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Protist

PROTIST NEWS

Regulation of *Dictyostelium* Guanylyl Cyclases

Introduction

Dictyostelium discoideum is a preferred organism to study complicated processes like chemotaxis and signal transduction. *Dictyostelium* has a haploid genome, facilitating the construction of knock-out strains. Furthermore it has a highly reproducible developmental program enabling simple detection of phenotypic defects. Vegetative *Dictyostelium* cells are chemotactically sensitive to folic acid (FA), produced by their natural food source, bacteria. Upon starvation, chemoattractant sensitivity is shifted from folic acid to cAMP, which is periodically secreted by starving cells. Using cAMP as a homing beacon, up to 10^5 cells can move towards each other, forming an aggregate. The multicellular aggregate then further develops into a fruiting body consisting of a stalk of lysed cells with a spore head on top. Under favourable conditions, the spores can germinate to enter the vegetative state and complete the developmental cycle. While cAMP is used as the extracellular signal molecule during chemotaxis, one of the intracellular second messengers used in this developmental program is cGMP. Conversion of GTP to cGMP is catalysed by specific enzymes called guanylyl cyclases (GCs). Recently, all guanylyl cyclases in *Dictyostelium* have been identified, giving new insights into its cyclic GMP signalling pathway. In this review we will discuss both the characteristics and regulation of the *Dictyostelium* guanylyl cyclases.

Signal Transduction in *Dictyostelium*

In the first step of chemotaxis, the chemoattractant binds to a cell surface receptor. *Dictyostelium* expresses folic acid receptors during the vegetative state, while four different cAMP receptors are expressed throughout development (Devreotes 1994). The FA and cAMP receptors are coupled to heterotrimeric G-proteins, consisting of a Gα, Gβ and Gγ subunit. Upon binding of an extracellular ligand to the receptors, guanylyl cyclase is activated in a G-protein dependent pathway (Fig. 1). Concentrations of cGMP start to rise with a lag time of about 1 second and reach a maximum at 10 seconds after stimulation (Mato et al. 1977a, 1977b). cGMP mediates its signal through cGMP binding proteins. *Dictyostelium* expresses two of those proteins, GbpC and GbpD (Bosgraaf et al. 2002a; Goldberg et al. 2002). GbpD contains two cyclic nucleotide binding

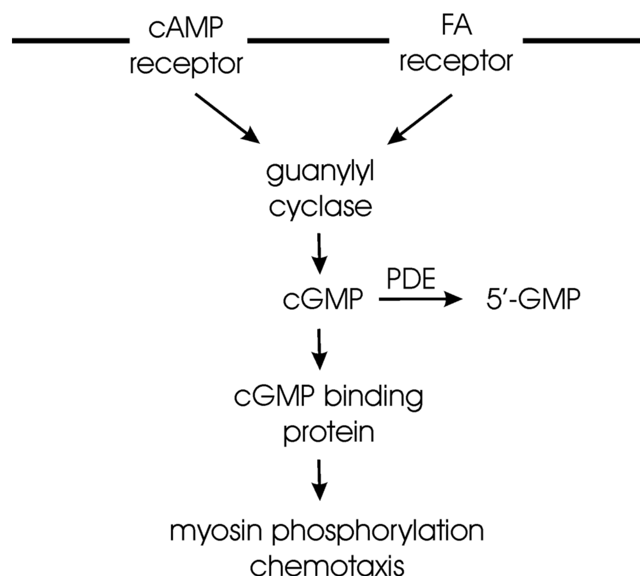

Figure 1. Overview of the cGMP pathway in *Dictyostelium*. Upon extracellular ligand binding, the membrane bound chemoattractant receptors for cAMP and FA activate the same pool of guanylyl cyclase. Produced cGMP may activate specific cGMP-binding proteins, which are known to phosphorylate myosin, leading to cytoskeletal rearrangements and chemotaxis. The cGMP signal can be turned off by phosphodiesterases which hydrolyse cGMP to 5′-GMP.

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domains and a RasGEF domain. GbpC is homologous to GpbD but contains an additional Ras and MAPKKK domain at the N-terminus. All high affinity cGMP binding activity is lost in *gbpC*⁻ mutants, indicating that GbpC is the major effector enzyme in cGMP signalling.

Extracellular cAMP induces the phosphorylation of myosin regulatory light chain (RLC) and myosin heavy chain (MHC). Phosphorylation of RLC and MHC is essential for efficient chemotaxis (Stites et al. 1998; Zhang et al. 2002). Mutant cell strains expressing a non-phosphorylatable RLC move more efficiently in a gradient and show prolonged maintenance of cell polarity. In contrast, strains expressing a non-phosphorylatable MHC display a decrease in directionality. Phosphorylation of RLC and MHC in response to chemoattractant stimulation is reduced in mutant *gbpC*⁻/*gbpD*⁻. Cell strains deficient in functional GbpC and GbpD are also unable to recruit myosin to the cytoskeleton in response to a cAMP stimulus, further confirming the involvement of GbpC and GbpD in rearrangements of myosin (Bosgraaf et al. 2002a).

