# ELUCIDATING THE RAS ACTIVATION PATHWAYS IN RESPONSE TO CYCLIC AMP IN THE AGGREGATION OF *DICTYOSTELIUM DISCOIDEUM*

by

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# ABSTRACT

Ras proteins are highly conserved molecular switches that regulate cellular responses to external stimuli. *Dictyostelium discoideum* contains an extensive family of Ras proteins that function in growth, cell type differentiation, and more. While there is much genetic evidence for the involvement of Ras proteins in these behaviours, little is known about the events that lead to their activation.

The primary objective of this thesis project was to develop an efficient and rapid assay that allowed the measurement of activated *Dictyostelium* Ras proteins. Modification of an assay used successfully to measure activated Ras in mammalian cells demonstrated that the Ras Binding Domain (RBD) of the *Schizosaccharomyces pombe* Ras effector protein, Byr2, was able to bind to RasB, RasC, and RasG. Furthermore, the use of dominant negative Ras mutants and nucleotide loading of cellular lysates showed that Byr2 (RBD) bound preferentially to Ras-GTP (activated Ras) over Ras-GDP (quiescent Ras). The 'RBD binding assay' was applied to elucidate the Ras activation pathways stimulated by cAMP during *Dictyostelium* aggregation.

Previous experiments have suggested that RasC is involved in cAMP stimulated signalling events during *Dictyostelium* aggregation, and this was confirmed using the RBD binding assay, as levels of RasC-GTP displayed a rapid and transient increase upon cAMP stimulation. Similarly, activated RasG also showed an increase in activation in response to cAMP. Through the use of various mutant strains, it was demonstrated that RasC and RasG activation is downstream of the cAMP receptor and its associated heterotrimeric G-protein.

This thesis also provides evidence that the *Dictyostelium* Ras guanine nucleotide exchange factor (RasGEF) proteins can each act on specific Ras proteins. RasC activation was

abolished in a *gefA*⁻ mutant, while RasG activation was normal, indicating that RasGEFA activates RasC, but not RasG. Conversely, RasC activation was normal in *gefM*⁻ and *gefR*⁻ mutants, whereas RasG activation was reduced in each strain, indicating that RasGEFM and RasGEFR activate RasG. Analysis of PKB activation suggests that RasGEFM is necessary for the activation of RasG in the pathways leading to PKB activation. Observing the streaming behaviour of aggregating *gefR*⁻ cells has suggested that RasGEFR may play a role in regulating chemotaxis.

"If we could just tap into the collective unconscious of the amoeba -- then we'd know how it all began."

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# List of Abbreviations

| | |
|---|---|
| A | ampere or amp, unit measure for electric current |
| ACA | adenylyl cyclase |
| AMP | adenosine monophosphate |
| ATP | adenosine triphosphate |
| b, bp | base, base pair |
| cAMP | adenosine 3',5'-cyclic monophosphate |
| cAR | cAMP receptor |
| CFP | cyan fluorescent protein |
| cGMP | guanosine 3′,5′-cyclic monophosphate |
| CRAC | cytosolic regulator of adenylyl cyclase |
| DAG | diacylglycerol |
| dNTP | deoxynucleoside triphosphate |
| EDTA | ethylene diamine tetraacetic acid |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| ERK | extracellular regulated protein kinase |
| g | gravity, unit measure for centrifugal force |
| GAP | GTPase activating protein |
| GC | guanylyl cyclase |
| GDP | guanosine 5'-diphosphate |
| GEF | guanine nucleotide exchange factor |
| GFP | green fluorescent protein |
| GPCR | G-protein coupled receptor |
| GST | glutathione-S-transferase |
| GTP | guanosine 5'-triphosphate |
| GTPγS | non-hydrolysable GTP analogue |
| h | hour(s) |
| HRP | horseradish peroxidase |

| | |
|---|---|
| HVR | hypervariable region |
| kDa | kilo Dalton |
| $KK_2$ | potassium based non-nutrient buffer |
| LPA | lysophosphatidic acid |
| MAP | mitogen activated protein |
| MEK | MAPK/ERK kinase |
| mGDP | fluorescent GDP analogue |
| min | minute(s) |
| PAGE | polyacrylamide gel electrophoresis |
| PH | pleckstrin homology |
| $PI(4,5)P_2$ | phosphatidylinositol 4,5-bisphosphate |
| $PI3,4,5)P_3$ | phosphatidylinositol 3,4,5-trisphosphate |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PTEN | phosphatase and tensin homolog |
| PTK | protein tyrosine kinase |
| RBD | Ras binding domain |
| RGS | regulator of G-protein signalling |
| rpm | revolutions per minute |
| RTK | receptor tyrosine kinase |
| s | second(s) |
| SDS | sodium dodecyl sulphate |
| SH2 | src homology 2 |
| SH3 | src homology 3 |
| SOS | Son of sevenless, a RasGEF |
| TLC | thin layer chromatography |
| V | volt(s), unit measure for electric potential |
| YFP | yellow fluorescent protein |

# ACKNOWLEDGEMENTS

I can clearly remember sitting in my third year industrial microbiology class, realizing that much of what is known about yeast genetics came about because we wanted to make better tasting beers. And that was the moment that my appetite for scientific research was first whetted. Many years, many beers, and many tears later, the long haul of my post-graduate degree is finally coming to a conclusion. And of course, there are many people who helped me along the way.

I'm not sure if I am writing this if my lovely and caring wife, Amy, wasn't with me every step of the way. Always there with heartfelt encouragement and thoughtful motivation, she is the driving force behind all of the positive things that have happened to me since I have met her. I can only hope to provide her with the same type of support that she has shown me through the years. And to my Lil' Man, for helping me keep my life in perspective, and for teaching me more than I could possibly teach him.

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Mahalo.

# 1 INTRODUCTION

## 1.1 Ras Superfamily

The Ras Superfamily of proteins comprises a family of small (20-40 kDa) GTP binding proteins. Members of this superfamily are highly conserved, and orthologs are found in all metazoans, yeast, slime mould, and plants (Takai et al., 2001). The Ras superfamily can be subdivided into 5 subfamilies on the basis of structural and functional similarities: **Ras** (gene expression, proliferation, differentiation); **Rho** (cytoskeletal rearrangement); **Rab** and **Arf** (vesicular transport, membrane trafficking); **Ran** (nuclear trafficking) (reviewed in Takai et al., 2001). The genes encoding Ras superfamily proteins are often mutated in cancers (Bourne et al., 1991), and thus Ras proteins have become a target for potential therapeutic agents (Fritz and Kaina, 2006; Shirai et al., 2007).

Proteins are grouped in the Ras superfamily if they share ~30% amino acid identity, specific conserved domains for GTP binding, and a small domain that is necessary for binding to downstream effector proteins known as the 'effector domain' (Bourne et al., 1991). Ras, Rho, and Rab members have a C-terminal motif that undergoes post-translational prenylation, serving as a membrane anchor (Section 1.5). Arf proteins have an N-terminal glycine residue that is modified with myristic acid and serves the same purpose as C-terminal prenylation, while Ran proteins are not subject to such post-translational modifications (Takai et al., 2001).

The Ras subfamily proteins were the first characterized members of the Ras superfamily, and have been the subject of intensive research due to their prominent involvement in oncogenesis. For the most part, Ras subfamily proteins serve to transduce extracellular signals into a wide array of intracellular signalling pathways by interacting with a diverse and catalytically distinct group of effector proteins. Members of the subfamily share ~50% amino

acid identity, and include H-Ras, N-Ras, K-Ras, R-Ras, Rap, Ral, and Rheb (Colicelli, 2004). The remainder of this thesis will deal with the topic of the Ras subfamily proteins, and hence the term 'Ras' will be used in reference to the Ras subfamily, unless otherwise noted.

## 1.2 Ras as a molecular switch

The function of Ras GTPases is to couple extracellular signals into intracellular responses. This function is carried out by adopting two conformations, one of which allows for transmission of the extracellular signal, whereas the other conformation does not. Thus, Ras GTPases are often referred to as molecular switches in that they function in a way that is analogous to a household light switch.

### 1.2.1 Monomeric GTPases

The signalling function of Ras is carried out by cycling between a GDP bound and a GTP bound conformation (Figure 1A). GDP release from Ras results in the binding of GTP due to the fact that GTP is more abundant in the cell (Van Dyke et al., 1977), and binding of GTP results in a conformational change to two regions of Ras referred to as Switch 1 (residues 21-40 relative to H-Ras) and Switch 2 (residues 57-75) (Figure 2) (Quilliam et al., 2002). As this conformational change facilitates binding of Ras with its various downstream effector proteins, Ras-GTP is typically viewed as being turned 'On' or activated. Hydrolysis of GTP to GDP by Ras returns Ras-GTP to Ras-GDP, effectively turning Ras 'Off.' Interconversion between Ras-GDP and Ras-GTP can happen spontaneously, but the half-times of these reactions are typically too slow to be physiologically relevant (Neal et al., 1988). Thus, the Ras activation cycle is catalyzed by two groups of proteins: guanine nucleotide exchange factors (GEFs) promote the release of GDP, and GTPase activating proteins (GAPs) enhance the GTPase activity of Ras-GTP. Therefore, the

concerted actions of these two groups of proteins are responsible for regulating the Ras activation cycle.

### 1.2.2 Heterotrimeric GTPases

The alpha subunit of the heterotrimeric G-protein family is related to the Ras protein superfamily, with a GTP binding domain that is structurally identical (Hamm, 1998). Given the importance of G-protein mediated signalling in *Dictyostelium* (Section 1.11), a brief description of the heterotrimeric GTPase cycle will be presented (Figure 1B). Heterotrimeric G-proteins are trimers consisting of alpha, beta, and gamma subunits (abbreviated as Gα, Gβ, and Gγ, respectively) and are associated with G-protein coupled receptors (GPCR). GPCRs are a large family of proteins with a canonical serpentine seven transmembrane domain, an intracellular cytoplasmic tail, and an extracellular ligand binding domain (reviewed in McCudden et al., 2005). Binding of a ligand induces activation of the Gα subunit by guanine nucleotide exchange, which is the GTP binding protein that also functions to couple the heterotrimeric G-protein to its GPCR, and activation of Gα leads to its dissociation from the Gβγ dimer. Gα and Gβγ can regulate signalling pathways independently, leading to the activation of enzymes such as adenylyl cyclases, phospholipases, and ion channels (Marinissen and Gutkind, 2001). The cycle is complete when GTP is hydrolysed to GDP, catalyzed by a group of proteins referred to as Regulators of G-protein signalling, or RGS. Gα-GDP then reassociates with Gβγ, terminating the signal.

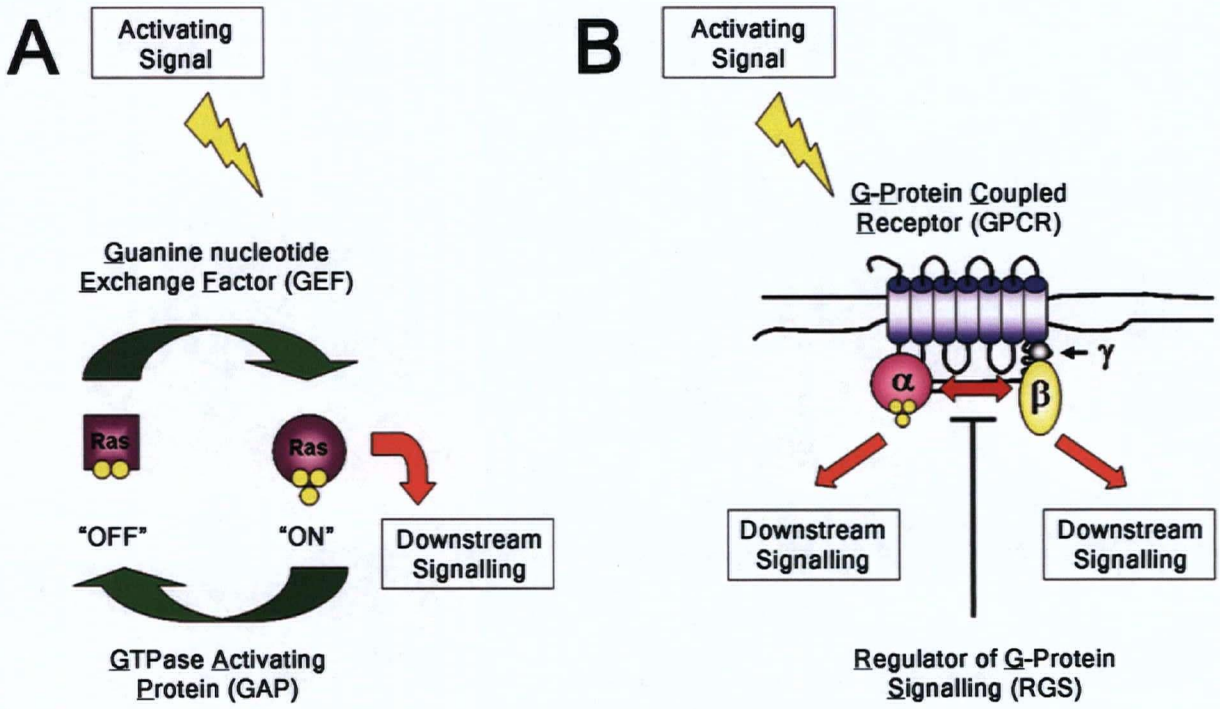


**Figure 1. The GTPase as a cellular switch.** (A) Model depicting the GTPase cycle of monomeric Ras superfamily proteins. Stimulation by an activating signal leads to displacement of GDP (depicted as two yellow circles) by GTP (depicted as three yellow circles), catalyzed by guanine nucleotide exchange factors (GEFs). GTP binding to Ras promotes a structural change facilitating the binding of Ras to various downstream effectors, propagating the signal. Ras is subsequently turned off through the action of GTPase activating proteins (GAPs), which enhance the intrinsic GTPase activity of Ras proteins. (B) Model depicting the GTPase cycle of heterotrimeric G-proteins. Heterotrimeric G-proteins consist of Gα, Gβ, and Gγ subunits. Gα couples the G-protein to a serpentine G-protein coupled receptor (GPCR), and Gα-GDP is associated with Gβ and Gγ, which functions as a dimer. Agonist binding to the GPCR, results in displacement of GDP by GTP to Gα, and Gα-GTP dissociates from the Gβγ dimer. Both subunits can participate in signalling events. The cycle is completed by regulators of G-protein signalling, which catalyze the Gα GTPase.

