleotides.

This thesis deals with the role of cyclic nucleotides in the development of the social amoeba *Dictyostelium discoideum*. In this introduction, I will first discuss the present knowledge of cyclic nucleotides and nucleotidyl cyclases in other systems, briefly in bacteria and extensively in eukaryotes, followed by an overview of cyclic nucleotide signalling in the eukaryote *Dictyostelium discoideum*.

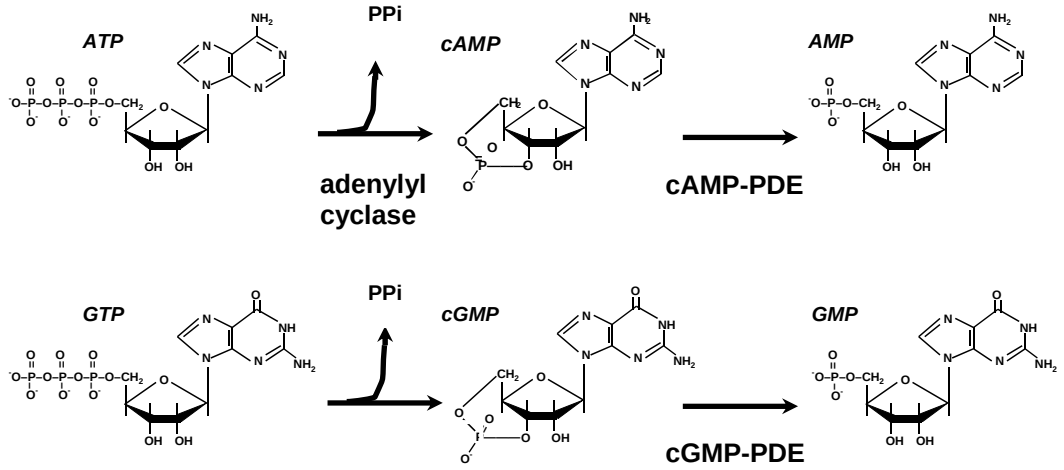

Fig. 1.1. Cyclic nucleotide metabolism.

1.2. The bacterial cyclase gene families

To date, three major cyclase families have been identified, which share no significant protein sequence homology (Danchin, 1993; Peterkofsky *et al.*, 1993). The class I and II families consist only of adenylyl cyclases and their presence is restricted to prokaryotes. The class III family members are found in eukaryotes and in some prokaryotes and this family also contains guanylyl cyclases. The number of gene families could expand, as recently novel bacterial adenylyl cyclases were identified that have no sequence homology with any of the three classes. The *Aeromonas hydrophila* adenylyl cyclase 2 displays homology with gene products from hyperthermophilic archaebacteria and might be the result of gene transfer, although lysates of *E. coli* strains expressing the *Archae* genes displayed no AC activity (Sismeiro *et al*., 1998). The novel adenylyl cyclase in *Prevotella ruminicola* is the first identified cyclase from an anaerobic bacterium (Cotta *et al*., 1998).

1.2.1 The bacterial class I adenylyl cyclases

These enzymes are found in enterobacteria and related species. In *E. coli*, cAMP plays a major role in catabolite repression, the mechanism by which specific sugars repress the expression of genes required for the degradation of other sugars. Here, cAMP binds to and activates a transcriptional regulator knows as cAMP receptor protein (CRP), that induces expression of genes for proteins that are involved in the metabolism of alternative carbohydrates. The *E. coli* adenylyl cyclase (cya) consists of a single N-terminal catalytic domain and a C-terminal regulatory domain. One of the main regulators of cya activity is the phosphoenolpyruvate:sugar system (PTS). The PTS is a multi-step phosphorelay system in which a phosphate is transferred from phosphoenolpyruvate (PEP) via intermediate protein components to sugars that are subsequently imported. Sugar depletion leads to an increase in phosphorylated intermediates which are considered to upregulate cya activity (Cases and De Lorenzo, 1998; Notley-McRobb *et al.*, 1997; Reddy and Kamireddi, 1998).

1.2.2 The pathogenic bacterial class II adenylyl cyclase

The second class of adenylyl cyclases are found in virulent bacteria such as *Bordetella pertussis* and *Bacillus anthracis*, which cause whooping cough and anthrax respectively (Escuyer *et al*., 1988; Ladant and Ullmann, 1999; Leppla, 1982). These enzymes are toxins that penetrate the eukaryotic host plasma membrane, where they are strongly stimulated by endogenous calmodulin, a calcium binding signal transduction protein. The high levels of cAMP cause edema and in neutrophils impair phagocytosis. The *B. anthracis* toxin consists of a complex of three proteins: a protective antigen (PA) that is required for receptor binding and for internalization of the other factors, the calmodulin-stimulated edema factor adenylyl cyclase (EF) and a peptidase-containing lethal factor (LF) that targets MAPKK and prevents MAPK signalling (Duesbury *et al.*, 1998; Escuyer *et al.*, 1988). The structure of EF has now been elucidated and it bears no resemblance to the mammalian adenylyl cyclases (Drum *et al.*, 2002). Binding of calmodulin induces a conformational change and stabilization of a disordered loop in the catalytic core, leading to activation of the cyclase.

1.2.3 Bacterial members of the class III cyclase superfamily

The bacterial class 3 adenylyl cyclases form a superfamily with the eukaryotic nucleotidyl cyclases (see later) and are found in cyanobacteria, myxobacteria, mycobacteria and rhizobia. In these bacteria, cAMP is involved in the regulation of a wide range of physiological processes, such as control of growth in response to nitrogen levels, light-on/light-off signals and pH-shifts (Ohmori, 1988; Ohmori *et al.*, 1989), respiration (Ohmori *et al.*, 1993) and cell motility (Terauchi and Ohmori, 1999). The class III cyclases have one catalytic domain that is conserved throughout these species. However, there is substantial variation in the domain organization of the different proteins. For example, the cya enzymes from *Anabaena* sp. strain PC7120 have respectively two N-terminal transmembrane domains, interspersed by a large extracellular domain (CyaA), cGMP-binding motifs (CyaB), response regulator and histidine kinase domains (CyaC) or a forkhead-associated protein-protein interaction domain (CyaD) (Katayama *et al.*, 1995; Katayama and Ohmori, 1997).

In *Mycobacterium tuberculosis*, the adenylyl cyclase has an N-terminal six transmembrane domain region followed by a single catalytic domain. (Guo *et al.*, 2001) This enzyme functions as a homodimer with 12 transmembrane segments and could therefore represent an evolutionary intermediate between simple bacterial adenylyl cyclases and mammalian 12 transmembrane adenylyl cyclases. The class III bacterial enzymes are most likely ancestral to eukaryote guanylyl cyclases. The adenylyl cyclase *CyaG* from *Spirulina platensis* harbours a dimerization domain that is highly conserved in mammalian guanylyl cyclases and three mutations can convert the enzyme fully into a cGMP-producing activity (Kasahara *et al.*, 2001). Indeed, cGMP has been found in many bacterial species, especially in cyanobacteria. Cya2 from *Synechocystis* is the first example of a bacterial guanylyl cyclase (Ochoa de Alda *et al.*, 2000). It is however more homologous to the cyanobacterial adenylyl cyclases than mammalian guanylyl cyclases, which suggests that the conversion of substrate specificity has occurred several times in evolution.

1.3 Cyclic nucleotides and nucleotidyl cyclases in eukaryotes

Cyclic nucleotides participate in many physiological responses in most eukaryotes. In yeast, adenylyl cyclase is activated in response to glucose and intracellular acidification and controls growth, metabolism and developmental choices, such as sporulation or hyphal growth. (Thevelein and De Winde, 1999). In metazoans, cAMP controls a plethora of processes, ranging from metabolism to cell differentiation and neurological functions, such as memory, learning and odour sensing. A wide range of signals affect cyclic nucleotide production in a highly cell type specific manner. Not surprisingly, animal cells have many different isozymes for the generation and transduction of the cyclic nucleotide signals, which allow regulation at different levels to produce the correct output in response to the many signals the cells perceive.

1.3.1 The adenylyl and guanylyl cyclase superfamily

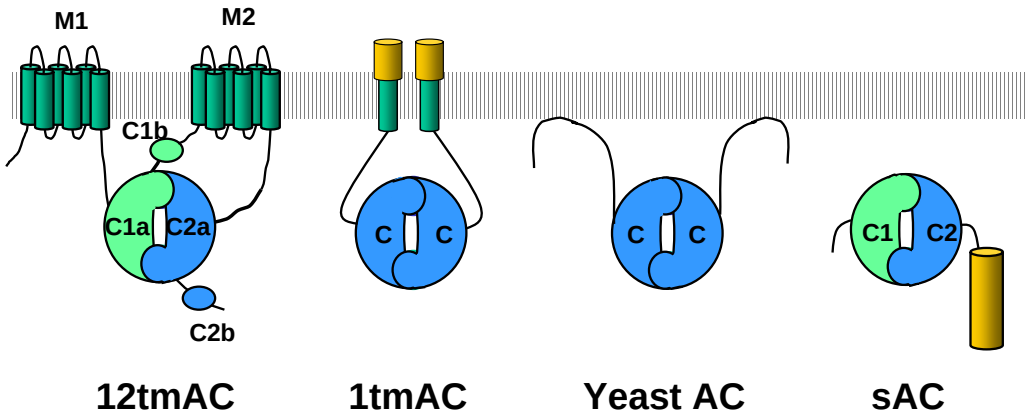

Fig. 1.2. Structure of eukaryotic adenylyl cyclases.

1.3.1.1 Adenylyl cyclases

Although studied for decades, the low abundance in cells and instability of the proteins made it extremely difficult to purify and sequence cyclases. The cloning of a cDNA encoding an adenylyl cyclase from bovine brain (Krupinski *et al.*, 1989) revealed the first structural and sequence data of these enzymes in mammalians and facilitated the cloning of different isozymes from various species. The similarity in the catalytic domains places all eukaryotic adenylyl and guanylyl cyclase genes in the single class III multigene family. However, the overall structure of the proteins shows considerable differences (fig. 1.2). The G-protein stimulated twelve transmembrane adenylyl cyclases (12tmACs) in mammals consist of two conserved domains (C1 and C2) separated by two stretches of six putative transmembrane segments (M1 and M2). The conserved domains are further divided into a and b domains, in which the C1a and C2a domain form the catalytic cores, whereas the C1b and C2b regulate activity. In mammals, 9 different tmAC (AC1-AC9) isoforms have been cloned, which differ in regulation and tissue-specific expression. Orthologues of 12tmACs have been found in other systems. In *Drosophila melanogaster*, the *rutabaga* gene encodes a 12tmAC involved in memory (Levin *et al.*, 1992). In *C. elegans*, the adenylyl cyclases SGS-1 and ACY-2 are most homologous to AC9 and AC2 respectively and have a role in neuronal signalling (Korswagen *et al.*, 1998). The *Dictyostelium* aggregation

specific adenylyl cyclase, ACA is responsible for the cAMP relay that controls aggregation (Pitt *et al.*, 1992).

The germination-specific adenylyl cyclase (ACG) in *Dictyostelium* has only one catalytic domain that shows sequence homology to mammalian ACs and a single transmembrane spanning domain (Pitt *et al.*, 1992). In the parasites *Leishmania* and *Trypanosoma*, single transmembrane receptor-like adenylyl cyclases with single catalytic domains (1tmACs) have also been found (Pays *et al.*, 1989; Ross *et al.*, 1991; Sanchez *et al.*, 1995; Taylor *et al.*, 1999). The amino acid sequence of the catalytic domains of these enzymes is more similar to those of the ACs from various species of fungi (Gold *et al.*, 1994; Kataoka *et al.*, 1995; Kore-eda *et al.*, 1991; Yamawaki-Kataoka *et al.*, 1989; Young *et al.*, 1989; Young *et al.*, 1991). However, fungal ACs are not transmembrane proteins, but are anchored at the membrane (Mitts *et al.*, 1990). They can be activated either by small G-proteins of the Ras-family or by heterotrimeric G-proteins (Thevelein and De Winde, 1999).

Recently, a soluble adenylyl cyclase (sAC) was purified and cloned from rat testis (Buck *et al.*, 1999). sAC has two N-terminal conserved domains. Interestingly, the conserved domains are more homologous to the ones found in cyanobacterial ACs than those in mammalian cyclases. The conserved domains are followed by a P-loop nucleotide binding domain and a long C-terminal domain with as yet unknown function.

1.3.1.2 Guanylyl cyclases

In animals, guanylyl cyclases (GCs) are present both in the cytosol and in the membrane (fig. 1.3) (reviewed in Lucas *et al.*, 2000). Soluble GCs (sGCs) are heterodimers composed of α- and β-subunits that are both required for catalytic activity. These cyclases carry a heme group in the regulatory N-terminus, which functions as a sensor for nitric oxide (NO), an activator of the enzyme.

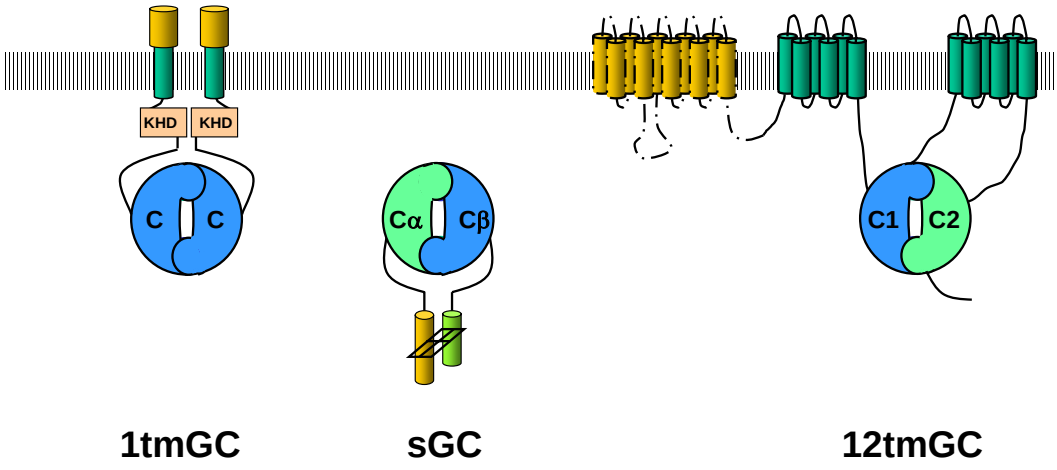

Fig. 1.3. Structure of eukaryotic guanylyl cyclases. Dashed structure in the 12tmGC resemble the Ca^{2+}-ATPase that is found in some but not all 12tmGCs

The single transmembrane or receptor guanylyl cyclases (1tmGCs) have single transmembrane domains and form homodimers. They all possess a kinase homology

domain (KHD) between the transmembrane and the catalytic domain, which serves as a regulator between extracellular ligand-receptor coupling and intracellular catalytic activity (see later). Only in retinal GC the KHD was shown to display intrinsic kinase activity (Aparacio and Applebury, 1996). Recently, a novel GC was found in the insect *Manduca* with highest homology to 1tmGCs, although it lacks the transmembrane and extracellular domains (Simpson *et al.*, 1999).

Guanylyl cyclases with the topology of 12tmACs have recently been identified in a number of lower eukaryotes, such as *Dictyostelium*, *Plasmodium*, *Paramecium* and *Tetrahymena* (Carucci *et al*., 2000; Linder *et al*., 1999; Roelofs *et al.*, 2001a). The catalytic domains of these proteins are inverted compared to 12tmACs, i.e. the C1 domains are most homologous to the C2 domains in 12tmACs and vice versa. The enzymes are further characterized, except for the *Dictyostelium* protein, by an N-terminally located Ca^{2+}-ATPase with hitherto unknown function.

1.3.1.3 Structure of the adenylyl cyclase catalytic core

The recent elucidation of the crystal structure of the catalytic core (Tesmer *et al.*, 1997; Zhang *et al.*, 1997) has lead to a detailed understanding of the catalytic mechanism of ACs and GCs (reviewed in Tang and Hurley, 1998; Tesmer and Sprang, 1998; Hurley, 1998). Tang and Gilman (1995) constructed a soluble form of mammalian AC by linking the C1 and C2 domain of AC1 and AC2 respectively. When expressed in *E. coli*, not only did the chimera show enzyme activity, but similar to the intact enzyme it was also activated by the stimulatory G_{α}-subunit $G_{s\alpha}$ and the diterpene forskolin and inhibited by $G_{\beta\gamma}$ subunits and adenosine (Tang and Gilman, 1995; Dessauer and Gilman, 1996). This meant that the conserved regions contain all the residues required for catalytic activity and direct activation. The transmembrane domains appear to serve primarily to anchor the enzyme at the site of activation and to bring the separate catalytic domains at close enough proximity to allow rapid formation of the catalytically active configuration. Mixing the separate catalytic domains will allow a heterodimer to form as well, which is activated by $G_{s\alpha}$ (Whisnant *et al.*, 1996; Yan *et al.*, 1996). A crystal structure of the stable heterodimer formed by AC5 C1a and AC2 C2 in complex with active $G_{s\alpha}$ showed that the domains form heterodimers in an antiparallel fashion, in which two nucleotide binding sites are generated in the central cleft, while the conserved regions donate residues to both sites (Tesmer *et al.*, 1997). One of the sites can bind the substrate; the second catalytically inactive site can bind forskolin (Dessauer *et al.*, 1997). This structure was used as a template to model other structures, such as ACG from *Dictyostelium* and the soluble and transmembrane GCs, showing similar folds for these homo- and heterodimeric enzymes (Liu *et al.*, 1997). In the homodimeric cyclases, both catalytic sites are active, since a mutation in the monomer would affect both sites.

1.3.1.4 Substrate specificity and catalysis

The structure determination of the catalytic core provided insight in the domains and residues involved in catalysis and substrate specificity. Mammalian ACs and GCs can be interconverted, which requires only two or three key mutations (Beuve, 1999; Sunahara *et al.*, 1998; Tesmer *et al.*, 1997; Tucker *et al.*, 1998). An invariable lysine and aspartate in the C2 domain of 12tmACs recognize the aminogroup at the 6 position and the unprotonated N_1 of the adenine respectively and form a salt bridge with each other. The counterparts are a glutamate and a cysteine in GC, which bind the protonated N_1 and form a weak hydrogenbond with the O_6 of GTP respectively. Changing these residues into the ones specific for AC converted the retinal GC into a

cAMP producing enzyme (Tucker *et al.*, 1998). The same substitutions in the heterodimeric sGC also completely changed substrate specificity from GTP to ATP, whereas activation by nitric oxide remained unaffected (Beuve, 1999; Sunahara *et al.*, 1998).

An asparagine and an arginine residue that are invariable throughout all the homodimeric and C2 domains of heterodimeric cyclases are absolutely essential for catalysis (Yan *et al.*, 1997a). These residues are involved in binding of the α- and β-phosphate groups and stabilization of intermediate structures during catalysis, whereas the γ-phosphate is bound by a lysine. Two Mg^{2+}-ions are also required for phosphate binding and catalysis (Tesmer *et al.*, 1999; Zimmermann *et al.*, 1998).The binding site is formed by two aspartates and a backbone carboxyl group that in 12tmACs are located in the C1 domain. During catalysis, the 3'-hydroxyl group is attacked by one metal ion and both ions share in transition state stabilization. Interestingly, the structure of the catalytic sites and the two-metal-mechanism are strikingly similar to what has been found for T7 and most other DNA polymerases, although sequence homology is virtually absent (Doublie *et al.*, 1998). This would indicate functional similarity, although it is unclear whether these enzymes share a common ancestor or are the result of convergent evolution.

1.3.2 Regulation of adenylyl cyclase by signal transduction molecules

cAMP signalling by 12tmACs is fine-tuned through tissue-specificity of expression and differential regulation by signal transduction molecules (Table 1.1; for references see reviews by Hamoune and Defer, 2001; Simonds, 1999; Sunahara *et al.*, 1996; Tang and Hurley, 1998; Tesmer and Sprang, 1998; Zippin *et al*., 2001). Some isozymes, for example AC4, AC7 and AC9 are widely expressed, whereas the expression of others is more restricted. For example, AC1 and AC8 are only expressed in brain and AC3 is very prominently expressed in olfactory neurons. The activity of all 12tmACs is modulated by G-protein coupled receptors (GPCRs) and other signal transduction components in their own unique way. This diversity in expression and regulation provides the adequate cAMP response required in cells specialised for different functions.

Table 1.1: Regulatory properties and main tissue distribution of mammalian adenylyl cyclases

Isoform	$G_{\alpha i}$	$G_{\beta\gamma}$	Ca^{2+}	PKA	PKC	Tissue distribution
AC1	↓	↓	↑ (CaM) ↓ (CaMKIV)		↑	Brain, adrenal, medula, (retina)
AC2	-	↑	-		↑	Brain, skeletal muscle, lung, (heart)
AC3	↓	-	↑ (CaM, *vitro*) ↓(CaMKII, *vivo*)		↑	Olfactory epithelium
AC4	↓	↑	-		↓ (G_{α}-stim. activity)	Brain, kidney
AC5	↓	↓ (β1γ2)	↓ (free Ca^{2+})	↓	↑	Heart, brain
AC6	↓	↓ (β1γ2)	↓ (free Ca^{2+})	↓	↓	Widespread
AC7	↓	↑	-		↑	Brain, platelets
AC8	↓		↑			Brain, lung
AC9	↓	-	↓ (calcineurin)			Brain, skeletal muscle
sAC	-	-	-			Testis

1.3.2.1 G-proteins

All 12tmAC isozymes have a basal activity, which is strongly enhanced by $G_{s\alpha}$ which, like forskolin, increases the affinity of C1 for C2, without altering the K_m for ATP (Whisnant *et al.*, 1996; Yan *et al.*, 1996). $G_{s\alpha}$ binds mainly on residues of the C2 domain (Yan *et al.*, 1997b) and likely induces a slight conformational change, thereby re-orientating the C1 and C2 domain with respect to each other. Tesmer and Sprang (1998) suggested a model in which $G_{s\alpha}$ brings the complex from a basal state with low activity into an activated ground state. ATP can bind to the protein in either state and results in collapse of residues around the substrate, followed by cAMP production. Other stimulatory or inhibitory effectors would merely influence collapse upon ATP binding, rather than affecting the affinity of the two domains for each other.

Most isozymes are inhibited by $G_{i\alpha}$ in a non-competitive fashion (Taussig *et al.*, 1994), whereas some are also inhibited by other α-subunits, such as $G_{o\alpha}$ and $G_{z\alpha}$ (Kozasa and Gilman, 1995; Taussig *et al.*, 1994). $G_{i\alpha}$ binds to the catalytic core on residues of the C1 domain at the opposite site of $G_{s\alpha}$ (Dessauer *et al.*, 1998; Wittpoth *et al.*, 1999) and possibly prevents the collapse of the complex around ATP (Tesmer and Sprang, 1998).

Released $G_{\beta\gamma}$-subunits have differential effects on tmAC activity, depending on the AC isozyme. They inhibit $G_{s\alpha}$-stimulated AC1 activity, but this inhibition requires higher concentration of $G_{\beta\gamma}$ than that of $G_{s\alpha}$ to activate the cyclase. This means that the source of $G_{\beta\gamma}$ is not the G_s complex, but likely the dissociation of an abundant G-protein, thus providing a mechanism for cross-talk (Tang and Gilman, 1991). AC2, 4 and 7 are stimulated by $G_{\beta\gamma}$ when $G_{s\alpha}$ is present. However, at least in the case of AC2 this depends on the combination of β- and γ-subunits. Bayewitch *et al.* (1998) showed that $G_{\beta1\gamma2}$ stimulated AC2 activity, but that $G_{\beta5\gamma2}$ is inhibitory. In contrast, both combinations inhibited AC1 equally well. This differential modulation adds another level of complexity to the regulation of cAMP production.

Apart from being regulated by G-proteins, 12tmACs also influence G-protein activity. Both receptor-induced guanine-nucleotide exchange and hydrolysis of G-protein bound GTP is enhanced by addition of soluble engineered AC5 or the C2, but not C1 domain of AC5 only (Scholich *et al.*, 1999). The effect is specific for $G_{s\alpha}$ and occurs only when the cyclase is in the active conformation, providing rapid onset and termination of signalling. Likewise, on $G_{i\alpha}$ GTP/GDP exchange mediated by inhibitory receptors and GTPase activity are stimulated by the C1 domain (Wittpoth *et al.*, 2000).

1.3.2.2 Protein kinases and calcium

Several protein kinases modulate 12tmAC activity. AC5 and AC6 are phosphorylated by the downstream effector protein kinase A (PKA) (Iwami *et al.*, 1995; Chen *et al.*, 1997a). This attenuates basal and $G_{s\alpha}$-stimulated activity and thus provides a mechanism for desensitization. Phosphorylation by PKA in AC6 occurs on a specific serine residue in the region that involves G-protein stimulation and possibly interferes with $G_{s\alpha}$-binding (Chen *et al.*, 1997a).

The phospholipase C pathway provides cross-talk to cAMP production through protein kinase C (PKC). Classical PKCs are activated by diacylglycerol, produced by phospholipase C, and Ca^{2+}.The effect of phosphorylation by PKC on activity depends on the 12tmAC isozyme. Phosphorylation by PKCα isozyme on a threonine residue in the C2a domain of AC2 and AC5 enhances basal and stimulated AC activity (Bol *et al.*, 1997; Levin and Reed, 1995; Zimmermann and Taussig, 1996). In contrast, $G_{s\alpha}$-stimulated AC4 activity is reduced by PKCα (Zimmermann and Taussig, 1996).

Calcium and cAMP signalling are coupled in some regulatory systems, for example neuromodulation or olfactory detection (Xia and Storm, 1997). The effects of Ca^{2+} on AC activity are mediated through calmodulin (CaM) or calmodulin kinase (CaM kinase), although evidence exists for activation of AC5 and AC6 by free Ca^{2+}. Both AC1 and AC8 are stimulated by Ca^{2+}/CaM. These ACs are specifically expressed in brain and are concentrated in post-synaptic dendrites that are involved in long-term potentiation (LTP) or synaptic plasticity, which is the development of neuronal connections in an activity-dependent fashion. Ca^{2+}-influx in response to activation of neurotransmitter sensing glutamate receptors triggers stimulation of AC activity and subsequent activation of downstream effectors, eventually resulting in altered gene expression. This has been supported by transgenic mice defective in AC1 and AC8 which have impaired LTP and long term memory defects (Wong *et al.*, 1999). AC3 mRNA is highly abundant in olfactory neurones. AC3 is involved in sensing of many odours and hence *AC3* homozygote mice fail several olfaction-based behavioural tests (Wong *et al.*, 2000). Although stimulated by Ca^{2+} *in vitro*, hormone-induced stimulation of AC3 is inhibited by intracellular Ca^{2+} (Wayman *et al.*, 1995b) This inhibition is modulated via CaM kinase II, which directly phosphorylates AC3 *in vivo* (Wei *et al.*, 1996; Wei *et al.*, 1998).

1.3.2.3 Regulation of sAC

The regulation of the recently discovered mammalian soluble adenylyl cyclase (sAC) differs greatly from 12tmACs in that neither G-proteins nor forskolin affect the activity (Buck *et al.*, 1999). Although the sequence predicts a 187 kD protein, the initially purified protein was only 48 kD and consisted of the N-terminal catalytic region, indicating possible proteolytic processing. The activity of this truncated form is ~20 fold higher than of the full length protein, which suggests an autoregulatory mechanism in the full length protein.

sAC is activated by bicarbonate, an important signal molecule for sperm maturation (Chen *et al.*, 2000). As predicted by its homology to sAC, the AC CyaC from the cyanobacteria *Spirulina platensis* is also activated by bicarbonate, implying a function that has been conserved for more than a billion years of evolution. Since sAC is also expressed in many other tissues, a function in other bicarbonate regulated processes, especially the regulation of metabolism, cannot be excluded (Zippin *et al.*, 2001).

1.3.3 Regulation of guanylyl cyclases by signal transduction proteins

In contrast to 12tmACs, mammalian GCs are not regulated by G-proteins. The tmGCs GC-A and GC-B function as receptors for and are activated by vasodilatory natriuretic peptides (ANP, BNP and CNP) (Potter and Hunter, 2001; Lucas *et al.*, 2000) and are also referred to as natriuretic peptide receptor A and B (NPR-A and NPR-B). These enzymes already exist as dimers and oligomers in the inactive state, thus activation is not achieved by bringing the monomers together as is the case for growth-factor receptors. Mutational and biochemical analysis has suggested a multistep activation mechanism (Potter and Hunter, 2001). First ANP binds to the dimer in a 2:2 ANP:monomer stochiometry (Misono *et al.*, 1999; Van den Akker *et al.*, 2000), resulting in dimerization of the juxtamembrane regions of the extracellular domains (Labrecque *et al.*, 2001). This in turn induces a conformational change of the kinase homology domains (KHD), which allows binding of ATP and proper orientation of the dimer. As an adaptation mechanism, ATP binding also results in loss of affinity for the ligand and subsequently dephosphorylation of the KHD leading

to inactivation of the cyclase. GC-C expression is limited to intestinal cells. The first ligands identified for GC-C were heat-stable enterotoxins (STs) from bacteria that colonize the intestine, such as *Escherichia coli*. Binding of ST activates GC-C and increases intracellular cGMP-levels in intestinal cells, which causes acute diarrhea. Disruption of the gene encoding GC-C results in mice that are resistant to STs (Mann *et al.*, 1997; Schulz *et al.,* 1997). Endogenous ST-like peptides have been isolated from mammals. They may play a role in the regulation of intestinal fluids and electrolytes. However GC-C knock-out mice appear to develop normally with normal intestinal functions (Mann *et al.*, 1997; Schulz *et al.,* 1997).

Two tmGCs are present in the retina, retGC-1 and retGC-2 (also known as ROSGC-1 and ROSGC-2 or GC-E and GC-F) where they play an important role in vision (Stryer, 1991; Lucas *et al.*, 2000). Photo excitation of rhodopsin increases cGMP phosphodiesterase activity. The decrease in cGMP levels results in closure of cGMP-gated ion channels, causing hyperpolarization which transduces the signal to the optical nerves. In addition, closure of the channels decreases intracellular [Ca^{2+}]. Lowering of calcium levels activates retGC which contributes to recovery from photo excitation and light adaptation of the photoreceptors. Though receptor-like molecules, retGCs are activated by calcium binding guanylyl cyclase activator proteins (GCAP) that bind at the intracellular site of the cyclase. The mechanism by which GCAPs activate retGCs is not fully understood. The current model suggests that GCAPs bind to retGCs, irrespective of [Ca^{2+}]. At high [Ca^{2+}] the GCAP monomers bind Ca^{2+} and have low affinity for each other. At low [Ca^{2+}], GCAP forms homodimers that promotes homodimerization and subsequently activation of retGC (Olshevskaya *et al*, 1999.; Yu *et al.*, 1999; Sokal *et al.*, 1999) The binding site on retGC for GCAP has homology with the binding sites of 12tmACs for $G_{s\alpha}$ and similarly it has been suggested that GCAPs promotes the formation of a high activity state of the catalytic centre around the substrate (Sokal *et al.*, 1999).

sGCs are activated by nitric oxide (NO), a regulator of vasodilatation and neuronal signal transduction. sGC is a heterodimeric protein, consisting of an α- and a β-subunit. Both subunits contain a regulatory heme binding, a dimerization and a catalytic domain. Binding of NO to the heme group creates a conformational change that activates the cyclase. Similar to 12tmACs, each subunit contributes specific residues to a single catalytic site (Liu *et al.*, 1997). Dimerization is mediated by specific domains, proximal to the catalytic domains, which share homology with the dimerization domains of receptor-guanylyl cyclases. The catalytic and dimerization domains alone are sufficient for basal activity (Wedel *et al.*, 1995). The activity of sGC is negatively regulated by Ca^{2+}. This inhibition reflects the antagonistic physiological effects of cGMP and Ca^{2+}, for example vasodilatation vs. vasorestriction. Ca^{2+} inhibits non-competitively, likely through an interaction with either substrate or one of the products (Parkinson *et al.*, 1999).

1.3.4 Cyclic Nucleotide Phosphodiesterases

In addition to the cyclases, cyclic nucleotide levels are regulated by phosphodiesterases (PDEs), which degrade these compounds to their respective monophosphates. Three different classes of PDEs exist, that share no obvious sequence homology. Most eukaryotic enzymes belong to the class 1 enzymes. Class 2 enzymes have mainly been found in yeast and fungi, but also the extracellular PDE in Dictyostelium belongs to this group. The class 3 PDEs are only present in bacteria.

Far from being static cyclic nucleotide removers, PDEs are regulated by a variety of signal transduction pathways and targeted to specific locations in the cell. In

mammalians, 12 different gene families have been identified and many of the members have different splice variants (reviewed by Houslay and Milligan, 1997; Soderling and Beavo, 2000; Francis et al., 2001; Houslay, 2001). The isozymes differ in substrate specificity i.e. some are specific for only cAMP or cGMP, whereas others can degrade both nucleotides.

Fig. 1.4. Domain structure of class 1 phosphodiesterases.

The catalytic domain resides in the C-terminus in all isozymes (fig. 1.4). The K_m values differ widely between PDEs. It is thought that the high affinity PDEs play a scavenging role to keep cAMP levels low when ACs are not stimulated, whereas the others play a role in regulation of local cAMP concentrations when ACs become activated. Recently the structure of the catalytic domain of PDE4B was solved (Xu et al., 2000). The active site contains a binuclear binding motif in which binding of two metal ions, Zn^{2+} in the first and Zn^{2+}, Mg^{2+} or Mn^{2+} in the second site, is coordinated by a conserved sequence motif of histidine and aspartate residues and a number of water molecules

The N-terminal region varies widely throughout the families and is the site of regulation and targeting (fig. 1.4). For example, PDE2, 5, 6, 10 and 11 all have GAF domains. GAF domains are cGMP binding motifs that are found in all kingdoms of life in a variety of signalling proteins, among which are cGMP-regulated PDEs, cyanobacterial ACs and the bacterial transcription factor FhlA. (Ho et al., 2000). In PDE2, cGMP binding stimulates the activity of the catalytic subunit, whereas in PDE5 it regulates accessibility for phosphorylation.

The retinal PDE6 is unique in that it is the only G-protein regulated PDE. The rod cell holoenzyme consists of two homologous catalytic subunits (α and β), in complex with two inhibitory γ-subunits. Light absorption by the photoreceptor rhodopsin activates the G-protein transducin. The released G_α-subunits of transducin interact with and displace the PDE6γ-subunits, thereby relieving the inhibition and causing a rapid increase in PDE activity (Francis et al., 2001).

One of the most studied isoforms is PDE4 (Houslay, 2001; Houslay and Adams, 2003). Four PDE4 genes exist (A to D) and alternative splicing generates long, short and supershort isoforms with differential cellular localization and regulatory properties. PDE4D contains docking sites for ERK2 (extracellular signal-regulated kinase 2) and can be phosphorylated in the catalytic domain (Houslay and Kolch, 2000). In the long isoforms, this results in inhibition of activity regulated internally by two conserved regions (UCR1 and UCR2) in the N-terminus. This inhibition in turn is under control of a negative feedback loop, as PKA phosphorylation in the N-terminus abolishes the inhibition by ERK2. However, in the short isoform which lacks UCR1, ERK2 phosphorylation stimulates PDE activity. This isoform is exclusively associated with the membrane fractions, whereas the long form is found in the cytosol and cortical cytoskeleton. The differences between the isoforms in regulation and

targeting could well govern differential activation of PKA in different compartments in the cell.

1.3.5 Targets of cAMP and cGMP

1.3.5.1 Protein kinase A

The best studied cyclic nucleotide target is the cAMP-dependent protein kinase (protein kinase A or PKA) (Feliciello *et al.*, 1997; Francis and Corbin, 1999). PKA was first described in the 1960s as the cAMP-dependent glycogen synthase kinase that mediates inhibition of glycogen synthesis. Since then, roles for PKA in many cellular processes, such as metabolism, synaptic transmission, ion channel function, gene transcription and differentiation have been described.

The mammalian PKA holoenzyme consists of a tetramer of two catalytic (C) and two regulatory (R) subunits. In the absence of cAMP the complex is tightly held together. In this state the pseudosubstrate domain of the regulatory subunit binds to the catalytic cleft of the catalytic subunit, thereby keeping the enzyme inactive. Upon binding of two molecules of cAMP to each regulatory subunit the complex dissociates and the catalytic subunits can phosphorylate serine and threonine residues in target proteins. In mammalians, two major R subunit isoforms (RI and RII) subdivided in α- and β-forms and two catalytic subunits exist, while a third catalytic subunit is only found in primates.

Genetic studies in mice and *Drosophila* have elucidated the involvement of PKA in metabolic, neuronal and developmental processes. Transgenic mice lacking a functional RIIβ-subunit have reduced body fat, higher metabolic rate and elevated body temperature (Cummings *et al.*, 1996). These mice also show defective neural gene expression and experience-dependent locomotor behaviour (Brandon *et al.*, 1998). Inactivation of the RIβ gene yields mice that are defective in neuronal plasticity (Hensch *et al.*, 1998). In these mice, the lack of Rβ subunits is however compensated by overexpression of RIα, which could suppress a more severe phenotype. *Drosophila* mutants carrying lesions in the regulatory or catalytic subunits display neuronal abnormalities as well. For example, disruption of the *PKA-RI* gene causes learning and short-term memory defects (Goodwin *et al.*, 1997) whereas mutants in *PKA-RII* display circadian abnormalities (Park *et al.* 2000). This correlates with similar phenotypes for mutants in other components of the cAMP signalling system in *Drosophila*, such as the AC *Rutabaga* (Levin *et al.*, 1992) and the PDE *dunce* (Byers *et al.*, 1981).

1.3.5.1.1 PKA and development

PKA activity is essential for development of the embryo. Mice that have a lesion in both catalytic subunits die in an early embryonic stage. Progressive reduction of PKA activity yields mice that have increasing defects in the posterior part of the neural tube (Huang *et al.*, 2002). RIα is essential for early embryogenesis. Mice lacking RIα have unrestrained PKA activity. In these embryos, the primitive streak is reduced and mesoderm derived structures, such as the heart, show strong deficits (Amieux *et al.*, 2002). In *Drosophila*, development of the wing imaginal disc is dependent on PKA activity. The secreted protein Hedgehog (Hh) triggers expression of a number of genes, among which are the signalling molecules Wingless (Wg) and the transcription factor Cubitus Interruptus (Ci). In the absence of Hh, PKA phosphorylates Ci which is then proteolytically cleaved into a shorter form that represses expression of target

genes (Kiger Jr. and O'Shea, 2001; Price and Kalderon, 1999). This process is overruled by Hh through a separate pathway, leading to increased full length Ci (Ohlmeyer and Kalderon, 1997). As a consequence, transgenics that have down-regulated PKA activity by overexpression of dominant-negative C- or R-subunits show ectopic *wingless* expression and wing duplication (Kiger Jr. and O'Shea, 2001; Price and Kalderon, 1999).

1.3.5.1.2 Compartmentalization of PKA signalling by AKAPs

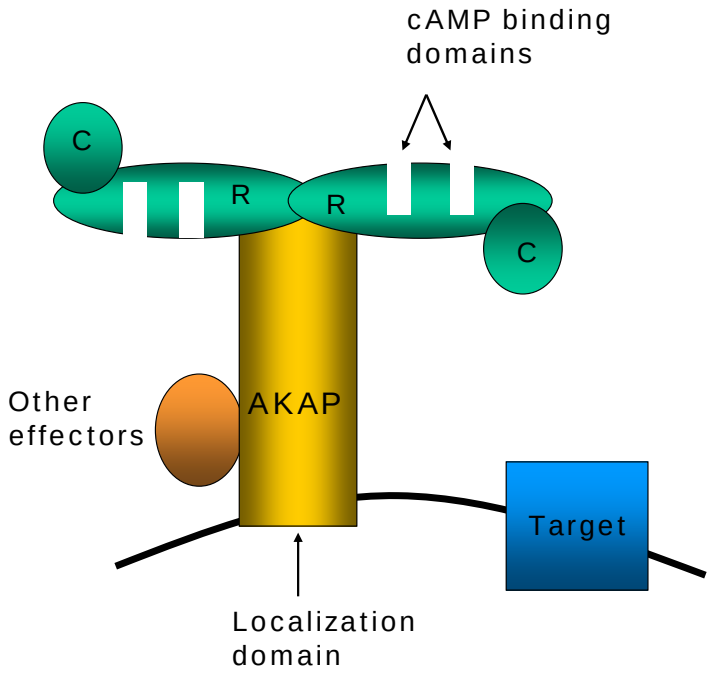

Fig. 1.5. Basic structure of signalling modules recruited by AKAPs.

In the cell, spatial and temporal cAMP signalling is achieved through the subcellular localisation of the different components (Houslay and Milligan, 1997). PKA-RII is almost exclusively associated with particulate cell fractions through its interaction with A-kinase anchoring proteins (AKAPs) (fig. 1.5) (Bauman and Scott, 2002; Feliciello *et al.*, 1997; Michel and Scott, 2002). AKAPs are functionally homologous proteins that recruit PKAII to specific targets in different organelles by binding the dimerization domains of RII subunits. Besides PKA-RII, AKAPs bind and are affected by other modulators, such as Ca^{2+} and PKC, allowing cross-regulation with other signal transduction cascades. Many AKAPs form complex local signalling modules. The muscle-selective AKAP (mAKAP) binds the long isoform PDE4D3 as well as PKA-RII holoenzyme at perinuclear regions (Dodge *et al.*, 2001). When cAMP concentrations are high, the PKA-C subunit dissociates from the PKA holoenzyme. Phosphorylation of the associated PDE4D3 stimulates the PDE activity, thereby degrading cAMP to basal levels and favouring re-association of the PKA holoenzyme. Thus, the module forms a negative feedback loop that controls the local pool of free C-subunit.

1.3.5.1.3 Gene regulation by PKA

In some cells, cAMP activates transcription of genes that are under control of the cAMP response element (CRE) (Daniel *et al.*, 1998). This element is recognized specifically by the transcription factors CREB (CRE binding protein) and ATF-1 (activator of transcription 1). These proteins are activated when phosphorylated on serine residues by PKA, which turns on gene expression. These factors are often part of multiprotein complexes and can be activated by different signalling pathways. For example, CREB is also activated by other serine/threonine kinases, such as CaM kinase, GSK3 (glycogen synthase kinase 3) and the ERK target Rsk.

cAMP signalling also feeds into the classical MAPK/ERK pathway through PKA. This pathway is activated by growth factors and involved in proliferation, differentiation, development and apoptosis. MAPK pathways consist of a module of three kinases: the serine/threonine kinase MAPK (mitogen activated protein kinase) which is activated by an upstream MAPK kinase (MAPKK), that is in turn activated by the MAPKK kinase (MAPKKK) Raf. Raf activity is controlled by small G-proteins such as Ras. PKA inhibits the ubiquitously expressed Raf-1 and therefore in many cell types cAMP acts negatively on the MAPK pathway. However, in some cell types the cAMP second messenger system stimulates the ERK pathway, most notably in neuronal cells (Houslay and Kolch, 2000). For example, in hippocampal neurons, long term potentiation and memory requires CREB-dependent transcription (Impey *et al.*, 1999). Instead of directly phosphorylating CREB, PKA triggers ERK nuclear localization, resulting in sequential phosphorylation of Rsk2 and CREB (Impey *et al.*, 1998). The reason why ERK is in some cases stimulated instead of inhibited by cAMP is still a matter of debate.

1.3.5.2 Epac, a cAMP regulated GEF

A few years ago, a number of studies showed that not all effects of cAMP depend on PKA. Activation of the small GTPase Rap1 is mediated by a number of second messengers, such as Ca^{2+}, diacylgycerol and cAMP. These effects are controlled by guanine exchange factors (GEFs) that contain binding sites for Ca^{2+} and diacylgycerol (CalDAG-GEF) and for cAMP (Epac; exchange protein directly activated by cAMP) (Springett *et al.*, 2004).

Fig. 1.6. Domain architecture of Epac1. Epac2 contains an additional cyclic nucleotide binding motif at the extreme N-terminus. DEP: Dishevelled/Egl-10/pleckstrin domain; cAMP: cAMP binding domain; REM: Ras-exchange motif; GEF: guanine nucleotide exchange factor.

Epac1 contains a DEP (Dishevelled/Egl-10/plecskstrin) domain, a single cAMP binding domain, a Ras exchange motif (REM) and a GEF domain (fig. 1.6) (De Rooij *et al*. 1998). The cAMP binding domain has a relatively low affinity for cAMP with a Kd in the micromolar range (De Rooij *et al.*, 2000). The close homolog Epac2 contains an additional cNMP binding motif that does not seem to be required for its regulation by cAMP (Kawasaki *et al*., 1998a). Epac1 is expressed in all connective tissues, whereas Epac2 expression is more restricted to brain, liver and adrenal glands (Kawasaki *et al*., 1998b).

