Universiteit Leiden
The Netherlands

Regulation of dynamic cyclic nucleotide signalling in social amoebas

Weening, K.E.

Citation
Weening, K. E. (2010, April 13). *Regulation of dynamic cyclic nucleotide signalling in social amoebas*. Retrieved from https://hdl.handle.net/1887/15209

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/15209

Note: To cite this publication please use the final published version (if applicable).

Regulation of Dynamic Cyclic Nucleotide Signalling in Social Amoebas

Proefschrift

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op 13 April 2010 klokke 13.45 uur

door

Karin Esther Weening

geboren te Hazerswoude-dorp in 1972

Promotiecommissie:

Promoteres:	Prof. dr. P.J.J. Hooykaas
	Prof. Dr. P. Schaap (University of Dundee, UK)
Overige leden:	Prof. Dr. B. van Duijn,
	Prof. Dr. H.P Spaink
	Prof. Dr. J. den Hertog
	Dr. M. Wang

To my family.
Particularly to my understanding and patient husband, Steve, who has put up with these many years of research. But also to my parents for their fullest support.

Printed by:
Cover: From Ernst Haeckel's "Kunstformen der Natur" (1904), illustrating organisms classified as Mycetozoa.

CONTENTS

Chapter 1 **General Introduction**

1. Cell signalling
2. Cyclic AMP signalling
3. *Dictyostelium discoideum*
4. Cyclic AMP signalling in *dictyostelium*
5. Aims and outline of this thesis
6. References

Chapter 2 **Contrasting Activities of the Aggregative and Late *PdsA* Promoters in *Dictyostelium Development***

Abstract
Introduction
Materials and Methods
Results
Discussion
References

Chapter 3 **Evolution of extracellular phosphodiesterases in *dictyostelids***

Abstract
Introduction
Materials and Methods
Results
Discussion
References

Chapter 4 **Adenylyl cyclase G is transiently activated by cAMP receptors in developing *Dictyostelium discoideum* cells**

Abstract
Introduction
Materials and Methods
Results
Discussion
References

Chapter 5 **Cyclic GMP controls developmental timing in *Dictyostelium***

Abstract
Introduction
Experimental Procedures
Results
Discussion
References

Chapter 6 **Conclusions**

References

Chapter 7 **Non-scientific part**

Summary (in Dutch)
Curriculum Vitae
Publications
Thank you

Chapter 1

General Introduction

1. CELL SIGNALLING

1.1 Cell communication

Environmental sensing is an essential attribute of all living organisms. Unicellular organisms constantly monitor the environment to find food or avoid stress and predation, and more occasionally to find mates. The component cells of multicellular organisms additionally monitor each other to maintain an appropriate balance of metabolic activities, cell proliferation, muscle contraction and organ function in the adult stage, and to coordinate cell differentiation and cell division during embryogenesis. In all circumstances, cells depend on complex mechanisms for regulated production and sensing of a wide range of stimuli.

1.2 Intercellular signalling

Cells can sense and respond to physical signals (e.g. light, temperature, touch) or chemical signals (e.g. nutrients, hormones and neurotransmitters). Chemical signalling is most commonly used for intercellular communication (Stoka 1999). In vertebrates, signalling by secreted molecules can be divided into three types, based on the distance over which they act. Some cells respond to signalling molecules that they themselves produce (autocrine signalling), whilst other cells respond to signals that are produced locally by neighbouring cells (paracrine signalling), or are released by glands in the blood stream (endocrine signalling).

Structurally, signalling molecules can range from gasses to proteins. Gasses may cross membranes and affect neighbouring cells directly. Hydrophobic molecules, such as steroids, can enter cells by diffusing across the plasma membrane and bind to intracellular receptors which are localized in the cytoplasm or nucleus of the cell. In contrast, hydrophilic molecules such as neurotransmitters, peptide hormones, neuropeptides and growth factors bind to cell surface receptors, as they are unable to cross the plasma membrane.

1.3 Detection of signals

Hydrophilic molecules are the most common means of intercellular communication and they are detected by a broad variety of structurally and functionally distinct receptors that are located on the plasma membranes of cells.

Cell surface receptors can be subdivided into several classes: I. Ligand-gated ion-channels. These receptors are protein pores in the plasma membrane that open and close in response to the binding of a signal molecule to the intrinsic ligand-binding domain. They directly control the permeability of the plasma membrane to ions. II. Receptors with intrinsic enzyme activity. Here binding of the signal molecule activates or inactivates the enzyme, which can be a tyrosine-, histidine- or

serine/threonine protein kinase, or a nucleotidyl cyclase that produces the intracellular messengers cAMP or cGMP. III. Receptors that are coupled to intracellular proteins, such as the heterotrimeric G-proteins or JAK tyrosine kinases that further process the response.

The G-protein coupled receptors or GPCRs are the largest and most versatile family of cell surface receptors. They are structurally similar with seven transmembrane α-helices and send signals to intracellular targets by activation of a diverse family of heterotrimeric guanine nucleotide-binding proteins (G-proteins) (Strader, Fong et al. 1995). Activation of the G-proteins is achieved by the binding of a signal molecule to the GPCR, which then alters the conformation of the G-protein α subunit to promote release of GDP and binding of GTP (Bourne 1997). Subsequently, the G-protein dissociates into its α and βγ subunits, which can independently activate intracellular effectors, such as an enzyme or an ion channel. Hydrolysis of GTP to GDP by the intrinsic GTP-ase activity of the α subunit terminates the response.

Activated effector enzymes next produce intracellular molecules, called second messengers, which in turn regulate the activity of other proteins. As a result of this reaction cascade, the original message is greatly amplified and the response time is greatly reduced. This model whereby second messengers are used to relay the action of extracellular stimuli, generally hormones, was first recognized by Sutherland and Rall in 1960 (Haynes, Sutherland et al. 1960; Sutherland and Rall 1960). The first second messenger identified was adenosine 3'-5'-cyclic monophosphate (cAMP) (Robison, Butcher et al. 1968). Since then many others have been identified such as guanosine'-5'-cyclic monophosphate (cGMP), calcium (Ca^{2+}), inositol trisphosphate (IP_3), diacylglycerol (DAG) and arachidonic acid.

2. CYCLIC AMP SIGNALLING

2.1 Discovery of cAMP

The discovery of cAMP, now more than 50 years ago, provided the paradigm for the second messenger concept (Sutherland and Rall 1958). Sutherland and Rall were interested in how the adrenal gland hormone epinephrine regulates the formation of glucose in liver and muscle cells (glycolysis). Using a cell-free system where epinephrine could control glycolysis, they isolated a small heat-stable molecule that was able to activate glycogen phosphorylase (Rall, Sutherland et al. 1957). Around the same time scientists interested in adenosine tri-phosphate (ATP), isolated cAMP after boiling ATP with barium hydroxide, which proved to be identical to the molecule identified by Sutherland and Rall (Cook, Lipkin et al. 1957).

2.2 Function of cAMP

The molecule cAMP is conserved throughout the tree of life although its existence in higher plants remains controversial. However, functions of cAMP and the key components that are required for its synthesis, detection and degradation can vary greatly amongst the diverse organisms. In prokaryotes, the functions for cAMP include regulation of gene expression in enterobacteria, regulation of virulence in *Bordetella pertussis* (pertussis) and *Bacillus anthracis* (anthrax), phagocytosis, and pH regulation, motility and respiration in cyanobacteria (Ullmann and Monod 1968; Pastan and Perlman 1970; Kasahara and Ohmori 1999).

In the lower eukaryotes, such as yeast, cAMP is involved in stress resistance, positive control of cell cycle, sporulation and negative regulation of meiosis and metabolism (Smith, Dickinson et al. 1990; Singh, Sharma et al. 2007). In pathogenic protists, cAMP is crucial for growth and differentiation, for example differentiation of bloodstream forms in *Trypanosoma* and for sexual stage development (gametogenesis) in *Plasmodium* (Seebeck, Schaub et al. 2004). In the higher eukaryotes, cAMP long-term effects are mostly through the action of cAMP-dependent protein kinase A (PKA) leading to gene expression, neurological processes, such as learning and memory, and a myriad of metabolic processes, including regulation of the digestive and reproductive system, glycogenolysis and lipogenesis, muscle contraction, cell growth or cancer, and apoptosis (Dudai 1997; Montminy 1997; Daniel, Walker et al. 1998). In addition, cAMP can be effective through cyclic nucleotide-gated (CNG) ion channel, in for example olfactory and taste neurons, or via cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), which are also called exchange proteins directly activated by cAMP (cAMP-Epacs) (Ronnett and Moon 2002; Bos 2003). (See §1.2.5)

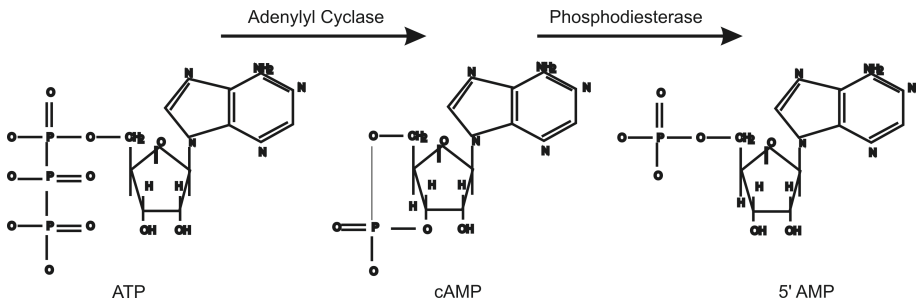


Figure 1: cAMP synthesis and degradation

2.3 cAMP synthesis

Given the importance of cAMP in hormone action, it is not surprising that the enzymes that regulate intracellular cAMP levels have been intensively studied. Sutherland demonstrated in 1960 that cAMP was produced from ATP by the enzyme adenylyl cyclase (Sutherland and Rall 1960). However it took almost 20 years before the first adenylyl cyclase (AC) genes were cloned and characterized. It is clear at present that there are six classes of ACs, of which five are only present in prokaryotes (Danchin 1993). The six classes do not show any significant protein sequence similarity with each other and have very different structures. The class III (universal) cyclases are found in both eukaryotes and prokaryotes (Tang, Yan et al. 1998; Shenoy, Srinivasan et al. 2002).

The first Class I adenylyl cyclase gene was cloned and sequenced from the enterobacterium, *E. coli* (Aiba, Kuwamukai et al. 1983). In *E. coli*, glucose depletion leads to synthesis of cAMP, which binds to and activates the catabolite gene activator protein (CAP) that subsequently triggers expression of the lactose (lac) operon (Kawamukai, Kishimoto et al. 1985). The class I ACs are highly conserved in the enterobacteria and related species. The glucose-sensitive regulatory domain is located at the carboxyl-terminus and the catalytic domain is located at the amino-terminus (Mock, Crasnier et al. 1991).

In 1988, the first class II ACs were identified from the pathogenic bacteria *Bordetella pertussis* (whooping cough) and *Bacillus anthracis* (anthrax*)* (Glaser, Danchin et al. 1988; Mock, Labruyere et al. 1988). Both cyclases showed no sequence homology with the class I cyclases and were shown to be activated by calmodulin, a calcium-binding protein that is only expressed in eukaryotes. Once activated, these adenylyl cyclases become highly active, raising intracellular cyclic AMP (cAMP) to toxic levels that will alter the functions of their host cells. Recently novel toxic cyclases have been cloned from *Yersinia pestis* (plague), *Yersinia Pseudotuberculosis* (gastrointestinal syndromes) and *Vibrio Cholerae* (massive diarrhoea) that are activated via an alternative host cell factor than calmodulin (Moriarty and Turnberg 1986).

When the first eukaryote class III AC was finally cloned from *Saccharomyces cerevisae*, it proved to be strikingly different from the other two classes, both with respect to protein sequence and domain organization. The yeast catalytic domain is located at the C-terminus whereas it can be found at the N-terminus in *E. coli* (Kataoka, Broek et al. 1985). At the same time, Garbers et al. recognized that guanylyl cyclases (GCs), which synthesize another second messenger, cGMP, from GTP, are derived from the same ancestor as the yeast AC. Subsequently many other genes encoding ACs or GCs, belonging to the ‘universal’ class III, were isolated and sequenced from both eukaryotes and prokaryotes (Shenoy, Srinivasan et al. 2002; Schaap 2004; Sinha and Sprang 2006).

The first mammalian AC was cloned in 1989 from bovine brain and 9 more gene families have been identified since, amongst which a soluble cytoplasmic enzyme (sAC) (Sunahara, Dessauer et al. 1996; Chen, Cann et al. 2000). The majority of mammalian AC isoforms are membrane proteins characterized by two bundles of six transmembrane helices each, which separate the two-tandem class III cyclase domains (C1 and C2) that make up the catalytic site of the enzyme. The C1 and C2 sub-domains each have specific residues that are critical for enzyme activity. These residues are required for both metal co-factor binding (C1) and nucleotide binding (C2). Since the substrate ATP binds at the interface of the asymmetric C1 and C2 domains the formation of an intramolecular C1-C2 heterodimer is essential for enzyme activity (Tang and Hurley 1998; Kamenetsky, Middelhaufe et al. 2006; Sinha and Sprang 2006). In contrast, many ACs from lower organisms only have a single cyclase domain, which form homodimers with a second AC molecule. These proteins also show a large variety in domain architectures (Sanchez, Zeoli et al. 1995; Taylor, Muhia et al. 1999; Baker and Kelly 2004; Shenoy and Visweswariah 2006). The class III cyclases are subdivided in 4 subclasses (a-d) according to the conserved amino-acid motifs within their cyclase domains (Linder and Schultz 2003). Moreover, the mammalian class IIIa enzymes are further subdivided 5 types based on their additional regulatory domains (Iyengar 1993).

More recently, three new cyclase classes (class IV-VI) were identified that each consists of only one or a few prokaryotic enzymes (Cotta, Whitehead et al. 1998; Sismeiro, Trotot et al. 1998; Tellez-Sosa, Soberon et al. 2002).

2.4 cAMP degradation

Hormone-induced responses are usually transient and it is therefore of great importance to control the intensity and duration of the cAMP signal. Inactivation of the signal is performed by the cyclic nucleotide phosphodiesterase (PDE) enzymes, which hydrolyse cAMP to 5’AMP. Cyclic nucleotide PDE activity was first described in 1962 (Butcher and Sutherland 1962).

Three classes of PDEs with structurally distinct catalytic domains have been identified. The class I PDEs are found in almost all eukaryotes and have high-affinity for cAMP (Beavo 1995). Class II low-affinity PDEs are found in some prokaryotes and in unicellular eukaryotes such as yeast and social amoebae (Nikawa, Sass et al. 1987; Bader, Kortholt et al. 2007). The completely unrelated class III high-affinity PDEs are confined to prokaryotes (Richter 2002).

At present, based on sequence homology, substrate specificity, modes of regulation and pharmacological properties, mammalian class I PDEs can be further subdivided into 11 families (PDE1-PDE11). Each family contains several members, resulting in a total of 21 different PDE genes. Most of these genes display a complex structure with multiple promoters, which together with alternate mRNA splicing, direct expression of a large number of isoforms (thus far 96 PDE isoforms have been

reported) in a tissue- and differentiation-specific manner (Conti and Beavo 2007).

PDE type I enzymes share a common structure with a conserved catalytic domain flanked by two regulatory domains at the N-terminus and the C-terminus. The substrate specificity of the PDE families includes cAMP-specific (PDE4, 7 and 8), cGMP-specific (PDE5, 6 and 9) and dual-specific PDEs (PDE1, 2, 3, 10 and 11).

Within families the regulatory domains show moderate homology and thus give each of the isoforms their unique regulation and function (Mehats, Andersen et al. 2002). The N-terminus includes sites for nucleotide binding, calmodulin binding, phosphorylation or intracellular targeting. Modifications at the N-terminus caused by ligand binding, dimerization and/or phosphorylation via kinases, result in conformation changes of the catalytic domain. Changes in conformation lead to changes in affinity to substrate, kinetics and interaction with inhibitors. Several properties of the C-terminal domains have been described and are thought to have a role in kinase docking and dimerization (Conti and Beavo 2007; Salter and Wierzbicki 2007).

2.5 cAMP detection and compartmentalization

For many years, all effects of cAMP were considered to be mediated by cAMP-dependent protein kinase A (PKA). (See §1.2.2) However, it is now clear that cells have more than one detection system, including cyclic nucleotide gated (CNG) channels and cAMP-specific guanine nucleotide exchange factors (GEFs), which are also known as exchange proteins directly activated by cAMP (Epacs) (Tasken and Aandahl 2004). Interestingly, the cAMP-binding domains of these proteins are highly homologous to the cyclic nucleotide binding (CNB) domains of PKA-R subunit and that of the CAP (also called cAMP receptor protein or CRP), a soluble *E. coli* transcription factor. Another cyclic nucleotide binding domain, the GAF domain (found in c**G**MP phosphodiesterases, **A**denylate cyclases, and **F**ormate hydrogen lyase transcription factor (FhlA)), which is present in some PDEs, shows no structural or sequence homology to the CNB domain. Proteins containing GAF domains appear to function as dimerization or regulatory domains that bind cyclic nucleotides or other small molecules. The GAF domain of mammalian PDE10 has been shown to bind cAMP (Zoraghi, Corbin et al. 2004).

CNG channels were first discovered in the retinal photoreceptors and olfactory sensory neurons but they are also found in chemosensory cells and a variety of tissues, including kidney, heart and brain (Kaupp and Seifert 2002). In the photoreceptors and olfactory neurons, CNG channels are targets in vision and smell detection. These channels are directly activated by binding of cGMP or cAMP, which are respectively produced in response to light or odor. Binding to a CNB site on the channel protein opens the channel and allows passage of cations, primarily sodium and calcium ions, through the plasma membrane pore into the cell.

Epac proteins contain either one (Epac1) or two (Epac2) cAMP binding domains at the N-terminal regulatory region and a GEF domain at the C-terminal catalytic region (Bos 2003; Bos 2006). In the inactive state, the regulatory region blocks the catalytic site. Direct binding of cAMP to Epac causes a conformational change, hereby exposing the GEF-domain. The GEF domain facilitates activation of Rap1, a small guanosine exchange protein, involved in a variety of processes, such as phagocytosis, cell adhesion, cell-cell contacts and exocytosis (de Rooij, Zwartkruis et al. 1998; Kawasaki, Springett et al. 1998; Bos, de Bruyn et al. 2003).

Despite the presence of these alternative targets, the majority of the effects of cAMP in eukaryotic cells are mediated by cAMP-dependent protein kinase (PKA). In vertebrates, the inactive form of PKA is a tetramer consisting of two regulatory (R) subunits and two catalytic (C) subunits. The activity of two distinct C subunits (Cα and Cβ) is regulated by four different R-subunit isoforms (RIα, RIβ, RIIα and RIIβ) and for both alternative splice variants have been reported. The R-subunits consist of a conserved organisation of sub-domains with a dimerization/docking domain at the N-terminus, and two-tandem cAMP binding domains (A- and B-site) at the C-terminus. Binding of 4 molecules of cAMP, two to each R-subunit of PKA, triggers a conformational changes, which causes the R-subunits to dissociate from the C-subunits. Only the free C-subunits are enzymatically active and are then able to phosphorylate a large variety of targets including transcription factors. Changes in gene expression and cell metabolism are the outcome (Francis and Corbin 1994; Chin, Yang et al. 2002; Skalhegg and Tasken 2000).

2.6 cAMP compartmentalization

Within the same cell, several distinct hormones can elicit specific cAMP-mediated responses, which raises the question how freely diffusible molecules like cAMP can have specific effects. This problem has only recently been solved by the realization that cAMP signalling in mammalian cells is highly compartmentalised (Baillie, Scott et al. 2005). Specificity of cAMP signalling is achieved through the generation of local pools of high cAMP (microdomains) and activation of PKA at distinct subcellular locations. Intracellular microdomains are controlled by the rate of cAMP production by ACs and its degradation by the PDEs. However, it is predominantly localized PDE activity that is responsible for creating intracellular gradients of cAMP. By rapidly degrading cAMP from selected compartments, PDE limits cAMP diffusion so that the cAMP signal can be distributed among their effectors at distinct locations (Houslay and Milligan 1997; Cooper 2003; Tasken and Aandahl 2004; Karpen and Rich 2005; Jarnaess and Tasken 2007).

Given the large diversity of PKA targets within the cell, a precisely regulated and restricted activity of PKA is essential for specificity of response. Intracellular targeting and compartmentalisation of PKA, is mediated by A-kinase anchoring proteins (AKAPs) (Edwards and Scott 2000; Feliciello, Gottesman et al. 2001;

Carlisle Michel and Scott 2002; Wong and Scott 2004; Beene and Scott 2007; Jarnaess and Tasken 2007). Through these anchoring proteins, PKA is available at distinct locations where it can respond rapidly to microdomains of cAMP and in close vicinity to targets. Accordingly, each substrate has its own anchored pool of PKA and its anchored pool of cAMP. Over the past decade over 50 anchoring proteins have been identified, which contain a PKA binding domain and a unique targeting domain.

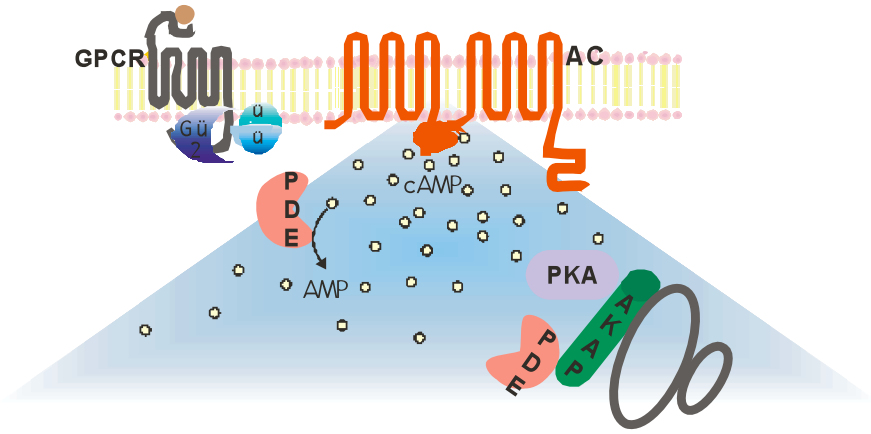


Figure 2: cAMP microdomain

In addition to anchoring of the R-subunit of PKA, some AKAPs are able to form multiprotein complexes. These multiprotein complexes contain signal-termination enzymes, such as PDEs and signal transduction enzymes, such as kinases. For example, the mAKAP complex in the heart brings together three cAMP effectors, PKA, the long PDE4 isoform (PDE4D3) and Epac1 (Dodge, Khouangsathiene et al. 2001). Each of these enzymes, are activated at different concentrations of cAMP and they communicate with each other. As cAMP increases, PDE4D3 is activated by PKA-mediated phosphorylation. Activated PDE decreases concentration of cAMP and allows reorganization of the inactive PKA holoenzyme. In addition, as cAMP levels drop, the Epac1 mediated inhibition of ERK5 pathway is blocked, and ERK will phosphorylate PDE43D hereby causing a decrease of PDE activity which allows accumulation of cAMP (Dodge, Khouangsathiene et al. 2001; McConnachie, Langeberg et al. 2006). Thus adding PDEs into anchoring complexes not only spatially restricts PKA activity but also makes sure that PKA activity is rapidly reduced by the local degradation of cAMP by PDE (Houslay and Baillie 2005; Conti and Beavo 2007; Rich, Xin et al. 2007).

3. *DICTYOSTELIUM DISCOIDEUM*

3.1 About the organism

Dictyostelium discoideum is a member of the social amoebas or *Dictyostelia*, which belong to the supergroup of amoebozoans, the closest sister group to the opisthokonts, which also include the animals and fungi (Baldauf 2003).

D. discoideum was first isolated in the 1930s by Raper from decaying leaves in the mountains of Northern Carolina (Raper 1935). Soon after he described how this on first sight simple unicellular eukaryote undergoes a transformation into a multicellular structure, which is remarkably similar to coordinated cell and tissue movement seen during metazoan morphogenesis (Raper 1940).

Next, Bonner demonstrated that cells in the aggregation centres produce a chemical termed acrasin, which functions as chemoattractant for the population (Bonner 1947). Later, this acrasin was recognized to be cAMP (Konijn, Van De Meene et al. 1967).

These early discoveries soon established *D. discoideum* as an attractive model for studies on cell motility, chemotaxis and cell signalling. More recently, it has been shown to be an equally useful system for studies of apoptosis, mechanisms of action of drugs and evolution (Kessin 2001; Bonner 2003; Adley, Keim et al. 2006; Kosta, Laporte et al. 2006; Urushihara 2008).

For this a wide range of cell biological, molecular genetic and biochemical techniques is available. The 34 Mb genome of *Dictyostelium discoideum* has been sequenced and is readily accessible at Dictybase, the online resource centre about *Dictyostelium* (Eichinger, Pachebat et al. 2005; Fey, Gaudet et al. 2006).

3.2 The *D. discoideum* life cycle

In their natural habitat, the soil, single amoebae feed by phagocytosis of bacteria. These bacteria secrete compounds such as folic acid, which serve as attractants that trigger chemotaxis in the *Dictyostelium* amoebas (Pan, Hall et al. 1972). The amoebas engulf the bacteria, grow and multiply by mitosis, as long as bacteria are available. Starvation stress initiates a transition to multicellular development that ultimately produces a dormant spore stage.

Upon food depletion, some amoebas in the population start to secrete cAMP pulses, which attract surrounding cells. Aggregates are formed which transform into slug-shaped cell masses and finally into fruiting bodies. The cells differentiate into two cell types: stalk cells, which are fated to die and spores, which remain viable for very long times. When food is once more available, the spores germinate and the cycle is repeated.

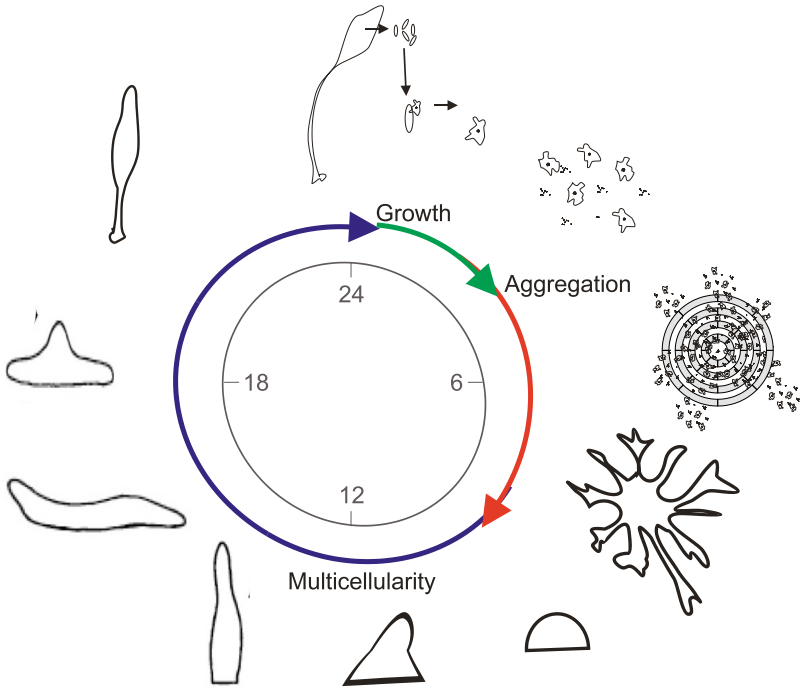


Figure 3: Life cycle of *D. discoideum*

3.3 Signalling during development

During the entire course of development secreted signal molecules play a vital role. Growing cells are able to sense availability of food in relation to cell density by a process called quorum sensing. Cell density is monitored measuring the threshold values of two secreted peptide factors, pre-starvation factor (PSF) and condition medium factor (CMF). Vegetative cells continuously secrete PSF, and when food supply has run down PSF production decreases and CMF increases. In succession, they initiate expression of early genes and genes required for aggregation and hence trigger the onset of the developmental stage (Mehdy and Firtel 1985; Gomer, Yuen et al. 1991; Rathi and Clarke 1992; Clarke and Gomer 1995). Amongst the early aggregative genes are cAMP receptors (cARs) and adenylyl cyclase A (ACA) that allow the cells to detect, produce and secrete cAMP.

Some starving cells begin to release cAMP pulses at about 6 min intervals. Neighbouring cells respond by moving chemotactically towards the cAMP source and by releasing a cAMP pulse themselves. This process is called cAMP relay (Parent and Devreotes 1996). As a result, waves of cAMP propagate through the cell

population and the cells stream towards the original source of the pulses to form an aggregate (Goldbeter 2006). The aggregate can contain up to a million cells and its centre continues to emit cAMP pulses. As a consequence this region is pushed upwards by the movement of cells underneath forming first a distinctive tip and later a finger-shaped "slug". The slug topples over by gravity, allowing it to migrate towards light and warmth, which leads it to the top layer of the soil (Fisher 1997). Once favourable conditions are found, the slug will culminate into a fruiting body.

After completion of aggregation, cAMP can reach micromolar concentrations, which induce expression of postaggregative genes and prespore genes. Moreover, micromolar cAMP induces synthesis of differentiation inducing factor (DIF), a chlorinated hexaphenone that induces expression of the prestalk genes and stalk cell differentiation, and also inhibits prespore differentiation (Morris, Taylor et al. 1987; Kay 1997). Prespore cells and prestalk cells at first differentiate intermixed in a 3:1 ratio, but sort out while the slug is being formed. Prespore cells will sort out to the posterior region of the slug while the prestalk cells will sort to the anterior tip. This occurs because the prestalk cells are more chemotactically responsive towards the cAMP pulses emitted by the tip (Nicol, Rappel et al. 1999).

High concentrations of ammonia, present in the tip of the slug, inhibit the prestalk to stalk transition. This inhibition is removed when the slug tip moves upwards at early culmination and ammonia is lost by gaseous diffusion (Feit, Medynski et al. 2001). The prestalk cells first form a cellulose tube, crawl into the tube and differentiate into stalk cells. The prespore cells climb up the stalk and differentiate into immobile spores (Dormann, Siegert et al. 1996; Maree and Hogeweg 2001).

The timing of spore differentiation is under tight control, since it should only occur once morphogenesis is complete. Spore cell formation is induced by two spore differentiation factors, SDF1 and SDF, which are produced and secreted by the newly formed stalk cells. SDF2 and discadenine, a plant cytokinin derivative, jointly stimulate encapsulation of the prespore cells into dormant cells (Anjard, Chang et al. 1998). However, they do this through separate signalling pathways.

The spores are kept dormant by high osmolarity and discadenine until the right environmental conditions are met. Germination activated by exposure to food results in the spread of unicellular amoebae.

4. CYCLIC AMP SIGNALLING IN *DICTYOSTELIUM*

4.1 Function of cAMP

Cyclic AMP in eukaryotes and prokaryotes is generally used as a second messenger acting on downstream effectors such as PKA. Yet, as already revealed in the previous paragraph, in *D. discoideum* cAMP has multiple roles. It acts extracellularly as a signal to control chemotaxis, expression of aggregative genes and

prespore genes. In addition, it also functions intracellularly, acting on PKA, controlling almost all major developmental transitions, including initiation of development, spore and stalk maturation and spore germination (Saran, Meima et al. 2002). Interestingly, environmental cues, such as quorum sensing, ammonia and SDF levels, high osmolarity and discadenine all control intracellular cAMP levels. (See §1.4.4.2)

It is therefore not surprising that many of the key proteins essential for cAMP signalling in other organisms are also present in *D. discoideum.* The role of the proteins used to detect, synthesise and degrade cAMP in development and functioning are discussed in turn.

4.2 cAMP detection

4.2.1 Detection of intracellular cAMP

In *Dictyostelium*, intracellular cAMP is detected by a single PKA regulatory subunit (PKA-R), which interacts with a single PKA catalytic subunit (PKA-C). PKA-C null mutants cannot aggregate, while disruption of PKA activity, by overexpression of dominant-negative regulatory subunits under stage- and cell-type specific promoter inhibits initiation of development and differentiation of spores and stalk cells (Mann and Firtel 1991; Harwood, Hopper et al. 1992; Schulkes and Schaap 1995). On the other hand, deletion mutants of PKA-R or mutants that constitutively express PKA-C show accelerated postaggregative development and precocious spore and stalk cell differentiation (Anjard, Pinaud et al. 1992; Mann, Yonemoto et al. 1992).

Thus, PKA activation is needed for initiation of development, control of developmental timing, differentiation of prespore cells, the maturation of stalk cells (Harwood, Hopper et al. 1992; Loomis 1998). In addition, PKA activity is required for maintenance of spore dormancy.

4.2.2 Detection of extracellular cAMP

Extracellular cAMP is detected by classical G-protein coupled seven transmembrane receptors. Four cAMP receptor (cAR1-4) genes have been identified that differ in ligand affinity and spatio-temporal expression (Saxe III, Johnson et al. 1991; Johnson, Van Haastert et al. 1992).

cAR1 is expressed during early aggregation whereas cAR3 is maximal during the mound stage. These are followed by cAR2 and cAR4, which are expressed exclusively in the prestalk cells in the slug and culmination stages. cAR1 was identified first and disruption of its gene blocks aggregation and subsequent development (Klein, Sun et al. 1988). Deletion of cAR3 had no obvious effect. However, when both cAR1 and cAR3 are deleted, all responses to extracellular cAMP are lost, suggesting that cAR1 and cAR3 have overlapping roles (Insall, Soede et al. 1994; Soede, Insall et al. 1994). Furthermore, overexpression of cAR2 or cAR3

in *cAR1-/cAR3-* cells rescues many of the functions of cAR1, suggesting that each receptor can couple to the same signalling pathway (Kim, Borleis et al. 1998). The other two receptors cAR2 and cAR4 are required for normal tip formation and pattern formation (Saxe III, Ginsburg et al. 1993; Kim, Harwood et al. 2002).

Ligand binding to cAR1 leads to activation of the associated heterotrimeric G-proteins. *Dictyostelium* has eleven G-protein α subunits, which are all expressed at different stages of development (Brzostowski and Kimmel 2001). Only Gα2 couples to cAR1 as deletion of Gα2 subunit leads to defects in many cAR1-mediated responses including chemotaxis to cAMP and *in vivo* adenylyl cyclase activity.

Binding of cAMP to cAR1 mediates the releases of Gα2 and the Gβγ subunit. A single *β* and *γ* have been cloned in *Dictyostelium* (Lilly, Wu et al. 1993; Zhang, Long et al. 2001). Null mutants in the Gβ-subunit loose all responses to cAMP and to other chemoattractants, such as folic acid. In addition, in the Gβ null mutant GTPγS is unable to activate adenylate cyclase A, while it can do so in Gα2- mutants. This indicates that the Gβγ subunit is required for activation of adenylyl cyclase (Wu, Valkema et al. 1995). The interaction between G*α*2 and *βγ* in response to cAMP receptor mediated activation has been stylishly shown by a fluorescence resonant energy transfer study (FRET) (Janetopoulos, Jin et al. 2001).

4.3 cAMP production

The *D. discoideum* genome encodes for three adenylyl cyclases, ACA, ACG and ACB. Their catalytic domains are related, but they differ considerably in their membrane topology, and show distinctive expression patterns, regulation and function (Kriebel and Parent 2004).

The *acgA* gene and the *acaA* gene were identified by PCR through their homology with the catalytic domain of mammalian adenylyl cyclases (Pitt, Milona et al. 1992). The ACB gene, *acrA*, was obtained later in a morphological mutant screen; although earlier its activity was established by biochemical assays (Kim, Chang et al. 1998; Soderbom, Anjard et al. 1999). ACA is expressed highest during aggregation, followed by ACB, with maximal expression at culmination and finally by ACG (Meima and Schaap 1999; Kriebel and Parent 2004).

4.3.1 ACA

The aggregation adenylyl cyclase (ACA) is homologous to the G-protein coupled mammalian type III cyclases. Similar to those enzymes, its structure also consists of 12 transmembrane domains and two catalytic domains that make up the catalytic site.

ACA is required for oscillatory cAMP production during aggregation. Oscillatory cAMP secretion requires regulation of the cAMP production process by a positive and a negative feedback loop (Tang and Othmer 1994; Goldbeter 2006). The positive

loop is provided by cAMP receptor mediated activation of ACA, which requires G-proteins and multiple cytosolic components.