Thirty seconds after stimulation cGMP concentrations return to their basal level. The degradation of cGMP is catalysed by phosphodiesterases (PDEs) that hydrolyse cGMP into 5′-GMP. Three PDEs that are able to hydrolyse cGMP have been identified by screening the genomic *Dictyostelium* database (Bosgraaf et al. 2002a, 2002b; Kuwayama et al. 2001). The first cGMP PDE identified was DdPDE3, an enzyme showing highest sequence identity with mammalian PDE9. The enzyme has a very high affinity for cGMP (K_m = 0.22 μM) and a low V_{max} (2 pmol/min/mg) suggesting the biological function of this PDE is to maintain a low basal cGMP concentration. In contrast cGMP specific PDE5 has a much higher processivity (V_{max} = 390 pmol/min/mg) that can be stimulated by cGMP. The cGMP response in *pde5*⁻ cells resembles that of the stmF mutant, suggesting PDE5 activity is absent in the stmF mutant (Bosgraaf et al. 2002b; Meima et al. 2002). Finally, PDE6 is a is a dual specificity enzyme with a nine-fold higher activity towards cAMP. Its activity can be stimulated both by cAMP and cGMP, allowing cross talk between both induced responses.

Identification of *Dictyostelium* Guanylyl Cyclases

Conversion of GTP into cGMP is catalysed by guanylyl cyclases. The enzymes are part of the adenylyl and guanylyl cyclase superfamily. Catalytic domains of members of this family show a high degree of similarity. Guanylyl cyclase activity requires dimerisation of two cyclase domains in an antiparallel fashion. Both cyclase domains can either be present in one enzyme or be part of different enzymes. If both domains reside on different proteins, they can be functional either as homodimer or as heterodimer. Each cyclase domain contains one nucleotide binding pocket. Upon dimerisation two potential catalytic sites are formed, α and β sites. In homodimers, both sites are identical, but in heterodimers usually only one pocket shows catalytic activity while the other pocket may serve a regulatory function.

In vertebrates, there are two predominant groups of guanylyl cyclases based on their number of transmembrane segments (Wedel and Garbers 2001). Enzymes with no apparent transmembrane segments are usually referred to as soluble guanylyl cyclases, acting as the intracellular receptors of NO and CO. Soluble GCs are shown to function as heterodimers, consisting of an α and β subunit (Koesling 1999). The second group of vertebrate GCs consists of enzymes with a single transmembrane segment and one cyclase domain that are active as homodimers. The transmembrane segment separates an extracellular ligand binding domain from an internal kinase homology domain and a cyclase homology domain. Like soluble GCs, single transmembrane GCs act as receptors. Natriuretic peptides have been identified as ligands for some single transmembrane GCs, where they serve as regulators of blood pressure and heart and renal functions (Tremblay et al. 2002). Unexpectedly, guanylyl cyclases in *Dictyostelium* do not fall in either of these two well established groups of guanylyl cyclases, and the enzymes have no known function as receptors for intra- or extracellular ligands.

The property that the catalytic domains of adenylyl and guanylyl cyclases are well conserved, was used to identify the first guanylyl cyclase in *Dictyostelium* (Roelofs et al. 2001b). By means of a PCR with degenerate primers on conserved regions of known cyclase sequences, a small DNA fragment could be amplified. This fragment was used to screen a cDNA bank. One full length cDNA clone was obtained containing an open reading frame coding for a 1486 amino acid protein. The expressed protein was shown to possess guanylyl cyclase activity but no adenylyl cyclase activity, and therefore the protein was called GCA. Remarkably, hydropathy analysis indicated that it contained two hydrophobic regions, consisting of six transmembrane spanning helices each. This topology is typical for mammalian adenylyl cyclases that are regulated by G-proteins. In GCA the cyclase C_1 and C_2

domain are swapped relative to mammalian ACs. Being a heterodimer, GCA contains two non-identical potential catalytic sites, the α-site and the β-site (see Fig. 2). Sequence alignment with mammalian adenylyl cyclases indicates the α-site of GCA to be catalytically active, while in mammalian ACs the β-site is active. Guanylyl cyclases with similar topology have been identified in *Paramecium tetraurelia, Tetrahymena pyriformis* and *Plasmodium falciparum*, giving rise to a third small group of multiple transmembrane guanylyl cyclases (Linder et al. 1999; Linder and Schultz 2002).

A knock-out of GCA did not result in a significant decrease of GC activity. Therefore, at least one other guanylyl cyclase must be present in *Dictyostelium*. By screening the genomic *Dictyostelium* database with consensus AC and GC sequences a novel gene with highest similarity to the mammalian soluble adenylyl cyclase (sAC) was revealed. Unexpectedly, disruption of the *Dictyostelium* gene resulted in a dramatic decrease of guanylyl cyclase activity, while

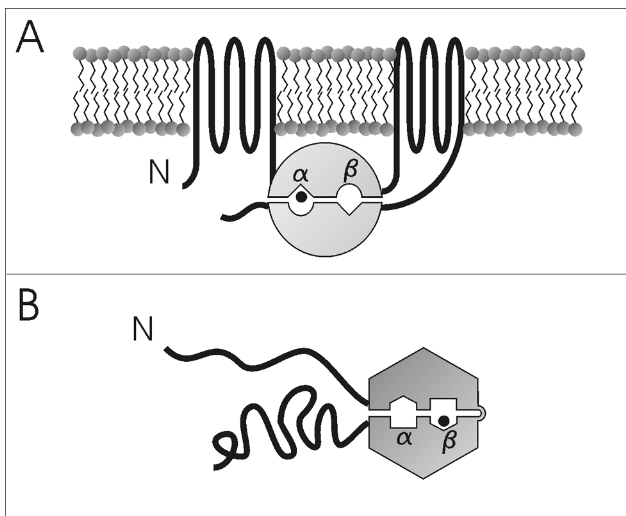

Figure 2. Schematic topology of *Dictyostelium* guanylyl cyclase GCA and sGC. (**A**) The topology of GCA resembles that of mammalian 12 transmembrane adenylyl cyclases. The non-identical cyclase domains C1 and C2 are associated in an antiparallel fashion, giving rise to two potential catalytic sites, α and β. Sequence alignment shows that GTP is hydrolysed in the α pocket of GCA. (**B**) The topology of *Dictyostelium* sGC resembles that of mammalian soluble adenylyl cyclase. The catalytic domain of about 800 amino acids is flanked at the N-terminus by about 1000 amino acids displaying low complexity. The soluble Cyclase Kinase Homology domain resides at the C-terminus and is about equal in size as the N-terminal domain. Sequence alignment has shown the β site of sGC to be catalytically active.