The function of Epac and Rap1 is a matter of ongoing debate. Some studies suggested a role for Rap1 in modulation of ERK activity. Ectopic expression of Rap1 antagonizes ERK activation through inhibition of the Ras effector Raf-1. In addition, it was found that Rap1 can induce ERK activation in certain cell types, such as PC12 neuronal cells, by binding and activation of B-Raf (Vossler *et al*., 1997; York *et al*., 1999). A novel cAMP analogue (8CPT-2Me-cAMP) was recently designed that is selective for Epac and does not activate PKA (Enserink *et al*., 2002). Although this analogue activates Rap1 in many cell types, no effect on ERK activity has ever been observed, suggesting that modulation of ERK activity by cAMP is independent of the Epac-Rap1 pathway. A role for Rap1 in cell adhesion was established by several groups. Cell adhesion to extracellular matrix proteins is mediated by integrins, which are heterodimeric transmembrane adhesion molecules. Many signalling pathways induce activation and clustering of integrins, a process called inside-out signalling. Increased Rap1 signalling, either trough ectopic Rap1 expression or activation of Epac with 8CPT-2Me-cAMP induces integrin signalling, while inhibition of Rap1 blocks integrin ligation (Bos *et al*., 2003). Epac2 is involved in insulin secretion. An oral glucose load elicits a quantity of insulin secretion that is larger compared to an intravenal load. This process, which is known as the incretin response, is controlled by the release of the glucagons-like peptide Glp-1 from the gut. Glp-1 binds to receptors on pancreatic β-cells, which results in activation of adenylyl cyclase. Elevated cAMP levels in these cells triggers exocytosis of insulin independently of PKA. Down-regulation of Epac2 mRNA with siRNA or expression of a dominant negative form blocks the incretin response, indicating the direct involvement of Epac2 in this process (Kashima *et al*., 2001; Ozaki *et al*., 2000). More functions of the cAMP-Epac-Rap1 pathway will likely be discovered in the future and some roles of cAMP that were previously ascribed to activation of PKA might be mediated by Epacs instead.

1.3.5.3 Protein kinase G

cGMP-dependent protein kinase (PKG) represents the main target of cGMP. PKGs are homologous to the PKA-complex, but here the nucleotide binding domains and catalytic domains are located in the same enzyme (Francis and Corbin, 1999). Two forms of PKG occur in mammalian tissue. PKG I is a soluble enzyme, whereas PKG II is membrane-bound. The two forms have complementary rather than overlapping patterns of expression and significantly different targets.

Activation of guanylyl cyclase in vascular smooth muscle cells by ANF and nitric oxide increases cGMP levels, which decreases muscle tone (Ignarro and Kadowitz, 1985). Mice deficient for PKG I have elevated blood pressure. In addition, the motor function of the gastrointestinal tract is severely affected (Pfeifer *et al.*, 1998). Vascular smooth muscle relaxation is mediated by cGMP and PKG I through the reduction of Ca^{2+}-concentrations and desensitization of the contractile apparatus to Ca^{2+}. This is achieved via a number of mechanisms: i. reduction of Ca^{2+}-influx through inhibition of L-type Ca^{2+}-channels , ii. activation of Ca^{2+}-efflux through the activation of exchangers and Ca^{2+}-ATPases in the plasma membrane, iii. sequestering of Ca^{2+} through activation of the sarcoplasmic reticulum Ca^{2+}-ATPase phospholamban and iv. decreasing Ca^{2+}-mobilization by inhibiting IP_3 receptors, which are involved in release of Ca^{2+} from the sarcoplasmatic reticulum (Lucas *et al.*, 2000). PKG I also inhibits platelet aggregation through phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) (Waldmann *et al.*, 1987; Aszódi *et al.*, 1999).

PKG II regulates fluid homeostasis at the cell membrane. Mice lacking PKG II are insensitive to heat-stable enterotoxin-induced diarrhea (Pfeifer *et al.*, 1996). This indicates the link between the production of cGMP by GC-C and PKG II function. The only established target for PKG II so far is the chloride channel CTFR (Vaandrager *et al.*, 1997). Phosphorylation of CTFR opens the channel, resulting in efflux of Cl^- and subsequent secretion of water in the intestine. In addition, PKG II is implicated in regulation of ion reabsorption and renin release in kidney and bone formation (Wagner *et al.*, 1998; Pfeifer *et al.*, 1996). The latter is supported by the phenotype of PKG knock-out mice which exhibit dwarfism.

1.3.5.4 Cyclic nucleotide gated channels

Cyclic nucleotide gated (CNG) channels are voltage-gated cation-channels located in the plasma membrane that contain cyclic nucleotide binding domains, similar to those in PKA and PKG. A classical example is CNG1 in retina which regulates influx of Na^+ and Ca^{2+}. Lowered cGMP levels upon transducin-mediated stimulation of PDE6 results in closure of CNG1, thereby inducing hyperpolarization. This also reduces Ca^{2+} levels, which results in retinal guanylyl cyclase activation by GCAPs and subsequently recovery of the system (Pugh Jr *et al.*, 1999). CNG4 or the olfactory channel is involved in olfactory sensing. This Ca^{2+}-channel is opened by odorant-induced elevation of cAMP through AC3. Elevated intracellular Ca^{2+} not only inhibits AC3 (Wei *et al.*, 1996; Wei *et al.*, 1998), but also activates PDE1 and desensitizes CNG4 through calmodulin (Bradley *et al.* 2001; Munger *et al.*, 2001). These negative feedback loops are likely responsible for the Ca^{2+}-and cAMP-oscillations during hormone stimulation of olfactory neurons (Jaworksy *et al.*, 1995; Wayman *et al.*, 1995a).

1.4. Cyclic nucleotide signalling in *Dictyostelium discoideum*

1.4.1 The *Dictyostelium* life cycle

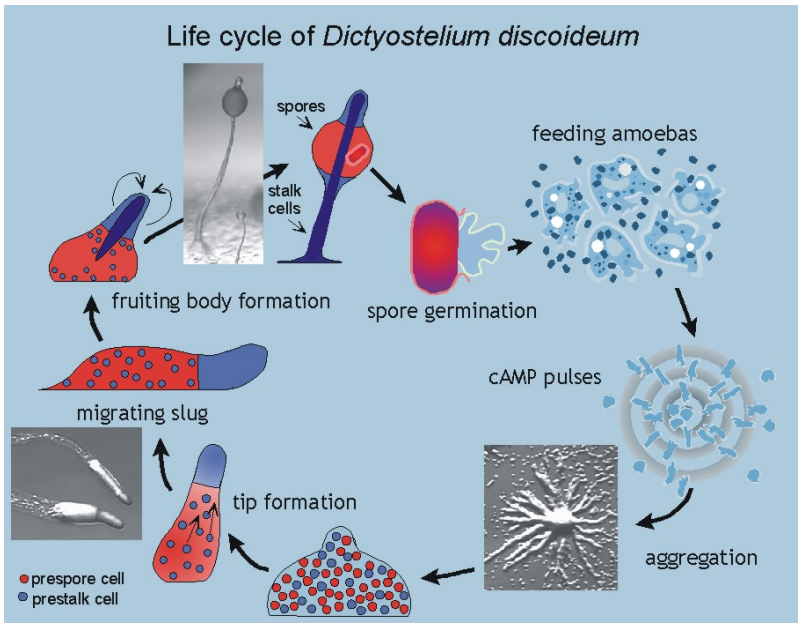

Fig. 1.7. Life cycle of *Dictyostelium discoideum.*

Cells of the social amoeba *Dictyostelium* live in the soil where they feed on bacteria. Upon starvation, a developmental program is initiated, ultimately resulting in the formation of a fruiting body in which cells can survive as spores (fig. 1.7). Some starving cells start to secrete pulses of cAMP. Surrounding cells respond by chemotaxis and relay of the signal, which results in the formation of an aggregate of up to 10^5 cells. The aggregate forms a tip, and subsequently elongates and falls over to become a migrating slug. This structure is capable of thermo- and phototaxis to find a suitable place for culmination. When this happens, a fruiting body is formed in which 80% of the cells have differentiated into spores, which are carried as a globular mass on top of a stalk of dead, highly vacuolized cells. Under favourable conditions, the spores germinate to yield vegetative cells.

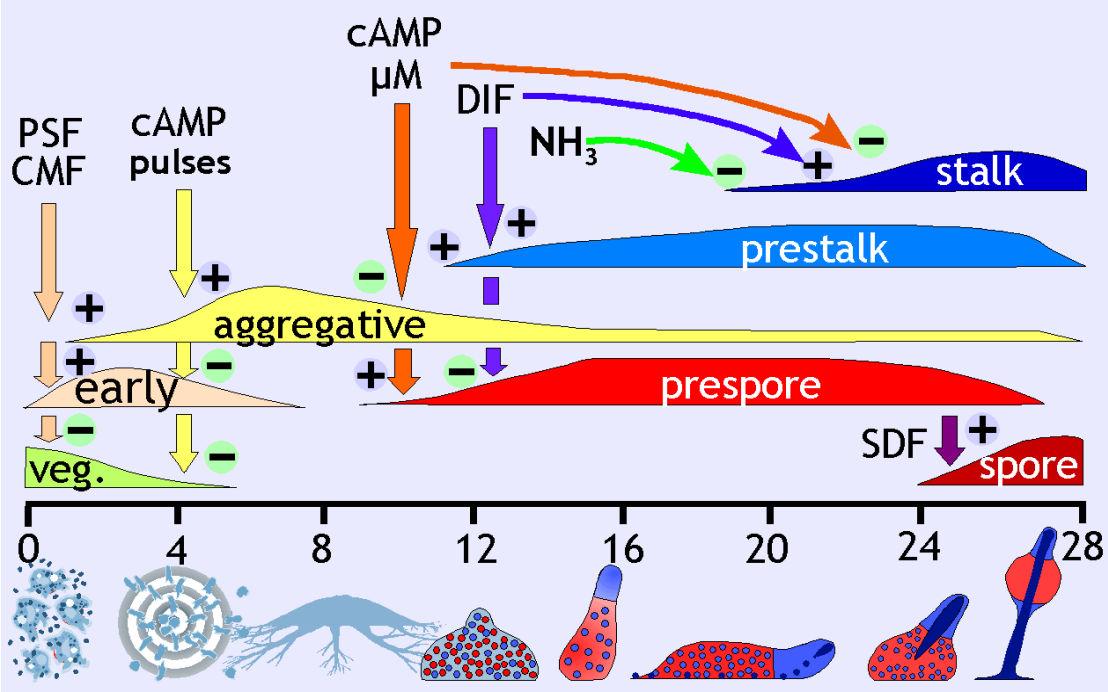

Fig. 1.8. Regulation of developmental gene expression by extracellular signals in *Dictyostelium*

1.4.2 Regulation of developmental gene expression

The developmental cycle is regulated by a number of extracellular signals (fig. 1.8). Early during starvation, cells secrete density-sensing factors, to determine whether the cell-density is high enough to initiate multicellular development. These signals trigger a low level of expression of early and aggregative genes that encode the machinery required for the process of aggregation, among which are cAMP receptors and the aggregation-specific adenylyl cyclase, ACA. Aggregative gene expression is strongly upregulated by the nanomolar pulses of cAMP during aggregation.

In the aggregate or mound, cells randomly differentiate into the precursors of the later spores and stalk cells. Prespore gene expression is induced by micromolar concentrations of cAMP that are assumed to be present in the mound. The prespore cells sort out to the posterior region of the slug. In addition, nanomolar concentrations of cAMP induce the expression of non-cell type-specific post-aggregative genes, such as *CP2*, which encodes a cysteine protease, and *RasD*. The differentiation inducing factor (DIF), a chlorinated hexaphenone, induces formation of prestalk cells (Kay and Jermyn, 1983; Morris *et al.*, 1987; Williams *et al.*, 1987). The majority of the prestalk

cells sort out to the tip. A small population of cells with prestalk cell properties, named anterior-like cells (ALCs) are found scattered throughout the prespore region. The ALCs replenish the cells that are lost from the tip during slug migration.

In the tip of the slug, high concentrations of ammonia prevent terminal stalk cell differentiation. Under favourable dry conditions, diffusion of ammonia relieves this inhibition and culmination occurs. During culmination, prestalk cells move into the newly formed stalk tube. They then differentiate into stalk cells, during which they vacuolize and become elongated. In the process, the prespore cells are lifted off the substratum and differentiate into spores. Sporulation is induced by spore differentiation factors (SDFs) that are secreted by the forming stalk cells. The ALCs and rearguard cells form the tissues that anchor the fruiting body to the substratum (basal disc) and support the spore mass (upper and lower cup).

1.4.2 cAMP as an extracellular first messenger

1.4.2.1 cAMP receptors

In *Dictyostelium discoideum*, four serpentine cAMP receptors (cARs) have been cloned, that differ in ligand affinity (Johnson *et al.*, 1992) and timing and cell-type specificity of expression (reviewed in Rogers *et al.*, 1997). The high affinity receptor cAR1 is predominantly expressed during aggregation (Klein *et al.*, 1988). Expression of the other high affinity receptor, cAR3, first appears in the aggregate and is during late development only observed in prespore cells (Johnson *et al.*, 1993; Yu and Saxe, 1996). cAR1 and cAR3 functionally overlap, since gene-disruptants for *car1* but not for both *car1* and *car3* can complete development after exposure to high concentrations of cAMP (Soede *et al*., 1994). *cAR3* disruptants have no apparent developmental phenotype (Johnson *et al.*, 1993). cAR2 and cAR4 are both low affinity receptors. *cAR2* expression is first detected in the tip of aggregates and later in the anterior part of the prestalk region. Expression is absent from ALCs and they are therefore only found in the stalk and upper cup of the fruiting body (Saxe III *et al.*, 1996). It was initially found that *car2* gene disruptants fail to form tips and are arrested at the mound stage (Saxe III *et al.*, 1993). However, this has been disputed by others, who found no phenotype for independently created knock-outs (C.J. Weijers, *unpublished results*). *cAR4* expression is first evident at the first finger stage and is maximal during culmination. Expression is present in all cell-types but prestalk-enriched. *car4* null-mutants are impaired in slug migration and fruiting body formation, with ~25% of the structure culminating into short fruiting bodies with enlarged spore heads. These defects are probably caused by the increase of prespore over prestalk cells and extension of prespore gene expression well into the prestalk zone (Louis *et al*., 1994).

When overexpressed in *car1*$^-$/*car3*$^-$, cAR1, cAR2 and cAR3 or chimeras are all capable of mediating the same responses, such as adenylyl cyclase activation, chemotaxis, actin polymerization and induction of cAMP mediated gene expression. The responses triggered by the different receptors have the same kinetics but depend on different concentrations of cAMP, according to their affinities (Kim *et al.*, 1998; Verkerke-Van Wijk *et al.*, 1998). However, adenosine, which in wild-type cells inhibits cAMP induced prespore gene expression, could only do so in cAR1 overexpressing cells, indicating that cAR1 normally mediates prespore differentiation. Furthermore, in cAR2 overexpressing cells, DIF-induced prestalk gene expression was 10-fold higher than in cAR1- or cAR3-overexpressors, suggesting a role for

cAR2 in induction of DIF competence (Verkerke-Van Wijk *et al.*, 1998). These results suggest that the different receptors are mainly tuned to detect different cAMP concentrations at specific stages, but in some instances are tuned to initiate a specific response.

1.4.2.2 Heterotrimeric G-proteins

At least 9 different G_α-proteins are expressed at different stages during *Dictyostelium* development (Brzostowksi *et al.*, 2002; Hadwiger *et al.*, 1991; Pupillo *et al.*, 1989; Wu and Devreotes, 1991), whereas only one G_β-subunit seems to be present that is expressed at equal levels throughout development (Lilly *et al.*, 1993). Recently a G_γ-subunit was identified from the *Dictyostelium* database that is expressed in parallel with G_β (Zhang *et al.*, 2001). G-protein signalling is essential during early development, because mutants that lack a functional G_β-subunit fail to aggregate (Lilly *et al.*, 1993; Wu *et al.*, 1995b). These cells show no chemoattractant-stimulated adenylyl and guanylyl cyclase activity or aggregative gene expression and are incapable of polymerizing actin for chemotaxis or phagocytosis. (Jin *et al.*, 1998; Wu *et al.*, 1995b). The $G_\alpha2$ subunit mediates most cAMP responses during aggregation (Kumagai *et al.*, 1989; Kumagai *et al.*, 1991), such as pulse-induced gene expression, activation of guanylyl cyclase (Okaichi *et al.*, 1992), phospholipase C (Okaichi *et al.*, 1992; Bominaar and Van Haastert, 1994) and adenylyl cyclase, the latter via coupled βγ-subunits rather than the $G_\alpha2$-subunit itself (Wu *et al.*, 1995b). *$g_\alpha1$* knock-outs are defective in adaptation of phospholipase C (Bominaar and Van Haastert, 1994), but develop normally. This is not surprising, since *plc* null mutants have no phenotype either (Drayer *et al*., 1994). Brazill *et al.* (1998) suggested that $G_\alpha1$ is coupled to CMF receptors and mediates CMF-stimulated PLC activation. $G_\alpha3$ is required for expression of genes encoding the cAMP signal machinery during early development (Brandon and Podgorski, 1997). *$G_\alpha4$* and *$G_\alpha5$* are both primarily expressed during post-aggregative development (Hadwiger *et al.*, 1991). $G_\alpha4$ mediates folic acid induced activation of guanylyl and adenylyl cyclase during early development (Hadwiger *et al.*, 1994). In multicellular stages, it is expressed in anteriorlike cells and probably involved in the generation of signals required for prespore gene expression and spore formation (Hadwiger and Firtel, 1991; Hadwiger *et al.*, 1994). *$G_\alpha5$* has a similar expression pattern as *$G_\alpha4$* and is required for proper timing of tip formation (Hadwiger *et al.*, 1996). *$G_\alpha5$ $g_\alpha7$* and *$g_\alpha8$* knock-outs have no apparent phenotype. $G_\alpha9$ has been implicated to regulate an inhibitory pathway during early development (Brzostowksi *et al.*, 2002). The role of the growth specific G_α-subunit $G_\alpha6$ has not been investigated (Wu and Devreotes, 1991; Wu *et al.*, 1994).

1.4.2.3 G-protein independent cAMP signalling

In mammalian cells, some signal transduction pathways are activated through serpentine receptors independently of G-protein activity. Similarly, in *Dictyostelium* some cAMP induced responses appear to be mediated by cARs in the absence of functional G-proteins (reviewed in Brzostowksi and Kimmel, 2001). During aggregation, cAMP-stimulated Ca^{2+} influx occurs in the absence of G-protein activity (Milne *et al.*, 1995). Activation of the mitogen-activated protein (MAP) kinase ERK2 by cAMP is normal in *g_β* or *$g_\alpha2$* null-mutants that overexpress cAR1 (Maeda *et al*., 1996) or cells that only express a temperature-sensitive G_β-subunit (tsG_β) (Schenk *et al.*, 1999). The ERK2 pathway is involved in late development as well. Conditional mutants in ERK2 lack prespore gene induction by cAMP, but other cell-type specific genes are expressed normally (Gaskins *et al.*, 1996). Many signalling events after

aggregation do not require G_β. In cells which only express tsG_β, prespore gene expression and repression of stalk gene expression by cAMP and DIF-induction of prestalk gene expression is normal at the restrictive temperature (Jin *et al.*, 1998). The transcription factor GBF is required for post-aggregative gene expression. GBF is activated by cAMP, independent of G-protein activity (Schnitzler *et al*., 1995). In addition, cAMP also strongly upregulates GBF expression via an autoregulatory loop. Similarly, tyrosine phosphorylation and nuclear localization of the transcription factor STATa by cAMP occurs in the absence of functional G-proteins (Araki *et al.*, 1998; Williams, 1999)

1.4.3 cAMP as an intracellular second messenger

The intracellular target for cAMP, the cAMP dependent protein kinase (PKA), plays a critical role in a number of transitions during *Dictyostelium* development (Loomis, 1998). In *Dictyostelium*, the holoenzyme consists of a heterodimer of the catalytic (PKA-C) and regulatory subunit (PKA-R), the latter containing two binding sites for cAMP. The *Dictyostelium* catalytic subunit harbours a long N-terminal domain (Anjard *et al.*, 1993) of which only the residues closest to the catalytic domain are important for activity (Dammann *et al.*, 1998). A possible regulatory role of the N-terminal domain remains unclear. Null mutants of the subunits have indicated the importance for development. Development in *pka-C*$^-$ is blocked before aggregation, whereas mutants that lack the R-subunit (*rdeC*) (Abe and Yanagisawa, 1983; Simon *et al.*, 1992) or overexpress *PKA-C* (Anjard *et al.*, 1992) have a rapid developing and sporogeneous phenotype.

Initiation of *Dictyostelium* development depends on PKA activity, as cells overexpressing dominant-negative regulatory subunits under the constitutive *actin15* promotor (*actin15-PKA-Rm*) fail to express early and aggregative genes (Schulkes and Schaap, 1995; Primpke *et al.*, 2000). A crucial factor in the transition from growth to development is the protein kinase YakA, which responds to prestarvation factors. YakA activity inhibits the translational regulator PufA. Active PufA inhibits translation of PKA-C, thus YakA activity allows PKA-C protein to accumulate (Souza *et al.*, 1999).

Overexpression of *ACA* in *pka-C*$^-$ from a constitutive promotor restores cAMP production but not aggregation (Mann *et al.*,1997), which means that PKA activity is not only required for ACA expression but also other aspects of aggregation. Pulse-induced expression of aggregative genes is not entirely lost in *actin15-PKA-Rm* (Schulkes and Schaap, 1995). This indicates that aggregative gene expression is not entirely dependent on PKA activity. How PKA is activated during aggregation is as yet unclear. ACA is not required for PKA activation, since *aca* null mutants have normal pulse-induced gene expression (Pitt *et al.*, 1993).

During multicellularity, PKA activity is involved in gene expression and terminal differentiation. cAMP initially induces expression of post-aggregative genes. PKA activity is not essential at this stage, although levels are reduced in *pka-c* null mutants (Mann *et al.*, 1997). However, later gene expression events are PKA dependent. Overexpression of *PKA-Rm* under the prespore promotor *pspA* inhibits expression of most prespore-specific genes and prevents spore formation (Hopper *et al.*, 1993b; Hopper and Williams, 1994; Hopper *et al.*, 1995). Likewise, expression of *PKA-Rm* under the prestalk-specific *ecmA* promotor blocks full expression of prestalk and stalk genes and prevents stalk formation (Harwood *et al.*, 1992; Hopper *et al.*, 1993a; Zhukovskaya *et al.*, 1996).

Once prespore cells have differentiated, PKA activity is sufficient for spore formation, as expression of *PKA-C* under a prespore promotor results in precocious, cell-autonomous sporulation (Simons *et al.*, 1992; Mann *et al.*, 1994) and addition of the cell-permeable PKA agonist 8Br-cAMP to prespore cells in a monolayer can trigger spore formation (Kay, 1989). Similarly, addition of 8Br-cAMP to prestalk cells results in stalk formation (Yamada and Okamoto, 1994), but prestalk cells overexpressing *PKA-C* also require release of the cAMP repression of stalk gene expression to differentiate into stalk cells (Hopper *et al.*, 1993a).

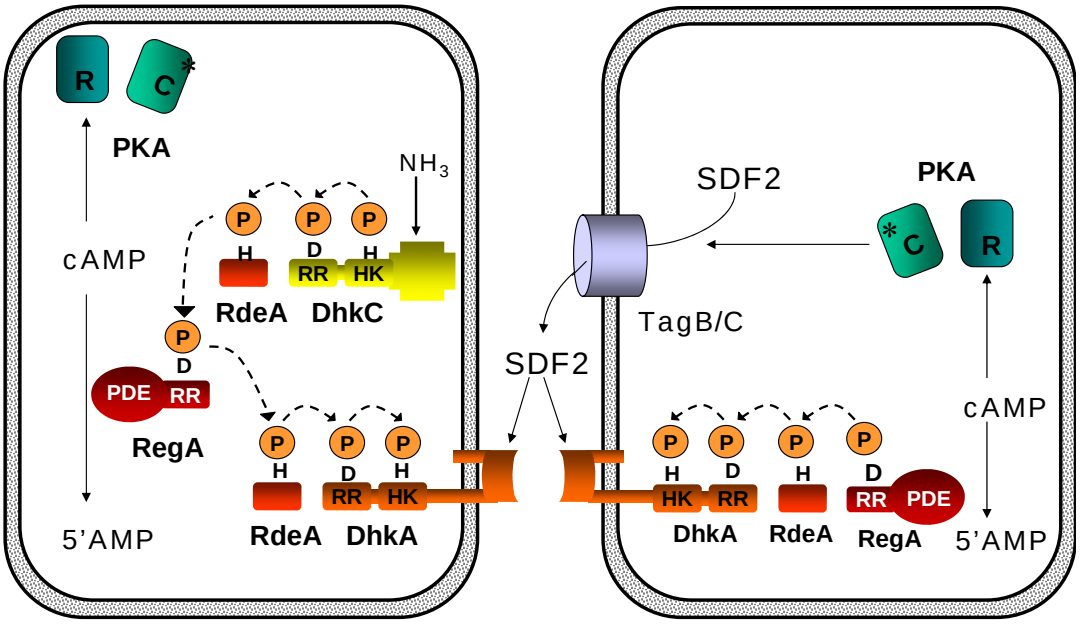

Fig. 1.9. Control of terminal differentiation by two-component and cAMP signalling cascades. See text for explanation. HK = histidine kinase, RR = response regulator.

Terminal differentiation is controlled by a unique rendezvous of the PKA pathway and two-component signal transduction cascades (fig. 1.9) (reviewed in Aubry and Firtel, 1999; Loomis, 1998; Thomason *et al.*, 1999a). These cascades are commonly found in prokaryotes, but also in plants and in yeast (Thomason and Kay, 2000). The system consists of a sensor histidine kinase and a response regulator. Detection of a signal by the sensor kinase results in auto- or transphosphorylation of a histidine residue. This phosphate is then transferred to an aspartate residue on a response regulator, which modulates the activity of a coupled output domain. More intermediate histidine and aspartate transfers are possible, thereby allowing a higher order of regulation. A well known example of such a phosphorelay cascade is the HOG MAP kinase pathway in yeast, which controls production of glycerol in response to the environmental high osmolarity. The MAP kinase pathway is under control of a multicomponent phosphorelay cascade that consists of i. the osmosensing histidine kinase Sln1p coupled to a response regulator, ii. an intermediate component, YPD1, which is phosphorylated on a histidine and iii. SSK1, a mediator of the

MAPKKKs SSK2 and SSK22, which contains a response regulator (Posas *et al.*, 1996).

In *Dictyostelium*, *rdeA* and *regA* mutants harbour lesions in phosphorelay proteins and display a rapid developing phenotype: they form short stalks and sporulate precociously, similar to *rdeC* (Abe and Yanagisawa, 1983). The *regA* null was identified in two independent studies; as a suppressor of the *tagB* null mutant, which fails to make spores (Shaulsky *et al.*, 1996), and in a screen for mutants that can make spores in a cell-autonomous fashion (Thomason *et al.*, 1998b). The gene encodes a cAMP-specific phosphodiesterase, coupled to a response regulator (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998). REGA controls PKA activity, and therefore terminal differentiation, through cAMP degradation. RDEA is an intermediate, similar to YPD1 in the HOG pathway, in the multistep phosphorelay to REGA (Chang *et al.*, 1998). Phosphotransfer between a histidine on RDEA and an aspartate on REGA is a reversible process *in vitro*. Phosphorylation of REGA results in a 20-fold increase in its PDE activity (Thomason *et al.* 1999b).

The hybrid histidine kinase DHKC was suggested to control REGA activity (Singleton *et al.*, 1998). *DhkC* null mutants have a rapid developing phenotype and culminate directly after the first finger stage. Slugs of cells overexpressing a dominant positive allele of *DhkC* that contains only the histidine kinase domain do not culminate but migrate until the source of energy is depleted. This 'slugger' phenotype depends on the presence of REGA and can be rescued by addition of 8Br-cAMP. Whereas ammonia can prolong migration of wild-type slugs, *dhkC*$^-$ cells are insensitive to ammonia. These results indicate that DHKC functions as an ammonia sensor and regulates the choice between slug migration and terminal differentiation through the modulation of REGA activity.

During culmination, prestalk cells descend into the prespore mass and induce spore gene expression from the top of the sorus downwards (Richardson *et al.*, 1994). Sporulation is controlled by two spore differentiation factors (SDF1 and SDF2) (Anjard *et al.*, 1997; Anjard *et al.*, 1998). These factors are secreted by prestalk cells, most likely by the *TAGB* and *TAGC* gene products, which form a putative ATP-transporter with protease activity (Shaulsky *et al.* 1995). *TAGB* is exclusively expressed in prestalk cells, but the null mutant has a sporulation defect, which can be overcome by overexpressing a constitutively active form of the histidine kinase DHKA (Wang *et al.*, 1996), suggesting that the factors produced by TAGB/C in prestalk cells are ligands for DHKA in prespore cells. SDF1 can be phosphorylated by PKA and its action requires protein synthesis in prespore cells, presumably to make the cells competent for encapsulation (Anjard *et al.*, 1997). One of these proteins is possibly PKA, as PKA expression in prespore cells increases strongly during culmination (Mann *et al.*, 1994). SDF2 induces rapid sporulation, independent of protein synthesis. Release of SDF2 from prestalk cells requires PKA activity and is strongly upregulated by a positive feedback loop (Anjard *et al.*, 1998). Cells expressing a DHKA protein with an inserted myc-epitope in its extracellular domain require a 100-fold higher concentration of SDF2 to encapsulate spores, indicating that SDF2 signalling occurs through activation of DHKA (Wang *et al.*, 1999). It has been proposed that DHKA negatively regulates REGA activity, thereby activating PKA and triggering sporulation (Loomis, 1998).

1.4.4 Adenylyl cyclases in *Dictyostelium discoideum*

1.4.4.1 ACA, the regulator of aggregation

Pitt *et al*. (1992) cloned two genes encoding adenylyl cyclases. ACG (germination-specific adenylyl cyclase) has a single catalytic domain and a single transmembrane domain. It is expressed in spores (Pitt *et al.*, 1992), where it controls spore germination (Van Es *et al.*, 1996) (see below). ACA (aggregation-specific adenylyl cyclase) has the topology of mammalian tmACs, with two catalytic domains, interspersed by two stretches of six transmembrane domains. *ACA* gene expression requires the presence of $G_{\alpha}3$ (Brandon and Podgorski, 1997), the transcription factor DdMyb2 (Otsuka and Van Haastert, 1998) and PKA (Schulkes and Schaap, 1995; Mann *et al.*, 1997) and is highest during aggregation (Pitt *et al.*, 1992).

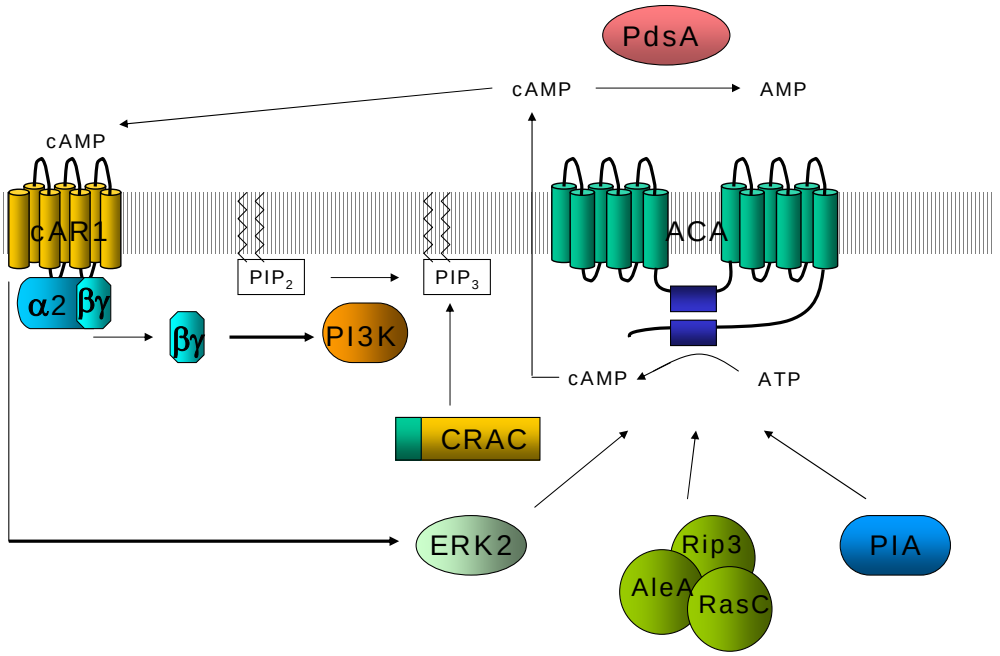

Fig. 1.10. Signalling molecules regulating ACA activity.

ACA is responsible for the cAMP-relay that controls aggregation. The regulation of ACA is complex, involving G-proteins, a MAP kinase pathway, a cytosolic regulator and proteins with unknown function (fig. 1.10) (reviewed in Parent and Devreotes, 1996; Aubry and Firtel, 1999). $G_{\beta\gamma}$-subunits released by G2 upon cAMP binding to cAR1 activate PI3-kinase, which results in the formation of the phophatidylinositol lipid PI(3,4,5)P_3 (PIP_3) (Jin *et al*., 2000; Zhang *et al*., 2001). PIP_3 recruits the cytosolic regulator of adenylyl cyclase (CRAC) to the plasma membrane by binding to its pleckstrin homology domain (Dormann *et al*., 2002). The translocation of CRAC is required for activation of ACA (Lilly and Devreotes, 1994; Insall *et al.*, 1994; Lilly an Devreotes, 1995). ACA activation also requires the presence of the Ras-GEF (guanine nucleotide exchange factor) aimless (AleA) (Insall *et al.*, 1996) and RasC (Lim *et al.*, 2001). A Ras-interacting protein, RIP3, was recently cloned (Lee *et al.*, 1999) which is also required for activation of ACA. If and to what extend RasC, AleA and RIP3 interact is not yet clear. In cells lacking the

MAP kinase ERK2, cAMP stimulation of ACA is abolished, but CRAC translocation is normal (Segall *et al.*, 1995). ERK2 activation is normal in *aleA* and *rasC* knock-outs, implying that these proteins function in different pathways. Finally, null mutants in *pia* (*Pianissimo*), a cytosolic protein with unknown function, are also defective in cAMP or GTPγS induced ACA activity (Chen *et al.*, 1997b).

The cAMP produced by ACA is rapidly secreted via a yet unknown mechanism, where it can activate cARs but is also rapidly degraded by a phosphodiesterase (PdsA) (Lacombe *et al.*, 1986). PdsA is either secreted or attached to the extracellular site of the plasmamembrane and is essential for aggregation (Sucgang *et al.*, 1997). The process of ACA activation/adaptation and secretion/degradation of cAMP results in the production of the cAMP pulses that are required for proper aggregation. Adaptation of cAMP production is still poorly understood. Persistent stimulation with cAMP results subsequentially in loss of affinity of the cAMP receptors, uncoupling of receptors from target proteins and finally internalization and degradation of receptors. (Van Haastert *et al.*, 1992). In *car1*$^{-}$, ACA stimulation can be taken over by cAR3, but requires higher concentrations of cAMP and adaptation of the response is absent, indicating that both excitation and adaptation are mediated by cAR1 (Pupillo *et al.*, 1992). Stimulation with cAMP results in reversible phosphorylation of cAR1. Receptors in which all the phosphorylation sites have been mutated show no sequestration upon cAMP stimulation (Caterina *et al.*, 1995a; Caterina *et al.*, 1995b), but have normal ACA, GC, F-actin and chemotactic responses, indicating that sequestration is not involved in adaptation of these responses (Parent and Devreotes, 1996; Kim *et al.*, 1997).

Adaptation is not mediated by modulation of stimulating G-proteins either. Janetopoulos *et al.* (2001) looked at fluorescence resonance energy transfer (FRET) between GFP-labelled $G_{\alpha}2$ and $G_{\beta\gamma}$ subunits. This study showed that the G2-protein remains dissociated during continuous exposure to cAMP, while cAMP relay subsides after a few minutes. Cells pretreated with pertussis-toxin have lost the adaptative response of ACA. Furthermore, a rapid second pulse of cAMP can trigger another cAMP relay response. These results suggest the presence of a pertussis-toxin sensitive inhibitory G-protein, with slower kinetics than the stimulatory pathway (Snaar-Jagalska and Van Haastert, 1990; Snaar-Jagalska *et al.*, 1991). Recently, an inhibitory G_{α}-subunit, $G_{\alpha}9$, was identified (Brzostowksi *et al.*, 2002). Deletion of *$G_{\alpha}9$* results in formation of more signalling centres and relative resistance to compounds that inhibit cAMP signalling. However, a direct role in ACA adaptation needs to be established. In addition, *$g_{\alpha}9^{-}$* cells still display pulsatile signalling, which means that other adaptation mechanisms are present.

After aggregation, *ACA* expression becomes restricted to the tip of the slug and a few cells scattered in the prespore region (Verkerke-Van Wijk *et al.*, 2001). During slug migration ACA regulates tip-specific gene expression of *CudA* (Verkerke-Van Wijk *et al.* 2001). *CudA* encodes a nuclear factor that is required for culmination and full expression of a number of prespore genes. It is expressed both in the prespore region of the slug and in the pstA population of prestalk cells in the tip of the slug. However, it is absent in the pstO cells, a population of prestalk cells posteriorly from the pstA zone (Fukuzawa *et al.*, 1997). Tip-specific expression of *cudA* is driven by nuclear StatA (Fukuzawa and Williams, 2000), which translocates in response to cAMP to the nucleus, in the slug specifically in pstA cells (Araki *et al.*, 1998). When *ACA* is ectopically expressed in all cells of the slug, both StatA nuclear translocation and CudA protein are present throughout the slug, indicating that extracellular cAMP produced by ACA controls gene expression events via StatA regulation.

1.4.4.1 ACG and regulation of spore dormancy

ACG is involved in the regulation of spore dormancy (fig. 1.11). Spore germination is one of the most critical steps in the *Dictyostelium* life cycle. Unconstrained germination could render emerging cells exposed to hostile conditions, such as low food supply, heat- and pH-stress or high osmolarity. However, such stress conditions are restrictive to spore germination. In the fruiting body, spores are kept dormant by the ambient osmolarity, caused by high concentrations of ammonium phosphate (Cotter, 1977; Cotter *et al.*, 1999) and the auto-inhibitor discadenine that is secreted by young spores (Cotter *et al.*, 1992). After activation of spore germination, either artificially (i.e. heat-shock or detergent) or by an auto-activator that is normally secreted and detected only by older spores, the spores enter a post-activation lag-phase. Here they can decide whether to proceed to germination or return to dormancy. If they proceed, the spores take up water and start synthesis of proteins in the swelling phase. Finally, the spores emerge and enter the vegetative stage.

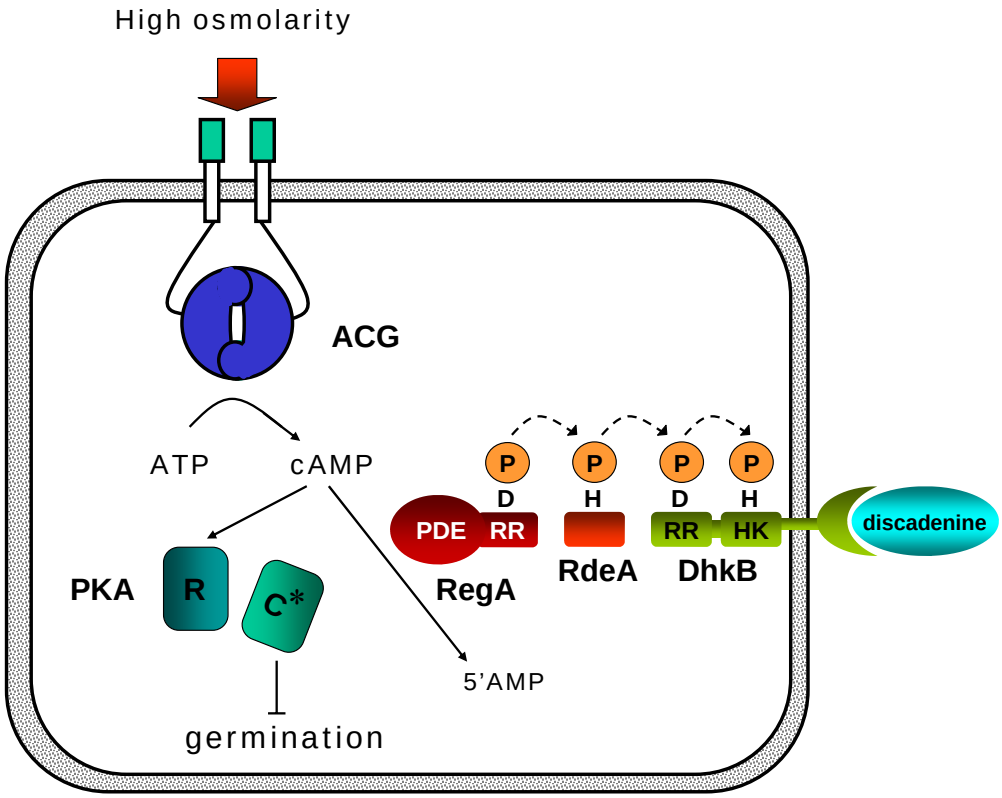

Fig. 1.11. Signalling pathways that control spore dormancy in *Dictyostelium*.

The control of spore germination in response to the osmotic pressure of the environment is controlled by intracellular cAMP levels (fig. 1.11) (Van Es *et al.* 1996; Virdy *et al.*, 1999). In spores, the cAMP levels are 10-fold higher than in vegetative cells. When high osmolarity is applied to heat-shock activated spores, the spores fail to germinate. This correlates with the maintenance of high levels of cAMP in the spore (Virdy *et al.*, 1999). Van Es *et al.* (1996) found that spores of a mutant lacking a functional *ACG* gene can germinate in the presence of high osmolarity with the same efficiency as wild-type spores. Furthermore, no heat-shock is required, although germination is still inhibited by discadenine (Van Es *et al.*, 1996). These results indicate that ACG controls spore germination in response to the environmental

osmolarity. Indeed, when overexpressed in *aca*$^-$ cells, constitutive ACG activity is 4-fold increased in the presence of 100 mM NaCl or 200 mM sucrose (200 milliosmolar) (Van Es *et al.*, 1996). Control of spore dormancy by cAMP is regulated through the activation of PKA. Germination is inhibited in the presence of the PKA-specific cAMP analogue 8-bromo-cAMP (Virdy *et al*. 1999). Also, spores of the strain *rdeC*, which lacks the R-subunit and of a strain overexpressing *PKA-C* under a spore-specific promotor germinate very poorly (Van Es *et al*., 1996; Virdy *et al*., 1999).

In addition to ACG, spore dormancy is also controlled by a two-component signal transduction cascade. Zinda and Singleton (1998) cloned a gene encoding a transmembrane sensor kinase DHKB, which contains both a histidine kinase and response regulator domain. Disruption of the *dhkB* gene yielded a mutant in which the spores germinated in the spore head in the presence of discadenine. The spores contained lower concentrations of cAMP and the phenotype could be reversed by overexpression of *PKA-C*, The authors suggested a model in which DHKB is the sensor for discadenine and controls cAMP levels and PKA activity in the spores, possibly via control of REGA activity. Indeed, spore germination is strongly delayed and inefficient in *regA*$^-$, indicating that phosphodiesterase activity is required to lower cAMP concentrations below a threshold level that allows spore germination (Meima and Schaap, *unpublished results*; Shaulsky *et al.*, 1998). It is however likely that DHKB controls more pathways and is somehow dominant, as spore germination in *acg*$^-$ is still inhibited by discadenine (Van Es *et al.*, 1996), but *dhkB* spores will germinate under high osmolarity. Finally, also DHKC is likely involved in modulation of REGA activity in spores, as *dhkC*$^-$ spores germinate poorly (Singleton *et al.* 1998).

1.4.4.3 Which adenylyl cyclase for which event?

The expression pattern and timing of the adenylyl cyclase and phenotypes of the knock-out mutants have left some functions of cAMP during *Dictyostelium* development yet unaccounted for. Initiation of development requires PKA, however neither *ACA* nor *ACG* are expressed during growth and the knock-outs are not blocked in the transition from growth to development (Pitt *et al.*, 1992; Pitt *et al.*, 1993). Development of *aca* null mutants can be rescued and yield viable spores by overexpression of *ACG*, mixing with wild-type cells or treatment with cAMP, indicating that the requirement for ACA is not cell-autonomous. This means that ACA is required for the production of extracellular cAMP but does not play a major role in activation of PKA during any stage of development (Pitt *et al.*, 1993). Some PKA-mediated responses, such as cAR1 down-regulation (Van Haastert, 1994) and ERK2 adaptation (Knetsch *et al.*, 1996) are lost in *aca*$^-$ cells, indicating some involvement in PKA activation. On the other hand, overexpression of the catalytic subunit in *aca*$^-$ cells results in near-normal development and it was suggested by the authors that extracellular cAMP regulated responses are controlled by the activation of PKA via ACA, whereas other signals regulate patterning and morphogenesis (Wang and Kuspa, 1997).