Binding of cAMP to the cAR1 receptor leads not only to dissociation of its associated G-protein G2 into its Gα2 and the βγ subunits, but also causes RasC activation, possibly through the Ras-GEF aimless (AleA) (Wu, Valkema et al. 1995; Insall, Borleis et al. 1996; Lim, Spiegelman et al. 2001; Kae, Kortholt et al. 2007). Co-activation of βγ subunit and RasC instigate recruitment of the phosphatidylinositol 3' kinase (PI3K) to the plasma membrane, where it phosphorylates the membrane lipid phosphatydyl-inositol 2-phosphate (PIP2) to form phosphatydyl-inositol 3-phosphate (PIP3).

PIP3 can bind to the pleckstrin homologue (PH) domain of the cytosolic regulator of adenylyl cyclase (CRAC) (Lilly and Devreotes 1994; Funamoto, Meili et al. 2002; Comer, Lippincott et al. 2005). This binding causes CRAC to translocate from the cytosol to the membrane where it activates ACA (Insall, Kuspa et al. 1994; Lilly and Devreotes 1995; Dormann, Weijer et al. 2002).

Additionally, optimal ACA activation requires input from the preformed multi-protein complex, TORC2, which includes Pianissimo (homolog of Raptor), Ras interacting protein 3 (homolog of AVO1) and Lst8 (Chen, Long et al. 1997; Lee, Parent et al. 1999; Lee, Comer et al. 2005).

Lastly, the Map kinase Erk2 has been shown to be essential for ACA activation possibly through its inhibition of the intracellular PDE RegA (Segall, Kuspa et al. 1995; Maeda, Lu et al. 2004). (See §1.4.4.2)

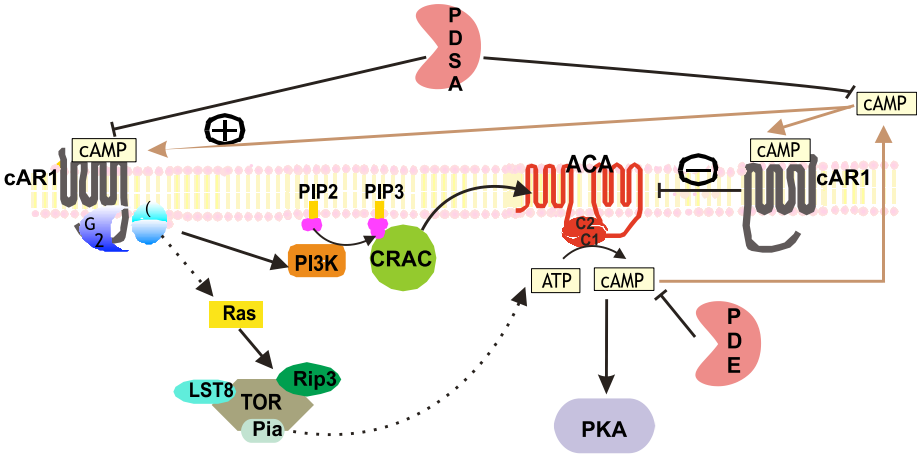


Figure 4: ACA signalling

Constant stimulation by cAMP will eventually make the cells insensitive to further stimulation, a process called adaptation (Devreotes and Steck 1979; Zhang, Goswami et al. 2005). This adaptation process of ACA is controlled via a yet poorly understood mechanism, although several models have been proposed, including uncoupling of receptors from G-proteins, ligand-induced phosphorylation of cAR1 and activation of intracellular cAMP phosphodiesterases (Tang and Othmer 1994; Kim, Soede et al. 1997; Laub and Loomis 1998; Meima, Weening et al. 2003). Removal of the stimulus, by the extracellular phosphodiesterase, PdsA, reverses the process of adaptation and will make cells responsive again (Lacombe, Podgorski et al. 1986). Oscillations involve cycles, each lasting about 6 minutes, of alternating activation, adaptation and de-adaptation (Gerisch and Wick 1975; Dinauer, MacKay et al. 1980; Dinauer, Steck et al. 1980; Dinauer, Steck et al. 1980).

Deletion mutants of ACA do not secrete cAMP and so are unable to aggregate (Pitt, Milona et al. 1992). However, development can be partly restored when cells are stimulated with extracellular cAMP or by overexpression of the PKA-C subunit (Pitt, Brandt et al. 1993).

ACA is expressed maximally during aggregation, but after aggregation ACA expression decreases and becomes restricted to the tip of the slug (Meima and Schaap 1999; Verkerke-van Wijk, Fukuzawa et al. 2001). Thus ACA is not required for the production of the high cAMP levels, which are essential during later development for PKA activation (Pitt, Brandt et al. 1993).

4.3.2 ACB

ACB activity was first identified in intact cells that lack either the intracellular cAMP-phoshodiesterase RegA or its activator, the histidine phosphotransferase protein RdeA, which suggests that ACB activity is closely associated with both proteins (Kim, Chang et al. 1998).

The ACB gene has seven transmembrane domains, a histidine kinase domain, two response regulator domains and a single catalytic domain (Soderbom, Anjard et al. 1999). ACB sequence and structure is most similar to that of some cyanobacterial cyclases (CyaA). It belongs to the subclass IIIb, which apart from many prokaryote cyclases also contains mammalian sAC and Dictyostelium sGC (Roelofs, Meima et al. 2001); Linder, 2003 #605; Schaap, 2005 #250].

The cyanobacterial adenylyl cyclases are activated by light through a two-component phosphorelay system and by bicarbonate ions through direct activation of the catalytic domain (Kasahara and Ohmori 1999). Bicarbonate ions have also been shown to activate the mammalian soluble cyclase (sAC) in a pH dependent manner (Chen, Cann et al. 2000; Kobayashi, Buck et al. 2004).

Thus far, it is unclear how ACB is regulated or what the functions of the histidine kinase and response regulator domains are. The histidine kinase domain is unlikely to be functional as it lacks the required histidine residue for

autophosphorylation (Soderbom, Anjard et al. 1999). However, recently it has been suggested that the histidine kinase, DhkB directly can activate ACB by forming a heterodimer with the histidine kinase domain of ACB (Anjard and Loomis 2008). Similar to CyaA and mammalian sAC, the cyclase catalytic domain of ACB has a threonine residue at the highly conserved aspartate, which mediates activation by bicarbonate in these cyclases (Kobayashi, Buck et al. 2004).

ACB is expressed at low levels in growth and early development with expression increasing during slug formation to its maximum at early culmination. Deletion mutants in the *acrA* gene, coding for ACB, are defective in spore formation, but prespore gene expression and stalk differentiation are normal. Hence, this cyclase is needed for cAMP production that triggers maturation of stalk and spore formation via PKA activation (Meima and Schaap 1999; Soderbom, Anjard et al. 1999).

4.3.3 ACG

The germination adenylyl cyclase (ACG) is structurally similar to the ACs found in the kinetoplastic protists, such as the parasites *Trypanosoma* and *Leishmania*, with one or two transmembrane (TM) domain, a large extracellular domain and a single catalytic domain (Seebeck, Schaub et al. 2004). However, ACG is a class IIIa enzyme, whereas the parasite cyclases are members of class IIIc (Pitt, Milona et al. 1992; van Es, Virdy et al. 1996; Ross, Raibaud et al. 1991). ACG mRNA is expressed at low levels throughout development, but is strongly up-regulated in maturing spores. ACG protein is localised in the posterior of the slug, the prespore and spore cells region, whilst the other ACA and ACB are restricted to the prestalk region (Alvarez-Curto, Saran et al. 2007) Here, ACG produces the cAMP needed for prespore differentiation (Alvarez-Curto, Saran et al. 2007).

In the mature spores, ACG is activated by high osmolarity that is present in the spore head. It controls spores germination, which is inhibited by high osmolarity, by acting on PKA (van Es, Virdy et al. 1996). Spore dormancy is also under control of the autoinhibitor discadenine and the histidine kinase DokA through separate signalling pathways. (See §1.4.5.2)

4.4 cAMP degradation

D. discoideum has seven PDEs of which three are responsible for extracellular cAMP degradation (PdsA/PDE1, PDE7 and PDE4) and two for intracellular cAMP degradation (RegA/PDE2, PdeE/PDE6). Their catalytic domains are homologous to the type I PDE (RegA and PDE4) or type II PDE (PdsA, PDE7 and PdeE).

Biochemically they can be distinguished through their distinct substrate affinity (Km) and enzyme-specific inhibitors (Bader, Kortholt et al. 2007).

4.4.1 Degradation of extracellular cAMP

During aggregation, degradation of extracellular cAMP by PdsA is important to support oscillatory cAMP signalling (Lacombe, Podgorski et al. 1986; Podgorski, Faure et al. 1988). After aggregation PdsA is required for coordinated cell movement and fruiting body morphogenesis (Wu, Franke et al. 1995; Sucgang, Weijer et al. 1997).

PdsA has a PDE II catalytic domain and is homologous to the yeast PDE II gene (Bader, Kortholt et al. 2007). It can be either secreted (ePDE) or targeted to the plasma membrane (mPDE) and degrades both cAMP and cGMP. The secreted form of PdsA is inhibited by a glycoprotein, PDI, which is secreted during aggregation when cAMP levels decrease (Wu and Franke 1990; Franke, Faure et al. 1991).

The *PdsA* gene is regulated by three promoters; the vegetative, aggregative and the late promoter, which direct gene expression during growth, aggregation and multicellular development, respectively (Faure, Franke et al. 1990). A deletion mutant of *PdsA* is deficient in aggregation while an overexpression mutant shows accelerated aggregation with a block in late development (Darmon, Barra et al. 1978; Faure, Podgorski et al. 1988). Expression of *PdsA* from both the aggregation promoter and the late promoter are required for normal development (Hall, Franke et al. 1993; Sucgang, Weijer et al. 1997).

Additionally, extracellular cAMP is degraded by PDE4 and PDE7. PDE4 has two transmembrane domains and an extracellular PDE class I domain that is highly homologous to the mammalian cAMP-specific PDE8. Null mutants show normal aggregation, but impaired development from the mound stage, which correlates with its expression levels that are low during aggregation but increases during the mound stage (Bader, Kortholt et al. 2006).

PDE7 is a close homologue of PdsA, which also gets secreted or targeted to the plasma membrane. Similarly to PdsA it is a dual specificity PDE that can degrade both cAMP and cGMP, though its Km is 15 times lower than that of PdsA (Bader, Kortholt et al. 2007).

4.4.2 Degradation of intracellular cAMP

The intracellular cAMP-specific phosphodiesterase, RegA, acts antagonistically with the adenylyl cyclases during late development. Inactivation of RegA provides an alternative way to increase intracellular cAMP and thus the activity of cAMP-dependent PKA to the levels required during late development for spore and stalk-cell development. Indeed, deletion of the *RegA* gene speeds up development into spores, yet it also affects proper morphogenesis and so leads to abnormal fruiting bodies (Shaulsky, Fuller et al. 1998).

RegA contains an N-terminal response regulator domain and a C-terminal class I PDE domain. RegA is regulated by phosphorylation of its response regulator

domain through a His-Asp phosphorelay system (Thomason, Traynor et al. 1999). Additionally, it is controlled via cAR-mediated phosphorylation of the MAP kinase homologue ERK2 (Mohanty, Lee et al. 2001). Activated ERK2 phosphorylates RegA, possibly on more then one site, which results in inhibition of RegAs PDE activity but also leads to ubiquitination and degradation of RegA by the proteasome (Mohanty, Lee et al. 2001; Maeda, Lu et al. 2004). Both would lead to cAMP accumulation and increased PKA activity. Interestingly, PKA has been suggested to inhibit ERK2 activity, which in turn lifts the inhibition of RegA and subsequently causes a decrease of cAMP levels (Laub and Loomis 1998).

His-Asp phosphorelay is a very common signal transduction system of prokaryotes, which is also found in fungi and plants but not in metazoans. Phosphorelay is initiated by a hybrid histidine kinase (HK), which is linked to a sensor and a response regulator domain. In the canonical pathway, activation of the sensor by physical or chemical stimuli activates the histidine kinase, which phosphorylates itself on a histidine residue followed by transfer of the phosphoryl group to a conserved aspartate in the response regulator. In eukaryotes the phosphoryl group is subsequently transferred to the conserved histidine of a phosphotransfer protein and finally to the aspartate of a second response regulator, which controls the activity of an attached enzyme or transcription factor. The histidine kinase also has phosphatase activity, which can alternatively be activated by stimuli and cause reverse phosphorelay (Thomason and Kay 2000; Yamamoto, Hirao et al. 2005; Anjard and Loomis 2008).

In the final stages of development, *D. discoideum* uses sensor HKs to increase intracellular cAMP levels and subsequent PKA activity. In *D. discoideum*, activation of a histidine kinase leads to transfer of phosphate one by one via the intermediate histidine phosphotransferase protein, RdeA to the response regulator of RegA (Thomason, Traynor et al. 1999). Several proteins containing histidine kinase domains have been identified, of which 4 have been shown to affect RegA activity via RdeA: DhkA, DhkB, DhkC and DokA. Earlier mentioned factors, ammonia, SDF2, osmolarity and discadenine, act through sensor HKs. (See §1.4.2)

DhkC senses ammonia levels in migrating slugs. Autophagic processes in particularly the prestalk cells of slugs produce large amounts of ammonia, which prevent stalk cell differentiation. High levels of ammonia activate DhkC, which leads to phosphorylation of RegA via RdeA. Activated RegA hydrolyses cAMP and therefore inactivates PKA. When the slug moves upwards during early culmination, ammonia is lost by diffusion. DhkC and therefore RegA are no longer activated. This leads to cAMP accumulation and activation of PKA (Singleton, Zinda et al. 1998).

The spore differentiation factor, SDF2 and the cytokinin-like discadenine, together stimulate spore formation. However, they do this through separate signalling pathways (Anjard, Zeng et al. 1998). SDF-2 activates the phosphatase activity of the histidine kinase DhkA, leading to dephosphorylation and inhibition of RegA activity (Wang, Soderbom et al. 1999). Discadenine was previously proposed to activate

DhkB, which maintains spore dormancy and inhibits germination (Zinda and Singleton 1998). However, more recently discadenine has been found not to activate DhkB, but to activate ACB directly by forming a heterodimer with the pseudo-histidine kinase domain of ACB (Anjard and Loomis 2008). (See §1.4.3.2) Both pathways, either through inhibition of a cAMP-PDE (RegA) or direct activation of an AC (ACB), lead to an increase of cAMP and hence PKA activity.

High osmolarity increases intracellular cAMP via two alternative independent pathways. High osmolarity, available in the sorus, increases intracellular cAMP through ACG or by acting on the histidine phosphatase DokA leading to inactivation of RegA (Ott, Oehme et al. 2000).

In addition to RegA, a second intracellular phosphodiesterase PdeE was identified. It has two cyclic nucleotide binding motifs and a binuclear Zn^{2+} binding domain that forms the catalytic center of metallo-β-lactamases (Goldberg, Bosgraaf et al. 2002; Meima, Biondi et al. 2002). Studies revealed that the *PdeE* gene encodes a cAMP-stimulated dual specificity phosphodiesterase with preference for cAMP that is mainly expressed during aggregation (Bosgraaf, Russcher et al. 2002; Meima, Weening et al. 2003).

5. AIMS AND OUTLINE OF THIS THESIS

Over the past years, much information has been gathered about the importance of the individual key components that control cAMP signalling in eukaryotes and prokaryotes. In mammalians, several different classes of ACs produce cAMP and much is known about their mechanism of stimulation and function. In contrast, much less is known about the enzymes that control degradation, the cAMP-PDEs and their regulatory mechanism. Nonetheless, it has become evident that cAMP is degraded by many isoforms of PDEs and that PDEs play a crucial role in controlling dynamic cAMP signalling.

Throughout my PhD I have been interested in several aspects of cAMP signalling in the model organism *Dictyostelium discoideum* with particular emphasis on the roles of the phosphodiesterases in dynamic cAMP signalling. PdsA is the most critical enzyme in the control of aggregation and post-aggregative morphogenesis. It is controlled by three promoters; the vegetative, aggregative and the late promoter, which direct expression during growth, aggregation and late development, respectively. Despite its known crucial role in multicellular development not much is known about the regulation of *PdsA* or its expression after aggregation. In chapter 2, I present studies on the patterns of the aggregative and late promoter activity throughout development, the signals that regulate promoter activity and the promoter sequences that mediate regulation by these signals.

D. discoideum and related species use extracellular cAMP as chemoattractant for aggregation, but most other species use other chemoattractants than cAMP. In chapter 3, I identified orthologues of the *PdsA* gene throughout the *Dictyostelid*

phylogeny and studied their expression during development. Additionally, I show that one of them encodes a fully functional PDE.

Cyclic AMP waves propagate throughout the *Dictyostelium* slug. This is somewhat enigmatic since ACA, the enzyme considered to be responsible for pulsatile cAMP signalling, is only expressed at the slug anterior. ACG is expressed at the posterior region of the slug, where it was shown to be responsible for induction of prespore differentiation. In chapter 4, I describe experiments showing that similar to ACA, ACG is transiently activated by stimulation of cAMP receptors. This could explain why cAMP waves can propagate in slug posteriors.

In *Dictyostelium*, cGMP is associated with chemotaxis and plays a major role in myosin II regulation during cytoskeletal reorganisation. In the chapter 5, I describe experiments showing that modulation of the expression of the *PdeD* gene, a cGMP-stimulated cGMP-PDE, has a pronounced effect on the expression of early genes, necessitating re-evaluation of the role of cGMP in *Dictyostelium* chemotaxis.

6 REFERENCES

Adley, K. E., M. Keim, et al. (2006). "Pharmacogenetics: defining the genetic basis of drug action and inositol trisphosphate analysis." Methods Mol Biol **346**: 517-34.

Aiba, H., M. Kuwamukai, et al. (1983). "Cloning and promoter analysis of the *Escherichia coli* adenylate cyclase gene." Nucleid Acids Res. **11**: 3451-3465.

Alvarez-Curto, E., S. Saran, et al. (2007). "cAMP production by adenylyl cyclase G induces prespore differentiation in Dictyostelium slugs." Development **134**(5): 959-66.

Anjard, C., W. T. Chang, et al. (1998). "Production and activity of spore differentiation factors (SDFs) in Dictyostelium." Development **125**: 4067-4075.

Anjard, C. and W. F. Loomis (2008). "Cytokinins induce sporulation in Dictyostelium." Development **135**(5): 819-27.

Anjard, C., S. Pinaud, et al. (1992). "Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of Dictyostelium discoideum." Development **115**: 785-790.

Anjard, C., C. J. Zeng, et al. (1998). "Signal transduction pathways leading to spore differentiation in dictyostelium discoideum." Developmental Biology **193**: 146-155.

Bader, S., A. Kortholt, et al. (2006). "DdPDE4, a novel cAMP-specific phosphodiesterase at the surface of dictyostelium cells." J Biol Chem **281**(29): 20018-26.

Bader, S., A. Kortholt, et al. (2007). "Seven Dictyostelium discoideum phosphodiesterases degrade three pools of cAMP and cGMP." Biochem J **402**(1): 153-61.

Baillie, G. S., J. D. Scott, et al. (2005). "Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract." FEBS Lett **579**(15): 3264-70.

Baker, D. A. and J. M. Kelly (2004). "Structure, function and evolution of microbial adenylyl and guanylyl cyclases." Mol Microbiol **52**(5): 1229-42.

Baldauf, S. L. (2003). "The deep roots of eukaryotes." Science **300**(5626): 1703-6.

Beavo, J. A. (1995). "Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms." Physiol Rev **75**(4): 725-48.

Beene, D. L. and J. D. Scott (2007). "A-kinase anchoring proteins take shape." Curr Opin Cell Biol **19**(2): 192-8.

Bonner, J. T. (1947). "Evidence for the formation of aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum." J.Exp.Zool. **106**: 1-26.

Bonner, J. T. (2003). "Evolution of development in the cellular slime molds." Evol Dev **5**(3): 305-13.

Bos, J. L. (2003). "Epac: a new cAMP target and new avenues in cAMP research." Nat Rev Mol Cell Biol **4**(9): 733-8.

Bos, J. L. (2006). "Epac proteins: multi-purpose cAMP targets." Trends Biochem Sci **31**(12): 680-6.

Bos, J. L., K. de Bruyn, et al. (2003). "The role of Rap1 in integrin-mediated cell adhesion." Biochem Soc Trans **31**(Pt 1): 83-6.

Bosgraaf, L., H. Russcher, et al. (2002). "Identification and characterization of two unusual cGMP-stimulated phoshodiesterases in dictyostelium." Mol Biol Cell **13**(11): 3878-89.

Bourne, H. R. (1997). "How receptors talk to trimeric G proteins." Curr Opin Cell Biol **9**(2): 134-42.

Brzostowski, J. A. and A. R. Kimmel (2001). "Signalling at zero G: G-protein-independent functions for 7-TM receptors." Trends in Biochem. Sci. **26**(5): 291-297.

Butcher, R. W. and E. W. Sutherland (1962). "Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine." J Biol Chem **237**: 1244-50.

Carlisle Michel, J. J. and J. D. Scott (2002). "AKAP mediated signal transduction." Annu Rev Pharmacol Toxicol **42**: 235-57.

Chen, M. Y., Y. Long, et al. (1997). "A novel cytosolic regulator, pianissimo, is required for chemoattractant receptor and g protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in dictyostelium." Genes And Development **11**: 3218-3231.

Chen, Y. Q., M. J. Cann, et al. (2000). "Soluble adenylyl cyclase as an evolutionary conserved bicarbonate sensor." Science **289**: 625-628.

Chin, K. V., W. L. Yang, et al. (2002). "Reinventing the wheel of cyclic AMP: novel mechanisms of cAMP signalling." Ann N Y Acad Sci **968**: 49-64.

Clarke, M. and R. H. Gomer (1995). "PSF and CMF, autocrine factors that regulate gene expression during growth and early development of Dictyostelium." Experientia **51**: 1124-1134.

Comer, F. I., C. K. Lippincott, et al. (2005). "The PI3K-mediated activation of CRAC independently regulates adenylyl cyclase activation and chemotaxis." Curr Biol **15**(2): 134-9.

Conti, M. and J. Beavo (2007). "Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signalling." Annu Rev Biochem **76**: 481-511.

Cook, W. H., D. Lipkin, et al. (1957). J. Am. Chem. Soc. **79**: 3607.

Cooper, D. M. (2003). "Regulation and organization of adenylyl cyclases and cAMP." Biochem J **375**(Pt 3): 517-29.

Cotta, M. A., T. R. Whitehead, et al. (1998). "Identification of a novel adenylate cyclase in the ruminal anaerobe, Prevotella ruminicola D31d." FEMS Microbiol Lett **164**(2): 257-60.

Danchin, A. (1993). "Phylogeny of adenylyl cyclases." Adv Second Messenger Phosphoprotein Res **27**: 109-62.

Daniel, P. B., W. H. Walker, et al. (1998). "Cyclic AMP signalling and gene regulation." Annu Rev Nutr **18**: 353-83.

Darmon, M., J. Barra, et al. (1978). "The role of phosphodiesterase in aggregation of Dictyostelium discodeum." J.Cell Sci. **31**: 233-243.

de Rooij, J., F. J. Zwartkruis, et al. (1998). "Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP." Nature **396**(6710): 474-7.

Devreotes, P. N. and T. L. Steck (1979). "Cyclic 3'5' AMP relay in Dictyostelium discoideum II Requirements for the intiation and termination of the response." J.Cell Biol. **80**: 300-309.

Dinauer, M. C., S. A. MacKay, et al. (1980). "Cyclic 3',5'-AMP relay in Dictyostelium discoideum III. The relationship of cAMP synthesis and secretion during the cAMP signalling response." J Cell Biol **86**(2): 537-44.

Dinauer, M. C., T. L. Steck, et al. (1980). "Cyclic 3',5'-AMP relay in Dictyostelium discoideum IV. recovery of the cAMP signalling response after adaptation to cAM." J.Cell Biol. **86**: 545-553.

Dinauer, M. C., T. L. Steck, et al. (1980). "Cyclic 3',5'-AMP relay in Dictyostelium discoideum V. Adaptation of the cAMP signalling response during cAMP stimulation." J Cell Biol **86**(2): 554-61.

Dodge, K. L., S. Khouangsathiene, et al. (2001). "mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signalling module." Embo J **20**(8): 1921-30.

Dormann, D., F. Siegert, et al. (1996). "Analysis of cell movement during the culmination phase of Dictyostelium development." Development **122**: 761-769.

Dormann, D., G. Weijer, et al. (2002). "Visualizing PI3 Kinase-Mediated Cell-Cell Signalling during Dictyostelium Development." Curr Biol **12**(14): 1178.

Dudai, Y. (1997). "Time to remember." Neuron **18**(2): 179-82.

Edwards, A. S. and J. D. Scott (2000). "A-kinase anchoring proteins: protein kinase A and beyond." Curr Opin Cell Biol **12**(2): 217-21.

Eichinger, L., J. A. Pachebat, et al. (2005). "The genome of the social amoeba Dictyostelium discoideum." Nature **435**(7038): 43-57.

Faure, M., J. Franke, et al. (1990). "The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains three promoters specific for growth, aggregation, and late development." Mol Cell Biol **10**(5): 1921-30.

Faure, M., G. J. Podgorski, et al. (1988). "Disruption of Dictyostelium discoideum morphogenesis by overproduction of cAMP phosphodiesterase." Proc Natl Acad Sci U S A **85**(21): 8076-80.

Feit, I. N., E. J. Medynski, et al. (2001). "Ammonia differentially suppresses the cAMP chemotaxis of anterior-like cells and prestalk cells in Dictyostelium discoideum." J Biosci **26**(2): 157-66.

Feliciello, A., M. E. Gottesman, et al. (2001). "The biological functions of A-kinase anchor proteins." J Mol Biol **308**(2): 99-114.

Fey, P., P. Gaudet, et al. (2006). "dictyBase and the Dicty Stock Center." Methods Mol Biol **346**: 51-74.

Fisher, P. R. (1997). "Genetics of phototaxis in a model eukaryote, Dictyostelium discoideum." Bioessays **19**: 397-407.

Francis, S. H. and J. D. Corbin (1994). "Structure and function of cyclic nucleotide-dependent protein kinases." Annu Rev Physiol **56**: 237-72.

Franke, J., M. Faure, et al. (1991). "Cyclic nucleotide phosphodiesterase of Dictyostelium discoideum and its glycoprotein inhibitor: structure and expression of their genes." Dev Genet **12**(1-2): 104-12.

Funamoto, S., R. Meili, et al. (2002). "Spatial and temporal regulation of 3-phosphoinositides by PI 3- kinase and PTEN mediates chemotaxis." Cell **109**(5): 611-623.

Gerisch, G. and U. Wick (1975). "Intracellular oscillations and release of cyclic AMP from Dictyostelium cells." Biochem.Biophys.Res.Commun. **65**: 364-370.

Glaser, P., A. Danchin, et al. (1988). "Bordetella pertussis adenylate cyclase: the gene and the protein." Tokai J Exp Clin Med **13 Suppl**: 239-52.

Goldberg, J. M., L. Bosgraaf, et al. (2002). "Identification of four candidate cGMP targets in Dictyostelium." Proc Natl Acad Sci U S A **99**(10): 6749-54.

Goldbeter, A. (2006). "Oscillations and waves of cyclic AMP in Dictyostelium: a prototype for spatio-temporal organization and pulsatile intercellular communication." Bull Math Biol **68**(5): 1095-109.

Gomer, R. H., I. S. Yuen, et al. (1991). "A secreted 80x10 3 Mr protein mediates sensing of cell density and the onset of development in Dictyostelium." Development **112**: 269-278.

Hall, A. L., J. Franke, et al. (1993). "The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde." Dev Biol **157**(1): 73-84.

Harwood, A. J., N. A. Hopper, et al. (1992). "Multiple roles for cAMP-dependent protein kinase during Dictyostelium development." Dev. Biol. **149**: 90-99.

Haynes, R. C., Jr., E. W. Sutherland, et al. (1960). "The role of cyclic adenylic acid in hormone action." Recent Prog Horm Res **16**: 121-38.

Houslay, M. D. and G. S. Baillie (2005). "Beta-arrestin-recruited phosphodiesterase-4 desensitizes the AKAP79/PKA-mediated switching of beta2-adrenoceptor signalling to activation of ERK." Biochem Soc Trans **33**(Pt 6): 1333-6.

Houslay, M. D. and G. Milligan (1997). "Tailoring cAMP-signalling responses through isoform multiplicity." Trends Biochem Sci **22**(6): 217-24.

Insall, R., A. Kuspa, et al. (1994). "CRAC,a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G-protein-mediated activation of adenylyl cyclase in Dictyostelium." J.Cell Biol. **126**: 1537-1545.

Insall, R. H., J. Borleis, et al. (1996). "The aimless RasGEF is required for processing of chemotactic signals through G-protein-coupled receptors in Dictyostelium." Curr.Biol. **6**: 719-729.

Insall, R. H., R. D. Soede, et al. (1994). "Two cAMP receptors activate common signalling pathways in Dictyostelium." Mol Biol Cell **5**(6): 703-11.

Iyengar, R. (1993). "Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases." Faseb J **7**(9): 768-75.

Janetopoulos, C., T. Jin, et al. (2001). "Receptor-mediated activation of heterotrimeric G-proteins in living cells." Science **291**(5512): 2408-11.

Jarnaess, E. and K. Tasken (2007). "Spatiotemporal control of cAMP signalling processes by anchored signalling complexes." Biochem Soc Trans **35**(Pt 5): 931-7.

Johnson, R. L., P. J. Van Haastert, et al. (1992). "The cyclic nucleotide specificity of three cAMP receptors in Dictyostelium." J Biol Chem **267**(7): 4600-7.

Kae, H., A. Kortholt, et al. (2007). "Cyclic AMP signalling in Dictyostelium: G-proteins activate separate Ras pathways using specific RasGEFs." EMBO Rep **8**(5): 477-82.

Kamenetsky, M., S. Middelhaufe, et al. (2006). "Molecular details of cAMP generation in mammalian cells: a tale of two systems." J Mol Biol **362**(4): 623-39.

Karpen, J. W. and T. C. Rich (2005). "High-resolution measurements of cyclic adenosine monophosphate signals in 3D microdomains." Methods Mol Biol **307**: 15-26.

Kasahara, M. and M. Ohmori (1999). "Activation of a cyanobacterial adenylate cyclase, CyaC, by autophosphorylation and a subsequent phosphotransfer reaction." J Biol Chem **274**(21): 15167-72.

Kataoka, T., D. Broek, et al. (1985). "DNA sequence and characterization of the S. cerevisiae gene encoding adenylate cyclase." Cell **43**(2 Pt 1): 493-505.

Kaupp, U. B. and R. Seifert (2002). "Cyclic nucleotide-gated ion channels." Physiol Rev **82**(3): 769-824.

Kawamukai, M., J. Kishimoto, et al. (1985). "Negative regulation of adenylate cyclase gene (cya) expression by cyclic AMP-cyclic AMP receptor protein in Escherichia coli: studies with cya-lac protein and operon fusion plasmids." J Bacteriol **164**(2): 872-7.

Kawasaki, H., G. M. Springett, et al. (1998). "A family of cAMP-binding proteins that directly activate Rap1." Science **282**(5397): 2275-9.

Kay, R. R. (1997). DIF signalling. Dictyostelium. A model system for cell and developmental biology, Universal Academy Press, Inc. and Yamada Science Foundation**:** 279-291.

Kessin, R. H. (2001). Dictyostelium: Evolution, cell biology and the development of multicellularity. Cambridge, UK, Cambridge University Press.

Kim, H. J., W. T. Chang, et al. (1998). "A novel adenylyl cyclase detected in rapidly developing mutants of Dictyostelium." J Biol Chem **273**(47): 30859-62.

Kim, J. Y., J. A. Borleis, et al. (1998). "Switching of chemoattractant receptors programs development and morphogenesis in dictyostelium: receptor subtypes activate common responses at different agonist concentrations." Developmental Biology **197**: 117-128.

Kim, J. Y., R. D. Soede, et al. (1997). "Phosphorylation of chemoattractant receptors is not essential for chemotaxis or termination of G-protein-mediated responses." J Biol Chem **272**(43): 27313-8.

Kim, L., A. Harwood, et al. (2002). "Receptor-dependent and tyrosine phosphatase-mediated inhibition of GSK3 regulates cell fate choice." Developmental Cell **3**(4): 523-532.

Klein, P. S., T. J. Sun, et al. (1988). "A chemoattractant receptor controls development in Dictyostelium discoideum." Science **241**: 1467-1472.

Kobayashi, M., J. Buck, et al. (2004). "Conservation of functional domain structure in bicarbonate-regulated "soluble" adenylyl cyclases in bacteria and eukaryotes." Dev Genes Evol **214**(10): 503-9.

Konijn, T. M., J. G. Van De Meene, et al. (1967). "The acrasin activity of adenosine-3',5'-cyclic phosphate." Proc Natl Acad Sci U S A **58**(3): 1152-4.

Kosta, A., C. Laporte, et al. (2006). "How to assess and study cell death in Dictyostelium discoideum." Methods Mol Biol **346**: 535-50.

Kriebel, P. W. and C. A. Parent (2004). "Adenylyl cyclase expression and regulation during the differentiation of Dictyostelium discoideum." IUBMB Life **56**(9): 541-6.

Lacombe, M.-L., G. J. Podgorski, et al. (1986). "Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*." J. Biol. Chem. **261**: 16811-16817.

Lacombe, M. L., G. J. Podgorski, et al. (1986). "Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum." J Biol Chem **261**(36): 16811-7.

Laub, M. T. and W. F. Loomis (1998). "A molecular network that produces spontaneous oscillations in excitable cells of Dictyostelium." Mol. Biol. Cell **9**: 3521-3532.

Lee, S., F. I. Comer, et al. (2005). "TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium."Mol Biol Cell **16**(10): 4572-83.

Lee, S., C. A. Parent, et al. (1999). "A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate signal relay in Dictyostelium." Molecular Biology of the Cell **10**(9): 2829-2845.

Lilly, P., L. Wu, et al. (1993). "A G-protein beta-subunit is essential for Dictyostelium development." Genes Dev. **7**: 986-995.

Lilly, P. J. and P. N. Devreotes (1994). "Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in Dictyostelium." J. Biol. Chem. **269**: 14123-14129.

Lilly, P. J. and P. N. Devreotes (1995). "Chemoattractant and GTPtS-mediated stimulation of adenylyl cyclase in Dictyostelium requires translocation of CRAC to membranes." J.Cell Biol. **129**: 1659-1665.

Lim, C. J., G. B. Spiegelman, et al. (2001). "RasC is required for optimal activation of adenylyl cyclase and Akt/PKB during aggregation." Embo J **20**(16): 4490-9.

Linder, J. U. and J. E. Schultz (2003). "The class III adenylyl cyclases: multi-purpose signalling modules." Cell Signal **15**(12): 1081-9.

Loomis, W. F. (1998). "Role of pka in the timing of developmental events in dictyostelium cells." Microbiology And Molecular Biology Reviews **62**: 684.

Maeda, M., S. Lu, et al. (2004). "Periodic signalling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA." Science **304**(5672): 875-8.

Mann, S. K. O. and R. A. Firtel (1991). "A developmentally regulated, putative serine/threonine protein kinase is essential for development in Dictyostelium." Mech. Dev. **35**: 89-101.

Mann, S. K. O., W. M. Yonemoto, et al. (1992). "DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase." Proc. Natl. Acad. Sci. USA **89**: 10701-10705.

Maree, A. F. and P. Hogeweg (2001). "How amoeboids self-organize into a fruiting body: multicellular coordination in Dictyostelium discoideum." Proc Natl Acad Sci U S A **98**(7): 3879-83.

McConnachie, G., L. K. Langeberg, et al. (2006). "AKAP signalling complexes: getting to the heart of the matter." Trends Mol Med **12**(7): 317-23.

Mehats, C., C. B. Andersen, et al. (2002). "Cyclic nucleotide phosphodiesterases and their role in endocrine cell signalling." Trends Endocrinol Metab **13**(1): 29-35.

Mehdy, M. C. and R. A. Firtel (1985). "A secreted factor and cyclic AMP jointly regulate cell-type- specific gene expression in Dictyostelium discoideum." Mol.Cell.Biol. **5**: 705-713.

Meima, M. E., R. M. Biondi, et al. (2002). "Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants." Mol Biol Cell **13**(11): 3870-7.