adenylyl cyclase activity was unaffected (Roelofs et al. 2001a). The encoded protein was therefore called soluble guanylyl cyclase (sGC). Mammalian sAC shows homology to *Dictyostelium* sGC over the entire sequence, but the *Dictyostelium* sGC contains an extra N-terminal region of about 1000 amino acids. This extra sequence contains large stretches of poly(N) and poly(Q) repeats and shows no significant homology to any known protein. The N-terminal region is followed by two cyclase catalytic domains. Like GCA both domains are present in the same enzyme, resulting in a heterodimer. Mutational analysis of mammalian adenylyl and guanylyl cyclases has revealed the amino acids that are interacting with the purine, ribose and pyrophosphate groups (Liu et al. 1997). Comparison of this analysis with the C_1 and C_2 domain of sGC indicates that the amino acids involved in substrate binding are only present in the C_2 domain. GTP is therefore thought to bind at the β site of sGC in contrast to GCA, where the substrate binds at the α site. The long 1000 amino acid C-terminal segment following the cyclase domains of sGC shows a high degree of sequence identity with the C-terminal segment of mammalian sAC along its entire sequence. The region also shows significant identity with a part of a protein from *Anabaena spirulensis* that contains not a cyclase domain, but a kinase instead. The region is therefore referred to as a soluble Cyclase Kinase Homology (sCKH) domain. The function of the sCKH domain is as yet unknown. A functional splice variant of mammalian sAC, missing the entire sCKH domain, shows enhanced activity suggesting the domain has an inhibitory effect and may function as a receiver of regulatory signals (Buck et al. 1999; Jaiswal and Conti 2001). The sCKH domain contains a P-loop motif very close to the second catalytic domain, followed by an AAA domain. P-loop motifs are able to bind to phosphate groups of purine nucleosides and are found in a variety of unrelated protein families (Saraste et al. 1990). The motif has been conserved in distinct evolutionary branches of the sCKH domain as it is also present in the sCKH domain of cyanobacteria (Roelofs and Van Haastert 2002b). It therefore seems likely that the loop will have a biological function.

Characterisation of GCA and sGC in vitro

Inactivation of both the *gca* and *sgc* gene results in a cell strain with neither basal nor stimulated GC activity, indicating that there are no more GCs present in *Dictyostelium* (Roelofs and Van Haastert 2002a). The lack of any additional consensus cyclase se-

quences in the nearly completed *Dictyostelium* genome confirms this assumption. Identification of both cyclases allows the investigation of the regulation of the individual enzymes. A knock-out of *gca* enables the characterisation of the remaining sGC and *vice versa* GCA can be characterised in the knockout of *sgc*.

Enzyme Kinetics

Biochemical analysis has revealed the kinetic properties of both enzymes. GCA has a K_M for GTP of 250 μM and a V_{max} of 6.9 pmol/min/mg protein (Roelofs and Van Haastert 2002a). GTPγS, an in vitro activator of cyclase activity, can stimulate this activity both by lowering the K_M to 66 μM GTP and increasing the V_{max} to 9.8 pmol/min/mg protein. With a K_M of 414 μM, sGC has a lower affinity for GTP than GCA, but its processivity is higher with a V_{max} of 16 pmol/min/mg protein. In a similar fashion as GCA, GTPγS can stimulate sGC by lowering the K_M to 112 μM GTP and increasing the V_{max} to 27 pmol/min/mg protein.

Metal Ion Dependency

Adenylyl and guanylyl cyclases are known to require a divalent metal ion for enzymatic activity. Characterisation of metal ion dependency of the individual enzymes in the *gca*⁻ and *sgc*⁻ cell strains shows that the optimal metal ion concentration is 2 mM for each guanylyl cyclase. However, GCA is about 5-fold more active with optimal Mg^{2+} concentrations than with optimal Mn^{2+} concentrations. In contrast, sGC is about 6-fold more active with 2 mM Mn^{2+} than 2 mM Mg^{2+}. Being a soluble enzyme, about 80% of Mn^{2+}-dependent sGC activity is found in the cytosol. Curiously, no significant activity can be detected in the cytosolic fraction using Mg^{2+} as a metal ion. The particulate fraction of sGC is equally active with either Mg^{2+} or Mn^{2+}. It has been proposed that Mn^{2+} reveals the intrinsic activity of sGC, while enzyme activity with Mg^{2+} can only be obtained in the presence of required physiological cofactors. The physiological concentrations of Mn^{2+} and Mg^{2+} in *Dictyostelium* have been determined by mass spectrometry and show to be 10 μM Mn^{2+} and 2 mM Mg^{2+} (Padh and Brenner 1984). Thus, although in vitro soluble sGC enzymatic activity is higher with manganese than with magnesium, the in vivo soluble sGC is not likely to be involved in producing cGMP. It is suggested that only the particulate fraction of sGC is active in vivo.

Activation by GTPγS

In vivo, receptor stimulation causes activation of guanylyl cyclase in a G-protein dependent pathway (Wu et al. 1995). Upon receptor activation, the receptor coupled heterotrimeric G-protein exchanges its bound GDP for GTP. The Gα subunit then dissociates from the Gβγ subunit. Both subunits can potentially activate guanylyl cyclase activity. Upon hydrolysis of the bound GTP to GDP the Gα and Gβγ subunit re-associate, terminating the signal.