This interpretation raises many questions. Because *aca*$^-$ development can be rescued by extracellular cAMP and ACG appears to be only expressed in mature fruiting bodies, what is the source of the cAMP required for PKA mediated cell-type specific gene expression and terminal differentiation? There are also several arguments against a role for extracellular cAMP solely in activation of PKA. Overexpression of PKA under the prespore promotor *psA* is insufficient to induce full

expression of prespore genes, for which it requires extracellular cAMP (Hopper *et al.*, 1995). Furthermore, *ACA* expression is almost completely restricted to the tip of the slug (Verkerke-Van Wijk *et al.*, 2001), which raises the question where the cAMP, both extracellular and intracellular, that controls prespore gene expression is generated. Similarly, an explanation for the role of cAMP in stalk cell differentiation remains absent. Even if PKA is expressed under a prestalk promotor, precocious stalk formation is still repressed by the presence of cAMP (Hopper *et al.*, 1993a), indicating a PKA-independent pathway. Finally, there is also evidence that suggests that the production of enzymes involved in DIF production and degradation are controlled by extracellular cAMP, which could explain the patterning of prespore and prestalk cells (Kay and Thompson, 2001).

Several suggestions have been made, for example cAMP-independent activation of PKA via its unusual N-terminal domain (Anjard *et al.*, 1993) or differential expression of the R- and C-subunit, but evidence for this is lacking. Another possibility is residual or ectopic of expression of *ACG* in the mutants, which has been observed in cells transformed with vectors harbouring the Neomycine selection marker (Schulkes *et al.*, 1995). Finally, there could be another adenylyl cyclase that is involved in the cAMP regulated processes that are still unaccounted for.

1.4.5 The role of cGMP in chemotaxis and osmoprotection

Mounting evidence over the years has implicated a role for cGMP in chemotaxis of *Dictyostelium* cells (fig. 1.12) (reviewed in Van Haastert and Kuwayama, 1997). Stimulation with folic acid or cAMP results in a rapid and transient cGMP response; maximum levels are reached after 10 seconds and basal levels after 30 seconds (Mato *et al.* 1977). Guanylyl cyclase activation is G-protein dependent and can be mimicked by addition of GTPγS to lysates. cAMP induced activation is regulated by cAR1 or cAR3 (Insall *et al.*, 1994) and $G_{\alpha}2$ (Okaichi *et al.*, 1992; Carrel *et al.*, 1994), whereas folic stimulated guanylyl cyclase activation is mediated via an unknown folic acid receptor (αFAR) and $G_{\alpha}4$ (Hadwiger *et al.*, 1994). With Mg^{2+}-GTP as substrate, *in vitro* guanylyl cyclase activity is exclusively found in the particulate fraction, but requires the presence of the cytosolic fraction, suggesting the involvement of a cytosolic factor (Schulkes *et al.*, 1992). The activity is strongly inhibited by Ca^{2+} ions, with half-maximal inhibition around 50 nM. Inhibition by Ca^{2+} is supposed to be required for adaptation of the response (Valkema and Van Haastert, 1992). Based on a collection of chemical chemotaxis mutants with aberrant cGMP responses and binding activities, Kuwayama and Van Haastert suggested the presence of a cGMP binding protein, which in its free state is required for full activation of guanylyl cyclase, but in the cGMP-bound state becomes inhibitory (Kuwayama *et al.*, 1993; Kuwayama *et al.*, 1995; Kuwayama and Van Haastert, 1996).

Most of the evidence for the involvement of cGMP in chemotaxis comes from these mutants (KI-1-KI-10) and the mutant *streamer F* (*stmF*). The chemotaxis mutant KI-8, isolated by Kuwayama and Van Haastert (1993), produces no detectable amounts of cGMP. KI-8 cells fail to aggregate due to their inability to chemotax. *StmF* has a prolonged cGMP response, due to a defect in a cGMP-dependent cGMP-phosphodiesterase. These cells form large aggregation streams and large aggregates when developing as colonies on bacterial lawns (reviewed in Newell and Liu, 1992).

The time course of the cGMP response correlates with rounding up of cells and loss of direction (cringe response) when myosin II heavy and regulatory light chains (MHC and RLC) become phosphorylated. Phosphorylation of MHC destabilizes the myosin filaments, allowing the filaments to redistribute according to the chemotactic

signal, whereas RLC phosphorylation is required for motor function (Liu and Newell, 1991). Indeed, in *stmF* the cringe response is prolonged and myosin phosphorylation delayed, whereas in the KI-mutants myosin phosphorylation remains unaltered in response to cGMP (Liu *et al*., 1993; Liu and Newell, 1994). Myosin II is probably not the only target for cGMP, since the myosin II heavy chain mutant (*mhcA*) can still perform chemotaxis, although less efficiently and the cells move slower (De Lozanne and Spudich, 1987).

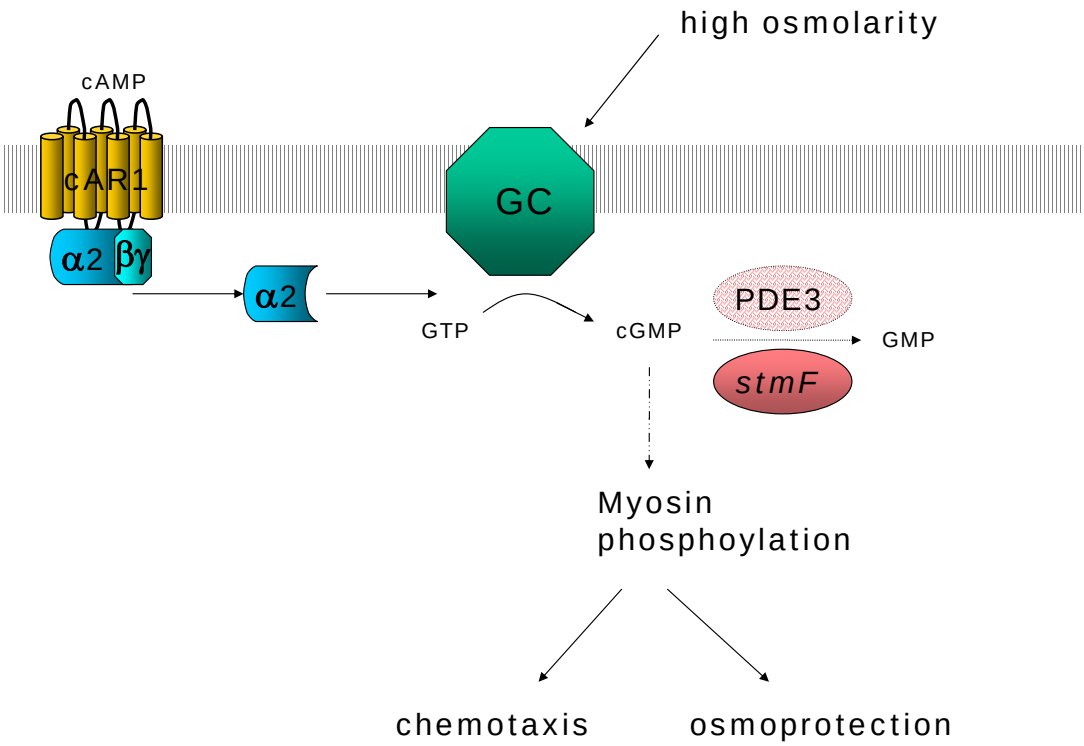

Fig. 1.12. cGMP signalling in *Dictyostelium*.

Besides chemotaxis, cGMP is also involved in resistance to osmotic shock in *Dictyostelium* (reviewed in Insall, 1996; Van Haastert and Kuwayama, 1997). Hypertonic stress induces an almost 50% reduction in cell volume (Oyama, 1996) and results in cell death after prolonged exposure with 50% of the cells dying after 1 hr (Kuwayama *et al.*, 1996). Osmotic stress results in cGMP accumulation which peaks after 10 min. and phosphorylation of myosin II heavy chain (Kuwayama *et al.*, 1996; Oyama, 1996). KI-8, *mhc* or cells expressing a myosin II heavy chain in which the phosphorylation sites are mutated are hypersensitive to osmotic shock with half of the cells dying after 5-10 min., but survival and myosin phosphorylation in KI-8 can be rescued by adding 8-bromo-cGMP (Kuwayama *et al.*, 1996; Kuwayama and Van Haastert, 1997). Upon osmostimulation, myosin II filaments form a cortex in a layer underneath an actin-rich cortex, which provides physical resistance (Kuwayama *et al.*, 1996). It is still unclear whether guanylyl cyclase has intrinsic osmosensing activity or is activated via a different osmsosensor. A *Dictyostelium* histidine kinase, DokA, is required for osmoresistance, but in *dokA*$^{-}$ the cGMP response toward osmotic shock is similar to wild-type (Schuster *et al.*, 1996).

Much of the work on cGMP signalling has long been hampered by the lack of sequence data. Recent progress however has resulted in the cloning of two genes

required for cGMP metabolism. *DdPDE3* encodes a cGMP-specific phosphodiesterase most homologous to the human cGMP-phosphodiesterase PDE9 and the cAMP-phosphodiesterase REGA (Kuwayama *et al.*, 2001). The gene is equally expressed throughout development and is probably different from the *stmF* gene product. Overexpressing *DdPDE3* has no effect on the phenotype and chemotaxis, although the cAMP-induced cGMP-response is somewhat lower. DdGCA is a guanylyl cyclase with the topology of mammalian tmACs and probably forms a group with other 12 transmembrane guanylyl cyclases that were recently cloned from *Plasmodium*, *Paramecium*, and *Tetrahymnia* (Carucci *et al.*, 2000; Linder *et al.*, 1999; Roelofs *et al.*, 2001a). As discussed before, in these cyclases the catalytic domains are transposed compared to tmACs. *DdGCA* expression is developmentally regulated, with highest levels during growth and post-aggregative development. Neither *DdGCA* overexpressors nor *ddgca*$^-$ cells show a phenotypical or chemotaxis defect. The null mutant has normal *in vitro* guanylyl cyclase activity and *in vivo* cGMP responses when stimulated with cAMP, folic acid or osmotic shock. This indicates that there should be at least one other guanylyl cyclase in *Dictyostelium*.

2. cAMP production

ACB, a novel adenylyl cyclase activity in *Dictyostelium* that is involved in terminal differentiation.

2a. A novel adenylyl cyclase detected in rapidly developing mutants of *Dictyostelium*.

Kim, H.J., Chang, W.T., Meima, M., Gross, J.D. and Schaap P.

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2b. Fingerprinting of adenylyl cyclase activities during *Dictyostelium* development indicates a dominant role for adenylyl cyclase B in terminal differentiation.

Meima, M.E. and Schaap, P.

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2a A novel adenylyl cyclase detected in rapidly developing mutants of *Dictyostelium*

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2a.1 Abstract

Disruption of either the *RDEA* or *REGA* genes leads to rapid development in *Dictyostelium*. The *RDEA* gene product displays homology to certain H2-type phosphotransferases, while *REGA* encodes a cAMP phosphodiesterase with an associated response regulator. It has been proposed that RDEA activates REGA in a multistep phosphorelay. To test this proposal, we examined cAMP accumulation in *rdeA* and *regA* null mutants and found that these mutants show a pronounced accumulation of cAMP at the vegetative stage that is not observed in wild-type cells. This accumulation was due to a novel adenylyl cyclase and not to the known *Dictyostelium* adenylyl cyclases, aggregation stage adenylyl cyclase (ACA) or germination stage adenylyl cyclase (ACG), since it occurred in an *acaA/rdeA* double mutant and, unlike ACG, was inhibited by high osmolarity. The novel adenylyl cyclase was not regulated by G-proteins and was relatively insensitive to stimulation by Mn^{2+} ions. Addition of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) permitted detection of the novel adenylyl cyclase activity in lysates of an *acaA*/*acgA* double mutant. The fact that disruption of the *RDEA* gene as well as inhibition of the REGA-phosphodiesteraseby IBMX permitted detection of the novel AC activity supports the hypothesis that RDEA activates REGA.

2a.2 Introduction

Mutants exhibiting an altered rate of development can provide useful insight into rate-limiting events in development (Ambros and Moss, 1994). There are three classes of rapidly developing mutants in *Dictyostelium*:*rdeA, rdeC*, and *regA. RdeC* mutants lack a functional regulatory subunit of cAMP-dependent protein kinase (PKA)[1], so that PKA activity is constitutive (Simon *et al*., 1992). PKA plays an important role in many aspects of metazoan development (Blair, 1995) as well as in learning and memory (Abel *et al*., 1997), and genetic manipulation has demonstrated that it is essential for gene expression throughout development in *Dictyostelium* (see Mann *et al*., 1997 and references therein). Thus the level of PKA activity appears to have a

[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; PKA-R, PKA regulatory subunit; ACA, aggregation stage adenylyl cyclase; ACG, germination stage adenylyl cyclase; IBMX, 3-isobutyl-1- methylxanthine; PDE, phosphodiesterase; 2'H-cAMP, 2'-deoxyadenosine 3',5'-monophosphate; GDPβS, guanosine 5'-*O*-(2-thiodiphosphate); GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); DTT, dithiothreitol; PB, phosphate buffer; ACB, adenylyl cyclase B.

profound influence on the rate of development in this organism. With regard to the two other classes of rapidly developing mutants, it has been reported that *rdeA* mutants have elevated intracellular cAMP levels during vegetative growth and possibly during development (Coukell *et al*., 1980; Abe and Yanagisawa, 1983). The *RDEA* gene product displays some homology to H2-type phosphotransferases in multicomponent signaling systems (Chang *et al*., 1998). The C terminus of REGA is a cAMP-phosphodiesterase (cAMP-PDE), while the N-terminal region is homologous to the well characterized response regulators of bacterial and eukaryotic two-component signaling systems (Shaulsy *et al*, 1996; Loomis *et al*., 1997; Thomason *et al*., 1998). It has therefore been proposed that RDEA activates the REGA-PDE in a multistep phosphorelay (Chang *et al*., 1998; Thomason *et al*., 1998).

We have examined cAMP accumulation in *rdeA* and *regA* mutants to explore certain implications of this proposal. During the course of this work we discovered a novel adenylyl cyclase. Until now genes coding for two structurally distinct forms of adenylyl cyclase have been identified in *Dictyostelium*. The so-called aggregative enzyme (ACA) displays negligible activity in growing (vegetative) cells and accumulates early in development with a maximum at the time of aggregation. Disruption of *ACA* results in a failure to aggregate (Pitt *et al*., 1992). ACA activity is stimulated by extracellular cAMP via a G-protein dependent pathway and rapidly adapts (Theibert and Devreotes, 1986). Transcripts of the other cyclase, ACG, are normally detected only during spore germination (Pitt *et al*., 1992). However, the enzyme can be readily assayed in vegetative cells when expressed under the control of the actin15 promoter. It is strongly stimulated by high osmolarity and functions as an osmosensor controlling spore germination (Van Es *et al*., 1996). We report the identification of a novel adenylyl cyclase and present evidence for the idea that RDEA activates REGA in a multistep phosphorelay.

2a.3 experimental procedures

2a.3.1 Materials and cell lines

2'-Deoxyadenosine 3',5'-monophosphate (2'H-cAMP), guanosine 5'-*O*-(2-thiodiphosphate) (GDPβS), guanosine5'-*O*-(3-thiotriphosphate) (GTPγS), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), and G418 were from Sigma, blasticidin was from ICN, and [^{3}H]cAMP was from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

The cell lines *acaA*, *acaA/ACG* (Pitt *et al*., 1992), DH1/*rdeA*, AX2/*rdeA*, *acaA/rdeA* (Chang *et al*., 1998), and AX2/*regA* (Thomason *et al*., 1998) were grown in standard axenic medium, which was supplemented with 20 μg/ml G418 for the *acaA/ACG* line. The *acaA/ACG* line harbors a gene fusion of the constitutive actin15 promoter and the entire coding sequence of the ACG gene, causing ACG to be expressed during the entire course of development (Pitt *et al*., 1992). The *acaA*/*acgA* line was obtained by transforming the *acaA* line with the *ACG* gene, in which an internal *Xba*I-*Eco*RV fragment was replaced by a blasticidin expression cassette (Sutoh, 1993). Nine blasticidin-resistant transformants were selected, and three mutants carrying an *acgA* gene disruption were identified by polymerase chain reaction and Southern blot analysis.

2a.3.2 Assays for cAMP accumulation by intact cells

Cells were harvested from growth medium, washed with 10 mM sodium/potassium phosphatebuffer, pH 6.5 (PB), and either resuspended directly in PB at 10^8cells/ml or plated on PB agar at 2.5×10^6 cells/cm^2, starved for 6 h at 22 °C, and subsequently collected and resuspended in PB. Cells were stimulated with 5 µM 2'H-cAMP and/or 5 mM DTT (final concentrations), and after variable time periods, the reaction was terminated by adding an equal volume of 3.5% (v/v) perchloric acid. Lysates were neutralized with $KHCO_3$, and cAMP levels were determined by isotope dilution assay, using purified PKA regulatory subunit (PKA-R) from beef heart as cAMP-binding protein (Van Haastert, 1984).

2a.3.3 *In vitro* adenylyl cyclase assays

Cells were harvested, washed once with PB, resuspended in ice-cold lysis buffer (2 mM $MgCl_2$ and 250 mM sucrose in 10 mM Tris, pH 8.0), and lysed through nuclepore filters (pore size, 3 µM). Aliquots of 10 µl cell lysate were added to 5 µl of variables and incubated at 0 °C for 5 min. The reaction was started by adding 5 µl of assay mix (2 mM ATP, 40 mM DTT in 2 × concentrated lysis buffer) and transferring the samples to a 22 °C water bath. The reaction was terminated by adding 10 µl of 0.2 M EDTA, pH 8.0, and by boiling the samples for 1 min. cAMP levels were assayed by isotope dilution assay. In a number of experiments an aliquot of sample was preincubated for 30 min with beef heart cAMP-PDE and then boiled for 1 min; this removed all activity competing with [^{3}H]cAMP for binding to PKA-R in the cAMP assay.

2a.4 Results

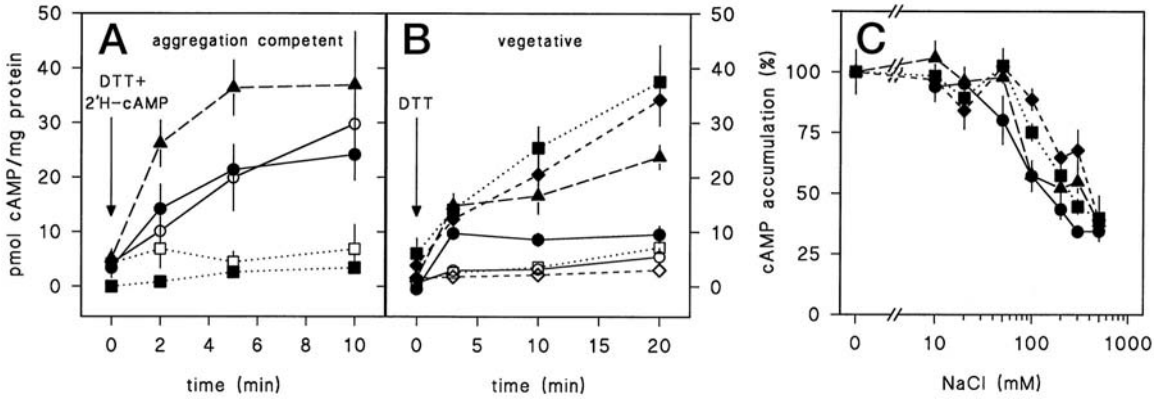

Fig 2a.1. cAMP accumulation in rdeA and regA mutants. *A*), cAMP relay response in aggregation competent cells. Parent strains (AX2, acaA) and rdeA and regA mutants were starved for 6 h at 22 °C, resuspended in PB, and stimulated with 5 µM 2'H-cAMP and 5 mM DTT. At the indicated time periods, the reaction was terminated with perchloric acid, and total cAMP levels were measured in the neutralized lysates. Data are standardized on the total protein content of the cell suspension. *B)*, cAMP accumulation by vegetative cells. Parent strains (AX2, DH1, *acaA*) and *rdeA* and *regA* mutants were harvested from growth medium, resuspended in PB, exposed to 5 mM DTT, and assayed for total accumulated cAMP levels at the indicated time periods. *C)*, effects of high osmolarity on cAMP accumulation. Washed vegetative *rdeA* and *regA* mutant cells were incubated with 5 mM DTT in the presence of the indicated concentrations of NaCl. After 20 min of incubation, total cAMP levels were determined. Data are expressed as percentage of cAMP levels accumulated in the absence of NaCl. All data represent the means and S.E. of two experiments performed in triplicate. Symbol key: ○, AX2; ●, AX2/*rdeA*; ▲, AX2/*regA*; ◊, DH1; ♦, DH1/*rdeA*; □, *acaA*; ■, *acaA*/*rdeA*.

2a.4.1 *In vivo* cAMP accumulation in *rdeA* and *regA* mutants

To investigate cAMP accumulation in *rdeA* and *regA* null mutants, we first performed a standard assay for the ACA-mediated cAMP relay response (Van Haastert, 1984). Aggregation-competent cells were stimulated with the cAMP receptor agonist 2'H-cAMP in the presence of DTT, an inhibitor of *extracellular* PDE (Henderson, 1975). Fig. 2a.1A shows that the presence of the *rdeA* or *regA* lesions had no pronounced effect on 2'H-cAMP-induced cAMP accumulation. As expected, there was little or no cAMP synthesis in *acaA* cells (Pitt *et al*., 1992) or in an *acaA/rdeA* double mutant. However, when cAMP accumulation was examined in washed vegetative cells, the *rdeA* and *regA* mutants both accumulated substantial amounts of cAMP, unlike wild-type (AX2, DH1) or *acaA* cells (Fig. 2a.1B). Remarkably, this accumulation was also observed in an *acaA/rdeA* double mutant, indicating that it was not dependent on ACA.

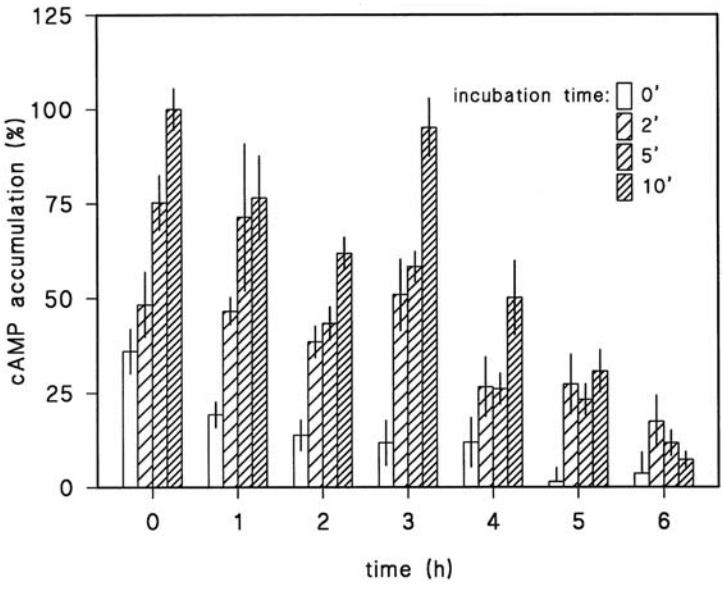

Fig. 2a.2. *In vivo* adenylyl cyclase activity during starvation of *acaA/rdeA* cells. *acaA/rdeA* cells were either used directly from growth medium (t = 0 h), or cells were first plated on PB agar and incubated for 1, 2, 3, 4, 5, or 6 h at 22 °C. Subsequently cells were resuspended in PB to 10^8 cells/ml, exposed to 5 mM DTT for 0, 2, 5, or 10 min, and assayed for total cAMP levels. Data were standardized on the protein level of the cell suspensions and are expressed as percentage of cAMP accumulated after 10 min in cells at $t = 0$ h. Means and S.E. of three experiments performed in triplicate are presented.

In vivo ACG activity is up to 4-fold stimulated by high osmolarity with an optimum at 200 mosM, followed by inhibition at higher solute concentrations. ACA activity displays only inhibition by high osmolarity (Schaap *et al*., 1995). As shown in Fig. 2a.1C, the activity in the rapidly developing mutants did not resemble ACG as it showed only inhibition by high osmolarity. Fig. 2a.2 shows that the activity was greatest in vegetative *acaA/rdeA* cells and declined progressively over the first 6 h of starvation. This decline was also evident when cAMP production by vegetative *acaA/rdeA* cells (Fig. 2a.1B) was compared with that of 6-h starved cells (Fig. 2a.1A).

2a.4.2 Detection and characterization of the novel AC activity in cell lysates

ACA activity can be measured in cell lysates provided that cAMP or GTPγS are present during lysis to activate an associated G-protein (Theibert and Devreotes, 1986). ACG activity is not dependent on G-proteins (Pitt *et al*., 1992). Fig. 2a.3 shows that an activity could be measured in lysates of vegetative *acaA/rdeA* cells, which presumably corresponded to the activity observed in intact cells. We will further refer to this activity as ACB. It differed from ACA in being neither stimulated by GTPγS nor inhibited by GDPβS (Fig. 2a.3A). ACB activity showed a fairly broad pH opti-

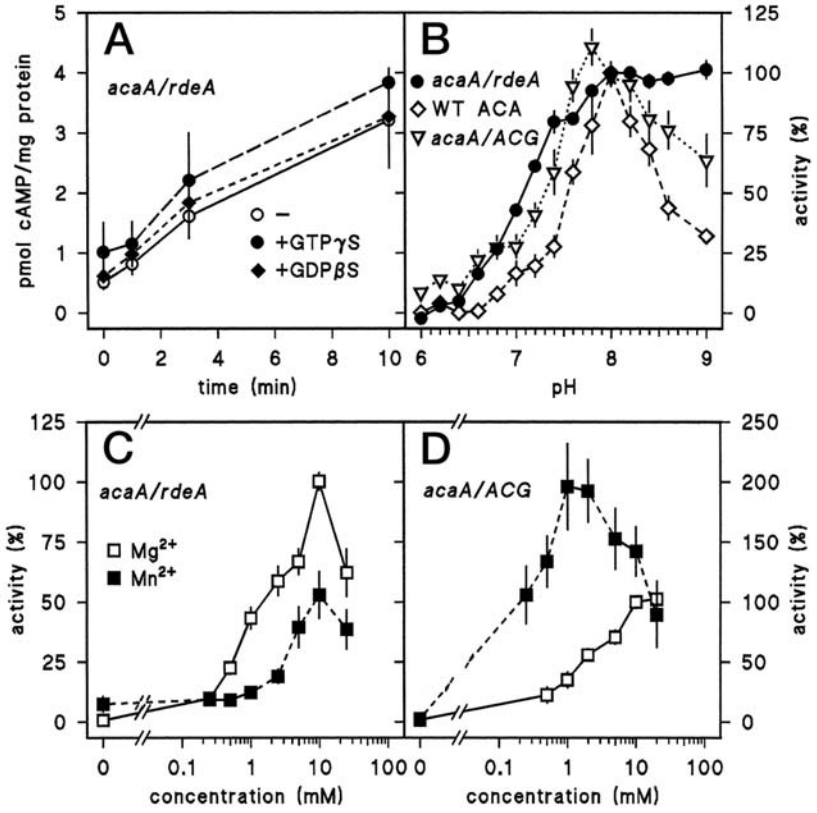

Fig. 2a.3. Detection and characterization of ACB activity in cell lysates. *A)*, possible G-protein dependence of ACB activity. Vegetative *acaA/rdeA* cells were lysed in the presence and absence of 30 μM GTPγS or 30 μM GDPβS. Lysates were incubated at 22 °C with 0.5 mM ATP and 10 mM DTT for the incubated time periods and assayed for cAMP. *B)*, pH dependence of ACB activity. *acaA/rdeA* cells were lysed in lysis buffer containing 0.5 mM instead of 10 mM Tris·HCl, and lysates were incubated with 0.5 mM ATP and 10 mM DTT in 250 mM potassium phosphate buffer of the indicated pH values. After 10 min of incubation, reactions were terminated and total cAMP levels determined. The data for *acaA/ACG*, and for ACA in starved wild-type cells stimulated with GTPγS, are retrieved from Schaap *et al.* (1995). Data are expressed as percentage of activity at pH 8.0. *C* and *D)*, Mg^{2+} and Mn^{2+} dependence of ACB and ACG activity. *acaA/rdeA* and *acaA/ACG* cells were lysed in lysis buffer without $MgCl_2$. Lysates were incubated with ATP and DTT with the indicated concentrations of Mg^{2+} or Mn^{2+} ions and assayed for cAMP. Data are expressed as percentage of activity obtained at 10 mM Mg^{2+}. Data in all panels represent means and S.E. of two experiments performed in triplicate

mum, while ACA and ACG showed distinct peaks of activity at pH 8.0 and 7.8, respectively (Fig. 2a.3B). In addition, ACB was clearly distinguishable from ACA and ACG in its response to divalent cations. The catalytic activity of most adenylyl cyclases is stimulated by the binding of divalent cations to a presumed allosteric site (Somkuti *et al*., 1982), and the relative efficacy of $Mg^{2+}$and Mn^{2+} differs widely between different enzymes. The activity in lysates of vegetative *acaA/rdeA* cells, due to ACB, is much more efficiently stimulated by Mg^{2+} than by Mn^{2+} (Fig.2a.3C) and in this respect is reminiscent of the recently described soluble adenylyl cyclase of Sf9 insect ovary cells (Kawabe *et al*., 1996) as well as of the adenylyl cyclase of *Escherichia coli* (Yang and Epstein, 1983). In contrast, the activity of ACG (Fig. 2a.3D) and of ACA (Pitt *et al*, 1992) is more responsive to Mn^{2+}, like standard mammalian forms of AC (Pieroni *et al*., 1995) and certain other adenylyl cyclases (Braun and Dods, 1975; Da Silveira *et al*., 1977; Heideman *et al*., 1987).

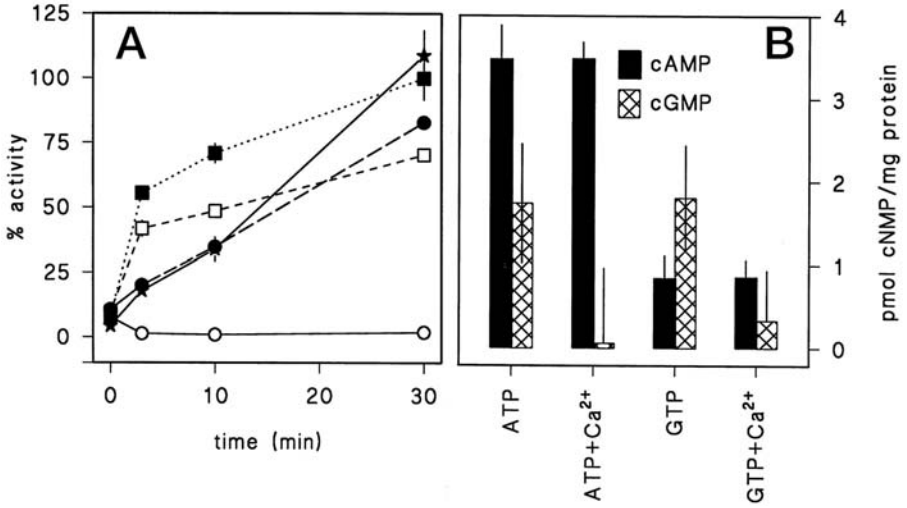

Fig. 2a.4. Further identification of ACB as a novel enzyme activity. *A)*, effect of a cAMP-PDE inhibitor on AC activity. *acaA, acaA/rdeA*, and *acaA*/*acgA* cells were lysed through nuclepore filters, incubated with ATP and DTT in the presence and absence of 0.2 mM of the cAMP-PDE inhibitor IBMX for the indicated time periods, and assayed for cAMP. Data are expressed as percentage of cAMP accumulation at $t = 30$ min by *acaA/rdeA* mutants in the presence of IBMX (4.2 ± 1.7 pmol·mg protein^{-1}). Symbol key: ○, *acaA*; ●, *acaA* + IBMX; □, *acaA*/*rdeA*; ■, *acaA*/*rdeA* + IBMX; ★, *acaA*/*acgA* + IBMX. *B)*, comparison of ACB and guanylyl cyclase activity. *acaA*/*acgA* lysates were incubated with 0.2 mM IBMX, 10 mM DTT, and either 0.5 mM ATP or 0.5 mM GTP in the presence and absence of 10 μM $CaCl_2$. After 30 min cAMP and cGMP levels were measured by isotope dilution assay and radioimmunoassay (Steiner *et al*., 1972)), respectively. The cAMP assay is about 1000 × less sensitive for cGMP than cAMP, and the reverse is true for the cGMP assay. *A* and *B* represent means and S.E. of two experiments performed in triplicate

2a.4.3 Relationship of ACB to the *rdeA* and *regA* lesions

If the novel activity can be detected in *rdeA* and *regA* cells because they lack a cAMP-PDE activity, it should be possible to detect the same activity in the lysates of *acaA* cells by inhibiting the cAMP-PDE by pharmacological means. To do this we employed IBMX, a well known inhibitor of mammalian cAMP-PDEs (Barber and Butcher, 1980), which has also been shown to inhibit the REGA-PDE (Thomason *et al*., 1998). Fig.2a.4A shows that no cAMP synthesis could be detected in *acaA* lysates in the absence of IBMX. However, in its presence *acaA* lysates accumulated as much cAMP as *acaA/rdeA* lysates. There appeared to be a slight stimulation of activity when the *acaA/rdeA* lysate was supplemented with IBMX, which could be due to the presence of an additional IBMX-sensitive cAMP-PDE or to residual REGA-PDE activity in *rdeA* cells. The use of IBMX also allowed us to detect ACB activity in other cell lines that do not carry the *rdeA* or *regA* lesions. In particular, to provide conclusive evidence that ACB is a novel adenylyl cyclase, we made a double knockout mutant in the *ACA* and *ACG* genes (*acaA*/*acgA*). Fig. 2a.4A shows that lysates of this cell line also accumulated considerable amounts of cAMP in the presence of IBMX. *Dictyostelium* cells also display a guanylyl cyclase activity, which is stimulated by GTPγS and strongly inhibited by Ca^{2+} ions (Janssens *et al*., 1989). To examine whether guanylyl cyclase could be responsible for cAMP production, we measured guanylyl cyclase in *acaA*/*acgA* lysates and the effect of Ca^{2+} ions on cAMP and cGMP production. Fig. 2a.4B shows that cGMP production is 2-fold lower than cAMP production in the presence of added GTP or ATP, respectively. Some production of cGMP or cAMP occurred in the presence of added ATP or GTP, respectively, which is possibly due to interconversion of the triphosphates. cAMP

accumulation is not affected by Ca^{2+} ions, while cGMP accumulation is completely inhibited. This and the low level of guanylyl cyclase activity in the *acaA*/*acgA* lysates make it very unlikely that cAMP synthesis is catalyzed by a guanylyl cyclase.

2a.5 Discussion

We have detected a novel adenylyl cyclase activity (ACB) in *rdeA* and *regA* cells that is distinct from ACA and ACG. Unlike ACA and guanylyl cyclase, ACB is not regulated by G-proteins, and unlike ACG it is not stimulated by high osmolarity. ACB is more effectively stimulated by Mg^{2+} than by Mn^{2+} ions, which is exactly the reverse of ACG, ACA, and guanylyl cyclase (Pitt *et al*., 1992; Janssens *et al*., 1989). The most convincing evidence that ACB is a novel enzyme is the observation that ACB activity can be detected in lysates of the double mutant *acaA*/*acgA* in the presence of IBMX. As noted in the Introduction, it has recently been proposed that the *rdeA* gene encodes a phosphotransferase that relays a phosphoryl group from an unknown histidine kinase to the response regulator of REGA and in so doing activates the REGA-PDE (Chang *et al*., 1998; Thomason *et al*., 1998). Since according to that view both *rdeA* and *regA* mutants would be expected to lack this cAMP-PDE activity, our finding that ACB can be detected in intact *regA* and *rdeA* cells, but not in wild-type cells, is evidence in its favor. This inference is further supported by the finding that the cAMP-PDE inhibitor IBMX, that is known to inhibit the REGA cAMP-PDE, permits detection of a similar activity in lysates of the *acaA* and *acaA*/*acgA* cell lines. However a direct demonstration of phosphoryl group transfer from the RDEA to the REGA protein will be necessary before the proposed relay scheme can be accepted with confidence.

The discovery of ACB may help to explain how PKA is activated in *Dictyostelium*. It has been shown that a number of early and late genes whose expression is dependent upon PKA can nonetheless be expressed (under appropiate conditions) in *acaA* cells (Mann *et al*., 1997; Schulkes and Schaap, 1995; Wu *et al*., 1995a; Endl *et al*., 1996; Pitt *et al*., 1993). Since the PKA regulatory subunit has a very high affinity for cAMP (De Gunzburg *et al*., 1984), a low level of cAMP production by ACB could account for this even if much of the cAMP produced was degraded by the active REGA-PDE. This argument would appear to apply at least to PKA-dependent gene expression early in development, when we can be confident that ACB is present (Fig. 2a.2). It may also apply later in development, since preliminary data suggest that ACB is present in slugs of wild-type cells (M. Meima and P. Schaap, unpublished results). Moreover, as mentioned, a quite low level of adenylyl cyclase activity may be sufficient to activate PKA.

Acknowledgments

We are grateful to Peter Devreotes for gifts of the *acaA* and *acaA/ACG* cell lines and for *ACG* DNA, as well as to Peter Thomason for the AX2/*regA* cell line. We also thank Raymond Brandt for excellent technical assistance.

2b Fingerprinting of adenylyl cyclase activities during *dictyostelium* development indicates a dominant role for adenylyl cyclase b in terminal differentiation

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2b.1 Abstract

Activation of cAMP-dependent protein kinase (PKA) triggers terminal differentiation in *Dictyostelium,* without an obvious requirement for the G-protein-coupled adenylyl cyclase, ACA, or the osmosensory adenylyl cyclase, ACG. A third adenylyl cyclase, ACB, was recently detected in rapidly developing mutants. The specific characteristics of ACA, ACG, and ACB were used to determine their respective activities during development of wild-type cells. ACA was highly active during aggregation, with negligible activity in the slug stage. ACG activity was not present at significant levels until mature spores had formed. ACB activity increased strongly after slugs had formed with optimal activity at early fruiting body formation. The same high activity was observed in slugs of ACG null mutants and ACA null mutants that overexpress PKA (*acaA/PKA*), indicating that it was not due to either ACA or ACG. The detection of high adenylyl cyclase activity in *acaA/PKA* null mutants contradicts earlier conclusions (B. Wang and A. Kuspa, *Science* 277, 251–254, 1997) that these mutants can develop into fruiting bodies in the complete absence of cAMP. In contrast to slugs of null mutants for the intracellular cAMP-phosphodiesterase REGA, where both intact cells and lysates show ACB activity, wild-type slugs only show activity in lysates. This indicates that cAMP accumulation by ACB in living cells is controlled by REGA. Both REGA inhibition and PKA overexpression cause precocious terminal differentiation. The developmental regulation of ACB and its relationship to REGA suggest that ACB activates PKA and induces terminal differentiation.

Key Words: adenylyl cyclase B; protein kinase A; rapidly developing mutants; terminal differentiation; *Dictyostelium discoideum.*

2b.2 Introduction

cAMP is one of the major intermediates of hormone action in metazoans and plays additional crucial roles in long-term memory storage (Frey *et al.,* 1993) and cell fate determination during embryogenesis (Jiang and Struhl, 1995). In pathological protozoans such as *Trypanosoma brucei* and *Plasmodium falciparum* cAMP triggers differentiation of the infectious epimastigotes (Fraidenraich *et al.,* 1993) and the gametes (Read and Mikkelsen, 1991), respectively. In almost all cases intracellular cAMP production is due to stimulation of adenylyl cyclases by extracellular signals, while the effects of cAMP are mediated by cAMP dependent protein kinases (PKAs). During the life cycle of the social protozoan *Dictyostelium,* both intracellular and

extracellular cAMP control cell differentiation and morphogenesis. Starving *Dictyostelium* amoebae secrete cAMP pulses that act as a chemoattractant to induce aggregation and transformation of aggregates into migrating slugs and fruiting bodies. After aggregation cells differentiate into prespore and prestalk cells, the precursors of the terminally differentiated stalk cells and spores of the mature fruiting body. Extracellular cAMP acting on surface cAMP receptors (cARs) also functions as a hormone-like signal to induce the expression of aggregative genes and prespore genes. Intracellular cAMP acting on PKA controls the transition from growth to early development and triggers the terminal differentiation of stalk and spore cells (Firtel, 1995).

Two adenylyl cyclases have been cloned in *Dictyostelium:* the G-protein-coupled enzyme ACA is responsible for producing the cAMP pulses that mediate aggregation. The G-protein-independent adenylyl cyclase ACG is structurally homologous to the peptide-activated guanylyl cyclases in vertebrates and the peptide-activated adenylyl cyclase of *Trypanosoma cruzi* (Pitt *et al.,* 1992; Ross *et al.,* 1991; Garbers *et al.,* 1994). ACG transcripts have only been found in spores and the enzyme acts as an osmosensor that controls spore germination (Van Es *et al.,* 1996). Remarkably, despite the fact that spore and stalk cell formation require PKA, both processes can occur in null mutants for either ACA or ACG in the presence of cAR specific cAMP analogs (Pitt *et al.,* 1992, 1993). Recently a third adenylyl cyclase activity (ACB) was detected in growing cells of *rdeA* and *regA* null mutants (Kim *et al.,* 1998). The *REGA* gene encodes a cAMP phosphodiesterase that is activated by phosphorylation of an attached response regulator, homologous to response regulators of bacterial phosphorelay systems (Shaulsky *et al.,* 1996; Thomason *et al.*, 1998). The *RDEA* gene encodes an H2-type phosphotransferase that acts as a phosphorelay intermediate to activate REGA (Chang *et al.,* 1998). Characterization of the novel adenylyl cyclase activity in *acaA/rdeA* double null mutants showed that it was not activated by high osmolarity like ACG, not activated by G-proteins like ACA, and not stimulated by Mn^{2+} ions as common to both ACG and ACA.

To establish to what extent ACA, ACB, and ACG may control cAMP-regulated processes during *Dictyostelium* development we have used the unique biochemical characteristics of each enzyme to measure their activity and activation during the entire course of development. Our results confirm that ACA and ACG are almost exclusively active in the aggregation and spore stages, respectively. ACB activity increases very strongly after slugs have formed to reach an optimum at early culmination. These data suggest that ACB is probably the long-sought-for activity that activates PKA and triggers terminal differentiation.

2b.3 Materials and methods

2b3.1 Materials

Adenosine 3',5'-monophosphate (cAMP), 2'-deoxyadenosine 3',5'-monophosphate (2'H-cAMP), guanosine 5'-*O*-(2-thiodiphosphate) (GDPβS), guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), G418, and bovine heart cAMP phosphodiesterase were obtained from Sigma (St. Louis, MO); blasticidin was from ICN (Costa Mesa, CA); nuclepore filters (pore size 3 μm) were from Corning (New York, NY); and [2,8-^{3}H]adenosine 3',5'monophosphate ([^{3}H]cAMP) was obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). Bradford reagent was from Bio-Rad (Hercules, CA). The

reverse-phase Jordi- Gel C18-DVB HPLC column was from Alltech Associates, Inc. (Deerfield, IL).

2b.3.2 Cell culture and induction of development

Wild-type NC4 cells were grown in association with *Klebsiella aerogenes* on glucose–peptone agar. The axenic strains AX3, AX4, AX4/*regA* (Shaulsky *et al.*, 1996), *acaA*/*PKA* (Wang and Kuspa, 1997), and *acgA* (Pitt *et al.*, 1992) were grown in axenic medium, which was supplemented with 20 μg/ml G418 for *acaA*/*PKA* cells. To induce multicellular development, cells were washed with 10 mM phosphate buffer, pH 6.5 (PB) to remove nutrients, distributed on nonnutrient agar (1.5% agar in PB) at 3×10^6 cells/cm^2, and incubated at 22°C until the desired developmental stage had been reached.

2b3.3 Assay for cAMP accumulation by intact cells

Cells or developing structures were harvested from nonnutrient agar plates and when necessary dissociated into single cells by multiple passages through a 19-gauge needle. Cells were resuspended in PB at 10^8 cells/ml and aerated for 10 min prior to assay. cAMP accumulation was initiated by addition of DTT to a final concentration of 5 mM in the presence of variables as indicated in the figure legends and terminated by adding an equal volume of 3.5% perchloric acid (v/v). Lysates were neutralized with $KHCO_3$ and cAMP levels were determined by isotope dilution assay, using purified beef heart PKA regulatory subunit as cAMP binding protein (Gilman, 1972; Van Haastert, 1984).