Meima, M. E. and P. Schaap (1999). "Fingerprinting of adenylyl cyclase activities during Dictyostelium development indicates a dominant role for adenylyl cyclase B in terminal differentiation." Dev Biol **212**(1): 182-90.

Meima, M. E., K. E. Weening, et al. (2003). "Characterization of a cAMP-stimulated cAMP phosphodiesterase in Dictyostelium discoideum." J Biol Chem **278**(16): 14356-62.

Mock, M., M. Crasnier, et al. (1991). "Structural and functional relationships between Pasteurella multocida and enterobacterial adenylate cyclases." J Bacteriol **173**(19): 6265-9.

Mock, M., E. Labruyere, et al. (1988). "Cloning and expression of the calmodulin-sensitive Bacillus anthracis adenylate cyclase in Escherichia coli." Gene **64**(2): 277-84.

Mohanty, S., S. Lee, et al. (2001). "Regulated protein degradation controls PKA function and cell-type differentiation in Dictyostelium." Genes Dev **15**(11): 1435-48.

Montminy, M. (1997). "Transcriptional regulation by cyclic AMP." Annu Rev Biochem **66**: 807-22.

Moriarty, K. J. and L. A. Turnberg (1986). "Bacterial toxins and diarrhoea." Clin Gastroenterol **15**(3): 529-43.

Morris, H. R., G. W. Taylor, et al. (1987). "Chemical structure of the morphogen differentiation inducing factor from Dictyostelium discoideum." Nature **6133**: 811-814.

Nicol, A., W. Rappel, et al. (1999). "Cell-sorting in aggregates of Dictyostelium discoideum." J Cell Sci **112**(Pt 22): 3923-9.

Nikawa, J., P. Sass, et al. (1987). "Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of Saccharomyces cerevisiae." Mol Cell Biol **7**(10): 3629-36.

Ott, A., F. Oehme, et al. (2000). "Osmotic stress response in Dictyostelium is mediated by cAMP." Embo J **19**(21): 5782-92.

Pan, P., E. M. Hall, et al. (1972). "Folic acid as second chemotactic substance in the cellular slime moulds." Nature New Biol. **237**: 181-182.

Parent, C. A. and P. N. Devreotes (1996). "Molecular genetics of signal transduction in Dictyostelium." Annu. Rev. Biochem. **65**: 411-440.

Pastan, I. and R. Perlman (1970). "Cyclic adenosine monophosphate in bacteria." Science **169**(943): 339-44.

Pitt, G. S., R. Brandt, et al. (1993). "Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenylyl cyclase (ACA)." Genes Dev **7**(11): 2172-80.

Pitt, G. S., N. Milona, et al. (1992). "Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development." Cell **69**(2): 305-15.

Podgorski, G. J., M. Faure, et al. (1988). "The cyclic nucleotide phosphodiesterase of Dictyostelium discoideum: the structure of the gene and its regulation and role in development." Dev Genet **9**(4-5): 267-78.

Rall, T. W., E. W. Sutherland, et al. (1957). J. Biol. Chem. **224**: 463.

Raper, K. B. (1935). "Dictyostelium discoideum, a new species of slime mold from decaying forest leaves." J.Agric.Res. **50**: 135-147.

Raper, K. B. (1940). "Pseudoplasmodium formation and organization in Dictyostelium discoideum." J. Elisha Mitchell Scient. Soc. **56**: 241-282.

Rathi, A. and M. Clarke (1992). "Expression of early developmental genes in Dictyostelium discoideum is initiated during exponential growth by an autocrine- dependent mechanism." Mech.Dev. **36**: 173-182.

Rich, T. C., W. Xin, et al. (2007). "Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells." Am J Physiol Cell Physiol **292**(1): C319-31.

Richter, W. (2002). "3',5' Cyclic nucleotide phosphodiesterases class III: members, structure, and catalytic mechanism." Proteins **46**(3): 278-86.

Robison, G. A., R. W. Butcher, et al. (1968). "Cyclic AMP." Annu Rev Biochem **37**: 149-74.

Roelofs, J., M. Meima, et al. (2001). "The Dictyostelium homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase." Embo J **20**: 4341-8.

Ronnett, G. V. and C. Moon (2002). "G proteins and olfactory signal transduction." Annu. Rev. Physiol. **64**: 189-222.

Ross, D. T., Raibaud, A., Forent, I. C., Sather, S., Gross, M. K., Storm, D. R. and Eisen, H. (1991). ‘’The trypanosome VSG expression site encodes adenylate cyclase and a leucine-rich putative regulatory gene.’’ Embo J **10**: 2047-53.

Salter, E. A. and A. Wierzbicki (2007). "The mechanism of cyclic nucleotide hydrolysis in the phosphodiesterase catalytic site." J Phys Chem B **111**: 4547-52.

Sanchez, M. A., D. Zeoli, et al. (1995). "A family of putative receptor-adenylate cyclases from Leishmania donovani." J Biol Chem **270**: 17551-8.

Saran, S., M. E. Meima, et al. (2002). "cAMP signalling in *Dictyostelium* - Complexity of cAMP synthesis, degradation and detection." Journal of Muscle Research and Cell Motility **23**: 793-802.

Saxe III, C. L., G. T. Ginsburg, et al. (1993). "CAR2, a prestalk cAMP receptor required for normal tip formation and late development of Dictyostelium discoideum." Genes Dev. **7**: 262-272.

Saxe III, C. L., R. L. Johnson, et al. (1991). "Expression of a cAMP receptor gene of Dictyostelium and evidence for a multigene family." Genes Dev. **5**: 1-8.

Schaap, P. (2004). "Non-metazoan class III nucleotidyl cyclases: novel forms and functions." IUBMB Life **56**: 527-8.

Schulkes, C. and P. Schaap (1995). "cAMP-dependent protein kinase activity is essential for preaggregative gene expression in Dictyostelium." FEBS Lett **368**(2): 381-4.

Seebeck, T., R. Schaub, et al. (2004). "cAMP signalling in the kinetoplastid protozoa." Curr Mol Med **4**: 585-99.

Segall, J. E., A. Kuspa, et al. (1995). "A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in Dictyostelium." J.Cell Biol. **128**: 405-413.

Shaulsky, G., D. Fuller, et al. (1998). "A cAMP-phosphodiesterase controls PKA-dependent differentiation." Development **125**: 691-699.

Shenoy, A. R., N. Srinivasan, et al. (2002). "The ascent of nucleotide cyclases: conservation and evolution of a theme." J Biosci **27**: 85-91.

Shenoy, A. R. and S. S. Visweswariah (2006). "Mycobacterial adenylyl cyclases: biochemical diversity and structural plasticity." FEBS Lett **580**: 3344-52.

Singh, A., S. Sharma, et al. (2007). "cAMP regulates vegetative growth and cell cycle in Candida albicans." Mol Cell Biochem **304**: 331-41.

Singleton, C. K., M. J. Zinda, et al. (1998). "The histidine kinase dhkC regulates the choice between migrating slugs and terminal differentiation in *Dictyostelium discoideum*." Dev. Biol. **203**: 345-357.

Sinha, S. C. and S. R. Sprang (2006). "Structures, mechanism, regulation and evolution of class III nucleotidyl cyclases." Rev Physiol Biochem Pharmacol **157**: 105-40.

Sismeiro, O., P. Trotot, et al. (1998). "Aeromonas hydrophila adenylyl cyclase 2: a new class of adenylyl cyclases with thermophilic properties and sequence similarities to proteins from hyperthermophilic archaebacteria." J Bacteriol **180**: 3339-44.

Skalhegg, B. S. and K. Tasken (2000). "Specificity in the cAMP/PKA signalling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA." Front Biosci **5**: D678-93.

Smith, M. E., J. R. Dickinson, et al. (1990). "Intracellular and extracellular levels of cyclic AMP during the cell cycle of Saccharomyces cerevisiae." Yeast **6**: 53-60.

Soderbom, F., C. Anjard, et al. (1999). "An adenylyl cyclase that functions during late development of Dictyostelium." Development **126**: 5463-71.

Soede, R. D., R. H. Insall, et al. (1994). "Extracellular cAMP can restore development in Dictyostelium cells lacking one, but not two subtypes of early cAMP receptors (cARs). Evidence for involvement of cAR1 in aggregative gene expression." Development **120**: 1997-2002.

Stoka, A. M. (1999). "Phylogeny and evolution of chemical communication: an endocrine approach." J Mol Endocrinol **22**: 207-25.

Strader, C. D., T. M. Fong, et al. (1995). "The family of G-protein-coupled receptors." Faseb J **9**: 745-54.

Sucgang, R., C. J. Weijer, et al. (1997). "Null mutations of the Dictyostelium cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates." Dev Biol **192**(1): 181-92.

Sunahara, R. K., C. W. Dessauer, et al. (1996). "Complexity and diversity of mammalian adenylyl cyclases." Annu Rev Pharmacol Toxicol **36**: 461-80.

Sutherland, E. W. and T. W. Rall (1958). "Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles." J Biol Chem **232**: 1077-91.

Sutherland, E. W. and T. W. Rall (1960). "Formation of adenosine-3,5-phosphate (cyclic adenylate) and its relation to the action of several neurohormones or hormones." Acta Endocrinol Suppl (Copenh) **34(Suppl 50)**: 171-4.

Tang, W. J. and J. H. Hurley (1998). "Catalytic mechanism and regulation of mammalian adenylyl cyclases." Mol Pharmacol **54**(2): 231-40.

Tang, W. J., S. Yan, et al. (1998). "Class III adenylyl cyclases: regulation and underlying mechanisms." Adv Second Messenger Phosphoprotein Res **32**: 137-51.

Tang, Y. and H. G. Othmer (1994). "A G protein-based model of adaptation in Dictyostelium discoideum." Math. Biosciences **120**: 25-76.

Tasken, K. and E. M. Aandahl (2004). "Localized effects of cAMP mediated by distinct routes of protein kinase A." Physiol Rev **84**(1): 137-67.

Taylor, M. C., D. K. Muhia, et al. (1999). "Trypanosoma cruzi adenylyl cyclase is encoded by a complex multigene family." Mol Biochem Parasitol **104**(2): 205-17.

Tellez-Sosa, J., N. Soberon, et al. (2002). "The Rhizobium etli cyaC product: characterization of a novel adenylate cyclase class." J Bacteriol **184**(13): 3560-8.

Thomason, P. and R. Kay (2000). "Eukaryotic signal transduction via histidine-aspartate phosphorelay." J. Cell Sci. **113**(Pt 18): 3141-3150.

Thomason, P. A., D. Traynor, et al. (1999). "The RdeA-RegA system, a eukaryotic phospho-relay controlling cAMP breakdown." J. Biol. Chem. **274**(39): 27379-27384.

Ullmann, A. and J. Monod (1968). "Cyclic AMP as an antagonist of catabolite repression in Escherichia coli." FEBS Lett **2**(1): 57-60.

Urushihara, H. (2008). "Developmental biology of the social amoeba: history, current knowledge and prospects." Dev Growth Differ **50 Suppl 1**: S277-81.

van Es, S., K. J. Virdy, et al. (1996). "Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores." J Biol Chem **271**(39): 23623-5.

Verkerke-van Wijk, I., M. Fukuzawa, et al. (2001). "Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression." Dev Biol **234**(1): 151-60.

Wang, N., F. Soderbom, et al. (1999). "SDF-2 induction of terminal differentiation in Dictyostelium discoideum is mediated by the membrane-spanning sensor kinase DhkA." Mol Cell Biol **19**(7): 4750-6.

Wong, W. and J. D. Scott (2004). "AKAP signalling complexes: focal points in space and time." Nat Rev Mol Cell Biol **5**(12): 959-70.

Wu, L. and J. Franke (1990). "A developmentally regulated and cAMP-repressible gene of Dictyostelium discoideum: cloning and expression of the gene encoding cyclic nucleotide phosphodiesterase inhibitor." Gene **91**: 51-56.

Wu, L., J. Franke, et al. (1995). "The phosphodiesterase secreted by prestalk cells is necessary for Dictyostelium morphogenesis." Dev Biol **167**(1): 1-8.

Wu, L., R. Valkema, et al. (1995). "The G protein beta subunit is essential for multiple responses to chemoattractants in Dictyostelium." J Cell Biol **129**(6): 1667-75.

Yamamoto, K., K. Hirao, et al. (2005). "Functional characterization in vitro of all two-component signal transduction systems from Escherichia coli." J Biol Chem **280**(2): 1448-56.

Zhang, M., M. Goswami, et al. (2005). "Constitutively active G protein-coupled receptor mutants block dictyostelium development." Mol Biol Cell **16**(2): 562-72.

Zhang, N., Y. Long, et al. (2001). "Ggamma in dictyostelium: its role in localization of gbetagamma to the membrane is required for chemotaxis in shallow gradients." Mol Biol Cell **12**(10): 3204-13.

Zinda, M. J. and C. K. Singleton (1998). "The hybrid histidine kinase dhkB regulates spore germination in *Dictyostelium discoideum*." Dev. Biol. **196**: 171-183.

Zoraghi, R., J. D. Corbin, et al. (2004). "Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins." Mol Pharmacol **65**(2):267-78.

Chapter 2

Contrasting Activities of the Aggregative and Late *PdsA* Promoters in *Dictyostelium Development*

Karin E. Weening[1], Irene Verkerke-Van Wijk[2], Christopher R. Thompson[3], Richard H. Kessin[4], Gregory J. Podgorski[5] and Pauline Schaap[1†]

1 School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK
2 Institute for Molecular Plant Sciences, Leiden University, 2333 AL Leiden, The Netherlands
3 MRC Laboratory of Molecular Biology, Cambridge, CB2 2QH, UK
4 College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA
5 Utah State University, Logan UT 84322-5305, USA
† To whom correspondence should be addressed. E-mail: P.schaap@dundee.ac.uk

ABSTRACT

Expression of the *Dictyostelium PdsA* gene from the aggregative (*PdA*) and late (*PdL*) promoter is essential for aggregation and slug morphogenesis respectively. We studied the regulation of the *PdA* and *PdL* promoters in slugs using labile β-galactosidase (gal) reporter enzymes. *PdL* was active in prestalk cells as was also found with stable gal. *PdA* activity decreased strongly in slugs from all cells except those at the rear. This is almost opposite to PdA activity traced with stable gal, where slugs showed sustained activity with highest levels at the front. *PdA* was down-regulated after aggregation irrespective of stimulation with any of the factors known to control gene expression. *PdL* activity was induced in cell suspension by cAMP and DIF acting in synergy. However, a DIF-less mutant showed normal *PdL* activity during development, suggesting that DIF does not control *PdL* in *vivo.* Dissection of the *PdL* promoter showed that all sequences essential for correct spatio-temporal control of promoter activity are downstream of the transcription start site in a region between -383 and -19 nucleotides relative to the start codon. Removal of nucleotides to position -364 eliminated responsiveness to DIF and cAMP, but normal *PdL* activity in prestalk cells in slugs was retained. Further 5' deletions abolished all promoter activity. This result also indicates that the induction by DIF and cAMP as seen in cell suspensions is not essential for *PdL* activity in normal development.

Key words: *Dictyostelium discoideum* / cyclic nucleotide phosphodiesterase / promoter analysis / morphogenetic signaling / differentiation inducing factor

INTRODUCTION

Extracellular cAMP signaling controls morphogenetic movement and prespore gene expression during multicellular development of *Dictyostelium discoideum* (Wang, Van Driel et al. 1988; Bretschneider, Siegert et al. 1995; Anjard, Soderbom et al. 2001). Three adenylyl cyclases, ACA, ACB and ACG have been identified that synthesize cAMP and one extracellular phosphodiesterase (PDE) that degrades it (Lacombe, Podgorski et al. 1986; Pitt, Milona et al. 1992; Kim, Chang et al. 1998; Soderbom, Anjard et al. 1999). The single *PdsA* gene, that encodes PDE, is regulated at the transcriptional level by three promoters that control expression during growth, aggregation and multicellular development respectively (Faure, Franke et al. 1990). The multicellular (late) promoter element is only active in the prestalk cell population (Hall, Franke et al. 1993). PDE enzyme activity is regulated by PDI, a secreted PDE inhibitor (Wu and Franke 1990).

It has not been possible to associate any of the adenylyl cyclases with a specific role in either slug morphogenesis or prespore gene expression, because null mutants in any of them can still form migrating slugs and express prespore genes, although in case of ACA null mutants this requires overexpression of PKA or prestimulation with cAMP (Pitt, Milona et al. 1992; Pitt, Brandt et al. 1993; Wang and Kuspa 1997; Soderbom, Anjard et al. 1999). This is probably due to partial overlap in the function of these enzymes (Anjard, Soderbom et al. 2001). In contrast, more severe effects are observed when PDE expression is altered. Loss of function mutants in PDE cannot aggregate at all (Darmon, Barra et al. 1978). Aggregation can be restored by expression of PDE from the aggregative promoter (*PdA*), while restoration of slug and fruiting body formation requires expression from the late promoter (*PdL*) (Sucgang, Weijer et al. 1997). The essential role of PDE in slug morphogenesis was also demonstrated by experiments where PDI was expressed from a prestalk promoter. This inhibited late PDE activity and blocked of slug morphogenesis (Wu, Franke et al. 1995). Gain of function of PDE is equally disruptive in the slug stage. Overexpression of PDE accelerates the aggregation process, but arrests tip formation and cell differentiation (Faure, Podgorski et al. 1988).

Despite the crucial role of correct PDE expression for multicellular development, little is known about the regulation of the gene by the *PdL* promoter. It is also not clear whether expression from the *PdA* promoter plays a role in the slug stage. Similar to control of the ACA gene, transcription controlled by the *PdA* promoter is downregulated after aggregation (Faure, Franke et al. 1990; Pitt, Milona et al. 1992). In case of ACA, this was due to selective cessation of transcription in posterior cells resulting in exclusive expression in the tip region (Verkerke-van Wijk, Fukuzawa et al. 2001). Other components of the oscillatory signaling system such as cAR1, Gα2, Gβ, Aimless and CRAC become similarly enriched in the tip/prestalk region and this may also be the case for *PdA* (Tsujioka, Yokoyama et al. 2001).

To gain insight in the regulation of *PDE* expression during multicellular development, we studied the patterns of *PdA* and *PdL* promoter activity by using labile β-galactosidase reporter proteins (Detterbeck, Morandini et al. 1994). *PdA* is downregulated after aggregation in most cells except those at the rear-guard and basal disc. *PdL* is active in prestalk cells and is synergistically activated by cAMP and DIF in disaggregated cells. However, deletion studies of the *PdL* promoter and analysis of DIF-less mutants show that at least DIF is not the activating signal in normal development.

MATERIALS AND METHODS

Materials

Differentiation Inducing Factor (DIF) was obtained from Affinity Research Products (Exeter, UK), chlorophenylred ß-D-galactopyranoside (CPRG) was from Roche (Burgess Hill, UK). Exonuclease III, mung bean nuclease and pLITMUS29 were from New England Biolabs (Ipswich, USA). The DNA sequencing kit Sequenase 2.0 was obtained from US Biochemical (Cleveland, USA).

Promoter deletions and plasmid construction

The 1.5 kb *PdA* promoter was amplified by PCR from vector pGalA (Hall, Franke et al. 1993) using oligonucleotides that generate *Xba*I and *Bgl*II sites at the 5' and 3' end of the promoter respectively. The XbaI/BglII digested product was ligated into the XbaI/BglII digested vector 63-iDQgal to generate *PdA*-ile-gal.

The GalL cell line used previously (Hall, Franke et al. 1993) was cotransformed with plasmid pB10T, which carries the neomycin selection cassette and plasmid pGalL, which contains a gene fusion of *PdL* and *lacZ*. In our hands β-galactosidase expression in this cell line was weak and declined progressively during cell culture. We made two new vectors *PdL*-gal and *PdL*-ile-gal in which *PdL* was fused to stable and labile variants of *LacZ* respectively. *PdL*-gal was made by replacing the KpnI/XhoI *lacZ* fragment of pDdgal17 (Harwood and Drury 1990) with the KpnI/XhoI *PdL-lacZ* fragment from pGalL. *PdL*-ile-gal was made subsequently by fusing the XbaI-BglII fragment from *PdL*-gal that contained the *PdL* promoter to ubi-ile-gal in the XbaI/BglII digested vector 63-iDQgal.

To construct promoter deletions, the 0.72 kb *PdL* promoter was subcloned into the pLITMUS29 vector using the EcoRI/HindIII restriction sites. This yielded the plasmid *PdL*-pLIT29, which contained an exonuclease III protected 3' overhang (NsiI) and an exonuclease III digestible 5' overhang (EcoRI). Five µg of *PdL*-pLIT29 plasmid, digested with NsiI/EcoRI, was treated under standard conditions for the preparation of exonuclease III-generated 5' deletions (Ausubel 1990). Samples from selected time points from the deletion reactions were used to transform *E. coli* DH5 cells. Estimates of the deletion endpoint were made by agarose gel electorophoresis and precisely determined by DNA sequencing. The BglII/HindIII promoter fragments of selected deletion constructs were fused to the *lacZ* gene in vector pDdgal17. Promoter fragments were also constructed using PCR. PCR reactions were performed using the oligonucleotide primers indicated in figure 7. All 5' primers contained a XbaI site and all 3' primers contained an BglII site to allow fusion of the amplified DNA to the *lacZ* gene in pDdgal17or 63iDQgal. Correct amplification of all PCR generated constructs was determined by DNA sequencing.

Dictyostelium AX3 cells were transformed with all constructs and AX2 and HM1030 cells (Thompson and Kay 2000) were transformed with *PdL*-ile-gal.

Growth and incubation conditions

All cell lines were grown in standard axenic medium, which was supplemented with 20 µg/ml G418 for cells transformed with Pde promoter fusions with stable or labile gal and with 5 µg/ml blasticidin for the DIFless mutant HM1030. To modulate *PdA* or *PdL* promoter activity in cell suspension, exponentially growing cells were harvested, washed in 10 mM Na/K-phosphate buffer pH 6.5 (PB) and distributed on 1.5% agar in PB at $3x10^6$ cells/cm^2. Cells were incubated for 22°C until aggregation territories or loose aggregates had formed. Aggregates were dissociated by vigorous pipetting, resuspended in PB at $3x10^6$ cells/ml and challenged with variables at 22°C and 150 rpm during 6-10 hours.

For detection of *PdA or PdL* promoter activity in developing structures, cells were harvested from growth medium, distributed at 10^6 cells/cm^2 over nitrocellulose filters, supported by PB agar, and incubated at 22°C until the stage of interest had been reached.

Spectrophotometric and histochemical β-galactosidase assays

For spectrophotometric quantitation of β-galactosidase activity, cells were lysed by freeze-thawing and 100 µl aliquots of lysate were incubated at 22°C in microtiterplate wells with 30 µl of 2.5 x Z-buffer and 20 µl of 40 mM CPRG (Dingermann, Reindl et al. 1989). The OD_{574} was measured at regular time intervals using a microtiter plate reader. Activity was expressed as the increase of OD_{574} per hour per mg protein.

For histochemical staining, nitrocellulose filters supporting developing cells were fixed for 20 minutes with 0.25% glutaraldehyde and 2% Tween-20 in Z-buffer, washed thoroughly and subsequently incubated with 1 mM X-gal in Z-buffer containing 5 mM $K_3[Fe(CN)]_6$ and 5 mM $K_4[Fe(CN)]_6$ for 1-4 hours (Dingermann, Reindl et al. 1989).

RESULTS

Expression patterns of *PdA* and *PdL*

The patterns of activity of the aggregative and late promoters of the PDE gene have been studied previously using a reporter gene that encodes a stable form of β-galactosidase (Hall, Franke et al. 1993). In cases where promoter activity is transient, stable reporters can provide misleading information because the activity of the reporter protein persists, when the mRNA has already dissappeared (Detterbeck,

Morandini et al. 1994). To avoid this problem the *PdA* and *PdL* promoters were fused to a chimeric DNA encoding ubiquitin-isoleucine-β-galactosidase to give vectors PdA-ile-gal and PdL-ile-gal. After translation the ubiquitin is cleaved off and exposes the isoleucine at the N-terminus of β-galactosidase, which reduces its half-life to about 60 min (Bachmair, Finley et al. 1986; Gonda, Bachmair et al. 1989; Detterbeck, Morandini et al. 1994). The ile-gal constructs cannot be used when promoter activity is low, because they yield considerably lower absolute activities of β-galactosidase than the stable constructs. For deletion studies of the PdL promoter, we therefore also used a stable PdL-gal construct.

When measured with the ile-gal reporter *PdA* activity becomes detectable after a few hours of starvation and is visible in most of the cells when they are collecting into aggregation streams (Fig. 1A). Once cells have formed tight mounds, expression begins to decline. This decline is first visible in the tip region (Fig. 1B) and later spreads over the anterior 75% of the slug. During culmination, *PdA* activity is concentrated at the basal disc/lower cup area (Fig. 1D, arrow) and becomes restricted to the basal disc when fruiting bodies have formed (Fig. 1E, arrow). The prestalk and stalk cells show almost no *PdA* activity (Fig. 1C,E). This pattern is quite different from that observed with the stable gal reporter. In this case all cells continue to show high activity after aggregation, but particularly those at the tip (Hall, Franke et al. 1993).

The pattern of *PdL* promoter activity measured with the ile-gal reporter is not very different from that observed with the stable gal reporter (Hall, Franke et al. 1993). *PdL* is first active at the tip and in a few scattered cells in mounds (Fig. 2B). During slug formation activity becomes most pronounced in the anterior prestalk region and in cells scattered throughout the prespore region (Fig. 2C). In fruiting bodies, only the apical prestalk cells show activity and there is no significant activity in the stalk and basal disc (Fig 2D).

A quantitative assay for β-galactosidase in cell lysates shows that *PdA* activity increases after 4 hours of starvation to reach a peak at 10 h of development. *PdL* activity increases after cells have formed mounds, to reach a maximum during fruiting body formation (Fig. 3). These temporal expression patterns closely follow those of the *PdA* and *PdL* mRNAs, respectively (Faure, Franke et al. 1990), which indicates that the labile reporter gene traces promoter activity reliably.

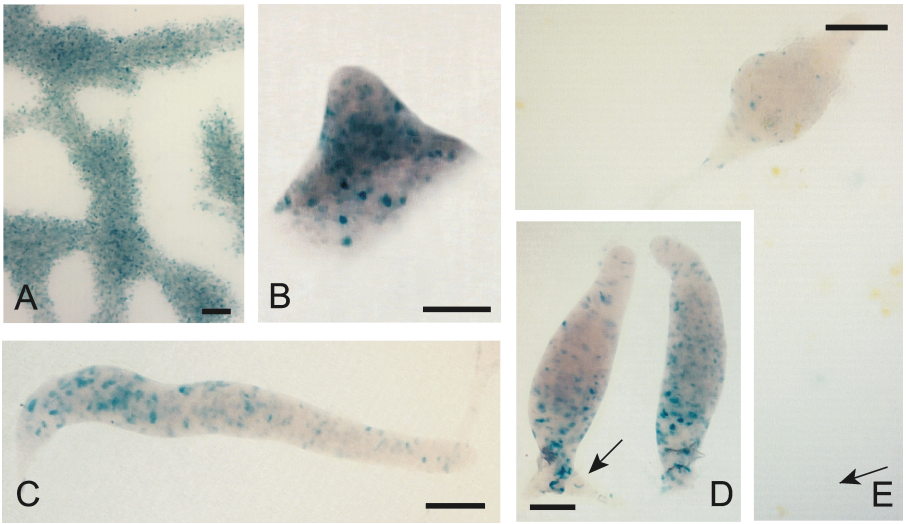


Figure 1: Patterns of *PdA* promoter activity during normal development
PdA-ile-gal cells were distributed on nitrocellulose filters supported by PB agar and incubated at 22°C. Cells were fixed and stained with X-gal after they had formed streaming aggregates (A), tipped aggregates (B), migrating slugs (C), early culminants (D) and mature fruiting bodies (E). Scale bar: 100 µM.

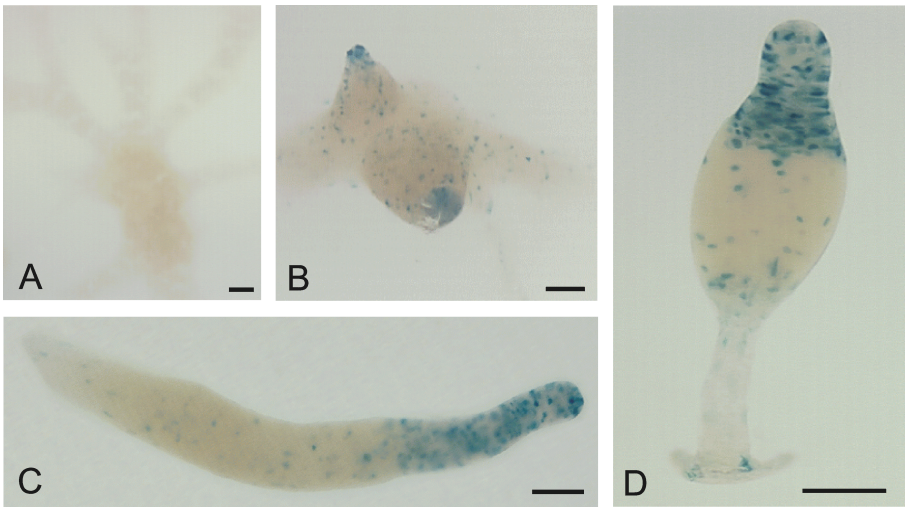


Figure 2: Patterns of *PdL* promoter activity during normal development
PdL-ile-gal cells were incubated at 22°C on nitrocellulose filters and stained with X-gal after they had formed streaming aggregates (A), tipped aggregates (B), migrating slugs (C) and mature fruiting bodies (D). Scale bar: 100 µM

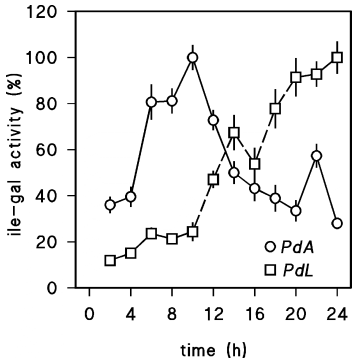


Figure 3: Developmental regulation of *PdA* and *PdL* promoter activity.
PdA-ile-gal and *PdL*-ile-gal cells were distributed on PB agar andv incubated for 24 h at 22°C. Cells were harvested at two hour intervals and β-galactosidase activity was measured in cell lysates. Data were standardized on the protein content of the cell lysate and are pressed as percentage of enzyme activity at T=10 h for *PdA* and at T=24 h for *PdL*. Means and s.e.m. of three time courses, assayed in quadruplicate are presented.

Signals that up- and down-regulate *PdA* and *PdL* promoter activity

Upon starvation *PdA* activity is induced by the prestarvation factor PSF (Clarke and Gomer 1995), 1995) and further up-regulated by cAMP (Franke, Podgorski et al. 1987). After aggregation, *PdA* activity is down-regulated dramatically in the prestalk cell population, while *PdL* activity is induced in these cells. We examined the signals most likely to trigger both events. The predominant signals for post-aggregative gene regulation are micromolar cAMP, which induces prespore gene expression (Kay 1982; Wang, Van Driel et al. 1988), and DIF, which induces prestalk gene expression (Morris, Taylor et al. 1987) (Williams, Ceccarelli et al. 1987). We also included nanomolar cAMP pulses, isopropylidene adenosine (IPA) and a combination of micromolar cAMP and DIF. cAMP acts in synergy with DIF to induce the prestalk gene *ecmA*; cAMP pulses are likely to be responsible for tip-specific expression of *ACA* in slugs (Verkerke-van Wijk, Fukuzawa et al. 2001), while adenosine acts as an inhibitor of prespore differentiation (Weijer and Durston 1985; Schaap and Wang 1986). IPA is a metabolically stable analog of adenosine (Soede, Hopper et al. 1996). Cells expressing the *PdA* or *PdL* ile-gal constructs were developed to the loose aggregate stage. The aggregates were dissociated into single cells and incubated for 10 h with the different stimuli. Figure 4A shows that *PdA* activity decreased strongly regardless of any added stimulus. However, after an initial decline cAMP pulses induced a modest transient increase in activity. A stronger but also transient increase was induced by the combination of cAMP and DIF. *PdL* was induced to moderate levels with micromolar cAMP. Neither DIF alone, nor any of the other stimuli induced any significant *PdL* activity. However, high levels of activity were induced by the combination of cAMP and DIF (Fig. 4B). This combinatorial effect of cAMP and DIF can either be due to the signals acting in synergy, or to one signal inducing responsiveness to the other. After prestimulation of cells with cAMP or DIF, maximal induction still required both signals acting together.

Furthermore, responsiveness of the cells to either signal was decreased rather than increased (data not shown). This suggests that cAMP and DIF act in synergy on PdL activation.

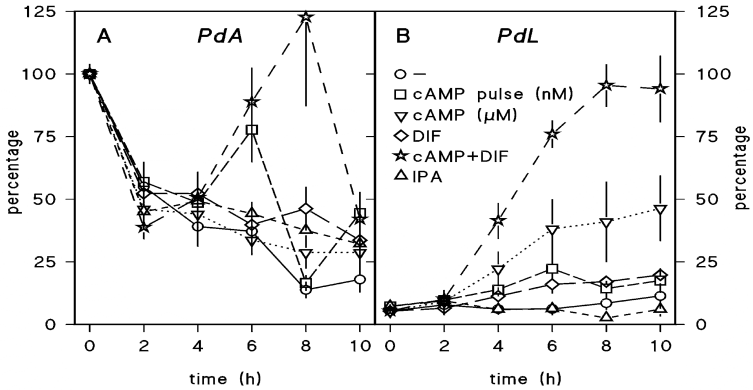


Figure 4: Effects of cAMP, DIF and IPA on PdA and PdL promoter activity
PdA-ile-gal and PdL-ile-gal cells were incubated on PB agar until loose aggregates had formed (T=10 h). Cells from dissociated aggregates were resuspended in PB and incubated during 10 h with 30 nM cAMP pulses, 300 μM cAMP/hour, 100 nM DIF added once, 300 μM cAMP/hour plus 100 nM DIF, 1 mM IPA or without additive. Cells were lysed and assayed for β-galactosidase activity, which was standardized to the protein content of the lysates. Data are expressed as percentage of activity at t=0 h for PdA and activity at t=10 h after the cAMP plus DIF treatment for PdL. Means and s.e.m. of 3 experiments performed in quadruplicate are presented

Is *PdL* regulated by DIF and cAMP during normal development?

To answer this question we introduced the *PdL*-ile-gal construct into a mutant that cannot synthesize DIF (Thompson and Kay 2000). Figure 5 shows that in the DIF-less mutant HM1030, *PdL* activity is high in anterior prestalk cells and scattered cells in the prespore region of slugs and in the apical region of fruiting bodies. This is identical to the *PdL* activity pattern in wild-type cells and suggests that DIF is not required to induce *PdL* expression in the developing organism.

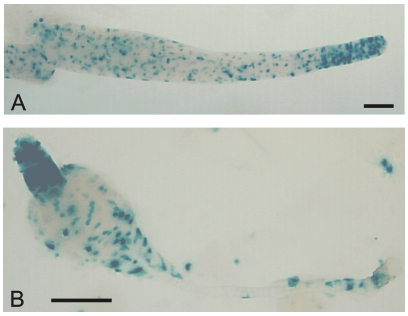


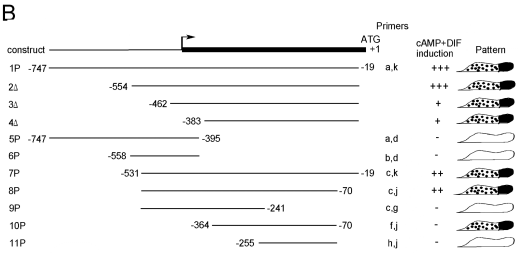
Figure 5: *PdL* promoter activity in the DIFless mutant
The DIFless mutant HM1030 was transformed with the *PdL*-ile-gal vector. Transformed cells were incubated at 22°C on nitrocellulose filters and stained with X-gal after they had formed migrating slugs (A) and mature fruiting bodies (D). Scale bar: 100 μM.