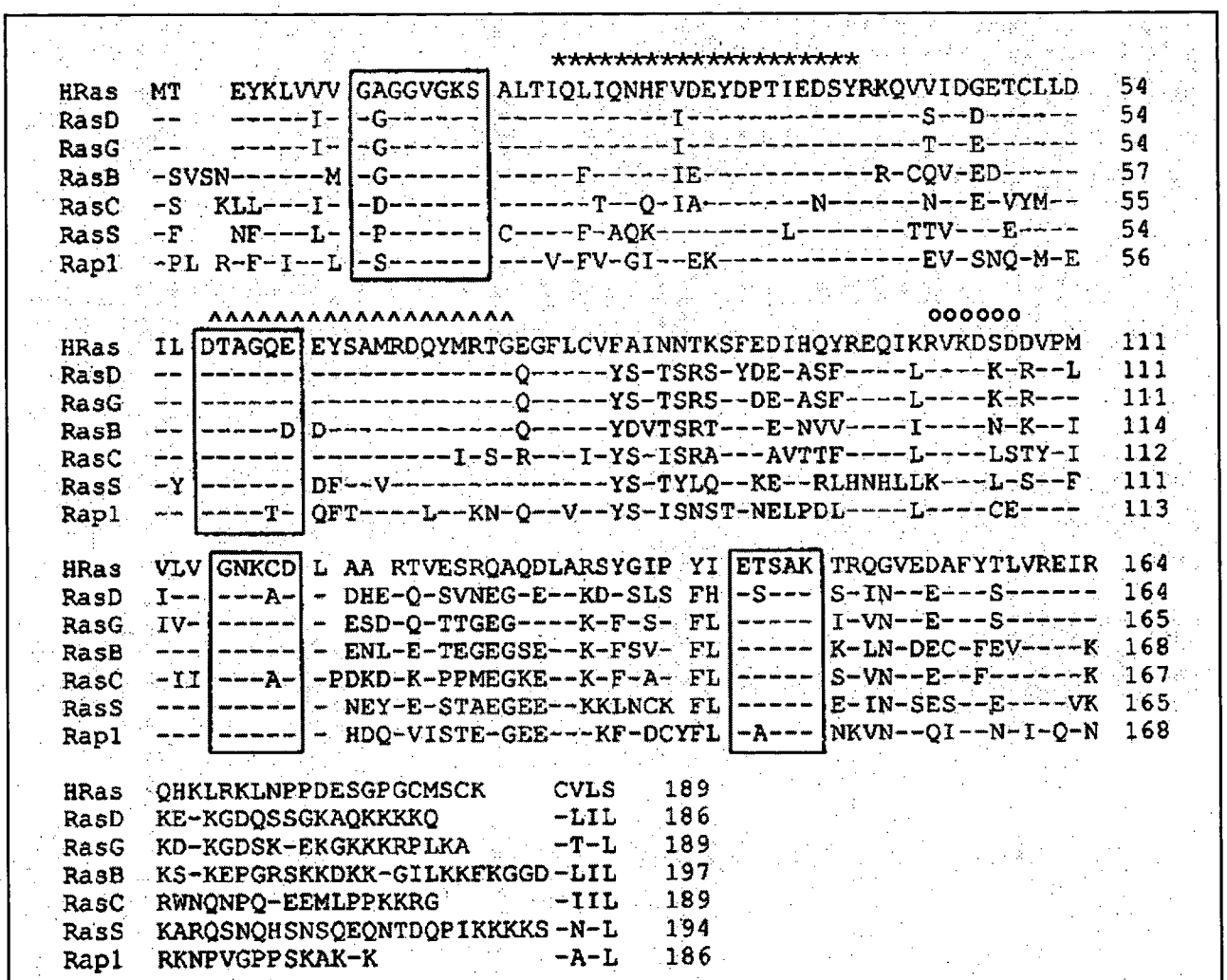


**Figure 2. Alignment of Ras protein sequences.** Gapped alignment of protein sequences of 6 *Dictyostelium* Ras subfamily proteins (RasB, RasC, RasD, RasG, RasS, Rap1) with mammalian H-Ras. Numbers indicate position of amino acids in protein, dashes indicate amino acids that are identical to those in H-Ras, and boxes refer to the conserved GTP-binding domain. Residues marked with a (*) represent the Switch 1 region, residues marked with a (^) represent the Switch 2 region, and residues marked with a (o) represent the α-helix 3 region. Figure modified from Daniel et al. (1995).

## 1.3 Activators of Ras proteins

The first RasGEF (CDC25p) was discovered in *Saccharomyces cerevisiae* in a genetic screen for components upstream of Ras (Broek et al., 1987). Biochemical confirmation of nucleotide exchange activity was shown a few years later (Jones et al., 1991). The first mammalian RasGEF cloned was SOS (Bowtell et al., 1992), discovered as a homologue of the *Drosophila* RasGEF, followed closely by the discoveries of the RasGEF, $CDC25^{Mm}$ (also known as RasGRF1) (Martegani et al., 1992), the RalGEF, RalGDS (Albright et al., 1993), and the RapGEF, C3G (Knudsen et al., 1994). As mutations in Ras contribute to a large number of diseases, it should come as no surprise that several RasGEFs have been implicated as well. Renal carcinoma cell lines display increased Ras activation, and the amplification of SOS has been suggested to play a role in this process (Shinohara et al., 1997). Insertional mutagenesis has revealed that amplification of the RapGEF, GRP2, induces acute myeloid leukemia in mice (Dupuy et al., 2001). Given that RasGEFs are often the limiting factor in Ras protein activation, their study has received much attention.

While RasGEFs are typically multi-domain containing proteins, one common feature is the presence of a catalytic domain with significant homology to CDC25p, and this domain is often referred to as the CDC25 domain. Sequence alignment of the ~250 residue domain was found to contain five structurally conserved regions (scr1-5) (Boguski and McCormick, 1993), and solution of a crystal structure of a SOS-Ras complex revealed that scr1-3 form the Ras binding pocket (Boriack-Sjodin et al., 1998). RasGEFs preferentially bind to Ras-GDP over Ras-GTP, and three regions of Ras have been implicated in the RasGEF-Ras interaction: Switch 1, Switch 2, and α-helix 3 (residues 102-107) (Figure 2). Mutational analysis has revealed that

the Switch 2 region is important for tight binding to the RasGEF, and this binding results in the displacement of Switch 1, leading to the disruption of the nucleotide binding site and ultimately to GDP release (Hall et al., 2001; Wittinghofer, 1998).

The family of RasGEFs is diverse and can be grouped into more than 10 different classes based on substrate specificity and structural similarities. SOS is ubiquitously expressed throughout mammalian tissues and cells, and believed to be the most commonly employed RasGEF, activating Ras in response to various growth factors via receptor tyrosine kinases (RTKs), GPCRs, and immune cell receptor activation (Nimnual and Bar-Sagi, 2002). RasGEFs can also be regulated by intracellular secondary messengers, creating a network of intertwined signalling pathways. For example, RasGRF1, expressed primarily in brain tissue, is regulated by the $Ca^{2+}$ binding protein calmodulin through an IQ (ilimaquinone) domain at its N-terminus (Farnsworth et al., 1995). The RasGRP family is regulated by binding to diacylglycerol (DAG), which serves to translocate RasGRP to the membrane. RasGRP is downstream of Phospholipase C $\gamma$-1 activation, and has been implicated in the growth and development of immune cells (Dower et al., 2000; Ebinu et al., 2000). The Epac (also referred to as cAMP-GEF) class of RasGEFs were isolated in a search of the human genome for sequences that contained both a RasGEF domain and a cAMP binding domain, and it has been shown in CHO cells that cAMP induces Rap1 activation by Epac1 (de Rooij et al., 1998). RasGEFs can also serve as dual specificity GEFs, catalyzing the activation of more than one GTPase. SOS, for example, contains a catalytically active RacGEF domain as well as its RasGEF domain, and expression of activated Ras (G12T) in COS-1 cells stimulates the RacGEF activity of SOS, suggesting that the RacGEF activity of SOS is regulated by its RasGEF activity (Nimnual et al., 1998). The dual specificity of SOS provides a mechanism for the regulation of Rac mediated cytoskeletal

rearrangement by Ras, and in general, dual specificity GEFs can link GTPase pathways, creating a network of GTPase signalling cascades.

## 1.4 Inhibitors of Ras activation

While much is known about GEFs and their role as regulators of Ras function, less is known about the GAPs that inhibit Ras. The reason for this is that GAPs were often thought to play a secondary role in Ras signalling, with their function being considered less important than that of the GEFs.

For the most part, GAPs for the different Ras subfamilies don't share much sequence similarities, whereas GAPs for Ras proteins within each subfamily are highly related (Boguski and McCormick, 1993). As a result of this similarity, GAPs are predicted to exhibit a high substrate preference for members of one Ras subfamily. Analysis of a crystal structure of a RasGAP-Ras-GTP complex revealed that RasGAPs bind to Ras-GTP at the Switch 1 and Switch 2 regions via a ~220 amino acid catalytic domain (Scheffzek et al., 1997). An 'arginine finger' (R789 in mammalian p120GAP) that is conserved in all RasGAPs (Bernards, 2003) inserts itself into the active site of Ras to help stabilize the transition state formed during GTP hydrolysis (Scheffzek et al., 1997).

Not much is known about how RasGAP activity is regulated *in vivo*, but like RasGEFs, RasGAP regulation is diversified (reviewed in Bernards, 2003). The most well studied mammalian RasGAP is p120 RasGAP, also referred to simply as RasGAP, a multidomain protein that includes SH2 (src homology 2), SH3 (src homology 3), and PH (pleckstrin homology) domains outside of the GAP catalytic domain. One study demonstrated that the SH2 domain mediates the recruitment of p120 RasGAP to activated RTKs in CHO cell lines (Kaplan

et al., 1990), although how this affects p120 RasGAP activity is unknown. However, if this translocation does facilitate GAP activation, this mechanism implies that the activation of Ras is coupled to the activation of its negative regulator since RTKs are often upstream of Ras activation. The RhoGAP, p190B, is regulated by phosphorylation, and it has been shown in mouse embryo-derived fibroblasts that p190B can be directly phosphorylated by the insulin receptor IGF-1 (Sordella et al., 2003). Phosphorylation of p190B induces the translocation of p190B to lipid raft containing plasma membrane, where activated Rho has been shown to accumulate. Again, this mechanism joins activation of a GTPase with the activation of its GAP.

## 1.5 Sub-cellular localization of Ras

The C-terminal sequences of Ras proteins are highly divergent, and hence the C-terminal ~25 amino acids of Ras is often called the hypervariable region (HVR) (Takai et al., 2001). This is also the domain that is responsible for membrane anchoring of Ras. The 4 C-terminal amino acid residues are referred to as the CaaX motif, where 'C' is a cysteine, 'a' refers to an aliphatic amino acid, and 'X' is any amino acid residue. Isoprenoids, such as farnesyl or geranylgeranyl, are incorporated at the cysteine residue following cleavage of the last three amino acids, and the nature of the prenylation motif can influence the localization of Ras (Pechlivanis and Kuhlmann, 2006). Fatty acids, especially palmitate, can also be added to Ras proteins at cysteine residues other than the terminal cysteine, also contributing to sub-cellular localization (Schroeder et al., 1997).

The fact that the HVR is so divergent suggests that Ras proteins exhibit different sub-cellular localization, and this has been shown to be true. H-Ras is associated equally with lipid raft and non-raft plasma membrane, and activation of H-Ras results in a shift to non-raft

membrane (Chen and Resh, 2001; Prior et al., 2001). K-Ras, on the other hand, is localized exclusively to non-raft plasma membrane (Prior et al., 2001), providing two separate platforms for the function of H-Ras and K-Ras. Ras activation has also been observed in non-plasma membrane domains. While Ras proteins have been visualized on the Golgi and ER membranes (Choy et al., 1999), it was not initially clear whether they contributed to signalling or exist there temporarily while being shuttled to the plasma membrane. The advent of fluorescent probes that can monitor Ras activation in live cells has demonstrated that the former is correct. EGF stimulation of COS-1 cells expressing wild type H-Ras demonstrated that Ras activation initially occurs at the plasma membrane, followed by a subsequent activation of Ras at the Golgi membrane (Chiu et al., 2002), demonstrating that Ras protein activation is not limited to the plasma membrane.

## 1.6 Ras activation by extracellular ligands

The canonical mammalian Ras activation pathway is that of the epidermal growth factor receptor (EGFR), belonging to the RTK family of proteins (Tan and Kim, 1999). RTKs consist of an extracellular domain required for ligand binding, one transmembrane domain that anchors the receptor to the membrane, and a cytoplasmic catalytic domain. The general mechanism for RTK activation begins with ligand binding, which induces homo-dimerization of the RTK and activation of the cytoplasmic kinase domain (reviewed in Olson and Marais, 2000). Activation of the kinase results in tyrosine phosphorylation of the cytoplasmic tails, and these phosphorylated tyrosines serve as docking sites for SH2 domain containing proteins, in particular Grb2, translocating these proteins from the cytosol to the activated receptor at the membrane. Grb2 is an adaptor protein consisting of a SH2 domain flanked by two SH3 domains, one of

which is constitutively associated with the RasGEF, SOS, through an interaction with a proline rich domain found at the C-terminus of SOS. If Ras is co-localized with the EGFR, translocation of Grb2 to the EGFR effectively serves to bring SOS in close proximity to Ras, allowing SOS to catalyze nucleotide exchange, thereby activating Ras.

Ras activation can also be mediated by antigen receptors such as those found on B-cell and T-cell lymphocytes (Downward et al., 1990). These receptors are hetero-dimers consisting of a large extracellular fragment, one transmembrane domain, and a short cytoplasmic tail. Ligand binding to the receptor recruits cellular protein tyrosine kinases (PTKs), which phosphorylate the cytoplasmic tails on tyrosine residues referred to as ITAMs (immunoreceptor tyrosine-based activation motifs). These phosphorylated tyrosine residues serve as docking sites for various SH2 domain containing proteins, ultimately leading to the recruitment of the Grb2-SOS complex to the membrane.

A third Ras activation pathway begins with GPCRs. One of the first GPCR agonists connected to Ras activation was the mitogen Lysophosphatidic acid (LPA) (Luttrell et al., 1997). The mechanism by which LPA binding to its receptor activates Ras is unclear, but a number of the signalling components have been elucidated. One model that has been proposed is referred to as 'transactivation' (Marinissen and Gutkind, 2001). This involves the activation of cellular PTKs, such as Src or Pyk2, which phosphorylate the EGF receptor, leading to recruitment of the Grb2-SOS complex, and activating Ras. Another pathway involves PI3K signalling. Yart et al. (2002) demonstrated that blocking PI3K$\gamma$ activity in Vero kidney epithelial cell lines through the use of dominant negative mutants results in a significant reduction in LPA stimulated Ras activation. Based on the reduction it was postulated that p110$\gamma$ activity generates phosphatidylinositol (3,4,5) trisphosphate ($PI(3,4,5)P_3$) at the membrane, which serve as docking

sites for the adaptor protein Gab1, and in turn leads to the recruitment of the Grb2-SOS complex to the site of Ras.