2b3.4 Adenylyl Cyclase assays in cell lysates

Cells were resuspended in ice-cold lysis buffer (250 mM sucrose in 10 mM Tris–HCl, pH 8.0) (LB) at 5×10^7 cells/ml and lysed through nuclepore filters (pore size 3 μm) in the absence and presence of 30 μM GDPβS or 30 μM GTPγS. Aliquots of 10 μl lysate were added at 0°C to 10 μl of variables in 2× assay mix [1 mM ATP, 20 mM DTT, 0.4 mM IBMX, and 4 mM of either $MgCl_2$ (Mg^{2+} assay mix) or $MnCl_2$ (Mn^{2+} assay mix) in LB]. Reactions were initiated by transferring the samples to a 23°C water bath and were terminated by addition of 10 μl 0.4 M EDTA and by boiling the samples for 1 min. cAMP levels were determined by isotope dilution assay. All assays were standardized on the protein content of the cell suspension or cell lysate as determined with Bradford reagent.

2b3.5 HPLC of slug lysates

Slug lysate was incubated for 30 min at 23°C with an equal volume of Mg^{2+} assay mix. The reaction was terminated by addition of EDTA and boiling, and a 200-μl aliquot of the boiled mixture was supplemented with 3000 cpm [^{3}H]cAMP and injected on a reverse-phase Jordi-Gel HPLC column. The mobile phase was 12% methanol in 1 mM phosphate buffer, pH 6.5, and the column was eluted at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected; 100 μl of each fraction was used to measure [^{3}H]cAMP by liquid scintillation counting; and the remaining 400 μl was lyophilized and resuspended in 45 μl 2 mM $MgSO_4$ in 10 mM imidazole, pH 7.5. One 20-μl aliquot of each sample was incubated for 30 min at 30°C with 10^{-4} units of beef-heart cAMP phosphodiesterase and another with an enzyme preparation inactivated by boiling. Both samples were then boiled for 1 min and cAMP levels were determined by isotope dilution assay.

2b.3.4 Results

The three *Dictyostelium* adenylyl cyclases ACA, ACG, and ACB show distinct differences in regulatory properties, which are summarized in Table 2b.1.

Table 2b.1.
Regulatory Properties of the Adenylyl Cyclases ACA, ACG, ACB

	Intact cells		Cell lysates			
Enzyme	cAMP or 2'H-cAMP	High osmolarity (100 mM NaCl)	GTPγS	GDPβS	Mg^{2+}	Mn^{2+}
ACA	+ + +	-	+ + +	-	+	+ + +
ACG	0	+ + +	0	0	+	+ + +
ACB	0	-	0	0	+ + +	+

Note, 0, no effect; +, stimulatory; + + +, strongly stimulatory; - inhibitory

In intact cells ACA is stimulated by cAMP receptor ligands, such as 2'H-cAMP, which is not the case for ACG and ACB (Theibert and Devreotes, 1986; Pitt *et al.*, 1992; Kim *et al.*, 1998). ACG is stimulated in intact cells by high osmolarity, while both ACA and ACB are inhibited by this variable (Van Es *et al.*, 1996). In cell lysates, the G-protein-regulated enzyme ACA can be activated by GTPγS and inhibited by GDPβS, while both ACG and ACB are not affected by these guanine nucleotides. In lysates, both ACA and ACG are much more strongly stimulated by Mn^{2+} than by Mg^{2+} ions, while ACB is more strongly stimulated by Mg^{2+} ions (Pitt *et al.*, 1992; Kim *et al.*, 1998). We have used the different regulatory properties of ACA, ACB, and ACG to identify their activities during development.

To measure the three adenylyl cyclases in intact cells and cell lysates during the entire course of development, wildtype NC4 cells were starved on nonnutrient agar for 21 h. Every 3 h, a batch of cells was harvested and cAMP production by intact cells and cell lysates was measured during a period of 30 min under the following conditions: (i) intact cells were incubated with DTT to inhibit extracellular phosphodiesterase (PDE) and with 5 μM 2'H-cAMP to activate ACA, 100 mM NaCl (high osmolarity) to activate ACG, or nothing (control); (ii) cell lysates were incubated with ATP in the presence of DTT and IBMX to inhibit PDE and the intracellular cAMP phosphodiesterase REGA, respectively. The lysates were exposed to 30 μM GTPγS in the presence of 2 mM Mg^{2+} to detect ACA activity or to GDPβS (to inhibit ACA) in the presence of 2 mM Mg^{2+} or 2 mM Mn^{2+} to detect ACB or ACG activity, respectively. For reasons of brevity we only show the assay kinetics of cAMP accumulation by intact cells (Figs. 2b.1B and D) and cell lysates (Figs. 2b.1F and H) at the more relevant stages of development (T = 6, 9, or 21 h). For the other stages we have plotted cAMP levels at the time points (t) in the assay, when the response reaches its optimum. This was 2 min for ACA activation by 2'H-cAMP in intact cells (Fig. 2b.1A), 5 min for ACA activation by GTPγS in lysates (Fig. 2b.1E), and 30 min for cAMP accumulation by ACB and ACG in intact cells (Fig. 2b.1C) or lysates (Fig. 2b.1G) which occurred linearly over time.

Figures 2b.1A, C, E, and G show that at the onset of starvation (T = 0 h) intact cells and cell lysates showed little cAMP accumulation. cAMP synthesis induced by 2'H-cAMP could be detected after 3 h of starvation and reached an optimum at 9 h, when cells had collected into loose aggregates. In responsive cells, 2'H-cAMP caused a rapid cAMP increase which peaked at 2 min and then slowly declined (Fig. 2b.1B).

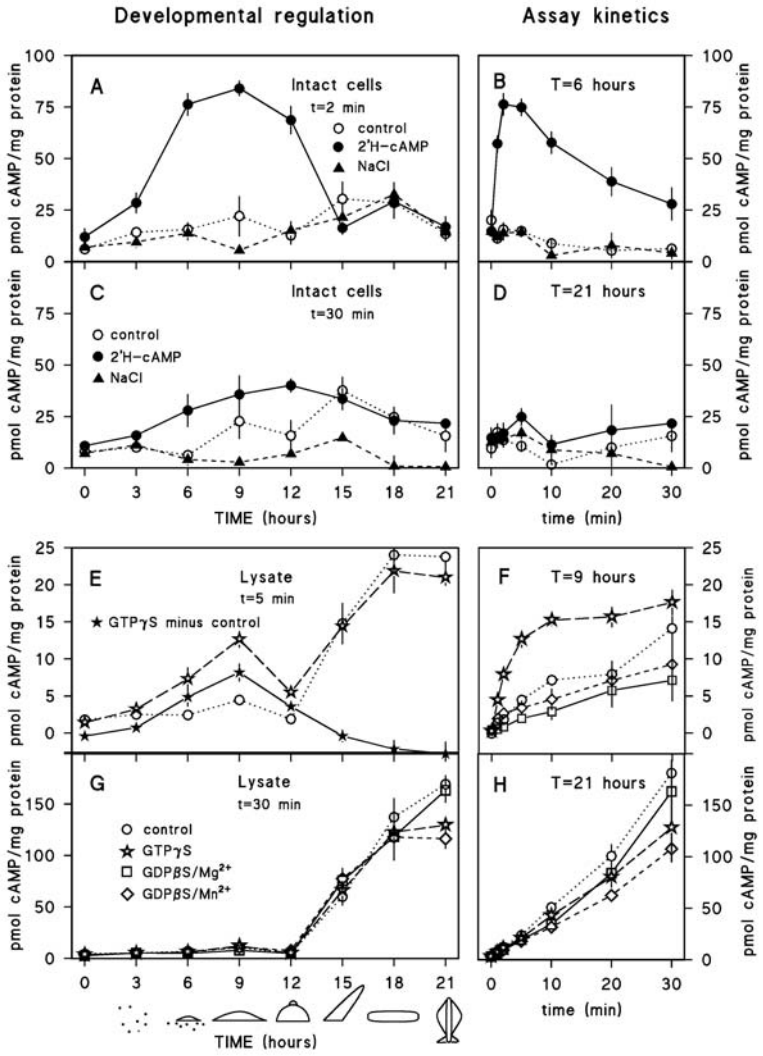

Fig. 2b.1. ACA, ACG, and ACB activity during *Dictyostelium* development. Wild-type NC4 cells were incubated on nonnutrient agar plates at 22°C. Every 3h a batch of cells was harvested and, when necessary, multicellular structures were dissociated. (A–D) cAMP accumulation by intact cells. Cells were resuspended in PB at 10^8 cells/ml and aliquots were incubated with 5 mM DTT (control), 5 μM 2'H-cAMP and 5 mM DTT (2'H-cAMP), or 100 mM NaCl and 5mM DTT (NaCl) for 0, 1, 2, 5, 10, 20, and 30 min. The reaction was terminated by addition of an equal volume of 3.5% (v/v) perchloric acid and total accumulated cAMP levels were measured by isotope dilution assay. Data in B and D represent the entire incubation period of cells harvested after 6 and 21 h of development, respectively. A and C represent the developmental time courses of cAMP levels that accumulated after respectively 2 and 30 min of incubation with DTT and variables in the assay. (E–H) cAMP accumulation by cell lysates. Cells were resuspended in lysisbuffer at 5×10^7 cells/ml and divided in three equal portions. Portion 1 was lysed at 0°C without further additions (control) and incubated with Mg^{2+} assay mix (0.5 mM ATP, 10 mM DTT, 0.2 mM IBMX, and 2 mM $MgCl_2$, final concentrations). Portion 2 was lysed in the presence of 30 μM GTPγS and was also incubated with Mg^{2+} assay mix (GTPγS). Portion 3 was lysed in the presence of 30 μM GDPβS and one part was incubated with Mg^{2+} assay mix (GDPβS/Mg^{2+}) and the other with Mn^{2+} assay mix (GDPβS/Mn^{2+}), in which 2 mM $MgCl_2$ was replaced by 2 mM $MnCl_2$. Reactions were started by transfer to a 23°C water bath and terminated at 0, 1, 2, 5, 10, 20, and 30 min by addition of EDTA to a final concentration of 0.13 M. Total accumulated cAMP levels were measured by isotope dilution assay. Data in F and H represent the entire incubation period of lysates made from cells that developed for 9 or 21 h of development, respectively. E and G represent the developmental time courses of cAMP levels that accumulated after respectively 5 and 30 min of exposure to the different variables. To enhance clarity the GDPβS-treated lysates are not shown in E (the profile for these variables is the same as at the 30-min time points in G). All data are standardized on the protein content of the cell suspension or lysate. Means and SE of three independent time courses with assays performed in triplicate are presented.

At the tipped aggregate stage (12 h) responsiveness to 2'H-cAMP was still considerable, but thereafter it decreased abruptly. Addition of 100 mM NaCl to intact cells had either no effect or caused a small decrease of cAMP accumulation during the entire course of development, suggesting either that ACG did not contribute significantly to cAMP accumulation by intact cells or that the inhibitory effects of high osmolarity on ACA and ACB affected cAMP levels more strongly than stimulatory effects on ACG.

The developmental regulation of cAMP accumulation induced by 2'H-cAMP in intact cells (Fig. 2b.1A) was almost completely mirrored by the developmental regulation of GTPγS-induced cAMP synthesis in cell lysates (Fig. 2b.1E). Also here some activation could be observed after 3 h of starvation, while maximum activation occurred at 9 h and then decreased. Similar to the 2'H-cAMP-induced accumulation in intact cells, stimulation by GTPγS in lysates was transient and arrested after 5 min (Fig. 2b.1F). Previous work showed that this is due to adaptation (Dinauer *et al.,* 1980; Pupillo *et al.,* 1992). Both basal and GTPγS-stimulated cAMP accumulations are inhibited by GDPβS, as is common for G-protein-regulated responses. However, even in the presence of GDPβS some residual activity could be detected.

After 12 h, cAMP production by cell lysates increased very strongly. Both the kinetics and regulation of the response were completely different from the response observed at 6 to 12 h. cAMP now increased linearly over time, resulting in accumulated cAMP levels of about 170 pmol/mg protein after 30 min of incubation. The activity was neither stimulated by GTPγS nor inhibited by GDPβS, indicating that it was not due to ACA. The activity is about 1.5-fold higher in the presence of Mg^{2+} than in the presence of Mn^{2+} ions, indicating that it was most likely not ACG, which is about 4-fold more active with Mn^{2+} than with Mg^{2+} ions.

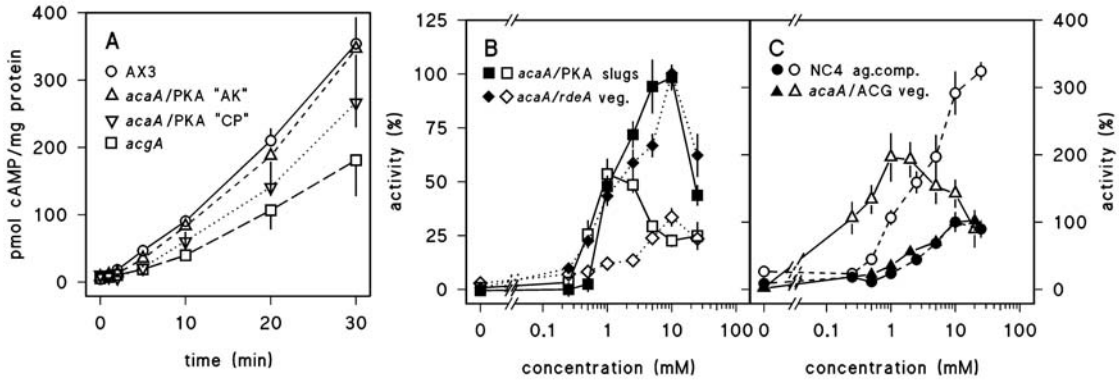

Fig. 2b.2. ACB activity in lysates of *acaA*/PKA and *acgA* slugs. (A) cAMP accumulation by AX3, *acgA,* and *acaA*/*PKA* slug lysates. Cells of parent strain AX3, mutant *acgA,* and two different lines of mutant *acaA*/*PKA* ("AK" and "CP") were incubated for about 20 h on nonnutrient agar until migrating slugs had formed. Slugs were dissociated into single cells, resuspended in 30 μM GDPβS in LB, and lysed through nuclepore filters. The slug lysates were incubated for 0, 1, 2, 5, 10, 20, and 30 min with Mg^{2+} assay mix and assayed for cAMP. Means and SE of two to three experiments performed in triplicate are presented. (B, C) Comparison of Mg^{2+}/Mn^{2+} dependence of AC activities. Cells from dissociated *acaA*/*PKA* slugs, vegetative *acaA*/*rdeA* cells, and aggregation-competent wild-type NC4 cells were resuspended in 30 μM GDPβS (*acaA*/*PKA, acaA*/*rdeA*) or GTPγS (NC4) in lysis buffer respectively and lysed. Cell lysates were incubated for 30 min (*acaA*/*PKA, acaA*/*rdeA*) or 5 min (NC4) with assay mix containing Mg^{2+} (solid symbols) or Mn^{2+} ions (open symbols) at the indicated concentrations and assayed for cAMP. The data for ACG in *acaA*/*ACG* cells are derived from Kim *et al.* (1998). Means and SE of two experiments performed in triplicate are presented.

Remarkably, the high activity that could be detected in cell lysates was not observed during incubation of intact cells (compare Figs. 2b.1D and H), although there seemed to be a small increase in unstimulated cAMP levels after 12 h of development (Fig. 2b.1C). This means that the activity may be latent in intact cells and requires an activator or removal of an inhibitor. Alternatively it is possible that cAMP produced in intact cells either is not secreted at all or is degraded before it can be secreted (and protected by phosphodiesterase inhibitors).

2b3.4.1 cAMP accumulation in slugs of *acaA* and *acgA* mutants

To confirm that the high levels of cAMP production by slug lysates were not due to ACA or ACG, we measured the activity in null mutants for these enzymes. *acgA* null mutants are defective in inhibition of spore germination by high osmolarity but show normal development up to the fruiting body stage (Pitt *et al.,* 1992; Van Es *et al.,* 1996). *acaA* null mutants cannot aggregate and form slugs, but these defects can be overcome by overexpression of PKA under the actin15 promoter and plating the cells at high cell density (Pitt *et al.,* 1992; Wang and Kuspa, 1997). Intact *acaA*/*PKA* cells have been reported to show no 2'H-cAMP stimulated cAMP accumulation and lysates to show no Mn^{2+}-stimulated adenylyl cyclase activity. A second *acaA*/*PKA* mutant was constructed (*C. A.* Parent, unpublished results). This mutant can also aggregate at high cell density, but then arrests at the mound stage, where the majority of the cells develop into spores. Mound arrest and precocious spore formation are also observed when wild-type cells overexpress PKA (Anjard *et al.,* 1992) and the difference between the Kuspa and Parent mutants is probably due to PKA gene dosage. To test whether any of the *acaA*/*PKA* or the *acgA* mutants show AC activity in the slug stage, we incubated cells on nonnutrient agar until migrating slugs had formed or in the case of the Parent *acaA*/*PKA* strain up to the time when wild-type cells had formed slugs. We then measured cAMP accumulation by cell lysates in the presence of GDPβS and 2 mM Mg^{2+}. Figure 2b.2A shows that lysates of slugs of axenic strain AX3, the *acgA* mutant, and both *acaA*/*PKA* mutants showed similar levels of cAMP accumulation as wild-type NC4 slugs (Fig. 2b.1H). To test whether the activity in the *acaA*/PKA mutants was the same as ACB and dissimilar from ACG and ACA, we compared the effects of Mg^{2+} and Mn^{2+} on cAMP accumulation in lysates of *acaA*/*PKA* slugs and growing cells of *acaA*/*rdeA* with those of ACA and ACG. Fig. 2b.2C shows that both ACA, measured in NC4 cells, and ACG, measured in vegetative *acaA* cells that overexpress ACG under the actin15 promoter (*acaA*/*ACG*), were much more strongly stimulated by Mn^{2+} (open symbols) than by Mg^{2+} ions (solid symbols). Especially low concentrations of Mn^{2+} (0.2 mM) stimulated ACG very strongly compared to Mg^{2+}. In both growing cells of *acaA*/*rdeA* and slugs of *acaA*/*PKA,* Mg^{2+} was more active to stimulate cAMP synthesis than Mn^{2+}. However, the *acaA*/*PKA* slugs showed a small peak activity at 1 mM Mn^{2+}, which was not observed in *acaA*/*rdeA* vegetative cells. We assume that this represents a small amount of ACG activity, which is very strongly stimulated by Mn^{2+} at this concentration. Our detection of high adenylyl cyclase activity in *acaA*/*PKA* slug lysates does not agree with earlier findings by Wang and Kuspa, showing the complete absence of adenylyl cyclase activity in lysates during the entire course of evelopment (Wang and Kuspa, 1997).

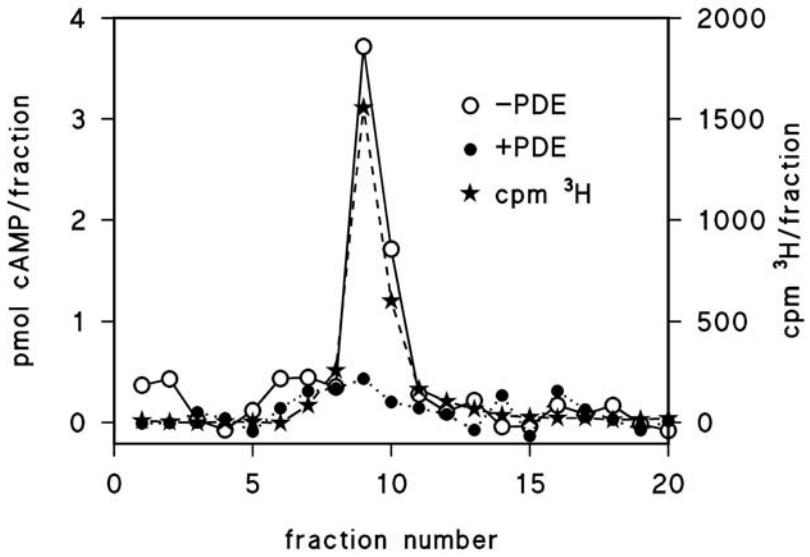

Fig. 2b.3. HPLC of cAMP in *acaA*/*PKA* slug lysates. *acaA*/*PKA* "AK" slug lysate (0.5 ml) was incubated for 30 min at 23°C with an equal volume of Mg^{2+} assay mix. The reaction was terminated by addition of 0.5 ml 0.4 M EDTA. cAMP levels were determined directly in 20-µl aliquots by isotope dilution assay. A 200-µl aliquot that contained 6.2 pmol cAMP and 0.12 mg protein was supplemented with 3000 cpm [^{3}H]cAMP (0.062 pmol) and injected on an HPLC reverse-phase column. Fractions (0.5 ml) were collected of which 100 µl was used to measure ^{3}H and the remaining part was divided in two parts. One part was treated with active cAMP phosphodiesterase (+PDE) and the other with boiled enzyme (-PDE) and both parts were then assayed for cAMP by isotope dilution assay. The amount of cAMP recovered in fractions 9, 10,11 was in total 5.7 pmol, which was 92% of the input. Comparable results were obtained with an AX3 slug lysate and an *acaA*/*PKA* "CP" mound lysate.

To confirm the identity of cAMP synthesized by the *acaA*/*PKA* lysates, we subjected samples to HPLC reverse-phase chromatography to test whether the activity measured by isotope dilution assay cochromatographed with commercial [^{3}H]cAMP. Fig. 2b.3 shows that all the product that was synthesized when *acaA*/*PKA* slug lysates were incubated with Mg^{2+} and ATP, and that competed with [^{3}H]cAMP for binding to the PKA-R subunit, cochromatographed with [^{3}H]cAMP. Treatment of the column fractions with a cAMP-specific phosphodiesterase removed all activity detected in the isotope dilution assay. This convincingly demonstrated that the product synthesized by the *acaA*/*PKA* lysates was indeed cAMP and indicated that the *acaA*/*PKA* mutant does display considerable adenylyl cyclase activity in the slug stage.

2b3.4.2 The role of REGA in cAMP production by intact slug cells

cAMP produced by ACA or ACG can be measured in intact cells, because it is secreted and accumulates in the extracellular medium, where it can be protected by the PDE inhibitor DTT (Van Haastert, 1984; Schaap *et al.,* 1995). ACB could not be detected in intact wild-type cells (compare Figs. 2b.1D and H), suggesting that the activity was latent or that cAMP produced by ACB either was not secreted or was degraded before it could be secreted. To study whether intracellular degradation was responsible, we compared cAMP accumulation by slug lysates and intact cells in null mutants for the intracellular cAMP phosphodiesterase REGA (Shaulsky *et al.,* 1996). Fig. 2b.4A shows that lysates of *regA* slugs accumulated comparable levels of cAMP as lysates of the parent strain AX4. How- ever, basal cAMP levels of intact *regA* null cells were considerably higher than those of AX4 and the *regA* cells accumulated considerable amounts of cAMP when exposed to DTT (Fig. 2b.4B). This accumula-

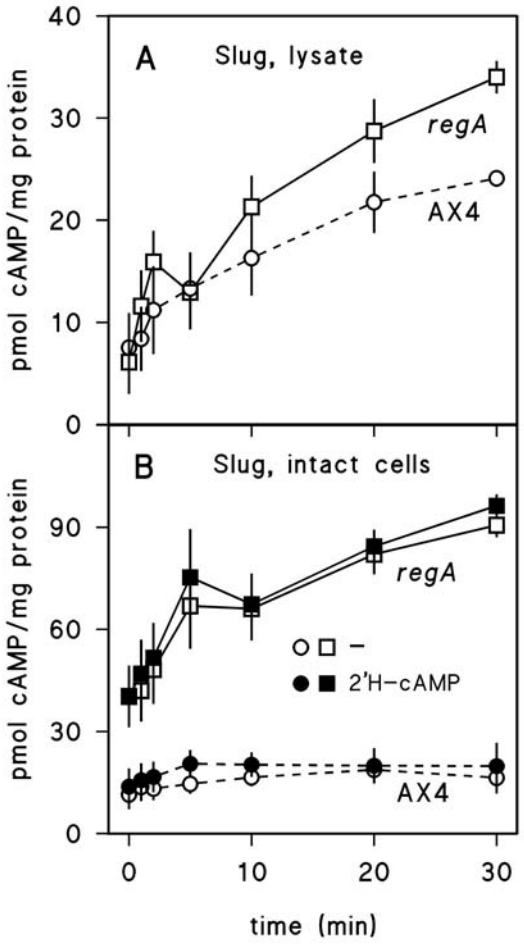

Fig. 2b.4. ACB activity in *regA* null mutants. *RegA* null cells and parent strain AX4 were developed on nonnutrient agar until slugs had formed, which were dissociated and resuspended in PB or in 30 μM GDPβS in lysis buffer. (A) The cells in GDPβS/lysis buffer were lysed and incubated with Mg^{2+} assay mix for 30 min. (B) The cells in PB were incubated for 30 min with 5 mM DTT in the presence and absence of 5 μM 2'H-cAMP. Total accumulated cAMP levels in cell lysates and cell suspensions were measured at the indicated time points. The means and SE of two experiments performed in triplicate are presented.

lation was not stimulated by 2'H-cAMP, indicating that it was not due to ACA. These data indicate that ACB is most likely active in intact slug cells, but that most of the cAMP that it produces is degraded by REGA.

2b3.5 Discussion

The different modes of regulation of ACA, ACB, and ACG were utilized to establish the contribution of the three enzymes to cAMP production during development. The G-protein-regulated enzyme ACA is predominantly active during aggregation, where, as was shown earlier, it produces the cAMP oscillations that coordinate the aggregation process (Pitt *et al.*, 1992). cAMP oscillations are also considered to coordinate slug morphogenesis (Siegert and Weijer, 1992). This does not seem to be borne out by our present observation that ACA activity decreases strongly after aggregation. However, in a parallel study we found that in slugs the ACA promoter remains only active in the tip cells and some scattered cells in the posterior of slugs, in total not amounting to more than 5% of the total cell population (I. Verkerke-Van Wijk, P. N. Devreotes, and P. Schaap, in preparation). This agrees with a supposed role for the slug tip as autonomous cAMP oscillator, but makes specific detection of ACA activity in total slug cell suspensions or lysates very difficult.

We did not detect any stimulation of cAMP accumulation by high osmolarity. This could mean either that ACG is not active at all prior to spore formation or that the negative effects of high osmolarity on ACA and ACB overrule a potential positive effect on ACG. The small peak of Mn^{2+}-stimulated activity found in *acaA*/*PKA* slug lysates (Fig. 2b.2B) seems to suggest that some ACG activity may be present.

A high level of adenylyl cyclase activity was detected in lysates of migrating slugs and early culminants, which was more strongly stimulated by Mg^{2+} than by Mn^{2+} ions. This is also the case for the activity that we previously characterized as ACB in growing *acaA*/*rdeA* cells (Kim *et al.,* 1998) and is in general common to enterobacterial adenylyl cyclases (Yang and Epstein, 1983), but not to most eukaryote adenylyl cyclases, where the reverse is true. The same high level of activity was also detected in *acgA* and *acaA*/*PKA* slugs, indicating that by genetic criteria ACB is dissimilar from ACG and ACA. Using HPLC and reaction product degradation by a cAMP-specific phosphodiesterase, we demonstrated that the supposed adenylyl cyclase activity in *acaA*/*PKA* mutants synthesized genuine cAMP. This contradicts earlier findings by Wang and Kuspa (1997) that no adenylyl cyclase activity was present in lysates of *acaA*/*PKA* mutants during the entire course of development, which led them to conclude that cAMP was not required for development. We assume that the suboptimal assay conditions (high Mn^{2+} concentrations and short incubation times) that were used by these workers were prohibitive for the detection of ACB activity and consider their conclusion of *Dictyostelium* development in the absence of cAMP as not valid.

In our previous work with *acaA*/*rdeA* mutants we measured a low activity (0.2 pmol/min mg protein) in lysates of growing cells, which decreased during starvation. This profile was not obvious in the wild-type cells studied here, presumably because the low level of ACB in growing cells is overwhelmed by the appearance of ACA during starvation. In growing *acaA* cells, ACB could only be detected in the presence of the REGA inhibitor IBMX (Kim *et al.,* 1998). In slug lysates, the amount of cAMP produced by ACB was so high (5 pmol/min mg protein) that addition of IBMX was not really required to detect it. In the absence of IBMX accumulated cAMP levels were about 5–10% lower than in its presence (data not shown).

ACB activity appeared to peak during early culmination. In later stages it could not be measured because the maturing stalk cells and spores cannot be lysed anymore by methods that do not compromise ACB detection. In wildtype cells the activity could only be detected in cell lysates; intact slug cells show elevated basal cAMP levels, but no significant accumulation of cAMP in the presence of the PDE inhibitor DTT. The REGA inhibitor IBMX was never found to promote cAMP accumulation by intact cells (data not shown), indicating that REGA activity is only present inside the cells. Intact cells from *regA* null slugs did accumulate large amounts of cAMP when exposed to the extracellular PDE inhibitor DTT. This indicated that (i) cAMP produced by ACB can be secreted and (ii) in wild-type cells cAMP produced by ACB is under control of REGA and is degraded before it can be secreted. It should be noted that this is not the case for cAMP produced by ACA during aggregation. Here *regA* null mutants show at most 20–40% higher accumulated cAMP levels than wild-type cells (Kim *et al.,* 1998). Since REGA protein is actually more abundant in aggregating cells than slugs (Thomason *et al.*, 1998) this could mean that REGA and ACB, but not ACA, may be colocalized giving REGA preferential access to cAMP produced by ACB.

Several lines of evidence indicate that PKA activity is required for both initiation of development and terminal differentiation of spores and stalk cells. Dominant

negative PKA inhibitors expressed under prestalk and prespore promoters inhibit spore and stalk cell formation, respectively (Harwood *et al.,* 1992; Hopper *et al.,* 1993b). Mutants that overexpress PKA or show constitutive PKA activity due to null mutations in the R-subunit show rapid development and precocious spore differentiation (Anjard *et al.,* 1992; Mann *et al.,* 1994; Simon *et al.,* 1992). A similar phenotype is shown by *regA* null mutants and mutants that lack the phosphotransferase RDEA (Shaulsky *et al.,* 1996; Thomason et al., 1998; Chang *et al.,* 1998). REGA is activated by phosphorylation of an aspartic acid residue in an attached response regulator. The phosphate is donated by a histidine residue on RDEA, which is considered to receive the phosphate from a currently unidentified histidine kinase (Thomason et al., 1998). The fact that REGA inhibition and PKA overexpression yield a similar phenotype indicates that elevation of intracellular cAMP levels is required for activation of PKA. This seemingly obvious conclusion was not borne out by earlier studies showing that null mutants for the only known adenylyl cyclases ACA and ACG could differentiate into mature spores and stalk cells (Pitt *et al.,* 1992, 1993).

The developmental regulation of ACB and its apparently close interactions with REGA strongly suggest that this enzyme produces the cAMP that is required for PKA activation. It is as yet somewhat enigmatic why in spite of the fact that slug lysates show high ACB activity, cAMP production by intact slug cells is low. However, it should be realized that during normal development, stalk and spore cell differentiations are triggered only at specific locations in the organism. Locally secreted peptides have been implicated in induction of terminal differentiation (Anjard *et al.,* 1997) and some lines of evidence indicate that one of these peptides activates a signal transduction cascade that results in inhibition of REGA (Anjard *et al.,* 1998). In our adenylyl cyclase assays in cell suspensions, the peptides can probably not reach a sufficiently high concentration to inactivate REGA. If REGA is eliminated by mutation, intact slug cells are found to produce large amounts of cAMP (Fig. 2b.4B), which indicates that inhibition of REGA is probably sufficient to allow cAMP accumulation by ACB, activation of PKA, and terminal differentiation. We can however at present not exclude that there may be additional signals that regulate ACB activity directly, such as for instance the catabolite ammonia, an inhibitor of fruiting body formation (Gross, 1994). In future studies we will try to gain more insight in the regulation of this intriguing enzyme by signal molecules and its interaction with other signal transduction components as REGA and PKA.

Acknowledgments

We thank Adam Kuspa and Carole Parent for their kind gifts of the *acaA*/*PKA* "AK" and *acaA*/*PKA* "CP" lines, respectively. The AX4, AX4/*regA,* and *acaA*/*rdeA* cell lines were kindly provided by Gadi Shaulsky and Julian Gross, respectively. We are grateful to Peter van Haastert and André Wijfjes for advice and assistance with HPLC and to Raymond Brandt for excellent technical assistance.

3. cGMP production

A novel type of soluble guanylyl cyclase from *Dictyostelium*

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3 A novel type of soluble guanylyl cyclase from *Dictyostelium*

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3.1 Abstract

A novel *Dictyostelium* cyclase gene was identified that encodes a protein (sGC) with high similarity to mammalian soluble adenylyl cyclase (sAC). Orthologues of sGC and sAC are present in bacteria and vertebrates, but are absent from the completely sequenced genomes of *Drosophila*, *C. elegans*, *S. cerevisae* and *Arabidopsis*. sGC gene expression levels are highest during aggregation and late development. Null mutants in *sGC* aggregate less efficiently under stringent conditions than wild-type. Adenylyl cyclase activity is unaffected in the mutant, but chemoattractant-induced cGMP production and guanylyl cyclase activity are more then ten-fold reduced in aggregating cells. Guanylyl cyclase activity is only slightly reduced in growing cells and is most likely due to the other *Dictyostelium* guanylyl cyclase, GCA. Most of the sGC activity is soluble with Mn^{2+}/GTP as substrate, but with the more physiological Mg^{2+}/GTP the activity is restricted to the membranes and stimulated by GTPγS.

3.2 Introduction

Cyclic nucleotides play an essential role in signal transduction in both prokaryotes and eukaryotes. These molecules are synthesized by a multigene family of adenylyl and guanylyl cyclases (ACs and GCs). In mammalians, cAMP is produced in response to hormones and neurotransmitters by at least nine different twelve transmembrane adenylyl cylases (12tmACs), that are regulated by serpentine receptor coupled G-proteins. (Sunahara *et al*., 1996) Recently, a soluble AC (sAC) with homology to the cyanobacterial ACs was discovered in mammalian testis (Buck *et al*., 1999), which functions as a bicarbonate sensor and is involved in sperm maturation (Chen *et al*., 2000). cGMP is produced in mammalian cells by single transmembrane and soluble GCs (1tmGCs and sGCs) and is involved in vasoregulation, fluid and electrolyte homeostasis and phototransduction (Lucas *et al.*, 2000).

In the social protozoan *Dictyostelium discoideum*, cAMP is required extracellularly as chemoattractant during aggregation and morphogen for regulation of differentiation. Intracellular cAMP functions as a second messenger through the activation of the cAMP-dependent protein kinase (PKA) and is required for gene expression events during different stages of development, such as the initiation of development and terminal differentiation of spore and stalk cells (Loomis, 1998). cAMP is synthesized by at least three different ACs. ACA, a homologue of the mammalian 12tmACs, is mainly expressed during aggregation (Pitt *et al*., 1992) and is responsible for the cAMP production that regulates aggregation. Ligand stimulated activation of ACA is mediated by the $G_{\beta\gamma}$-subunit (Wu *et al*., 1995b) and also involves a cytosolic factor (CRAC) (Insall *et al*., 1994), a RAS-GEF (*aimless*) (Insall

et al., 1996) the MAP kinase ERK2 (Segal *et al*., 1995) and a protein with unknown function, Pianissimo (Chen *et al*., 1997b). In the slug, ACA expression is restricted to the tip, where it functions as an organiser and controls tip-specific expression of the nuclear factor CudA via tip-specific nuclear translocation of the transcription factor StatA (Verkerke-Van Wijk *et al*., 2001). ACG has the topology of 1tmGCs and is expressed in spores (Pitt *et al*., 1992; Meima and Schaap, unpublished observations), where it functions as an osmosensor controlling spore germination (Van Es *et al*., 1996). ACB harbours a single catalytic domain that is closely related to the catalytic region of cyanobacterial ACs and a putative response regulator domain. It is mainly active during post-aggregative development and essential for spore maturation (Söderbom *et al*., 1999; Meima and Schaap, 1999).

cGMP is involved in chemotaxis and phosphorylation of myosin chains (Van Haastert and Kuwayama, 1997). cAMP stimulation of aggregating cells results in a rapid and transient cGMP response and requires heterotrimeric G-protein activity (Janssens *et al*., 1989; Schoen *et al*., 1996; Wu *et al*., 1995b). A mutant lacking significant GC activity, KI-8, is non-chemotactic (Kuwayama *et al*., 1993), whereas *stmF*, which is defected in cGMP phosphodiesterase activity and therefore has a prolonged cGMP response, shows prolonged chemotaxis and myosin phoshorylation (Newell and Liu, 1992). Recently, a *Dictyostelium* GC with the topology of 12tmACs, DdGCA, was identified (Roelofs *et al*., 2001a).

Null mutants in *ddgca* have no apparent phenotype and substantial GC activity. In addition, the phenotypes of the different AC knock-out mutants suggest the possibility of a fourth AC. We screened the *Dictyostelium* genome and cDNA databases and report here the identification of a GC that is similar to the mammalian soluble adenylyl cyclases, sAC.

3.3 Materials and methods

3.3.1 Materials

Adenosine 3':5'-monophosphate (cAMP), 2'-adenosine 3':5'-monophosphate (2H'-cAMP), guanosine 3':5'-monophosphate (cGMP), guanosine 5'-O-(3-thiophosphate) (GTPγS), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), G418 and Bradford reagent were obtained from Sigma (St. Louis, USA). Blasticidin was from ICN (Costa Mesa, USA) and nucleopore filters, pore size 3 μm, were from Whatmann (Maldstone, United Kingdom). [2,8-^{3}H] adenosine 3':5'-monophospate (^{3}H-cAMP) and [8-^{3}H] guanosine 3':5'-monophosphate (^{3}H-cGMP) were from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom), Gene Screen was obtained from NEN (Boston, USA) and the random primed labelling kit was from Roche Diagnostics GmbH (Mannheim, Germany).

3.3.2 Cell growth and development

Wild-type *D. discoideum* strain NC4 was grown in association with *Klebsiella aerogenes* on SM agar plates. All other cells were grown in standard axenic medium, which was supplemented with 5 μg/ml blasticidin for *sgc*$^-$. For development, cells were harvested from growth medium, washed in phosphate buffer (PB, 10 mM Na/K-phosphate, pH 6.5), plated on non-nutrient agar (1.5% agar in PB) at 2.5x10^6 cells/cm^2 and incubated at 22°C until the desired developmental stages were reached. For development under buffer, cells were plated at different densities submerged in 1 ml PB in 40 mm diameter petridishes.

3.3.3 Bioinformatics

The *Dictyostelium* genome database was screened using consensus sequences for AC catalytic domains. A large contig (contig CHR2.0.71028, assembled by the Dictyostelium genome project at the genome sequencing centre Jena, Germany (http://genome.imb-jena.de/dictyostelium) was identified that contained sequence encoding two cyclase domains at the 5' end of a long ORF. In addition, 3 putative upstream ORFs were present which were confirmed as exons of the gene by RT-PCR (see below). The complete *sGC* ORF was deposited in DDBJ/EMBL/GenBank under accession number AF361947. The BLAST (http://www.ncbi.nlm.nih.gov/Blast) and PFAM (http://www.sanger.ac.uk/Software/Pfam/) programs were used to identify homologous sequences and characterize the domain architecture. Multiple sequences alignments were created with ClustalW (http://www.ebi.ac.uk/clustalw/) and adjusted by eye. Protein distance matrices were calculated with ProtDistance and Fitch and phylogenetic trees created with Drawtree (Phylip 3.5 package, http://bioweb.pasteur.fr/cgi-bin/seqanal (Felsenstein, 1993).

3.3.4 RNA isolation, RT-PCR and Northern Blot analysis

Total RNA was isolated from developing NC4 cells at 2 hrs intervals of development using an RNeasy kit (Qiagen) according to the manufacturer's protocol. RT-PCR was performed with the primers SGC-RT5 (CCCACTGATGAATCTCT CATTG) and SGC-RT3 (CTTTCTGTTAGTGATGTAAATCCTG) using the Qiagen 1 step RT-PCR kit according to the manufacturer's protocol. For Northern analysis, RNA was size-fractionated on a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to a Gene Screen membrane. For a probe, a *sgc* fragment from nt 5232 to 6190, which comprises a large part of the catalytic domain, was amplified by PCR using the oligonucleotides SGC1 (CTGGGATCCATTCATCATGTGATTATTTGC) and SGC2 (CTGGAATTCAAAACATTTACCACAATCTCC). The probe was ^{32}P-labeled using the random primer labelling method. The membrane was hybridized according to standard conditions and exposed to an X-ray film.

3.3.5 Vector construction and transformation

To disrupt the *sgc*-gene, a knock-out construct was designed in which the catalytic domain is substituted for the blasticidin cassette. *sgc* fragments from nt position 5768-6558 (α) and 3446-4464 (β) were amplified by PCR using the oligonucleotides SGC 3(CGTAGATCTTGAAGAAACCTCGAAAACTGC) and SGC4 (GTCGGATCCTT GAGGGACGGTAATGAAATG) that yielded a 5'-*BglII* and a 3'-*BamHI* site for α and SGC8 (TGCGGATCCATCATCCAAAATCATTAGAGTC) and SGC6 (GCTGA ATTCATGTGGTGGTACAATTGGAG) that gave a 5'-*BamH*I and a 3'-*EcoRI* site for β. The fragments were cloned in tandem into the *BamHI* and *BamHI*/*EcoRI* sites of the plasmid pUC118bsrΔBamHI that harbours the blasticidin selection marker. Linearization of this construct with BamHI yielded a fragment in which the entire plasmid is flanked by the *sgc* fragments α and β. 12 µg of linearized fragment was transformed into the *Dictyostelium* wild-type cell line AX2 by electroporation (Pang *et al.*, 1999) and selected for growth on 5 µg/ml blasticidin. Individual clones were obtained by plating cells on bacterial lawns and were analysed for homologous recombination by Southern blotting.

3.3.6 Guanylyl and adenylyl cyclase assays

Vegetative cells were harvested from growth medium and either used directly or starved on PB agar plates. To measure AC and GC activity, cells were resuspended in ice-cold lysisbuffer (250 mM sucrose in 10 mM Tris.HCl, pH 8.0) (LB) at $1x10^8$ cells/ml and lysed through a nucleopore filter in the presence or absence of 30 μM GTPγS. In some experiments, the lysate was first fractionated. 0.5 ml of lysate were first centrifuged for 15 min at 16.000xg, the pellet was washed once with 1.5 ml LB and then resuspended in 0.5 ml LB for the particulate fraction. The supernatant was further centrifuged for 5 min at 180,000 x *g* for the soluble fraction. Aliquots of 10 μl lysate or fractions were added to 10 μl 2x assay buffer (10 mM DTT, 0.4 mM IBMX, 1 mM ATP for AC and 1 mM GTP for GC activity respectively and either Mg^{2+} or Mn^{2+} at the concentrations as indicated in the legends). Reactions were initiated by transferring the samples to a 23°C waterbath and terminated by adding 10 μl 0.4 M EDTA and boiling for 1 min.

To measure cAMP or cGMP responses, cells were resuspended at $1x10^8$ cells/ml in PB and aerated for 5 min. 25 μl of cells were stimulated with 3 μl 50 μM 2'H-cAMP in 50 mM DTT and reactions were terminated at the indicated time points by adding 30 μl 3.5% perchloric acid (PCA) (v/v). Samples were neutralized with 15 μl 50% saturated $KHCO_3$ and 75 μl cAMP assay buffer (150 mM K-phosphate, 4 mM EDTA, pH 7.5). cAMP levels were determined with an isotope dilution assay using purified beef heart regulatory PKA subunit as binding protein. cGMP levels were determined with a radioimmunoassay assay using a cGMP antibody (Schulkes *et al.*, 1992). To standardize the levels of cAMP and cGMP, protein content of whole cells and lysates were determined with a Bradford assay according to the manufacturer's protocol.

3.4 Results

3.4.1 Identification of a novel cyclase in *Dictyostelium*

To find novel adenylyl cyclases, the *Dictyostelium* genome DNA databases were screened with the peptide sequence of the ACB catalytic domain, revealing an ORF on the 18 kb contig CHR2.0.71028. Blast searches with this sequence against the entire Swiss-Prot database showed a high degree of similarity with mammalian soluble adenylyl cyclase (sAC) (Buck *et al*., 1999). As discussed below, the gene proved to encode a guanylyl cyclase. We therefore have named it *sgcA* and the protein DdsGC.

The cyclase domains are located at the 5' end of a ~5.4 kb ORF. A start codon however was absent, suggesting that the ORF was probably preceded by an upstream intron and (an) additional exon(s). Further analysis indicated the presence of at least three additional upstream exons. To investigate this, we performed RT-PCR on aggregation stage mRNA with primers that flank the three putative introns the genomic sequence. Sequence analysis of the RT-PCR products confirmed the presence of a total of 4 exons in the *sGC* gene. The first exon consists of 1947 base pairs and is followed by a second exon of only 70 base pairs that is required to maintain the reading frame. This is followed by an intron of 1176 base pair, which is unusually long for *Dictyostelium*. The last two exons encode 373 and 1796 amino acids respectively interspersed by a short AT-rich intron.