In vitro Mg^{2+} dependent guanylyl cyclase activity requires the presence of physiological cofactors for optimal activity. It has been investigated whether guanylyl cyclase activity can be stimulated by G-proteins in vitro. G-proteins are irreversibly activated by binding GTPγS, an unhydrolysable GTP analogue. Incubation of lysates with GTPγS has been shown to activate the Mg^{2+} dependent guanylyl cyclase activity in vitro both for GCA and sGC

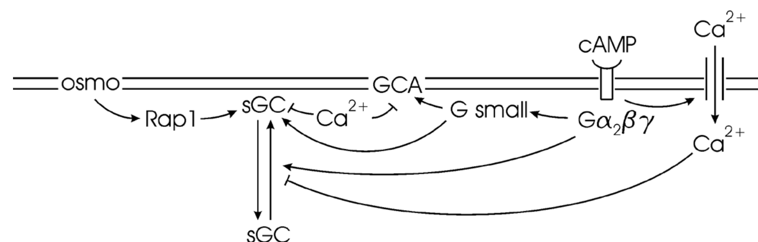

Figure 3. Regulation of *Dictyostelium* GCA and sGC activity. Extracellular ligand binding to the cAMP receptor leads to the dissociation of $G\alpha_2$ and Gβγ. The dissociated heterotrimeric G-protein promotes association of sGC to the membrane. Activity of GCA and membrane-associated sGC is stimulated by a small monomeric G-protein. Parallel to this activation, cAMP receptor stimulation results in a transient influx of Ca^{2+} ions in a heterotrimeric G-protein-independent pathway. Guanylyl cyclase activity is inhibited by Ca^{2+} via two mechanisms. Firstly it promotes dissociation of sGC from the membrane and secondly it inhibits enzymatic activity of both GCA and sGC. The cGMP response induced by osmotic shock is independent of heterotrimeric G-proteins in general and is only known to activate membrane bound sGC.

(Janssens and de Jong 1988; Roelofs and Van Haastert 2002a). Half maximal stimulation occurs at 11 µM GTPγS for GCA and at 8 µM GTPγS for sGC. Because of the similar concentrations of required GTPγS, it is assumed that GCA and sGC are activated by the same G-protein.

The target of GTPγS can be heterotrimeric G-proteins but also small monomeric G-proteins; GTPγS may also interact with guanylyl cyclase itself via a nucleotide binding pocket. Efforts have been made to identify the target of GTPγS. Guanylyl cyclase in a cell lysate prepared in the presence of GTPγS is constitutively active, even if GTPγS is removed by washing the membranes before measuring activity, suggesting that transient exposure to GTPγS is sufficient for guanylyl cyclase activation (Schulkes et al. 1992). Guanylyl cyclase in the particulate fraction prepared in the absence of GTPγS can be stimulated by adding supernatant that is prepared in the presence of GTPγS. The supernatant loses this ability either upon heating for 5 minutes at 90 °C or incubation with proteases, indicating that GTPγS stimulation involves binding to a protein and does not interact with guanylyl cyclase directly. In knockouts of either $G\alpha_2$, $G\alpha_4$ or Gβ, it is observed that GTPγS is still able to stimulate GC activity (Wu et al. 1995). As all heterotrimeric G-protein-dependent second messenger responses are lost in a $g\beta^-$ cell strain, this implicates that the target of GTPγS in respect to guanylyl cyclase activation is most likely not a heterotrimeric G-protein. It has been proposed that GTPγS activates a factor downstream of heterotrimeric G-proteins, possibly a small monomeric G-protein (Fig. 3); this conclusion has been made for the activation of both sGC and GCA (Roelofs and Van Haastert 2002a) .

Inhibition by Calcium Ions

Calcium, a potential activator of cyclases in many tissues, strongly inhibits the in vitro Mg^{2+} dependent guanylyl cyclase activity in *Dictyostelium* (Janssens and de Jong 1988; Padh and Brenner 1984). The observation that electropermeabilised cells are also sensitive to external calcium concentrations suggests a physiological role for calcium inhibition (Valkema and Van Haastert 1992). Using the gca^- and sgc^- null strains to investigate calcium inhibition of the individual guanylyl cyclases, it has been found that with an inhibition constant of 50 nM GCA is more sensitive to Ca^{2+} than sGC which has a K_i of 200 nM.

One of the second messenger responses elicited by cAMP stimulation is a transient influx of extracellular calcium ions (Wick et al. 1978). An increase in cytosolic calcium concentration may function as a physiological inhibitor of guanylyl cyclase. Because of the existence of intracellular calcium stores it is difficult to determine the free cytosolic calcium concentration by standard means like mass spectroscopy. Attempts to determine calcium concentrations by scrape loading of calcium dyes have led to contradictory results (Abe et al. 1988; Nebl and Fisher 1997; Yumura et al. 1996). Harsh treatment of the cells during loading and sequestering of calcium dyes into intracellular vesicles complicates the interpretation of obtained results. By using fura-2-labelled bovine serum albumin or the extrachromosomal expression of aequorin, the encountered problems might be circumvented. Results show a resting concentration of free calcium of ~50 nM. Stimulation with cAMP results in a transient increase with a maximum of 150–200 nM. Different times for the onset and the maximum of the Ca^{2+} response have been reported, ranging from 2.5 to 6.5 seconds for the onset and from 8 to 25 seconds for the peak values (Nebl and Fisher 1997; Yumura et al. 1996).