As a second approach we identified regions in the *PdL* promoter that mediate its expression during normal development and its induction by cAMP and DIF in cell suspension. A set of *PdL* promoter modifications was prepared as nested 5' deletions (Δ constructs) or as PCR-amplified promoter fragments (P constructs) (Fig. 6) and fused to stable or labile reporter genes.

A

```
(3380)
        a→
-747    gatatctatt gtgtgtaaca attaggggtt ttattatttc ctttttttt aaaaaaattt tttttttttt tttatttact actatttttta aaaaaaaaaa
                                                                                                  b→  →2Δ
-647    aaaaaaaaaa aaaaaaaaaa agataattta ttttgatctt ttatgtgttg atacaccttt tcatgtttac acacaaacaa aaaacactcg ataaatttta
                        c→                                                                          →3Δ
-547    ttattatttt ttatttgttt ttaatttaat tgatattaaa aaaaaaaaaa ttaaaaaaaa aaaaaaaaaa aaattttaag tgatatataa tttttaaaat
                                                          ←d            →4Δ                f→                  ←e
-447    tttttattta TTTATTTTTT TAAAAAATTA ATTTTTCAAA CAATAATACA TTCAAAATTA AAACAATAAT AACAGATTCA AAATATCATT TGGTTTTGTT
                                                                                                                h→
-347    TTTTTTTTTT TTTTTATTTA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAATTAT ACATATAAAT ATTTATAACA ATAACCCACA AACGCCACAC
          ←g                                                                                      i→
-247    ACTCACTTAT TTTTTTTAAT TTTAAATTTT ATTTATTTTA TTTATTTATT TATTTATTTA ATCAAAAAAA AAAAAAAAAA AAAAACTAAA ATTAAAATTA
                                                                                          ←j
-147    CTAACTTATT TTTTTTTTTA TAAATATATA TAAAAAAAAA AAAAAAATAA AATTTTTTTT TTTTCCAATA TCTTTATCTT TTTTTTTTAT TATAAAAGAA
(4080)                                  ←k                  +1
-47     GAAAGAATTA ATATAGTTCG AATAACTACA AAAAATATAC AAAAAAAATG
```

B



C

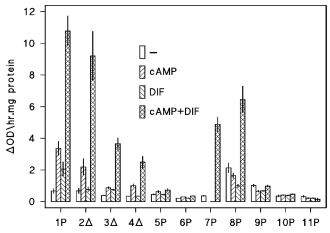


Figure 6: Overview of promoter deletions and their effect on expression patterns and induction

A. *Nucleotide sequence of the PdL promoter.* The promoter sequence was retrieved from (Faure et al., 1990) and renumbered for convenience with the start codon at position +1. The original numbering is indicated between parentheses. Deletion endpoints of ExoIII-generated constructs 2Δ, 3Δ and 4Δ are indicated by arrows. The sequences of PCR primers, a - k, are indicated in bold and their direction by arrows. The TATA box predicted by (Faure et al., 1990) is underlined. Transcribed regions are shown in upper case and non-transcribed regions in lower case.

B. *Schematic of deletions.* The first column provides construct names, the second column shows a schematic view of the constucts and, in the case of PCR-generated constructs, the position of primers used in their creation. The next column indicates relative induction by 100 μM cAMP plus 100 nM DIF (- no significant induction; +, ++, +++: moderate, strong and very strong induction respectively). The last column summarizes the expression pattern of β-galactosidase in the transformants.

C. *Response of deletion constructs to cAMP and DIF in vitro*

Dissociated loose aggregates of cells transformed with deletion constructs were incubated without additives (-), 100 μM cAMP/hr, 100 nM DIF or a combination of 100 μM cAMP/hr with 100 nM DIF (cAMP+DIF) and assayed for β-galactosidase activity and protein levels. Two or three independently transformed clones were tested twice for each construct in an assay performed in triplicate. The absolute β-galactosidase activities (ΔOD/hr.mg protein) obtained after incubation of individual clones for each construct with the different variables was averaged and means and s.e.m. of the pooled data are indicated.

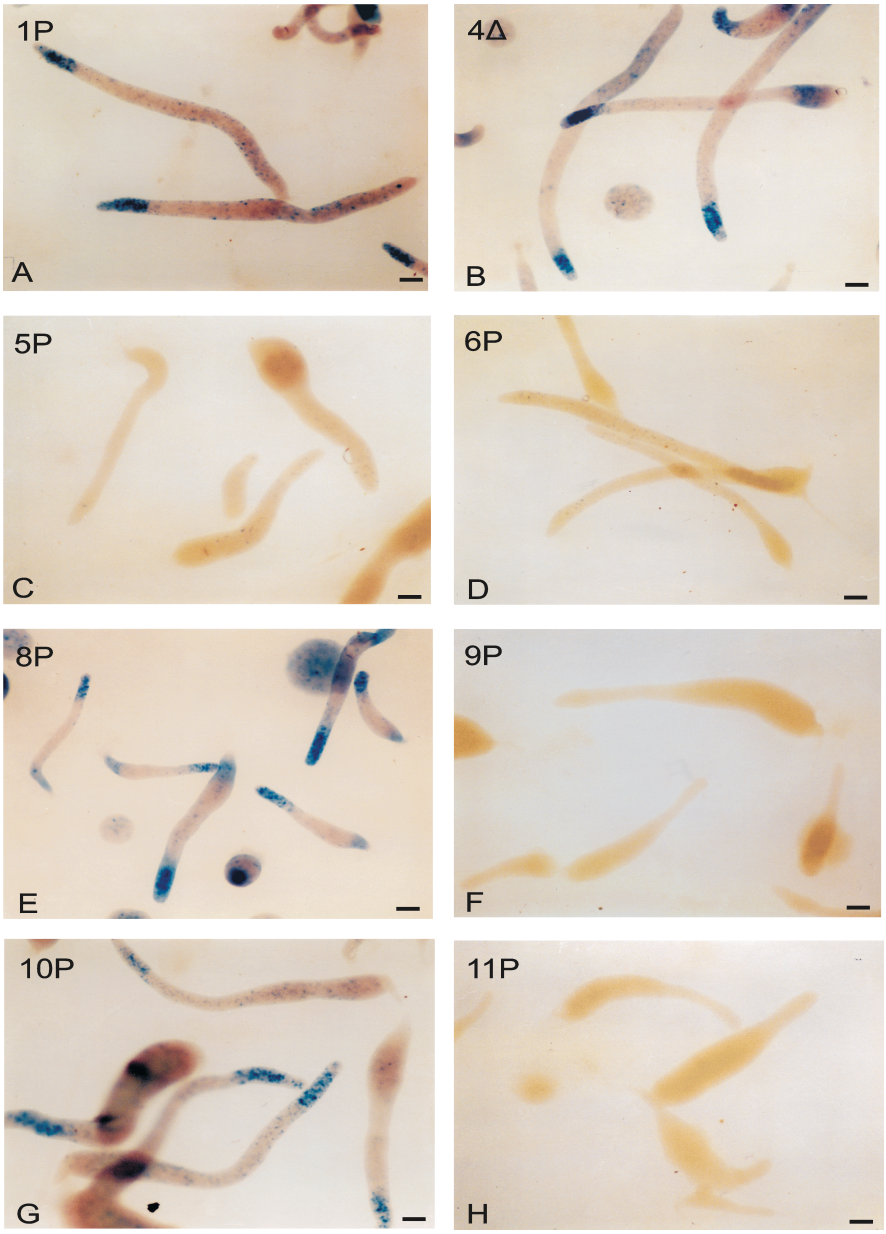


Figure 7: Expression patterns of *PdL* promoter deletion constructs
Cells transformed with the different deletion constructs were developed on nitrocellulose filters until migrating slugs had formed and stained for β-galactosidase activity. The construct code (see figure 7B) is indicated on each photograph. The transformant carrying the full length *PdL* promoter was photographed after 1 hour of staining and all others after 4 hours. Scale bar: 100 µM

We measured the activity of the promoter fragments during normal development and during induction in cell suspensions with DIF and cAMP. All 5' deletion constructs up to 4Δ in which the entire untranscribed region is deleted, showed the same pattern of expression in slugs as the intact *PdL* promoter (Figs 7A,B), with high levels of expression in the prestalk region and in the anterior-like cells.

However, the intensity of staining decreased with the shorter constructs and was too low for the ile-gal gene fusions to remain detectable. During incubation in suspension, all constructs showed high levels of induction by the combination of DIF and cAMP and some induction by cAMP alone. The absolute levels of β-galactosidase decreased 3-4 fold when the region between -554 and -462 bp was deleted (Fig. 6C). In constructs 5P and 6P that lacked most of the transcribed region β-galactosidase activity was undetectable in slugs and could no longer be induced by cAMP and DIF. This indicates that regions downstream of the start site of transcription are essential for promoter function. Constructs 7P and 8P, which contain the predicted TATA box and most of the 5' leader sequence, showed the same expression pattern in migrating slugs as the intact promoter as well as good inducibility by cAMP plus DIF (Figs. 6C, 7E).

Expression in slugs and inducibility by cAMP plus DIF was completely lost from the shorter construct 9P (Figs. 6C, 7F), indicating that the essential promoter sequences reside between -70 and -383 bp from the start codon. A deletion from -383 to -364 (compare constructs 4Δ and 10P) retained the normal expression pattern in migrating slugs, but lost cAMP and DIF inducibility (Figs 6C, 7G). In a further deletion up to -255 all promoter activity was lost (Fig. 7H). This indicates that one region of the promoter controls normal expression in slugs, and another is implicated in induction by cAMP plus DIF in cell suspension.

DISCUSSION

Opposite expression patterns for the aggregative and late PdsA promoters

The *PdsA* gene, that encodes PDE, has an elaborate structure with different promoters controling expression of the gene during growth, aggregation and post-aggregative development (Faure, Franke et al. 1990). The function of PDE during growth is not clear, but it is essential for aggregation and later morphogenesis (Darmon, Barra et al. 1978; Wu, Franke et al. 1995; Sucgang, Weijer et al. 1997).

When measured with a stable gal reporter, *PdA* driven β-galactosidase activity increases strongly during aggregation and remains very pronounced in slugs with highest apparent activity in the anterior prestalk cells (Hall, Franke et al. 1993). A labile reporter presents quite a different pattern. In this case *PdA* also increases during aggregation, but decreases once mounds have formed. This decrease spreads as an anterior to posterior wave until only the rear-guard and basal disc cells show activity (figure 1). *PdA* transcripts also decrease markedly after

aggregation (Faure, Franke et al. 1990), so the activity measured with the labile ile-gal construct is likely to follow *PdA* promoter activity closely. The prestalk-enriched expression that was detected with the stable gal is most likely a memory effect, reflecting a tendency for prestalk sorting in cells that strongly expressed *PdA* during aggregation and never lost the stable β-galactosidase enzyme later. This is supported by findings that prestalk sorting and high *PDE* expression during aggregation are both properties of cells that enter development in early cell cycle phase (Weijer, Duschl et al. 1984; Wang, Aerts et al. 1988). No significant differences were found for the stable and labile lacZ versions of the *PdL* promoter (figure 2) (Hall, Franke et al. 1993). This is probably due to the fact that this promoter is expressed so late that memory effects do not have time to become established.

The pattern of expression of *PdL* is exactly opposite to that of *PdA* with predominant expression in the prestalk region and in the anterior-like cell population. These different distributions are in good agreement with the distribution of PDE enzyme activity in slugs as detected by microdissection. Also here high activity was detected in both the anterior prestalk region and rear-guard/basal disc region with little activity in the intervening prespore region (Brown and Rutherford 1980). *In situ* hybridization also shows the presence of *PdsA* mRNA in the prestalk and rear-guard/basal disc regions (Tsujioka, Yokoyama et al. 2001).

Signals that regulate postaggregative *PdA* and *PdL* promoter activity

Consensus models for regulation of cell-type specification in *Dictyostelium* indicate micromolar cAMP and DIF as inducers of prespore and prestalk cell differentiation respectively. Micromolar cAMP can potentially be generated by the adenylyl cyclases ACA, ACB and ACG, which are all expressed in slugs (Alvarez-Curto and Schaap, unpublished results) (Soderbom, Anjard et al. 1999; Verkerke-van Wijk, Fukuzawa et al. 2001). DIF is generated by prespore cells to trigger conversion of a proportion of their number into anterior-like cells (Kay and Thompson 2001). These cells then sort to the anterior to form the prestalk region (Sternfeld and David 1982). Earlier work showed that *PdL* mRNA was elevated in cells that were treated with DIF after a 10 h pretreatment with 5 mM cAMP (Franke, Faure et al. 1991). We show here that DIF also induces *PdL* activity in cells that have developed into mounds under more physiological conditions. However, they only do so when cAMP is present at the same time.

We attempted to identify the signal that triggers the precipitous decline in PdA promoter activity once aggregates have formed. We found that dissociating aggregated cells did not block the decline in PdA promoter activity, making it unlikely that cell-cell contact or a diffusible signal secreted in the aggregate is the trigger.

DIF activates *PdL* in cell suspension, but not in the intact organism

PdL is strongly upregulated by DIF and cAMP in suspensions of dissociated cells, but two lines of evidence indicate that at least DIF does not regulate expression of the gene in slugs: i. a mutant that is defective in DIF synthesis showed normal expression of *PdL* in slugs and fruiting bodies (figure 6), ii. Deletion analysis of the *PdL* promoter indicated that a region from -383 to-364, relative to the start codon, is required for DIF/cAMP induction in disaggregated cells. However, when this region was deleted, *PdL* expression in prestalk and anterior-like cells in slugs was not diminished. The *PdL* promoter showed another unusual feature; primary transcriptional regulatory elements lie in the 5' leader sequence of the gene. Deletions of this region eliminated promoter activity, whereas deletions that removed regions upstream of the transcription only reduced the efficiency of transcription.

Prestalk genes share no common inductive signal

A number of genes, such as *ecmA*, *ecmB*, *rasD*, *carB* and *PdL* are first expressed in the prestalk cell population (Reymond, Gomer et al. 1984; Williams, Ceccarelli et al. 1987; Saxe III, Ginsburg et al. 1993). Other genes, such as *gpaB (Gα2), gpbA (Gβ), carA, acaA, dagA* (CRAC) and *aleA* (aimless) are expressed in most cells during aggregation and later become prestalk specific (Carrel, Dharmawardhane et al. 1994; Tsujioka, Yokoyama et al. 2001; Verkerke-van Wijk, Fukuzawa et al. 2001). This is most likely because their expression is down-regulated in prespore cells.

In contrast to prespore genes, which are dependent on cAMP for expression in cell suspension and in intact slugs (Anjard, Soderbom et al. 2001)(Kay 1982)(Wang, Van Driel et al. 1988), the prestalk genes share no common mode of induction. *EcmA, ecmB* and *PdL* are all DIF-inducible in suspension (Williams, Ceccarelli et al. 1987), but only the pstO subdomain of the *ecmA* promoter requires DIF for expression in slugs (Thompson and Kay 2000). *carB* and *rasD* are induced by micromolar cAMP in suspension (Reymond, Gomer et al. 1984; Saxe III, Ginsburg et al. 1993), while *AcaA* expression is maintained in dissociated slug cells by cAMP pulses (Verkerke-van Wijk, Fukuzawa et al. 2001).

This heterogeneity in regulation is most likely a consequence of the multifunctionality of cells in the anterior region of the slug. The posterior prespore cells loose the chemotactic functions of aggregative cells and show a concerted pattern of gene activities that prepare them for encapsulation. In contrast, the anterior cells continue to play a very active role in cAMP signaling and chemotaxis, in addition they acquire novel roles in phototaxis, thermotaxis and synthesis of the mucopolysaccharide sheath. The gene products that are required for this plethora of activities are probably under control of several regulatory feedback loops, using any of a number of intercellular interactions.

The overriding characteristic of the prestalk region may be that these cells are inhibited to enter the spore pathway and can therefore remain fully competent for signaling and motility. The function of DIF in this scheme could be to act as a prespore inhibitor rather than a prestalk inducer. The expression of *PdL* and probably other prestalk genes are likely to require signalling interactions that remain to be identified.

ACKNOWLEDGEMENT

We thank Harry MacWilliams for his kind gift of the 63-iDQgal vector. This research was supported by grants 805-31-051 and 805-17048 from the Life Sciences Foundation of the Netherlands Organisation for Scientific Research to I.V and K.E.W. respectively and by Wellcome Trust University Award Grant 057137. RHK is supported by NIH grant GM33136.

REFERENCES

Anjard, C., F. Soderbom, et al. (2001). "Requirements for the adenylyl cyclases in the development of Dictyostelium." Development **128**(18): 3649-54.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (1990). Current Protocols in Molecular Biology, John Wiley & Sons, New York.

Bachmair, A., D. Finley, et al. (1986). "In vivo half-life of a protein is a function of its amino-terminal residue." Science **234**(4773): 179-86.

Bretschneider, T., F. Siegert, et al. (1995). "Three-dimensional scroll waves of cAMP could direct cell movement and gene expression in Dictyostelium slugs." Proc. Natl. Acad. Sci. USA **92**: 4387-4391.

Brown, S. S. and C. L. Rutherford (1980). "Localization of cyclic nucleotide phosphodiesterase in the multicellular stages of Dictyostleium discoideum." Differentiation **16**: 173-183.

Carrel, F., S. Dharmawardhane, et al. (1994). "Spatial and temporal expression of the Dictyostelium discoideum Ga protein subunit Ga2: Expression of a dominant negative protein inhibits proper prestalk to stalk differentiation." Mol.Biol.Cell **5**: 7-16.

Clarke, M. and R. H. Gomer (1995). "PSF and CMF, autocrine factors that regulate gene expression during growth and early development of Dictyostelium." Experientia **51**: 1124-1134.

Darmon, M., J. Barra, et al. (1978). "The role of phosphodiesterase in aggregation of Dictyostelium discodeum." J.Cell Sci. **31**: 233-243.

Detterbeck, S., P. Morandini, et al. (1994). "The 'prespore-like cells' of Dictyostelium have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters." Development **120**: 2847-2855.

Dingermann, T., N. Reindl, et al. (1989). "Optimization and in situ detection of Escherichia coli beta-galactosidase gene expression in Dictyostelium discoideum." Gene **85**: 353-362.

Faure, M., J. Franke, et al. (1990). "The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains three promoters specific for growth, aggregation, and late development." Mol Cell Biol **10**(5): 1921-30.

Faure, M., G. J. Podgorski, et al. (1988). "Disruption of Dictyostelium discoideum morphogenesis by overproduction of cAMP phosphodiesterase." Proc Natl Acad Sci U S A **85**(21): 8076-80.

Franke, J., M. Faure, et al. (1991). "Cyclic nucleotide phosphodiesterase of Dictyostelium discoideum and its glycoprotein inhibitor: structure and expression of their genes." Dev Genet **12**(1-2): 104-12.

Franke, J., G. J. Podgorski, et al. (1987). "The expression of two transcripts of the phosphodiesterase gene during the development of Dictyostelium discoideum." Dev Biol **124**(2): 504-11.

Gonda, D. K., A. Bachmair, et al. (1989). "Universality and structure of the N-end rule." J Biol Chem **264**(28): 16700-12.

Hall, A. L., J. Franke, et al. (1993). "The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde." Dev Biol **157**(1): 73-84.

Harwood, A. J. and L. Drury (1990). "New vectors for expression of the E.coli lacZ gene in Dictyostelium." Nucl.Acids Res. **18**: 4292.

Kay, R. R. (1982). "cAMP and spore differentiation in Dictyostelium discoideum." Proc.Natl.Acad.Sci.USA **79**: 3228-3231.

Kay, R. R. and C. R. Thompson (2001). "Cross-induction of cell types in Dictyostelium: evidence that DIF-1 is made by prespore cells." Development **128**(24): 4959-66.

Kim, H. J., W. T. Chang, et al. (1998). "A novel adenylyl cyclase detected in rapidly developing mutants of Dictyostelium." J Biol Chem **273**(47): 30859-62.

Lacombe, M. L., G. J. Podgorski, et al. (1986). "Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum." J Biol Chem **261**(36): 16811-7.

Morris, H. R., G. W. Taylor, et al. (1987). "Chemical structure of the morphogen differentiation inducing factor from Dictyostelium discoideum." Nature **6133**: 811-814.

Pitt, G. S., R. Brandt, et al. (1993). "Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenylyl cyclase (ACA)." Genes Dev **7**(11): 2172-80.

Pitt, G. S., N. Milona, et al. (1992). "Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development." Cell **69**(2): 305-15.

Reymond, C. D., R. H. Gomer, et al. (1984). "Developmental regulation of a Dictyostelium gene encoding a protein homologous to mammalian ras protein." Cell **39**: 141-148.

Saxe III, C. L., G. T. Ginsburg, et al. (1993). "CAR2, a prestalk cAMP receptor required for normal tip formation and late development of Dictyostelium discoideum." Genes Dev. **7**: 262-272.

Schaap, P. and M. Wang (1986). "Interactions between adenosine and oscillatory cAMP signaling regulate size and pattern in Dictyostelium." Cell **45**(1): 137-44.

Soderbom, F., C. Anjard, et al. (1999). "An adenylyl cyclase that functions during late development of Dictyostelium." Development **126**(23): 5463-71.

Soede, R. D. M., N. A. Hopper, et al. (1996). "Extracellular cAMP depletion triggers stalk gene expression in Dictyostelium: Disparities in developmental timing and dose dependency indicate that prespore induction and stalk repression by cAMP are mediated by separate signaling pathways." Dev.Biol. **177**: 152-159.

Sternfeld, J. and C. N. David (1982). "Fate and regulation of anterior-like cells in Dictyostelium slugs." Dev.Biol. **93**: 111-118.

Sucgang, R., C. J. Weijer, et al. (1997). "Null mutations of the Dictyostelium cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates." Dev Biol **192**(1): 181-92.

Thompson, C. R. and R. R. Kay (2000). "The Role of DIF-1 Signaling in Dictyostelium Development." Mol Cell **6**(6): 1509-1514.

Tsujioka, M., M. Yokoyama, et al. (2001). "Spatial expression patterns of genes involved in cyclic AMP responses in Dictyostelium discoideum development." Dev Growth Differ **43**(3): 275-83.

Verkerke-van Wijk, I., M. Fukuzawa, et al. (2001). "Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression." Dev Biol **234**(1): 151-60.

Wang, B. and A. Kuspa (1997). "Dictyostelium development in the absence of cAMP." Science **277**: 251-254.

Wang, M., R. J. Aerts, et al. (1988). "Cell cycle phase in Dictyostelium discoideum is correlated with the expression of cyclic AMP production, detection, and degradation. Involvement of cyclic AMP signaling in cell sorting." Dev Biol **125**(2): 410-6.

Wang, M., R. Van Driel, et al. (1988). "Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in Dictyostelium discoideum slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation." Development **103**: 611-618.

Weijer, C. J. and A. J. Durston (1985). "Influence of cyclic AMP and hydrolysis products on cell type regulation in Dictyostelium discoideum." J.Embryol.Exp.Morph. **86**: 19-37.

Weijer, C. J., G. Duschl, et al. (1984). "Dependence of cell-type proportioning and sorting on cell cycle phase in Dictyostelium discoideum." J.Cell Sci. **70**: 133-145.

Williams, J. G., A. Ceccarelli, et al. (1987). "Direct induction of Dictyostelium prestalk gene expression by DIF provides evidence that DIF is a morphogen." Cell **49**: 185-192.

Wu, L. and J. Franke (1990). "A developmentally regulated and cAMP-repressible gene of Dictyostelium discoideum: cloning and expression of the gene encoding cyclic nucleotide phosphodiesterase inhibitor." Gene **91**: 51-56.

Wu, L., J. Franke, et al. (1995). "The phosphodiesterase secreted by prestalk cells is necessary for Dictyostelium morphogenesis." Dev Biol **167**(1): 1-8.

Wu, L., J. Franke, et al. (1995). "The phosphodiesterase secreted by prestalk cells is necessary for Dictyostelum discoideum." Dev.Biol. **167**: 1-8.

Chapter 3

Evolution of extracellular phosphodiesterases in *dictyostelids*

Karin E. Weening[1,2], Yoshinori Kawabe[1] and Pauline Schaap[1†]

[1] College of Life Sciences, Division of Cell and Developmental Biology, University of Dundee DD1 5EH, United Kingdom
[2] present address: School of Biosciences, Department of Molecular Biology, Cardiff University CF10 3AX, United Kingdom
[†] To whom correspondence should be addressed. E-mail: p.schaap@dundee.ac.uk

ABSTRACT

Cyclic AMP (cAMP) is an important intracellular messenger in all almost all domains of life that mediates the effects of a broad variety of external physical and chemical stimuli that regulate development and physiology. The social amoeba *Dictyostelium discoideum* not only uses cAMP intracellularly, but also secretes cAMP in an oscillatory manner to coordinate the processes of cellular aggregation and fruiting body morphogenesis. For this function, cAMP is produced by the adenylyl cyclase A and detected by G-protein coupled cAMP receptors (cARs). The extracellular phosphodiesterase, PdsA, plays an important role in degrading cAMP between pulses and in generating steep chemotactic gradients. To understand how the extracellular role of cAMP evolved, we are retracing the origins and changes in function of the proteins that are critical for extracellular cAMP signalling, such as PdsA. The *Dictyostelia* can be subdivided into four major taxon groups. We amplified *PdsA* gene fragments from representative taxa of each of these groups and cloned a full-length *PdsA* gene from the group 3 taxon *D. minutum*. The *PdsA* gene appeared to be conserved in all tested species with independent events of gene duplication occurring in groups 1, 2 and 4. The *D. minutum PdsA* restored the phenotype of a *D. discoideum pdsA* null mutant but showed a lower affinity for cAMP than the *D. discoideum* enzyme. There were significant differences between the test species in the developmental regulation of *PdsA* genes, which suggests group-specific elaboration of PdsA function.

Key words: *Dictyostelia*, oscillatory cAMP signalling, Phosphodiesterase

INTRODUCTION

The social amoebas or *Dictyostelia* are members of the supergroup of amoebozoans, the closest sister group to the opisthokonts that contain the animals and fungi (Baldauf 2003). A recently constructed molecular phylogeny divides all known *Dictyostelia* into 4 major groups (Schaap, Winckler et al. 2006). The most derived group 4 contains *Dictyostelid* species that use cAMP as chemottractant for aggregation (acrasin), including the model organism *D. discoideum*. *Dictyostelia* from the other groups use other attractants, such as glorin for *Polysphondylium violaceum*, *P. pallidum* and *D. caveatum* and folic acid for *D. minutum* (Shimomura, Suthers et al. 1982; De Wit and Konijn 1983).

Earlier studies revealed that even species that do not use cAMP for aggregation would use cAMP to coordinate slug and fruiting body morphogenesis after aggregation (Schaap, Konijn et al. 1984; Schaap, Van Lookeren Campagne et al. 1986; Fukushima and Maeda 1991; Dormann, Weijer et al. 1997). This suggests that extracellular cAMP signalling has a conserved universal role to coordinate fruiting body formation and a derived role in aggregation. In agreement with this

hypothesis a recent study showed that cAMP receptor (cAR) orthologs are only expressed after aggregation in early diverging species, but became expressed during aggregation from a second distal promoter in group 4 taxa (Alvarez-Curto, Rozen et al. 2005).

In its role as chemoattractant, cAMP is typically secreted in an oscillatory manner. In *D. discoideum,* some starving amoebae start to secrete cAMP pulses which are relayed by surrounding cells and trigger chemotactic movement towards the cAMP source and consequently aggregation (Goldbeter 2006). After aggregation, oscillatory cAMP secretion continues from the tip region of slugs and now coordinates the cell movement that shapes the slugs and fruiting bodies (Weijer 2004). Oscillatory cAMP signalling requires cAR-mediated activation of adenylyl cyclase A (ACA), which synthesizes cAMP that is rapidly secreted. Continued stimulation leads to desensitization of cARs blocking further cAMP production (Zhang, Goswami et al. 2005). The extracellular cyclic nucleotide phosphodiesterase, PdsA degrades cAMP, allowing the cells to become re-sensitized for the next cAMP pulse. In addition PdsA generates steep chemotactic gradients around the cells and is therefore an essential component of dynamic cAMP signalling (Lacombe, Podgorski et al. 1986).

The expression of the *D. discoideum PdsA* gene is controlled by three different promoters. The proximal late promoter directs expression in the prestalk region of slugs, the central, vegetative promoter directs expression during growth, while the distal aggregative promoter directs expression during aggregation (Faure, Franke et al. 1990). Expression from the aggregative and late promoters is essential for aggregation and post-aggregative morphogenesis respectively (Wu, Franke et al. 1995). It has been proposed that as cAMP acquired more roles additional promoters were added to the primitive proximal promoter (Faure, Franke et al. 1990; Alvarez-Curto, Rozen et al. 2005). To add to this complexity PdsA enzyme activity is regulated by a secreted inhibitory glycoprotein (PDI) (Wu and Franke 1990).

To understand how the role of PdsA in the Dictyostelia evolved, we studied conservation and change in *PdsA* genes throughout the *Dictyostelium* phylogeny. We next examined the developmental regulation of *PdsA* genes in group-representative taxa and we used complementation to assess conservation of PdsA function.

MATERIALS AND METHODS

Cell culture and development

D. rosarium M45 (*Dros*), *D. purpureum* (*Dpur*), *D. mucoroides* S28 (*Dmuc*), *P. Violaceum P6 (Pvio)*, *D. minutum* 71-2 (*Dmin*), *D. rhizopodium (Drhi), P. pallidum TNS-C-98 (Ppal), Acytostelium anastomosans (Aana)*, *D. aureo-stipes YA6 (Daus)*

and *D. fasciculatum* SH3 (*Dfas*) spores were grown in association with *E. coli* 281 on 0.1% lactose/peptone (LP) agar plates [(Raper 1984) To obtain large quantities of cells, 1.5×10^8 amoebas were inoculated in 300 ml 10 mM sodium/phosphate buffer, pH 6.5 (PB), supplemented with a resuspended *E.coli* 281 pellet from a 300 ml stationary culture in LB. The suspension was shaken at 200 rpm until the amoeba had reached a density of about $3\text{-}5\text{x}10^6$ cells/ml. *D. discoideum* cells were grown in HL5 medium.

For developmental time courses, growing cells were washed free from bacteria with PB, and incubated at 22° C on non-nutrient (NN) agar (1.5 % agar in PB) at 5×10^6 cells/cm^2. In some cases, pellets of activated charcoal were placed in the lids to improve synchronous development (Fridborg, Pedersén et al. 1978). To examine development after complementation, cells were harvested from growth medium, washed and resuspended in PB to cell densities of 10^8 or 10^7 cells/ml. The different cell concentrations were deposited as 10 µl droplets on black nitrocellulose membrane filters (Millipore; 0.45 µM) supported by NN agar and incubated for 24 hours at 22°C.

Primer	Amino Acids	DNA sequence
PDEF1	LGHSHL/FDHV	5'-CCTCGGTCACAGTCATYTNGAYCAYGT-3'
PDER1	MFGHLRPR	5'-GGTCTTGGACGGAGRTGNCCRAACAT-3'
PDERN1	FS/C/WDTGVPS	5'-GGATGGAACACCAGTRTCNCWRAA-3'
PdsA-51A	FSDTGV	5'-TTYTCNGAYACNGGNGTNCC-3'
PdsA-51B	FSDTGV	5'-TTYAGYGAYACNGGNGTNCC-3'
PdsA-3E	PEQGEPI	5'-ATIGGNWCNCCYTGYTCNGG-3'
PdsA-3N	PNQGV/D/EI	5'-ATIGGNWCNCCYTGRTTNGG-3'
PdsA-32	IPNQGD	5'-GGRTCNCCYTGRTTNGGDAT-3'
DminPdsAEx5'		5'-CCCGGATCCAAATCGACACTTAACCTTTTCAAGATAATTATAATT ATTATCCTCATAAATCAAATTTCAACAAATGAGACAGCTGATC -3'
DminPdsAEx3'		5'-CCCCTCGAGTTATATACAAATTGGTTCACCTTGTT CAGG -3'

Table 1: Designed Oligonucleotides
A) Degenerative primers for amplification of *PdsA* homologous across the Dictyostelid phylogeny. Primers were designed against conserved amino acid region of the phosphodiesterase domain of the *PdsA* gene.
B) Primers for D. minutum expression construct. Primers contain BamHI and XhoI restriction sites for cloning into the X plasmid.

Gene identification and analysis

Genomic DNA (gDNA) of species that represent all *Dictyostelid* groups was isolated as earlier described (Schaap, Winckler et al. 2006; Kawabe, Morio et al. 2009). Degenerate oligonucleotide primers were designed to match amino acid sequences that are conserved between the PDE-II domains of *Ddis* PdsA and a bacterial and 3 fungal PDEII enzymes (Genbank: EDV10448, P32782, P36599, Q56686). Primers PDEF1 and PDER1 (Table I) yielded 633 nt products from *Dmuc*, *Dros* and *Dmin* gDNAs, which were validated first by nested PCR with primer pair PDEF1 and PDER1N and next by DNA sequencing.

The *Dmin* PCR product was used to screen a gDNA library, resulting in cloning of a full length *Dmin PdsA* ORF (see below). Conserved regions between the *Dmin* and *Ddis* ORF could then be used to design primer combinations that successfully amplified *PdsA*s from more basal species. A combination of forward primers PdsA-51A and PdsA51B with reverse primers PdsA-3E and PdsA-3N yielded around 300 bp products from *Pvio, Drhi, Aana, Daus* and *Dfas* gDNAs. Full-length *Dfas*- and *Ppal PdsA* genes could very recently be retrieved from ongoing genome sequencing projects in these species (http://genome.imb-jena.de/dictyostelium). The PCR program was composed of 35 cycles (95° C, 30 sec; 50° C, 30 sec; 68° C, 30 sec). The amplified PCR fragments were sub-cloned in the pGEM-T Easy vector (Promega) and their sequence was determined from 3 independent clones.

Cloning and expression of *DminPdsA*

The *Dmin PdsA* PCR product was used to screen a custom-made λZAPII library (Stratagene) of sheared *D. minutum* gDNA and yielded 6 positive plaques. Their pBluescript phagemids were isolated by *in vitro* excision according to manufacturer's instructions and their 4.71, 3.82, 4.74, 4.90, 6.50 and 6.53 kb inserts were sequenced. The sequences could be assembled into a 9.02-bp contig, which included *Dmin PdsA* with three flanking ORFs. Blast searches of the entire GenBank database revealed that these ORFs were most similar to *D. discoideum DDB0218780*, *DDB0218778* and *DdisAbcF1*.

To generate an expression construct for gene complementation a 1.3-kb fragment, which includes the complete coding region of *Dmin PdsA* without its single intron, was amplified from λZAPII clone P6 using oligonucleotides Dmin-PdsA-Ex5 and Dmin-PdsA-Ex3 (Table 1) that contained BamHI and XhoI restriction sites respectively. After BamHI/XhoI digestion the PCR product was cloned into similarly digested vector pDV-CYFP (Meima, Weening et al. 2007), which places a *DminPdsA-YFP* gene fusion downstream of the constitutively active *D. discoideum* actin 15 promoter, yielding plasmid A15:DminPdsA. The plasmid was verified by digestions and by sequencing.

The *D. discoideum PdsA* null mutant *Uk7-* (Sucgang, Weijer et al. 1997) was transformed by electroporation with two independently sequenced plasmid clones, 420 and 421 (Knecht and Ming Pang 1995). Briefly, cells were harvested, washed twice in H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO4, 5 mM NaHCO3 and 1 mM NaH2PO4.H_2O) and resuspended to $5x10^7$ cells/ml. 100 µl aliquots of cell suspension were pulsed twice with 6 µg of DNA at 0.65 V. Transformants were selected by growth in HL5 medium supplemented with 40 µg/ml G418 (Sigma, St.Louis, MO, USA).

Phylogenetic analysis

Deduced amino-acid sequences of partial and full length *Dictyostelid* PdsA and non-Dictyostelid PDEII sequences were aligned using CLUSTALW (Chenna, Sugawara et al. 2003). Stretches of sequence that could not be unambiguously aligned were deleted. Bayesian inference was used to construct a PdsA phylogeny (Ronquist and Huelsenbeck 2003). A mixed amino-acid model was used with rate variation across sites estimated by a gamma distribution with a proportion of invariable sites and four rate categories. The analysis was run for 500.000 generations at which point the average standard deviation of split frequencies was 0.0123. Posterior probabilities were averaged over the final 75% of trees.