## 1.7 Ras effector signalling pathways

A large number of proteins have been characterized as Ras effector proteins, regulating many cellular processes. The first mammalian Ras effector, Raf1, was isolated in a yeast two-hybrid screen (Vojtek et al., 1993). Raf1 is a serine/threonine kinase, and it was demonstrated that Raf1 interacted with H-Ras (G12T), but not H-Ras (S17N) (Vojtek et al., 1993). Raf1 is recruited to the plasma membrane upon receptor stimulated Ras activation, and activated Raf1 initiates a phosphorylation cascade that proceeds to MEK, and then leads to activation of the ERK proteins (Arbabi and Maier, 2002). Activated ERKs then translocate to the nucleus to activate various transcription factors such as Elk1 and JUN, coupling Ras activation at the membrane to gene expression.

Another well characterized Ras effector is Phosphoinositide 3-Kinase (PI3K) (reviewed in Katso et al., 2001). A lipid kinase, activated PI3K phosphorylates $PI(4,5)P_2$ at the 3' position to generate $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ serves as a binding site for pleckstrin homology (PH) domain containing proteins, leading to the translocation of these proteins, usually from the cytosol, to the site of PI3K activity (Blomberg et al., 1999). An important effector of PI3K is the protein kinase PKB, also referred to as Akt (Vanhaesebroeck and Waterfield, 1999). While PH domain dependent translocation is required for PKB to function (Bellacosa et al., 1998), activation of PKB is stimulated by phosphorylation on two sites, T308 and S473. Phosphorylation on T308 is mediated by the PH domain containing kinase, PDK1 (Alessi et al., 1997), and phosphorylation on S473 is mediated by a complex of proteins, TOR Complex 2 or

TORC2 (Sarbassov et al., 2005). Activated PKB then phosphorylates downstream target proteins, including the pro-apoptotic group of transcription factors of the Forkhead family, inhibiting their function (Dijkers et al., 2002). PI3K products have also been shown to regulate RacGEF activity both *in vitro* (Han et al., 1998) and *in vivo* in COS-7 cell lines (Michiels et al., 1997), providing a mechanism for the role of PI3K in cell motility by inducing cytoskeletal rearrangement.

The RalGDS family, which includes RalGDS, Rgl, Rlf, and Rgr, functions as GEFs for the Ral family of GTPases, and it has been demonstrated that RalGDS promotes the activation of Ral in COS-7 cells expressing activated H-Ras, but not activated R-Ras or Rap1 (Urano et al., 1996), thereby stimulating mitogenesis. Phospholipase Cε (PLCε) is another important Ras effector. Expressing activated H-Ras in COS-7 cells stimulates PLCε activity (Kelley et al., 2001). The PLC family of proteins, of which only PLCε harbours a RBD, cleaves $PI(4,5)P_2$ at the plasma membrane to generate two important intracellular second messengers, diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP3). DAG subsequently leads to the activation of the protein kinase PKC, whereas IP3 stimulates the increase of intracellular $Ca^{2+}$. Thus, Ras can integrate DAG and IP3 stimulated pathways through the activation of PLCε. Other examples of Ras effectors are AF6 (inhibitor of cell proliferation in HEK293 cell lines;(Radziwill et al., 2003), Nore1 (tumor suppressor in A549 human lung tumor cell lines; (Vos et al., 2003), and p120 RasGAP (Ridley et al., 1993), which has been implicated in cytoskeletal rearrangement via the activation of the Rho family GTPases in Swiss 3T3 fibroblast cell lines. Thus, the cellular role of Ras involves a complex balance of a number of signalling pathways.

## 1.8 *Dictyostelium* as a model organism

*Dictyostelium discoideum* is a soil living amoeba where it feeds on bacteria in the wild, and is affectionately referred to as the 'social amoeba' because starvation initiates a developmental process that unites the normally solitary amoeba into a multicellular fruiting body containing ~100,000 cells. Belonging to the class Mycetozoa, rRNA analysis suggests that early Dictyostelids diverged before the evolution of plant, animals, and fungi (Embley and Martin, 2006), while protein based analyses place the divergence closer to the point of divergence of animals and plants (Baldauf, 2003).

While the evolutionary origin of *Dictyostelium* is somewhat cloudy, it does not deter from its role as a model organism. *Dictyostelium* is easily grown in the lab on both solid surfaces and in shaken culture with bacteria as a food source, and the generation of axenic strains has allowed for cultivation in liquid media, bypassing the need for providing bacteria as a food source. The *Dictyostelium* genome is haploid for most of its life cycle, and coupled with the tractability of its genome, allows for the targeted disruption of genes of study. The nature of the life cycle (Section 1.9) permits for growth and differentiation to be treated as independent processes, allowing for the characterization of growth specific or developmental specific genes. The *Dictyostelium* genome, which has been fully sequenced (Eichinger et al., 2005), encodes for many of the same components that are involved in human signalling, and it has proven to be useful for studying processes such as cytoskeletal organization, cell motility and chemotaxis, phagocytosis, and signal transduction (Affolter and Weijer, 2005; Aubry and Firtel, 1999; Franca-Koh et al., 2006; Lee et al., 2001; Manahan et al., 2004; Rupper and Cardelli, 2001). Finally, given the similarity of its feeding mechanism to that of human phagocytes, *Dictyostelium* has proven useful for studying human diseases caused by intracellular pathogens

such as *Legionella pneumophila*, *Mycobacterium marinum*, and *Salmonella typhimurium* (Williams et al., 2006).

## 1.9 The life cycle of *Dictyostelium discoideum*

The following section provides a brief overview of the *Dictyostelium discoideum* life cycle (Figure 3) (reviewed in Kessin, 2001). *Dictyostelium* lives as a single celled amoeba during the vegetative growth stage, feeding on bacteria, or in the case of the laboratory axenic strains, taking up nutrients from liquid media via pinocytosis. When the food source is depleted, cells within the population initiate the starvation response. Cells begin to emit pulses of cAMP ~6-7 minutes apart, which elicits a variety of responses, including the activation of the aggregation stage adenylyl cyclase (ACA), stimulating the synthesis of cAMP that functions as both an intracellular second messenger and as an extracellular signal to neighbouring cells, and the induction of the expression of genes required for aggregation and development. cAMP also functions as a chemoattractant. The relay of cAMP pulses allows for cell to cell communication such that up to 100,000 cells from as far away as 1 cm can coalesce together to form a 'loose aggregate.' Upon forming the aggregate, the mound tightens up and develops a tip (the 'tipped aggregate'). The tip elongates to form a vertical finger like structure, sometimes referred to as a 'standing slug,' which proceeds to fall over onto the surface and becomes a phototactic and thermotactic motile slug. In the slug, cells undergo a differentiation process that results in two general populations of cells, those destined to become spore cells (prespore cells), and those destined to become stalk cells (prestalk cells), with the tip of the slug consisting primarily of prestalk cells, and the rear ¾ of the slug consisting primarily of prespore cells. Development finalizes with the 'culmination' stage, a process that describes the transition from the slug stage

to the final fruiting body. This involves a rounding up of the slug, invagination of the stalk cells through the middle of the round disc, and a resulting lifting of the prespore cells off the substratum by the elongating stalk tube. During this time the cells undergo the final differentiation process; the prestalk cells make a cellulose sheath, vacuolate, and eventually die, whereas the prespore cells are encapsulated in a wall of polysaccharides and cellulose. The final fruiting body consists of a sorus of spores held up by a long stalk tube, supported at the bottom by a basal disc. The spores can be spread through the action of passing animals or insects, for example, and in response to favourable growth conditions, the spores germinate into vegetative amoebae. The full process of development from amoeba to fruiting body requires about 24 hours to complete.

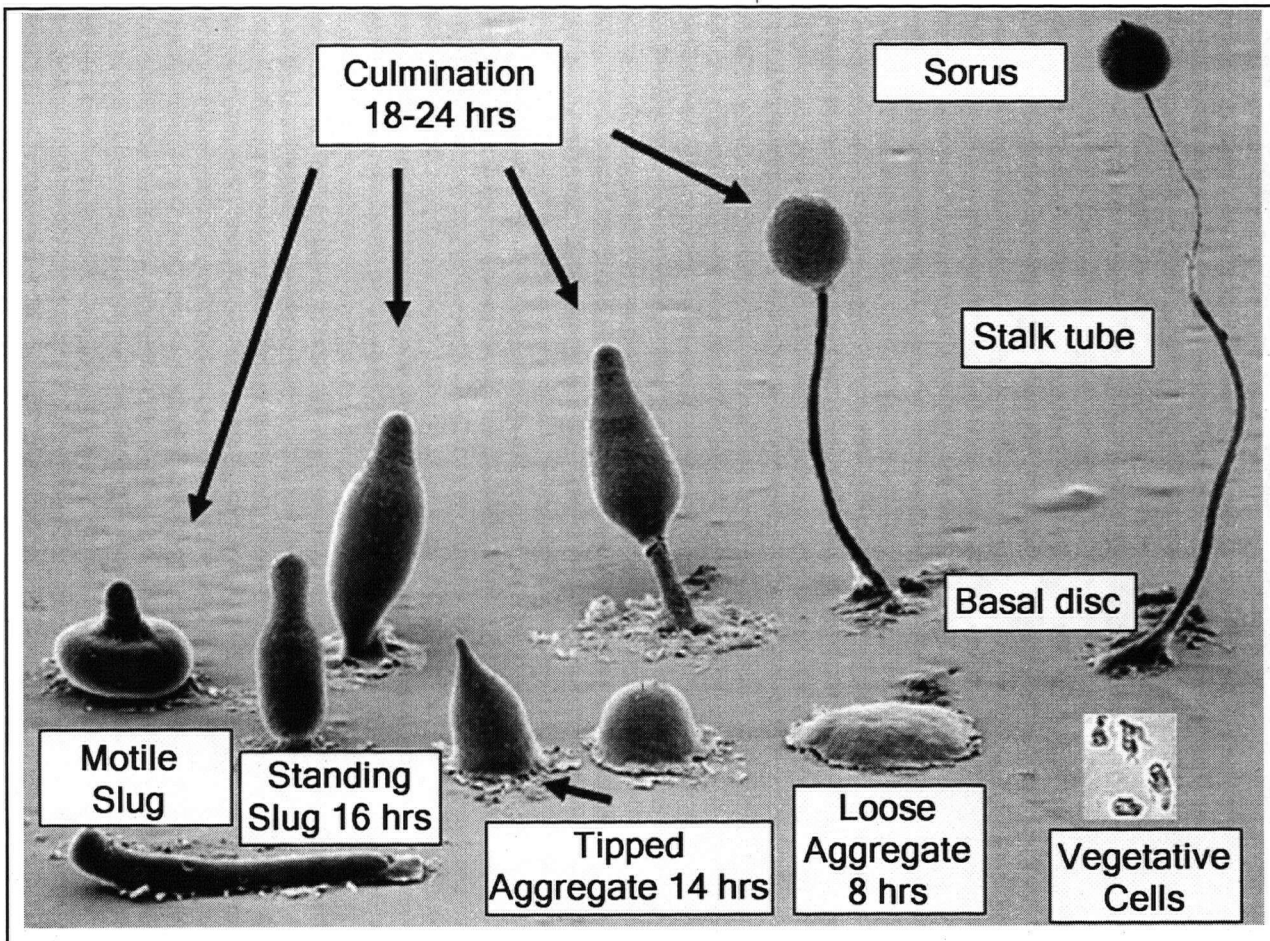


**Figure 3. The life cycle of *Dictyostelium discoideum*.** Scanning electron micrograph depicting multicellular developmental structures *Dictyostelium discoideum*. Life cycle begins with 'vegetative cells' at the lower right corner of the figure, and proceeds clockwise. Electron micrograph is by Mark J. Grimson and R. Lawrence Blanton of the Biological Sciences Electron Microscopy Laboratory, Texas Tech University. Printed with permission from R. Lawrence Blanton. 

## 1.10 *Dictyostelium* Ras proteins

The *Dictyostelium discoideum* genome contains 6 genes encoding for Ras subfamily proteins (Figure 2) that have been characterized to some extent: *rasB*, *rasC*, *rasD*, *rasG*, *rasS*, and *rapA* (summarized in Table 1), with at least 9 other putative *ras* genes present in the genomic database, including two more *rap* genes and one *rheb* gene (Weeks et al., 2005). This large number of *ras* genes is quite peculiar in that this number is almost half as many *ras* genes found in human (35) (Colicelli, 2004) despite the relatively small size of the *Dictyostelium* genome (~35 Mb) versus that of the human genome (~3.2 Gb). The *Dictyostelium* Ras proteins exhibit > 50% amino acid identity with mammalian H-Ras, with RasD and RasG sharing the highest similarity, 65% and 68%, respectively (Daniel et al., 1995). While the effector domains (residues 32-40 relative to H-Ras) of RasB, RasD, RasG and Rap1 are identical to H-Ras (Daniel et al., 1995), a number of variations exist when including the residues that account for the Switch 1 region (Figure 2). As residues from Switch 1 contribute to effector binding specificity, these differences would suggest that each Ras protein displays differential affinities for the same effector protein. The temporal regulation of expression is different for each of the characterized genes (Daniel et al., 1995), suggesting that each gene is required for mediating a specific set of functions during specific stages of the life cycle. Genetic analyses by targeted gene disruption have supported the notion that the *Dictyostelium* Ras proteins are not completely functionally redundant (reviewed in Weeks and Spiegelman, 2003; reviewed in Wilkins and Insall, 2001).