Despite the contradictory results that have been found in the kinetics of the calcium response, the Ca^{2+} concentration and timeframe of the response make it a potential physiological moderator of GC activity. Washed cell membranes are still sensitive to calcium inhibition, suggesting that calcium can either interact directly with guanylyl cyclase or via a firm membrane bound protein (Schulkes et al. 1992).

Regulation of GCA and sGC in vivo

Activation by cAMP, Folic Acid and Osmotic Shock

Exposure of *Dictyostelium* cells to hypertonic stress induces a transient increase of cytosolic cGMP concentrations with a maximum after about 10–15 minutes (Oyama 1996). The response is completely abolished in the sgc^- mutant, while it is unaltered in the gca^- mutant, indicating sGC is the only cyclase activated by osmotic shock (Roelofs and Van Haastert 2002a). The mechanism of this activation is as yet largely unknown. Stimulation with osmoshock and folic acid is additive, suggesting these two pathways of activation are distinct (Oyama 1996). The observation that a cell strain lacking functional Gβ is still resistant to osmotic stress and induces a good GC response shows that, unlike chemoattractant stimulation, the activation via osmotic shock is independent of heterotrimeric G-proteins (Kuwayama and Van Haastert 1998). The histidine kinase DokA was shown to be involved in survival of

Dictyostelium cells during osmotic stress, but disruption of the gene did not affect cGMP accumulation (Schuster et al. 1996). Recently evidence was provided that the small monomeric G-protein Rap1 may mediate the osmotic shock induced cGMP response (Kang et al. 2002). A knock-out of the encoding rapA gene seems to be lethal. Investigation of the properties of Rap1 using anti-sense RNA expression reveals that the expression levels of Rap1 and the induced cGMP response are proportionally related. The timeframe of activation of Rap1 and cGMP response are also similar, indicating the possibility of a direct relation between Rap1 and sGC activation (Fig. 3).

Significantly more data is available on the chemoattractant stimulated cGMP response than the osmoshock response. Folic acid is the most used chemoattractant in research on chemotaxis of vegetative *Dictyostelium* cells, but it actually represents a broader variety of pterin derivatives (Pan et al. 1972; Rifkin 2002). In a cell strain lacking $G\alpha_4$, folic acid stimulation of vegetative cells no longer results in an accumulation of cGMP, while responses via cAMP receptors are not affected, indicating that FA receptors use the $G\alpha_4$ subunit to activate guanylyl cyclase (Hadwiger et al. 1994). cAMP-induced responses of starved cells are mediated by cAMP receptors. Four different cAMP receptors have been identified, which are sequentially expressed throughout development (Hereld and Devreotes 1992). Each receptor is coupled to a heterotrimeric G-protein. Disruption of the G-protein subunit $G\alpha_2$ results in the loss of all cAMP-stimulated cGMP accumulation, suggesting $G\alpha_2$ is the only cAMP receptor-coupled a subunit that mediates guanylyl cyclase activation (Kumagai et al. 1991). A cell strain missing the Gβ subunit is deficient in both folic acid and cAMP-mediated cGMP accumulation, in good accordance with the assumption that the Gβ subunit is essential for G-protein signalling and that *Dictyostelium* expresses only a single Gβ subunit (Wu et al. 1995).

Expression of GCA and sGC

Upon development, mRNA expression of GCA decreases about 5 fold during aggregation and subsequently rises, reaching a maximum during the slug stage. In a complementary fashion, sGC is mainly expressed during the aggregative phase. Combining the expression data with the kinetics of both enzymes shows that GCA contributes ~50% to the basal cGMP levels in vegetative cells and ~40% to basal cGMP in aggregative cells. cAMP and FA stimulate GCA in *sgc*$^-$ cells about 2.5-fold and sGC in the *gca*$^-$ cell strain about 8-fold. Using these data it can be calculated that sGC provides the major guanylyl cyclase activity in *Dictyostelium*, contributing about 90% of the chemoattractant induced cGMP response (Roelofs and Van Haastert 2002a).

Desensitisation of cGMP Response

An early observed property of the chemoattractant-stimulated cGMP response is the rapid desensitisation of guanylyl cyclase activity to a second stimulus of an equal or lower concentration (Mato et al. 1977a). Deadaptation of the response with a half life of about 90 seconds can only be triggered by removal of the stimulus. Cells that are adapted to folic acid are not adapted to cAMP stimulus and vice versa (Van Haastert 1983). This implies that adaptation takes place before both signals merge, possibly at the level of the chemoattractant receptors. Using cAMP binding experiments, it has been shown that upon binding of cAMP, the receptors rapidly interconvert between fast and slow dissociating forms. This transition has been associated with the activation of G-proteins. Desensitisation of guanylyl cyclase stimulation is proposed to be related to inhibition of receptor interconversions (van Haastert et al. 1986).

It has also been investigated whether desensitisation of guanylyl cyclase is caused by phosphorylation of cAR1, the main cAMP receptor expressed during cell aggregation. cAMP binding induces receptor phosphorylation, which reduces the affinity of the receptor for cAMP (Caterina et al. 1995; Vaughan and Devreotes 1988; Xiao et al. 1999). However, cells expressing an unphosphorylatable mutant of cAR1 show normal adaptation to cAMP, indicating that receptor phosphorylation is not involved in desensitisation of the cGMP response (Kim et al. 1997).