RNA isolation and Northern blot analysis

Total RNA was isolated from cells developing on NN agar at 2 hours intervals until fruiting bodies had been formed. RNA was size fractionated on 1.5 % agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Genescreen). Membranes were hybridised at 60° C to [α-^{32}P]dATP (Amersham) labelled *DdisPdsA*, *DrosPdsA*, *PvioPdsA*, *DminPdsA*, *PpalPdsA*, *DfasPdsA* and *DausPdsA*. After washes under high stringency, conditions blots were exposed to X-ray film. The same blots were stripped by boiling for 30 minutes in 0.1 % SDS and re-probed with the *DdisIG7* gene that is expressed constitutively throughout *D. discoideum* development.

PDE assays

To measure cAMP hydrolysis, cells were starved in PB for 5 h at a density of 10^7 cells/ml. Cells were pelleted, resuspended to 10^8 cells/ml and diluted 1:10 or 1:100 times in PB. Aliquots of 16 µl cell suspensions were incubated for 30 minutes at 22 °C with 100 nM [2,8-^{3}H]AMP (Amersham) in a total volume of 20 µl in the absence or presence of unlabeled cAMP as indicated in the figure legends.

Reactions were terminated after 30 minutes by boiling the suspensions for 1 min. The product, [2,8-^{3}H]AMP, was further hydrolysed to [2,8-^{3}H]adenosine by incubation for 30 min at 37° C with 10 µg of *Naja messambica* snake venom which has 5'nucleotidase activity (SA venom suppliers, Louis Trichardt, SA). Finally, 100 µl of the anion exchange resin Dowex (AG1×2) was added to remove the remaining unhydrolysed [2,8-^{3}H]cAMP from [2,8-^{3}H]adenosine. After 10 min of incubation, under regular mixing, samples were centrifuged for 2 min at 14000 g and 50 µl of the supernatant was measured by scintillation counting. Assays were performed in triplicate and expressed as percentage of hydrolysis obtained in the absence of cAMP.

RESULTS

Identification of *PdsA* genes in group representative taxa

We used a PCR approach with degenerate oligonucleotide primers to amplify homologs of the *D. discoideum* (*Ddis*) *PdsA gene* from group representative taxa. Figure 1A, shows a schematic representation of the SSU rRNA phylogeny of the Dictyostelia and illustrates the position of the test species. The first set of primers was designed to match regions conserved between the PDE-II domain of DdisPdsA and non-dictyostelid PDE-II type enzymes. This set only yielded products from two other group 4 taxa, *D. mucoroides (Dmuc)* and *D. rosarium (Dros)* and from a group 3 taxon *D. minutum (Dmin)*.

The *Dmin* PdsA product was subsequently used to screen a genomic library. This yielded a full-length *PdsA* gene (see below), the sequence of which was used to design more specific primers for dictyostelid PdsAs. These primers yielded a single sequence from another group 3 taxon *D. rhizopodium* (*Drhi*), the group-intermediate taxon *P. violeceum* (*Pvio*), the group 1 taxa *D. aureo-stipes* (*Daus*) and *D. fasciculatum* (*Dfas*), and triple sequences from the group 2 taxon *A. anastamosans* (*Aana*). Complete coding sequences for the *Dpur-*, *Ppal-* and *DfasPdsA* or *-PDE7* genes were recently obtained from ongoing genome sequencing projects. All derived partial and complete *PdsA* amino acid sequences were aligned with previously published *D. discoideum PdsA* and *PDE7* sequences and with the closest *PdsA* homologs in non-dictyostelid organisms, which are two PDEII sequences from *Legionella pneumophila* and *Flavobacterium johnsoniae* (see supplement 1).

The sequence alignment was used to determine phylogenetic relationships between the PdsA sequences by Bayesian inference (Fig. 1B).

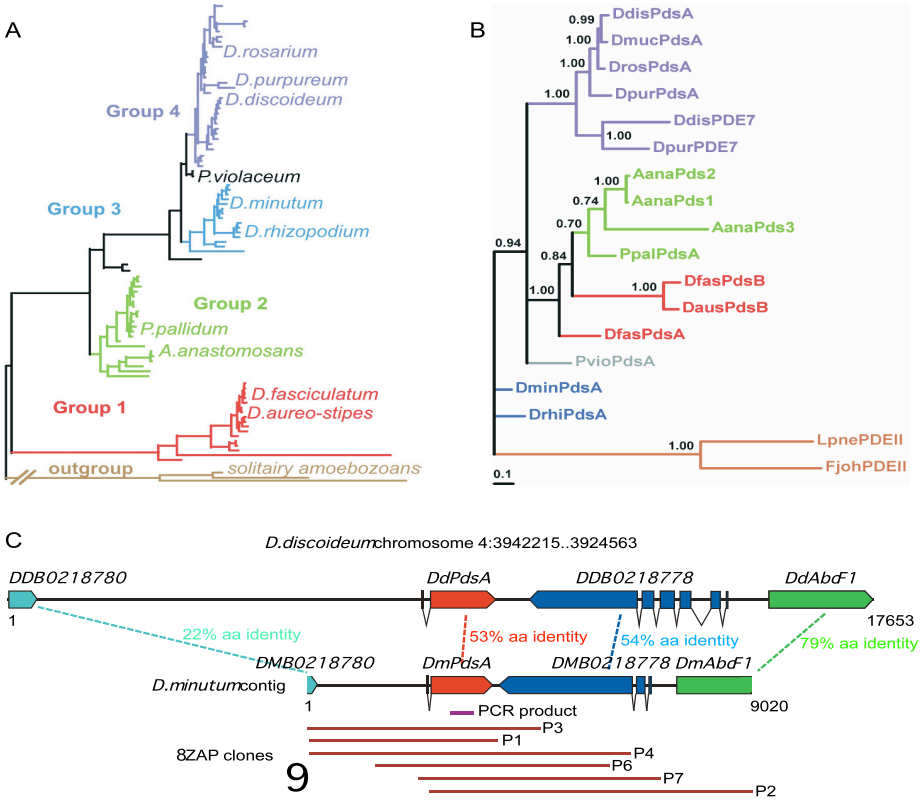


Figure 1: PdsA identification and phylogeny

A) Schematic of molecular phylogeny of Dictyostelia based on small subunit rRNA sequences. The tree shown illustrates the position of the test species and sequences from solitary amoebae were used to root the tree. The major groups are colour coded: purple= group 4; blue= group 3; green= group 2; and red= group 1.

B) PdsA phylogeny. Phylogenetic relationships between sequences were determined by Bayesian inference from 100 (partial PCR products) to 450 (full length protein) well-aligned positions (Ronquist and Huelsenbeck 2003). The tree was rooted on the bacterial PDEII sequences. The Bayesian inference posterior probabilities of nodes are indicated. Branch lengths are drawn to scale as indicated by the scale bar at the lower left. The taxonomic position of the source species is colour coded according to the major groups.

C) Screening of a D. minutum genomic DNAlibrary with theDminPdsA PCR product yielded a 9.02-kb contig. This contig contains DminPdsA and three flanking genes, which we denote DmB0218780, DmB0218778 and DmAbcF1. These genes are most similar to the D. discoideum genes DdB0218780, DdB0218778 and DdAbcF1 respectively, which occupy the same positions relative to DdisPdsA on chromosome 4.

The analysis showed that the clustering of PdsA sequences follows the clustering of species within the four taxon groups. However, the order of divergence of group-specific clusters is not clearly resolved. The *PdsA* gene appears two have duplicated twice in the group 2 taxon *Aana* and there is a second independent duplication in the group 4 taxa *Ddis* and *Dpur*. Only a single *PdsA* gene is present in another group 2 taxon *Ppal* for which we have now complete genome sequence (Gloeckner, Schaap et al., in preparation). This suggests that the two gene duplications of *Aana* are restricted to the clade of *Acytostelids*.

The partial *DminPdsA* PCR product was used to screen a *Dmin* λZAP genomic library. Six positive clones were identified, which could be assembled into a 9.02 kb contig (Fig. 1C). This contig contains *DminPdsA* and three flanking genes, which we denote *DMB0218780*, *DMB0218778* and *DmAbcF1*. These genes are most similar to the *D. discoideum* genes *DDB0218780*, *DDB0218778* and *DdAbcF1* respectively, which occupy the same positions relative to *DdisPdsA* on chromosome 4. Thus there is synteny between the *Ddis and Dmin* genomes and *DminPdsA* is a true ortholog of *DdisPdsA*.

Developmental regulation of *PdsA* expression

We next investigated the developmental regulation of the identified *PdsA* genes are expressed in the different taxa. For some delicate species such as Drhi and Aana it proved to be impossible to obtain sufficiently synchronized development for this purpose. The other test species were incubated on NN agar until fruiting bodies had been formed. Total RNA was extracted at 2-h intervals, and the progression of development was photographed. Northerns of total RNA isolated were hybridized to [α-^{32}P]dATP labelled -*DdisPdsA*, -*DrosPdsA*, -*PvioPdsA*, -*DminPdsA*, -*PpalPdsA*, -*DfasPdsA* or –*DausPdsA*, respectively.

Figure 2 shows that in the group 3 taxon *D min* single PdsA mRNA appears shortly after aggregation. This is in agreement earlier data showing that PdsA activity in *D. minutum* was only detectable after aggregation (Schaap and Spek 1984). In the group 2 taxon *Ppal*, two different mRNAs are predominantly transcribed after aggregation. However, the same blot after re-probing with *PpalIG7* also shows a lack of IG7 transcript before aggregation, which is a direct indication of lack of mRNA (see supplement 2). Surprisingly, in the group 1 taxon *Dfas*, PsdA expression is highest during growth and declines gradually during development. In the second group 1 taxon *Daus*, expression of 2 mRNAs is also highest during growth, with one transcript re-appearing during aggregation. In the group 4 taxa *Dros* and *Ddis* two transcripts are visible throughout development and this is also the case for Pvio. Evidently, there is considerable variation in the complexity of PdsA transcription that does not show any obvious evolutionary progression.

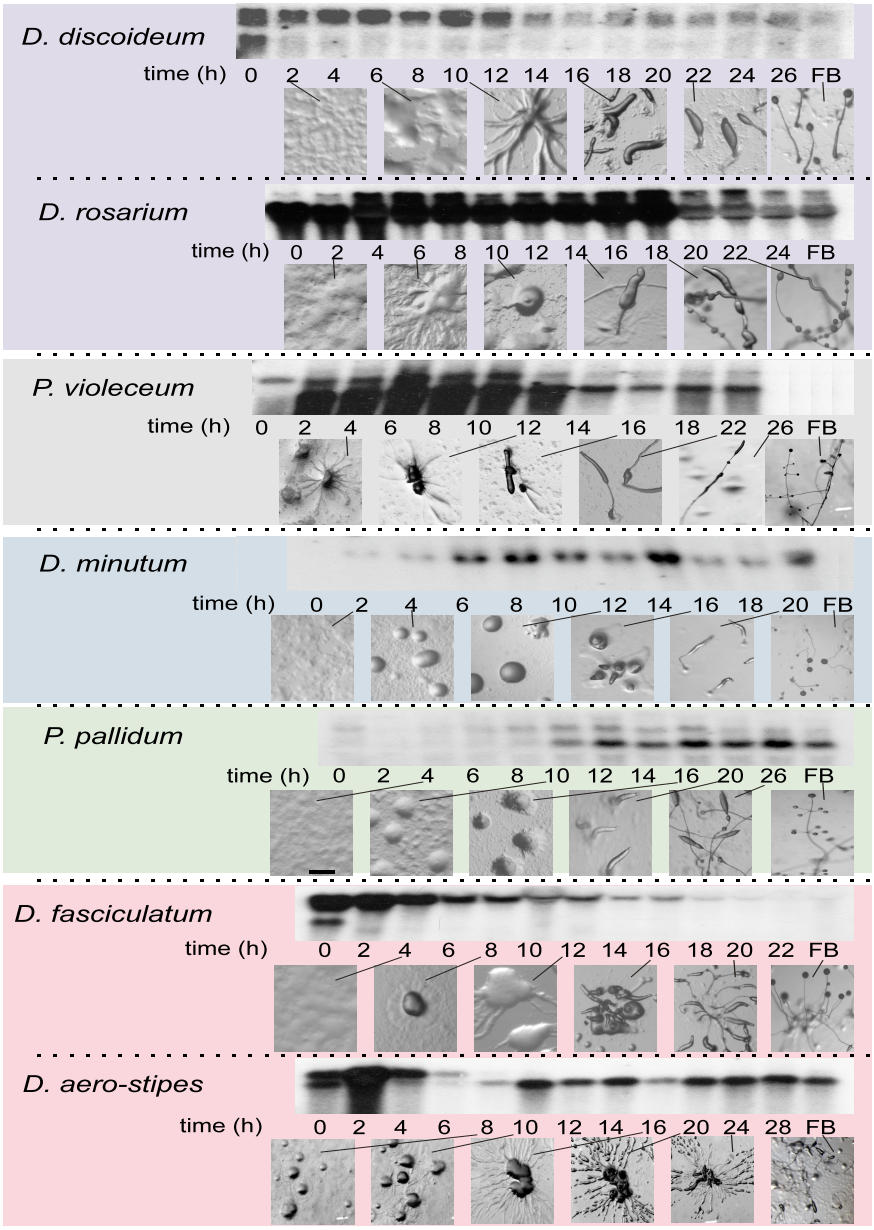


Figure 2: Developmental regulation of PdsA gene expression

Cells of the indicated three species were incubated on agar until fruiting bodies had formed. Total RNA was extracted at 2-h intervals, and the progression of development was photographed. Northern blots were probed at 60°C with [32P]dATP -*DdisPdsA*, -*DrosPdsA*, -*PvioPdsA*, -*DminPdsA*, -*PpalPdsA*, -*DfasPdsA* and -*DausPdsA* PCR products, respectively, and washed at high stringency. The same blots were re-probed with the *DdisIG7* gene that is expressed constitutively throughout *Ddis* development (see supplement 2).

Functional analysis of *DminPdsA*

To investigate whether *DminPdsA* is a functional cAMP-PDE, we fused *DminPdsA* to the constitutive A15 promoter and tested whether this construct could restore the aggregation deficient phenotype of *Ddis* mutant Uk7 that harbours a disruption in the *PdsA* gene. Figure 3A shows that expression of A15:*DminPdsA* in *Uk7-* cells (Uk7:DminPdsA) restores aggregation and fruiting body formation.

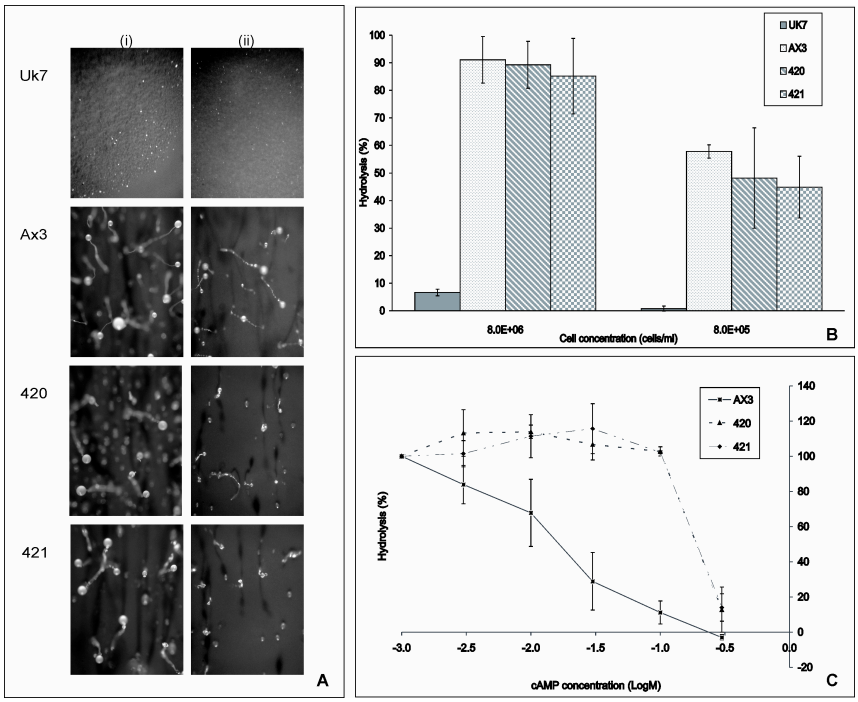


Figure 3: Rescue of function

A construct was made that harbors the *DminpdsA* gene under the actin 15 promoter. Subsequently, the *A15:DminPdsA* gene fusion constructs 420 and 421 were introduced into the *D. discoideum PdsA-* mutant, *UK7-*.which yielded Uk7:*DminPdsA* cellines 420 and 421.

(*A*) Restoration of development. The *UK7-*, AX3, 420 and 421 cellines were developed on filters at 22°C and photographed after 24 hours. Panel (i) shows cells at higher cell density and (ii) at lower cell density.

(B) Restoration of hydrolytic activity. The *Uk7-*, AX3, 420 and 421 were starved for 5 hours at 10^7 cells/ml in PB. Subsequently cells were pelleted, resuspended to 10^8 cells/ml, diluted 1:10 or 1:100 and assayed for [2,8-^{3}H]cAMP hydrolysis. Data are means +/- SD derived from four experiments and expressed as percentage of the maximum hydrolysis of [2,8-^{3}H]cAMP obtained with undiluted AX3.

(C) Effect of varying concentrations of non-radiolabelled cAMP on the hydrolysis of the 3H-cAMP by *DdisPdsA* (AX3) and *DminPdsA* (420 and 421). Data are means +/- SEM derived from two experiments and expressed as percentage of the maximum hydrolysis of [2,8-^{3}H]cAMP obtained with the lowest concentration of cAMP used.

However, whereas complete rescue was obtained at a cell density of $2x10^6$ cells/cm^2, at lower densities smaller and slightly aberant fruiting bodies are formed. This effect might be due to the fact that the constitutive A15 promoter causes ectopic overexpression of *DminPdsA.* Defects in late morphogenesis were also caused by overexpression of *DdisPdsA* from the A15 promoter (Faure, Podgorski et al. 1988). Because the *DminPdsA* fully restores aggregation and development of the Uk7 mutant, it is likely that *DminPdsA* encodes for an active extracellular PDE.

To test this directly, we measured the hydrolysis of 100 nM [2,8-^{3}H]cAMP by progressive dilutions of *Uk7-,* AX3, and the Uk7:*DminPdsA* cells. Figure 3B shows that while the *Uk7* cells only show significant cAMP hydrolysis at high cell densities, the two Uk7:*DminPdsA* cell lines 420 an 421 show similar hydrolytic activity as wild-type AX3 cells. The residual hydrolysis of Uk7 cells has been reported to be caused by DdisPDE7 activity, but this is apparently insufficient to restore aggregation (Bader, Kortholt et al. 2007).

To assess whether DminPdsa has a similar affinity (Km) for substrate as DdisPdsA, we measured [2,8-^{3}H]cAMP hydrolysis in the presence of increasing concentration of unlabeled cAMP. Figure 3C shows that at least 10x higher cAMP concentrations are required to compete for [2,8-^{3}H]cAMP hydrolysis by Dmin PdsA than for Ddis PdsA, indicating that *DminPdsA* has a much lower affinity for cAMP than *DdisPdsA*.

DISCUSSION

PdsA genes are deeply conserved in Dictyostelid evolution

Extracellular cAMP pulses coordinate the aggregation of starving amoebae and fruiting body morphogenesis in the model organism *D. discoideum*. Extracellular cAMP is produced by an adenylyl cyclase A, and degraded by the extracellular phosphodiesterase PDE, PdsA. Together with the cAMP receptors, cARs, these enzymes are essential for oscillatory cAMP signalling. *D. discoideum* is a member of the most recently diverged group 4 of the *Dictyostelia* and all tested species in this group use extracellular cAMP as chemoattractant for aggregation. However, this is not the case for species in groups 1-3, which use folic acid, glorin, pterin or unknown compounds to aggregate (Shimomura, Suthers et al. 1982; De Wit and Konijn 1983). Remarkably, cAMP receptors are present group 1-3 species. However, they are only expressed during post-aggregative development and ablation of cAMP receptor function blocks fruiting body formation and not aggregation in these species. This suggests that all species use extracellular cAMP signalling to coordinate post-aggregative morphogenesis and that the use of cAMP as chemoattractant is a recent innovation (Schaap, Konijn et al. 1984; Schaap, Van Lookeren Campagne et al. 1986; Fukushima and Maeda 1991; Dormann, Weijer et al. 1997; Alvarez-Curto, Rozen et al. 2005; Kawabe, Morio et al. 2009).

During aggregation, degradation of extracellular cAMP by PdsA is critical to support oscillatory cAMP signalling (Lacombe, Podgorski et al. 1986; Podgorski, Faure et al. 1988), while after aggregation PdsA is required to sustain oscillatory cAMP signalling for coordinated cell movement and fruiting body morphogenesis (Wu, Franke et al. 1995; Sucgang, Weijer et al. 1997).

To substantiate the evidence that secreted cAMP signalling is a universal signal for control of morphogenesis, we investigated the conservation of of the PdsA gene throughout the *Dictyostelid* phylogeny. By using a PCR approach we identified *PdsA* homologs in all investigated group-representative species, with independent events of gene duplication in a subset of group 1, 2 and 4 species. A full-length *PdsA* gene was cloned from the group 3 species *D. minutum,* which has the same flanking genes as the *D. discoideum PdsA*, indicating that it is a true ortholog of this gene. Full-length *PdsA* genes were also retrieved from ongoing *P. pallidum* and *D. fasciculatum* genome sequencing projects. However, these genomes show very limited synteny with the *D. discoideum* genome, which precludes confirmation of orthology between conserved genes.

D.minutum PdsA encodes a functional cAMP phosphodiesterase

To investigate whether the lower *PdsA* genes encoded functional phosphodiesterases we complemented the *D. discoideum PdsA* null mutant Uk7 with the *D.minutum PdsA* gene. Complementation with DminPdsA partially restored aggregation and fruiting body formation of *Uk7-*. The Uk7:*DminPdsA* cells also regained the ability to hydrolyze extracellular cAMP, although the Km of DminPdsA appeared to be significantly lower than that of DdisPdsA. This could be related to the fact that secreted cAMP can accumulate to higher concentrations in aggregates, where DminPdsA is active, than in a field of starving cells, where DdisPdsA additionally operates.

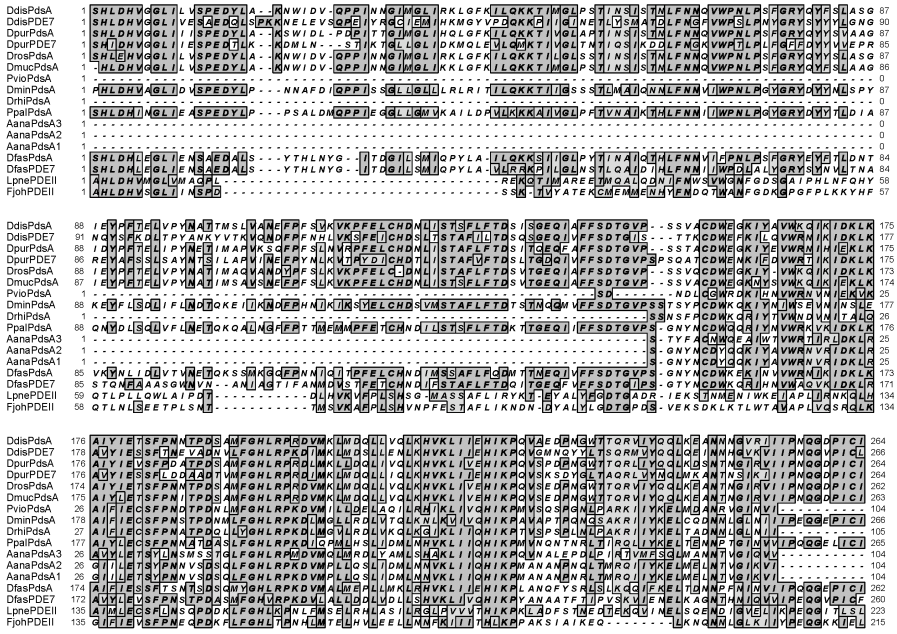
Developmental regulation of PdsA expression throughout the phylogeny

The regulation of the *PdsA* gene in *D.discoideum* is complex with 1.9, 2.4 and 2.2 kb mRNAs expressed from growth, aggregation and late promoters respectively (Faure, Franke et al. 1990). Except for *Dmin* which appears to express a single mRNA after aggregation, all other taxa also express at least two transcripts with different developmental regulation. Most strikingly is the strong expression of *PdsA* during aggregation of the group 1 taxa *Dfas* and *Daus*. These taxa do not express cAMP receptors at this stage and do not use cAMP for aggregation. This raises a conundrum of what might be the function of an extracellular cAMP phosphodiesterase at this stage. Generation of PdsA null mutants in these taxa will be required to resolve this problem.

ACKNOWLEDGEMENT

This research was supported by Wellcome Trust grant 76618 and BBSRC grant BB/D013453/1.

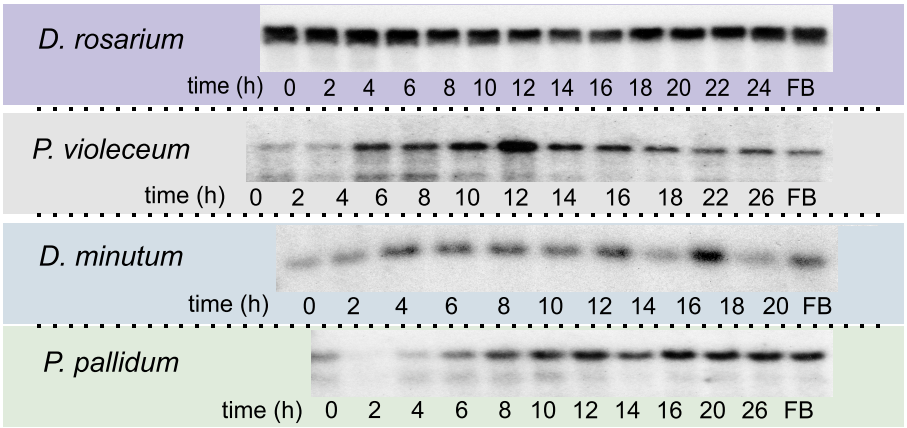
SUPPLEMENTS



Supplement 1: Aminoacid alignment

Alignment of all test species PdsA and bacterial PDEII amino acid sequences with *D. discoideum* PdsA and PDE7 sequences by using CLUSTALW. Partial 100-630 bp *PdsA* sequences were amplified by PCR from genomic DNA of test species. Complete coding sequences for DminPdsA were obtained by screening a Dmin λZAP genomic library with the *DminPdsA* PCR product. Complete coding sequences for the *Dpur-*, *Ppal-* and *DfasPdsA* genes were obtained from ongoing genome sequencing projects. Aminoacid residues that are identical are shaded grey.

Genbank accession numbers for published sequences are: *D. discoideum* PdsA: XP_637948; *D. discoideum* PDE7: XP_636383; *Legionella pneumophila* PDEII: YP_095961; *Flavobacterium johnsoniae* PDEII: YP_001193575.



Supplement 2:
Cells of the indicated four species were incubated on agar until fruiting bodies had formed. Total RNA was extracted at 2-h intervals, and the progression of development was photographed. Northern blots were probed first at 60°C with [32P]dATP -*DrosPdsA*, -*PvioPdsA*, -*DminPdsA*, -*PpalPdsA* and -*DfasPdsA*, respectively, and washed at high stringency (See figure 2). The same blots were re-probed with the *DdisIG7* gene that is expressed constitutively throughout *Ddis* development (See supplement 2).

REFERENCES

Alvarez-Curto, E., D. E. Rozen, et al. (2005). "Evolutionary origin of cAMP-based chemoattraction in the social amoebae." Proc Natl Acad Sci U S A 102(18): 6385-90.

Bader, S., A. Kortholt, et al. (2007). "Seven Dictyostelium discoideum phosphodiesterases degrade three pools of cAMP and cGMP." Biochem J 402(1): 153-61.

Baldauf, S. L. (2003). "The deep roots of eukaryotes." Science 300(5626): 1703-6.

Chenna, R., H. Sugawara, et al. (2003). "Multiple sequence alignment with the Clustal series of programs." Nucleic Acids Res 31(13): 3497-500.

De Wit, R. J. W. and T. M. Konijn (1983). "Identification of the acrasin of Dictyostelium minutum as a derivative of folic acid." Cell Differ. 12: 205-210.

Dormann, D., C. Weijer, et al. (1997). "Twisted scroll waves organize dictyostelium mucoroides slugs." Journal Of Cell Science 110 Part 16 : 1831-: 16-1837.

Faure, M., J. Franke, et al. (1990). "The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains three promoters specific for growth, aggregation, and late development." Mol Cell Biol 10(5): 1921-30.

Faure, M., G. J. Podgorski, et al. (1988). "Disruption of Dictyostelium discoideum morphogenesis by overproduction of cAMP phosphodiesterase." Proc Natl Acad Sci U S A 85(21): 8076-80.

Fridborg, G., M. Pedersén, et al. (1978). "The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis." Physiol.Plant. 43: 104-106.

Fukushima, S. and Y. Maeda (1991). "Whorl formation in Polysphondylium violaceum: Relevance to organization by cyclic AMP." Develop.Growth Differ. 33: 525-533.

Goldbeter, A. (2006). "Oscillations and waves of cyclic AMP in Dictyostelium: a prototype for spatio-temporal organization and pulsatile intercellular communication." Bull Math Biol 68(5): 1095-109.

Kawabe, Y., T. Morio, et al. (2009). "Activated cAMP receptors switch encystation into sporulation." Proc Natl Acad Sci U S A 106(17): 7089-94.

Knecht, D. and K. Ming Pang (1995). Electroporation of Dictyostelium discoideum. Methods in Molecular Biology. J. A. Nickoloff. Totowa, NJ, Humana Press Inc.: 321-329.

Lacombe, M.-L., G. J. Podgorski, et al. (1986). "Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*." J. Biol. Chem. 261: 16811-16817.

Meima, M. E., K. E. Weening, et al. (2007). "Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium." Protein Expr Purif 53(2): 283-8.

Podgorski, G. J., M. Faure, et al. (1988). "The cyclic nucleotide phosphodiesterase of Dictyostelium discoideum: the structure of the gene and its regulation and role in development." Dev Genet 9(4-5): 267-78.

Raper, K. B. (1984). The Dictyostelids. Princeton, New Jersey, Princeton University Press.

Ronquist, F. and J. P. Huelsenbeck (2003). "MrBayes 3: Bayesian phylogenetic inference under mixed models." Bioinformatics 19(12): 1572-4.

Schaap, P., T. M. Konijn, et al. (1984). "cAMP pulses coordinate morphogenetic movement during fruiting body formation of Dictyostelium minutum." Proc Natl Acad Sci U S A 81(7): 2122-2126.

Schaap, P. and W. Spek (1984). "Cyclic-AMP binding to the cell surface during development of Dictyostelium discoideum." Differentiation 27: 83-87.

Schaap, P., M. M. Van Lookeren Campagne, et al. (1986). "Postaggregative differentiation induction by cyclic AMP in Dictyostelium: intracellular transduction pathway and requirement for additional stimuli." Dev Biol 118(1): 52-63.

Schaap, P., T. Winckler, et al. (2006). "Molecular phylogeny and evolution of morphology in the social amoebas." Science 314(5799): 661-3.

Shimomura, O., H. L. B. Suthers, et al. (1982). "Chemical identity of the acrasin of the cellular slime mold Polysphondylium violaceum." Proc.Natl.Acad.Sci.USA 79: 7376-7379.

Sucgang, R., C. J. Weijer, et al. (1997). "Null mutations of the Dictyostelium cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates." Dev Biol 192(1): 181-92.

Weijer, C. J. (2004). "Dictyostelium morphogenesis." Curr Opin Genet Dev 14(4): 392-8.

Wu, L. and J. Franke (1990). "A developmentally regulated and cAMP-repressible gene of Dictyostelium discoideum: cloning and expression of the gene encoding cyclic nucleotide phosphodiesterase inhibitor." Gene 91: 51-56.

Wu, L., J. Franke, et al. (1995). "The phosphodiesterase secreted by prestalk cells is necessary for Dictyostelium morphogenesis." Dev Biol 167(1): 1-8.

Zhang, M., M. Goswami, et al. (2005). "Constitutively active G protein-coupled receptor mutants block dictyostelium development." Mol Biol Cell 16(2): 562-72.

Chapter 4

Adenylyl cyclase G is transiently activated by cAMP receptors in developing *Dictyostelium discoideum* cells

Karin E. Weening[1, 2] and Pauline Schaap[1†]

[1] College of Life Sciences, Department of Cell and Developmental Biology, University of Dundee DD1 5EH, United Kingdom
[2] present address: School of Biosciences, Department of Molecular Biology, Cardiff University CF10 3AX, United Kingdom
[†] To whom correspondence should be addressed. E-mail: p.schaap@dundee.ac.uk

ABSTRACT

In the model organism *Dictyostelium discoideum*, intracellular and secreted cAMP control cell differentiation and morphogenesis throughout development. Aggregation and development is initiated by pulsatile production and secretion of cAMP. After aggregation, extracellular cAMP binding to surface cAMP receptors and intracellular cAMP binding to cAMP-dependent protein kinase (PKA) is required to induce prespore differentiation. Later, a second episode of PKA activation triggers stalk and spore maturation. cAMP is produced by three structurally distinct adenylyl cyclases, ACA, ACG and ACB. Adenylyl cyclase ACA, which is expressed at high levels during aggregation, is responsible for cAMP production at this stage. Adenylyl cyclase B (ACB) produces cAMP for maturation, but the cAMP source for prespore induction was for a long time obscure. Traditionally, ACG was thought to be only expressed in spores, where it functions as an osmosensor controlling spore germination by acting on PKA. But more recently, ACG was shown to produce the cAMP needed for prespore induction. We show here that ACG activity becomes increasingly non-linear while cells are starving which is remarkably similar to ACA activation. Furthermore, we show that this response is mediated through the cAMP receptors, but unlike ACA, does not require heterotrimeric G-proteins. Finally we provide hypotheses about the molecular mechanism and the developmental role of this transient ACG mediated cAMP production.

Key words: *Dictyostelium discoideum* / adenylyl cyclase / activation kinetics / prespore differentiation / cAMP receptor

INTRODUCTION

Throughout development of the social amoebae *Dictyostelium discoideum*, intracellular and secreted cAMP play crucial roles in controlling cell movement and gene regulation (Saran, Meima et al. 2002). When food is depleted, some amoebae begin to release pulses of cAMP. Neighbouring cells respond by chemotactic movement towards the cAMP source. In addition, they themselves will start to secrete cAMP pulses. As a result, waves of cAMP propagate through the cell population and the cells stream towards the original source of the pulses to form an aggregate, which can consist of up to 10^5 cells. Following aggregation, cells construct a multicellular slug and differentiate into prespore and prestalk cells in a 3:1 ratio. Prestalk cells will sort to the anterior of the slug, while prespore cells occupy the posterior region of the slug. Eventually, the slugs transforms into a fruiting body consisting of a mass of spores supported by a stalk.

In addition to chemotaxis, the cAMP pulses also upregulate expression of aggregation genes, while after aggregation, secreted cAMP induces the expression of prespore genes and inhibits stalk gene expression (Wang, Van Driel et al. 1988;

Soede, Hopper et al. 1996). cAMP is detected in *Dictyostelium* by 4 homologous G-protein coupled cAMP receptors (cARs 1-4). The four cARs differ in ligand affinity, and show different spatio-temporal expression patterns (Kim, Borleis et al. 1998).