**Table 1. Summary of *Dictyostelium* Ras proteins and their proposed function.**

| Protein | Gene | Function |
|---|---|---|
| RasB | *rasB* | Inability to generate knockout suggests gene is essential for viability. Reduced expression of *rasB* coincides with reduced growth. Expression of activated RasB leads to increased rate of nuclear division. |
| RasC | *rasC* | *rasC*$^-$ cells fail to aggregate due to inability to activate ACA during early development. |
| RasD | *rasD* | Expression of activated RasD suggests a role in cell type differentiation in late development. Phenotype of *rasD*$^-$ slug also suggests a role in photo- and thermotaxis. |
| RasG | *rasG* | Expressed primarily during growth, *rasG*$^-$ cells display defects in cytokinesis, cytoskeletal regulation, and growth in shaken suspension. |
| RasS | *rasS* | Loss of *rasS* leads to growth defects associated with a reduction in fluid phase endocytosis. |
| Rap1 | *rapA* | Inability to generate knockout suggests gene is essential for viability. Knockdown of *rapA* expression coincides with reduced growth rate and cell viability. |

The first *Dictyostelium ras* gene to be cloned in *Dictyostelium* was *rasD* (Reymond et al., 1984). The *rasD* mRNA is not expressed during vegetative growth, and only expressed at low levels during the aggregation stage (~8 hours from the onset of development), with maximal expression during culmination (~18-24 hours). When cells expressing activated RasD (G12T) are induced to undergo multicellular development, the aggregates exhibit an abnormal

morphology, with multiple tips protruding from the tipped aggregate (versus one tip for wild type cells), and development proceeding no further (Louis et al., 1997). Another consequence of expressing RasD (G12T) is an enhancement of stalk cell specific genes, and a concomitant reduction in spore cell specific genes (Louis et al., 1997). These observations suggested that RasD is intimately involved in the developmental process, and in particular responsible for regulating spore and stalk cell differentiation (Table 1). Thus, it was somewhat of a surprise that a population of *rasD*⁻ knockout cells developed normally with a wild type stalk cell to spore cell distribution, and the only obvious phenotype was a motile slug that was not thermo- or phototactic (Wilkins et al., 2000). The role of RasD in cell type differentiation may have been masked by partial compensation by the highly homologous RasG (87 % amino acid identity to RasD) as there is an increase in RasG protein level in strains lacking *rasD* (Khosla et al., 2000).

RasG is almost identical to RasD over the first 111 amino acids with only 4 differences (Figure 2) (Daniel et al., 1995). *rasG* mRNA is expressed predominantly during growth, with transcript levels coming down during aggregation, and not detectable by the 'standing slug' stage (16 hours) (Daniel et al., 1995). Consistent with the mRNA expression pattern that suggests RasG is important during vegetative growth, gene ablation of *rasG* results in cells that exhibit severe growth defects when grown axenically in suspension (Tuxworth et al., 1997). When grown in axenic medium on a solid surface, cells grow large and become multinucleate, indicating that *rasG*⁻ cells have defects in cytokinesis (Table 1). *rasG*⁻ cells also have defects in actin cytoskeleton regulation, in particular formation of lamellipodia, filopodia, and reduced cell motility (Tuxworth et al., 1997). RasD can compensate for some RasG functions, as expressing *rasD* from the *rasG* promoter in *rasG*⁻ cells results in rescue of the vegetative growth and cytokinesis defects (Khosla et al., 2000). However, RasD cannot fully compensate for the loss of

RasG as the expression of *rasD* from the *rasG* promoter in *rasG*⁻ cells does not rescue the defects in cell motility.

RasB is a unique protein in that it has been linked to the regulation of mitosis and immunofluorescence studies have revealed that RasB localizes to the nucleus (Sutherland et al., 2001), both novel attributes for Ras subfamily proteins. For most of the cell cycle, RasB is found in the nucleus; however, from metaphase to telophase of mitosis, RasB exits the nucleus to the cytosol, returning only once mitosis is complete. Expression of activated RasB (G15T), the equivalent of the activating H-Ras (G12T) mutation, results in cells that display an increased rate of nuclear division relative to the rate of cell division. Lending more evidence to the importance of RasB in regulating the cell cycle is the apparent inability to disrupt the *rasB* gene (Sutherland et al., 2001). An initial population of what appeared to be *rasB*⁻ cells grew extremely slowly, and displayed undetectable levels of RasB protein. Eventually, a subpopulation of cells was able to outgrow the slow growing cells, and this reversion to normal growth coincided with the restoration of RasB protein levels. Subsequent genetic studies demonstrated that this population of cells was able to undergo a recombination event that restored the expression of the *rasB* gene while still maintaining antibiotic resistance. These data suggest that RasB is essential and required for maintenance of normal cell division (Table 1).

RasC is required for aggregation of *Dictyostelium*, as evidenced by the phenotype of a strain with a disruption of the *rasC* gene (Lim et al., 2001). Further analysis demonstrated that the aggregation defect is due to an inability to produce cAMP upon receptor stimulation (Table 1). Supplying *rasC*⁻ cells with an exogenous supply of cAMP rescued the aggregation defect, suggesting that the primary function for RasC is to regulate ACA activity. *rasC*⁻ cells that have been starved without cAMP pulses chemotax to cAMP poorly. Supplying cAMP exogenously to

starving *rasC*⁻ cells results in a hyper-polar cell, relative to wild type (Lim et al., 2001), suggesting that RasC may also regulate aspects of cell polarity or cytoskeletal organization during chemotaxis.

The expression pattern of *rasS* mRNA is similar to that of *rasG*, and disruption of *rasS* leads to cells that have defects in growth, endocytosis, and motility (Chubb et al., 2000). Unlike *rasG*⁻ cells, whose growth defects are primarily due to defects in cytokinesis, the growth defect of *rasS*⁻ cells is a dramatically reduced rate of fluid phase endocytosis. *rasS*⁻ cells are also elongated and exhibit a faster rate of motility, which coincides with an abundance of F-actin localized to the pseudopod. Chubb et al. (2000) proposed a model in which RasS regulates F-actin re-arrangement, and the need for actin in both feeding and motility means that one behaviour will be compromised by the function of the other. Thus, the high motility of *rasS*⁻ cells is proposed to inhibit endocytosis for feeding (Table 1).

The gene encoding Rap1, *rapA*, exhibits a biphasic expression pattern with one peak during aggregation (~8 hours) and a second peak during culmination (18 hours) (Robbins et al., 1990). Like *rasB*, attempts at disrupting *rapA* have been unsuccessful, suggesting that *rapA* is essential for cell survival (Kang et al., 2002). Studies using inducible antisense constructs showed that a decrease in Rap1 protein levels coincides with a reduction in growth rate and cell viability, supporting a role for Rap1 in cell viability (Table 1).

## 1.11 cAMP signalling during *Dictyostelium* aggregation

As this thesis is primarily concerned with the events that occur during aggregation (the term 'early development' will be used interchangeably with the aggregation stage), this section will provide an overview of the cAMP stimulated signalling events during this time (summarized in Figure 4). cAMP signal transduction is required for the cAMP relay (ACA activation) and for chemotaxis and motility. The latter employs components orthologous to that of mammalian signalling (reviewed in Manahan et al., 2004).

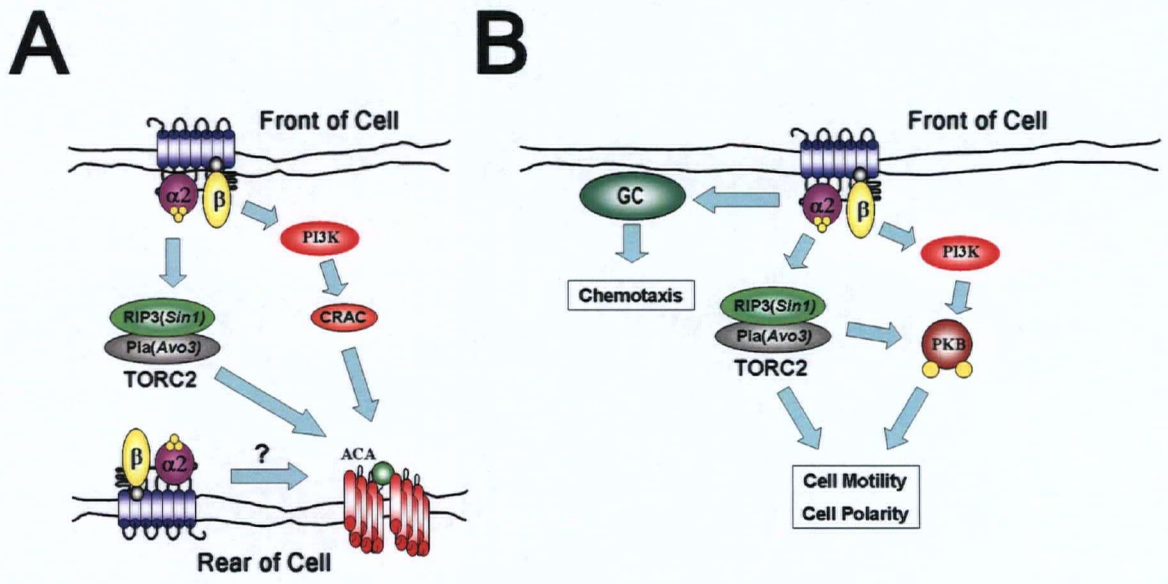


**Figure 4. The cAMP stimulated pathways during *Dictyostelium* aggregation.** (A) Schematic of the cAMP stimulated signalling pathway leading to the activation of ACA. Both the PI3K and TORC2 pathways are required for ACA activation downstream of the cAMP receptor complex. PI3K activity results in the translocation of the PH domain containing protein CRAC from the cytosol to the membrane. RasC is also required for ACA activation, but it is not clear how RasC interacts with these pathways as both PI3K and TORC2 appear to be downstream of RasG (Section 1.12). As ACA activation occurs at the rear of cells, it is possible that a signal is transmitted by RasC to ACA at the rear. (B) Schematic of the cAMP simulated pathways regulating chemotaxis, cell motility and cell polarity. As with the pathways to ACA activation, both the PI3K and TORC2 are involved in cell motility and establishing cell polarity. PI3K activity results in the translocation of the PH domain containing protein PKB from the cytosol to the membrane. A separate pathway leading to the activation of GC is also required for the chemotaxis behaviour.

The primary receptor for cAMP at the aggregation stage, cAR1, is a typical GPCR, and is thought to mediate all cAMP stimulated events during aggregation (Pupillo et al., 1992). cAR1 is predominantly associated with the heterotrimeric G-protein involving the Gα2 and Gβγ subunits (Parent and Devreotes, 1996). Binding of cAMP stimulates the exchange of GDP for GTP on Gα2, which in turn leads to dissociation of Gα2 from Gβγ, and each of these are thought to initiate a separate signalling pathway; Gβγ is thought to activate PI3K and ACA (Parent and Devreotes, 1996; Wu et al., 1995), whereas Gα2 is thought to stimulate the pathway leading to the activation of the aggregation stage guanylyl cyclases, sGC and GCA (Roelofs et al., 2001; Roelofs and Van Haastert, 2002). ACA, like the mammalian counterpart, consists of six membrane spanning domains, two large cytoplasmic domains, and functions as a homodimer. However, unlike the mammalian adenylyl cyclase, the *Dictyostelium* ACA does not contain a consensus Gβγ binding site, and how Gβγ activates ACA is not certain. What is known is that two cytosolic components are required, the Cytosolic Regulator of Adenylyl Cyclase (CRAC) and Pianissimo (PiaA) (Chen et al., 1997; Lilly and Devreotes, 1994), and both of these proteins are necessary for ACA activation. CRAC is a PH domain containing protein and translocates to the front of migrating cells in response to cAMP, and this translocation is dependent on PI3K activity (Huang et al., 2003). PiaA is an orthologue of AVO3, and is a part of the TORC2 complex, along with TOR, LST8 and the *Dictyostelium* homologue of mammalian Sin1, RIP3 (Lee et al., 2005). The TORC2 complex is required for ACA activation, cell motility, and cell polarity, and there is evidence that demonstrates that RasG is involved in the regulation of this complex (Section 1.12).

cAMP stimulation of aggregation stage cells leads to a rapid production of cGMP, peaking ~10 s after stimulation. *Dictyostelium* expresses 2 guanylyl cyclases during aggregation,

GCA and sGC (Roelofs and Van Haastert, 2002), homologous to 12 transmembrane domain and soluble guanylyl cyclases, respectively, and collectively referred to as GC. The evidence suggests that sGC is the primary GC during aggregation as *gcaA*$^-$ cells produce normal levels of cGMP whereas *sgcA*$^-$ cells have a more dramatic reduction in cGMP production when stimulated with cAMP; a *sgcA*$^-$/*gca*$^-$ strain produces no measurable level of cGMP (Roelofs and Van Haastert, 2002). The role of cGMP as a second messenger is less clear. What is known is that cGMP is required for mobilizing myosin II to the rear of chemotaxing cells, and cells unable to produce cGMP have severe defects in chemotaxis efficiency (see below) (Bosgraaf et al., 2005; Roelofs and Van Haastert, 2002). A bioinformatic screen looking for cGMP binding proteins found only one protein, GbpC, with a demonstrated cGMP binding ability (Goldberg et al., 2002), and *gbpC*$^-$ cells have a phenotype similar to cells that are unable to produce cGMP (Bosgraaf et al., 2005), suggesting that GbpC is needed to transduce the cGMP signal. Mutational analysis indicates that a small G-protein is upstream of GC and downstream of Gα2 (Roelofs et al., 2001), and analysis of cAMP stimulated cGMP production in a *rasG*$^-$ strain suggests that RasG mediates GC activity (Bolourani et al., 2006).

An important mediator of the intracellular cAMP signal is the cAMP dependent protein kinase, PKA. PKA is a heterodimer consisting of a catalytic kinase subunit, PKA-C, and a regulatory subunit, PKA-R (Saran et al., 2002). In the absence of a signal, PKA-C and PKA-R are associated, inhibiting PKA activity. Binding of cAMP to PKA-R forces dissociation of PKA-R from PKA-C, leading to the activation of PKA, and deletion of *pkaR* removes the cAMP dependent regulation of PKA activity, resulting in constitutive PKA activity (Zhang et al., 2003). PKA is required for all stages of development, including the transition from growth to development (Schulkes and Schaap, 1995), regulating intracellular cAMP degradation (Shaulsky

and Loomis, 1993), and terminal maturation of spore and stalk cells (Harwood et al., 1992; Mann et al., 1994). One role of PKA during aggregation is as a regulator of RegA, an intracellular cAMP specific phosphodiesterase that breaks down the cAMP generated by ACA (Shaulsky and Loomis, 1993). PKA is also involved in chemotaxis and cell motility, as constitutive PKA activity results in cells that display reduced cell speed and a loss of cell polarity (Zhang et al., 2003). However, the lack of known downstream substrates has prevented elucidating the role of PKA in chemotaxis.

cAMP stimulation of the PI3K pathway has been suggested to be downstream of Gβγ (Sasaki and Firtel, 2006). The pathway is complicated because *Dictyostelium* encodes at least five proteins that are similar to mammalian type I PI3Ks (Hoeller and Kay, 2007; Zhou et al., 1995). *Dictyostelium* PI3K1 and PI3K2 (encoded by *pikA* and *pikB*, respectively) are closely related orthologues of mammalian p110 PI3Ks (Zhou et al., 1995), and cAMP stimulation of aggregation stage cells leads to an increase in $PI(3,4,5)P_3$ at the front of the cell, and a concomitant translocation of PH domain containing proteins such as PKB and CRAC to the site of PI3K activity (Huang et al., 2003; Loovers et al., 2006; Meili et al., 1999). A *pikA*$^-$/*pikB*$^-$ strain exhibits dramatically reduced $PI(3,4,5)P_3$ accumulation, and a defect in the cAMP stimulated activation of ACA (Funamoto et al., 2002), presumably due to the loss of CRAC function in this mutant.