Using a FRET pair of Gβ and $G\alpha_2$ it has been demonstrated that an external cAMP stimulus induces the dissociation of the $G\alpha_2$ and Gβγ subunits, which is expected to be associated with the activation of the G-protein (Janetopoulos et al. 2001). Unexpectedly, the subunits remain dissociated for prolonged periods when receptor-mediated activation of adenylyl and guanylyl cyclases are already desensitised. Receptor phosphorylation appears to have no effect on subunit dissociation. These results suggest that adaptation takes place at a point downstream of receptor binding and heterotrimeric G-protein activation, but before guanylyl cyclase.

Even though prolonged stimulation results in a continuous dissociation of the $G\alpha_2$ and Gβγ subunit,

Dictyostelium does use G-protein subunits to control both kinetics and magnitude of the cGMP response. Until now, eleven different Gα subunits, one Gβ and one Gγ subunit have been identified (Zhang et al. 2001). Research on these subunits has provided a wealth of information that unfortunately is not easily fitted into one model. Deletion of $G\alpha_1$ results in a delayed cGMP response, while overexpression of $G\alpha_1$ displays the kinetics of a wild-type response, but with a lower maximum (Dharmawardhane et al. 1994). A $G\alpha_5$ overexpressor lacks a folic acid-induced cGMP response, indicating it may be an antagonist of $G\alpha_4$ (Natarajan et al. 2000). Finally, $G\alpha_9$ has been implied in receptor desensitisation (Brzostowski et al. 2002). This variety of observations suggests *Dictyostelium* uses heterotrimeric G-proteins as an instrument to transduce and to regulate second messenger responses.

Further data on adaptation of the cGMP response have been acquired in studies using the membrane protrusive chemical 2,3-dimercapto-1-propanol (BAL). Upon incubation of BAL with aggregation competent cells, guanylyl cyclase becomes permanently activated (Oyama 1991). This suggests that BAL can activate guanylyl cyclase without activating its inhibitory pathway. Stimulation of cells with both BAL and cAMP restores the transient behaviour of the induced cGMP response (Oyama et al. 1991). Intriguingly, cAMP is unable to terminate the continuous effect of BAL in a cell strain lacking the $G\alpha_2$ subunit. This indicates that activation of guanylyl cyclase with BAL is $G\alpha_2$ independent, but the cAMP-mediated termination of this response is dependent on $G\alpha_2$.

Other experiments present evidence for regulation of guanylyl cyclase by a negative feedback loop. A chromatographic fraction of cytosolic proteins containing high affinity cGMP-binding activity stimulates cyclase activity of the particulate fraction (Kuwayama and Van Haastert 1996). Adding the cGMP-binding fraction along with Mg^{2+} and ATP has an opposite effect, inhibiting guanylyl cyclase activity. Addition of 8-bromo-cGMP further enhances this inhibitory effect. A model has been proposed in which a cGMP binding protein is thought to have an intrinsic GC stimulating activity, while the cGMP bound form is also able to inhibit GC by phosphorylating it (Kuwayama and Van Haastert 1996). Protein kinase inhibitors enhance the stimulated cGMP response, confirming this model (Oyama 1991). This negative feedback regulation can be further investigated now it is known that the high affinity cGMP-binding activity is associated with GbpC and that the greater part of the chemoattractant induced cGMP response can be attributed to sGC.

Translocation of sGC

Dictyostelium expresses two guanylyl cyclases. GCA, a membrane bound protein and sGC, a soluble enzyme. sGC is the more active protein, contributing about 90% to the chemoattractant-induced cGMP response. Approximately 80% of sGC resides in the cytosol. All attempts to activate this cytosolic fraction in vitro under physiological conditions have failed. Thus soluble sGC is inactive with Mg^{2+}, and GTPγS has no effect. In contrast, the particulate sGC fraction shows strong activity with Mg^{2+} and can readily be stimulated by GTPγS. The presence of a large inactive pool of sGC in the cytosol allows a speculative mechanism of activation by translocation to the membrane. This mechanism of activation in addition to stimulation of the existing particulate pool could explain the discrepancy between the 8-fold increase of GC activity after stimulation with cAMP in vivo and the 3.5-fold increase of activity after stimulation with GTPγS in vitro (Roelofs and Van Haastert 2002a). Translocation of inactive cytosolic sGC to the membrane could potentiate the cGMP response and account for the difference between the in vivo and in vitro stimulation.

Reconstitution experiments have confirmed that sGC can reversibly bind to the membrane. The particulate fraction of cAMP-stimulated cells contains more sGC activity than the particulate fraction of unstimulated cells. Titration of the membrane fraction of *gca*$^-$/*sgc*$^-$ cells with sGC from the supernatant of *gca*$^-$ cells reveals the number of sGC binding sites in the membrane to be limiting. Thus, translocation of sGC to the membrane could be the result of a cAMP-induced increase of sGC binding sites (Roelofs et al. unpublished observations).

The affinity of sGC for the membrane binding sites is very sensitive to inhibition by calcium. The transient influx of calcium after chemoattractant stimulation may play a physiological role in the regulation of the cGMP response by dissociating sGC from the membrane and thereby inactivating it. On the other hand, GTPγS is unable to shift the ratio of membrane bound and soluble sGC. Activation of GC activity is proposed to be a combination of translocation regulated by calcium and activation regulated by GTPγS (see Fig. 3).

Perspectives

The identification of presumably all guanylyl cyclases and cGMP targets in *Dictyostelium* allows us to reinterpret data that have previously been col-

lected in the research of regulation of guanylyl cyclase activity. Earlier postulated models can now be tested using knockout cell strains obtained by homologous recombination. The guanylyl cyclases of *Dictyostelium* and metazoa appear to have evolved from adenylyl cyclases independently from each other. The targets of cGMP in *Dictyostelium*, GbpC and GbpD, also seem to have a different evolutionary origin than their metazoan counterparts, cGMP-dependent protein kinase and cGMP-regulated channels. This suggests that the cGMP signalling pathway evolved separately in both lineages (Roelofs et al. in press).