In its classical role as intracellular second messenger, cAMP induces the maturation of stalk and spore cells, and maintains spore dormancy under conditions that are unfavorable for cell growth. These functions are all mediated by cAMP dependent protein kinase (PKA) (Loomis 1998).

cAMP is produced by three structurally distinct adenylyl cyclases, ACA, ACG and ACB. The aggregation-associated adenylyl cyclase A (ACA) is structurally similar to the mammalian G-protein regulated cyclases with 12 transmembrane helices (Pitt, Milona et al. 1992; Kriebel and Parent 2004). ACA is maximally expressed during aggregation and it is thereafter down-regulated in all cells except those at the slug tip (Pitt, Milona et al. 1992; Verkerke-van Wijk, Fukuzawa et al. 2001). ACA produces the cAMP pulses that are required for aggregation and post-aggregative morphogenesis (Pitt, Milona et al. 1992). It is activated in a complex indirect manner through cAMP binding to cARs. Binding leads to dissociation of the G-protein G2 into its α and βγ subunits and also results in activation of the small GTPase RasC (Pupillo, Insall et al. 1992; Wu, Valkema et al. 1995; Lim, Spiegelman et al. 2001). Co-activation of βγ subunit and RasC via distinct mechanisms causes recruitment of _phosphatidylinositol 3' kinases (PI3K) to the plasma membrane, where it phosphorylates the membrane lipid phosphatydyl-inositol-bis-phosphate (PIP2) to form phosphatydyl-inositol-tris-phosphate (PIP3) (Kae, Kortholt et al. 2007). PIP3 then causes translocation of the Cytosolic Regulator of Adenylyl Cyclase (CRAC) to the plasma membrane by binding to the pleckstrin homologue (PH) domain of CRAC, which in turn activates ACA (Comer, Lippincott et al. 2005). Prolonged stimulation with cAMP causes desensitization of cARs and cessation of ACA activation (Dinauer, Steck et al. 1980; Van Haastert 1987). The mechanisms of this adaptation process are as yet poorly understood.

Adenylyl cyclase B (ACB), encoded by *AcrA*, is most related to the adenylyl cyclases from cyanobacteria. ACB is expressed at low levels during growth and expression increases strongly in the slug stage to reach a maximum at culmination (Soderbom, Anjard et al. 1999). ACB produces cAMP that is needed for spore and stalk maturation (Meima and Schaap 1999).

The germination adenylyl cyclase (ACG) is expressed at low levels during the entire course of development with a strong increase at the spore stage. Remarkably, ACG protein is absent during aggregation to increase strongly in the posterior region of the slug (Alvarez-Curto, Saran et al. 2007). ACG has two transmembrane domains that flank an extracellular domain, and an intracellular catalytic domain. ACG activity is upregulated by high osmolarity, a condition that keeps spores dormant in the spore head. This effect is mediated by ACG acting on PKA (van Es, Virdy et al. 1996; Saran and Schaap 2004). In addition, recently ACG

was shown to produce the cAMP needed for prespore differentiation (Alvarez-Curto, Saran et al. 2007).

In this work we investigated the kinetics of ACG activation in living cells. We found that ACG synthesizes cAMP at a constant rate when expressed in vegetative cells. However ACG activity becomes increasingly more transient while cells are starving.

We show that this is due to positive and negative feedback on activation by secreted cAMP. Similar to ACA activation, this response requires cAMP receptors, but unlike ACA it does not depend on activation of heterotrimeric G-proteins.

MATERIALS AND METHODS

Materials

G418, Dithiothreitol (DTT), 2'3'isopropylidene adenosine (IPA), 2'-deoxyadenosine 3':5' monophosphate (2'deoxy cAMP) and guanosine 5'-[γ-thio]triphosphate (GTPγS) were from Sigma (St.Louis, MO, USA); nucleopore filters (pore size 3 µm) were from Corning (New York, USA); and [2,8-^{3}H]cAMP was from Amersham Biosciences (Little Chalfont, Bucks., UK).

Cell transformation, culture and development

For the first set of experiments, *aca-* cells that express plasmid pGSP1 were used (*aca-*/ACG). This plasmid harbors full-length ACG under the actin15 promoter. (Pitt, Milona et al. 1992) For the next sets of experiments, *aca-* cells (Pitt, Milona et al. 1992) and *car1car3* cells (Insall, Soede et al. 1994) were transformed with a slightly different plasmid A15::ACG-YFP. For this we amplified genomic ACG DNA from vector pBS-ACG with primers Co-DdACG-XhoI (AACCTCGAGCTTTTTTTGATTCTACATTTTCGTC) and N-BamHI-DdACG (AAAGGATCCAAAACATTTGTAAAGATACTATCTC). The BamHI-XhoI digested fragment was used to place ACG downstream of the actin15 promoter and start codon and upstream of the yellow fluorescence protein (YFP) gene into vector pBS17S.

Transformation was done by electroporation according to Knecht. (Knecht and Ming Pang 1995) Briefly, cells were harvested, washed twice in H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO4, 5 mM NaHCO3 and 1 mM NaH2PO4.H_2O) and resuspended as $5*10^7$ cells/ml. 100 µl of cells with 6 µg of DNA were pulsed twice at 0.65 V.

All cell lines were grown in HL5 axenic medium supplemented with 5 µg/µl blasticidine for *car1/car3* and 50 µg/ml of G418 for *aca-*/ACG and *aca-/*ACG-YFP.

ACG assay in intact cells

Exponentially growing *aca*/ACG cells were harvested from axenic medium and resuspended to 10^8 cells/ml in 10 mM sodium/phosphate buffer, pH 6.5 (PB). To induce development, cells were incubated at 10^7 cells/cm^2 on NN agar (1.5 % agar in PB) at 22° C. Next cells were harvested and resuspended to 10^8 cells/ml in 10 mM phosphate buffer, pH 6.5 (PB).

25 µl aliquots of cell suspension were incubated with or without 10 mM IPA in a total volume of 30 µl in microtiter plate wells. Reactions were started by adding 5 µl 50 µM 2-deoxy cAMP in 50 mM DTT, and terminated at variable time intervals with 30 µl 3.5% (v/v) perchloric acid. Lysates were neutralized with 15 µl 50% saturated $KHCO_3$ and 75 µl cAMP buffer (4 mM EDTA in 150 mM sodium phosphate, pH 7.5). cAMP levels were determined by isotope dilution assay.

Assay of ACG in cell lysates

Cells were harvested, washed and resuspended in ice-cold lysis buffer (250 mM sucrose in 10 mM Tris-HCl, pH 8.0) at 10^8 cells/ml and lysed on ice through 3 µm nucleopore filters, in the presence or absence of 30 µM GTPγS. Aliquots of 10 µl cell lysate were added on ice to 5 µl of assay mix (2 mM ATP, 0.8 mM IBMX and 40 mM DTT in lysis buffer), with addition of either 5 ul 8 mM $MgCl_2$ (Mg^{2+} assay mix) or $MnCl_2$ (Mn^{2+} assay mix). After 5 min of incubation on ice, reactions were started by transferring the samples to a 22 °C water bath. Reactions were terminated by adding 10 µl of 0.4 M EDTA (pH 8.0) followed by boiling for 1 min. cAMP levels were assayed directly in the boiled lysate by isotope dilution assay. For all assays the cAMP levels were standardized on the protein content of the lysate.

RESULTS

cAMP production by ACG becomes non-linear in starving cells

Spores are not accessible to direct assays for ACG activity, but the enzyme can be conveniently measured when expressed from the actin 15 promoter in *aca*-cells (*aca*-/ACG). *Dictyostelium* cells secrete almost all cAMP that they synthesize, and after addition of dithiothreitol (DTT), which inhibits the extracellular cAMP phosphodiesterase (PdsA) (Orlow, Shapiro et al. 1981), ACG activity is evident by linear accumulation cAMP production (Fig. 1A). The rate of cAMP accumulation is about 2-fold higher in the presence of either, 0.2 M sucrose or 0.1 M sorbitol, as reported previously (van Es, Virdy et al. 1996).

After cells have starved for 2 hours, the kinetics of cAMP accumulation becomes very different. Without solute stimulation, cAMP levels peaks at 5-10 min and thereafter decreases (Fig. 1B). In the presence of solute, cAMP levels increase to about 2 fold higher levels in 5 minutes, and thereafter remain constant. After 4 h of starvation, the addition of DTT triggers even more pronounced transient accumulation of cAMP in the absence of solute. With solute, cAMP levels increase more rapidly, but reach only a modest peak before decreasing again (Fig.1C).

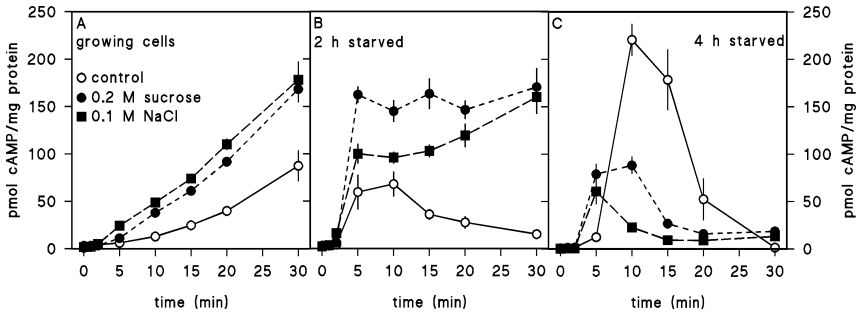


Figure 1: Effect of osmolarity on ACG activity in *aca*-/ACG cells
Vegetative (A), 2 hours starved (B) or 4 hour starved (C) *aca*-/ACG cells were collected and resuspended to 10^8 cells/ml. Subsequently, aliquots of cells were incubated with 5 mM DTT in the presence of 100 mM NaCl (■), 200 mM sucrose (●) or without additives (○). At indicated time periods, reactions were terminated and assayed for total accumulated cAMP by isotope dilution assay. Means and SD of three time courses, assayed in quadruplicate are presented.

ACG mediated cAMP production in starving cells is inhibited by IPA

The transient ACG mediated cAMP production in starving cells is remarkably similar to ACA mediated cAMP production in response to cAMP stimulation. (Dinauer, Steck et al. 1980) Although cAMP was not added in our assays, it will inevitably aacumulate extracellularly once DTT has been added. The effect of cAMP is mediated by cAMP receptors (cARs) and can be inhibited by purine nucleosides, such as 2'3'isopropylidene adenosine (IPA), which inhibits the binding of cAMP to cARs. (Van Lookeren Campagne, Schaap et al. 1986)

To determine if DTT induced ACG mediated cAMP accumulation is similarly dependent on cARs, we measured the effect of IPA on this response in vegetative and starving cells. For this experiment we used *aca*- cells that were freshly transformed with a slightly different construct in which ACG was fused to YFP at the carboxyl-terminus (*aca-*/ACG-YFP). Figure 2A shows that in growing cells IPA has no effect on the linear accumulation of cAMP. However, in 4 hours starved cells IPA effectively inhibits the transient cAMP accumulation by ACG. This suggests that ACG is upregulated by cARs in starved cells.

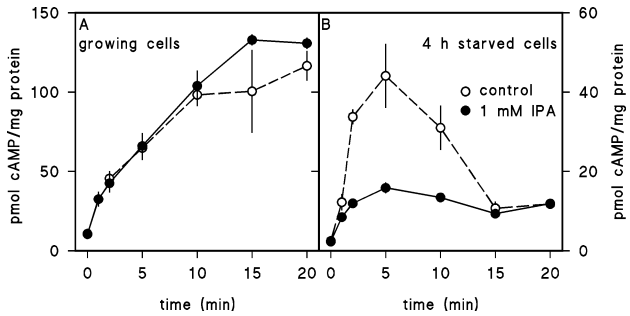


Figure 2: Effect of IPA on ACG activity in *aca-*/ACG cells
Vegetative (A), or 4 hour starved (B) *aca-*/ACG-YFP cells were collected and resuspended to 10^8 cells/ml. Subsequently, aliquots of cells were incubated with 5 mM DTT, with (●) or without 0.1 mM IPA (○). cAMP was assayed at the indicated time intervals. Means and SD of three time courses, assayed in quadruplicate are presented.

Effects of 2'H-cAMP and GTPγS on ACG activity in starved cells

To confirm that the apparent stimulatory effect of DTT results from preventing cAMP hydrolysis and not from more direct interference with ACG activity, we also tested whether a cAR agonist, 2'H-cAMP, can activate ACG in the absence of DTT. Figure 3A, shows that 2'H-cAMP induces transient cAMP accumulation in *aca-*/ACG-YFP cells although the accumulation is much higher when cAMP hydrolysis is inhibited by DTT.

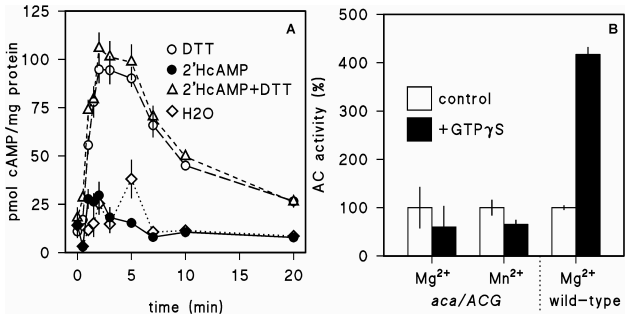


Figure 3: Effects of 2'HcAMP and GTPγS on ACG activity in developing cells
A). 4 Hour starved *aca-*/ACG-YFP cells were collected and resuspended to 10^8 cells/ml. Subsequently, aliquots were incubated in the presence of 5 mM DTT (○), 5 µM 2'HcAMP (●) or 5 µM 2'HcAMP and 5 mM DTT (▲). At the indicated time intervals, reactions were terminated and assayed for cAMP accumulation. B). 4 Hour starved wild-type and *aca-*/ACG-YFP cells were collected and resuspended in lysis buffer at 10^8 cells/ml and subsequently lysed on ice through nucleopore filters, in the presence (■) or absence (□) of 30 µM GTPγS. Subsequently aliquots of cell lysate were incubated with Mg^{2+} or Mn^{2+} assay mix as indicated. Reactions were started, by transferring the samples to a 22 °C water bath and terminated by addition of EDTA. cAMP was assayed and standardized on the protein content of the lysate. Means and SD of three time courses, assayed in quadruplicate are presented.

In addition to cAR1, the activation of ACA also requires the heterotrimeric G-protein G2. (Pupillo, Insall et al. 1992) To test whether ACG activation is also mediated by a G protein, we tested the effect of GTPγS, a direct activator of G-proteins, on ACG activity in cell lysates. Figure 3B shows that GTPγS activates ACA effectively in 4 hour starved cells, but has no effect on ACG activity. This suggests that ACG activity is not under G-protein regulation.

ACG activity in cAR null mutants

The observation that IPA inhibits cAMP production in developing cells but not in growing cells suggests that ACG activation is mediated by binding of cAMP to cARs, as. *D. discoideum* has four different cARs, of which cAR1 and cAR3 are important for the activation of ACA. (Insall, Soede et al. 1994) To provide further evidence that ACG activation is dependent on cARs, we transformed a *car1car3* null mutant with the A15::ACG-YFP construct (*car1car3*/ACG-YFP).

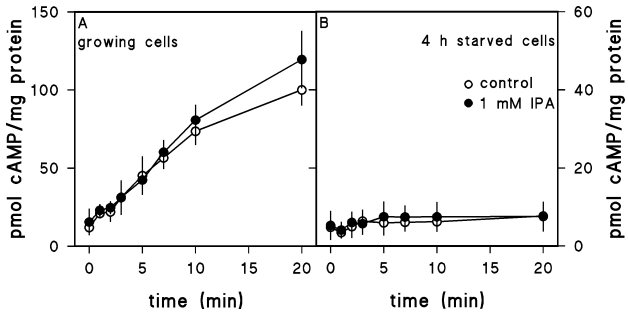


Figure 4: ACG activity in *car1-/car3-* null mutants
Vegetative (A), or 4 hour starved (B) *car1car3*/ACG-YFP cells were collected and resuspended to 10^8 cells/ml. Subsequently, aliquots of cells were stimulated with 5 μM 2'HcAMP and 5 mM DTT, in the presence (●) or absence (○) of 0.1 mM IPA. cAMP accumulation was assayed at the indicated time periods. Means and SD of three time courses, ssayed in quadruplicate are presented.

Next we measured the effect loss of cARs on cAMP accumulation in growing and 4 hour starving *car1car3*/ACG-YFP cells.

Figure 4A shows that in growing cells loss of cARs has no effect on the linear accumulation of cAMP, and that IPA does not effect cAMP production by ACG at this stage. However in 4 hours starved cells, cAMP accumulation by ACG is completely lost, indicating that ACG is stimulated by cAMP via cAR1 or cAR3 (Fig.4B).

DISCUSSION

Aggregation and development is initiated by pulsatile production and secretion of cAMP. ACA, which is expressed at high levels during aggregation, is responsible for cAMP production at this stage (Pitt, Milona et al. 1992). Its activity is upregulated by a complex mechanism that is initiated by cAMP binding to cARs leading in succession to G-protein activation, PI3-kinase translocation to the plasma membrane, PIP3 production, CRAC translocation, and finally ACA activation (Pupillo, Insall et al. 1992; Wu, Valkema et al. 1995; Comer, Lippincott et al. 2005; Kae, Kortholt et al. 2007). Continued cAR occupation leads to cessation of ACA activation, in a process that does not require ACA to be active, but is otherwise not understood (Dinauer, Steck et al. 1980; Van Haastert 1987).

Loomis and coworkers suggested a model in which intracellular cAMP activates the cAMP phosphodiesterase RegA by PKA mediated inhibition of the MAP kinase ERK2, which relieves inhibition of RegA by ERK2 (Laub and Loomis 1998; Maeda, Lu et al. 2004). This model is supported by a fair body of evidence, but does not agree with the earlier studies showing that ACA need not be itself active to be desensitized to by continued occupancy of cARs. Our results provide a new twist to this debate. They indicate that cAMP can also be transiently produced in response to cAR activation by other adenylyl cyclases than ACA.

ACG was initially shown to be expressed in spores, where it acts as an osmosensor controlling spore germination (van Es, Virdy et al. 1996). Later, ACG protein levels were found to be markedly upregulated in the posterior region of slugs, where it triggers prespore differentiaton (Alvarez-Curto, Saran et al. 2007). Our experiments were performed under artificial conditions where ACG is expressed at high levels from an actin15 promoter throughout development. Earlier and current studies showed that in vegetative cells, ACG produces a steady output of cAMP, which shows an increased rate in the presence of high osmolality (van Es, Virdy et al. 1996). At this stage, ACG is not regulated by cAR1. However, after 4 hours of starvation, ACG mediated cAMP production becomes rapidly upregulated through positive feedback from secreted cAMP that accumulates when extracellular cAMP phosphodiesterase is inhibited. Both disruption of the cAR1 and cAR3 genes and acute inhibition of cAR function by IPA prevent cAMP-induced ACG activation, indicating that cARs mediate the response. However, ACG cannot be activated by GTPγS, suggesting that unlike ACA, heterotrimeric G-proteins do not activate ACG.

The MAP kinase ERK2 is activated by cAMP acting on cARs and this response also does not require heterotrimeric G-proteins (Maeda, Aubry et al. 1996) (Brzostowski and Kimmel 2001). This suggests a mechanism by which transient ACG mediated cAMP production might occur. In this mechanism, cAR mediated RegA inhibition; rather cAR mediated ACG activation causes cAMP to accumulate. As proposed by Loomis and Laub, cAMP activation of ERK2 leads to inhibition of the intracellular cAMP phosphodiesterase RegA. This allows intracellular cAMP to

accumulate and be secreted, but also to activate PKA. In the Laub/Loomis model PKA then inactivates ERK2, causing RegA activation and cAMP degradation (Laub and Loomis 1998). To test the validity of this model, it should be established whether the presence of RegA, ERK2 or PKA are crucial for the cAR-induced ACG mediated cAMP accumulation.

Our result raises the question whether the transient cAR mediated activation of ACG is of any developmental significance? Cyclic AMP waves can be visualised as optical density waves propagating through aggregation territories and slugs throughout development (Siegert and Weijer 1992; Dormann, Vasiev et al. 1998; Dormann and Weijer 2001). The cAMP waves coordinate both the aggregation process and the cell movement that shape the slug and fruiting body (Weijer 2004). In the slug stage, cells in the prestalk region show spiral movement, while cells in the prespore region move forward in the direction of slug migration (Bretschneider, Vasiev et al. 1999). This suggested that the tip continues to produce spiral waves of cAMP, which propagate as scroll waves in the anterior prestalk region and as standing waves in the posterior prespore zone. Furthermore, ACA was thought to be the cyclase responsible for this cAMP production. However, Verkerke et al., showed that ACA expression is lost from all cells except from those at the tip of the slug and ACB expression is also restricted to the slug anterior (Verkerke-van Wijk, Fukuzawa et al. 2001; Alvarez-Curto, Saran et al. 2007). Since ACG is expressed throughout the slug posterior, it could potentially act to propagate cAMP waves in this region. Imaging of cell movement in *acg-* slugs could resolve this issue. Unfortunately, even in wild-type slugs waves of cell movement or often poorly or not at all visible, which makes their absence in *acg-* slugs extremely difficult to ascertain.

ACKNOWLEDGEMENT

We thank Peter Devreotes (John Hopkins University, Medical School, Baltimore) for the *aca* and *car1car3* cell line. The latter was kindly provided by Jakob Franke from the Dicty stock Center (Columbia University, New York). This research was supported by NWO grant 805-17048 to K.E.W. and Wellcome Trust grant 76618.

REFERENCES

Alvarez-Curto, E., S. Saran, et al. (2007). "cAMP production by adenylyl cyclase G induces prespore differentiation in Dictyostelium slugs." Development **134**(5): 959-66.

Bretschneider, T., B. Vasiev, et al. (1999). "A model for Dictyostelium slug movement." Journal of Theoretical Biology **199**(2): 125-136.

Brzostowski, J. A. and A. R. Kimmel (2001). "Signaling at zero G: G-protein-independent functions for 7-TM receptors." Trends Biochem Sci **26**(5): 291-7.

Comer, F. I., C. K. Lippincott, et al. (2005). "The PI3K-mediated activation of CRAC independently regulates adenylyl cyclase activation and chemotaxis." Curr Biol **15**(2): 134-9.

Dinauer, M. C., T. L. Steck, et al. (1980). "Cyclic 3',5'-AMP relay in Dictyostelium discoideum V. Adaptation of the cAMP signaling response during cAMP stimulation." J Cell Biol **86**(2): 554-61.

Dormann, D., B. Vasiev, et al. (1998). "Propagating waves control dictyostelium discoideum morphogenesis." Biophysical Chemistry **72**: 21-35.

Dormann, D. and C. J. Weijer (2001). "Propagating chemoattractant waves coordinate periodic cell movement in Dictyostelium slugs." Development **128**(22): 4535-43.

Insall, R. H., R. D. Soede, et al. (1994). "Two cAMP receptors activate common signaling pathways in Dictyostelium." Mol Biol Cell **5**(6): 703-11.

Kae, H., A. Kortholt, et al. (2007). "Cyclic AMP signalling in Dictyostelium: G-proteins activate separate Ras pathways using specific RasGEFs." EMBO Rep **8**(5): 477-82.

Kim, J. Y., J. A. Borleis, et al. (1998). "Switching of chemoattractant receptors programs development and morphogenesis in dictyostelium: receptor subtypes activate common responses at different agonist concentrations." Developmental Biology **197**: 117-128.

Knecht, D. and K. Ming Pang (1995). Electroporation of Dictyostelium discoideum. Methods in Molecular Biology. J. A. Nickoloff. Totowa, NJ, Humana Press Inc.**:** 321-329.

Kriebel, P. W. and C. A. Parent (2004). "Adenylyl cyclase expression and regulation during the differentiation of Dictyostelium discoideum." IUBMB Life **56**(9): 541-6.

Laub, M. T. and W. F. Loomis (1998). "A molecular network that produces spontaneous oscillations in excitable cells of Dictyostelium." Mol. Biol. Cell **9**: 3521-3532.

Lim, C. J., G. B. Spiegelman, et al. (2001). "RasC is required for optimal activation of adenylyl cyclase and Akt/PKB during aggregation." Embo J **20**(16): 4490-9.

Loomis, W. F. (1998). "Role of pka in the timing of developmental events in dictyostelium cells." Microbiology And Molecular Biology Reviews **62**: 684.

Maeda, M., L. Aubry, et al. (1996). "Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in Dictyostelium." J Biol Chem **271**: 3351-3354.

Maeda, M., S. Lu, et al. (2004). "Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA." Science **304**(5672): 875-8.

Meima, M. E. and P. Schaap (1999). "Fingerprinting of adenylyl cyclase activities during Dictyostelium development indicates a dominant role for adenylyl cyclase B in terminal differentiation." Dev Biol **212**(1): 182-90.

Orlow, S. J., I. Shapiro, et al. (1981). "The extracellular cyclic nucleotide phosphodiesterase of Dictyostelium discoideum." J.Biol.Chem. **256**: 7620-7627.

Pitt, G. S., N. Milona, et al. (1992). "Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development." Cell **69**(2): 305-15.

Pupillo, M., R. Insall, et al. (1992). "Multiple cyclic AMP receptors are linked to adenylyl cyclase in Dictyostelium." Mol.Biol.Cell **3**: 1229-1234.

Saran, S., M. E. Meima, et al. (2002). "cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection." J Muscle Res Cell Motil **23**(7-8): 793-802.

Saran, S. and P. Schaap (2004). "Adenylyl cyclase G is activated by an intramolecular osmosensor." Mol Biol Cell **15**(3): 1479-86.

Siegert, F. and C. J. Weijer (1992). "Three-dimensional scroll waves organize Dictyostelium slugs." Proc.Natl.Acad.Sci.USA **89**: 6433-6437.

Soderbom, F., C. Anjard, et al. (1999). "An adenylyl cyclase that functions during late development of Dictyostelium." Development **126**(23): 5463-71.

Soede, R. D., N. A. Hopper, et al. (1996). "Extracellular cAMP depletion triggers stalk gene expression in Dictyostelium: disparities in developmental timing and dose dependency indicate that prespore induction and stalk repression by cAMP are mediated by separate signaling pathways." Dev Biol **177**(1): 152-9.

van Es, S., K. J. Virdy, et al. (1996). "Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores." J Biol Chem **271**(39): 23623-5.

Van Haastert, P. J. (1987). "Down-regulation of cell surface cyclic AMP receptors and desensitization of cyclic AMP-stimulated adenylate cyclase by cyclic AMP in Dictyostelium discoideum. Kinetics and concentration dependence." J Biol Chem **262**(16): 7700-4.

Van Lookeren Campagne, M. M., P. Schaap, et al. (1986). "Specificity of adenosine inhibition of cAMP-induced responses in Disctyostelium resembles that of the P site of higher organisms." Dev.Biol. **117**: 245-251.

Verkerke-van Wijk, I., M. Fukuzawa, et al. (2001). "Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression." Dev Biol **234**(1): 151-60.

Wang, M., R. Van Driel, et al. (1988). "Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in Dictyostelium discoideum slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation." Development **103**: 611-618.

Weijer, C. J. (2004). "Dictyostelium morphogenesis." Curr Opin Genet Dev **14**(4): 392-8.

Wu, L., R. Valkema, et al. (1995). "The G protein b subunit is essential for multiple responses to chemoattractants in Dictyostelium." J.Cell Biol. **129**: 1667-1675.

Chapter 5

Cyclic GMP controls developmental timing in *Dictyostelium*

Karin E. Weening[1,2], Marcel E. Meima[1] and Pauline Schaap[1,†]

[1] College of Life Sciences, Department of Cell and Developmental Biology, University of Dundee DD1 5EH, United Kingdom
[2] Present address: School of Biosciences, Department of Molecular Biology, Cardiff University CF10 3AX, United Kingdom
[†] To whom correspondence should be addressed. E-mail: p.schaap@dundee.ac.uk

ABSTRACT

Starving Dictyostelid amoebae secrete cAMP pulses which act as chemoattractant to cause aggregation and also accelerate the expression of genes that are required for aggregation. Mutants with defects in cGMP synthesis or cGMP detection show altered myosin phosphorylation and chemotactic responsiveness to cAMP, which has implicated cGMP as intermediate for the chemotactic response. We report here that loss-of-function mutants in the cGMP phosphodiesterase, PdeD precociously expressed aggregative genes, which resulted in early onset of chemotactic signalling and development. Conversely, PdeD gain-of-function mutants showed a pronounced delay in aggregative gene expression, which was also observed in double null mutants in the *Dictyostelium* guanylyl cyclases sGC and GCA. Stimulation with cAMP pulses restored the developmental timing defect of the PdeD overexpressors and *sgc/gca* double null mutants and also restored full chemotactic responsiveness. These data indicate a role for cGMP in control of early gene expression, which has a pleiotropic effect on the chemotactic response.

Keywords: cGMP-phosphodiesterase, guanylyl cyclase, heterochronicity, chemotaxis, developmental gene expression.

INTRODUCTION

The intracellular signalling intermediate cGMP is best known for its role in vasodilation and vision in humans (Olshevskaya, Ermilov et al. 2002; Friebe and Koesling 2003). In protists, its function has been associated with cell motility and gamete maturation. In ciliates, a cGMP controls ciliar movement, while in the related alveolate parasite *Plasmodium falciparum*, a cGMP increase triggers the exflagellation of male gametes (Kawamoto, Alejo-Blanco et al. 1990; Muhia, Swales et al. 2001). In the social amoeba *Dictyostelium discoideum* cGMP has a long history of association with the chemotactic response. Initial experiments showed that the two *Dictyostelium* chemoattractants cAMP and folic acid both induced a rapid increase in cGMP levels which peaked at 10 s after stimulation (Mato, Van Haastert et al. 1977; Wurster, Schubiger et al. 1977).

Involvement of cGMP in chemotaxis was further suggested by the identification of *stmF* mutants with an elevated and prolonged cGMP response, which was due to a defective cGMP-stimulated cGMP phosphodiesterase (Ross and Newell 1981; van Haastert, van Lookeren Campagne et al. 1982). These mutants formed extensive aggregation streams when grown as colonies on bacterial lawns. In response to autonomously produced cAMP waves in uniform cell lawns, *stmF* cells take a longer movement step at reduced speed, when compared to wild-type (Ross and Newell 1981). Upon cAMP stimulation, *stmF* cells displayed prolonged

association of myosin II with the triton-insoluble cytoskeleton, which was correlated with delayed myosin II heavy chain (MHC) phosphorylation (Liu and Newell 1988; Liu and Newell 1991). It was proposed that by inhibiting myosin II phosphorylation, cGMP prevented its dissociation from the cytoskeleton.

In the early 90s, other mutants with defective chemotaxis were isolated that showed no attractant-induced cGMP response (Kuwayama, Ishida et al. 1993). In one investigated mutant KI-10, attractant induced myosin II phosphorylation and its association with the cytoskeleton were also absent (Liu, Kuwayama et al. 1993). Later studies showed that the phosphorylation of myosin regulatory light chain (RLC) was also impaired in KI-10, thus strengthening the link between cGMP and cytoskeletal reorganization (Liu and Newell 1994).

The identification of the crucial genes that control cGMP synthesis, detection and degradation was one of the many important outcomes of the Dictyostelium genome and cDNA sequencing projects. There are two guanylyl cyclases GCA and sGC. Surprisingly, G*ca* null mutants show normal chemotaxis (Roelofs, Snippe et al. 2001), while *sgc* null mutants only show aggregation defects under very stringent conditions (Roelofs, Meima et al. 2001). However, a *sgc/gca* double null mutant does not polarize properly during chemotaxis, which is due to its inability to suppress the formation of lateral pseudopods. The *sgc/gca* mutant shows slightly reduced phosphorylation of myosin heavy and light chains and reduced cytoskeletal association of myosin (Goldberg, Wolpin et al. 2006). More recently, a study on null mutants that express a catalytic dead sGC and can therefore not produce cGMP has revealed two opposing roles for sGC in chemotaxis. The protein is required for pseudopodia formation at the leading edge, whereas it catalytic action of cGMP production is required for transient stimulation of myosin II formation, which subsequently suppresses lateral pseudopods (Veltman and Van Haastert 2006).

There is one cGMP target protein, cGbpC, which is a multidomain protein that apart from two cyclic nucleotide binding domains that can bind cGMP also contains RasGEF, Ras-GTPase and kinase domains (Bosgraaf, Russcher et al. 2002). The closely related cGbpD also contains two cNMP binding domains but has been shown not to bind cyclic nucleotides (Bosgraaf, Russcher et al. 2002).

The *gbpC* null mutant phenocopies the *sgc/gca* mutant, suggesting that both proteins interact closely. Indeed, studies have shown that phosphorylation of RLC during chemotaxis requires cGMP dependent activation of GdbpC but that the sGC protein may also interact directly with GdpC protein in a cGMP independent manner (Goldberg, Wolpin et al. 2006; van Egmond, Kortholt et al. 2008).

Two homologous genes *pdeD* (or *gbpA* or *DdPDE5*) and *pdeE* (or *gbpB* or *PDE6*) that encode unusual phosphodiesterases were identified (Bosgraaf, Russcher et al. 2002; Meima, Biondi et al. 2002; Meima, Weening et al. 2003). Both genes contained two cyclic nucleotide-binding domains and a metal binding domain that is common to a variety of hydrolases. The latter domain displays cGMP hydrolysing activity for PdeD and cAMP hydrolysing activity for PdeE, while the

cyclic nucleotide binding domains act as allosteric activators of the enzyme. PdeD appears to be the cGMP-stimulated cGMP phosphodiesterase that is lacking in the *stmF* mutant and the *pdeD* null mutant concordantly formed long aggregation streams while developing as colonies on bacterial lawns (Meima, Biondi et al. 2002). Double null mutants in both genes show slightly increased myosin light chain phosphorylation, but normal heavy chain phosphorylation, which does not quite equal the effects observed in the *stmF* mutant (Bosgraaf, Russcher et al. 2002).

In this work we explore an entirely novel aspect of the *pdeD* null mutant phenotype, its slow colonie growth on bacterial lawns and accelerated development. Our data indicate a new role for cGMP in the growth to development transition and the induction of gene expression during early development.

EXPERIMENTAL PROCEDURES

Cell culture and development

All cell lines were grown in HL5 medium which was supplemented with 200 µg/ml uracil for *uracil* null (*ura-*) auxotrophic strain DH1, with 5 µg/ml blasticidin for the *gca*/*sgc* null mutants and with 200 µg/ml G418 for AX2 cells transformed with the PdeD over-expression vector.
For developmental time courses, cells were harvested from growth medium, washed with PB (10 mM NaH2PO4/ K2HPO4, pH 6.5) buffer, plated at variable cell densities on PB agar (1.5% agar in PB) and incubated for indicated time at 22° C. For development under buffer, cells were plated at 5×10^4 cells/ml submerged in 1 ml PB in 3.5 cm^2 petridishes.

Quantitative cell growth

For growth on bacteria, cells were harvested and resuspended in PB buffer to 10^5 cells/ml. Subsequently 1 µl aliquots were spotted in quadruplicate, leaving enough space between the spots for growth, on SM agar plates with a Klebsiella aerogenes (KA) lawn. After incubation for 4 days at 22° C, measurements of the colony diameter were taken once a day for 4 days with a ruler.

For liquid growth culture, exponentially growing cells were harvested, diluted to 10^5 cells/ml in 30 ml of HL5 medium and incubated under continues shaking at 22° C. Cells were counted every morning and late afternoon until the cell density did not increase anymore.

Vector Construction and transformation

To obtain the new *pdeD* knock-out vector, a DNA fragment of *pdeD* comprising nucleotides 4-1050 was amplified by PCR using oligonucleotides that yielded a 5'-BamHI and 3'-EcoRI site. This fragment was used to replace the BamHI/EcoRI site of the earlier described PdeD knockout construct (Meima, Biondi et al. 2002).

To generate a *gca/sgc* double null mutant, first the *gca* gene was disrupted in the *uracil* auxotroph strain DH *gca*-gene and subsequently the *sgc*-gene was disrupted in several lines that carry the *gca* gene disruption. To disrupt the *gca* gene a knockout construct was created in which the pyr5/6 selection cassette replaces the catalytic domain. Two DNA fragments of *gca* comprising nucleotides 3168-4078 and 83-1240 were amplified by PCR using oligonucleotides that yielded a 5'-HindIII and a 3'-BamHI site on the first fragment and 5'-BamHI and 3'-XbaI site on the second fragment. These fragments were cloned in tandem into the HindIII/BamHI sites and XbaI/BamHI sites of URA vector PJB1 (gift of Peter N. Devreotes).

To disrupt the *scg* gene a previously created knockout construct of sGC was used that replaces both catalytic domains for the blasticidin cassette (Roelofs, Meima et al. 2001).

Transformation was done by electroporation according to Knecht (Knecht and Ming Pang 1995). Briefly, cells were harvested, washed twice in H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO4, 5 mM NaHCO3 and 1 mM NaH2PO4.H_2O) and resuspended as 5x 10^7 cells/ml.
For PdeD over-expression; 100 µl of cells were pulsed twice with 6 µg of the previously described PdeD-YFP fusion vector at 0.65 V (Meima, Biondi et al. 2002). Transformed AX2 cells were selected at 200 µg/ml G418.