There has been significant controversy surrounding the role of PI3K as a mediator of chemotaxis (reviewed in Sasaki and Firtel, 2006). PI3K1 and PI3K2 both translocate to the front of migrating cells when placed in a gradient of cAMP, and *pikA*$^-$/*pikB*$^-$ cells are poorly polarized and chemotax very slowly to the source of chemoattractant (Funamoto et al., 2002). Additionally, constitutive localization of PI3K1 to the entire cell periphery results in cells that

extend pseudopods in all directions (Funamoto et al., 2002). PTEN (encoded by the *ptenA* gene) is a lipid phosphatase that converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$, effectively reversing the action of PI3K. *Dictyostelium* PTEN shows an intracellular translocation that is reciprocal to PI3K, i.e. towards the rear and sides of migrating cells. Cells that over-express *ptenA* have suppressed pseudopod formation at the lateral sides of the cell while chemotaxing, appearing hyper-polar relative to wild type cells (Funamoto et al., 2002). This led to a model of 'Local Excitation, Global Inhibition' (LEGI) in which PI3K plays the central role in chemotaxis (Ma et al., 2004). In this model, PI3K orients the cell by generating $PI(3,4,5)P_3$ at the front of a chemotaxing cell (local excitation). This small gradient is then amplified through the action of PTEN (global inhibition), and the resulting polarity places the appropriate machinery into proper position to allow chemotaxis to occur.

Recent studies have refuted the role of PI3K as a mediator of chemotaxis. Chemotactic index is a quantitative measure of chemotaxis efficiency, and can be simply defined as the net displacement of a cell towards the source of the chemoattractant divided by the total displacement of the cell (Wessels et al., 2004), i.e. a cell that travels only in the direction of the chemoattractant will have a C.I. of one, whereas a cell that moves back and forth but makes no net movement towards the chemoattractant source will have a C.I. of zero. Thus, C.I. can be used to separate defects in motility from defects in chemotaxis. Quantitative analysis of chemotaxing cells revealed that there was no difference in C.I. between *pikA*$^-$*/pikB*$^-$ cells and wild type cells, but there was a significant loss in cell speed and cell polarity, indicating that the defects in chemotaxis were actually defects in motility and polarity (Chen et al., 2007). Hoeller and Kay corroborate this report by observing that a *Dictyostelium* strain in which all five type-1

PI3K homologues have been knocked out shows no defect in C.I. when chemotaxing to cAMP, but suffers a significant decrease in the rate of motility (Hoeller and Kay, 2007).

So what might be the controlling element for chemotaxis? A *gcaA*$^-$/*sgcA*$^-$ mutant that is defective in the production of cGMP, and a *gbpC*$^-$ mutant that is defective in transmitting signalling events downstream of cGMP production, both have a C.I. close to zero while chemotaxing to cAMP (Bosgraaf et al., 2002; Bosgraaf et al., 2005). These mutants demonstrate that cGMP is important for the chemotactic response. cGMP is required for mobilizing myosin II to the cell cortex, but cells lacking myosin II are still able to chemotax, albeit slower relative to wild type cells (Shelden and Knecht, 1995). This observation suggests that the role of cGMP in mobilizing the myosin II cytoskeleton is distinct from its role in chemotaxis. Based on a quantitative analysis of pseudopod generation, a new model of chemotaxis has been proposed (Andrew and Insall, 2007). The 'Informed Choice' model was based on the observation that cells protrude pseudopods randomly, and these pseudopods often split, or bifurcate, with the pseudopod that orients itself spatially closer to the chemoattractant source becoming the dominant pseudopod, and a subsequent retraction of the spatially further pseudopod. Thus, small improvements in the direction of the leading pseudopod over time lead to efficient chemotaxis. While treatment of wild type cells with the PI3K inhibitor LY294002 did not affect the C.I. of chemotaxing cells, it did dramatically decrease the rate of pseudopod generation (Andrew and Insall, 2007). The same chemotactic behaviour was observed in human neutrophils and mouse embryonic fibroblasts treated with LY294002 (Andrew and Insall, 2007), suggesting a conserved mechanism regulating chemotaxis. Taken together, these data would suggest that PI3K is important in regulating the machinery involved in cell motility, but not in selecting the direction of cells during chemotaxis.

## 1.12 Involvement of Ras in aggregation

Aside from the *Dictyostelium* PI3Ks, orthologues of well established Ras effectors in mammalian signalling pathways, a number of Ras signalling components have been implicated in ACA activation and chemotaxis during early development. The first was the RasGEF, RasGEFA, formerly known as Aimless or AleA, as *gefA*$^-$ cells do not activate ACA in response to cAMP (Insall et al., 1996). Subsequent isolation of a *rasC*$^-$ cell line with a similar phenotype suggested that RasGEFA was the activator of RasC (Lim et al., 2001); however, no further genetic or biochemical data was able to corroborate this. Another protein that was found to be necessary for ACA activation is Ras Interacting Protein 3, RIP3 (encoded by the *ripA* gene) (Lee et al., 1999). As the name implies, RIP3 harbours a Ras Binding Domain (RBD), and its involvement in ACA activation provided a possible signalling pathway consisting of RasGEFA to RasC to RIP3. However, RIP3 was shown to interact specifically with RasG, and not RasB, RasC, RasD, or RasS (Lee et al., 1999). This was somewhat surprising as there had been no prior indication that RasG was involved in ACA activation, with any evidence being tenuous at best, prior to this finding. RIP3 is part of the TORC2 complex of proteins, and along with the defects in ACA activation, *ripA*$^-$ cells don't aggregate and chemotax slower than wild type cells (Lee et al., 2005). A RIP3 protein that bears two point mutations in the RBD, K680E and R681E, does not bind to RasG (Lee et al., 2005). While over-expression of RIP3 rescues the phenotypes of a *ripA*$^-$ strain, over-expression of RIP3 (K680E, R681E) does not rescue the phenotypes of a *ripA*$^-$ strain (Lee et al., 2005), demonstrating that binding of RasG to RIP3 is necessary for the function of TORC2. As both RasG and TORC2 have been shown to be involved in the cAMP stimulated activation of PKB (Bolourani et al., 2006; Lee et al., 2005), it

may be that binding of RasG to RIP3 functions to localize TORC2 to the membrane, facilitating PKB activation.

Two RasGEFs have been identified recently, and both demonstrate a role in early development. RasGEFM was identified in a genomic search for RasGEF sequences, and is required for ACA activation, but not GC activation (Arigoni et al., 2005). *gefM*$^-$ cells chemotax to cAMP poorly and do not polarize well. The observation that GC activity is unaffected in *gefM*$^-$ cells suggests that the poor chemotaxis is due to defects in cell motility and/or polarity. Another RasGEF implicated in early development is GbpD, isolated in a search for cGMP binding proteins (Goldberg et al., 2002). While disruption of *gbpD* does not affect C.I. dramatically relative to wild type cells, *gbpD*$^-$ cells are hyper-polarized when chemotaxing to cAMP (Bosgraaf et al., 2005), suggesting that GbpD regulates aspects of determining cell polarity, perhaps as a negative regulator. Given the morphologic similarities of chemotaxing *gbpD*$^-$ and *rasC*$^-$ cells (Bosgraaf et al., 2005; Lim et al., 2001), it is tempting to speculate that GbpD functions in the RasC pathway, perhaps as an activator of RasC.

## 1.13 Thesis Objective

At the time I began my thesis project, much genetic and biochemical data was emerging that shed light on Ras signalling in *Dictyostelium*; however, not much was known about activation of the Ras proteins themselves. Many of the connections to pathways were speculative, for example the link between RasGEFA and RasC, and the number of Ras pathway proteins identified began to create some confusion as to what the role of Ras really was.

The objective of this thesis was to characterize the Ras activation pathway during *Dictyostelium* early development. This stage of the life cycle was chosen because the cAMP

signalling pathway was the best described, and given the implication of Ras in cAMP stimulated signalling pathways (Section 1.12), it was hypothesized that Ras was activated during this process. In order to confirm the role of Ras in the signalling events during aggregation, it must first be demonstrated that Ras is activated upon stimulation by cAMP. To begin such an undertaking, an assay was needed that was able to measure activated *Dictyostelium* Ras proteins. The first part of this thesis project was to modify an existing assay that was developed for monitoring mammalian Ras activation, and had been already modified for measuring not only Ras, but other small GTPases in both mammalian and other model systems. The second part of this project was to employ this assay to characterize the upstream factors that are involved in Ras protein activation in order to elucidate the Ras signalling pathways during early development. Based on the available data, it would be expected that RasC is activated downstream of cAR1 and the $G\alpha 2\beta\gamma$ heterotrimeric G-protein, and activated RasC mediates the pathway leading to ACA activation as *rasC*$^-$ cells are unable to activate ACA in response to cAMP. Furthermore, the striking similarities of the phenotypes of the *gefA*$^-$ and *rasC*$^-$ cells suggest that RasGEFA activates RasC. While there is not enough evidence to strongly implicate another Ras protein in the cAMP signalling pathway, the fact that *rasC*$^-$ cells are able to chemotax efficiently to cAMP suggests that RasC is not the sole Ras responsible for mediating the downstream effects of cAMP.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Reagents

Restriction endonucleases, DNA polymerases and other DNA modifying enzymes were obtained from Invitrogen (Burlington, ON). Growth media reagents were from Oxoid (Nepean, ON), BBL (Cockeysville, MD), or BD Sciences (Mississauga, ON). All chemicals were obtained from Fisher (Ottawa, ON), Invitrogen (Burlington, ON), or Sigma (Oakville, ON), unless otherwise stated. All cloning and sub-cloning steps employed the use of chemical or electro-competent *Escherichia coli* strain XL1-Blue MRF' (Stratagene, LaJolla, CA). All protein expression vectors were transformed in *Escherichia coli* BL21 DE3 (Invitrogen). Hybond P polyvinylidene difluoride (PVDF) membranes, horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody, enhanced chemiluminescence (ECL), and ECL Plus reagents for immunoblot analysis were obtained from GE Healthcare (Baie d'Urfe, PQ).

### 2.1.2 Services

All DNA sequencing was performed by the Nucleic Acid and Protein Services (NAPS) unit (University of British Columbia, Vancouver, BC). All oligonucleotide synthesis was performed by Alpha DNA (Montreal, PQ).

**Table 2. *Dictyostelium* strain descriptions.**

| Strain | Description | Selection | Reference |
|---|---|---|---|
| Ax2 | Parental axenic strain for following strains. | | |
| Ax2::pVEII-*rasB* (G15T) | Ax2 transformed with pVEII encoding activated *rasB* (G15T) expressed from a folate repressible *discoidin* promoter. | 10 µg/ml G418 | (Sutherland et al., 2001) (Blusch et al., 1992) |
| Ax2::*rasC*-*rasC* (G13T) | Ax2 transformed with pJLW25 encoding activated *rasC* (G13T) constitutively expressed from a *rasC* promoter. | 10 µg/ml G418 | (Lim, 2002) |
| Ax2::MB-*rasG* (G12T) | Ax2 transformed with pMB38 encoding activated *rasG* (G12T) expressed from a tetracycline repressible TRE-$P_{min}$ promoter. | 10 µg/ml G418<br>5 µg/ml Blasticidin S | (Secko et al., 2004) (Blaauw et al., 2000) |
| Ax2::pVEII-*rasG* (G10V) | Ax2 transformed with pVEII encoding dominant negative *rasG* (G10V) expressed from a folate repressible *discoidin* promoter. | 10 µg/ml G418 | |
| Ax2::pVEII-*rasG* (S17N) | Ax2 transformed with pVEII encoding dominant negative *rasG* (S17N) expressed from a folate repressible *discoidin* promoter. | 10 µg/ml G418 | (Khosla et al., 1996) |
| *gefM*$^-$ | Chromosomal KO of *gefM* gene in Ax2. | 5 µg/ml Blasticidin S | (Arigoni et al., 2005) |
| Ax3 | Parental axenic strain for following strains. | | |
| Ax3::*act15*-*gbpD* | Ax3 transformed with pMB74 encoding *gbpD* expressed from a constitutive high expression *act15* promoter. | 10 µg/ml G418 | (Kortholt et al., 2006) |
| *acaA*$^-$ | Chromosomal KO of *acaA* gene in Ax3. | | (Pitt et al., 1992) |
| *carA-/carC*$^-$ | Chromosomal KO of *carA and carC* genes in Ax3. | | (Soede et al., 1994) |
| *dagA*$^-$ | Chromosomal KO of *dagA* gene in Ax3. | | (Lilly and Devreotes, 1994) |
| *gbpD*$^-$ | Chromosomal KO of *gbpD* gene in Ax3. | | (Kortholt et al., 2006) |
| *gefA*$^-$ | Chromosomal KO of *gefA* gene in Ax3. | | (Insall et al., 1996) |
| *gefC*$^-$ | Chromosomal KO of *gefC* gene in Ax3. | 5 µg/ml Blasticidin S | (Wilkins et al., 2005) |
| *gefD*$^-$ | Chromosomal KO of *gefD* gene in Ax3. | 5 µg/ml Blasticidin S | (Wilkins et al., 2005) |
| *gefR*$^-$ | Chromosomal KO of *gefR* gene in Ax3. | 5 µg/ml Blasticidin S | (Secko et al., 2004) |
| *gpaB*$^-$ | Chromosomal KO of *gpaB* gene in Ax3. | | (Wu et al., 1995) |
| *gpbA*$^-$ | Chromosomal KO of *gpbA* gene in Ax3 | | (Kumagai et al., 1991) |
| *piaA*$^-$ | Chromosomal KO of *piaA* gene in Ax3. | | (Chen et al., 1997) |

### 2.1.3 *Dictyostelium* strains

Various *Dictyostelium* strains were sourced for use in this thesis. *gefA*$^-$, *gefC*$^-$, *gefD*$^-$, *gefR*$^-$ and their wildtype parental strains (Table 2) were from Dr. Robert Insall (University of Birmingham, Birmingham, U.K.). *gefM*$^-$ and its parental strain (Table 2) were from Dr. Salvatore Bozzaro (University of Torino, Orbassano, Italy). *gbpD*$^-$ and Ax3::*act15-gbpD* and their parental strains (Table 2) were obtained from Dr. Peter Van Haastert (University of Groningen, Haren, The Netherlands). *acaA*$^-$, *carA*$^-$/*carC*$^-$, *dagA*$^-$, *gpaB*$^-$, *gpbA*$^-$, *piaA*$^-$ and their respective parental strains (Table 2) were obtained from the Dicty Stock Center (Columbia University, New York, NY).