References

Abe T, Maeda Y, Iijima T (1988) Transient increase of the intracellular Ca^{2+} concentration during chemotactic signal transduction in *Dictyostelium discoideum* cells. Differentiation **39**: 90–96

Bosgraaf L, Russcher H, Smith JL, Wessels D, Soll DR, Van Haastert PJM (2002a) A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium*. EMBO J **21**: 4560–4570

Bosgraaf L, Russcher H, Snippe H, Bader S, Wind J, Van Haastert PJM (2002b) Identification and characterization of two unusual cGMP-stimulated phoshodiesterases in *Dictyostelium*. Mol Biol Cell **13**: 3878–3889

Brzostowski JA, Johnson C, Kimmel AR (2002) Galpha-mediated inhibition of developmental signal response. Curr Biol **12**: 1199–1208

Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR (1999) Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc Natl Acad Sci USA **96**: 79–84

Caterina MJ, Devreotes PN, Borleis J, Hereld D (1995) Agonist-induced loss of ligand binding is correlated with phosphorylation of cAR1, a G protein-coupled chemoattractant receptor from *Dictyostelium*. J Biol Chem **270**: 8667–8672

Devreotes PN (1994) G protein-linked signaling pathways control the developmental program of *Dictyostelium*. Neuron **12**: 235–241

Dharmawardhane S, Cubitt AB, Clark AM, Firtel RA (1994) Regulatory role of the G alpha 1 subunit in controlling cellular morphogenesis in *Dictyostelium*. Development **120**: 3549–3561

Goldberg JM, Bosgraaf L, Van Haastert PJM, Smith JL (2002) Identification of four candidate cGMP targets in *Dictyostelium*. Proc Natl Acad Sci USA **99**: 6749–6754

Hadwiger JA, Lee S, Firtel RA (1994) The G alpha subunit G alpha 4 couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in *Dictyostelium*. Proc Natl Acad Sci USA **91**: 10566–10570

Hereld D, Devreotes PN (1992) The cAMP receptor family of *Dictyostelium*. Int Rev Cytol **137B**: 35–47

Jaiswal BS, Conti M (2001) Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. J Biol Chem **276**: 31698–31708

Janetopoulos C, Jin T, Devreotes P (2001) Receptor-mediated activation of heterotrimeric G-proteins in living cells. Science **291**: 2408–2411

Janssens PM, de Jong CC (1988) A magnesium-dependent guanylate cyclase in cell-free preparations of *Dictyostelium discoideum*. Biochem Biophys Res Commun **150**: 405–411

Kang R, Kae H, Ip H, Spiegelman GB, Weeks G (2002) Evidence for a role for the *Dictyostelium* Rap1 in cell viability and the response to osmotic stress. J Cell Sci **115**: 3675–3682

Kim JY, Soede RD, Schaap P, Valkema R, Borleis JA, Van Haastert PJ, Devreotes PN, Hereld D (1997) Phosphorylation of chemoattractant receptors is not essential for chemotaxis or termination of G-protein-mediated responses. J Biol Chem **272**: 27313–27318

Koesling D (1999) Studying the structure and regulation of soluble guanylyl cyclase. Methods **19**: 485–493

Kumagai A, Hadwiger JA, Pupillo M, Firtel RA (1991) Molecular genetic analysis of two G alpha protein subunits in *Dictyostelium*. J Biol Chem **266**: 1220–1228

Kuwayama H, Van Haastert PJM (1996) Regulation of guanylyl cyclase by a cGMP-binding protein during chemotaxis in *Dictyostelium discoideum*. J Biol Chem **271**: 23718–23724

Kuwayama H, Van Haastert PJM (1998) Chemotactic and osmotic signals share a cGMP transduction pathway in *Dictyostelium discoideum*. FEBS Lett **424**: 248–252

Kuwayama H, Snippe H, Derks M, Roelofs J, Van Haastert PJM (2001) Identification and characterization of DdPDE3, a cGMP-selective phosphodiesterase from *Dictyostelium*. Biochem J **353**: 635–644

Linder JU, Schultz JE (2002) Guanylyl cyclases in unicellular organisms. Mol Cell Biochem **230**: 149–158

Linder JU, Engel P, Reimer A, Kruger T, Plattner H, Schultz A, Schultz JE (1999) Guanylyl cyclases with the topology of mammalian adenylyl cyclases and an N-terminal P-type ATPase-like domain in *Paramecium, Tetrahymena* and *Plasmodium*. EMBO J **18**: 4222–4232

Liu Y, Ruoho AE, Rao VD, Hurley JH (1997) Catalytic mechanism of the adenylyl and guanylyl cyclases:

modeling and mutational analysis. Proc Natl Acad Sci USA **94**: 13414–13419

Mato JM, Krens FA, van Haastert PJM, Konijn TM (1977a) 3′:5′-cyclic AMP-dependent 3′:5′-cyclic GMP accumulation in *Dictyostelium discoideum*. Proc Natl Acad Sci USA **74**: 2348–2351

Mato JM, Van Haastert PJM, Krens FA, Rhijnsburger EH, Dobbe FC, Konijn TM (1977b) Cyclic AMP and folic acid mediated cyclic GMP accumulation in *Dictyostelium discoideum*. FEBS Lett **79**: 331–336

Meima ME, Biondi RM, Schaap P (2002) Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants. Mol Biol Cell **13**: 3870–3877

Natarajan K, Ashley CA, Hadwiger JA (2000) Related Galpha subunits play opposing roles during *Dictyostelium* development. Differentiation **66**: 136–146