For disruption of the *pdeD*, *gca* and *sgc* gene by homologous recombinations a lineair knock-out fragments that contain either *pdeD*, *gca* or *sgc* flanking gDNA and the selection marker were obtained with BamHI. Subsequently, 100 µl of cells were pulsed twice with 6 µg of the linearized knockout vector, at 0.65 V. Wildtype AX2 that carries disruption of the *pdeD* gene was selected with 5 µg/ml blasticidin. The *uracil* auxotroph DH1 cells transformed with the GCA knock-out fragment were selected by growth in the absence of uracil whereas selected *gca*-cellines transformed with the sGC knock-out fragment were selected at 5 µg/ml blasticidin. Individual clones were obtained by plating cells on KA lawns and were identified for homologous recombination by PCR and Southern blotting.

RNA isolation and analysis

Total RNA was isolated from developmental plates at 1-hour intervals during first 8-9 hours, size fractionated on 1.5 % agarose gels containing 2.2 M formaldehyde and transferred to Genescreen membranes (Nellen, Datta et al. 1987). Membranes were hybridized to [α-^{32}P]dATP-labelled *cAR1*, *csA* and *PdsA*, according to standard procedures. After washes under high stringency conditions blots were exposed to film. In order to probe the same blots with the 3 different genes, blots were stripped by boiling for 30 minutes in 0.1 % SDS.

Guanylyl cyclase assays

Cells were resuspended to 10^8 cells/ml in lysis buffer (250 mM sucrose, 1.5 mM EGTA in 10 mM TRIS, pH 8.0) and lysed through nuclepore filters (pore size 3 µm) in the presence and absence of 30 µM GTPγS. Lysates were subsequently incubated at 23°C with 0.5 mM GTP, 10 mM dithiothreitol and 0.2 mM IBMX and alternatively with 2 mM $MgCl_2$ or 2 mM $MnCl_2$. The reactions were terminated by addition of an equal volume of 3.5% (v/v) perchloric acid. Lysates were neutralized with $KHCO_3$, and the accumulated cGMP levels were determined by radioisotope dilution assay.

Optical density waves in aggregation streams

Optical density or darkfield waves are diagnostic for propagating extracellular cAMP waves. Darkfield waves were visualized as previously described by Dormann and Weijer (Dormann and Weijer 2006). In brief, exponentially growing cells were harvested, washed twice and distributed on thin layer of 1% water agar as 5.2 x10^5 cells/cm^2, i.e. 5 x10^6 cells per 3.5 cm^2 plate. Once cells had settled, pieces of agar containing a homogenous cell layer of both wildtype and mutant cellines were excised and transferred to a 9 cm^2 petridish. Subsequently, the petridish was placed on a previously described dark-field system setup, so that all pieces of agar containing the diverse cellines can be observed in one macroscopic field. Brightfield and darkfield images were recorded every 20 s on a PC controlled with Optimas software (MediaCybernetics, version 6.1) until waves had passed (Siegert and Weijer 1989).

Chemotaxis assays with micropipette

Chemotaxis was studied by using the microcapillary assay. Cells were developed to the ripple stage (onset of aggregation) on NN agar as 7.9 x10^5 cells/cm^2 or in shaken suspensions as 10^7 cells/ml in KK2 buffer (20 mM KH_2PO_4/K_2HPO_4, pH 6.8), at 22° C. Shaken cultures were pulsed with 30 nM cAMP

every 6 minutes. After 5 hours, cells were harvested, resuspended to 10^7 cells/ml and incubated for a further 45 minutes on a rotator, in the presence or absence of 20 µM 5-chloromethyl-fluorescein diacetate (CMFDA, Cell Tracker Green, Molecular Probes, Inc.) per 10^7 cells, at 22 ºC. Subsequently, labeled cells and unlabeled cells were washed twice and mixed 1:1. Finally, mixed cells at a density of approximately 10^5 cells/cm2 were placed on a glass coverslip in a Attofluor chamber (Molecular Probes) submerged with 1 ml KK2 buffer and subjected to a micropipette diffusing cAMP. Micropipette was made from a glass capillary using a vertical electrode puller, which was subsequently filled with a microliter syringe with 10-4 M cAMP. The tip position of the micropipet was controlled by a micromanipulator attached to the microscope stage. Cells were imaged every 5 s for 20 min using a Leica TCS SP2 system with a 100100x/1.4NA immersion objective.

RESULTS

Developmental phenotype of *PdeD* loss-of-function mutant

A novel *pdeD* null mutant was generated in which blasticidin selection cassette replaced the catalytic domain of the gene instead of the first cyclic nucleotide-binding domain. Similar to the earlier *pdeD* null mutant, this mutant also displayed a *stmF*-type phenotype (Ross and Newell 1981; Meima, Biondi et al. 2002). When grown as colonies on rich bacterial lawns, they showed extensive formation of aggregation streams almost up to the feeding front (Fig 1A).

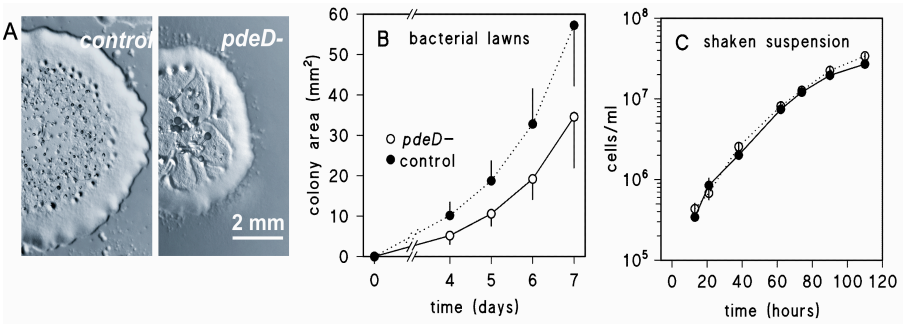


Figure 1: The streamer phenotype is associated with slow growth on bacterial lawns.
Aliquots of PdeD KO, RI and AX2 were spotted on SM agar plates with a *KA* lawn and incubated at 22° C. (A) Morphology of PdeD KO, RI and AX2 colonies grown on *KA* lawns. Note the streaming phenotype and significantly larger fruiting bodies of the KO clones. (B) After incubation for 4 days, the colony diameter was measured once a day for 5 days with a ruler. Colony diameter was plotted against time. (C) For liquid growth culture, exponentially growing cells were harvested, diluted to 10^5 cells/ml in 30 ml of HL5 medium and incubated under continues shaking at 22° C. Cells were counted every morning and late afternoon until the cell density did not increase anymore. The cell density was plotted against incubation time.

Wild-type cells do not normally form streams under such high cell density. Closer observation of the *pdeD* null colonies also revealed that border of quiescent cells inside the feeding front is narrower than that of the control strains and that colony size is reduced. The latter parameter was determined quantitatively by measuring the surface area of the clones over several days of growth. This indicated that the *pdeD* null clones increased in size about two times slower than the control cells (Fig 1B).
To test whether this was due to an intrinsic propagation defect of the *pdeD* cells, we also measured cell propagation in a homogeneous shaken suspension. However, under these circumstances no differences between null mutants and control lines were encountered (Fig 1C). Rather, the streaming phenotype, narrow feeding front and reduced clone growth suggested that feeding cells were precociously recruited into aggregates.

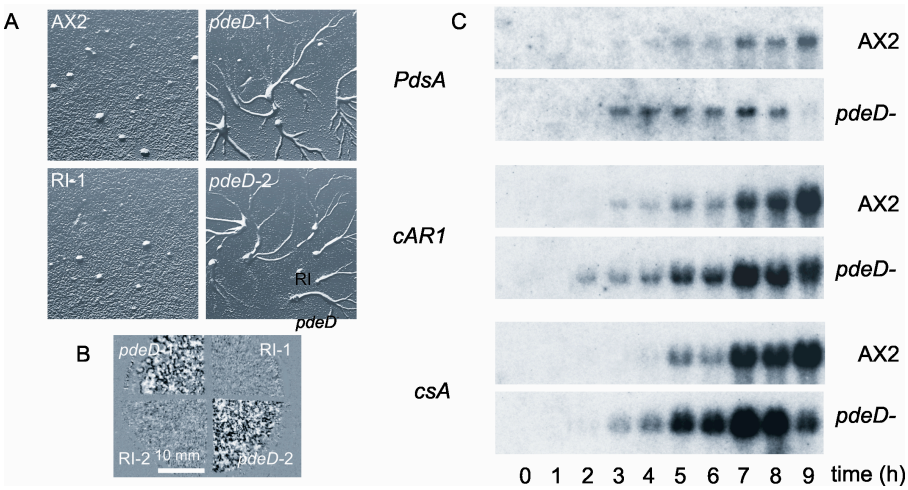


Figure 2: PdeD null mutants show accelerated early development and gene expression
(A) Phenotypes of two *pdeD* KO and two RI lines. Cells were plated on petridishes under phosphate buffer at densities of 5×10^4 cells/ml. (B) Time lapse movie of aggregation. A series of dark-field images of aggregating amoebae were taken every 20s apart. (C) Expression of cAR1, PdsA and csA during early KO and RI development. Cells were harvested, washed and incubated on NN agar at 2 $\times10^6$ cells/cm^2 for 9 hours. RNA was isolated every hour, and Northerns were probed with [^{32}P]dATP-labelled *carA*, *csA* or *PdsA*.

Developmental timing in *PdeD* loss-of-function mutant

To assess whether *pdeD* null cells showed accelerated early development, we followed the aggregation process of several *PdeD* knock-out (KO) clones with the parent AX2 and clones carrying a randomly integrated (RI) knock-out vector. Figure

2A shows that the *pdeD* knockout (KO) mutants started aggregation several hours earlier than the parent or random integrant (RI) cell lines. Time-lapse video microscopy of developing KO and RI cells showed that the KO clones initiated the dark field spiral waves, which are diagnostic for oscillatory cAMP signalling after about 2 h of starvation, whereas the RI clones only started at 5 h (Fig 2B). This suggests that the KO cells aggregated faster because cAMP signalling initiated earlier.

The most likely explanation for the early onset of cAMP signalling is precocious expression of genes involved in the aggregation process. We therefore followed the expression of the aggregative marker genes encoding the extracellular phosphodiesterase *pdsA*, the surface cAMP receptor (*car1*) and adhesive contact sites A (*csA*) during the first 8 hours of development. For all three markers precocious expression was found. Figure 2C shows that PdsA expression started about 4 h earlier in the pdeD KO cells, whereas expression of cAR1 and csA occurred about 2 h earlier.

Developmental timing and colony morphology of a PdeD gain-of-function mutant

If loss- of-function of PdeD accelerates development, then gain-of-function should delay it. To test this we fused the PdeD coding sequence to the constitutive actin 15 promoter and expressed the construct in AX2 cells. Figure 3A shows the progression of development in AX2 and A15:*pdeD* cells. Aggregation of the A15:*pdeD* cells is delayed by about 9 hours but thereafter the cells aggregate normally and form fruiting bodies. Time-lapse video microscopy showed that the A15:*pdeD* cells initiated the dark-field waves that are diagnostic for oscillatory signalling much later than the AX2 cells. These waves tended to propagate as target patterns rather than the spiral waves of the AX2 cells (Fig 3B). The expression of aggregative genes is concordantly also 9 hours delayed (Fig 3C).
The growth rate of A15:*pdeD* colonies on bacterial lawns was also compared to that of the parent strain. A15:*pdeD* colonies showed about 4 fold increase in growth rate compared to AX2 cells (Fig 4B). However, they only started aggregating after obtaining a very large diameter (Fig 4A).

Developmental timing and gene regulation in mutants defective in cGMP synthesis

PdeD is a multidomain protein that apart from the central catalytic region, which hydrolyses cGMP also contain two cGMP binding domains and a large uncharacterized N-terminal domain. Logically, the effect of overexpression of PdeD should be due to depletion of cGMP levels.

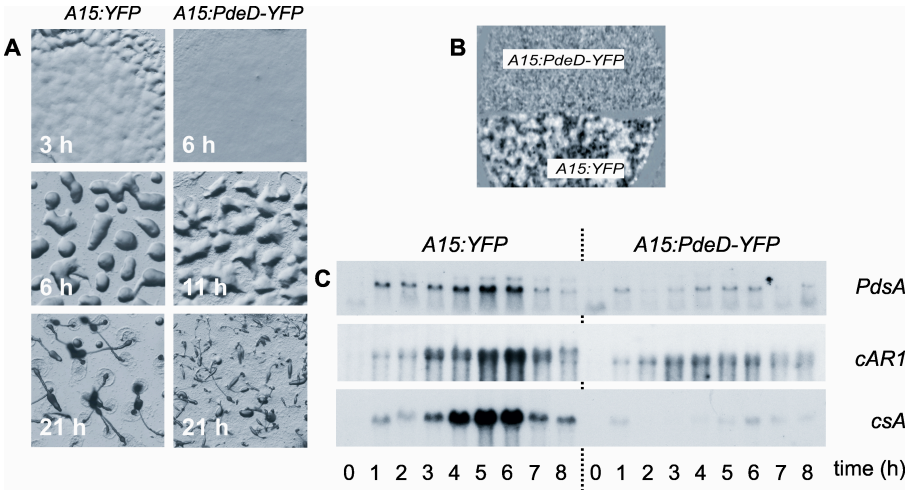


Figure 3: PdeD overexpressors show delayed development and gene expression.
A) Developmental phenotype of A15:*pdeD* overexpressor and AX2 parent. Cells were harvested, washed with PB buffer, plated at 2 x10^6 cells/cm^2 on PB agar and incubated for indicated time at 22° C. Note the strong delay in development and smaller fruiting body of the overexpressor celline. (B) Time lapse movie of aggregation. A series of dark-field images of aggregating amoebae were taken every 20s apart. (C) Expression of cAR1 and csA during early development in overexpressor and control. Cells were harvested, washed and incubated on NN agar at 2x10^6 cells/cm^2 for 8 hours. RNA was isolated every hour, and Northern were probed with radio labelled carA, csA or PdsA.

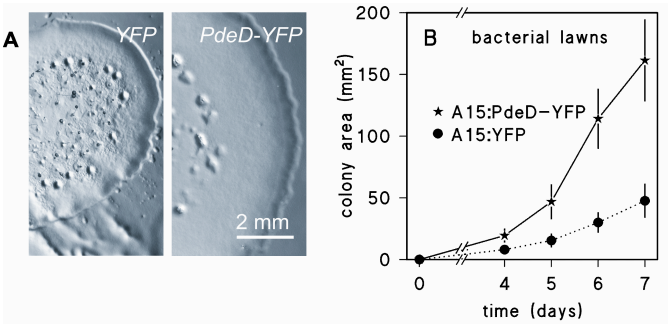


Figure 4: PdeD overexpressors show increased colony growth.
Aliquots of PdeD OE and AX2 were spotted on SM agar plates with a *KA* lawn and incubated at 22° C. A) Morphology of PdeD OE and AX2 colonies grown on klebsiella areogenes lawns. Note the significantly larger growth front and diameter of the PdeD OE. B) After incubation for 3 days, the colony diameter was measured once a day for 4 days with a ruler. Colony diameter was plotted against time.

To test this explicitly, we generated double null mutants in the two *Dictyostelium* guanylyl cyclases sGC and GCA. First the *gca* gene was disrupted by introducing a *gca* knockout construct into the *ura-* strain DH1, which replaces a region of about 640 bp by the URA selection cassette. Several cell lines carrying either *gca* gene disruptions (KO) or random integrations (RI) of the cassette were identified by PCR and Southern blotting. One of each of these lines was subsequently transformed with a *sgc* deletion construct in which 376 bp that includes both catalytic domains was replaced by the blasticidin selection cassette. Again, several *sgc* KO and RI lines were identified.

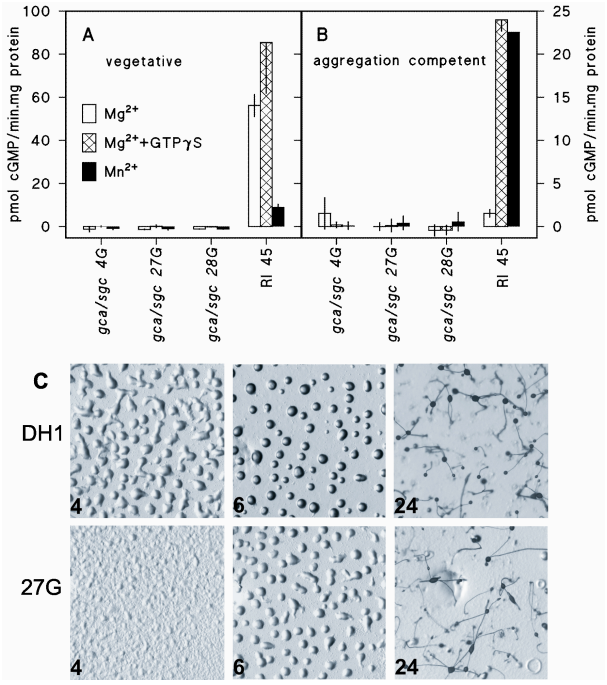


Figure 5: GC activity and development in *gca/sgc* mutants
GC activity in vegetative (A) and aggregative cells (B). Cells were either harvested directly from growth medium (vegetative) or starved for about 6-8 hours on non-nutrient agar until aggregation territories started to form (aggregation competent). After cell lysis in the presence and absence of 30 µM GTPγS, lysates were assayed for guanylyl cyclase activity with either 2 mM Mg^{2+} or 2 mM Mn^{2+}. C) Developmental phenotype of *gca/sgc* null mutant 27G and DH1 parent. Cells were harvested, washed with PB buffer, plated at 2 x10^6 cells/cm^2 on PB agar and incubated for indicated time at 22° C. Note the small delay of approximately 2 hours in development of the double guanylyl cyclase mutant.

As a further test for gene disruption, three *gca/sgc* double knockouts and one *gca/sgc* double RI line were assayed for GC activity. GCA and sGC are most active during growth and aggregation respectively. They can be distinguished by the fact

that sGC can use Mn^{2+}-GTP as substrate whereas GCA cannot. With Mg^{2+}-GTP as substrate both sGC and GCA require GTPγS for optimal activity. Figure 5A shows that in the vegetative stage, the double RI lines displayed high Mg^{2+} dependent GC activity that was further stimulated by GTPγS but only low Mn^{2+} dependent activity. This GCA-like activity was lacking in all *gca/sgc* double knockout lines. Aggregation competent RI cells also showed high Mn^{2+} dependent activity in addition to GTPγS dependent activity, reflecting the expression of sGC at this stage. (Fig 5B) Both activities were missing from the *gca/sgc* double knockout, which confirms that both GCA and sGC are no longer present.

We next investigated the developmental timing of the *gca/sgc* knockout cells. Figure 5C shows that *gca/sgc* null mutant showed a small delay in aggregation compared to the parent strain DH1, but otherwise formed normal aggregates and fruiting structures.

Is cGMP an intermediate for pulse-induced gene expression?

The expression of aggregative genes occurs in two phases - a basal level of expression is induced shortly after starvation, which makes it possible for cells to initiate oscillatory cAMP signalling. Once initiated the cAMP pulses themselves strongly accelerate the expression of aggregative genes. Because cAMP pulses give rise to transient increases in intracellular cGMP levels, cGMP could be a signalling intermediate for pulse-induced gene expression. To test this explicitly we compared pulse-induced gene expression in *gca/sgc* cells, PdeD overexpressors and their parent strains. Figure 6 shows that although the *gca/sgc* cells and PdeD gain-of-function mutants were a little slower to initiate pulse-induced genes expression, they nevertheless showed as vigorous a response to this stimulus as wild-type cells. This demonstrates that cGMP is not required for pulse-induced gene expression and most likely acts to promote the first phase of aggregative gene induction.

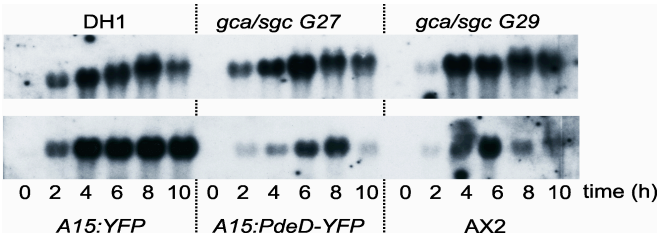


Figure 6: Sgc/gca mutants and A15PdeD cells are not defective in pulse-induced gene expression. Expression of csA in pulsed gca/sgc KO clones, PdeD OE and control/parent cells. Cells were developed for 10 hours in shaken suspension in the presence of exogenous 30 nM cAMP pulses every 6 minutes. RNA was isolated every 2 hours, and Northern were probed with radio labelled carA.

Is delayed development due to a chemotaxis defect?

Delayed development of *gca/sgc* cells was also observed by Van Haastert and coworkers whom attributed this to a defect in chemotaxis to cAMP. However, our data indicate that defective gene expression precedes the chemotaxis defect. Because pulsing cells can overcome defective gene expression, we tested whether the chemotaxis defect still persisted after cells had been pulsed to induce optimal expression of aggregative genes.

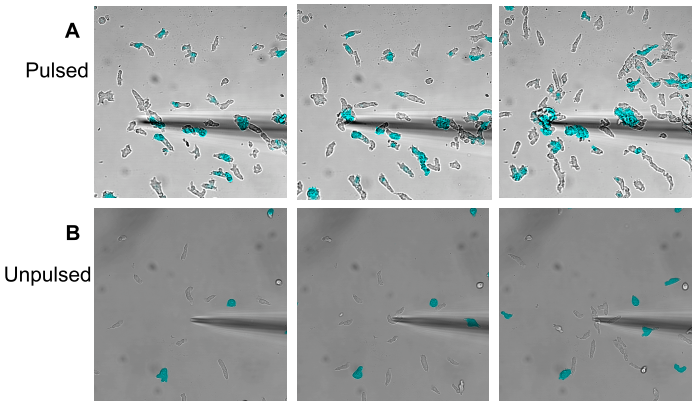


Figure 7: Chemotaxis in pulsed and unpulsed sgc/gca cells.
Time-lapse movie of mixture of labeled DH1 wild type and *gca/sgc* double null mutant cellines towards cAMP pipette. A) Cells were developed in shaken suspension in the presence of exogenous 30 nM cAMP pulses and tested for chemotaxis towards a microcapillary diffusing cAMP, as described in experimental procedures. B) Cells were developed on NN agar plates up to the ripple stage and tested for chemotaxis towards a microcapillary diffusing cAMP, as described in experimental procedures.

The time-lapse movie shown in Figure 7A shows that after pulsing both the *gca/sgc* null mutant and parent strain move towards the micropipette with equal efficiency. Cells of both strains are highly polarized. When cells are developed to the ripple stage as was done in the experiments described by Van Haastert, a distinct difference in cell orientation was observed. The *gca/sgc* cells orientated less efficiently, move slower towards the pipette and show more lateral pseudopods (Fig 7B)

DISCUSSION

PdeD null mutants show accelerated early development and increased cAMP signalling efficiency

PdeD, PdeE and PdeE/PdeD null mutants showed no gross morphological defects, but important alterations were found in the PdeD null mutants. These mutants initiated aggregation 2-3 h earlier than control cell lines and this was

accompanied by the precocious appearance of the optical density waves that result from oscillatory cAMP signalling. In addition, they showed precocious expression of cAMP receptors and extracellular PDE, two components of the cAMP signalling system. The actual duration of the aggregation process, once initiated, and the time course of slug and fruiting body formation were not altered in the PdeD null mutants (Meima, Biondi et al. 2002).

At an intermediate cell density both PdeD null mutants and controls formed streaming aggregates with well polarized cells, indicating that chemotactic cell movement is not impaired in the mutant. At 10-fold lower cell density, where wild-type and the control cell lines cannot aggregate any more, the PdeD null mutant still formed streaming aggregates. Stream formation is diagnostic for chemotaxis in a field where the cAMP signal is relayed (Dallon and Othmer 1997). At very high cell density where the confluent cell layer just compacts to form mounds, the PdeD null mutant formed 2-fold larger aggregates than the controls, which subsequently gave rise to larger fruiting bodies. Under high-density conditions, territory size depends less on cell movement than on oscillator dominance, i.e. the capacity for an autonomous oscillator to prevent competing oscillators to establish themselves. The relationship between accelerated development and higher efficiency of cAMP signalling is not clear. It is possible that asynchrony in expression of different components of the cAMP signalling system affects the intrinsic properties of the oscillatory system.

PdeD and chemotaxis

In Dictyostelium cGMP is generally associated with the chemotactic response. Chemoattractants, such as cAMP or folic acid cause a rapid increase in cGMP levels and mutants that are defective in cGMP metabolism show altered chemotaxis (Mato, Van Haastert et al. 1977; Wurster, Schubiger et al. 1977; Ross and Newell 1981). Streamer F (StmF) mutants, recognized by their propensity to form long aggregation streams when grown clonally on bacterial lawns, are defective in cGMP phosphodiesterase (van Haastert, van Lookeren Campagne et al. 1982). They show a prolonged cGMP response and a prolonged period, but reduced rate, of cell movement upon stimulation with chemotattractant (Ross and Newell 1981; Coukell and Cameron 1986; Segall 1992).

The PdeD null mutants also show extensive streaming when plated clonally with bacteria (Meima, Biondi et al. 2002). If, in analogy to PKA-R, PdeD would inhibit a protein kinase activity in the absence of cGMP, then PdeD null mutants could phenocopy stmF mutants. However, the altered cell movement properties of stmF cause very distinct banding patterns to form in aggregation streams and a reversal of the normally narrow band of moving cells and wide band of non-moving cells in optical density wave patterns (Newell and Liu 1992). Neither of these was observed in PdeD null mutants, while the StmF mutants do not show accelerated development

(Ross and Newell 1981). The streamer phenotype can probably be caused independently by altered cell movement or by precocious aggregation. The present study shows that cGMP is not solely involved in chemotaxis, but plays a crucial role in developmental gene regulation as well.

The downside of being fast

Accelerated development in mutants that lack the R-subunit of PKA or overexpress the catalytic subunit of PKA is accompanied by severe morphological anomalies because spore differentiation and fruiting body formation are no longer synchronized (Abe and Yanagisawa 1983; Anjard, Pinaud et al. 1992; Simon, Pelegrini et al. 1992). In PdeD null mutants no adverse effects of accelerated development are observed after vegetative growth. To the contrary, the mutation may even be beneficial by enabling aggregation at low cell density and in causing larger fruiting bodies to form at high cell density (Meima, Biondi et al. 2002). However, the early onset of aggregation results in a trade-off for growth of amoebal colonies on bacterial lawns. Whereas wild-type cells remain quiescent for a certain period after the density of the bacterial food source decreases, the PdeD null cells aggregate much earlier and the aggregates coerce cells away from the growth front. As a consequence, the growth rate of mutant colonies is reduced, despite the absence of a growth defect of the individual cells.

It is evident that in a mixed population of amoebal predators, slow colony growth rate would be a distinct disadvantage and such a mutation would be unlikely to invade a population of wild-type cells. We therefore propose that PdeD normally acts to prevent untimely aggregation and that its activity is tuned by an as yet unknown mechanism to offset the advantages of speedy aggregation with the disadvantages of reduced colony growth.

ACKNOWLEDGEMENT

We would like to thank Dr. Dirk Dormann for his help with the chemotaxis assays. This research was funded by Dutch Science Foundation (NWO) Project Grant 805.17.048 to K.E.W and by Wellcome Trust University Award Grant 057137.

REFERENCES

Abe, K. and K. Yanagisawa (1983). "A new class of rapid developing mutants in Dictyostelium discoideum: Implications for cyclic AMP metabolism and cell differentiation." Dev. Biol. **95**: 200-210.

Anjard, C., S. Pinaud, et al. (1992). "Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of Dictyostelium discoideum." Development **115**: 785-790.

Bosgraaf, L., H. Russcher, et al. (2002). "A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in Dictyostelium." Embo J **21**(17): 4560-70.

Bosgraaf, L., H. Russcher, et al. (2002). "Identification and characterization of two unusual cGMP-stimulated phoshodiesterases in dictyostelium." Mol Biol Cell **13**(11): 3878-89.

Coukell, M. B. and A. M. Cameron (1986). "Characterization of revertants of stmF mutants of Dictyostelium discoideum: Evidence that stmF is the structural gene of the cGMP-specific phosphodiesterase." Dev. Gen. **6**: 163-177.

Dallon, J. C. and H. G. Othmer (1997). "A discrete cell model with adaptive signalling for aggregation of Dictyostelium discoideum." Phil. Trans. R. Soc. Lond. B. **352**: 391-417.

Dormann, D. and C. J. Weijer (2006). "Visualizing signaling and cell movement during the multicellular stages of dictyostelium development." Methods Mol Biol **346**: 297-309.

Friebe, A. and D. Koesling (2003). "Regulation of nitric oxide-sensitive guanylyl cyclase." Circ Res **93**(2): 96-105.

Goldberg, J. M., E. S. Wolpin, et al. (2006). "Myosin light chain kinase A is activated by cGMP-dependent and cGMP-independent pathways." FEBS Lett **580**(8): 2059-64.

Kawamoto, F., R. Alejo-Blanco, et al. (1990). "Possible roles of Ca2+ and cGMP as mediators of the exflagellation of Plasmodium berghei and Plasmodium falciparum." Mol Biochem Parasitol **42**(1): 101-8.

Knecht, D. and K. Ming Pang (1995). Electroporation of Dictyostelium discoideum. Methods in Molecular Biology. J. A. Nickoloff. Totowa, NJ, Humana Press Inc.**:** 321-329.

Kuwayama, H., S. Ishida, et al. (1993). "Non-chemotactic Dictyostelium discoideum mutants with altered cGMP signal transduction." J. Cell Biol. **123**: 1453-1462.

Liu, G., H. Kuwayama, et al. (1993). "The role of cyclic GMP in regulating myosin during chemotaxis of Dictyostelium: evidence from a mutant lacking the normal cyclic GMP response to cyclic AMP." J.Cell Sci. **106**: 591-596.

Liu, G. and P. C. Newell (1988). "Evidence that cyclic GMP regulates myosin interaction with the cytoskeleton during chemotaxis of Dictyostelium." J.Cell Sci. **90**: 123-129.

Liu, G. and P. C. Newell (1991). "Evidence that cyclic GMP may regulate the association of myosin II heavy chain with the cytoskeleton by inhibiting its phosphorylation." J.Cell Sci. **98**: 483-490.

Liu, G. and P. C. Newell (1994). "Regulation of myosin regulatory light chain phosphorylation via cyclic GMP during chemotaxis of Dictyostelium." J.Cell Sci. **107**: 1737-1743.

Mato, J. M., P. J. Van Haastert, et al. (1977). "Cyclic AMP and folic acid mediated cyclic GMP accumulation in Dictyostelium discoideum." FEBS Lett **79**(2): 331-6.

Meima, M. E., R. M. Biondi, et al. (2002). "Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants." Mol Biol Cell **13**(11): 3870-7.

Meima, M. E., K. E. Weening, et al. (2003). "Characterization of a cAMP-stimulated cAMP phosphodiesterase in Dictyostelium discoideum." J Biol Chem **278**(16): 14356-62.

Muhia, D. K., C. A. Swales, et al. (2001). "The gametocyte-activating factor xanthurenic acid stimulates an increase in membrane-associated guanylyl cyclase activity in the human malaria parasite Plasmodium falciparum." Mol Microbiol **42**(2): 553-60.

Nellen, W., S. Datta, et al. (1987). Molecular Biology in Dictyostelium: Tools and applications. Methods in Cell Biology. J. A. Spudich. Orlando, FL, Academic Press. **28:** 67-100.

Newell, P. C. and G. Liu (1992). "Streamer F mutants and chemotaxis of Dictyostelium." Bioessays **14**: 473-478.

Olshevskaya, E. V., A. N. Ermilov, et al. (2002). "Factors that affect regulation of cGMP synthesis in vertebrate photoreceptors and their genetic link to human retinal degeneration." Mol Cell Biochem **230**(1-2): 139-47.

Roelofs, J., M. Meima, et al. (2001). "The Dictyostelium homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase." Embo J **20**(16): 4341-8.

Roelofs, J., H. Snippe, et al. (2001). "Guanylate cyclase in Dictyostelium discoideum with the topology of mammalian adenylate cyclase." Biochem J **354**(Pt 3): 697-706.

Ross, F. M. and P. C. Newell (1981). "Streamers: Chemotactic mutants of *Dictyostelium discoideum* with altered cyclic GMP metabolism." J. Gen. Microbiol. **127**: 339-350.

Segall, J. E. (1992). "Behavioral responses of streamer F mutants of Dictyostelium discoideum: effects of cyclic GMP on cell motility." J. Cell Sci. **101**: 589-597.

Siegert, F. and C. Weijer (1989). "Digital image processing of optical density wave propagation in Dictyostelium discoideum and analysis of the effects of caffeine and ammonia." J.Cell Sci. **93**: 325-335.

Simon, M.-N., O. Pelegrini, et al. (1992). "Mutation of protein kinase A causes heterochronic development of Dictyostelium." Nature **356**: 171-172.

van Egmond, W. N., A. Kortholt, et al. (2008). "Intramolecular activation mechanism of the Dictyostelium LRRK2 homolog Roco protein GbpC." J Biol Chem **283**(44): 30412-20.

van Haastert, P. J., M. M. van Lookeren Campagne, et al. (1982). "Altered cGMP-phosphodiesterase activity in chemotactic mutants of Dictyostelium discoideum." FEBS Lett **147**(2): 149-52.

Veltman, D. M. and P. J. Van Haastert (2006). "Guanylyl cyclase protein and cGMP product independently control front and back of chemotaxing Dictyostelium cells." Mol Biol Cell **17**(9): 3921-9.

Wurster, B., K. Schubiger, et al. (1977). "Cyclic GMP in Dictyostelium discoideum. Oscillations and pulses in response to folic acid and cyclic AMP signals." FEBS Lett **76**: 141-144.

Chapter 6

Conclusions

CONCLUSIONS

cAMP plays a ubiquitous role in controlling almost all aspects of *D. discoideum* development. As a secreted signal it regulates expression of different classes of genes, and coordinates cell movement during aggregation and morphogenesis. As an intracellular messenger it controls entry into development, spore and stalk cell maturation and spore germination (Saran, Meima et al. 2002). Its sister molecule cGMP is considered to play a more modest role as a signalling intermediate in the chemotactic response. The effects of both molecules on cellular responses do not follow straightforward dose – effect relationships. In most cases, the information is carried by the dynamic transient nature of the signal that, in the case of cAMP, evokes complex spatial patterns of signal propagation. Both the cyclases that synthesize cAMP and cGMP, and the phosphodiesterases that degrade them, contribute to dynamic signalling. In this thesis I have mainly focussed on the role of the phosphodiesterases in dynamic signalling, but I also identified novel transient activation of an adenylyl cyclase, that was previously thought to synthesize cAMP at a steady rate.

Expression pattern and regulation of the extracellular phosphodiesterase PdsA

PdsA degrades extracellular cAMP and plays an essential role in both aggregation and postaggregative morphogenesis. Its activity generates steep chemotactic gradients and by degrading cAMP between pulses, it causes resensitization of cells to the cAMP pulse (Darmon, Barra et al. 1978; Faure, Podgorski et al. 1988; Hall, Franke et al. 1993; Wu, Franke et al. 1995; Sucgang, Weijer et al. 1997). PdsA has three different promoters: the most distal vegetative promoter (PdV) is active during growth, while the middle aggregative (PdA) and the proximal late promoter (PdL) are active during and after aggregation, respectively (Faure, Franke et al. 1990). Earlier expression studies, using a stabile β-galactosidase reporter showed that the PdA promoter remained active in the entire slug with highest levels in the anterior tip region (Hall, Franke et al. 1993). This contradicted earlier findings that transcripts resulting from PdA promoter activity almost disappeared after aggregation (Faure, Franke et al. 1990).