### 2.1.4 Plasmids and DNA

Various plasmids and DNA were generously provided by the following investigators: pGEX-Raf encoding GST-Raf1 (RBD) from Dr. David Shalloway (Cornell University, Ithaca, NY); pSL333 and pSL335, encoding GST-PI3K1 (RBD) and GST-PI3K2 (RBD), respectively, from Dr. Richard A. Firtel (University of California at San Diego, La Jolla, CA); pGEX-RalGDS encoding GST-RalGDS (RBD) from Dr. Michael R. Gold (University of British Columbia, Vancouver, BC); Genomic DNA from *Schizosaccharomyces pombe* from Dr. Ivan Sadowski (University of British Columbia, Vancouver, BC).

## 2.2 Methods

### 2.2.1 Cell culture and development of *Dictyostelium discoideum*

*D. discoideum* cells were grown axenically in HL5 medium (14.3 g peptone, 7.15 g yeast extract, 15.4 g glucose, 0.96 g $Na_2HPO_4.7H_2O$ and 0.486 g $KH_2PO_4$ per litre of water) supplemented with 50 μg/ml streptomycin at 22°C, either in Nunclon tissue culture dishes (Nunc, Rochester, NY), or in suspension in flasks shaken at 160 rpm (Watts and Ashworth, 1970). Transformed strains were selected and maintained in HL5 media supplemented with the following antibiotics as appropriate: 5 μg/ml blasticidin S (Calbiochem, Darmstadt, Germany) or 10 μg/ml G418 (Table 2). Cells were counted using a haemocytometer. Cells were maintained on tissue culture dishes, and passaged by scraping confluent plates and diluting 10-40 times into fresh HL5 on tissue culture plates. For growing cells in shake suspension, confluent tissue culture plates were scraped and resuspended in 60 ml HL5, and shaken at 160 rpm. Experiments conducted on cells growing exponentially used cells at a density of 2-4 x $10^6$ cells/ml. For cryo-preservation, ~1 x $10^8$ cells were frozen in 1.0 ml aliquots of HL5 supplemented with 10% DMSO. All cells were maintained for no more than 8 passages before recovering fresh stocks from cryo-preserved stocks.

To observe aggregation streams, exponentially growing vegetative cells were washed three times with Bonner's Salts (10 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$), and seeded at 5 x $10^5$ cells/$cm^2$ in tissue culture dishes submerged under Bonner's salts.

Microscopic observations of streaming amoeba were performed using a Leica DM IL inverted microscope (Leica, Wetzler, Germany) fitted with a 4X objective lens. All images were captured using a Leica DFC350 F digital monochrome camera (Leica) in conjunction with Open Lab 4.0.4 software (Improvision, Lexington, MA).

### 2.2.2 Inducible expression of activated Ras proteins

Ax2::MB-*rasG* (G12T) (Table 2) expresses activated RasG from a tetracycline repressible promoter (Blaauw et al., 2000). Growth in HL5 supplemented with 5 μg/ml tetracycline represses expression of the protein. To induce expression, exponentially growing cells were harvested by centrifugation (500 x g, 5 min) and washed three times with HL5 without tetracycline. Cells were resuspended in HL5 without tetracycline at 2-4 x $10^6$ cells/ml, and shaken in flasks at 160 rpm. Maximal expression is in 4 hours (Secko et al., 2004).

Ax2::pVEII-*rasB* (G15T), Ax2::pVEII-*rasG* (G10V), Ax2::pVEII-*rasG* (S17N) (Table 2) express RasB and RasG mutants from a folate repressible *discoidin* promoter (Blusch et al., 1992). Growth in HL5 supplemented with 1 mM folate represses expression of the proteins. To induce expression, exponentially growing cells were harvested by centrifugation (500 x g, 5 min) and washed three times with HL5 without folate. Cells were resuspended in HL5 without folate at 5 x $10^5$ cells/ml, and shaken in flasks at 160 rpm. Maximal expression is within 48-60 hours (Khosla et al., 1996; Sutherland et al., 2001). As high cell density represses expression from the *discoidin* promoter, cells were diluted such that the cell density did not exceed 2 x $10^6$ cells/ml.

### 2.2.3 Preparation of pulsed cells

To prepare pulsed cells, exponentially growing cells were harvested by centrifugation (500 x g, 5 min), and washed three times with $KK_2$ (20 mM potassium phosphate, pH 6.1). Cells were resuspended at 5 x $10^6$ cells/ml in $KK_2$, and shaken in a flask at 160 rpm for 30 min. Cells were pulsed with 100 μl pulses of cAMP, as applied by using a Polystaltic pump (Buchler, Fort Lee, NJ), to a final concentration of 50 nM. Timing of the pulses was every 6 min, as controlled

by a Lab Controller timer (VWR, Mississauga, ON). After pulsing, cells were harvested by centrifugation (500 x g, 5 min), washed two times with $KK_2$, and resuspended at 5 x $10^7$ cells/ml in $KK_2$.

### 2.2.4 Cloning of GST-Byr2 (RBD)

The fragment encoding the RBD of the *byr2* gene product (encoding for amino acid residues 1-237) was amplified from *S. pombe* genomic DNA by PCR such that an *Eco*RI restriction site was engineered at the 5' end and a *Sal*I restriction site was engineered at the 3' end. The oligonucleotide primers used were as follows:

| | |
|---|---|
| Byr2 Forward | 5' GAA TTC ATG GAA TAT TAT ACC TCG AA 3' |
| Byr2 Reverse | 5' GTC GAC AAC GCC GAG AGT TTG ATA TTG T 3' |

Standard PCR reactions were conducted in 10 µl mixtures in glass capillary tubes containing 5.58 µl $H_20$, 1.0 µl 10X Idaho PCR buffer (500 mM Tris, pH 8.3, 2.5 mg/ml BSA, 20% (w/v) sucrose, 1 mM cresol red, 30 mM $MgCl_2$), 0.5 µl dNTPs (2.5 mM) (GE Healthcare), 1.0 µl of each oligonucleotide (2.5 mM), 1.0 µl *S. pombe* genomic DNA (250 ng/µl) and 0.12 µl *Taq* DNA polymerase (5 U/µl). The capillary tubes were set up in an Idaho Rapidcycler (Idaho Technologies, Idaho Falls, ID) and the following cycling parameters were employed:

- 2 cycles of D=92°C for 60 s, A=56°C for 7 s, E=72°C for 60 s
- 36 cycles of D=92°C for 1 s, A=56°C for 7 s, E=72°C for 60 s

- 1 cycle of E=72°C for 120 s

D is the denaturation temperature, A is the annealing temperature, E is the extension temperature. The temperature ramping rate, S, for all cycling was set at 6.0.

The PCR samples were resolved on 1.0 % agarose gel electrophoresis in TBE (89 mM Boric Acid, 89 mM Tris, 2 mM EDTA) buffer. The resulting ~700bp fragment was excised from the gel and extracted using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON), as per manufacturer's instructions. The extracted DNA fragment was ligated into a pGEM-T Easy (Promega, Madison, WI) cloning vector and transformed into electrocompetent *E. coli* XL1-Blue MRF'. The pGEM-T Easy-*byr2* (RBD) vector was purified from ampicillin resistant colonies using a QIAprep Spin Miniprep Kit (Qiagen), as per manufacturer's instructions, and sequenced to confirm the identity of the fragment. Plasmid DNA from one clone with an exact copy of the genomic *byr2* (RBD) was digested with *Eco*RI and *Sal*I to excise the DNA fragment. The pGEX 4T-1 expression vector (GE Healthcare) was digested with *Eco*RI and *Sal*I to allow for the directional cloning of the *byr2* (RBD) DNA fragment. The resulting digested DNA was resolved on 1 % agarose gel electrophoresis and the desired DNA fragments were extracted from the gel using a QIAquick Gel Extraction Kit. 100 ng of the digested pGEX 4T-1 were mixed with 42 ng of the *byr2* (RBD) DNA fragment (a 3:1 molar ratio) in a 10 µl ligation reaction using T4 DNA ligase and incubated overnight at 15°C, as per manufacturer's instructions. 4 µl of the ligation mixture were used to transform chemically competent *E. coli* BL21 DE3, and transformants were confirmed by sequencing.

### 2.2.5 Expression of GST-RBD fusion proteins

GST-RBD fusion proteins were prepared by the following procedure. One colony was picked and used to inoculate 5 ml of LB media supplemented with 50 μg/ml ampicillin. 2.5 ml of the overnight culture was used to inoculate 500 ml of LB media supplemented with 50 μg/ml ampicillin, and grown in shake flasks at 37°C until the $OD_{600}$ was 0.6–0.8. The cells were induced with 0.1 mM IPTG and grown for 18-20 hours at 22°C. Cells were harvested by centrifuging at 5,000 x g for 10 min. The bacterial pellet was resuspended in 10 ml STE buffer (10 mM Tris-CL (pH 8.0), 150 mM NaCl, 1 mM EDTA) and frozen overnight. The cell suspension was thawed and treated with lysozyme (0.05 mg/ml, final concentration) and incubated on ice for 30 min. 100 μl DTT (1 M) and 1.4 ml Sarkosyl (10 %) were added just prior to lysis. Cells were lysed on ice by sonication using an Ultrasonic processor XL (Misonix Inc., Farmingdale, NY) in 6, 10 s pulses at power setting 4, and then centrifuged at 17,000 x g for 30 min at 4°C. 2 ml Triton X-100 (20 %) was added to the supernatant and the total volume was brought up to 20 ml such that the final concentrations of Triton X-100 and Sarkosyl were 2 % and 0.7 %, respectively. The lysates were separated into 10 ml aliquots and snap frozen in a dry ice/ethanol bath.

### 2.2.6 Purification of GST-RBD fusion proteins

Glutathione-sepharose beads (GE Healthcare) are supplied in a 70 % bead, 30 % ethanol mixture. 665 μl of glutathione-sepharose beads (equivalent to 500 μl dry volume of beads) were washed three times with PBS (2.7 mM KCl, 1.4 mM $KH_2PO_4$, 4.3 mM $Na_2HPO_4 \cdot 12H_2O$, 137

mM NaCl, pH 7.5), and subsequently topped with 500 µl PBS to create a 50:50 bead to buffer slurry.

1 ml of a 50:50 glutathione-sepharose bead slurry was added to 10 ml bacterial lysates containing GST-RBD fusion protein, and tumbled end over end for 2 hours at 4°C. Beads were harvested by centrifugation (500 x g, 5 min, 4°C), washed 3 times with ice cold STE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA; 150 mM NaCl), and resuspended in 500 µl STE resulting in a 50:50 bead to buffer slurry. The quality of each preparation of the GST-RBD proteins was determined by testing the ability of each GST-RBD to bind to activated RasC and RasG over a range of increasing input cellular protein similar to that shown in Figure 10.

### 2.2.7 RBD Binding Assay

Exponentially growing cells were harvested by centrifugation (500 x g, 5 min), washed three times with $KK_2$, and resuspended at 5 x $10^7$ cells/ml in $KK_2$. Cells were lysed in an equal volume of cell suspension and ice cold 2X RBD-LB (20 mM Sodium Phosphate, pH 7.2, 2 % Triton X-100, 20 % Glycerol, 300 mM NaCl, 20 mM $MgCl_2$, 2 mM EDTA, 2 mM $Na_3VO_4$, 10 mM NaF, with two tablets of 'Complete protease inhibitor cocktail' (Roche, Basel, Switzerland) added per 50 ml buffer). For cAMP stimulated cells, 6 h pulsed cells were stimulated with 200 nM (final concentration) cAMP, and 400 µl aliquots were taken at the selected times, lysed in an equal volume of ice cold 2X RBD-LB, and incubated on ice for 5 min. Efficiency of cell lysis was estimated visually by using a haemocytometer. The lysates were cleared by centrifugation (13,000 x g, 10 min, 4°C). Protein concentrations were determined using the *DC* Protein Assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions, and lysates were diluted to a concentration of 1 mg/ml. 400 µg of protein lysates were incubated with 50 µl of 50:50 GST-

RBD bead slurry, and the mixture was tumbled end over end at 4°C for 1 h. Beads were harvested by centrifugation and washed 3 times in ice cold 1X RBD-LB. Beads were suctioned dry using a 22½G syringe needle (Beckton Dickinson, Franklin Lakes, NJ). 50 µL of 1X SDS-PAGE loading buffer (50 mM Tris-Cl, pH 6.8, 1.6 % (w/v) SDS, 100 mM DTT, 0.002 % (w/v) bromophenol blue, 5 % (v/v) glycerol) were added to the pelleted beads, and the mixture was heated at 100°C 5 min. A 10 µl sample of the lysates was added to 50 µl of 6X SDS-PAGE loading buffer. Approximately 8.3 µg of protein lysate were added to one lane of the SDS-PAGE gel and included as a load control to assess the level of total Ras protein. The samples from the RBD binding assay were loaded on the gel representing the pool of activated Ras from approximately 160 µg of total protein. Samples were analyzed for bound Ras proteins by immunoblot analysis.

### 2.2.8 Guanine nucleotide loading of Ras proteins

Guanine nucleotide loading of cell lysates was accomplished by modification of the protocol described by Taylor and Shalloway (1996). Exponentially growing cells were harvested by centrifugation (500 x g, 5 min), washed three times with $KK_2$, and resuspended at 5 x $10^7$ cells/ml in $KK_2$. Cells were lysed in an equal volume of cell suspension and ice cold 2X GTP-LB (20 mM Sodium Phosphate, pH 7.2, 2 % Triton X-100, 20 % Glycerol, 300 mM NaCl , 10 mM EDTA, 2 mM $Na_3VO_4$, 10 mM NaF, with two tablets of 'Complete protease inhibitor cocktail' (Roche) added per 50 ml buffer). Either 1 mM (final concentration) GDP or GTPγS, a non-hydrolysable GTP analogue, was added and incubated at room temperature for 10 min. $MgCl_2$ was added to a final concentration of 50 mM and incubated at room temperature for 30

min. Protein concentrations were determined using the *DC* Protein Assay, according to manufacturer's instructions, and subjected to the RBD binding assay.