Nebl T, Fisher PR (1997) Intracellular Ca^{2+} signals in *Dictyostelium* chemotaxis are mediated exclusively by Ca^{2+} influx. J Cell Sci **110**: 2845–2853

Oyama M (1991) Reducing reagent-induced activation of guanylate cyclase in the cellular slime mold, *Dictyostelium discoideum*. J Biochem (Tokyo) **110**: 934–938

Oyama M (1996) cGMP accumulation induced by hypertonic stress in *Dictyostelium discoideum*. J Biol Chem **271**: 5574–5579

Oyama M, Kubota K, Okamoto K (1991) Regulation of guanylate cyclase by a guanine nucleotide binding protein, G alpha 2, in *Dictyostelium discoideum*. Biochem Biophys Res Commun **176**: 1245–1249

Padh H, Brenner M (1984) Studies of the guanylate cyclase of the social amoeba *Dictyostelium discoideum*. Arch Biochem Biophys **229**: 73–80

Pan P, Hall EM, Bonner JT (1972) Folic acid as second chemotactic substance in the cellular slime moulds. Nat New Biol **237**: 181–182

Rifkin JL (2002) Quantitative analysis of the behavior of *Dictyostelium discoideum* amoebae: stringency of pteridine reception. Cell Motil Cytoskeleton **51**: 39–48

Roelofs J, Van Haastert PJM (2002a) Characterization of two unusual guanylyl cyclases from *Dictyostelium*. J Biol Chem **277**: 9167–9174

Roelofs J, Van Haastert PJM (2002b) Deducing the origin of soluble adenylyl cyclase, a gene lost in multiple lineages. Mol Biol Evol **19**: 2239–2246

Roelofs J, Meima M, Schaap P, Van Haastert PJM (2001a) The *Dictyostelium* homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase. EMBO J **20**: 4341–4348

Roelofs J, Snippe H, Kleineidam RG, Van Haastert PJM (2001b) Guanylate cyclase in *Dictyostelium discoideum* with the topology of mammalian adenylate cyclase. Biochem J **354**: 697–706

Roelofs J, Smith JL, Van Haastert PJM (2003) cGMP signalling: different ways to create a pathway. Trends Genet (in press)

Saraste M, Sibbald PR, Wittinghofer A (1990) The P-loop – a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci **15**: 430–434

Schulkes CC, Schoen CD, Arents JC, Van Driel R (1992) A soluble factor and GTP gamma S are required for *Dictyostelium discoideum* guanylate cyclase activity. Biochim Biophys Acta **1135**: 73–78

Schuster SS, Noegel AA, Oehme F, Gerisch G, Simon MI (1996) The hybrid histidine kinase DokA is part of the osmotic response system of *Dictyostelium*. EMBO J **15**: 3880–3889

Stites J, Wessels D, Uhl A, Egelhoff T, Shutt D, Soll DR (1998) Phosphorylation of the *Dictyostelium* myosin II heavy chain is necessary for maintaining cellular polarity and suppressing turning during chemotaxis. Cell Motil Cytoskeleton **39**: 31–51

Tremblay J, Desjardins R, Hum D, Gutkowska J, Hamet P (2002) Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. Mol Cell Biochem **230**: 31–47

Valkema R, Van Haastert PJM (1992) Inhibition of receptor-stimulated guanylyl cyclase by intracellular calcium ions in *Dictyostelium* cells. Biochem Biophys Res Commun **186**: 263–268

Van Haastert PJM (1983) Relationship between adaptation of the folic acid and the cAMP mediated cGMP response in *Dictyostelium*. Biochem Biophys Res Commun **115**: 130–136

Van Haastert PJM, de Wit RJ, Janssens PM, Kesbeke F, DeGoede J (1986) G-protein-mediated interconversions of cell-surface cAMP receptors and their involvement in excitation and desensitization of guanylate cyclase in *Dictyostelium discoideum*. J Biol Chem **261**: 6904–6911

Vaughan RA, Devreotes PN (1988) Ligand-induced phosphorylation of the cAMP receptor from *Dictyostelium discoideum*. J Biol Chem **263**: 14538–14543

Wedel B, Garbers D (2001) The guanylyl cyclase family at Y2K. Annu Rev Physiol **63**: 215–233

Wick U, Malchow D, Gerisch G (1978) Cyclic-AMP stimulated calcium influx into aggregating cells of *Dictyostelium discoideum*. Cell Biol Int Rep **2**: 71–79

Wu L, Valkema R, Van Haastert PJ, Devreotes PN (1995) The G protein beta subunit is essential for multiple responses to chemoattractants in *Dictyostelium*. J Cell Biol **129**: 1667–1675

Xiao Z, Yao Y, Long Y, Devreotes P (1999) Desensitization of G-protein-coupled receptors. agonist-in-

duced phosphorylation of the chemoattractant receptor cAR1 lowers its intrinsic affinity for cAMP. J Biol Chem **274**: 1440–1448

Yumura S, Furuya K, Takeuchi I (1996) Intracellular free calcium responses during chemotaxis of *Dictyostelium* cells. J Cell Sci **109**: 2673–2678

Zhang H, Wessels D, Fey P, Daniels K, Chisholm RL, Soll DR (2002) Phosphorylation of the myosin regulatory light chain plays a role in motility and polarity during *Dictyostelium* chemotaxis. J Cell Sci **115**: 1733–1747

Zhang N, Long Y, Devreotes PN (2001) Ggamma in *Dictyostelium*: its role in localization of gbetagamma to the membrane is required for chemotaxis in shallow gradients. Mol Biol Cell **12**: 3204–3213

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