To understand this discrepancy and to gain more information about the regulation of PdsA expression during multicellular development, I studied the expression patterns and regulation of the PdA and PdL promoter during multicellular development using a labile β-galactosidase reporter (Detterbeck, Morandini et al. 1994). When measured with the labile reporter, PdA showed highest activity in the mound stage after which it strongly declined, leaving only expression in the basal disc of the fruiting body. PdL was highly expressed in the anterior prestalk region of slugs and in the anterior-like cell population that is intermixed with the posterior prespore cells. This means that the two promoters direct expression in two cell populations that

differentiate into vacuolated stalk cells, but not in prespore cells. This is in agreement with the fact that cAMP induces prespore differentiation and inhibits stalk cell differentiation and suggests that the localization of PdsA plays a role in cell fate determination (Wang, Van Driel et al. 1988; Soede, Hopper et al. 1996).

Next we investigated which developmental signals regulate PdA and PdL expression after aggregation. Earlier studies have shown that PdA activity is induced in early development by the prestarvation factor (PSF), and is further up-regulated by cAMP. PdL activity was found to be upregulated by the differentiation inducing factor (DIF) after a pretreatment with cAMP (Franke, Podgorski et al. 1987; Franke, Faure et al. 1991; Clarke and Gomer 1995).

We found that after aggregation PdA activity decreased strongly, which was counteracted weakly by cAMP pulses and strongly by a combination of cAMP and DIF. PdL activity was low in cells derived from aggregates and was strongly upregulated by cAMP and DIF. We next investigated whether PdL is regulated by DIF and cAMP during normal development. First, we expressed the PdL labile reporter construct into a mutant that cannot synthesize DIF (Thompson and Kay 2000) and found a normal pattern of PdL activity. Second, regulatory sequences were identified in PdL that were essential for its regulation by cAMP and DIF in cell suspension. However, these sequences were not necessary for normal PdL activity in the prestalk region of slugs, indicating that the cAMP and DIF do not control PdL activity in normal development. More recently it was shown that DIF is only necessary for basal disc cell differentiation, where PdA is expressed, and that *Dictyostelium* cells synthesize other compounds that can induce stalk cell differentiation (Saito, Taylor et al. 2006).

Evolutionary conservation of the extracellular phosphodiesterase PdsA

Phylogenetic analysis has revealed that the *Dictyostelia* can be subdivided into 4 major groups with the model *D.discoideum* residing in the most derived group 4 (Schaap, Winckler et al. 2006). Fascinatingly, while *D.discoideum and other group 4 taxa* use extracellular cAMP pulses as chemoattractant for aggregation, species in groups 1-3 use other acrasins than cAMP to aggregate (Shimomura, Suthers et al. 1982; De Wit and Konijn 1983). However, earlier studies did present evidence for the use of extracellular cAMP in late development of some dictyostelid species that do not use extracellular cAMP for aggregation (Schaap, Van Lookeren Campagne et al. 1986; Fukushima and Maeda 1991; Dormann, Weijer et al. 1997). This suggested that extracellular cAMP signalling has a conserved universal role to coordinate fruiting body formation and a derived role in aggregation. In agreement with this hypothesis, recent studies showed that in early diverging species, cAMP receptors (cAR) are only expressed after aggregation and deletion or inhibition of cARs blocks late development but not aggregation (Alvarez-Curto, Rozen et al. 2005; Kawabe, Morio et al. 2009).

To understand how this novel extracellular role of cAMP evolved, we retraced the origins and changes of the extracellular phosphodiesterase PdsA in the *Dictyostelia.* Together with the cAMP receptors (cARs) and the adenylyl cyclase A (ACA), PdsA is essential for oscillatory cAMP signalling during aggregation (Saran, Meima et al. 2002). I show in chapter 3 that PdsA orthologs are present in all investigated group-representative taxa. The *PdsA* gene from the group 3 species *D. minutum* restored the ability to hydrolyze cAMP of a *D.discoideum PdsA* null mutant and also rescued loss of aggregation and fruiting body formation of the mutant. This indicated that the *D.minutum PdsA* is a fully functional ortholog of *DdisPdsA*.

The *D.minutum and P.pallidum PdsAs* were only expressed after aggregation, which agreed with the fact that in group 3 and group 2 taxa, extracellular cAMP regulates post-aggregative development and not aggregation. However, the group 1 taxa *D.fasciculatum* and *D.aureo-stipes,* which also do not use cAMP during aggregation, express *PdsA* strongly during growth and at lower levels during further development.

This suggests that in addition to post-aggregative morphogenesis, these taxa possibly use cAMP as a foodseeking chemoattractant, since the bacterial food source also secretes cAMP. Generation of PdsA null mutants in basal taxa will be required to test these hypotheses.

ACG can be activated by cAMP receptors

Post-aggregative morphogenesis in all Dictyostelia is most likely coordinated by cAMP oscillations that initiate in the tip region and propagate as waves through the structure (Bretschneider, Vasiev et al. 1999). The adenylate cyclase ACA is responsible for oscillatory cAMP signalling in *D. discoideum* aggregation and is expressed in the tip region of slugs (Pitt, Milona et al. 1992; Verkerke-van Wijk, Fukuzawa et al. 2001). Although this is in agreement with the tip's role as organizer of morphogenesis, it leaves unexplained how cAMP waves propagate in the slug posterior. It was recently shown that adenylate cyclase G is markedly upregulated in the posterior region of slugs, where it triggers prespore differentiaton (Alvarez-Curto, Saran et al. 2007). This enzyme was known to continuously synthesize cAMP at a low rate, which is upregulated by high osmolality.

We found by serendipity that ACG activity can be transiently upregulated by activation of cAMP receptors. In agreement with an earlier study we show that ACG produces cAMP at a constant rate in growing cells (van Es, Virdy et al. 1996). However, after 4 hours of starvation, ACG mediated cAMP production displayed transient kinetics and this was not stimulated by high osmolarity. This transient accumulation of cAMP resembled ACA mediated cAMP production in response to cAMP binding to cARs and suggested that activation of ACG also becomes mediated by cARs. Indeed we showed that disruption of both cAR1 and cAR3 genes and acute inhibition of cAMP binding to cAR1 with its antagonist 2'3'isopropylidene adenosine

(IPA) prevented cAMP-induced ACG activation. However, unlike ACA, ACG cannot be activated by GTPγS, suggesting that heterotrimeric G-proteins (G-proteins) do not mediate cAMP induced ACG activation. Some cAR1 mediated processes also do not require G-proteins, such as activation of the MAP kinase ERK2 (Maeda, Aubry et al. 1996; Brzostowski and Kimmel 2001). ERK2 has been proposed to play a role in transient cAMP accumulation. In this model, cAR mediated activation of ERK2 leads to inhibition of the intracellular cAMP phosphodiesterase RegA, which allows intracellular cAMP to accumulate and to be secreted, but also to activate PKA. PKA then inactivates ERK2, causing RegA activation and cAMP degradation (Laub and Loomis 1998). It is not yet known whether RegA, ERK2 or PKA are required for cAR-induced ACG mediated cAMP accumulation. However, since ACG is expressed throughout the slug posterior, our observation that ACG can be transiently activated by cAMP may resolve the conundrum how cAMP waves are propagated in slug posteriors. Imaging of cell movement in *acg-* slugs could resolve this issue.

Role for cGMP in gene expression

Extracellular cAMP acting on cARs not only leads to cAMP production by the adenyl cyclases but also to activation of guanylyl cyclases (GCs) and subsequent accumulation cGMP. cGMP is produced by two GCs, GCA and sGC and is detected by the cGMP binding protein, GbpC (Roelofs, Meima et al. 2001; Roelofs, Snippe et al. 2001; Bosgraaf, Russcher et al. 2002). Studies have shown that mutants with defects in cGMP synthesis or cGMP detection have altered chemotactic responsiveness to cAMP and myosin II phosphorylation, which has implicated cGMP as intermediate for the chemotactic response. In a search for cAMP binding proteins, two homologous genes were identified, PdeD (or GbpA or DdPDE5) and PdeE (or GbpB or DdPDE6), which encode unusual phosphodiesterases (Bosgraaf, Russcher et al. 2002; Meima, Biondi et al. 2002; Meima, Weening et al. 2003). Both genes contained two cyclic nucleotide-binding domains and a metal binding domain that is common to a variety of hydrolases. The latter domain displays cGMP hydrolysing activity for PdeD and cAMP hydrolysing activity for PdeE, while the cyclic nucleotide binding domains act as allosteric activators of the enzyme (Meima, Biondi et al. 2002)(Meima, Weening et al. 2003). PdeD appeared to be the cGMP-stimulated cGMP PDE that is lacking in the *stmF* mutant (Ross and Newell 1981; van Haastert, van Lookeren Campagne et al. 1982; Meima, Biondi et al. 2002).

Similar to the *stmF* mutant phenotype, a mutant that lacks PdeD forms long aggregation streams, while developing as colonies on bacterial lawns (Meima, Biondi et al. 2002). In addition, colony growth was reduced and the feeding front was relatively narrow, which suggested that feeding cells were precociously recruited into the streaming aggregates.

In chapter 4, I explore a potential role of *pdeD* in the timing of development. I found that the reduced growth and colony size of the *pdeD* null mutant was caused

by precocious expression of early and aggregative genes. Concordantly, a cell line that overexpressed PdeD showed the opposite phenotype with a delay in expression of aggregative genes and subsequent aggregation. We also explored the developmental timing in a mutant that lacks the two GCs, GCA and sGC to verify if the effect the PdeD overexpressor is due to depletion of cGMP levels. However, the double null mutants showed only a small delay in aggregation and formed normal aggregates and fruiting structures.

Earlier work attributed the delay in development of the *sgc-/gca-* mutants to a defect in chemotaxis to cAMP. However, our data suggested that the chemotaxis defect could be a consequence of the delayed aggregative gene expression. To test this we stimulated *sgc-/gca-* cells with cAMP pulses to induce optimal expression of aggregative genes. Under these conditions, the mutant and parent strain move towards a micropipette filled with cAMP gradient with equal efficiency. This indicates that proposed chemotactic defect of the *sgc-/gca-* mutant is actually due to a defect in early gene expression.

REFERENCES

Alvarez-Curto, E., D. E. Rozen, et al. (2005). "Evolutionary origin of cAMP-based chemoattraction in the social amoebae." Proc Natl Acad Sci U S A **102**(18): 6385-90.

Alvarez-Curto, E., S. Saran, et al. (2007). "cAMP production by adenylyl cyclase G induces prespore differentiation in Dictyostelium slugs." Development **134**(5): 959-66.

Bosgraaf, L., H. Russcher, et al. (2002). "A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in Dictyostelium." Embo J **21**(17): 4560-70.

Bosgraaf, L., H. Russcher, et al. (2002). "Identification and characterization of two unusual cGMP-stimulated phoshodiesterases in dictyostelium." Mol Biol Cell **13**(11): 3878-89.

Bretschneider, T., B. Vasiev, et al. (1999). "A model for Dictyostelium slug movement." Journal of Theoretical Biology **199**(2): 125-136.

Brzostowski, J. A. and A. R. Kimmel (2001). "Signaling at zero G: G-protein-independent functions for 7-TM receptors." Trends Biochem Sci **26**(5): 291-7.

Clarke, M. and R. H. Gomer (1995). "PSF and CMF, autocrine factors that regulate gene expression during growth and early development of Dictyostelium." Experientia **51**: 1124-1134.

Darmon, M., J. Barra, et al. (1978). "The role of phosphodiesterase in aggregation of Dictyostelium discodeum." J.Cell Sci. **31**: 233-243.

De Wit, R. J. W. and T. M. Konijn (1983). "Identification of the acrasin of Dictyostelium minutum as a derivative of folic acid." Cell Differ. **12**: 205-210.

Detterbeck, S., P. Morandini, et al. (1994). "The 'prespore-like cells' of Dictyostelium have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters." Development **120**: 2847-2855.

Dormann, D., C. Weijer, et al. (1997). "Twisted scroll waves organize dictyostelium mucoroides slugs." Journal Of Cell Science **110 Part 16 : 1831-**: 16-1837.

Faure, M., J. Franke, et al. (1990). "The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains three promoters specific for growth, aggregation, and late development." Mol Cell Biol **10**(5): 1921-30.

Faure, M., G. J. Podgorski, et al. (1988). "Disruption of Dictyostelium discoideum morphogenesis by overproduction of cAMP phosphodiesterase." Proc Natl Acad Sci U S A **85**(21): 8076-80.

Franke, J., M. Faure, et al. (1991). "Cyclic nucleotide phosphodiesterase of Dictyostelium discoideum and its glycoprotein inhibitor: structure and expression of their genes." Dev Genet **12**(1-2): 104-12.

Franke, J., G. J. Podgorski, et al. (1987). "The expression of two transcripts of the phosphodiesterase gene during the development of Dictyostelium discoideum." Dev Biol **124**(2): 504-11.

Fukushima, S. and Y. Maeda (1991). "Whorl formation in Polysphondylium violaceum: Relevance to organization by cyclic AMP." Develop.Growth Differ. **33**: 525-533.

Hall, A. L., J. Franke, et al. (1993). "The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde." Dev Biol **157**(1): 73-84.

Kawabe, Y., T. Morio, et al. (2009). "Activated cAMP receptors switch encystation into sporulation." Proc Natl Acad Sci U S A **106**(17): 7089-94.

Laub, M. T. and W. F. Loomis (1998). "A molecular network that produces spontaneous oscillations in excitable cells of Dictyostelium." Mol. Biol. Cell **9**: 3521-3532.

Maeda, M., L. Aubry, et al. (1996). "Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in Dictyostelium." J Biol Chem **271**: 3351-3354.

Meima, M. E., R. M. Biondi, et al. (2002). "Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants." Mol Biol Cell **13**(11): 3870-7.

Meima, M. E., K. E. Weening, et al. (2003). "Characterization of a cAMP-stimulated cAMP phosphodiesterase in Dictyostelium discoideum." J Biol Chem **278**(16): 14356-62.

Pitt, G. S., N. Milona, et al. (1992). "Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development." Cell **69**(2): 305-15.

Roelofs, J., M. Meima, et al. (2001). "The Dictyostelium homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase." Embo J **20**(16): 4341-8.

Roelofs, J., H. Snippe, et al. (2001). "Guanylate cyclase in Dictyostelium discoideum with the topology of mammalian adenylate cyclase." Biochem J **354**(Pt 3): 697-706.

Ross, F. M. and P. C. Newell (1981). "Streamers: Chemotactic mutants of *Dictyostelium discoideum* with altered cyclic GMP metabolism." J. Gen. Microbiol. **127**: 339-350.

Saito, T., G. W. Taylor, et al. (2006). "Identification of new differentiation inducing factors from Dictyostelium discoideum." Biochim Biophys Acta **1760**(5): 754-61.

Saran, S., M. E. Meima, et al. (2002). "cAMP signaling in *Dictyostelium* - Complexity of cAMP synthesis, degradation and detection." Journal of Muscle Research and Cell Motility **23**(7-8): 793-802.

Saran, S., M. E. Meima, et al. (2002). "cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection." J Muscle Res Cell Motil **23**(7-8): 793-802.

Schaap, P., M. M. Van Lookeren Campagne, et al. (1986). "Postaggregative differentiation induction by cyclic AMP in Dictyostelium: intracellular transduction pathway and requirement for additional stimuli." Dev Biol **118**(1): 52-63.

Schaap, P., T. Winckler, et al. (2006). "Molecular phylogeny and evolution of morphology in the social amoebas." Science **314**(5799): 661-3.

Shimomura, O., H. L. B. Suthers, et al. (1982). "Chemical identity of the acrasin of the cellular slime mold Polysphondylium violaceum." Proc.Natl.Acad.Sci.USA **79**: 7376-7379.

Soede, R. D., N. A. Hopper, et al. (1996). "Extracellular cAMP depletion triggers stalk gene expression in Dictyostelium: disparities in developmental timing and dose dependency indicate that prespore induction and stalk repression by cAMP are mediated by separate signaling pathways." Dev Biol **177**(1): 152-9.

Sucgang, R., C. J. Weijer, et al. (1997). "Null mutations of the Dictyostelium cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates." Dev Biol **192**(1): 181-92.

Thompson, C. R. and R. R. Kay (2000). "The Role of DIF-1 Signaling in Dictyostelium Development." Mol Cell **6**(6): 1509-1514.

van Es, S., K. J. Virdy, et al. (1996). "Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores." J Biol Chem **271**(39): 23623-5.

van Haastert, P. J., M. M. van Lookeren Campagne, et al. (1982). "Altered cGMP-phosphodiesterase activity in chemotactic mutants of Dictyostelium discoideum." FEBS Lett **147**(2): 149-52.

Verkerke-van Wijk, I., M. Fukuzawa, et al. (2001). "Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression." Dev Biol **234**(1): 151-60.

Wang, M., R. Van Driel, et al. (1988). "Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in Dictyostelium discoideum slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation." Development **103**: 611-618.

Wu, L., J. Franke, et al. (1995). "The phosphodiesterase secreted by prestalk cells is necessary for Dictyostelium morphogenesis." Dev Biol **167**(1): 1-8.

Chapter 7

Non-Scientific Part

Cel communicatie

Alle levende wezens, oftewel organismen moeten informatie uitwisselen met hun omgeving om zich lekker te voelen en om te overleven. Er bestaan twee hoofdgroepen organismen. Prokaryoten waaronder bacteriën vallen, zijn hele eenvoudige, meestal eencellige organismen. De meer ingewikkelde, eukaryoten, kunnen ofwel eencellig zijn ofwel, net als wij mensen, samengesteld zijn uit triljoenen cellen, die als gespecialiseerde eenheden zoals weefsels en organen worden georganiseerd.

Cellen zijn de bouwstenen van levende organismen; een cel bestaat uit water, zouten, suikers en eiwitten, omsloten door een celmembraan. De cel bevat ook het genetisch materiaal, DNA oftewel desoxyribonucleïnezuur, die de informatie voor de productie van eiwitten bevat. Gen-expressie is het proces waarbij een klein stukje code DNA, het gen, wordt gekopieerd naar RNA oftewel ribonucleïnezuur en dat vervolgens tot werkende eiwitten wordt afgeschreven**.**

De vorming van weefsels en organen vereist specialisatie van in aanvang identieke cellen tot alternatieve celtypen met specifieke functies. Dit proces, dat differentiatie wordt genoemd, wordt geregeld door communicatie tussen de cellen. Communicatie is de uitwisseling van informatie tussen de zender van de informatie en de ontvanger van die informatie. Informatie, in de vorm van kleine moleculen, afgescheiden eiwitten, temperatuur of licht, kan komen van binnen of van buiten het organisme.

Voor een doeltreffende communicatie beschikken cellen over een groot aantal signaalmoleculen oftewel boodschappers. Deze boodschappers worden herkend en opgepikt door specifieke receptoren die zich bevinden in de celmembraan. Na ontvangst van het signaal kunnen de nu geactiveerde receptoren zogenaamde enzymen in de cel aanzetten tot productie van zogenaamde tweede boodschappers die uiteindelijk de gewenste reactie bewerkstelligen. Dit kan zijn: celbeweging, secretie van diverse moleculen, cel groei en cel differentiatie. Signaaloverdracht is het gehele proces vanaf detectie van het signaal tot activatie van enzymen.

Dictyostelium discoideum als modelorganisme

Zeer veel processen van signaaloverdracht zijn al vroeg in de evolutie aangelegd en grotendeels bewaard gebleven tussen organismen. Ingewikkelde organismen, zoals wij mensen, delen veel van dezelfde processen met muizen, fruitvliegjes en eenvoudige levensvormen zoals schimmels, amoeben en zelfs bacterien. De sociale amoebe, *D. discoideum* is een eencellig organisme, dat over kan gaan in een meercellig, als er een gebrek aan voedsel is. Zij zijn verwant aan hogere soorten zoals de mens en hebben veel genen gemeenschappelijk, die meestal dezelfde functie hebben.

In hun natuurlijke omgeving, leven amoeben in plantaardig of dierlijk afval in de grond en eten van de normale rottingsbacteriën. Terwijl zij groeien en zich vermedigvuldigen door celdeling, houden zij hun eigen celdichtheid en die van hun voedingsbron bij. Hongersnood treedt op bij hoge celdichtheid en voedseltekort en dit zet cellen aan tot productie en afscheiding van een chemische stof. Naburige cellen worden door deze chemische stof aangetrokken en zij gaan zich daar dan naar toe bewegen. Dit aggregatie proces leidt tot de vorming van een veelcellig aggregaat, een klompje cellen van wel 100.000 cellen. Na aggregatie wordt hetdifferentiatie stadium ingegaan en ontwikkelen *D. discoideum* amoeben zich voornamelijk in twee celtypen, de toekomstige steel cellen en sporen. Bovendien ondergaan ze een serie van gecompliceerde bewegingen om uiteindelijk middels eerst een slakvormig stadium een structuur te vormen die het vruchtlichaam wordt genoemd. Dat vruchtlichaam bestaat uit een steel van dode cellen en een bolvormig hoofdje dat de sporen bevat.

Tijdens het slakstadium is het patroon van de toekomstige steel en spore cellen goed herkenbaar. De toekomstige steel cellen bevinden zich aan het voorste gedeelte in ‘de tip’ van de slak terwijl de prespore cellen het achterste driekwart gedeelte van de slak in beslag nemen. De steelcellen offeren zichzelf op om de voortgang van de verspreiding van sporen te vergemakkelijken als zich gunstige omstandigheden voordoen. Iedere spore kan zich ontwikkelen tot een nieuwe amoebe. Voor een tekening van hun levenscyclus wordt verwezen naar figuur 3 in hoofdstuk 1.

Het meercellige ontwikkeling stadium van *Dictyostelium* amoeben is te vergelijken met de vorming van weefsel en organen in meer ingewikkelde organismen, zoals mensen. Voor beiden is gecompliceerde signaaloverdracht noodzakelijk om morfogenetische en cellulaire differentiatie te coördineren. Bovendien, de manier waarop cellen bewegen tijdens de aggregatie, vertoont gelijkenis met hoe onze immuuncellen een ontstoken plek lokaliseren en er naartoe bewegen.

Deze overeenstemmingen tesamen met hun gebruiksgemak (o.a. zijn ze gemakkelijk op te groeien in het laboratorium en hun volledige ontwikkeling duurt maar 24 uur) en de beschikbaarheid van de *D. discoideum* genoomsequentie, de DNA-volgorde, maken hen tot een aantrekkelijk modelorganisme in de ontwikkelingsbiologie.

cAMP als signaalmolecule

De belangrijkste chemische boodschapper voor de ontwikkeling van *D. discoideum* is het cyclisch adenosinemonofosfaat (cAMP). Ongeveer een halve eeuw geleden werd cAMP in eukaryoten ontdekt als een molecuul dat binnen de cel wordt geproduceerd als tweede boodschapper. In deze rol wordt cAMP door alle

levende organismen gebruikt om een groot aantal signalen van buitenaf door te geven aan een verscheidenheid aan biologische reacties (zie hoofdstuk 1).

In *D. discoideum* fungeert cAMP niet alleen als intracellulaire boodschapper, maar ook als een extracellulair signaal. Zoals hiervoor al werd opgemerkt, scheiden hongerende amoeben een chemische stof af waar de cellen zich toe aangetrokken voelen en zij zich naartoe bewegen. Dit proces waarin amoeben bewegen naar een chemische stof, die in hun omgeving wordt afgescheiden, wordt “chemotaxis” genoemd.

De hongerende amoebe scheidt als chemo-attractant extracellulaire cAMP af in regelmatige pulsen die worden herkend en opgepikt door specifieke cAMP receptoren (cARs), welke zich bevinden in de celmembraan van naburige amoeben. De detectie van het cAMP signaal heeft tot gevolg dat deze amoeben zich gaan bewegen naar de cAMP bron, maar tevens ook dat de amoeben zelf cAMP gaan afscheiden en zo de informatie doorgeven (= relay signaal). Dit alles heeft tot gevolg dat golven van cAMP zich verspreiden door de celpopulatie en dat de cellen zich begeven naar het beginpunt van de cAMP pulsen om zich te verenigen in een aggregaat. Na aggregatie controleert extracellulair cAMP de specialisatie van de prespore voorlopercellen en morfogenese.

Binnen de cellen controleert cAMP de uiteindelijk specialisatie oftewel terminale differentiatie van de sporen en steelcellen en sporen ontkieming door de activering van het eiwitkinase (PKA). Een kinase is een specifiek enzym dat eiwitten verandert door er een fosfaat aan te koppelen waardoor de activiteit van het bewuste eiwit verandert.

Doel en conclusie van dit proefschrift

Tijdens mijn promotieonderzoek was ik geïnteresseerd in cAMP signalering in *Dictyostelium discoideum*. Om te begrijpen hoe dat in zijn werk gaat, moet men begrijpen hoe de eiwitten zich gedragen die nodig zijn voor de detectie, de productie en de afbraak oftewel degradatie van het cAMP signaal. In Hoofdstuk 1 geef ik een overzicht van de informatie die in de loop der tijd bekend gemaakt is, in zowel prokaryoten als eukaryoten, over deze belangrijke eiwitten die cAMP signalering controleren.

De productie en afbraak van cAMP is een ”dynamisch” proces. Veranderingen in cAMP concentraties zijn een kort leven beschoren in sommige cellen, maar kunnen uren duren in andere. In feite bepalen de duur en het niveau van concentratie het specifieke karakter van het antwoord van de cel. In theorie hangt het niveau van cAMP af van de snelheid waarmee het wordt geproduceerd door adenylylcyclasen en afgebroken door fosfodiesterasen. Echter, in de afgelopen jaren is duidelijk geworden dat fosfodiesterasen een uiterst kritische rol spelen bij de regulering, locatie en hoogte van het cAMP signaal. In dit proefschrift beschrijf ik

studies met betrekking tot de regulering van dynamische cAMP signalering in sociale amoeben met een speciale nadruk op de rol van fosfodiesterasen.

De afbraak van extracellulair cAMP door PdsA, een extracellulaire fosfodiesterase is essentieel voor aggregatie en de vorming van het vruchtlichaam, maar er het is nog niet bekend hoe expressie van PdsA precies gereguleerd wordt na het aggregatie stadium. Het *PdsA* gen is onder de controle van drie verschillende stukjes DNA, oftewel promotoren die elk gen-expressie op verschillende tijdstippen en plaatsen kunnen aanzetten gedurende de ontwikkeling. In Hoofdstuk 2 beschrijf ik studies met betrekking tot de gen-expressie patronen van de aggregatie en de late promotor gedurende de ontwikkeling. Hiertoe maakten we gebruik van een zogenaamd reporter gen, dat aangestuurd wordt door een der bestudeerde promoters. Wanneer de promoter actief is, leidt dit tot gen-expressie van het reporter gen en dus tot de productie van een eiwit. Dit eiwit product kan zowel met een biochemische als histo-chemische kleur reactie zichtbaar worden gemaakt. De gen-expressie patronen in zich ontwikkelende structuren (zie de blauwe kleuring in Hoofdstuk 2, figuren 1 en 2) tonen dat de aggregatie promotor het meest actief is gedurende het aggregatie stadium en de late promotor het meest actief is na aggregatie. Bovendien sturen de beide promotoren *PdsA* gen-expressie in bepaalde cellen, die tot de steel cellen behoren. Aldus controleert de afbraak van cAMP door PdsA op verschillende tijdstippen en plaatsen de differentiatie van de steelcellen. De signalen, die de activiteit van de promotor regelen gedurende de late ontwikkeling waren niet bekend, maar ik toon aan, dat in celsuspensie (*in vitro*) die activiteit sterk gestimuleerd wordt door een combinatie van hoge cAMP en DIF. DIF is een ander belangrijk chemisch signaal dat specifiek nodig is voor de ontwikkeling van steelcellen. Vervolgens veranderden, oftewel modificeerden we de promotor door het verwijderen van stukjes DNA, stukje voor beetje, om het gebied te vinden dat nodig is voor de stimulatie van gen-expressie door cAMP en DIF. Zo waren we in staat aan te tonen, dat het promotor gebied verantwoordelijk voor stimulatie door DIF en cAMP zoals waargenomen in celsuspensies, bij een normale ontwikkeling niet essentieel is voor de activiteit van de promotor.

In Hoofdstuk 3 ga ik de oorsprong na van PdsA, om de ontwikkeling van extracellulaire cAMP signalering te begrijpen. De sociale amoeben of *Dictyostelia* worden onderverdeeld in 4 hoofdgroepen op basis van hun verwantschap. *D. discoideum* behoort tot de meest ontwikkelde, de jongste groep en die groep kan van de andere *Dictyostelia* groepen worden onderscheiden omdat zij gebruik maken van extracellulaire cAMP signalering voor aggregatie. Echter, alle groepen maken gebruik van cAMP signalering na aggregatie voor het vormen van vruchtlichamen. Door de moleculaire variatie aan te tonen tussen de verschillende PdsA eiwitten van vertegenwoordigers van elke *Dictyostelia* subgroep was ik in staat hun verwantschap in de vorm van een stamboom weer te geven. Tevens kon ik aantonen wanneer het *PdsA* gen tot expressie wordt gebracht tijdens de ontwikkeling in vertegenwoordigers van elke subgroep. Verbazingwekkend lijkt PdsA

tot expressie te komen gedurende de gehele ontwikkeling ook bij soorten die geen cAMP gebruiken voor chemotaxis en pas de cAMP receptoren tot expressie brengen na aggregatie. Dit leidt tot de vraag wat de functie van PdsA is voornamelijk tijdens het groeistadium en dit vereist nader onderzoek.

cAMP golven worden waargenomen tijdens alle stadia van ontwikkeling. Drie verschillende adenylyl cyclasen (ACA, ACB en ACG) zijn verantwoordelijk voor de productie van cAMP op verschillende tijdstippen en plaatsen gedurende de ontwikkeling, hetgeen nauw verbonden is met hun rol gedurende de ontwikkeling. ACA produceert extracellulair cAMP nodig voor aggregatie, maar zijn expressie gaat merendeels verloren na aggregatie en is alleen nog maar aantoonbaar in het uiterste van de tip in de slak. Ook ACB komt alleen tot expressie in het voorste gedeelte van de slak en functioneert in de terminale differentiatie van de sporen en steel cellen. Dus na aggregatie kunnen zowel ACA als ACB niet verantwoordelijk zijn voor de productie van de cAMP golven in het achterste deel oftwel posterior van de slak. In hoofdstuk 4, maak ik gebruik van cellen waarin het gen voor ACA is uitgeschakeld en het gen voor ACG op hoog niveau tot expressie wordt gebracht gedurende de gehele ontwikkeling. Dus in deze cellen worden vele kopieën van het ACG mRNA aangemaakt hetgeen het meten van ACG gedurende de gehele ontwikkeling, zowel in groeiende cellen als ontwikkelende cellen mogelijk maakt. Als intacte groeiende cellen geincubeerd worden met extracellulair cAMP tezamen met een cAMP fosfodiesterase inhibitor, zag ik, zoals verwacht, een lineaire productie van cAMP. Echter onverwachts zag ik in hongerende cellen een afgifte voor korte duur van cAMP, hetgeen op een kortstondige activatie van ACG duidt. Deze ‘transiente’ activatie van ACG vertoont sterke gelijkenis met hoe ACA geactiveerd wordt middels de cAMP receptor. Voorts toon ik aan dat ook ACG geactiveerd wordt door cARs maar niet middels de heterotrimere G-eiwitten. Aangezien ACG in het achterste deel van de slak tot expressie wordt gebracht is het zeer waarschijnlijk dat dit het lang gezochte cyclase is dat verantwoordelijk is voor de productie van de cAMP golven in het achterste gedeelte van de slak. Hiervoor is echter nader onderzoek nodig.

Extracellulaire cAMP prikkeling van de cAMP receptor veroorzaakt ook de activering van de nauw verwante guanylyl cyclasen. Deze typen cyclasen produceren cGMP, een enigszins ander cyclisch nucleotide, dat een rol speelt bij chemotaxis. In Hoofdstuk 5 vond ik een potentieel nieuwe rol voor cGMP. Ik beschrijf experimenten die aantonen dat veranderingen in de expressie van het *PdeD* gen, een intracellulair fosfodiesterase, dat intracellulaire cGMP afbreekt, de expressie van vroege genen die nodig zijn voor aggregatie, doet veranderen.

CURRICULUM VITAE

Karin Esther Weening was born on 13-April-1972 and grew up in Hazerswoude-dorp, The Netherlands. In 1989 she received her HAVO secondary education diploma at the 'Bonaventura College' in Leiden. This facilitated access to the Higher Laboratory School from which she obtained a BSc specialized in Histo-Cyto-and Pathology in 1996. Doing her diagnostics project at Leyenburgh hospital (The Hague) then her research project at the Electron Microscopy unit in collaboration with the biomaterials department at Leiden University. These experiences enthused Karin for doing research, leading her to do her MSc Biology at the University of Leiden specializing in Life Sciences. Her first graduation research project was done at the Institute for Moleculair Plant Sciences (Leiden University) in the laboratory of Professor Schaap. It was here where *Dictyostelium* first seduced Karin and she continued to work on it during her second project that was done within the Biological Chemistry department of Johns Hopkins University (Baltimore, USA) in the laboratory of Professor Devreotes. After gaining her MSc in 1999 she started her PhD, again with Professor Schaap. This laboratory group relocated to the University of Dundee in September 1999 where Karin continued her PhD still registered at Leiden University. In 2003 she developed her research career first as Research Assistant and later as Scientific Officer. Around this point Karin met Stephen John Rae to whom she is now married. Currently, Karin is a Research Associate in the laboratory of Professor Harwood at Cardiff University, Wales (UK). Here she uses her *Dictyostelium* skills, logistically setting up and screening a large set of REMI mutants, specifically interested in their sensitivity or resistance to the mood disorder drug Lithium.

PUBLICATIONS

Alvarez-Curto, E., **Weening K. E**. and Schaap, P. (2007). "Pharmacological profiling of the Dictyostelium adenylate cyclases ACA, ACB and ACG." Biochem J 401(1): 309-16.

Meima, M. E., **Weening K. E**. and Schaap, P. (2003). "Characterization of a cAMP-stimulated cAMP phosphodiesterase in Dictyostelium discoideum." J Biol Chem 278(16): 14356-62.

Meima, M. E., **Weening K. E.**, and Schaap, P. (2007). "Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium." Protein Expr Purif 53(2): 283-8.

Saran, S., M. E. Meima, Alvarez-Curto, E., **Weening, K. E**., Rozen, D. E., and Schaap, P. (2002). "cAMP signaling in *Dictyostelium* - Complexity of cAMP synthesis, degradation and detection." Journal of Muscle Research and Cell Motility 23(7-8): 793-802.

van Es, S., **Weening K. E**., and Devreotes, P. N. (2001). "The protein kinase YakA regulates g-protein-linked signaling responses during growth and development of Dictyostelium." J Biol Chem 276(33): 30761-5.

Weening, K. E., Wijk I. V., ., Thompson, C.R., Kessin, R.H., Podgorski, G. and Schaap, P. (2003). "Contrasting activities of the aggregative and late PDSA promoters in Dictyostelium development." Dev Biol 255(2): 373-82.

EPILOGUE

I'd like to thank my committee members for critical reading of my manuscript and promoteres Paul Hooykaas and Pauline Schaap for their support and advice. Additionally to Pauline, thank you for introducing me to *Dictyostelium*, giving me the opportunity to do my PhD and helping me in the write up of this thesis. During my undergraduate project I had fun at the bench and enjoyed many 'nog één vaasje dàn' with Raymond, Marcel, Paul and Ellen, which continued when I started my PhD. At first it was sad to leave, but moving to Scotland (Dundee) with the lab was a great opportunity for scientific discovery and getting to know the country of my dreams as a wee lassie. Ta much to the Scottish hills for plenty of fresh air, the views and challenging me in different ways even though I could have done with 'some flatness' at times – cheers Dundee Rucksack club!
A thanks also goes to those that moved with the Lab, Elisa and Marcel for the fun days setting up the lab and exploring Scotland. I wonder if you, Elisa, still daydream of Nigel in his orange overall. Those whom made a particular difference for me were 'stimulating' Shweta, 'movie making' Dirk and 'traditional' Gerti. And there were others too, Yoshi, Allyson, Claire…those who helped in our shared working environment, those from the next-door labs for laughter, gossip and humanity shared over an americano and scone…and many more. Not forgetting my family. My brothers, nieces, nephews, mum and dad for their fullest support and believe in me. In particular, I am grateful to my understanding, patient husband and best friend Steve, who has put up with these many years of research, boring evenings and weekends. Together we make a great team! And finally, I just want to say to my late grandfather, it is smelling great!!