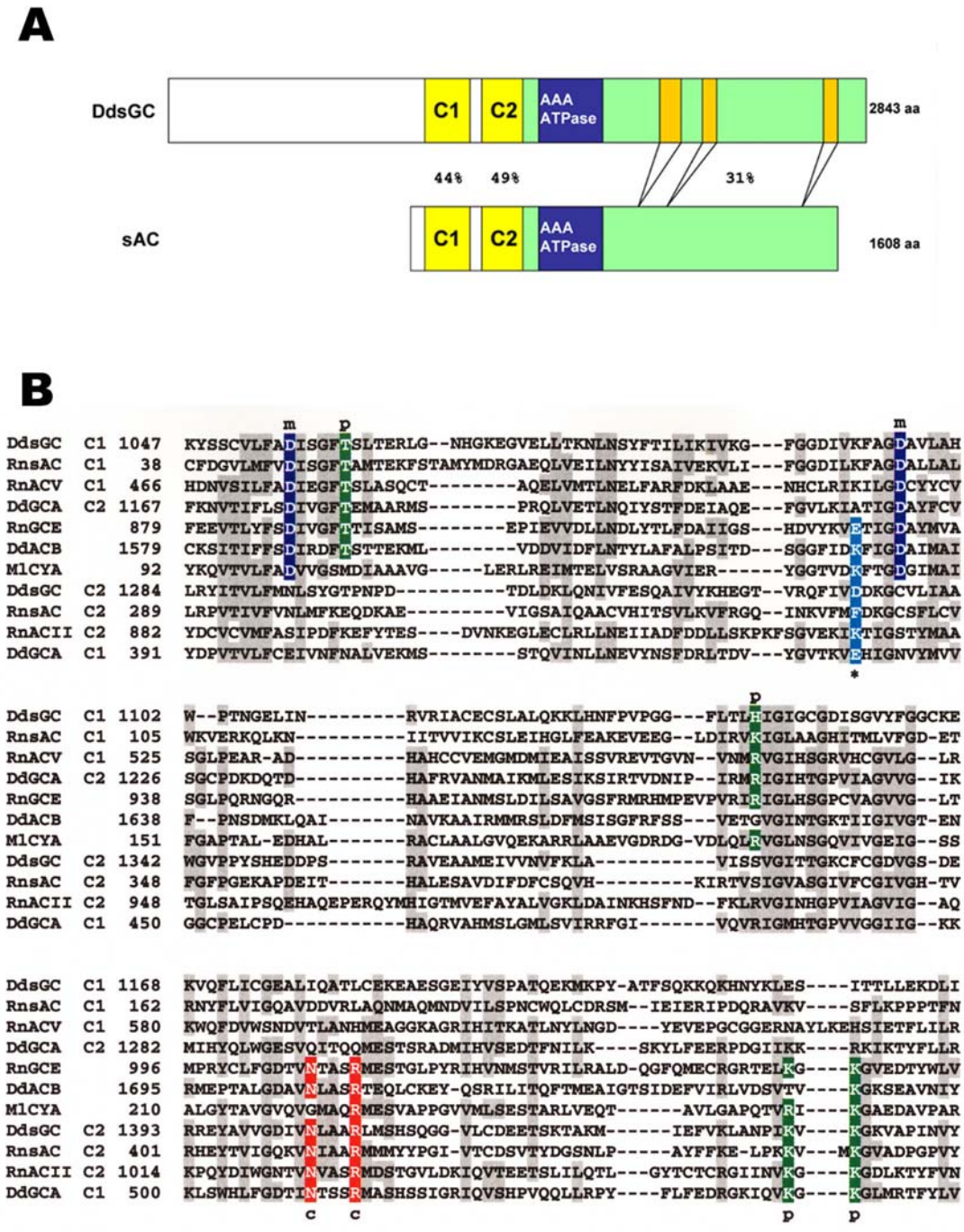

Fig. 3.1. Domain and sequence comparison of sGC with other cyclases. *A)* Comparison of the domain structure of sGC and the sAC. Cyclase domains are in yellow. A P-loop nucleotide binding domain is indicated in blue. For optimal sequence alignment of the C-terminal domains (green), low complexity sequence in sGC (orange) was omitted. Percentages indicate sequence similarity of the cyclase domains and the C-terminal domain. *B)* Alignment of the cyclase domains of several class III adenylyl and guanylyl cyclases, based on the crystal structure of the ACV-ACII dimmer (Tesmer *et al.*, 1997). Residues that are conserved in six or more cyclase domains are highlighted. Residues with specific functions are colour and letter coded. Blue (m), metal binding, green (p), phosphate binding, red (c), required for analysis, turquoise (*), substrate recognition through purine binding.

3.4.2 Domain architecture of sGC

The total open reading frame predicts a protein of 2843 amino acids without putative transmembrane helices. The N-terminal domain is rich in repetitive sequences and shows no homology to proteins with known function. The region between amino acids 1051-1410 contains two PFAM cyclase domains (C1 and C2) with highest homology to the catalytic domains of sAC (see below) (fig. 3.1A). An AAA ATPase with a P-loop nucleotide binding domain (Lupas and Martin, 2002) closely follows the second cyclase domains of sGC and sAC. Homology with sAC extends throughout the C-terminal domain, apart from a few stretches of repetitive sequence. In addition, the C-terminal domain has substantial homology over the first half to the C-terminal domain of the *Mycobacterium laepra* adenylyl cyclase and over its entire length to the central domain of a complex kinase from the cyanobacterium *Anabaena* (AAF445653) (not shown).

3.4.3 The sGC catalytic domains

The crystal structure of mammalian adenylyl cyclases shows that cyclase domains dimerize in an antiparallel fashion to form two catalytic pockets in which ATP or GTP can bind (Liu *et al.*, 1997; Tesmer *et al.*, 1997; Zhang *et al.*, 1997**)**. In 12tmACs, several mutations have rendered one of the binding sites inactive. In these dimers, residues of the C1 domain are predominantly involved in Mg^{2+} and ribose binding (m and r in fig. 3.1B), whereas residues of the C2 domain are involved in substrate specificity and catalysis (* and c). Recognition of the purine moiety of either ATP or GTP is determined by only a few residues. The most pronounced is a lysine in adenylyl cyclases (K938 in ACII) and a glutamate in guanylyl cyclases (E928 in GCE), that interact with the purine moiety of ATP and GTP respectively (Tesmer *et al.*, 1997). In sGC, this is a lysine (K1098) in the C1 and an aspartate (D1332) in the C2 domain. As in 12tmACs, the residues involved in metal and ribose binding are only conserved in the C1 domain and the asparagines and arginine residues required for catalysis are only present in the C2 domain. This would mean that substrate specificity of sGC is determined by the aspartate in the C2 domain and therefore that *sGC* encodes a guanylyl cyclase.

3.4.5 Phylogeny of the sGC cyclase domains

To demonstrate the phylogenetic relation of sGC to other cyclases, we aligned the cyclase domains with those of mammalian ACs and GCs, including sAC, cyanobacterial ACs and all other *Dictyostelium* cyclases and constructed a phylogenetic tree (fig. 3.2).

The cyclase domains of sGC are closely related to their counterpart in sAC. As previously reported for sAC, these cyclase domains group with the cyanobacterial ACs and ACB and are less related to mammalian cyclases. The C1 and C2 domains of sGC and sAC are more distant to each other than to some cyanobacterial cyclase domains, indicating that these cyclases result from the merging of two genes. Interestingly, no homologues for sAC and sGC were found in the *Drosophila, C. elegans*, yeast or *Arabidopsis* genome databases.

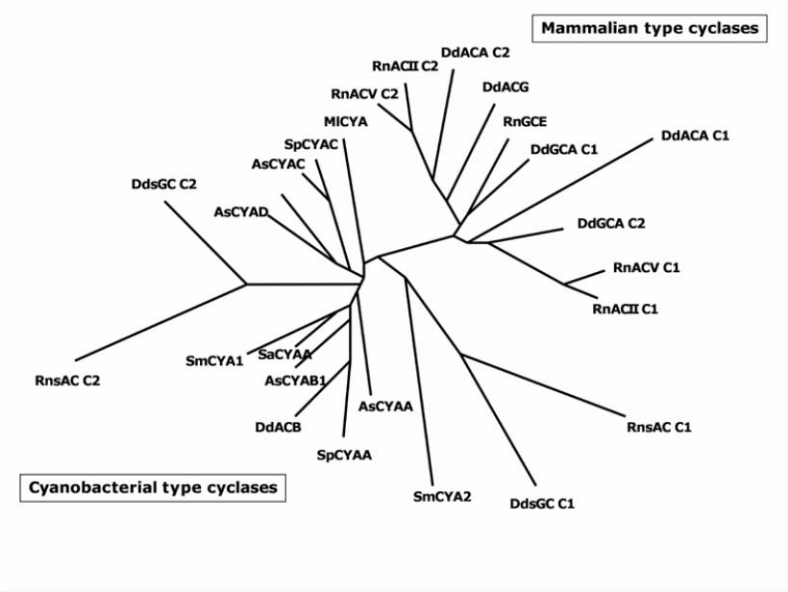

Fig. 3.2. Phylogenetic relationship between catalytic domains of sGC and other class III cyclases. Alignments were performed with CLUSTALW and an unrooted phylogenetic tree was constructed using ProtDistance and Fitch (Phylip 3.5) (Felstenstein, 1993). Accession numbers for the aligned sequences are: RnACII (*Rattus norvegicus* ACII: P26769), RnACV (*Rattus norvegicus* ACV: AAB39764), RnGCE (*Rattus norvegicus* GCE: P51840), RnsAC (*Rattus norvegicus* sAC:), DdACB (*Dictyostelium discoideum* ACB/ACR: AAD50121), DdACG (*Dictyostelium discoideum* ACG: Q03101), DdGCA (*Dictyostelium discoideum* GCA: CAB42641), DdsGC (*Dictyostelium discoideum* sGC: AAK92097), AsCYAA (*Anabaena* sp. CYAA: I39600), AsCYAB1 (*Anabaena* sp. CYAB1: BAA13998), AsCYAC (*Anabaena* sp. CYAC: BAA14000), AsCYAD (Anabaena *sp.* CYAD: BAA14001), SpCYAA (*Spirulina platensis* CYAA: BAA22996), SpCYAC (*Spirulina platensis* CYAC: BAA22997), SmCYA1 (*Sinorhizobium meliloti* CYA1: P19485), SmCYA (*Sinorhizobium meliloti* CYA2: S60684), MlCYA (*Mycobacterium leprae* CYA: CAA19149), SaCYAA (*Stigmatella aurantiaca* CYAA: P40137).

3.4.6 Developmental regulation of *sgcA* expression

The developmental regulation of *sgcA* expression was determined using Northern blot analysis. As can be seen in fig. 3.3, expression is nearly absent in vegetative cells, increases gradually when cells are starved and plateaus during late development. Around culmination, the single transcript is replaced by a smaller and a larger transcript.

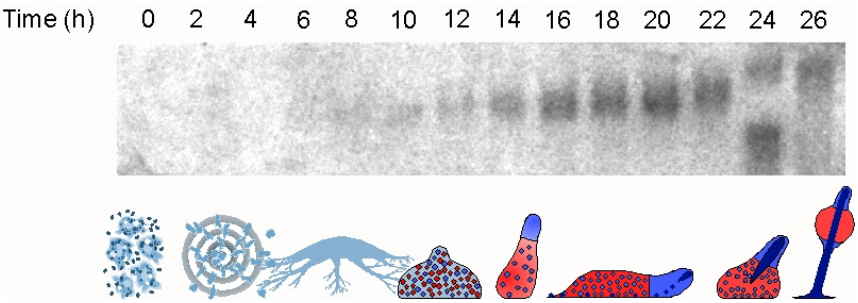

Fig.3.3. Developmental regulation of *sGC* expression. RNA was isolated from developing NC4 wild-type cells, size-fractionated and hybridized with a probe encoding the catalytic region of sGC.

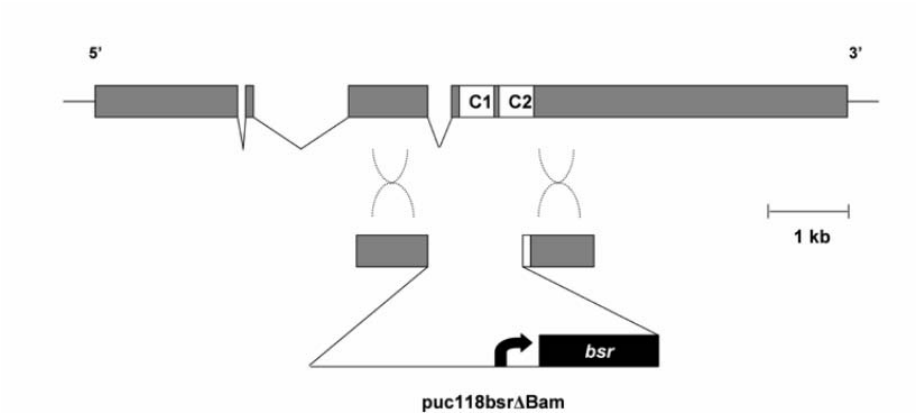

Fig. 3.4. Schematic of the *sgc* knock-out construct. The two cyclase domains are replaced by a blasticidine resistance cassette.

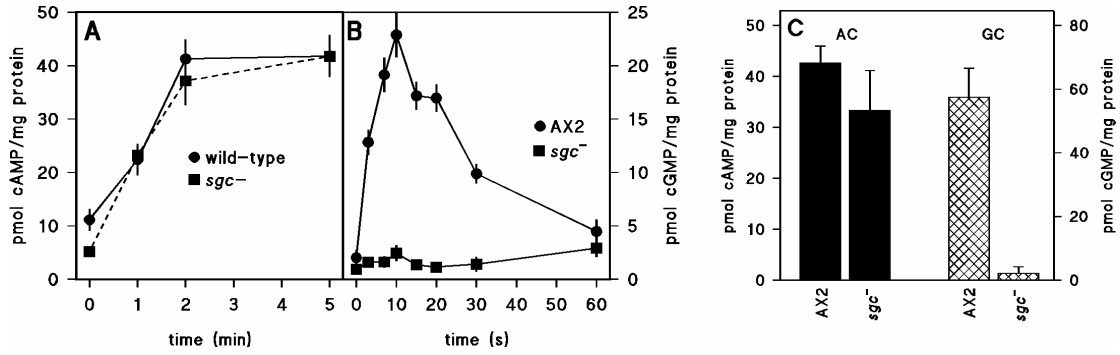

Fig. 3.5. Cyclic nucleotide production in aggregating AX2 wild-type and *sgc*⁻ cells. *A* and *B*) *In vivo* cAMP and cGMP response. AX2 wild-type or *sgc*⁻ cells were stimulated with 5 uM 2'H-cAMP and 5 mM DTT. At the indicated time points, reactions were terminated with PCA and cAMP and cGMP levels were determined. *C) In vitro* AC and GC activity. Cell lysates were incubated with 0.5 mM of either ATP or GTP in the presence of 2 mM Mn^{2+}. Reactions were terminated after 2 minutes by addition of EDTA and assayed for cAMP and cGMP levels. Average of two independent experiments.

3.4.7 Inactivation of *sgcA* by homologous recombination

To study the function of *sgcA* in *Dictyostelium*, the gene was inactivated by homologous recombination. A knock-out construct was generated in which the catalytic domain was replaced by the blasticidine resistance gene (fig. 3.4). The linearized DNA was transformed into the wild-type AX2 strain and blasticidine resistant clones were analyzed for homologous recombination by Southern blotting. The *sgcA* gene proved to be disrupted in 90% of the clones analysed. One clone was selected for further characterisation.

We first tested the null mutant for *in vivo* cAMP in cGMP production in aggregating cells in response to the receptor agonist 2'H-cAMP. In wild-type AX2 cells, cAMP accumulation reaches a plateau after 2 min of stimulation (fig. 3.5A). The cGMP response is fast and transient in wild-type cells. cGMP levels peak 10 sec after stimulation and reach baseline within 1 min (fig. 3.5B). Whereas cAMP accumulation is unaffected in *sgc*⁻, the cGMP response is 90% reduced. The residual activity shows the same kinetics and is most likely caused by low levels of GCA activity. GCA is expressed at high levels in vegetative cells and during post-

aggregative development, and at low levels during aggregation (Roelofs *et al*., 2001 a).

We next analysed AC and GC activity in cell lysates in the presence of Mn^{2+} (fig. 3.5C). AC activity is the same in *sgc*$^-$ and wild-type AX2 lysates and most likely represents the 12tmAC ACA, which is Mn^{2+}-stimulated and expressed during aggregation. GC is strongly reduced in the *sgc* null mutant. Together with the loss of cGMP accumulation *in vivo*, these data confirm that sGC functions as a guanylyl cyclase.

3.4.8 GC activities during growth and aggregation

In *Dictyostelium*, GC activation by cell surface cAMP receptors is mediated by heterotrimeric G-proteins. In lysates, the enzyme can therefore be activated by the non-hydrolysable analogue GTPγS. We measured GC activity with Mg^{2+}-GTP as substrate after lysis in the presence or absence of GTPγS. In addition, we also looked at activity in the presence of Mn^{2+}, a potent activator of some, but not all cyclases. In vegetative wild-type cells, total GC activity was 4-fold induced by GTPγS, but activity was similar with Mn^{2+} as compared to basal activity in the presence of Mg^{2+} (fig. 3.6A). In vegetative *sgc*$^-$ lysates, GTPγS-induced GC-activity was only slightly reduced, but the Mn^{2+} depended activity was lost (3.6B). In 7 hr starved wild-type cells, GC-activity was 3-fold induced by GTPγS and 6-fold by Mn^{2+}-ions (3.6C). In *sgcA*$^-$ cells, GTPγS-induced activity was 4-fold reduced as compared to wild-type, whereas Mn^{2+}-stimulated activity was completely absent (3.6D). In short, disruption

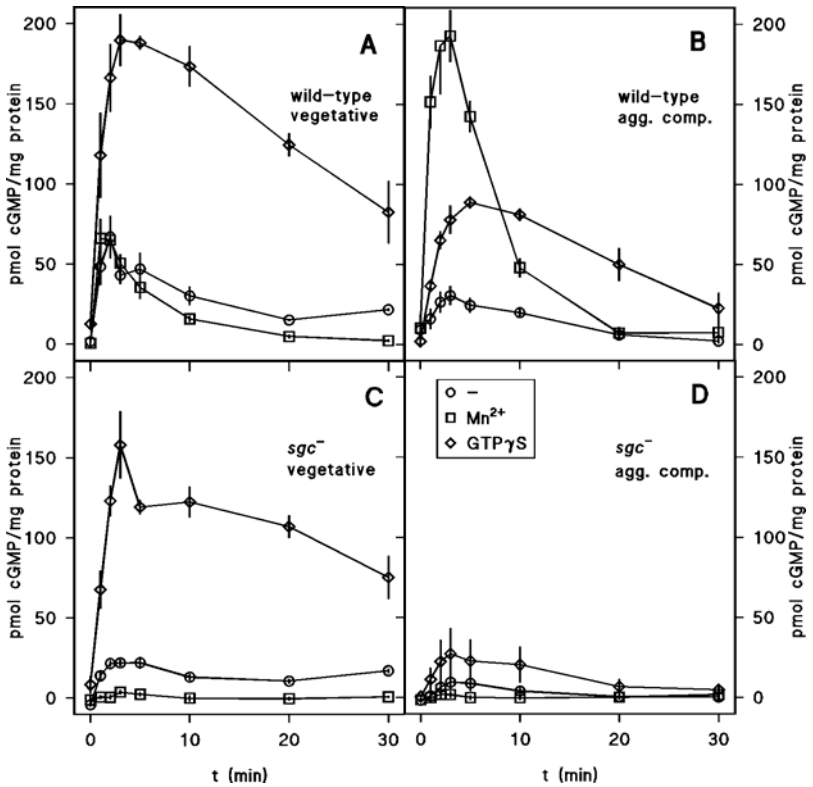

Fig. 3.6. GC activity in vegetative or aggregating AX2 wild-type cells (*A* and *C*) or *sgc*$^-$ cells (*B* and *D*). Cells were lysed in the presence or absence of 30 μM GTPγS and incubated with 0.5 mM GTP

in the presence of 2 mM Mg^{2+} or Mn^{2+}. At the indicated time points, reactions were terminated with EDTA and cGMP levels determined. Average of two independent experiments.

of *sGC* abolishes all Mn^{2+}-stimulated GC activity and strongly reduces GTPγS-stimulated in lysates of aggregating cells, but leaves activity in growing cells largely unaffected.

These results indicate the presence of two GCs with their peak activity at different stages of development. sGC is G-protein and Mn^{2+} stimulated and accounts for the majority of the activity in aggregating cells, but is also present at low levels during growth. A second GC is mainly active in vegetative cells and weakly during aggregation. This activity is also G-protein dependent, but insensitive to Mn^{2+}-ion and most likely resembles GCA, which is 3-fold higher expressed during growth than in aggregating cells (Roelofs *et al.*, 2001a).

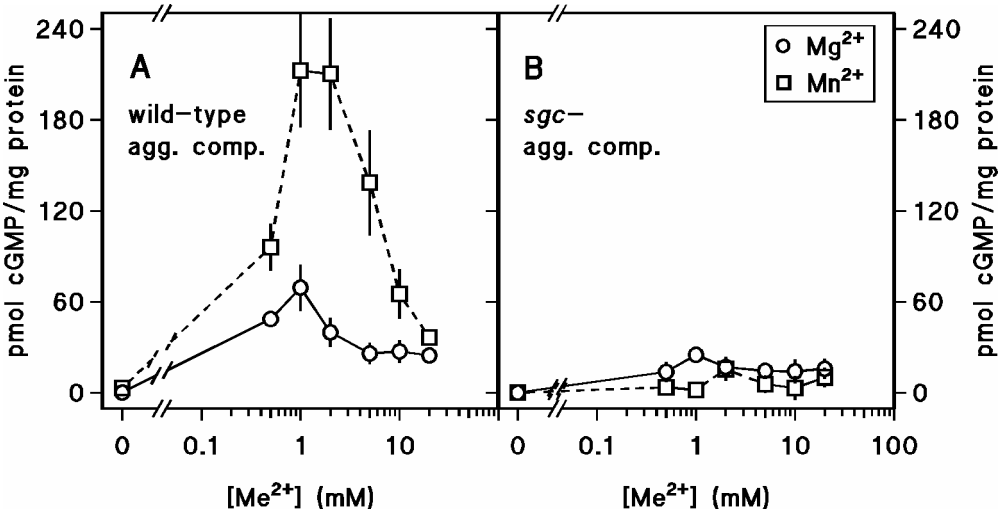

Fig. 3.7. Metal dependence of GC activity. Aggregating AX2 wild-type (*A*) or sgc$^-$ cells (*B*) were lysed, incubated for 2 minutes with 0.5 mM GTP in the presence of the indicated concentrations of Mg^{2+} or Mn^{2+} and assayed for cGMP levels. Average of two independent experiments.

3.4.9 Characterisation and subcellular localisation of sGC activity

We further characterized sGC activity in lysates of aggregating cells. We first looked at the dependence of activity on Mg^{2+} and Mn^{2+}. The dose response of sGC for both metal ions is the same, but the activity is 4-5 fold higher in the presence of Mn^{2+} (fig. 3.7A). These activities are derived from sGC, since activity is virtually absent in the null mutant at all metal concentrations tested (3.7B). To determine the subcellular localization of sGC, we fractionated lysates of aggregating cells into 180K particulate and soluble fractions and measured GC activity. To identify the G-protein activated pool, we also measured in fractions of cells lysed in the presence of GTPγS. In unstimulated cells, nearly all the GC activity resides in the soluble fraction with However, only the particulate fraction is stimulated by GTPγS and only when Mg^{2+}/GTP is used as substrate (3.8B). These results indicate that the majority of sGC is present in the cytosol, but that only a small membrane-associated pool is activated by G-proteins. The Mn^{2+}-stimulated activity is not significantly reduced in GTPγS treated lysates, which would mean that sGC does not translocate to the membrane upon G-protein activation.

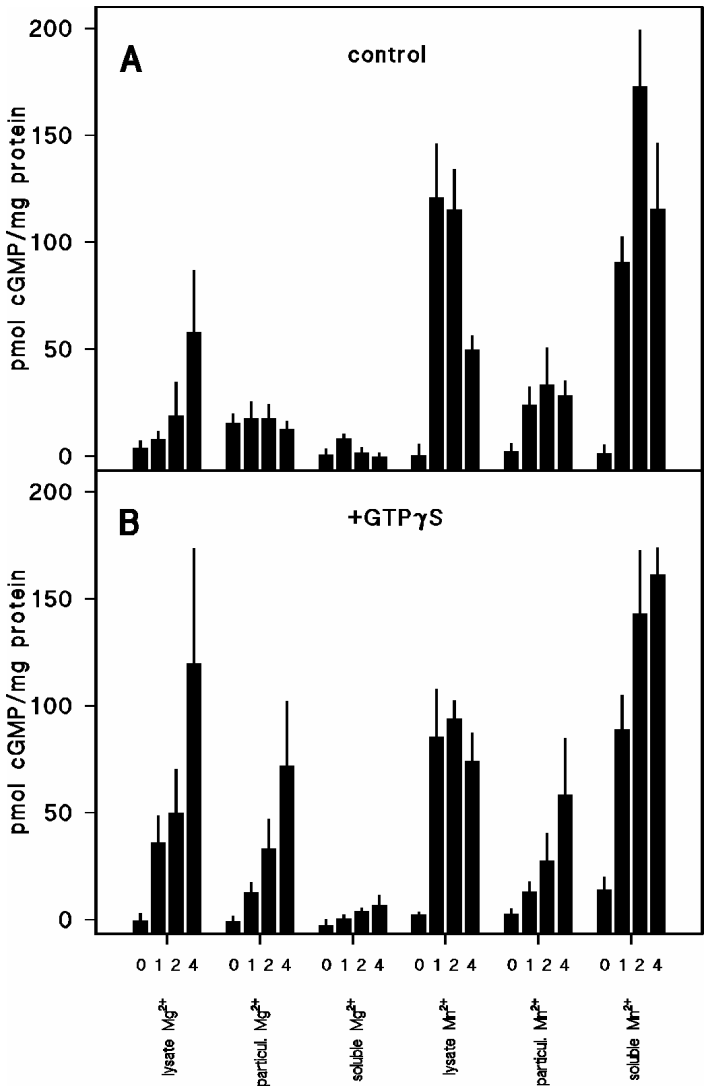

Fig. 3.8 Localisation of sGC activity. Cells were lysed in the absence *A)* or presence *B)* of 30 μM GTPγS, fractionated into pellet and 180K supernatants and incubated with 0.5 mM GTP in the presence of 2 mM Mg^{2+} or Mn^{2+}. Reactions were terminated by addition of EDTA at the indicated time-points and cGMP levels determined. Average of two independent experiments.

3.4.10 *sgc*$^-$ aggregation is impaired under stringent conditions

The *sgc* null mutant aggregated and developed into normal fruiting bodies on phosphate buffered agar at the same speed as wild-type. Also, *sgc*$^-$ cells expressing GFP constitutively from the actin15 promoter aggregated at the same speed as wild-type when mixed (not shown). Under these conditions the cells are closely packed and chemotactic abnormalities might not become detected. We then tested the cells' ability to develop at decreasing cell densities under a layer of phosphate buffer. Under these more stringent conditions where the secreted cAMP becomes rapidly diluted, *sgc*$^-$ aggregation was affected (fig. 3.9). Wild-type cells could still aggregate efficiently at $3x10^4$ cells/cm^2, whereas *sgc*$^-$ cells did not aggregate at all. Furthermore, even at densities of $1x10^6$ cells/cm^2, where *sgc*$^-$ cells could aggregate, this happened with a several hour delay as compared to wild-type. These results suggest that reduction of the cGMP response by 90% has no apparent effect under favourable conditions, but that chemotaxis becomes impaired at more stringent conditions.

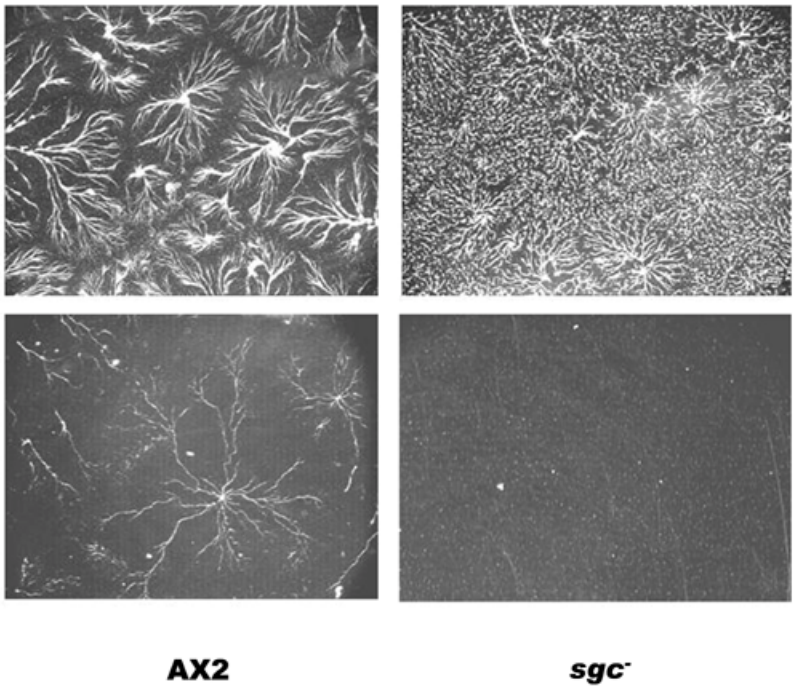

Fig. 3.9. Phenotype of the *sgc* knock-out. AX2 wild-type and *sgc*$^-$ cells were plated on petridishes under phosphate buffer at densities of 10^6 cells/ml and $5x10^4$ cells/ml (upper and lower panels respectively). A representative of two experiments is shown.

3.5 Discussion

3.5.1 sGC, a homologue of the mammalian soluble adenylyl cyclase

We demonstrated the existence of sGC, the closest homologue of the soluble mammalian adenylyl cyclase (sAC) in *Dictyostelium discoideum*. As in the *Dictyostelium* adenylyl cyclase ACB, the catalytic domains of these enzymes are closely related to the subgroup of cyanobacterial adenylyl cyclases and more distantly to the group of mammalian transmembrane cyclases to which also the *Dictyostelium* adenylyl cyclases ACA and ACG belong. This is supported by the significant homology of the C-terminal domain to bacterial gene products. In contrast to ACB and cyanobacterial adenylyl cyclases, sAC and sGC harbour two catalytic domains, rather than one. Homology of the separate catalytic domains is higher to other cyanobacterial cyclases than among the C1 and C2 domains, suggesting that these enzymes have their origin in the fusion of two independent genes, instead of duplication of a single gene. From an evolutionary point of view, the absence of homologues in yeast, nematodes or *Drosophila*, which are evolutionary all closer to mammalians (Baldauf *et al*., 2000) suggest that the gene has been lost independently at several occasions during evolution. Considering the homology with sAC and cyanobacterial ACs, it seems likely that a single mutation in the ancestral gene has caused the transition of an adenylyl to a guanylyl cyclase.

3.5.2 sGC has guanylyl cyclase activity

Disruption of the *sgcA* gene resulted in a 90% loss of the cAMP induced cGMP in aggregating cells and a similar reduction in the GTPγS stimulated activity in lysates, but adenylyl cyclase activity was unaffected. GC activity in lysates of growing cells is hardly affected, suggesting the presence of at least one other guanylyl cyclase. Similar to the sAC and the 12tm adenylyl cyclases, sGC is strongly activated by Mn^{2+}. In the *sgc* null mutant, both in vegetative and in aggregating cells all Mn^{2+} dependent activity is, but in vegetative cells, a significant activity remains, when Mg^{2+}-GTP is used as substrate. This supports the presence of two distinct G-protein dependent guanylyl cyclase activities in *Dictyostelium*. sGC is mainly active during aggregation and at low levels in growing cells and is strongly activated by Mn^{2+}-ions. The other activity is present mainly in vegetative cells and weakly during aggregation. This activity is insensitive to Mn^{2+} and most likely reflects the recently identified *Dictyostelium* guanylyl cyclase DdGCA. This enzyme has the 12tm topology of mammalian adenylyl cyclases and is homologous to a group of guanylyl cyclases recently identified in *Plasmodium*, *Tetrahymena* and *Paramecium* (Carucci *et al*., 2000; Linder *et al*., 1999). GCA is mainly expressed during growth and post-aggregative development, but only weakly in aggregating cells, which would correlate with the activities described here.

3.5.3 Localisation and regulation of sGC

The amino acid composition of sGC predicts no transmembrane domains. Concordantly, the bulk of the sGC activity resides in the cytosolic fraction. This is similar to the sAC, which was initially purified as a soluble protein with AC activity from rat testis (Buck *et al.*, 1999). The purified sAC protein had a molecular weight of 48 kD, whereas the cDNA predicted a 187 kD protein. The protein sequence of the purified protein suggested that it was proteolytically processed from the 187 kD precursor. When expressed in HEK293 cells, the truncated form appeared 20-fold more active than the full length protein, suggesting an autoregulatory mechanism present in the full length protein. It would be interesting to see whether sGC undergoes similar posttranslational processing. In contrast, whereas sAC activity is GTPγS-insensitive, sGC activity is G-protein regulated. Previous experiments showed the involvement of the α-subunit of G2 in the activation of guanylyl cyclase. Whether $G_{\alpha}2$ binds directly to sGC or acts through an intermediate is as yet unclear. Homology between sAC and sGC extends throughout the protein sequences, but sGC harbours a ~ 1000 aa. N-terminal region that is absent from sAC, which could be the target for G-protein activation. The G-protein stimulated activity resides mainly in the particulate fraction. This raises the possibility of sGC translocation from the cytosol to the plasma membrane as a means of activation. However, upon GTPγS stimulation we could not detect an increase in Mn^{2+} stimulated activity in the particulate fraction or a decrease in the cytosol (data not shown). GFP-fusions and mutational analysis should establish whether activity requires translocation of sGC or separate membrane associated and cytosolic pools exist.

3.5.4 Function of sGC in *Dictyostelium*

Previous studies have suggested a function for cGMP in chemotaxis and phosphorylation of myosin light and heavy chain in *Dictyostelium*. Most information has come from studies on chemical mutants. *StreamerF* (*stmF*) shows higher and prolonged cGMP production, due to a lowered cGMP-dependent cGMP-phosphodiesterase activity. This mutant is characterized by prolonged myosin

phosphorylation and chemotaxis and the formation of large streams during aggregation. A study into a set of non-chemotactic mutants showed most mutants to have an altered cGMP response, of which KI-8 had no detectable levels of cGMP production (Kuwayama *et al.*, 1993).

In this light, the apparently normal phenotype of the *sgc* null mutant, in which the cGMP response to cAMP in aggregating cells is 10-fold lower, seems remarkable. At favourable laboratory conditions, *sgc*$^-$ cells can aggregate at the same speed as wild-type, but under more stringent conditions, the residual GCA activity is probably insufficient for proper aggregation. As *sgc*$^-$, null mutants in *ddgca* have no apparent phenotype (Roelofs *et al*., 2001a). These cells chemotax normally towards cAMP and have a similar cGMP response as wild-type. The phenotype of the knock-out suggests a nearly entire overlap in function, which could be expected for components that are involved in a crucial process as chemotaxis. Alternatively, the chemotactic mutants could have been affected in multiple genes and the role of cGMP therefore been overestimated. The presence of another guanylyl cyclase in the *Dictyostelium* database seems, because of the status of the project, unlikely. The generation of a double knock-out would give a definite answer to these questions. Finally, the highest expression levels of *sgc* during late development imply functions other than single cell chemotaxis, for example slug photo- and thermotaxis. However, *sgc*$^-$ slugs can still move efficiently to light when plated on charcoal-containing PB-agar (results not shown).

4. cGMP degradation

Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic *stmF* mutants.

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4 Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic *stmF* mutants

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4.1 Abstract

StmF mutants are chemotactic mutants that are defective in a cGMP phosphodiesterase (PDE) activity. We identified a novel gene, *PdeD*, that harbors two cyclic nucleotide-binding domains and a metallo-β-lactamase homology domain. Similar to *stmF* mutants, *pdeD*-null mutants displayed extensively streaming aggregates, prolonged elevation of cGMP levels after chemotactic stimulation, and reduced cGMP-PDE activity. *PdeD* transcripts were lacking in *stmF* mutant NP377, indicating that this mutant carries a *PdeD* lesion. Expression of a PdeD-YFP fusion protein in *pdeD*-null cells restored the normal cGMP response and showed that PdeD resides in the cytosol. When purified by immunoprecipitation, the PdeD-YFP fusion protein displayed cGMP-PDE activity, which was retained in a truncated construct that contained only the metallo-β-lactamase domain.

4.2 Introduction

Mutants in cGMP metabolism have implicated cGMP as intermediate for ligand-induced chemotaxis in *Dictyostelium* (Ross and Newell, 1979, 1981; Kuwayama *et al.*, 1993). Mutants KI8 and KI10 show no ligand-induced cGMP response and cannot chemotax (Kuwayama *et al.*, 1993). Streamer F (*stmF*) mutants are defective in cGMP-PDE activity (Van Haastert *et al.*, 1982). These mutants show an elevated and prolonged cGMP response and prolonged association of myosin with the cytoskeleton (Ross and Newell, 1981; Liu and Newell, 1988, 1991, 1994). When grown as colonies on dense bacterial lawns, *stmF* mutants form aggregates with very pronounced aggregation streams, a phenotype that under these conditions is not displayed by wild-type cells.

Three PDE genes have been identified in *Dictyostelium*; two of those, RegA (Shaulsky *et al.*, 1996; Thomason *et al.*, 1998) and PDE3 (Kuwayama *et al.*, 2001), belong to the large class of HD-domain PDEs that is commonly found in vertebrates (Mehats *et al.*, 2002). The third enzyme, PdsA (Lacombe *et al.*, 1986), is a class II PDE that is also found in yeast (Nikawa *et al.*, 1987). None of these genes are associated with the *stmF* locus.

In our search for targets of cyclic nucleotides, we identified a gene, *PdeD*, with two putative cyclic nucleotide (cNMP)-binding domains and a binuclear Zn^{2+}-binding domain. The latter domain forms the catalytic center of bacterial metallo-β-lactamases, which hydrolyze an amide bond in the β-lactam ring of carbapenem antibiotics (Carfi *et al.*, 1995) and are a major cause for widespread bacterial antibiotic resistance (Payne, 1993). We show that *pdeD*-null mutants phenocopy *stmF*

mutants. One of the cNMP-binding domains of PdeD most likely functions as an allosteric activator of the enzyme, whereas the metallo-β-lactamase homology domain catalyzes the hydrolysis of cGMP.

4.3 Materials and methods

4.3.1 Cell growth and development

D. discoideum strains NC4, XP55, and NP377 were grown in association with *Klebsiella aerogenes* on SM agar plates, and all other strains were grown in HL5 medium, supplemented with 5 µg/ml blasticidin or 20-200 µg/ml G418 for strains transformed with pBsrΔBam or neomycin selection markers, respectively. For developmental time courses, cells were harvested from bacterial plates or growth medium, washed with 10 mM NaH_2PO_4/K_2HPO_4 buffer, pH 6.5 (PB), plated at variable cell densities on PB agar (1.5% agar in PB), and incubated at 22°C.

4.3.2 Bioinformatics

The *Dictyostelium* genome and cDNA databases were screened with various sequences for cNMP-binding domains. In addition to the protein kinase (PK) A regulatory subunit (Mutzel *et al.*, 1987), a 419-base pair (bp) fragment of another candidate gene was hit. Eight cycles of BLAST (Altschul *et al.*, 1990) searches initiated with the 419-bp fragment and sequence assembly yielded contiguous DNA sequence of 3473 bp with at least fourfold coverage at any particular region. As of July 8, 2002, the sequence is available on the 80.3-kilobase (kb) contig JC3a201c05.r1, which was assembled by the *Dictyostelium* genome sequencing project Jena, Germany (http://genome.imb-jena.de/dictyostelium/) and is located on chromosome II (Gloeckner *et al.*, 2002). The sequence shows an open reading frame (ORF) with two cNMPs when analyzed with SMART (Schultz *et al.*, 1998) for PFAM domains (Bateman *et al.*, 2000). The sequence upstream of the ORF was interrupted by two short AT-rich regions with stop codons in all reading frames. To determine whether these regions were introns, oligonucleotides were designed that flanked both regions simultaneously (CCCTGATATGATTAATTCAATCTCTACG and CCCGCTTCATGTAATACCACCG). These oligos were used to perform RT-PCR of mRNA of NC4 cells starved for 10 h by use of the Qiagen Onestep RT-PCR kit (Qiagen, Hilden, Germany). A band of 600 bp was obtained and sequenced. This showed the presence and position of a 96- and a 122-bp intron. The putative start codon could then be identified, which was preceded by a 1.56-kb AT-rich region and a 3-kb ORF with weak homology to a *Brassica campestris* pollen-coat protein. The start codon conformed well to the *Dictyostelium* Kozak sequence, and because introns >1 kb are very rare in *Dictyostelium*, the complete 2601-nucleotide coding sequence could be established with confidence. For reasons described below, we named the gene *PdeD*, and the sequence of its ORF is deposited in GenBank under accession number AY047363.

4.3.3 Gene inactivation

Two DNA fragments of *PdeD* comprising nucleotides 2289-3109 and 1402-2195 were amplified by PCR using oligonucleotides that yielded a 5'-*Xba*I and 3'-*Bam*HI site on the first fragment and 5'-*Bam*HI and a 3'-*Eco*RI site on the second fragment. These fragments were cloned in tandem into the *Bam*HI/*Eco*RI sites and *Xba*I/*Bam*HI sites of pBsrΔBam (Sutoh, 1993). The construct was linearized with *Bam*HI, which

yielded the pBsrΔBam plasmid flanked by ~800 bp of 5' and 3' *PdeD* DNA. Homologous recombination with this construct causes insertion of the entire plasmid at position 2195 and a deletion of 94 bp.

The knockout (KO) construct was introduced into wild-type AX2 cells by electroporation, and transformed cells were selected by growth in the presence of 5 μg/ml blasticidin. Selected clones were screened for homologous recombination by two separate PCR reactions and analysis of Southern blots of genomic digests. Three of 200 clones tested from two separate transformations proved to carry a gene disruption. Three KO lines and three lines carrying different random vector integrations (RIs) were used for further analysis.

4.3.4 PdeD-YFP fusion constructs

PdeD gene fragments were created by PCR using oligonucleotides that generated 5'-*Bam*HI and 3'-*Xho*I sites. These fragments corresponded to residues 2-867 for full-length PdeD, 524-867 for PdeDΔNΔlac, and 321-572 for PdeDΔNΔC. The fragments were cloned into the *Bam*HI/*Xho*I-digested vector pB17SYFP, which is a derivative of pDXA-HC (Manstein *et al.*, 1995) that contains the coding sequence for enhanced yellow fluorescent protein (YFP) (Miyawaki *et al.*, 1997) downstream of the constitutive actin15 promoter. The constructs yield fusion proteins with YFP at the C-terminus of PdeD. The vectors were transformed into parent strain AX2 and the *pdeD*-null mutant clone KO96. Cells were selected in HL5 medium with 20 or 200 μg/ml G418.

4.3.5 Immunoprecipitation

Mouse monoclonal green fluorescent protein (GFP) antibody (αGFP) (25 μg) (Roche, Welwyn Garden City, U.K.) was incubated for 1 h at 4°C with a mixture of 250 μl each of slurries of protein G linked to Sepharose 4B (Sigma, St. Louis, MO) and protein A linked to Affiprep (Bio-Rad, Hercules, CA). The αGFP-linked matrix was washed with PBS (0.7% NaCl in PB) and resuspended to the original concentration. *Dictyostelium* cells, resuspended at 2×10^8 cells/ml in PBS, were lysed through Nucleopore filters. Lysates were cleared by centrifugation for 10 min at 14,000 × *g*, and 1 ml of cleared lysate was incubated for 4 h with 100 μl of αGFP-matrix suspension. The matrix was washed thoroughly with PDE assay buffer and resuspended in the same buffer.

4.3.6 Western Blot analysis

Cleared lysates equivalent to 10^5 cells per lane were subjected to 8% SDS-PAA gel electrophoresis. Size-fractionated proteins were transferred to nitrocellulose and probed with a 1:1000 dilution of αGFP antibody. Detection was performed with the Supersignal chemoluminescence kit (Pierce, Rockford, IL) using a horseradish peroxidase conjugated goat-anti-mouse secondary antibody (Promega, Madison, WI) according to the manufacturer's instructions.

4.3.7 cGMP-PDE assay and cGMP response

To measure cGMP-PDE activity, cleared cell lysate or a suspension of matrix linked to αGFP immunoprecipitate was incubated for 30 min at 22°C with 10^{-8} M ^{3}H-cGMP, 5 mM dithiothreitol, 0.2 mM IBMX, and 1 mM $MgCl_2$ in 20 mM HEPES, pH 7.0 (Kuwayama *et al.*, 2001). The reaction was terminated by boiling. ^{3}H-5'-GMP was further hydrolyzed by incubation for 30 min at 37°C with 0.5 mg/ml *Ophiophagus*

hannah snake venom to [^{3}H]guanosine, which was separated from [^{3}H]cGMP by adsorption of the latter to Dowex anion exchange resin.