### 2.2.9 PKB phosphorylation assay

6 h pulsed cells were resuspended at 5 x $10^7$ cells/ml in $KK_2$ supplemented with 1 mM caffeine, and shaken at 200 rpm for 30 min (Jeon et al., 2007). Caffeine treatment has been shown to block the cAMP relay (Brenner and Thoms, 1984), and is used to prevent auto stimulation by inhibiting endogenous cAMP pulsing, as a means to synchronize cells prior to stimulation. Cells were stimulated with 15 μM (final concentration) cAMP, and 100 μl samples were taken at the indicated time points and lysed in 20 μl 6X SDS-PAGE loading buffer. Samples were heated to 100°C for 5 min and phosphorylated PKB was detected by immunoblot analysis using a polyclonal anti-phospho-threonine specific antibody (Cell Signaling Technology, Danvers, MA). Phosphorylated PKB appears as a ~51 kDa band, and the identity of this band has been previously confirmed as PKB with the use of an anti-*Dictyostelium* PKB antibody (Lim et al., 2001).

### 2.2.10 Immunoblot Analysis

Protein samples were prepared by collecting cells by centrifugation (500 x g, 5 min) and lysing directly in 1X SDS-PAGE loading buffer. Other times, cells in suspension in $KK_2$ buffer were directly lysed by mixing with 6X SDS-PAGE loading buffer in a volume ratio of 5:1. Soluble lysate fractions were collected by mixing with 6X SDS-PAGE loading buffer in a

volume ratio of 5:1. Samples were boiled at 100°C for 5 min and used for discontinuous SDS-PAGE or stored at -86°C.

Discontinuous SDS-PAGE gels consists of an upper protein stacking gel (3.9 % bis-acrylamide (1:19), 125 mM Tris-Cl, pH 6.8, 0.1 % SDS, 0.03 % APS, 0.1 % TEMED) and a lower protein resolving gel (10 % bis-acrylamide (1:19), 375 mM Tris-Cl, pH 8.8, 0.1 % SDS, 0.03 % APS, 0.07 % TEMED). Samples were electrophoresed at 100 V for 100 min. The electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3. Separated proteins were blotted onto Hybond P PVDF membranes using a Mini Protean II electroblot apparatus (Bio-Rad) at 0.2 A for 1 h submerged in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). The gels were stained with Coomassie blue solution (0.025 % w/v Coomassie G-250, 10 % v/v acetic acid) and examined visually for equal loading and even transfer.

Membranes were incubated for 1 h at 22°C with gentle agitation, in blocking buffer consisting of 5 % w/v milk (non-fat dried powder) in TBS-T (50 mM Tris-Cl, pH 7.4, 154 mM NaCl, 0.1 % Tween20) when using Ras isoform specific antibodies, or in 5 % w/v bovine serum albumin (BSA) in TBS-T when probing for phosphorylated PKB, and then incubated with anti-Ras isoform specific primary antibodies in 1 % w/v non-fat milk powder/TBS-T overnight at 22°C or with anti-phospho-threonine antibodies in 5 % w/v BSA/TBS-T overnight at 4°C on a rotary shaker. Membranes were washed 3 times for 5 min each with TBS-T and incubated with HRP conjugated goat anti-rabbit secondary antibody (GE Healthcare) at 1:5000 dilution in 1 % w/v milk/TBS-T for 1 h at 22°C on a rotary shaker. Membranes were washed 3 times for 5 min each with TBS-T, developed with ECL or ECL Plus, as indicated, according to manufacturer's instructions and exposed to X-OMAT XK-1 X-ray film (Eastman Kodak Company, Rochester, NY) for the appropriate amount of time.

# 3 RESULTS

## 3.1 Selection, cloning, and induction of GST-RBD bacterial constructs

Taylor and Shalloway developed an assay that is able to measure the level of activated Ras from cellular lysates (Taylor and Shalloway, 1996). This assay is not too labour intensive, requiring as much work as an ordinary immunoprecipitation, and it does not require the use of radioactivity like previous methods used to study Ras activation (Satoh et al., 1988). The assay, referred to here as the 'RBD binding assay' (Figure 5), exploits the specific nature of the interaction between Ras and its effectors. The region of the effector proteins that binds to Ras is called the Ras binding Domain (RBD), and the RBD binds to Ras-GTP with an affinity that is three orders of magnitude greater than its affinity for Ras-GDP (Herrmann et al., 1995). Taylor and Shalloway fused the RBD of mammalian Raf1 to a Glutathione-S-transferase (GST) affinity tag and incubated this fusion protein with cellular lysates. By virtue of the GST affinity tag, the GST-RBD-Ras-GTP complex can be purified from cell extracts using glutathione-sepharose beads, and the bound Ras protein can be detected via immunoblot analysis using anti-Ras antibodies.

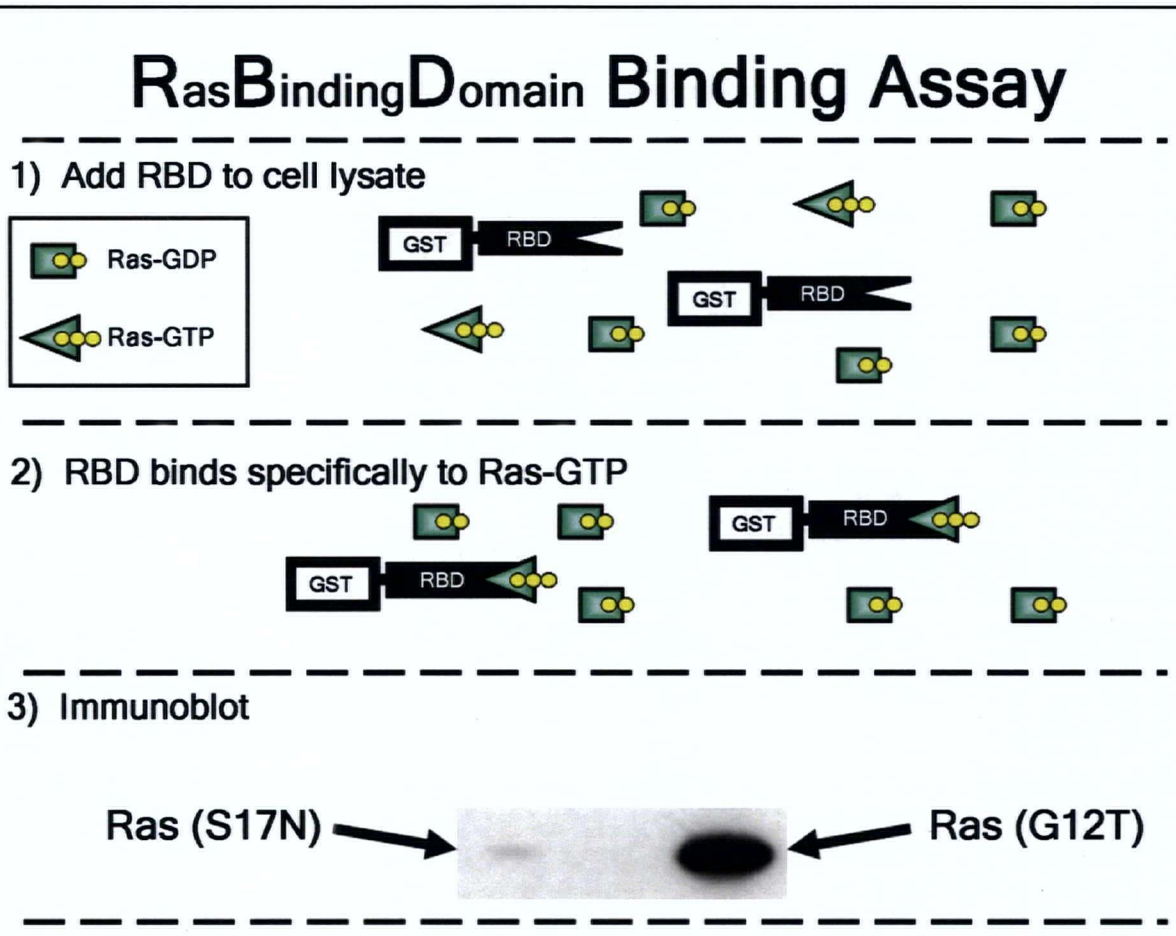


**Figure 5. The RBD Binding Assay.** The RBD Binding assay is based on the observation that the RBD of Ras effector proteins binds with a much higher affinity to Ras-GTP than Ras-GDP. The RBD is fused to a GST affinity tag, incubated with cellular lysates, and the GST-RBD-Ras-GTP complex can be purified using glutathione-sepharose beads. The affinity purified Ras can be detected by immunoblot analysis with anti-Ras antibodies.

The most important consideration in developing an assay to measure activated Ras was to find a RBD that was able to bind to the different *Dictyostelium* Ras proteins. In the *Dictyostelium* Ras proteins, the effector domains are almost identical to the effector domain of H-Ras, with only one amino acid difference in RasC (D38N, relative to H-Ras) and RasS (I36L) (Figure 2). Thus it seemed plausible that *Dictyostelium* Ras proteins would be able to bind to the RBD of mammalian effector proteins. As the RBDs of Raf1 and RalGDS had been successfully employed for Ras subfamily activation assays (Kang et al., 2002; Taylor and Shalloway, 1996), these two RBDs were chosen for initial study. *E. coli* strains expressing GST-Raf1 (RBD) and GST-RalGDS (RBD) (referred to as GST-Raf1 and GST-RalGDS, respectively, unless otherwise noted) were obtained as gifts from D. Shalloway and M. Gold, respectively, and protein was expressed and purified as described in Materials and Methods. Both GST-RBD proteins were expressed at high levels and the predominant protein band in the purified fraction corresponded to the predicted molecular weight of the fusion protein, based on amino acid sequence (Figure 6, Table 3).

**Table 3. Predicted mass of GST fusion proteins.**

| | GST alone | GST-Byr2 | GST-RalGDS | GST-Raf1 | GST-PI3K1 | GST-PI3K2 |
|---|---|---|---|---|---|---|
| **Predicted size of fusion protein, kDa** | 29.0 | 56.2 | 45.8 | 41.9 | 58.1 | 54.5 |

**Note:** Predicted molecular weight was calculated by amino acid sequence using ProtParam tool (http://us.expasy.org/tools/protparam.html).

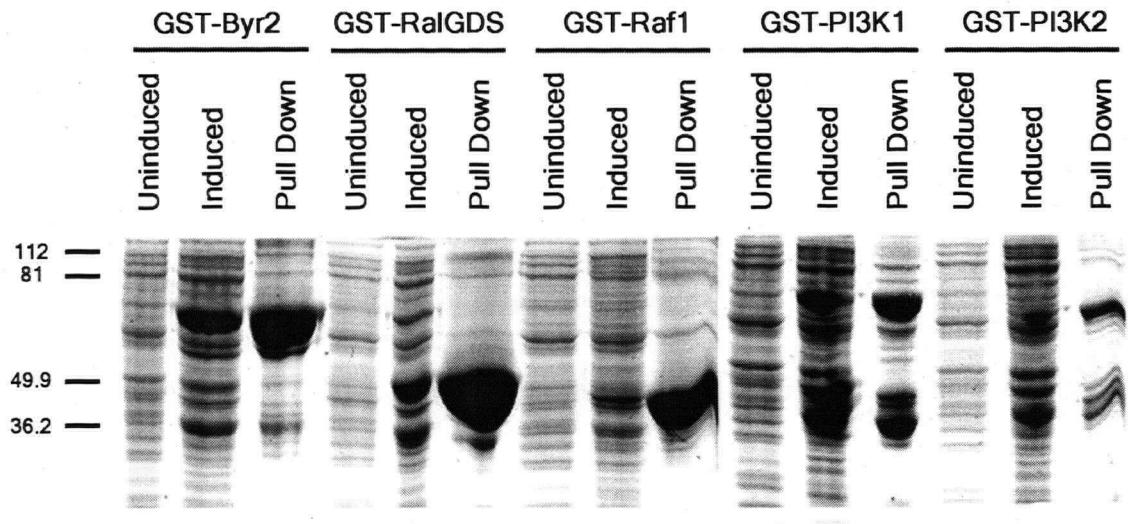


**Figure 6. Expression and purification of GST-RBD fusion proteins.** 500 mL of LB media were inoculated with 5 mL of an overnight culture of *E. coli* BL21 DE3 strains carrying plasmids that encoded GST-Byr2, GST-RalGDS, GST-Raf1, GST-PI3K1, and GST-PI3K2 fusion proteins as labelled on figure. Cells were grown at 37°C until $OD_{600}$ ~0.8, induced with 1 mM IPTG, and incubated for 18 h at 22°C. Samples were collected before adding IPTG (labelled 'Uninduced') and after induction (labelled 'Induced'). Cells were harvested by centrifugation (5000 x g, 10 min, 4°C), and lysed by sonication. Lysates were cleared by centrifugation (12,000 x g, 30 min, 4°C). 50 µl of a 50:50 glutathione-sepharose bead slurry were added to 1 mL bacterial cell lysates and incubated at 4°C for 2 hours. Beads were pelleted by centrifugation and washed three times with ice cold PBS. 50 µl of 1X SDS loading buffer were added to the pelleted beads, and protein was eluted by boiling for 5 min (labelled 'Pull Down'). 10 µl were loaded onto a SDS-PAGE gel (10% acrylamide). The gel was stained with Coomassie Blue. Protein size standards are shown, in kDa.

The ability of GST-Raf1 and GST-RalGDS to bind to activated Ras was tested in lysates of Ax2::pVEII-*rasB* (G15T), Ax2::*rasC-rasC* (G13T), and Ax2::MB-*rasG* (G12T), *Dictyostelium* strains that express the activated Ras mutants RasB (G15T), RasC (G13T), or RasG (G12T) (Table 2). These Ras mutants are the equivalent of the H-Ras (G12T) mutation that renders the GTPase activity non-functional, and locks the Ras protein in its GTP bound conformation. These three Ras proteins were chosen due to the availability of Ras isoform specific antibodies generated by our lab (Lim, 2002; Robbins et al., 1991; Sutherland, 2001). Cells were lysed in a phosphate buffer as described in Materials and Methods, as it has been shown that this type of buffer is efficient in immunoprecipitations of phosphorylated proteins (Yurchak et al., 1996). Both RasB (G15T) and RasG (G12T) were able to bind to GST-Raf1 and GST-RalGDS (Figure 7A, 7C). There was no binding detected to a GST alone control (Figure 7A, 7C), demonstrating that the contaminating protein bands present in the purified GST-RBD preparations recovered from *E. coli* (Figure 6) do not contribute to a non-specific binding of Ras. No detectable level of RasC (G13T) was able to bind to GST-RalGDS (Figure 7B), and binding of RasC (G13T) to GST-Raf1 could only be demonstrated if blots were over exposed (Figure 7B, data not shown).