To measure the cAMP-induced cGMP response, 10^8 cells/ml PB were stimulated with 10^{-7} M cAMP. Aliquots of cell suspension were rapidly mixed with an equal volume of 3.5% perchloric acid (vol/vol) at various intervals after stimulation. Lysates were neutralized with $KHCO_3$, and cGMP levels were measured by radioimmunoassay.

4.4 Results

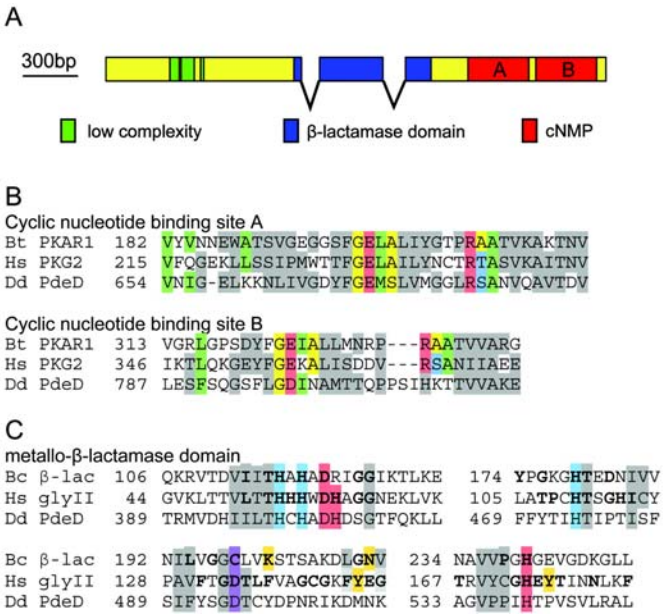

Fig. 4.1. PdeD gene structure and protein sequence. *A)* Schematic of the ORF of PdeD indicating the position of the introns, the metallo-β-lactamase domain, and the cyclic nucleotide-binding motifs. *B)* Alignment of the cNMP-binding motifs of PdeD, bovine PKA-RI, and human PKG2. Gray, conserved; green, hydrophobic pocket for purine binding; yellow, hydrogen bonding of backbone with exocyclic oxygens; red, side chain essential for hydrogen bond and ion-pair formation with ribose 2'-OH and exocyclic oxygens, respectively; blue, ser/thr that distinguishes guanine from adenine binding (Su *et al.*, 1995). *C)* Alignment of the β-lactamase domains of PdeD with those of the *B. cereus* β-lactamase 569/H/9 (Bc-βlac) and human glyoxylase II. Gray, conserved; bold letters, identical residues in either all β-lactamases or all glyoxylases; blue, binding to Zn^{2+}(I); red, binding to Zn^{2+}(II); purple, binding to water or to both Zn^{2+}(I) and Zn^{2+}(II); amber, binding to substrate (Carfi *et al.*, 1995; Concha *et al.*, 1996; Cameron *et al.*, 1999).

4.4.1 Structure of PdeD

Screening of *Dictyostelium* cDNA and genome databases with consensus sequences for cNMP-binding domains yielded a novel gene, *PdeD*, with an ORF of 2601 bp. RT-PCR revealed that *PdeD* harbors two introns of 96 and 122 bp, respectively, at positions 1108 and 1565 (Fig. 4.1A). Analysis of the domain architecture of the *PdeD* gene with SMART (Schultz *et al.*, 1998) suggests subdivision of the gene into three regions: 1) an N-terminal region rich in low-complexity sequence and containing no known functional domains. Such N-terminal regions are common features of *Dictyostelium* genes (Mann and Firtel, 1991; Roelofs *et al.*, 2001b); 2) a middle region, which contains a PFAM metallo-β-lactamase

domain (Bateman *et al.*, 2000); and 3) a C-terminal region, which contains two PFAM cNMP-binding domains.

Double cNMP-binding domains are typically found in the PKA regulatory subunit (PKA-R) and in PKG. In the latter protein, they are located upstream of the PK catalytic region (Francis and Corbin, 1999). cAMP-binding proteins, such as PKA-RI and PKA-RII, share a common folding pattern, as determined from cocrystal structures with cAMP (Diller *et al.*, 2001; Su *et al.*, 1995). Homologous cGMP-binding proteins are predicted to possess a similar structure (Francis and Corbin, 1999). Critical residues for cNMP binding in both cNMP domains of *PdeD* were aligned with equivalent residues in bovine PKA-RI and human PKG2 (fig. 4.1B). The major determinant for nucleotide specificity is Ala^{210} (site A) and Ala^{334} (site B) in bovine PKA-RI. The equivalent position in cGMP-binding proteins is a Ser or Thr (blue), which would allow hydrogen bond formation with the C^2-NH_2 group of the guanine base (Shabb *et al.*, 1991). Site A in PdeD harbors a serine at the position equivalent to Ala^{210}, suggesting that this site preferentially binds cGMP (fig.4.1B). Site B carries a Lys^{811} at the position equivalent to Ala^{334}, which is not likely to contribute to either cAMP or cGMP binding. Site B shows further deviations from the consensus binding motif. The essential arginine (in red) that forms an ion-pair with the equatorial exocyclic phosphate oxygen of cNMP (equivalent to Arg^{209} and Arg^{333} in PKA-RI) (Su *et al.*, 1995) is replaced by His. In addition, an insertion of three residues directly precedes this key residue in site B. Additional residues at this position are absent in all characterized cNMP-binding proteins.

The metallo-β-lactamase domain contains a binuclear Zn^{2+}-binding motif that was first found in the bacterial class B β-lactamases. These enzymes catalyze the hydrolysis of an amide bond in the β-lactam ring of penicillin-type antibiotics (Carfi *et al.*, 1995; Concha *et al.*, 1996). A similar domain is found in glyoxylase II. Here, it hydrolyses the thiolester bond between gluthathione and 2-hydroxycarboxylic acid during inactivation of toxic methylglyoxal, a side product of glycolysis (Cameron *et al.*, 1999). The crystal structure for both enzymes has been solved. Fig. 4.1C shows the alignment of the *PdeD* metallo-β-lactamase homology region with that of *Bacillus cereus* β-lactamase II (Bc-βlac) and human glyoxylase II. Apart from the conserved His and Asp residues that are involved in metal binding, the PdeD sequence does not conform to any of the other enzymes with respect to residues that are specifically conserved for that class of enzyme (in bold), particularly those that are involved in substrate binding (in amber). The yeast and *Dictyostelium* class II phosphodiesterases, PDE1 and PdsA, also harbor the highly conserved HxHxDHxxG motif, which is the most characteristic feature of metallo-β-lactamase domain. However, unlike PdeD, these proteins share little homology with this domain elsewhere in the protein or with PdeD itself. It is therefore not possible to deduce the function of the PdeD from its sequence with any confidence.

4.4.2 Developmental regulation and disruption of the PdeD gene

To gain more insight into the function of PdeD, we studied its developmental regulation and disrupted the gene. A Northern blot of *PdeD* probed to RNA isolated during the 28-h developmental life cycle shows that *PdeD* is transcribed into an mRNA of ~2.9 kb during growth and development, with the highest level of expression during aggregation (fig. 4.2A).

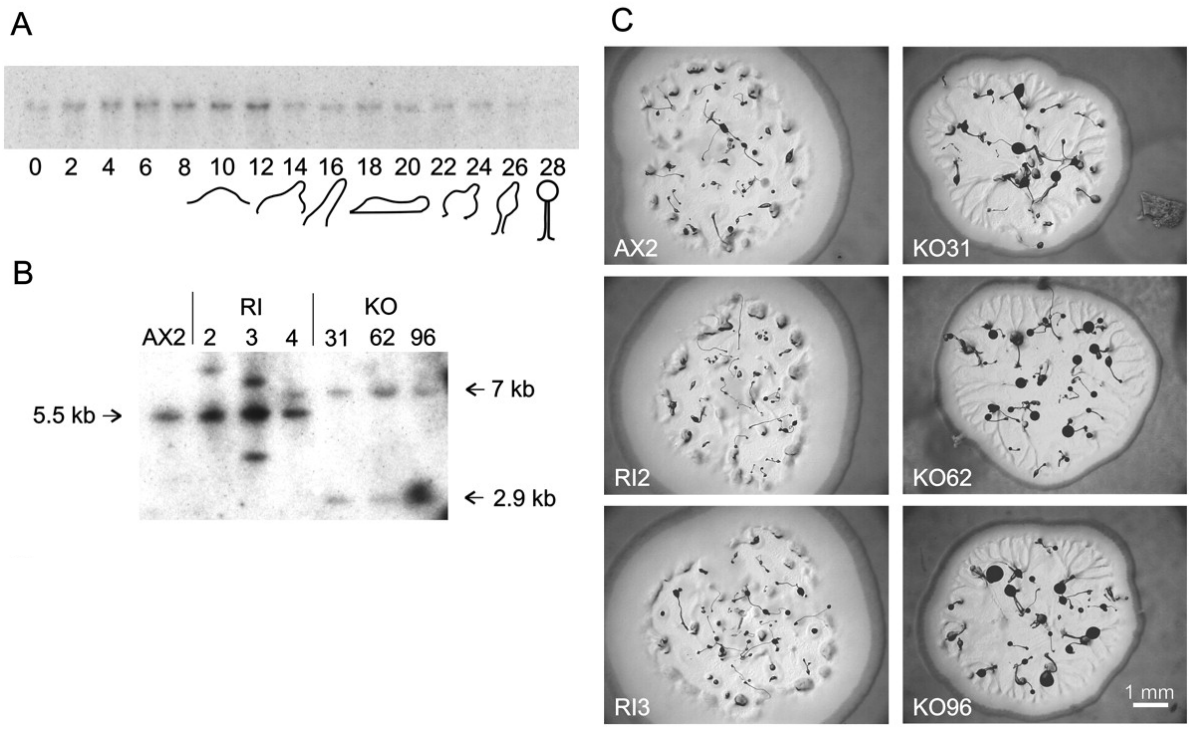

Fig. 4.2. Developmental regulation and gene disruption of *PdeD*. *A)* Developmental regulation. *D. discoideum* NC4 cells were washed free of bacteria and incubated on PB agar at 2×10^6 cells/cm^2 until mature fruiting bodies had formed. RNA was isolated at 2-h intervals, and Northern transfers were probed with a [^{32}P]dATP-labeled *PdeD* probe. RNA markers of 0.28-6.6 kb (Promega) were run on the same gel and stained with ethidium bromide to estimate the size of the *pdeD* mRNA, which appeared to be ~2.9 kb. *B)* Genomic digests of putative gene disruptants. AX2 cells were transformed with a linear construct of vector pBsrΔBam flanked by ~800 bp of 5' and 3' DNA of the *PdeD* gene. Clones 31, 62, and 96 were selected by two PCR reactions as putative *PdeD* gene knockouts (KO) and clones 2, 3, and 4 as random integrants (RI). Genomic DNA of the KO and RI clones was double-digested with *Xho*I and *Nsi*I, and Southern transfers were probed with *PdeD* DNA as described above. In AX2 and RI cells, a 5.5-kb band should be present, with additional bands of unknown size marking the random vector integrations. Insertion of pBsrΔBam in the *PdeD* gene by homologous recombination should yield bands of 2.9 and 7 kb. *C)* The morphology of colonies of parent strain AX2, two RI and three *pdeD* KO clones grown on *Klebsiella aerogenes* lawns. Note the streaming phenotype and significantly larger fruiting bodies of the KO clones.

The *PdeD* gene was inactivated by homologous recombination to generate cell line *pdeD*. Construct integration was verified by two separate PCR reactions and by Southern analysis of genomic digests (fig. 4.2B). Three KO constructs and three clones with randomly integrated vectors (RI) were selected for further analysis. All KO and RI clones formed normal-looking aggregates, slugs, and fruiting bodies when cells were plated on nonnutrient agar. However, the KO cells showed an abnormal morphology when plated out clonally on bacterial lawns (fig. 4.2C). Colonies of the parent strain AX2 and the control RI lines showed mound-shaped aggregates at some distance from the growth edge and fruiting bodies toward the center of the colony. Formation of aggregation streams is not evident at this high cell density. The KO clones showed marked formation of large aggregation streams. In addition, aggregate and fruiting body size was larger than in the control cell lines.

This phenotype resembles the phenotype of a class of chemotactic mutants called streamers, which fall into different complementation groups (Ross and Newell, 1979).

None of the mutated genes have been identified, but the most thoroughly characterized *stmF* mutants are defective in a cGMP-stimulated cGMP-PDE activity (Van Haastert *et al.*, 1982) that was first identified in a mutant defective in cAMP phosphodiesterase (Dicou and Brachet, 1980). Absence of the cGMP phosphodiesterase results in an elevated and prolonged cGMP response on stimulation with chemoattractant, which is assumed to cause the streamer phenotype (Ross and Newell, 1981).

4.4.3 The cGMP response and cGMP-PDE activity in the pdeD-null mutant

We first measured whether the *pdeD* mutant showed the characteristic elevated and prolonged cGMP response of the *stmF* mutants. Fig. 4.3A shows that the cGMP response in the *pdeD* KO lines was 3 times higher than in the RI lines. Moreover, cGMP levels were still elevated in the KO lines at 30 s after stimulation, when in the control lines, cGMP was already back to the basal level. This almost exactly mimics the kinetics of the cGMP response in the *stmF* mutants (Ross and Newell, 1981) and suggests that *pdeD* encodes the cGMP-PDE that is defective in these mutants.

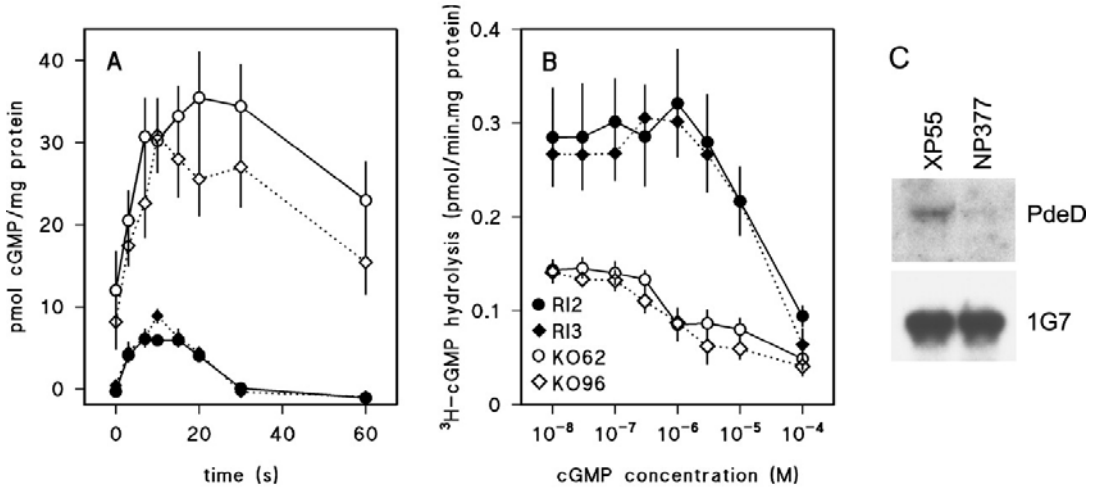

Fig. 4.3. Absence of cGMP-PDE in *pdeD-null* mutants and *PdeD* transcripts in *stmF*. *A)* cGMP response. Two *pdeD* KO cell lines and two control RI lines were developed on PB agar until aggregation territories had formed. Cells were stimulated with 10^{-7} M cAMP, and accumulated cGMP levels were determined by radioimmunoassay at the indicated time points. Data were standardized on the protein content of the cell suspension. Means and SEs of two experiments, each consisting of six time courses, are presented. *B)* cGMP-PDE activity. KO and RI cells were harvested and lysed while forming territories. Hydrolysis of ^{3}H-cGMP was determined in the presence of increasing concentrations of cGMP in lysates that were diluted to degrade not more than 30% of the ^{3}H-cGMP. Data were standardized on the protein content of the cleared lysate. Means and SEs of three experiments performed in triplicate are presented. *C) PdeD* mRNA. Total RNA was isolated from *stmF* mutant NP377 and parent strain XP55 after 8 h of starvation. A Northern transfer was first probed with ^{32}P-labeled *PdeD* DNA, then stripped and reprobed with the *1G7* rRNA gene, which is constitutively expressed throughout development (Williams *et al.*, 1987).

To test this directly, we measured cGMP-PDE activity in lysates of KO and RI cell lines in the presence of increasing concentrations of cGMP. Fig. 4.3B shows that in the control RI cell lines, unlabeled cGMP induced a modest stimulation of ^{3}H-cGMP hydrolysis between 3×10^{-8} and 10^{-6} M before it showed significant competition with ^{3}H-cGMP at 10^{-5} M. In the KO cell lines, total ^{3}H-cGMP hydrolysis was

strongly reduced and no stimulation by cGMP was evident. This suggests that the two KO lines lack a cGMP-PDE activity. The remaining activity is most likely the previously characterized cGMP phosphodiesterase PDE3, which is not stimulated by cGMP (Kuwayama *et al.*, 2001).

To obtain further evidence that *stmF* mutants are defective in PdeD, we probed mRNA extracted from *stmF* mutant NP377 and its parent strain XP55 (Ross and Newell, 1981) with ^{32}P-labeled *PdeD* DNA. Fig. 4.3C shows that *PdeD* mRNA is greatly reduced in *stmF* mutant NP377.

4.4.4 Expression of PdeD-YFP fusion constructs

The loss of cGMP-PDE activity from the *pdeD*-null cell lines can in theory be caused by an (indirect) inhibitory effect of PdeD on the expression of the gene that actually encodes a cGMP-PDE. We have tried to express His-tagged PdeD and GST-PdeD fusion proteins in *Escherichia coli* to measure the enzyme activity of purified PdeD directly. Although the tagged proteins were expressed in *E. coli*, we have not yet been able to isolate them in native form.

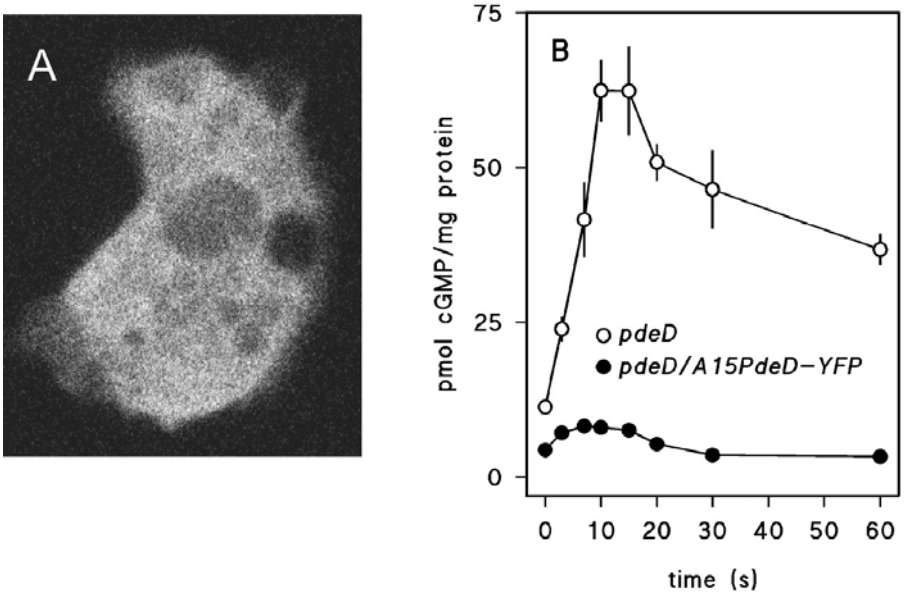

Fig. 4.4. Expression of PdeD-YFP in *pdeD-null* cells. *A)* PdeD-YFP expression. A fusion construct of YFP with full-length PDE was expressed under the actin 15 promoter in *pdeD* KO clone 96. An optical section of YFP fluorescence in a living *pdeD/A15PdeD-YFP* cell was obtained with a Leica DMRBE confocal laser scanning microscope. *B)* cGMP response. *pdeD* and *pdeD/A15PdeD-YFP* cells were harvested when forming aggregation territories, stimulated with 10^{-7} M cGMP, and assayed for accumulated cGMP levels. Means and SEs of two experiments, each consisting of six time courses, are presented.

As an alternative approach, we fused the *PdeD* gene to the gene for enhanced YFP (Miyawaki *et al.*, 1997) and expressed the fusion construct under control of the constitutive actin 15 promoter (A15) in the *pdeD*-null mutant. Confocal microscopy of *pdeD*/A15PdeD-YFP cells shows that the protein is present in the cytosol (Fig.4.4A). Expression of PdeD-YFP brought the cAMP-induced cGMP response in the *pdeD*-null mutant back to the level that is normal for wild-type cells, indicating that the phenotype of the null mutant is caused by loss of PdeD (Fig. 4.4B).

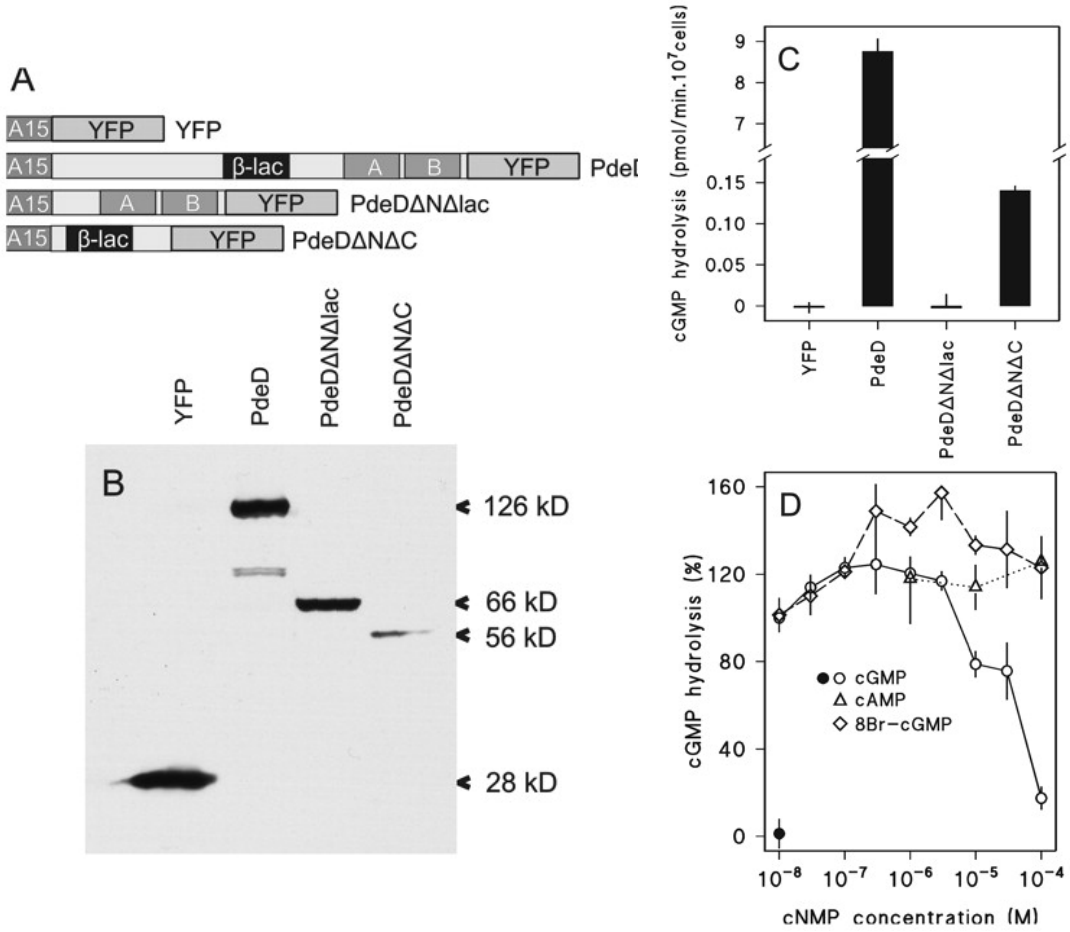

Fig. 4.5. Expression and activity of full-length and truncated YFP-PdeD constructs. *A)* Schematic of four fusion constructs of the actin 15 promoter, full-length or partial PdeD sequence, and YFP. Transformants of either of the four constructs were grown under selection with 200 µg/ml G418 to obtain YFP expression for the PdeDΔNΔC construct, which showed no YFP fluorescence under normal selection conditions. *B)* Western blots of expressed YFP fusion proteins. Cleared lysates of vegetative cells transformed with the four YFP constructs were size-fractionated by SDS-PAGE and immunoblotted with mouse monoclonal αGFP antibodies. The size of the bands as deduced from their position relative to protein markers is indicated. *C)* cGMP-PDE activity in transformants. Cleared lysates were assayed for hydrolysis of 10^{-8} M ^{3}H-cGMP at 10-, 30-, 100-, 300-, and 1000-fold dilutions. Activities were calculated from dilutions that did not hydrolyze >30% of the substrate. Means of two experiments performed in duplicate are presented. *D)* Immunoprecipitation of YFP fusion proteins. Cleared lysates of YFP (closed symbol) and PdeD-YFP (open symbols) -transformed cells were incubated with αGFP antibody linked to a mixed protein A/protein G coupled matrix. The matrix was thoroughly washed, then incubated with 10^{-8} M ^{3}H-cGMP and the indicated concentrations of cGMP, 8-Br-cGMP, and cAMP for 30 min and assayed for ^{3}H-5'-GMP. Data are expressed as percentage of ^{3}H-cGMP hydrolysis obtained from the PdeD-YFP immunoprecipitate in the absence of added cyclic nucleotides. Means of two experiments performed in duplicate are presented.

Two truncated forms of PdeD, ΔNΔlac with only the cNMP-binding domains and ΔNΔC with only the β-lactamase domain, were also expressed as YFP fusion proteins under the A15 promoter. A15YFP-transformed cells were included as control (Fig. 4.5A). Fig. 4.5B shows Western blots of the expressed proteins detected with GFP antibody. The YFP construct yielded the highest level of protein expression, followed by the full-length PdeD-YFP construct. The ΔNΔlac-YFP construct was also expressed to reasonable levels, but the ΔNΔC-YFP construct was expressed rather poorly. Lysates from cells transformed with full-length PdeD-YFP showed by far the

highest cGMP-PDE activity (Fig. 4.5C). No activity was found in either ΔNΔlac-YFP or A15-YFP lysates, but the ΔNΔC-YFP lysates showed significant cGMP-PDE activity despite the low levels of protein expression. The activity was lost within a few hours from the ΔNΔC-YFP lysates, whereas it was stable for at least 1 day for the full-length constructs. This led us to suppose that protein expression from the ΔNΔC-YFP construct may seem so low because the protein is unstable. Nevertheless, the ΔNΔC-YFP protein, with only the β-lactamase domain present, still displayed cGMP-PDE activity, whereas the ΔNΔlac-YFP protein, which was expressed to much higher levels, showed none. This strongly suggested that the PDE activity resides in the β-lactamase domain.

The full-length PdeD-YFP and YFP proteins were immunoprecipitated with anti-GFP antibody. There was no activity associated with the YFP immunoprecipitate. The cGMP-PDE activity in the PdeD-YFP immunoprecipitate was ~5% of the lysate from which it was derived. The immunoprecipitated activity was slightly activated by submicromolar cGMP concentrations and more strongly by 8-Br-cGMP, which is a good activator but a poor substrate for the stmF cGMP-PDE (Kesbeke *et al.*, 1985). cAMP was a very poor competitor for ^{3}H-cGMP hydrolyzing activity. This yields convincing evidence that *PdeD* encodes a cGMP-specific phosphodiesterase activity.

4.5 Discussion

A novel gene, *PdeD,* was identified from the sequencing project of *Dictyostelium discoideum* chromosome II (Gloeckner *et al.*, 2002). *PdeD* encodes a protein with two cNMP-binding domains and a binuclear Zn^{2+}-binding motif, which is common to the metallo-β-lactamases. The same gene was recently identified, but not functionally characterized, from the genome sequencing project by Goldberg *et al.* (2002) and named *GbpA*. These authors also detected three other proteins with cNMP-binding motifs (*GbpB-D*). We also found *GbpB*; knockout and overexpression studies show that this gene, which we have named *PdeE*, encodes a cAMP phosphodiesterase (Meima, Weening, and Schaap, P., unpublished observations).

The cNMP-binding site A of *PdeD* harbors a characteristic Ser for cGMP binding and has all the essential residues for binding to the purine, ribose, and cyclic phosphate moieties of cGMP (Fig.4.1B). Binding site B shows several deviations in critical residues as well as an insertion of three amino acids in a region that in PKA-R has critical interactions with both the ribose and cyclic phosphate ring of cAMP (Su *et al.*, 1995). Site B may therefore not be functional.

The function of the histidine-rich binuclear Zn^{2+}-binding motif was not immediately obvious. The domain forms the catalytic center of three unrelated enzymes: metallo-β-lactamase, glyoxylase II, and a less-well-characterized bacterial arylsulfatase, which hydrolyze amide, thiolester, and sulfate ester bonds, respectively (Barbeyron *et al.*, 1995; Carfi *et al.*, 1995; Cameron *et al.*, 1999). A histidine-rich metal-binding domain is also essential for catalysis in the cNMP phosphodiesterases, but the configuration of the histidines in this domain is entirely different (Xu *et al.*, 2000). However, two low-affinity PDEs, PdsA from *Dictyostelium* (Lacombe *et al.*, 1986) and PDE1 from *S. cerevisiae* (Nikawa *et al.*, 1987), harbor a histidine-rich motif that is similar to that of the metallo-β-lactamases, indicating that this motif can also mediate the hydrolysis of cNMPs. The observation that *pdeD*-null mutants phenocopied *stmF* mutants, which are defective in cGMP-PDE, suggested that the hydrolytic activity encoded by its β-lactamase domain degrades cGMP.

4.5.1 The PdeD gene is most likely defective in *stmF* mutants

The *stmF* mutants provided the first evidence that cGMP might mediate induction of chemotaxis by the *Dictyostelium* chemoattractants cAMP and folic acid (Ross and Newell, 1979, 1981). Selected for their propensity to form long aggregation streams when grown clonally, they were later shown to be defective in a cGMP-PDE activity (Van Haastert *et al.*, 1982; Coukell and Cameron, 1986). As a consequence, they show a prolonged cGMP response on stimulation with chemoattractant, which was correlated with a prolonged period but reduced rate of cell movement (Ross and Newell, 1981; Newell and Liu, 1992; Segall, 1992).

The *PdeD* mutants also showed extensive streaming when plated clonally with bacteria (Fig. 4.2C), and further biochemical analysis (Fig. 4.3) showed that their cGMP response was similarly prolonged and their cGMP-PDE activity similarly reduced, as is the case for the *stmF* mutants. The *stmF* genetic locus maps to chromosome II (Coukell and Cameron, 1985), and so does the *PdeD* sequence. In combination with the observation that the *stmF* mutant NP377 expressed almost no *PdeD* mRNA (Fig. 4.3C), this suggests that the defective gene in the *stmF* mutants is *PdeD*. However, we have not been able to find mutations in the promoter of the *stmF* PdeD gene, which could account for reduced transcription. Final evidence has to await complementation of the *stmF* mutants by *PdeD* and identification of the genetic lesion.

4.5.2 The PdeD catalytic activity resides in its Metallo-β-Lactamase domain

The full-length *PdeD* gene and *PdeD* truncations that either lacked the β-lactamase domain or contained only this domain were expressed in *Dictyostelium* cells as YFP fusion proteins (Fig.4.5). Only the full-length protein and the protein that contained the β-lactamase domain showed activity, indicating that the PDE-catalytic activity is provided by the β-lactamase domain. The full-length protein was purified by immunoprecipitation with GFP antibodies. The purified PdeD showed cGMP-PDE activity that was stimulated by cGMP and by 8-Br-cGMP, as was previously described for the enzyme lacking in *stmF* mutants (Kesbeke *et al.*, 1985). In our hands, the stimulation by cGMP was less pronounced than described by Kesbeke *et al.*, which is perhaps a result of the use of different parental strains. In the original biochemical characterization of the enzyme, stimulation by cGMP was also not noted (Dicou and Brachet, 1980). 8-Br-cGMP induced a more significant stimulation, because it is a poor substrate (Kesbeke *et al.*, 1985). cAMP was no competitor for ^{3}H-cGMP hydrolysis. This indicates that the *PdeD* gene product is a cGMP-specific phosphodiesterase.

4.5.3 The cNMP-binding domain A of PdeD may mediate allosteric activation

The cGMP-PDE that is lost in *stmF* mutants is allosterically activated by cGMP. Studies with cGMP analogues showed that cyclic nucleotide specificity of the activator and catalytic sites of the enzyme are not identical (Kesbeke *et al.*, 1985). Both sites are specific for cGMP, do not bind cAMP, and do not tolerate modification of the exocyclic oxygens. In addition, however, the catalytic site does not tolerate modification of cGMP at N^1H/C^6O, C^8, and $O^{3'}$, whereas the activator site does not tolerate modification of C^2-NH_2 and the 2'-OH. The latter two positions would characteristically interact with Ser^{681} and Glu^{671} in cNMP-binding domain A of PdeD (Fig. 4.1B). Binding site B does not contain the conserved Ser/Thr at the equivalent position and is, as discussed above, probably not functional as a cGMP-binding site.

8-Br-cGMP is a very poor PdeD substrate but a good agonist for the activator site. This also suggests that the activator site is homologous to eukaryotic cNMP-binding domains, which bind cyclic nucleotides in the *syn* conformation that is enforced by the bulky bromine at the 8-position of the purine ring (de Wit *et al.*, 1982). The nucleotide specificity of the catalytic domain does not resemble that of any known cGMP-binding protein. Elucidation of the crystal structure of PdeD will be necessary to understand its interaction with the substrate and the manner by which the cNMP-binding domain activates catalytic activity.

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5. cAMP degradation

Characterization of a cAMP-stimulated cAMP phosphodiesterase in *Dictyostelium discoideum*.

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5 Characterization of a cAMP-stimulated cAMP phosphodiesterase in *Dictyostelium discoideum*

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5.1 Abstract

A cyclic nucleotide phosphodiesterase, PdeE, that harbors two cyclic nucleotide binding motifs and a binuclear Zn^{2+}-binding domain was characterized in *Dictyostelium*. In other eukaryotes, the *Dictyostelium* domain shows greatest homology to the 73-kDa subunit of the pre-mRNA cleavage and polyadenylation specificity factor. The *Dictyostelium PdeE* gene is expressed at its highest levels during aggregation, and its disruption causes the loss of a cAMP-phosphodiesterase activity. The *pdeE* null mutants show a normal cAMP-induced cGMP response and a 1.5-fold increase of cAMP-induced cAMP relay. Overexpression of a PdeE-yellow fluorescent protein (YFP) fusion construct causes inhibition of aggregation and loss of the cAMP relay response, but the cells can aggregate in synergy with wild-type cells. The PdeE-YFP fusion protein was partially purified by immunoprecipitation and biochemically characterized. PdeE and its *Dictyostelium* ortholog, PdeD, are both maximally active at pH 7.0. Both enzymes require bivalent cations for activity. The common cofactors Zn^{2+} and Mg^{2+} activated PdeE and PdeD maximally at 10 mM, whereas Mn^{2+} activated the enzymes to 4-fold higher levels, with half-maximal activation between 10 and 100 μM. PdeE is an allosteric enzyme, which is ~4-fold activated by cAMP, with half-maximal activation occurring at about 10 μM and an apparent K_m of ~1 mM. cGMP is degraded at a 6-fold lower rate than cAMP. Neither cGMP nor 8-Br-cAMP are efficient activators of PdeE activity.

5.2 Introduction

In mammalian cells the inactivation of the ubiquitous second messengers cAMP and cGMP is achieved by no less than 11 different families of the class I phosphodiesterases (PDEs).[1] These families differ in substrate specificities, endogenous and exogenous regulators, and targeting to different subcellular compartments. All families share the same conserved catalytic domain at the carboxyl

[1] The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; cNMP, cyclic nucleotide monophosphate; YFP, yellow fluorescent protein; GFP, green fluorescent protein; CPSF, cleavage and polyadenylation specificity factor; PKA, cAMP-dependent protein kinase; PKG, cGMP dependent protein kinase; cAR, cAMP receptor; ACA, adenylyl cyclase A; 8-Br-cAMP, 8-bromoadenosine 3':5'-monophosphate; 8-Br-cGMP, 8-bromoguanosine 3':5'-monophosphate; DTT, dithiothreitol; IBMX, isobutylmethylxanthine; ORF, open reading frame; PFAM, protein family database of alignments and hidden Markov models; SMART, simple modular architecture research tool; KO, knockout; RI, random integrant.

terminus, whereas the domains required for targeting and regulation of enzyme activity are mainly localized at the amino terminus (Mehats *et al.*, 2002; Houslay, 2001; Houslay and Milligan, 1997).

In the social amoeba *Dictyostelium discoideum*, the class I PDEs are represented by the cAMP-PDE RegA (Shaulsky *et al.*, 1996; Thomason *et al*., 1998; Shaulsky *et al*., 1998) and the cGMP-PDE PDE3 (Kuwayama *et al.*, 2001). Lower eukaryotes additionally use class II PDEs, which have an entirely different catalytic center. These enzymes are exemplified by PDE1 from yeast (Nikawa *et al.*, 1987) and PdsA from *Dictyostelium* (Lacombe *et al*., 1986).

Recent screens of *Dictyostelium* genomic and cDNA data banks for proteins with cyclic nucleotide monophosphate (cNMP) binding motifs identified two proteins that each contained two cNMP binding motifs and the binuclear Zn^{2+}-binding domain that forms the catalytic center of metallo-β-lactamases (Meima et al., 2002; Goldberg et al., 2002; Carfi et al., 1995). The corresponding genes were named *PdeD* and *PdeE* by us (Meima *et al.*, 2002) and *GbpA* and *GbpB* by others (Goldberg *et al.*, 2002; Bosgraaf *et al.*, 2002a). Gene disruption and overexpression studies of *PdeD/GbpA* showed that the gene encodes the cGMP-stimulated cGMP phosphodiesterase, which is lacking in the chemotactic *stmF* mutants (Meima *et al.*, 2002; Bosgraaf *et al.*, 2002a; Ross and Newell, 1981). Analysis of truncated PdeD constructs revealed that the catalytic activity was provided by the metallo-β-lactamase domain (Meima *et al.*, 2002).

Here, we report gene ablation, overexpression, and biochemical characterization of the other gene, *PdeE/GbpB*. This gene encodes a cAMP-stimulated cAMP-PDE that is highly active during the aggregation stage of development. A bioinformatics approach showed that a subregion of the metallo-β-lactamase domain of PdeE and PdeD shares additional homology with a 73-kDa factor that is part of the complex for pre-mRNA cleavage and polyadenylation (Jenny *et al*., 1996). Because the endonuclease (phosphodiesterase) activity of this complex is still unknown (Wahle and Ruegsegger, 1999), we speculate that it is provided by the highly conserved metallo-β-lactamase domain of the 73-kDa factor.

5.3 Experimental procedures

5.3.1 Cell Growth and Development

D.discoideum strain NC4 was grown in association with *Klebsiella aerogenes* on SM agar plates, and all other strains were grown in HL5 medium, which was supplemented with 200 μg/ml uracil for the *ura*$^-$ strain DH1 (Caterina *et al.*, 1994) and 200 μg/ml G418 for the AX2 cells transformed with YFP or PdeE-YFP constructs. For developmental time courses, cells were harvested from bacterial plates or growth medium, washed with 10 mM PB buffer (NaH_2PO_4 and K_2HPO_4, pH 6.5), plated at variable cell densities on PB agar (1.5% agar in PB buffer), and incubated at 22 °C.

5.3.2 Gene Identification

The *Dictyostelium* genome and cDNA databases were screened with consensus sequences for cyclic nucleotide binding motifs. Two candidate genes, *PdeD* (Meima *et al.*, 2002) and *PdeE*, were identified. *PdeE* was assembled after several cycles of data base screening, yielding the complete coding sequence and 5'-untranslated region with at least 5-fold coverage. The gene is part of the recently published sequence of chromosome II (Glockner *et al.*, 2002) and was also identified by other workers

(Goldberg *et al.*, 2002). Similar to PdeD, PdeE showed an ORF with two PFAM cNMP binding motifs (Bateman *et al*., 2002) when analyzed with SMART (Schultz *et al.*, 1998), and the sequence upstream of the ORF was interrupted by two short AT-rich regions. To determine whether these regions were introns, oligonucleotides were designed that flanked the two regions (5'-ATGAATTCTAAATATGGGGATAA CATTATAG-3' and 5'-CCAATTCTTGCTGA AATCACTGC-3' for the 5'-intron and 5'-TGCAGATCATGATAGTGGTATCCTTC-3' and 5'-GATGGTAACTCCAAAA GGTTTGCC-3' for the 3'-intron) to perform reverse transcription PCR of the mRNA of 10-h starved NC4 cells. Bands of 192 and 410 bp, respectively, were obtained and sequenced, showing the presence of a 202 and a 80 bp intron. The 3288-kb ORF of PdeE could then be established, and its sequence has been in GenBank™ under accession number AY047364 since July 19, 2001.

5.3.3 Gene Inactivation

The PdeE gene was inactivated in a *ura*$^-$ cell line by homologous recombination (De Lozanne, 1987) with a linear construct that contains ~1 kb each of the 5'- and 3'-regions of the *PdeE* gene flanking the PJB1 vector, which contains the *URA+* selection cassette (Kalpaxis *et al.*, 1990). Two *PdeE* fragments comprising nucleotides 2961-4427 and 1689-2835 were amplified with oligonucleotides that yielded a 5'-*Pst*I and 3'-*Bam*HI on the first fragment and a 5'-*Bam*HI and 3'-*Xba*I site on the second fragment. The fragments were cloned in tandem in the *Pst*I-*Bam*HI- and *Xba*I-*Bam*HI-digested URA vector PJB1 (gift of Peter N. Devreotes). The two constructs were linearized with *Bam*HI, which yielded the PJB1 plasmid flanked by ~1 kb of 5'- and 3'-DNA of the *PdeE* gene. Homologous recombination with this construct causes insertion of the entire plasmid at position 2835 of *PdeE* and the deletion of about 100 nucleotides. The knockout construct was introduced into the *ura*$^-$ strain DH1, and transformed cells were selected by growth in FM medium (Franke and Kessin, 1977) in the absence of uracil. Selected clones were screened for homologous recombination by two separate PCR reactions and analysis of Southern blots of genomic digests (Fig. 5.2A). 11 of 90 clones derived from two transformations carried a gene disruption (*pdeE*). Three knockout (KO) and three random integrant (RI) clones were used for further analysis.

5.3.4 PdeE-YFP Fusion Constructs

The complete *PdeE* ORF was amplified by PCR using the Advantage™ genomic PCR kit (BD Biosciences) and the oligonucleotides 5'-CGGGGATCCAATTCTAAA TATGGGGATAACATTATAGATTTTC-3' and 5'-GGGCTCGAGTTAAACCTT CAATTGGATAAACTTTTCTTGG-3', which generate 5'-*Bam*HI and 3'-*Xho*I sites, respectively. The *Bam*HI/*Xho*I-digested PCR product was cloned into the *Bam*HI/*Xho*I-digested vector pB17SYFP, which is a derivative of pDXA-HC (Manstein *et al.*, 1995) that contains the coding sequence for the enhanced YFP (Miyawaki *et al.*, 1997) downstream of the constitutive actin15 promoter. The construct yields a 1335-amino acid fusion protein with YFP (239 amino acids) at the carboxyl terminus of PdeE (1096 amino acids) in which the carboxyl terminal Ser and Ile of PdeE are replaced by Thr and Arg and fused to Asp and the start Met of YFP. The integrity of the PdeE sequence was verified by DNA sequencing. Two errors were detected, *i.e.* a neutral AAT to AAC transition in Asn^{540} and an ATT to GTT transition, which transforms Ile^{1036} into Val^{1036}. The Ile^{1036} is not conserved, and there is often a Val at this position in homologous proteins; thus, protein function is unlikely to be affected. The actin15PdeE-YFP vector and pB17SYFP were

transformed into parent strain AX2. Cells were selected in HL5 medium with 200 µg/ml G418.

5.3.5 RNA Isolation and Analysis

Total RNA was isolated from 2×10^7 cells, size fractionated on 1.5% agarose gels containing 2.2 M formaldehyde (Nellen *et al.*, 1987), and transferred to nylon membranes. Membranes were hybridized to [^{32}P]dATP-labeled DNA probes according to standard procedures. 3 µl of 0.28-6.6-kb RNA markers (Promega) were run on the same gel and stained with ethidium bromide to estimate the size of the *PdeE* mRNA.

5.3.6 Cyclic Nucleotide Phosphodiesterase Assays

To measure cAMP or cGMP hydrolysis, cells were resuspended in 250 mM sucrose in 20 mM Hepes, pH 7.0 and lysed through Nuclepore filters (pore size 3.0 µM). Lysates were cleared by centrifugation for 20 min at 40,000 × *g*. Cleared cell lysates or a suspension of matrix linked to αGFP immunoprecipitate were incubated for 30 min at 22 °C with 10 nM or 10 µM [^{3}H]cGMP or [^{3}H]cAMP, 5 mM dithiothreitol (DTT), 0.2 mM IBMX, and 1 mM $MgCl_2$ in 20 mM Hepes, pH 7.0 and assayed for 5'-[^{3}H]AMP or 5'-[^{3}H] GMP levels as described previously (Meima *et al.*, 2002). All PDE assays in this work were performed in the presence of DTT and IBMX, which inhibit other *Dictyostelium* PDEs such as PdsA, RegA, and PDE3, but not PdeD or PdeE.

5.3.7 cAMP Relay and cAMP-induced cGMP Response

Cells were starved for 8 h, resuspended in PB buffer to 10^8 cells/ml, and stimulated with 0.1 µM cAMP for the cGMP response and 5 µM 2'-deoxy-cAMP in the presence of 5 mM DTT for cAMP-induced cAMP production (cAMP relay). Aliquots of cell suspension were rapidly mixed with an equal volume of 3.5% perchloric acid (v/v) at various intervals after stimulation. After neutralization of the lysates with $KHCO_3$, cGMP levels were measured by radioimmunoassay, and cAMP levels were measured by competition with [^{3}H]cAMP for binding to the bovine PKA regulatory subunit (Gilman, 1972).

5.3.8 Immunoprecipitation

25 µg of mouse monoclonal GFP antibody (αGFP; Roche Molecular Biochemicals) was incubated for 1 h at 4 °C with a mixture of 250 µl each of slurries of protein G linked to Sepharose 4B (Sigma) and protein A linked to Affiprep (Bio-Rad). The αGFP antibody also cross-reacts with and immunoprecipitates YFP. The αGFP-linked matrix was washed with phosphate-buffered saline (0.7% NaCl in PB buffer) and resuspended to the original concentration. 1 ml of cleared lysate, prepared in phosphate-buffered saline at 2×10^8 cells/ml, was incubated for 4 h with 100 µl of αGFP matrix suspension. The matrix was washed thoroughly with 0.2 M NaCl in PB buffer and resuspended in 20 mM Hepes, pH 7.0.

5.4 Results

5.4.1 Structure and Developmental Regulation of PdeE

Screening of *Dictyostelium* cDNA and genome databases with consensus sequences for cyclic nucleotide binding motifs yielded two novel genes of similar structure, *PdeD* (Meima *et al.*, 2002) and *PdeE*, which were also identified by others as *gbpA* and *gbpB* (Goldberg *et al.*, 2002; Bosgraaf *et al.*, 2002a). *PdeE* is transcribed into a 3.4 kb mRNA and shows low expression during growth and a very pronounced peak at 8-10 h of starvation, when cells are aggregating (Fig. 5.1A). This peak was not found for *gbpB* expression in earlier work (Goldberg *et al.*, 2002), which may be due to the fact that the 4-h interval used by these workers between successive points in the developmental time course was too large.

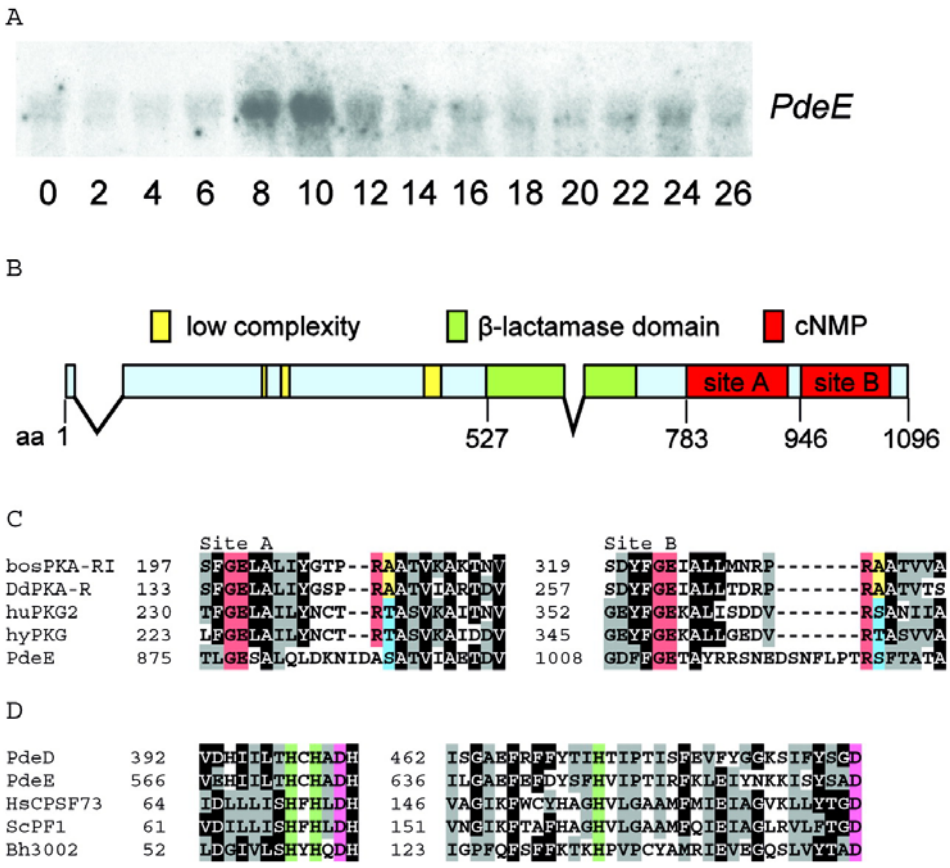

Fig. 5.1. Developmental regulation and structure of *PdeE*. *A)*, developmental regulation. *D. discoideum* NC4 cells were incubated on PB agar until fruiting bodies had formed. RNA was isolated at 2-h intervals and hybridized to a [^{32}P]dATP-labeled *PdeE* probe. RNA markers were run on the same gel to estimate the size of the *PdeE* mRNA, which was ~3.4 kb. *B)*, schematic of PdeE structure, indicating the position of the two introns, the metallo-β-lactamase domain, and the two cyclic nucleotide binding motifs. *C)*, alignment of the cNMP binding motifs of PdeE, bovine PKA-RI, *Dictyostelium* PKA-RI, human PKG2, and *Hydra* PKG. *Black*, identical; *gray*, functionally conserved; *red*, side chain essential for hydrogen bond and ion pair formation with ribose 2'-OH and exocyclic oxygens, respectively; *blue*, Ser/Thr that distinguishes guanine from adenine (*yellow*) binding (Su et *al.*, 1995). *D)*, alignment of PdeD and PdeE with the 73-kDa CPSF subunits from *Homo sapiens*, its homolog PF1 from *Saccharomyces cerevisiae*, and the *Bacillus halodurans* protein BH3002. *Green*/*magenta*, binding to Zn^{2+}(I) and Zn^{2+} (II), respectively, in *Bacillus cereus* metallo-β-lactamase and human glyoxylase II (Carfi et al., 1995, Cameron *et al.*, 1999).

The 1096-amino acid PdeE protein can be subdivided into an amino terminal region with no homology to known proteins, a middle region, containing the metallo-

β-lactamase domain that is common to a number of hydrolases (Melino *et al.*, 1998), and a carboxyl-terminal region that contains two cyclic nucleotide binding cNMP motifs (Bateman *et al.*, 2002) (*site A* and *site B* in Fig. 5.1B). PdeD has a similar domain architecture and encodes a cGMP-stimulated cGMP phosphodiesterase (Meima *et al.*, 2002; Bosgraaf *et al.*, 2002a).

cAMP and cGMP-binding domains are highly homologous and share a similar structure that was first determined for the regulatory subunit of bovine cAMP-dependent protein kinase (PKA RI) (Su *et al.*, 1995). The major determinant for nucleotide specificity is Ala^{210} (Fig. 5.1C, *site A*, *yellow*) and Ala^{334} (*site B*, *yellow*) in PKA RI (Shabb *et al.*, 1991). The equivalent positions in human cGMP-dependent protein kinase (huPKG2) are Thr^{243} and Ser^{367} (in *blue*), which allow hydrogen bond formation with the C^2-NH_2 group of the guanine base. Both sites A and B in PdeE harbor a serine (Ser^{890} and Ser^{1030}) at the positions equivalent to Ala^{210} or Ala^{334}, suggesting that PdeE binds cGMP (Fig. 5.1C). The putative PdeE cGMP-binding domains deviate rather strongly from the consensus motif. In site A, an Ala^{889} replaces the essential Arg^{209} (in *red*) of PKA RI that forms an ion pair with the equatorial phosphate oxygen of cNMP (Su *et al*., 1995). In addition, insertions of two and seven amino acids precede this key residue in sites A and B, respectively. Additional residues at this position are absent in all characterized cNMP-binding proteins.

5.4.2 Search for Putative Functional Homologues of PdeD and PdeE

The PFAM metallo-β-lactamase domain contains the H*X*H*X*D sequence, two more conserved His residues, and one Asp/Cys residue (Bateman *et al.*, 2002). These residues can bind one or two Zn^{2+} ions that are essential for catalysis (Carfi *et al*., 1995; Concha *et al.*, 1996). The metallo-β-lactamase domain is also present in the well characterized enzyme glyoxalase II (Cameron *et al.*, 1999) and in a variety of other proteins that usually function as hydrolases (Daiyasu *et al.*, 2001). Class II cAMP-PDEs, such as *Dictyostelium* PdsA, also contain the H*X*H*X*D sequence but do not otherwise conform to the PFAM metallo-β-lactamase domain (Bateman *et al.*, 2002). PdeE and PdeD do conform but have little else in common with any enzymes of known substrate specificity that carry this domain or with the class II PDEs. The closest homolog of PdeE outside *Dictyostelium* is a *Bacillus halodurans* protein (BH3002) of unknown function (Takami *et al.*, 2000). The homology between PdeE, PdeD, and BH3002 was greatest in a region preceding the conserved Asp^{669} in PdeE (Fig. 5.1, *magenta*) and showed the motif F*X*FF*X*T/S*X*H*XX*P*XXXXXX*E*XX*G*XXXX* YS/T*X*D (Fig. 5.1D). When GenBank™ was searched with this motif, a class of highly homologous proteins was detected. These proteins also carried the H*X*H*X*D motif of the metallo-β-lactamase domain upstream of the search motif and were either 73-kDa subunits of the pre-mRNA cleavage and polyadenylation specificity factor (CPSF) (Jenny *et al.*, 1996) or uncharacterized bacterial proteins (Fig. 5.1D). CPSF73 is one of a complex of factors that binds to the RNA sequence AAUAAA and causes 3'-endonucleolytic cleavage and the addition of the poly(A) tail a few bases downstream from this sequence. Another factor in the complex, CPSF100 (Jenny *et al.*, 1994), harbors a degenerate metallo-β-lactamase motif, which does not display the region of homology to PdeD and PdeE. CPSF100 also lacks an essential residue for binding to Zn(I). Despite the fact that all components of the polyadenylation complex have been cloned, the 3'-endonuclease has not yet been identified (Wahle and Ruegsegger, 1999; Proudfoot *et al.*, 2002). Because 3'-endonucleases cleave 3'-nucleotide phosphodiester bonds, as is the case for PdeD and PdeE, we consider it likely that CPSF73 provides the 3'-endonuclease activity. The bacterial homologs may likewise be 3'-nucleotidases.

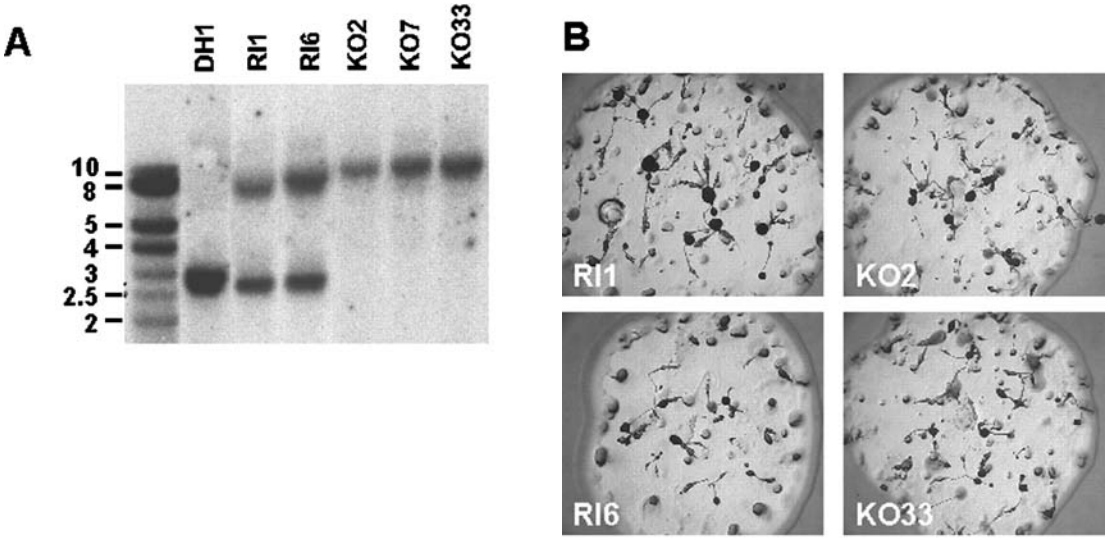

Fig. 5.2. Gene disruption of *PdeE*. *A*, genomic digests of putative knockouts. *A),*The *ura*$^-$ strain DH1 was transformed with a linear construct of the URA$^+$ vector, PJB1, flanked by 1.1 and 1.4 kb of the 5'- and 3'-PdeE sequences, respectively. Clones 2, 7, and 33 were identified by two PCR reactions as putative PdeE gene KOs, and clones 1, 3, and 6 as RIs. Genomic DNA of the parent strain DH1 and the KO and RI clones was digested with Bcl I. In DH1 and RI cells a 2.8-kb band should be present, with additional bands of unknown size marking the random vector integrations in RI cells. Insertion of vector PJB1 in the *PdeE* gene should yield a band of 9.4 kb. *B)*, the morphologies of colonies of RI clones 1 and 6 and KO clones 2 and 33 growing on *Klebsiella aerogenes* lawns are virtually identical.

5.4.3 Disruption of the PdeE Gene

The *PdeE* gene was inactivated by homologous recombination in the *ura*$^-$ strain DH1 to generate cell line *pdeE*. Construct integration was verified by two separate PCR reactions and Southern analysis of genomic digests (Fig. 5.2A). Three randomly selected KO and RI clones were compared in an analysis of phenotypes. Growth and developmental morphology on bacterial plates of the KO and RI clones were identical (Fig. 5.2B). We also could not distinguish differences in growth rate in axenic cultures or in the morphology and rate of development of cells plated on PB agar at cell densities of 10^4, 10^5, and 10^6 cells/cm^2 (data not shown).

Similar to its homolog *PdeD*, *PdeE* is likely to encode a cytosolic cyclic nucleotide hydrolyzing activity. We therefore tested whether such an activity might be lacking in the KO cell lines during the course of their development. Assays were performed in cleared lysates in the presence of DTT and IBMX to inhibit the PDE activities of PdsA, RegA, and PDE3 (Thomason *et al.*, 1998; Kuwayama *et al.*, 2001; Pannbacker and Bravard, 1972). Fig. 5.3A shows that, in the control cell line RI1, cytosolic [^{3}H]cAMP-hydrolyzing activity increased strongly to reach a peak at 8 h of starvation. The activity persisted at low levels during the slug stage of development and was completely absent in the *pdeE* null mutant KO2. There was no significant difference in [^{3}H]cGMP hydrolysis between the KO and RI cell lines (Fig. 5.3B), which suggests that PdeE encodes a cAMP phosphodiesterase. We next analyzed the dose dependence of the cAMP-PDE activity that was detected at t = 8 h in the control RI cells. Fig. 3*C* shows that [^{3}H]cAMP hydrolysis in the control cells is stimulated by micromolar concentrations of cAMP, whereas competition only occurs at millimolar concentrations. This does not resemble the dose-dependence of the known cAMP-PDEs, PdsA and RegA in *Dictyostelium*, which have a K_m of 10 µM and 5 µM, respectively (Thomason *et al.*, 1998; Shaulsky *et al.*, 1998; Kessin *et al.*, 1979).

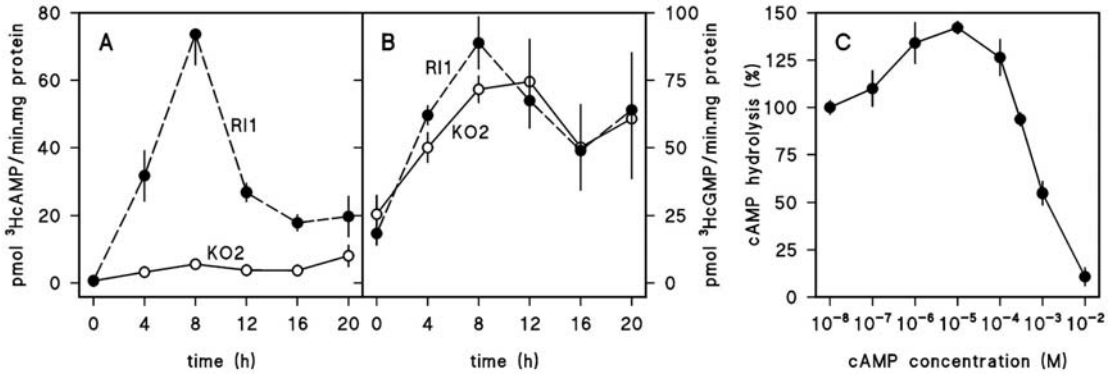

Fig. 5.3. Developmental regulation of cNMP hydrolyzing activity in *PdeE* mutants. *A* and *B)*, development. The *pdeE* null line KO2 (*open symbol*) and control line RI1 (*closed symbol*) were plated on PB agar at 2×10^8 cells/plate and incubated at 22 °C for 20 h. Every 4 h, cleared lysates of 10^8 cells/ml were prepared and tested at 1:0, 1:3, 1:10, and 1:30 dilutions for hydrolysis of 10^{-5} M [^{3}H]cAMP (*A*) or 10^{-5} M [^{3}H]cGMP (*B*) in the presence of the PdsA inhibitor DTT and the RegA and PDE3 inhibitor IBMX. Activities were calculated from the dilution that gave 10-20% hydrolysis of the substrate and standardized on the protein content of the cleared lysate. Means ± S.E. of two time courses assayed in triplicate are presented. *C)*, kinetics. The activity detected at $t = 8$ h in RI1 was used to perform a dose-response analysis of [^{3}H]cAMP hydrolysis. 1:3 diluted cleared lysate was incubated for 30 min at 22 °C with 10^{-8} M [^{3}H]cAMP and the indicated concentrations of cAMP and assayed for [^{3}H]cAMP hydrolysis. Data are derived from two experiments and expressed as percentage of hydrolysis achieved in the absence of added cAMP.

5.4.4 Effects of PdeE Gene Disruption on Cyclic Nucleotide Responses

The developmental regulation of putative PdeE activity agrees well with the appearance of *PdeE* transcripts, with maximum levels at the aggregation stage (Fig. 5.1A). At this stage, both cAMP and cGMP are transiently synthesized in response to stimulation with cAMP receptor ligands. To test whether PdeE is involved in regulating cAMP-induced cAMP production (cAMP relay) or the cGMP response, we measured the two responses in the KO and RI cell lines.

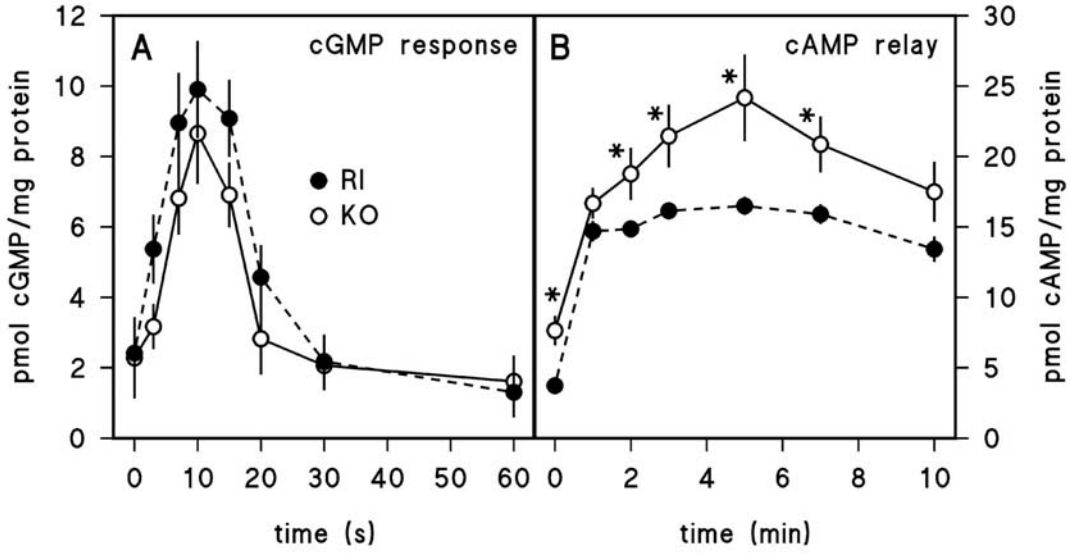

Fig. 5.4. cAMP relay and cGMP response in the *PdeE* mutants. *PdeE* KO and RI cells were starved for 6 h and stimulated with 0.1 µM cAMP for the cGMP response (*A*) and 5 µM 2'-deoxy-cAMP and 5 mM DTT for the cAMP relay response (*B*) and assayed at the indicated time points for cGMP and cAMP levels, respectively. The data represent means ± S.E. for two sets of three time courses for the cGMP response and two experiments performed in triplicate for cAMP relay. The *asterisks* (*) indicate that cNMP levels at individual time points are significantly different ($p > 0.95$) between RI and KO cells as determined by analysis of variance (Dunn *et al.*, 1987).

Fig. 5.4A shows that the cAMP-induced cGMP response and basal cGMP levels are not significantly different between KO and RI cells. However, basal cAMP levels are 2-fold elevated in the KO cells. The initial rate of cAMP accumulation is similar in KO and RI cells, but the KO cells continue to accumulate cAMP for a longer period, resulting in about 1.5-fold higher cAMP levels (Fig. 5.4B). These data suggest that PdeE has no function in degrading cGMP but may play a role in regulating the cAMP relay response.

5.4.5 Overexpression of a PdeE-YFP Gene Fusion

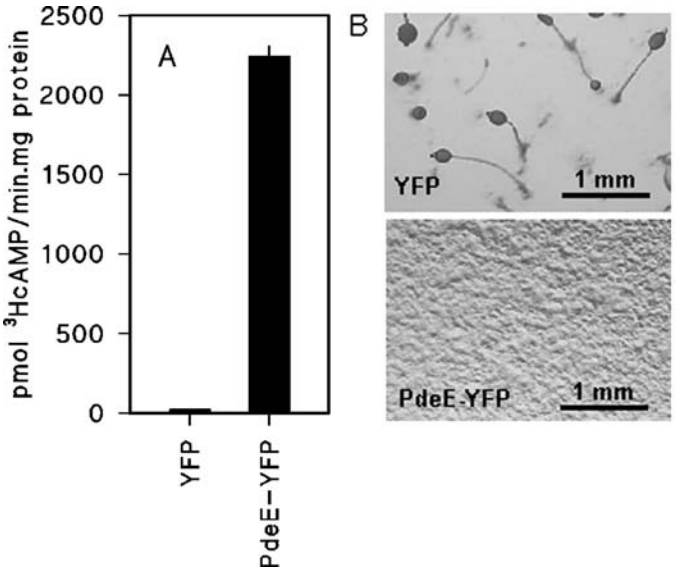

Fig. 5.5. Expression of a PdeE-YFP fusion construct. *A)*, PDE activity. AX2 cells expressing either *YFP* or *PdeE-YFP* from the constitutive actin15 promoter were harvested in growth phase and lysed at 10^8 cells/ml. Hydrolysis of 10^{-5} M [^{3}H]cAMP was measured during 30 min in progressive dilutions of cleared lysate. Enzyme activity was calculated from dilutions (1:300 for PdeE-YFP and 1:0 for YFP) that did not degrade more than 20% of the substrate and standardized on the protein content of the lysate. *B)*, phenotype. Vegetative AX2 cells expressing either *PdeE-YFP* or *YFP* were distributed on PB agar at 3×10^5 cells/cm^2. Cells were incubated for 22 h at 22 °C and photographed.

To provide further evidence that *PdeE* encodes the cAMP hydrolyzing activity that is lacking in the *pdeE* null mutants, we fused the full-length *PdeE* gene to the gene for the enhanced YFP (Miyawaki *et al.*, 1997) and expressed the fusion construct under control of the constitutive actin15 promoter (A15) in the wild-type strain AX2. [^{3}H]cAMP hydrolysis in growing PdeE-YFP cells was 100 times higher than that in control YFP cells (Fig. 5.5A). The YFP cell line developed normally into fruiting bodies within 22 h, but the PdeE-YFP line was blocked in early development (Fig. 5.5B). PdeE activity in the overexpressers (2.2 nmol/min/mg protein) is about 30-fold higher than the maximum activity reached during development of control cell lines (see Fig. 5.3A), and this could lead to significant depletion of intracellular cAMP. Early development and aggregation show a cell-autonomous requirement for PKA activation (Schulkes and Schaap, 1995; Mann *et al.*, 1997) and a non-cell-autonomous requirement for oscillatory cAMP signaling (Pitt *et al.*, 1993). Overexpressed PdeE could hydrolyze most of the intracellular cAMP and block either or both of these processes. To test whether defective development in the PdeE-YFP cells was cell-autonomous, we developed the cells in synergy with the wild-type AX2. Fig. 5.6A shows that the PdeE-YFP cells (detected by YFP fluorescence) co-aggregated with the AX2 cells and were incorporated into aggregates and slugs albeit that development was somewhat delayed. This suggests that oscillatory cAMP signaling was affected rather than the activation of PKA. To test this directly, we compared the cAMP relay response in the PdeE-YFP and the YFP overexpressers. Fig. 5.6B shows that the relay response in the PdeE-YFP cells was very strongly reduced.

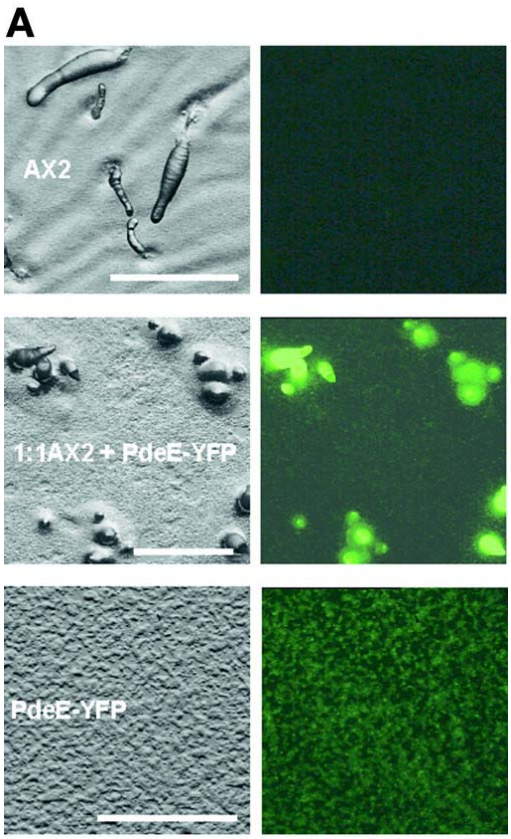

Fig. 5.6. Synergistic development and cAMP relay. *A)*, synergistic development. AX2 cells and PdeE-YFP cells were mixed at 1:0, 1:1, or 0:1 ratios and plated on PB agar at 3×10^5 cells/cm^2. Cells were photographed after 16 h at 22 °C under both bright field and UV illumination to monitor morphogenesis and YFP fluorescence simultaneously. *Bar* length equals 1 mm. *B)*, cAMP relay. AX2 cells expressing either YFP or PdeE-YFP were starved for 6 h, stimulated with 5 µM 2'-deoxy-cAMP and 5 mM DTT, and assayed for cAMP at the indicated time points. Means ± S.E. of two experiments performed in triplicate are presented

5.4.6 Metal and pH Dependence of PdeE

To establish optimal assay conditions for PdeE and its ortholog PdeD, we determined the pH and metal dependence of the two enzymes. Fig. 5.7A shows that the pH dependence of PdeD and PdeE is almost identical with optimal activity at pH 7.0. The catalytic activity of PdeD resides in its metallo-β-lactamase domain (Meima *et al.*, 2002), which requires the binuclear Zn^{2+} binding motif for hydrolysis (Carfi *et al.*, 1995; Cameron *et al.*, 1999). PdeE has a similar domain, and the activity of both PdeD and PdeE may therefore be expected to be dependent on Zn^{2+} ions. However, pilot experiments showed no stimulation of PdeD by Zn^{2+} ions. We assumed that the metal was tightly bound to the enzyme. To relieve PdeE-YFP and PdeD-YFP from their bound metals, we immunoprecipitated the enzymes with GFP antibodies and incubated the immunoprecipitate overnight with 0.2 M EDTA. After removal of EDTA, the enzymes were no longer active without added metals. Fig. 5.7, B and C show that EDTA-treated PdeE and PdeD were stimulated with equal effectiveness by Zn^{2+} or Mg^{2+}, showing maximal stimulation at 10 mM. No synergistic effects of Zn^{2+} and Mg^{2+} were observed (data not shown). Unexpectedly, Mn^{2+} ions activated both enzymes to at least 4-fold higher levels than Zn^{2+} or Mg^{2+}. Increased activation was already evident at a concentration (~10 µM) where Mn^{2+} is present in the cell. Co^{2+}, Cu^{2+}, and Cd^{2+} stimulated PdeD and PdeE to similar levels as Zn^{2+} and Mg^{2+} did (data not shown).

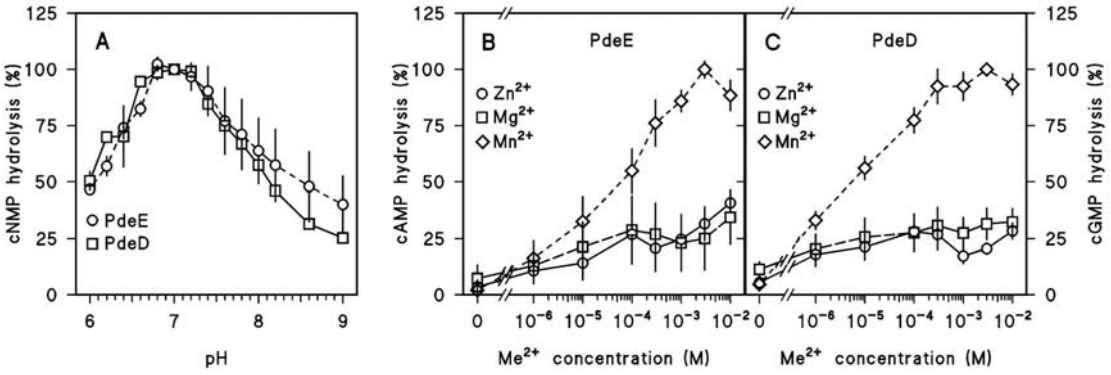

Fig. 5.7. pH and metal dependence of PdeE and PdeD. *A)*, pH. Cells expressing PdeE-YFP or PdeD-YFP (Meima *et al*., 2002) were lysed in 0.5 mM Hepes, pH 7.0. Lysates were diluted 1:300 in 50 mM potassium phosphate buffer at the indicated pH and tested for hydrolysis of 10^{-5} M [^{3}H]cAMP or [^{3}H]cGMP, respectively. Data are expressed as percentage of hydrolysis obtained at pH 7.0. Means ± S.E. of two experiments performed in triplicate are presented. *B* and *C)*, metal. Immunoprecipitates of PdeE-YFP (*B*) and PdeD-YFP (*C*) were incubated for 16 h at 0 °C with 0.2 M EDTA in 20 mM Hepes, pH 7.0. After thorough washing with 20 mM Hepes, the immunoprecipitates were tested for hydrolysis of 10^{-5} M [^{3}H]cAMP or [^{3}H]cGMP, respectively, in the presence of the indicated concentrations of $ZnCl_2$, $MgCl_2$, and $MnCl_2$. Data are expressed as percentage of hydrolysis obtained with 3×10^{-3} M $MnCl_2$. Means ± S.E. of two experiments performed in triplicate are presented.

5.4.7 Cyclic Nucleotide Specificity of PdeE

The experiments shown in Fig. 5.3 indicate that PdeE is not a major contributor to [^{3}H]cGMP hydrolysis by cytosolic cell fractions. This does not exclude cGMP as a putative PdeE substrate. We compared the efficiency of hydrolysis of [^{3}H]cAMP and [^{3}H]cGMP by PdeE-YFP at increasing substrate concentrations, and we also determined whether 8-Br-cAMP is as good an activator for PdeE as 8-Br-cGMP is for PdeD (Meima *et al*., 2002). Fig. 5.8 shows that [^{3}H]cAMP hydrolysis by PdeE-YFP was strongly stimulated by cAMP with half-maximal activation occurring around 10 μM. Competition by cAMP was half-maximal around 1 mM. The absolute level of stimulation was somewhat higher than observed for the endogenous enzyme (Fig. 5.3*C*). This could be due to the fact that the endogenous enzyme, because of its low activity, must be measured in concentrated lysates, which may contain some cAMP. cGMP and 8-Br-cAMP activated [^{3}H]cAMP hydrolysis less efficiently than cAMP and required 10-fold higher concentrations. When [^{3}H]cGMP was used as a substrate, the rate of hydrolysis was at least six times lower than that for cAMP over the entire concentration range. These data suggest that PdeE most likely functions as a cAMP-stimulated cAMP- phosphodiesterase.

5.5 Discussion

5.5.1 The Role of PdeE in Dictyostelium Development

We characterized a cAMP-stimulated cAMP phosphodiesterase that is expressed at high levels in aggregating cells. Ablation of the *PdeE* gene has no noticeable effects on growth and aggregation, but overexpression of *PdeE* blocks aggregation completely. This effect is not cell-autonomous, because *PdeE* overexpressers will aggregate when mixed with wild-type cells. The cAMP-induced cAMP relay response, which is essential for chemotactic signal propagation during aggregation, is strongly reduced in PdeE overexpressors. This is the most likely cause for their failure

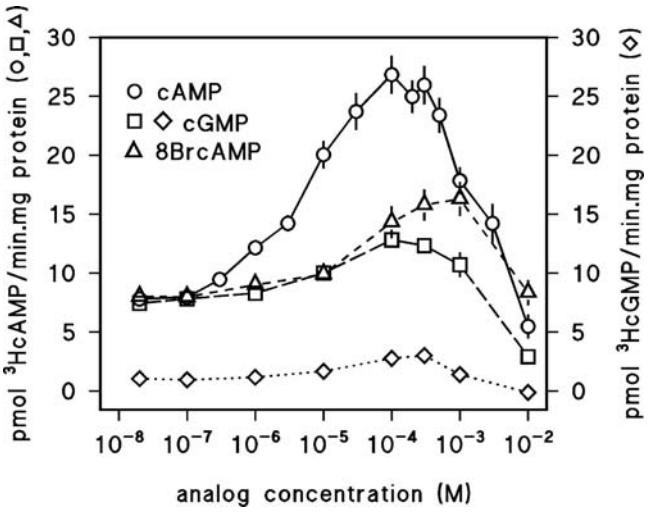

Fig. 5.8. Analog specificity of PdeE. 1:300 diluted, cleared lysates of cells expressing PdeE-YFP were incubated for 30 min at 22 °C with 2.10^{-8} M [^{3}H]cAMP or 2.10^{-8} M [^{3}H]cGMP in the presence of the indicated concentrations of cAMP, cGMP, and 8-Br-cAMP and tested for cyclic nucleotide hydrolysis. Data were standardized on the protein content of the cleared lysate. Means ± S.E. of two or three experiments performed in triplicate are presented.

to aggregate. Mutants defective in adenylyl cyclase A (ACA) or CracA, the adenylyl cyclase and its activating factor that are required for cAMP production, can also only aggregate in synergy with wild-type cells (Pitt *et al.*, 1993; Lilly and Devreotes, 1994). The *pdeE* null mutant shows a 1.5-fold increase in cAMP-induced cAMP relay. This does not markedly affect the aggregation process under standard conditions, but the very pronounced expression of the *PdeE* gene during aggregation (Fig. 5.1A) suggests a role in cAMP signaling at this stage.

Oscillatory cAMP secretion requires regulation of the cAMP production process by both positive and negative feedback loops (Martiel and Goldbeter, 1987; Tang and Othmer, 1994). The positive loop is provided by cAMP receptor (cAR)-mediated activation of ACA. The negative loop involves cAR-mediated adaptation of ACA, but the exact nature of this process has proven hard to resolve (Kim *et al.*, 1997). The negative feedback loop can, in theory, be provided by a cAMP-stimulated cAMP phosphodiesterase, and a model was proposed that was based on indirect activation of the cAMP-PDE RegA by cAMP (Laub and Loomis, 1998). However, cAMP activation of RegA has not yet been documented. PdeE is strongly activated by cAMP in a direct manner, and this could contribute to the transient nature of the cAMP relay response. cAR-mediated adaptation of ACA does not require cAMP production by ACA (Theibert and Devreotes, 1983), which rules out the possibility that the cessation of cAMP accumulation is solely due to a cAMP-stimulated PDE. However, PdeE, RegA, and cAR-mediated ACA adaptation may act together to terminate cAMP relay. This would make the oscillatory signaling system very robust but difficult to characterize by a single genetic lesion.

5.5.2 Metal Dependence of PdeE

PdeE and its *Dictyostelium* homolog, PdeD, both harbor a region with high homology to the binuclear Zn^{2+} binding motifs that were first characterized in the metallo-β-lactamases. The second Zn^{2+} ion is not essential for all metallo-β-lactamases, and Mg^{2+}, Cd^{2+}, and Co^{2+} can often replace Zn^{2+} (Fabiane *et al.*, 1998; de Seny *et al.*, 2001). We found that Zn^{2+} and Mg^{2+} were not very effective in supporting PdeE and PdeD activity but that Mn^{2+} stimulated activity to much higher levels. This was already evident at the micromolar concentrations that equate Mn^{2+} levels in cells. It is therefore possible that PdeD and PdeE use Mn^{2+} instead of Zn^{2+} as a cofactor for hydrolysis.

5.5.3 Cyclic Nucleotide Specificity of PdeE

Similar to PdeD, PdeE is an allosteric enzyme that is stimulated by its substrate. The K_A and K_m for cAMP lie around 10 µM and 1 mM, respectively. cGMP is degraded at a 6-fold lower rate than cAMP, and activation is also much less pronounced. Other workers recently reported that cGMP was degraded at a 9-fold lower rate and required three times higher concentrations for activation than cAMP (Bosgraaf *et al.*, 2002a). Their K_A and K_m for cAMP were at 0.7 µM and 0.2 mM, respectively, which are somewhat lower than the values found by us and could be due to the different parental strains (AX3 *versus* AX2) that were used in the two studies. The PdeE fusion with YFP may, in our case, also have slightly altered the kinetics of the enzyme.

For the cGMP-stimulated cGMP-PDE/PdeD, allosteric activation can be satisfactorily explained from the domain architecture of the protein. The metallo-β-lactamase domain harbors the cGMP hydrolytic activity (Meima *et al*., 2002), and the first of the two carboxyl-terminal cNMP binding motifs shows the consensus cGMP binding motif that is found in the cGMP dependent protein kinases (Meima *et al.*, 2002; Goldberg *et al.*, 2002; Su *et al.*, 1995). PKA and PKG bind 8-Br-substituted ligands with equal or greater affinity than cAMP or cGMP itself, because the bulky bromine forces the molecule in the *syn* conformation (purine above ribose) that is preferred by the binding site (Francis and Corbin, 1999; de Wit *et al.*, 1982). In agreement with this, 8-Br-cGMP is a better activator for PdeD then cGMP itself, whereas cAMP does not activate at all (Meima *et al.*, 2002; Kesbeke *et al.*, 1985).

For PdeE, the situation is less straightforward. The PdeE cNMP binding motifs both show the characteristic serine residue that confers specificity for cGMP rather than for cAMP (Fig. 5.1C). Nevertheless, cAMP is a more efficient activator of PdeE than cGMP. In addition, 8-Br-cAMP is a poor activator of PdeE activity. Furthermore, the PdeE cGMP binding motifs show severe deviations from the consensus motif, and it is questionable whether they can bind cGMP or cAMP at all. It is therefore possible that allosteric activation of PdeE is not provided by these motifs.

Ablation of PdeE function causes the loss of a cytosolic cAMP hydrolyzing activity during development and augmentation of the cAMP relay response. There is no significant reduction of [^{3}H]cGMP hydrolyzing activity, and the cGMP response is unaffected. This means that, within the context of cellular physiology, PdeE functions as a cAMP phosphodiesterase.

Acknowledgements

Sequence data for *D. discoideum* were obtained from the Genome Sequencing Centre Jena, website at genome.imb-jena.de/dictyostelium/. The German part of the *D. discoideum* Genome Project is carried out by the Institute of Biochemistry I, Cologne, and the Department of Genome Analysis, Institut für Molekulare Biotechnologie, Jena, with support by the Deutsche Forschungsgemeinschaft (No 113/10-1 and 10-2). We thank Dr. Inke Näthke for advice on immunoprecipitation procedures.

6 Discussion

6.1 ACB: A third adenylyl cyclase activity in *Dictyostelium*

The cAMP-PDE RegA is regulated by a phosphorelay cascade that uses the phosphotransfer protein RdeA as intermediate (Thomason *et al*., 1998; Thomason *et al.*, 1999b). Mutants in *regA* or *rdeA* display a rapid developing phenotype, due to elevated intracellular levels of cAMP. We found that disruption of RegA or RdeA unmasks a novel adenylyl cyclase activity in growing cells that we named ACB. This activity is not detectable in wild-type or *aca*$^-$ cells (Chapter 2). The cAMP-PDE inhibitor IBMX, which inhibits RegA, permits detection of ACB activity in lysates, indicating that RegA activity normally obliterates cAMP production by ACB.

Both the *in vivo* and *in vitro* regulation of ACB differs from that of the previously identified adenylyl cyclases, ACA and ACG. Unlike ACA, ACB is not stimulated by extracellular cAMP or regulated by G-proteins and in contrast to ACG, ACB is inhibited under high osmotic conditions. The most marked difference is the regulation by bivalent cations. ACA and ACG, like the mammalian adenylyl cyclases, are more strongly activated by Mn^{2+} than by Mg^{2+}. In contrast, ACB activity is 2-fold higher in the presence of Mg^{2+} than Mn^{2+}, which allows easy distinction from the other *Dictyostelium* AC activities.

6.2 ACB is predominantly active during late development and controls terminal differentiation

The different regulatory properties of the three *Dictyostelium* adenylyl cyclases provided the possibility to determine the contribution of each to the production of cAMP during development. ACA is highly active during aggregation. Significant ACG activity was not found during the stages measured. ACB activity strongly increased after tipped aggregate formation and reached a maximum in early culminants. This activity was also found in slugs of *acg*$^-$ and *aca*$^-$ cells that overexpress the catalytic subunit of PKA (*aca*$^-$*/PKA-C*), which provides also genetic evidence of the presence of ACB activity during post-aggregative development. The presence of adenylyl cyclase activity in *aca*$^-$*/PKA-C* slugs contradicts findings by Wang and Kuspa (1997), who reported that these cells cannot make cAMP. From this, the authors concluded that during late development extracellular cAMP is only required for the activation of ACA and subsequent activation of PKA, and that other signals are involved in differentiation and morphogenesis. They might have missed ACB activity because of suboptimal assay conditions, i.e. short incubation times and the presence of Mn^{2+}, rather than Mg^{2+}. Our detection of substantial levels of ACB activity during post-aggregative development means that their conclusions are not substantiated. In addition, we found a low level of Mn^{2+}-stimulated activity that is probably derived from ACG, which is expressed at low levels in slug prespore cells (Alvarez-Curtos and Schaap, in preparation).

In spite of the high levels of ACB activity in cell lysates in the presence of IBMX, cAMP accumulation is not detected in intact wild-type cells. However, significant accumulation can be detected in *regA*$^-$ cells, which suggests that the cAMP produced by ACB is normally quickly degraded by RegA. This tight regulation by RegA seems specific for ACB, as we measured only slightly elevated ACA derived cAMP accumulation in *regA* null mutants (see figure 2.1A). This would suggest that ACB and RegA are in close proximity in the cell. In mammalian, multiprotein signaling complexes are commonly found, most notably those

involving AKAPs (A-Kinase Anchoring Proteins) (Bauman and Scott, 2002; Michel and Scott, 2002). AKAPs bind to the dimerization domain of the regulatory subunit of PKA in addition to a wide variety of other signal transduction components, such as phosphodiesterases, kinases and phosphatases. No AKAPs have been identified in the *Dictyostelium* genome yet and the *Dictyostelium* PKA-R lacks a dimerization domain. The mammalian AKAPs however show homology in function and structure, but not in amino acid sequence. A *Dictyostelium* homologue might therefore not be readily identifiable from the sequence. Intriguingly, a study by Shaulsky *et al*. (1998) showed that RegA can bind to PKA-R and this interaction increases its PDE activity by 18-fold.

The presence of high ACB activity during late development suggests a function in terminal differentiation. The gene encoding ACB has been identified in a mutagenesis screen and named *acrA* (Söderbom *et al*., 1999). The *acr* gene encodes a protein with several different domains. A single AC catalytic domain is located in the C-terminus. The sequence of the catalytic domain reveals a higher homology with cyanobacterial ACs and mammalian soluble adenylyl cyclase (sAC), then with mammalian tmACs, ACA or ACG. The N-terminal part contains two putative transmembrane segments and domains related to histidine kinases and response regulators. The response regulator domain is well preserved, but mutations in the histidine kinase domain have made this domain probably dysfunctional.

The timing of *acr* expression correlates with the developmental regulation of ACB activity and mutants in *acr* are deficient in the late, Mg^{2+}-stimulated AC activity. Growth and development of the *acrA* knock-out mutant is normal up to the slug stage, but the cells form aberrant fruiting bodies with long, think stalks and almost no viable spores. Prespore and prestalk markers are expressed at normal levels, however expression of the spore-specific marker *spiA* is absent. The sporulation defect is cell-autonomous, confirming a role for ACB/ACR as the source of intracellular cAMP required for terminal differentiation.

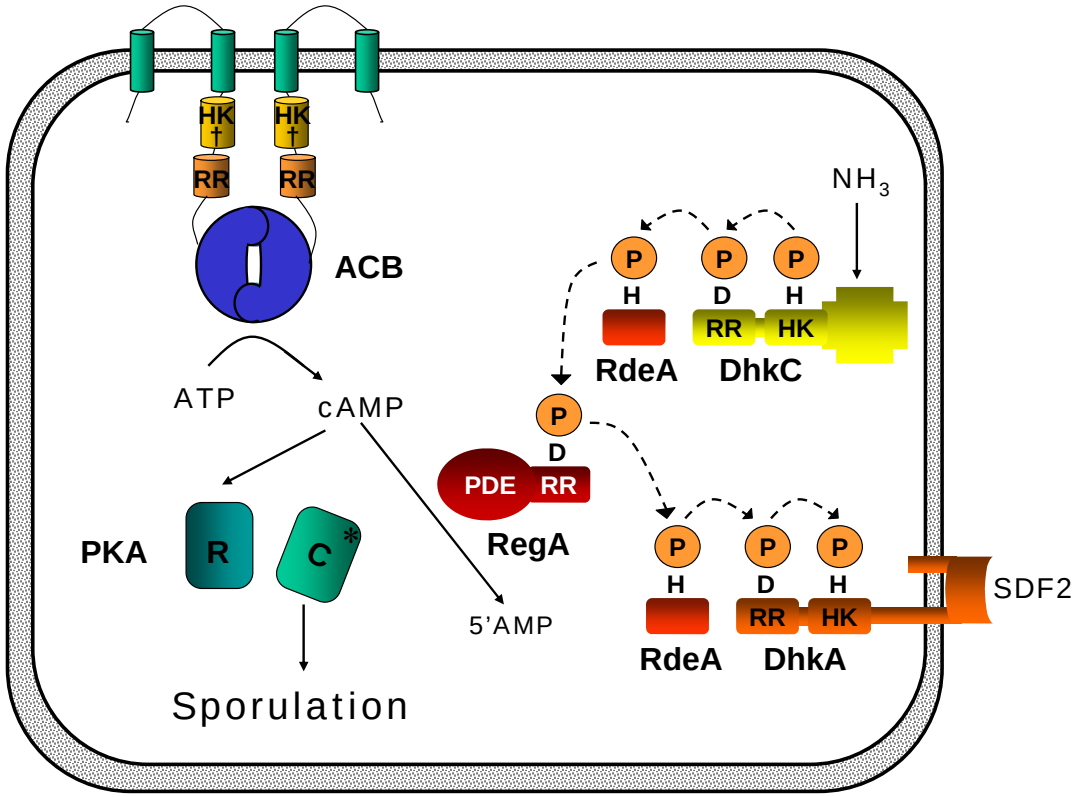

Fig. 6.1. Model for the regulation of terminal differentiation in *Dictyostelium* by cAMP. See text for details. HK = histidine kinase, HK† = non-functional HK, RR = response regulator.

Based on work by our and other groups, the following model has emerged (fig. 6.1) (Saran *et al*., 2003). In slug cells, ACB is required for the production of cAMP that controls terminal differentiation through the activation of PKA (Chapter 3; Söderbom *et al*., 1999). However, the levels of cAMP are tightly controlled by the phosphodiesterase REGA, which is activated by the phosphorylation of its response regulator domain (Thomason *et al.*, 1999b). REGA is regulated by two upstream histidine kinases. DHKC functions as a sensor for ammonia, a negative regulator of culmination (Singleton *et al.* 1998). This triggers a phosphate transfer from DHKC to REGA, resulting in a decrease of cAMP levels and inhibition of culmination. DHKA is proposed to be the sensor for the spore differentiation factor SDF2 (Wang *et al.* 1999), which is secreted by the forming stalk cells as they plunge into the prespore mass (Anjard *et al.*, 1998). It has been hypothesized that binding of SDF2 to DHKA results in an inverse phosphate flow from REGA to DHKA, resulting in elevated cAMP degradation and subsequent culmination (Anjard *et al.*, 1998; Loomis, 1998). Thus, the fate of the slug cells depends on the strength of the two opposing pathways. It is unclear which signals, if any, feed into ACB/ACR. The presence of a sequentially intact response regulator in ACB/ACR leaves the possibility of an upstream, as yet unidentified, phosphorelay cascade.

6.3 sGC, a novel guanylyl cyclase homologous to mammalian soluble adenylyl cyclase

Dictyostelium cells produce cGMP in response to chemoattractants, such as cAMP during and folic acid. Previous studies on chemical mutants have implied a role for cGMP in chemotaxis. The effects of cGMP are thought to be mediated through the phosphorylation of myosin followed by rearrangements of the myosin cytoskeleton (Van Haastert and Kuwayama, 1997). Recently the first gene encoding a *Dictyostelium* guanylyl cyclase, GCA, was cloned (Roelofs *et al.*, 2001). However, the *gca* null mutant showed no phenotype and still displayed a cGMP response when stimulated with cAMP, implying that a second guanylyl cyclase had to be present.

In Chapter 3, the identification of a novel gene encoding a guanylyl cyclase, sGC, is described. sGC is most homologous with mammalian soluble adenylyl cyclase (sAC). sGC accounts for ~90% of the cAMP-induced cGMP response in aggregating cells. GC activity in growing cells is only weakly reduced in an *sgc* null mutant. This means that GCA is mainly active during growth and sGC during aggregation, with little overlapping activities. Roelofs and van Haastert (2002) found that an *sgc*/*gca* double null mutant has no detectable GC activity at all developmental stages tested, which renders the presence of a third guanylyl cyclase in *Dictyostelium* unlikely.

sGC is stimulated by cAMP in a G-protein dependent fashion. sGC activity is strongest with Mn^{2+}/GTP as substrate and is under these conditions mainly found in the cytosolic fraction. However, GTPγS-stimulated activity is only found with Mg^{2+}/GTP as substrate and resides in the membrane fraction. Intracellular concentrations of Mn^{2+} are low compared to the prevailing Mg^{2+} concentration (Padh and Brenner, 1984). Mg^{2+}/GTP is therefore likely the physiological substrate which means that the majority of the enzyme is present in an inactive pool in the cytosol and that only membrane-bound sGC is activated by G-proteins. We have as yet found no evidence that sGC is recruited from the cytosol to the plasmamembrane upon G-protein activation.

The signal transduction cascade to sGC has still not been fully resolved. GC activation by cAMP requires the presence of the $G_{\alpha}2$-subunit (Carrel *et al.*, 1994; Okaichi *et al*., 1992). However, GC activity is still stimulated in lysates from a g_{β} null mutant (Wu *et al.*, 1995b). Biochemical evidence suggests that activation of sGC by G2 is not direct, but mediated by a

small G-protein (Roelofs and Van Haastert, 2002). This is supported by the observation that null mutants in the RasGEF *AleA* and the Ras interacting protein *RIP3* have a reduced cAMP-induced cGMP response (Lee *et al.*, 1999). The activity of both GCA and sGC are strongly down-regulated by Ca^{2+} (Chapter 3; Roelofs and Van Haastert, 2002; Valkema *et al.*, 1992). It has been hypothesized that Ca^{2+} regulates the magnitude of the cGMP response (Van Haastert and Kuwayama, 1997).

The phenotype of the *sgc* null mutant is surprisingly subtle. In a previous study by Kuwayama *et al.* (1993), a set of mutants were chemically created that were defective in chemotaxis and aggregation. Of these mutants, one (KI-10) lacked a cGMP response but produced basal levels of cGMP, whereas another (KI-8) had no detectable levels of cGMP. From this, it was concluded that cGMP is essential for chemotaxis and therefore aggregation. Even though the cGMP response is 10-fold lower in the *sgc* null mutant, the cells develop normally. Only under stringent conditions, i.e. under buffer, do the cells aggregate less efficiently. This is not due to redundancy with GCA, as a *sgc*/*gca* double null mutant also develops (Roelofs and Van Haastert, 2002). However, this mutant is impaired in cell speed and directionality in a chemotactic gradient. In addition, phosphorylation of myosin heavy chain II (MHC) and regulatory light chain (RLC) appears to be reduced and cAMP induced MHC association with the cytoskeleton is absent. This implies that cGMP is required for efficient chemotaxis, but not essential. The discrepancy with the KI-mutants could be due to multiple mutations or a mutation that affects both the cGMP pathway and another pathway that together are essential for chemotaxis.

Dictyostelium cells also produce cGMP when challenged with osmotic stress. This response is much more prolonged and peaks around 10 minutes (Kuwayama *et al.*, 1996). This response triggers the formation of a thick myosin cytoskeletal cortex that protects the cell against collapse under high osmotic conditions. KI-8 mutants or cells that harbour myosin heavy chain molecules in which the phosphorylation sites have been mutated are hypersensitive to osmotic pressure. The production of cGMP in response to osmotic stress is completely attributable to sGC, as this response is absent in sgc^- cells (Roelofs and Van Haastert, 2002). How sGC is stimulated by high osmolarity is as yet unknown. The response is still present in G_β^- and thus G-protein independent (Kuwayama and Van Haastert, 1998). A few years ago a histidine kinase, DokA, was identified that is involved in osmotic resistance (Schuster *et al.*, 1996). DokA mediates a cAMP response in to osmotic shock. However, cGMP signalling is unaltered in $dokA^-$ (Ott *et al.*, 2000).

Although similar in regulation, the phylogeny of the *Dictyostelium* GCs suggests that they are not closely related to each other or to mammalian GCs. It seems likely that they evolved independently from related ACs. It is intriguing that orthologues of sGC are present in bacteria and mammals, but absent from *C. elegans*, *Drosophila*, the yeast *S. cerevisae* and the plant *Arabidopsis*. Since the *Dictyostelids* branch of the lineage to animals before the yeast and fungi group (Baldauf *et al.*, 2000), this means that sGC orthologues have been subject to several independent gene loss events (Roelofs and Van Haastert, 2002).

6.4 PdeD and PdeE belong to a novel class of phosphodiesterases

Cyclic nucleotide degradation is as important for dynamic cyclic nucleotide signalling system as synthesis. In *Dictyostelium*, three cyclic nucleotide-specific phosphodiesterases had previously been identified. The extracellular enzyme PdsA belongs to the class 2 PDEs (Lacombe *et al.*, 1986), to which also the yeast PDE belongs (Nikawa *et al.*, 1987). RegA and DdPDE3 belong to the mammalian class 1 PDEs (Kuwayama *et al.*, 2001; Shaulsky *et al.*, 1996; Shaulsky *et al.*, 1998; Thomason *et al.*, 1998). In a search for cAMP targets, we

identified two similar genes, *PdeD* and *PdeE*, encoding novel phosphodiesterases. These genes were also identified by others (Goldberg *et al.*, 2002; Bosgraaf *et al*., 2002b) and named *GbpA* and *GbpB*. Both genes are mainly expressed during aggregation, with *PdeE* peaking strongly after 8-10 hours. The proteins contain two C-terminal cyclic nucleotide binding domains, a binuclear Zn^{2+} binding motif that is commonly found in metallo-β-lactamases and a N-terminal domain with no homology to proteins with known functions.

PdeD null mutants form extensive aggregation streams when cloned on bacterial lawns, similar to the chemotactic *stmF* mutants. These mutants were chemically created and are defective in a cGMP-activated cGMP-PDE (Liu and Newell, 1992; Ross and Newell, 1981; Van Haastert *et al.*, 1982). Like *stmF*, null mutants in *pdeD* have an elevated and prolonged cGMP response due to the loss of a cGMP-stimulated cGMP-PDE activity. Similar to the *stmF* locus, *PdeD* is located on chromosome 2 (Coukell and Cameron, 1985). Most importantly, in one of the *stmF* mutants, NP377, *PdeD* mRNA was nearly absent. This is strong evidence that *PdeD* resides on the *stmF* locus. We have however not found any mutations in the promotor region of *PdeD* in NP377. The loss of *PdeD* messenger could also be due to a mutation in the coding or 3' untranslated region with a destabilizing effect on the RNA, or in a transcription factor.

The *pdeE* null mutant displays a 1.5-fold elevated cAMP relay response, but the cGMP response is unaffected. Under conditions in which the cAMP-PDEs RegA and PdsA are inhibited, lysates of wild-type cells have cAMP hydrolysing activity that peaks after 8 hours. This activity is completely lost from *pdeE*¯. Using overexpressors, we found that PdeE has dual specificity, but preference for cAMP. Its activity is strongly upregulated by cyclic nucleotides, with cAMP being the better activator. These findings suggest that the physiological role of PdeE is degradation of cAMP.

Overexpression of *PdeE* causes a severe delay in development. Eventually, some fruiting bodies appear, but most cells fail to incorporate into aggregates. However, *PdeE* overexpressors can develop in synergy with wild-type cells. This is also the case for cells lacking ACA which cannot produce cAMP but are able to develop when the signal is delivered by wild-type cells (Pitt *et al*., 1993). We found that PDE overexpressors have a strongly decreased cAMP-induced cAMP response, which shows that the developmental phenotype is caused by a defect in relay rather than a cell-autonomous defect

The Zn^{2+} binding domains in PdeD and PdeE contain a conserved histidine-rich motif, HxHxD that is also found in bacterial metallo-β-lactamases, which hydrolyse the β-lactam ring in carbapenem antibiotics (Carfi *et al*., 1995; Concha *et al*., 1996), as well as in bacterial aryl sulfatases and eukaryotic glyoxylase II (Barbeyron *et al*., 1995; Cameron *et al*., 1999). Histidine-rich metal binding motifs are also conserved in mammalian PDEs, but the consensus of the motif is different from those in metallo-β-lactamases. Using different PdeD truncations fused to GFP, we identified the β-lactamase domain as the catalytic core of the PDE, which is a novel function for this domain. The HxHxD motif is also present in the class 2 enzyme PdsA (Lacombe *et al.*, 1986) and its homologue from yeast (Nikawa *et al.*, 1987), but homology outside these residues is very low. Database searches using the PFAM algorithm with this sequence failed to find these PDEs, but did produce metallo-β-lactamases. Gene bank searches with a subdomain of the metallo β-lactamase domain revealed homology with the 73 kD subunit of the premRNA cleavage and polyadenylation specificity factor (CPSF). CPSF73 is part of a multi-protein complex that binds to the polyadenylation site of premature RNA and regulates 3' endonucleolytic cleavage and addition of the polyA tail (Jenny *et al.*, 1996). The 3' endonuclease of the complex has not yet been identified (Wahle and Rügsegger, 1999). Since these enzymes cleave 3' nucleotide phosphodiester bonds, like PdeD and PdeE, it is likely that CPSF73 functions as the endonuclease.

The metal dependency of PdeD and PdeE is quite unusual. β-lactamase domains in proteins of which the structure has been resolved have a high and a low affinity binding site for Zn^{2+}. The enzymes are catalytically active with only the high affinity site occupied. Besides Zn^{2+}, the second site can also bind other bivalent cations (Fabiane *et al.*, 1998; Paul-Soto *et al.*, 1999; De Seny *et al.*, 2001). In both PdeD and PdeE, neither Zn^{2+} nor Mg^{2+} are very effective, but Mn^{2+} stimulates to much greater levels. Since Mn^{2+} stimulation already occurs in the micromolar levels that are present in the cell, it is possible that it functions as the physiological cofactor.

Both enzymes are allosterically activated by their substrate. The sequence of the cyclic nucleotide binding domains predicts cGMP preference for site A in PdeD and both sites in PdeE, whereas mutation of a critical residue in site B probably renders this site inactive. However, all sites except site A in PdeD have insertions of a stretch of amino acids in the binding loop, which likely prevents efficient cyclic nucleotide binding. Therefore, only site A in PdeD seems capable of cyclic nucleotide binding, with specificity for cGMP. This confirms the activation profile we observed for PdeD. 8Br-cGMP is a good activator, but serves as a poor substrate, as had previously been found in *stmF* (Kesbeke *et al.*, 1985). 8Br–substitutions on cAMP and cGMP force the purine ring in a syn-position relative to the ribose moiety. This is the preferred conformation for the binding sites in PKA and PKG, which bind these nucleotides equally well or better than the unmodified nucleotides (de Wit *et al*., 1982). This indicates that allosteric activation is mediated by site A of PdeD. cGMP binding to this domain however still needs to be confirmed. It is doubtful that PdeE is activated in a similar fashion. Although serine residues in the cyclic nucleotide binding domains would predict cGMP binding, the enzyme is best activated by cAMP. Furthermore, 8Br-analogues are not as good activators of PdeE activity as the unmodified nucleotides. This suggests that allosteric activation of PdeE is regulated by a different mechanism.

6.5 Concluding remarks and future prospects

6.5.1 cAMP signalling in *Dictyostelium*

Over the course of this study, there has been an enormous surge in our knowledge of cAMP metabolism and function in *Dictyostelium*. A decade ago, the genetic information was restricted to an extacellular phosphodiesterase (PdsA), two adenylyl cyclases (ACA and ACG) and protein kinase A. Since then another adenylyl cyclase (ACB), two cAMP-phosphodiesterases (RegA and PdeE) and many regulators of ACA have been identified. This list is still growing, as another class 1 PDE, PDE4 was identified from the database, although not characterized (Bosgraaf *et al.*, 2002b). It is however unlikely that more adenylyl cyclases are to be found in the database. These developments have both given answers and raised further questions.

Not all functions of cAMP have yet been accounted for by the different adenylyl cyclases. During early development, PKA activity is required for early and aggregative gene expression (Schulkes and Schaap, 1995). It is however unclear whether an adenylyl cyclase is involved. Neither of the adenylyl cyclase null mutants have a defect in the initiation of development (Pitt *et al.*1993; Söderbom *et al*., 1999). In addition, expression of *PKA-R* is absent in the first four hours of starvation (Mutzel *et al.*, 1987). This raises the surprising possibility that cAMP plays no role in the regulation of PKA activity during the growth-development transition. Instead, the control of PKA protein levels at the translational level by PufA-YakA pathway could be the sole mechanism (Souza *et al*., 1999).

In spite of the well-documented role of cAMP in the induction of prespore gene expression, its source has not been positively identified. ACA expression is restricted to the

tip of the slug (Verkerke-Van Wijk, 2001). ACG expression and activity are present at low levels in the prespore cells (Chapter 2b; Alvarez-Curto *et al.*, in preparation). The localization of ACB has not been reported. ACB is capable of producing high amounts of cAMP *in vitro*, but we could not detect cAMP accumulation from intact slug cells. Furthermore, both *acg* and *acr* knock-out mutants develop normally up to culmination and have normal levels of prespore gene expression (Pitt *et al.*, 1992; Söderbom *et al*., 1999), which indicates that neither enzyme can be solely responsible. An *acg/acg* double knock-out and an adenylyl cyclase dead mutant that can be manipulated with extracellular cAMP or *PKA-C* overexpression should be generated to investigate the different functions of cAMP during late development.

cAMP is obviously not the sole regulator of late development. There is clear evidence that other signals are involved. One example is the expression of the extracellular phosphodiesterase *PdsA* from its late promotor (*PdL*). In a study that used fusion of PdL-LacZ reporter constructs it was found that expression of the late promotor is restricted to the anterior of the slug and is strongly induced *in vitro* by cAMP and DIF in synergy (Weening *et al.,* 2003). However, the expression pattern was unaltered in a mutant that cannot produce significant amounts of DIF or when constructs were used in which the cAMP/DIF response elements had been deleted. This shows that other signals control PdsA expression of the late promotor in vivo. In another study, a protein factor named psi (ψ), was purified that can induce prespore differentiation (Nakagawa *et al.*, 1999). Many genes have been found with REMI (Restriction Enzyme Mediated Integration), the null mutants of which give a distinct developmental phenotype, but have not yet an identified place in existing signalling cascades. Furthermore, the *Dictyostelium* genome carries a surprisingly large number of genes (Eichinger and Noegel, 2003; Glockner *et al*., 2002). This could mean that the signalling mechanisms that control optimal development in *Dictyostelium* are more numerous and complex than previously anticipated.

Finally, even though many key events in *Dictyostelium* development depend on PKA activity, not a single target has been identified so far. In mammalians, cAMP-dependent gene expression is mediated by a number of transcription factor, most notably CREB, but CREB appears to be absent from the *Dictyostelium* genome database. A novel method named KESTREL has recently been developed to rapidly find kinase target (Knebel *et al.*, 2001). This method could be used for *Dictyostelium* PKA as well to fill up a large gap in our current understanding of the signalling events downstream of PKA. Another aspect of PKA signalling in mammalians is its targeting to subcellular compartments by AKAPs (Bauman and Scott, 2002; Michel and Scott, 2002). Even though AKAP homologues are absent from the genome database, functional homologues might exist. To establish the presence of AKAPs in *Dictyostelium* requires a proteomics approach. The tandem affinity purification (TAP) method has been used successfully to purify protein complexes from yeast and mammalian (Puig *et al*., 2001; Rigaut *et al*. 1999). This method uses two affinity tags to obtain pure complexes under native conditions. We have started to set up this system for *Dictyostelium*. Preliminary experiments indicate that TAP could be successfully applied in *Dictyostelium* to screen for protein complexes.

6.5.2 cGMP signalling in *Dictyostelium*

The study of cGMP metabolism and function has been frustrated for a long time by the lack of sequence data. This has changed dramatically in the last few years due to the *Dictyostelium* sequencing project. It has become clear that cGMP metabolism is mediated by a robust system consisting of two GCs (GCA and sGC) and two cGMP-PDEs (DdPDE3 and PdeD/GbpA). The dual-specificity PDE PdeE/GbpB can degrade cGMP as well, but its

cGMP hydrolysing activity becomes only significant in the absence of PdeD (Bosgraaf *et al*., 2002a).

The cGMP signalling system in *Dictyostelium* appears to have evolved independently from its counterpart in metazoans (Roelofs *et al*., 2003). GCA is most homologous to 12tmGCs found in other protozoa, whereas sGC seems to have evolved out of a cyanobacterial adenylyl cyclase. DdPDE3 is similar to mammalian PDEs, but both the domain structure of PdeD as its catalytic domain, which has highest homology to β-lactamases, is different from that found in mammalian PDEs.

This uniqueness of the cGMP signalling system in *Dictyostelium* is extended to two recently identified cGMP targets, GbpC and GbpD (Goldberg *et al*., 2002; Bosgraaf *et al.*, 2002a). The genes encode similar multidomain proteins that have two cyclic nucleotide binding domains, protein-protein interaction domains and a RasGEF. GbpC has in addition an N-terminal leucine-rich repeat motif, which is commonly involved in protein-protein interaction, a Ras and a kinase domain with highest homology to the MAPKK kinase MEKK. Of the *Dictyostelium* proteins that have cGMP-binding domains, GbpC seems the only one that can bind cGMP with high affinity (Bosgraaf *et al.*, 2002a). Double null mutants in *gbpC* and *gbpD* have a similar defect in chemotaxis and myosin phosphorylation as *sgc*$^{-}$/*gca.* RLC is phosphorylated by one or more myosin light chain kinases (MLCK), such as MLCK-A. This protein is activated by cGMP through phosphorylation by an upstream kinase (Silveira *et al*., 1998; Smith *et al*., 1996). This could either be GbpC itself or a kinase cascade that is activated by GbpC. Searches with cGMP binding domains homologous to those in PKA and PKG or GAF domains have not revealed other cGMP targets from the *Dictyostelium* genome database so far (Bosgraaf *et al.*, 2002a).

Mutants that can either not produce (*sgc*$^{-}$/*gca*$^{-}$) or not transduce (*gbpC*$^{-}$/*gbpD*$^{-}$) the cGMP signal are impaired in chemotaxis (Bosgraaf *et al.*, 2002a). In chemoattractant gradients the cells move more slowly and less persistent and have a reduced sense of directionality. This correlates with a reduced cell polarity and depleted association of myosin with the cytoskeleton. The cells can still aggregate under conditions in which the cAMP gradient is steep. However, in the wild where conditions are suboptimal the reduced chemotactic efficiency would be a severe disadvantage.

Recent developments suggest that cGMP is involved in other functions as well. High osmolarity induces tyrosine phosphorylation and nuclear translocation of the transcription factor StatC, which can be mimicked by 8Br-cGMP (Araki *et al.*, 2003). This is still observed in the *gbpC*/*gbpD* double knock-out, which indicates that other yet unidentified proteins respond to cGMP. Recently it was found in our lab that *pdeD* null mutants have a rapid developing phenotype, due to precocious expression of early and aggregative genes (Weening and Schaap, in preparation), thus cGMP could play a role in gene expression in response to density signals or cAMP as well. The recent identification of genes involved in cGMP signaling has now provided the toolkit to elucidate the many outstanding questions.

7 References

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8 Samenvatting

8.1 Signaal transductie

Om te kunnen functioneren in een complex milieu moeten cellen veranderingen in hun omgeving waar kunnen nemen. Dit geldt voor alle levensvormen, van bacteriën tot complexe organismen als mensen, waarin cellen in verschillende organen communiceren door middel van signaalmoleculen of hormonen. De signaalmoleculen worden herkend door eiwitten in de celmembraan, *receptoren* genoemd. De receptoren geven het signaal verder door in de cel door de activiteit van andere eiwitten te veranderen, een proces dat *signaalverwerking* of *signaaltransductie* wordt genoemd. Een goed voorbeeld is de werking van insuline dat tijdens rust de opname en opslag van bloedsuikers activeert, terwijl adrenaline juist de afgifte van suikers in het bloed tijdens inspanning stimuleert.

8.2 *Dictyostelium*

De sociale amoebe *Dictyostelium discoideum* is een model organisme voor de bestudering van signaaltransductie in hogere organismen. Vele van de signaal transductie componenten in hogere organismen zijn ook aanwezig in *Dictyostelium*. Onder hongerende condities ondergaat het doorgaans ééncellige organisme een ontwikkelingsprogramma met als eindresultaat een meercellig vruchtlichaampje van zo'n 100.000 cellen. In het vruchtlichaam is het merendeel van de cellen aanwezig als sporen in een massa ondersteunt door een steel van dode cellen. Onder gunstige condities kunnen de sporen weer kiemen tot cellen. Het proces waarbij verschillende celtypen met een specifieke functie ontstaan heet *differentiatie*, een proces dat gereguleerd wordt door het geheel aan signalen dat de cellen ontvangen.

8.3 cAMP

Cyclisch AMP (cAMP) is één van de meest bestudeerde signaalstoffen in de cel. Hormonen, zoals adrenaline, maar ook bijvoorbeeld zenuwprikkels en geurstoffen veroorzaken de aanmaak van cAMP, wat op zijn beurt vele processen in de cel activeert. Het cAMP signaal wordt in de cel doorgegeven aan andere eiwitten, de meest belangrijke het proteine kinase A (PKA). *Kinases* zijn eiwitten die een fosfaatgroep aan andere eiwit kunnen zetten (*kineren* of *fosforyleren*) en daarmee de activiteit van dat eiwit veranderen. PKA activeert verschillende processen, bijvoorbeeld differentiatie, vorming van geheugen, afbraak van dierlijk zetmeel (glycogeen) tot glucose tijdens inspanning, en vele andere.

cAMP speelt in *Dictyostelium* een belangrijke rol, zowel in (*intracellulair*) als buiten (*extracellulair*) de cel. Als *Dictyostelium* cellen hongeren scheiden ze cAMP uit. Andere cellen herkennen het cAMP signaal door middel van cAMP receptoren, waarop deze cellen naar de bron bewegen en zelf ook cAMP uitscheiden. Dit resulteert in de samenkomst (*aggregatie*) van de cellen. Daarnaast reguleert het na aggregatie de differentiatie van *prespoor* cellen, de voorlopers van de latere sporen. Intracellulair cAMP reguleert de uiteindelijke differentiatie van spoor- en steelcellen (*terminale differentiatie*) en de kieming van sporen via de activatie van PKA. cAMP wordt aangemaakt door een *enzym* (eiwit dat een reactie kan katalyseren) genaamd *adenylaat cyclase*. Ongeveer 10 jaar geleden werden twee

genen gevonden in *Dictyostelium* die coderen voor adenylaat cyclases[1]. Adenylaat cyclase A (ACA) is verantwoordelijk voor de uitscheiding van cAMP tijdens aggregatie. ACG is aanwezig in sporen en wordt geactiveerd door hoge concentratie opgeloste stoffen (osmolariteit) in het milieu, een conditie die nadelig is voor cellen, maar gemakkelijk is te overleven voor sporen. De activatie van ACG leidt tot de remming van sporenkieming.

8.4 ACB

Hoofdstuk 2 beschrijft de identificatie van een nieuwe adenylaat cyclase activiteit, ACB. ACB activiteit hebben wij in eerste instantie ontdekt in groeiende cellen (Hoofdstuk 2A). Echter, na aggregatie blijkt de activiteit vele malen hoger te zijn (Hoofdstuk 2B). Tijdens de late ontwikkeling is cAMP nodig voor terminale differentiatie. De hoge ACB activiteit tijdens dit stadium zou daarom kunnen duiden op een betrokkenheid bij de terminale differentiatie. Deze hypothese bleek juist te zijn, toen het gen dat voor ACB codeert door een andere groep werd geidentificeerd. Cellen waarin een functioneel ACB gen ontbreekt zijn niet in staat tot de terminale differentiatie van sporen. Het is op dit moment niet bekend of ACB activiteit tijdens de late ontwikkeling wordt gereguleerd. Er zijn aanwijzingen dat bepaalde signalen cAMP *afbraak* verminderen en dus terminale differentiatie induceren. Of deze of andere signalen ook cAMP productie door ACB veranderen of dat het enzym ongereguleerd cAMP produceert is voor nu nog onduidelijk.

8.5 sGC

De genetische code van *Dictyostelium* is nu bijna in zijn geheel bepaald door het *Dictyostelium genome sequence project*. Dit maakt de identificatie van nieuwe genen gemakkelijker. Hoofdstuk 3 beschrijft de identificatie van een gen, *sGC*, dat codeert voor een *guanylaat cyclase*, een eiwit dat cGMP, een op cAMP lijkende stof, produceert. cGMP reguleert in dierlijke systemen doorgave van visuele prikkels naar de hersenen, verwijding van bloedvaten (viagra remt de afbraak van cGMP!) en vochthuishouding in de dikke darm. cGMP speelt in *Dictyostelium* een belangrijke rol bij *chemotaxis*, het proces waarbij de cel zeer gericht in de richting van een signaalstof, *chemoattractant* geheten, beweegt. Een goed voorbeeld van zo'n chemoattractant is cAMP dat in *Dictyostelium* aggregatie reguleert. Een ander voorbeeld zijn formylmethionyl peptiden, kleine stukjes eiwitten die door bacteriën worden uitgescheiden en door witte bloedcellen (afweercellen) in het menselijk lichaam worden gedetecteerd.

We hebben een mutant gemaakt, waarin een stuk DNA in het *sGC* gen is geplaatst (*knock-out mutant*) en dus niet meer voor een compleet sGC-eiwit codeert. Via deze methode kan de functie en de bijdrage van sGC aan cGMP productie worden bepaald. *Dictyostelium* cellen

[1] Genen zijn stukken DNA die een blauwdruk vormen voor eiwitten. Het genoom is het geheel aan genen en de elementen die bepalen wanneer genen moeten worden vertaald tot eiwitten. Dit kan worden vergeleken met bijvoorbeeld een autofabriek. Een cel die bepaalde signalen ontvangt is te vergelijken met de opdracht tot het bouwen van een bepaald type auto. Een blauwdruk is inert, maar wanneer blauwdrukken voor de verschillende onderdelen worden uitgestuurd en de onderdelen worden geproduceerd kunnen deze worden geassembleerd tot een functionele auto. Op dezelfde manier leiden signalen van buiten de cel tot de afschrift (*genexpressie*) van bepaalde genen. Deze afschriften bestaan uit op DNA lijkende moleculen, *RNA* genoemd. RNA kan worden vertaald door bepaalde structuren in de cel, *ribosomen* genaamd, tot functionele eiwitten. Net zoals de opdracht kan worden gegeven tot het produceren van verschillende auto's kunnen verschillende signalen zorgen voor de aanmaak van verschillende eiwitten. Dit kan de aard van de cel definitief veranderen, oftewel de cel is dan gedifferentieerd.

produceren cGMP na stimulatie met extracellulair cAMP. Deze respons is voor 90% verdwenen in de knock-out mutant. Hieruit blijkt dat sGC voornamelijk actief is tijdens de aggregatie en wordt gestimuleerd door extracellulair cAMP.

De *sgc* knock-out ontwikkelt onder standaard condities als *wild-type* (niet-gemanipuleerde cellen). Echter, onder de condities waarbij het extracellulair cAMP snel diffundeert aggregeren de cellen minder efficient dan wild-type. Dit geeft aan dat chemotaxis minder efficient is onder moeilijker condities. cGMP lijkt dus nodig te zijn voor volledige chemotaxis maar is niet essentieel.

De aanwezigheid van sGC is *Dictyostelium* is vrij bijzonder. sGC lijkt het meest op het oplosbaar adenylaat cyclase, sAC, dat in verscheidene hogere dieren, inclusief de mens is gevonden. sAC en sGC zijn meer verwant aan adenylaat cyclases uit cyanobacteriën (ook wel blauwe algen genoemd) dan aan (andere) dierlijke cyclases. Echter, verwanten van sGC zijn niet gevonden in gist en fruitvliegen, soorten die evolutionair dichter bij de mens staan en waarvan de genetische code nu in zijn geheel is bepaald. Dit betekent dat waarschijnlijk tijdens de evolutie in een aantal organismen het gen is verdwenen. Daarnaast hebben mutaties (veranderingen in de genetische code) ervoor gezorgd dat in *Dictyostelium* het eiwit van een adenylaat cyclase in een guanylaat cyclase is veranderd**.**

8.6 PdeD en PdeE

Hoofdstuk 4 en 5 beschrijven de karakterisatie van twee nieuwe fosfodiesterases, PdeD en PdeE. Fosfodiesterases termineren signaal processen die worden gereguleerd door cAMP en/of cGMP doordat ze cAMP en cGMP afbreken. Terminatie is even belangrijk als initiatie: aangezien de meeste signaalprocessen tijdelijk van aard zijn moeten de signalen in de cel tijdig worden be-eindigd. Als dit niet gebeurt, bijvoorbeeld doordat door een mutatie een signaal eiwit altijd ‘aan’ staat, kan dit voor de cellen ernstige gevolgen hebben. Een goed voorbeeld zijn tumoren, waarin in sommige gevallen het signaal ‘celdeling’ constant wordt doorgegeven, ook al zijn de extracellulaire signalen die dit normaal gesproken induceren afwezig. Een voorbeeld in *Dictyostelium* is de *regA* knock-out mutant die versneld ontwikkeld doordat de concentraties cAMP in de cel zijn verhoogd en niet meer worden gecontroleerd.

PdeD en PdeE wijken qua structuur af van dierlijke fosfodiesterases. Wij hebben het domein in de enzymen gekarakteriseerd dat verantwoordelijk is voor de katalytische activiteit. Dit domein lijkt structureel het meest op β-lactamases, bacteriële enzymen die penicilline-achtige antibiotica afbreken en een bron zijn van resistentie tegen deze antibiotica. De fosfodiesterase activiteit in de *Dictyostelium* eiwitten is een nieuwe functie voor dit type domein.

PdeD breekt specifiek cGMP af. Daarnaast wordt de fosfodiesterase activiteit door hogere concentraties cGMP extra versterkt (dit wordt *allosterische activatie* genoemd). PdeD knock-out mutanten hebben een verhoogde cGMP respons door een verminderde cGMP afbraak. De cellen aggregeren in ongewoon grote stromen. Dit gedrag (*fenotype*) lijkt veel op dat van de mutant *stmF*. Deze mutant is over de jaren uitvoerig bestudeerd, maar het gen dat defect is was nooit geidentificeerd. Net als de *pdeD* knock-out heeft *stmF* een verlaagde cGMP afbraak. Wij hebben aangetoond dat in *stmF* het *PdeD* gen niet wordt afgeschreven en dus geen PdeD eiwit wordt aangemaakt. Hiermee is het gen dat defect is in *stmF* na meer dan 20 jaar eindelijk geidentificeerd.

PdeE heeft *duale specificiteit*, dwz het kan zowel cAMP als cGMP afbreken. Ook PdeE activiteit wordt allosterisch geactiveerd door cAMP en cGMP. Echter, zowel afbraak als activatie is hoger wanneer cAMP als *substraat* (uitgangsmateriaal voor het enzym) en

activator wordt gebruikt. *pdeE* knock-out mutanten scheiden iets meer cAMP uit dan wild-type cellen. Echter, wanneer vele kopieën van het *PdeE* gen in *Dictyostelium* cellen worden gebracht zodat deze veel meer PdeE-eiwit maken (*overexpressie*), aggregeren de cellen zeer slecht. Dit komt doordat PdeE bijna al het cAMP dat door de cellen wordt gemaakt afbreekt voordat het wordt uitgescheiden en de cellen dus niet meer kunnen communiceren. Dit defect kan worden overkomen door deze cellen met wild-type cellen te mengen die dan het cAMP signaal kunnen leveren (*synergie*).

8.7 Conclusie en toekomstige vraagstukken

In de afgelopen jaren is onze kennis omtrend de functie en productie van cAMP in *Dictyostelium* enorm toegenomen. Aan het begin van de jaren 90 waren twee adenylaat cyclases (ACA en ACG) en een (extracellulair) cAMP-fosfodiesterase bekend. Ondertussen zijn een nieuw adenylaat cyclase (ACB) en twee cAMP-fosfodiesterases (REGA en PdeE) gekarakteriseerd. Vele vragen zijn echter nog onbeantwoord. Zo zijn nog steeds niet alle functies van cAMP verbonden met een specifiek adenylaat cyclase. In sommige gevallen is het wel duidelijk. Bijvoorbeeld, alleen ACA is verantwoordelijk voor het cAMP dat de aggregatie stuurt en alleen ACG produceert cAMP in de sporen die met hoge osmolariteit worden behandeld. Maar we weten bijvoorbeeld nog steeds niet welk adenylaat cyclase het extracellulaire cAMP levert dat vereist is voor expressie van prespoorgenen. Het is mogelijk dat verschillende adenylaat cyclases deze functie kunnen uitvoeren. Om dit uit te zoeken moeten cellen worden gecreeerd waarin meerdere adenylaat cyclases zijn uitgeschakeld. Een ander braakliggend terrein is wat er gebeurt na PKA. Ondanks de vele functies van PKA weten we nog steeds welke eiwitten door PKA worden gefosforyleerd en hoe dit de afschrift van genen beinvloedt. Daarnaast is er bewijs dat de eiwitten voor de productie (ACB), afbraak (REGA) en doorgave (PKA) van het cAMP signaal dat terminale differentiatie reguleert dicht bij elkaar zijn en mogelijk in een eiwitcomplex bestaan. Recentelijk zijn methodes ontwikkelt waarbij de substraten van kinases relatief eenvoudig kunnen worden gevonden. Daarnaast zijn wij op dit moment bezig een techniek toe te passen in *Dictyostelium* die het mogelijk maakt om eiwitcomplexen op te zuiveren, waarna de individuele eiwitten in zo'n complex kunnen worden geidentificeerd.

Het onderzoek aan cGMP in *Dictyostelium* heeft eveneens geprofiteerd van het sequence project. Hoewel de productie van cGMP in *Dictyostelium* reeds in de mid jaren 70 werd aangetoond, waren de genen die nodig zijn voor cGMP productie en signaaltransductie lange tijd onbekend. Dit is in de laatste paar jaar drastisch veranderd met de indentificatie van genen voor twee guanylaat cyclases (GCA en sGC) en twee cGMP-fosfodiesterases (PDE3 en PdeD). Daarnaast zijn ook twee genen die coderen voor eiwitten die het cGMP signaal kunnen doorgeven (GbpC en GbpD) gekarakteriseerd. De meeste van deze eiwitten verschillen van de eiwitten uit dierlijke cellen met gelijksoortige functies. Het mechanisme voor cGMP signaaltransductie is dus onafhankelijk onstaan van dat in dieren tijdens de evolutie. Werk aan knock-out mutanten voor bovenstaande genen hebben de rol voor cGMP in chemotaxis bevestigd. Cellen die hetzij geen cGMP kunnen produceren of het signaal niet door kunnen geven vertonen minder efficiente chemotaxis, vooral onder moeilijker condities. Daarnaast heeft cGMP mogelijk ook andere functies. Wij hebben gevonden dat *pdeD* knock-out mutanten genen voor eiwitten die betrokken zijn bij aggregatie versneld tijdens de ontwikkeling afschrijven, dus cGMP is mogelijk ook betrokken bij genexpressie. Door de identificatie van de genen die betrokken zijn bij cGMP signaaltransductie en de generatie van mutanten waarin deze genen zijn uitgeschakeld is er nu de mogelijkheid om de rol van cGMP in *Dictyostelium* in detail te bestuderen.

Curriculum Vitae

Marcel Erwin Meima werd geboren op 8 Februari 1971 in Veendam. Hij behaalde het VWO diploma aan de Rijksscholengemeenschap Winkler Prins te Veendam in 1989 en kwalificeerde zich voor eindronde van de landelijke Chemie Olympiade dat jaar. Hij studeerde Scheikunde aan de Rijksuniversiteit Groningen, waar hij met zeer veel genoegen afstudeerde in de richting Biochemie in 1995. Het hoofdvakonderzoek werd verricht in het laboratorium van Professor Van Haastert, afdeling Biochemie, Faculteit Wiskunde en Natuurwetenschappen aan de regulatie van fosfolipase C in *Dictyostelium*. Het promotieonderzoek werd verricht in het Instituut voor de Moleculaire Plantkunde aan de Universiteit Leiden in het laboratorium van Professor Schaap. Vanwege de verhuizing van de groep van Professor Schaap naar de University of Dundee, Verenigd Koninkrijk, werd het promotiewerk daar afgerond, waarna hij tot 2003 als Research Assistant in dezelfde groep werkzaam was. Heden is hij werkzaam als Assistant Specialist in het laboratorium van Professor Barber aan de University of California, San Francisco in de Verenigde Staten, waar hij werkt aan signaal complexen geassocieerd met de Na^+/H^+-exchanger NHE1. Marcel is getrouwd met Suzanne Thérese Françoise Bosch en heeft een pasgeboren dochtertje, Jolanda Lynn.

Marcel Erwin Meima was born on February 8th 1971 in Veendam, The Netherlands. He received his pre-university high school certificate at the "Rijksscholengemeenschap Winkler Prins" in Veendam in 1989 and qualified for the final round of the National Chemistry Olympiade that year. He studied Chemistry at the University of Groningen, the Netherlands, where he B.S. with high honours, specialisation Biochemistry, in 1995. His graduation research project was done in the laboratory ofn Professor Van Haastert, Department of Biochemistry, on the regulation of Phospholipase C in *Dictyostelium*. His PhD research project was done at the Institute for de Moleculair Plant Sciences at the University of Leiden, The Netherlands, in the laboratory Professor Schaap. Due to the movement of Professor Schaap's group to the University of Dundee, United Kingdom, his PhD project was finished in Dundee, after which he worked till 2003 as Research Assistant in the same group. Nowadays, he works as an Assistant Specialist in the laboratory of Professor Barber at the University of California, San Francisco in the United States, where he studies signalling complexes associated with the Na^+/H^+-exchanger NHE1. Marcel is married to Suzanne Thérese Françoise Bosch and has a newborn daughter, Jolanda Lynn.

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