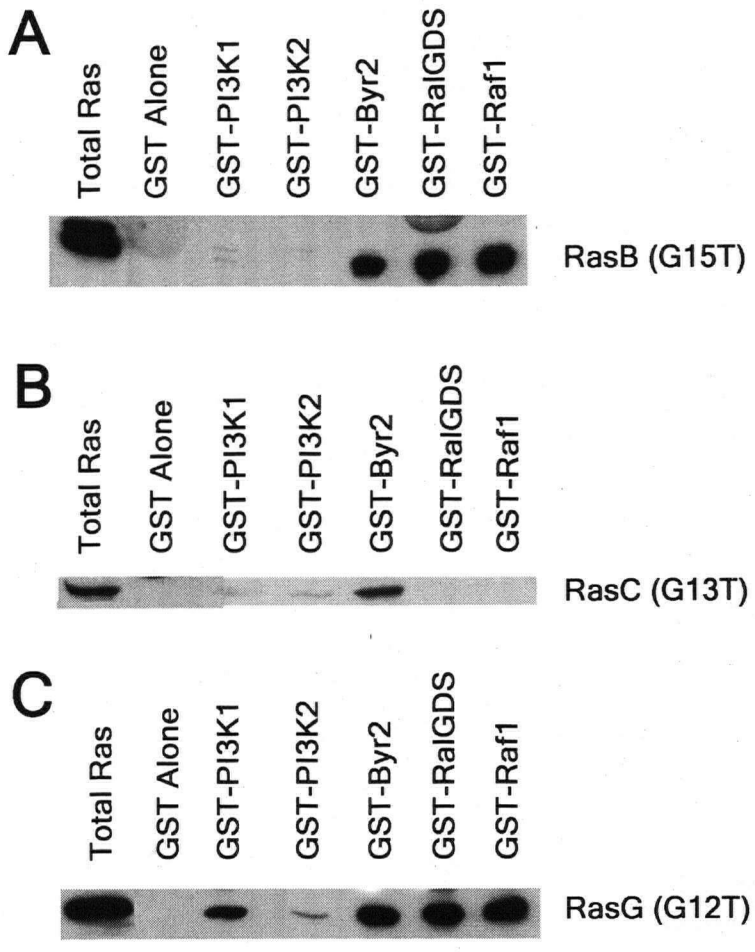


**Figure 7. Binding of RBDs to activated Ras mutants.** Ax2::pVEII-*rasB* (G15T) and Ax2::MB-*rasG* (G12T) (Table 2) were induced to express activated Ras protein. Ax2::*rasC-rasC* (G13T) cells (Table 2) constitutively express activated RasC. Exponential stage (2-4 x $10^6$ cells/ml) Ax2::pVEII-*rasB* (G15T), Ax2::*rasC-rasC* (G13T), and Ax2::MB-*rasG* (G12T) were harvested by centrifugation, and 2.5 x $10^7$ cells were lysed in 1 ml RBD-LB, incubated on ice for 10 min, and lysates were cleared by centrifugation (13,000 x g, 10 min, 4°C). 50 µl of GST-Raf1, GST-RalGDS, GST-Byr2, GST-PI3K1, GST-PI3K2, or GST only bound to glutathione-sepharose beads were added to 500 µg total cellular protein, and the resulting mixtures were subjected to the RBD Binding assay. After elution of the bound Ras protein in 50 µl of 1X SDS loading buffer, 10 µl of the samples were fractionated by SDS-PAGE (10 % acrylamide) and bound Ras protein was detected by immunoblot analysis. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. (A) RasB (G15T), (B) RasC (G13T), (C) RasG (G12T).

The *Dictyostelium* genome contains a number of genes encoding PI3Ks (Zhou et al., 1995), and it has been well established that mammalian Ras-GTP binds to and activates mammalian PI3K (Pacold et al., 2000; Rubio et al., 1997). In light of this link, we tested the ability of the RBDs of *Dictyostelium* PI3K1 and PI3K2 to bind to *Dictyostelium* Ras. These RBDs had previously been reported to bind to RasD and RasG, but not to any other *Dictyostelium* Ras proteins when tested in a yeast two-hybrid assay (Funamoto et al., 2002; Lee et al., 1999). *E. coli* strains expressing GST-PI3K1 (RBD) and GST-PI3K2 (RBD) (referred to as GST-PI3K1 and GST-PI3K2, respectively, unless otherwise noted) were obtained as a gift from R. Firtel, and protein was expressed and purified as described in Materials and Methods. Both GST-RBD proteins were able to be purified from bacterial extracts, but at levels lower than that of GST-Raf1 and GST-RalGDS (Figure 6). While a band was present in the purified fraction that corresponded to the predicted size of the fusion protein (Table 3), the fusion protein was subject to degradation, as evidenced by the presence of a number of other bands (Figure 6). As these bands were also present in the bacterial whole cell lysates, it would suggest that both GST-PI3K1 and GST-PI3K2 experienced degradation *in vivo* (Figure 6). GST-PI3K1 and GST-PI3K2 did not to bind RasB (G15T) and RasC (G13T) (Figure 7A, 7B), supporting the results of the yeast two-hybrid assay. While GST-PI3K1 and GST-PI3K2 were able to bind RasG (G12T), the relative amount of bound RasG was significantly lower when compared to that bound to GST-Raf1, GST-RalGDS, and GST-Byr2 (Figure 7C). The low amount of bound RasG may reflect the *in vivo* affinity of Ras for the RBDs of PI3K1 and PI3K2, or the relative instability of the GST-PI3K1 and GST-PI3K2 fusion proteins, rendering these fusion proteins inadequate for use in an assay to measure activated Ras.

While both Raf1 and RalGDS were very efficient at binding to RasB and RasG, neither of these RBDs were able to interact very well with RasC. Although RasC shares very high overall sequence identity with H-Ras, the amino acid difference at position 39 (the equivalent of position 38 in H-Ras), asparagine instead of aspartate, had been shown through a mutational analysis of H-Ras to abrogate binding to its effector proteins (Akasaka et al., 1996). This change may explain the inability of GST-Raf1, GST-RalGDS, GST-PI3K1, and GST-PI3K2 to bind to activated RasC. Another Ras effector, the *Schizosaccharomyces pombe* kinase Byr2, had been shown to retain the ability to bind to H-Ras containing the D38N substitution (Akasaka et al., 1996), and thus it was postulated that the Byr2 RBD may bind activated RasC as well. In order to test this hypothesis, the RBD of Byr2 was cloned from *S. pombe* genomic DNA via PCR, as described in Materials and Methods. Protein from the *E. coli* strain expressing GST-Byr2 (RBD) (referred to as GST-Byr2, unless otherwise noted) was expressed and purified as described in Materials and Methods, and the predominant protein band in the purified fraction corresponded to the predicted molecular weight of GST-Byr2 (Figure 6, Table 5). As predicted, Byr2 was able to bind to activated RasC (G13T) (Figure 7B). Furthermore, Byr2 was able to bind to RasB (G15T) and RasG (G12T) as well (Figure 7A, 7C). These results demonstrate that Byr2 can be used as a tool to measure the level of activated RasB, RasC, and RasG from *Dictyostelium* cell lysates.

To investigate the GTP dependence of the RBD-Ras interaction, two strains, Ax2::pVEII-*rasG* (G10V) and Ax2::pVEII-*rasG* (S17N) (Table 2), expressing dominant negative mutants were employed. These strains over-express RasG (G10V) and RasG (S17N), respectively. In mammalian Ras, these mutations result in an inability of the Ras protein to exchange GDP for GTP, even in the presence of a RasGEF (John et al., 1993), locking Ras in a GDP bound

conformation. Ax2::MB-*rasG* (G12T) (Table 2) was used as a positive control. GST-Raf1 and GST-Byr2 were used in this experiment as both GST-RBDs were effective at recovering RasG-GTP from cellular lysates (Figure 7C). Figure 8 shows that although RasG (G10V) and RasG (S17N) were highly expressed in cell lysates, they did not bind to either GST-Raf1 or GST-Byr2, as evidenced by the lack of a signal corresponding to RasG in the immunoblot. RasG (G12T) was effectively recovered by both GST-Raf1 and GST-Byr2 (Figure 8). A faint signal was seen when lysates of RasG (S17N) were incubated with GST-Raf1 or GST-Byr2. The signal could be representative of the activated pool of endogenous RasG, but the relative amount of this band is negligible when compared to RasG (G12T) (Figure 8). Furthermore, the amount of endogenous RasG-GTP would be expected to be low, as dominant negative Ras proteins have been shown to interfere with activation of endogenous Ras, at least in higher eukaryotes (Feig, 1999). These results show that the interaction between the GST-RBDs and Ras is dependent on Ras being in its GTP bound, or activated form.

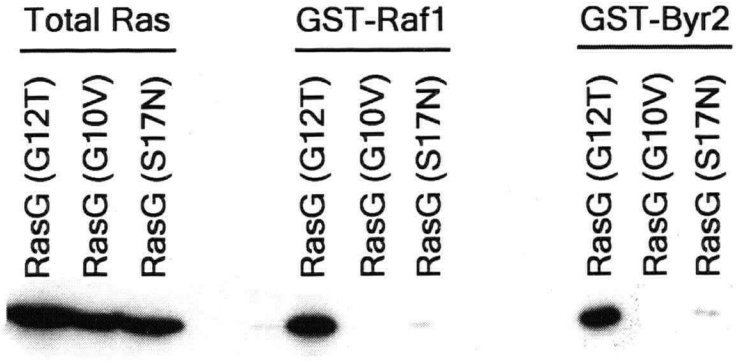


**Figure 8. Binding of GST-Raf1 and GST-Byr2 to activating and dominant negative RasG mutants.** Ax2::MB-*rasG* (G12T), Ax2::pVEII-*rasG* (G10V), and Ax2::pVEII-*rasG* (S17N) (Table 2) were induced to express activated Ras protein. Lysates were prepared as described in Materials and Methods and activated Ras was measured as described in Figure 7. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'GST-Raf1' refers to Ras-GTP bound to GST-Raf1, and 'GST-Byr2' refers to as Ras-GTP bound to GST-Byr2.

## 3.2 RBD interaction with endogenous levels of Ras

The experiments in Figure 7 and 8 were carried out with *Dictyostelium* strains that over-express activated Ras proteins in order to ensure that activated Ras was not a limiting factor. However, studies measuring Ras-GTP in NIH 3T3 cells have shown that physiological levels of activated endogenous Ras are much lower (1.3 fmol per mg of cellular protein) relative to NIH 3T3 cells that over-express H-Ras (G12T) (2049 fmol per mg of cellular protein) (Scheele et al., 1995). Thus, in order to determine whether or not the RBD-Ras interaction was strong enough to detect only endogenous Ras-GTP, protein lysates from wild type Ax3 cells were isolated and incubated with GTPγS or GDP to load Ras protein in the lysates with either nucleotide (Materials and Methods). Using GST-Raf1, GST-Byr2 and GST-RalGDS, RasG was detected from GTPγS loaded lysates (Figure 9C), in support of the data from Figure 7C. Contrary to the data seen in Figure 7C, GST-PI3K1 and GST-PI3K2 did not pull down detectable levels of RasG (Figure 9C). One possible explanation for this observation is that the apparent instability of GST-PI3K1 and GST-PI3K2 (Figure 6) had an effect on the structure of the fusion proteins, affecting their ability to bind to RasG. When lysates were loaded with GDP prior to incubation with GST-Byr2, the amount of RasG recovered was either reduced from the levels seen with GTPγS, or completely abolished (Figure 9C), which showed that the interaction was selective for RasG-GTP over RasG-GDP.

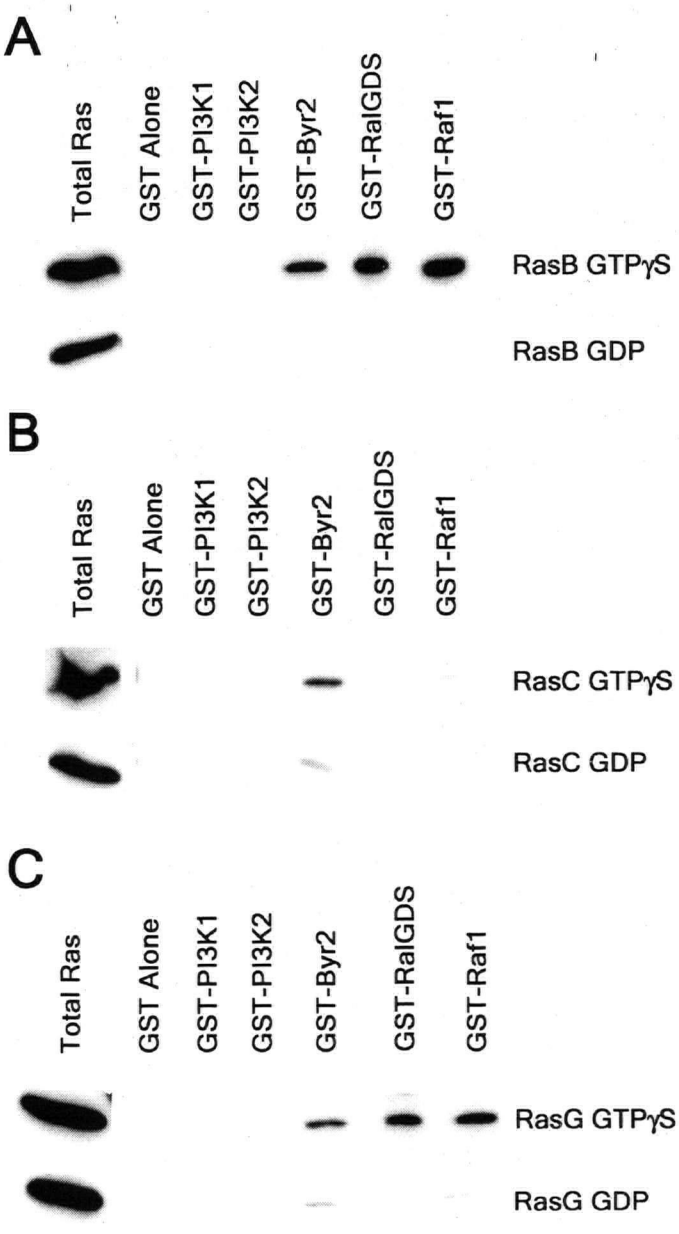


**Figure 9. GTP dependence of RBD-Ras interaction from endogenous levels of Ras.** Exponential stage (2-4 x $10^6$ cells/ml) Ax3 cells (Table 2) were harvested by centrifugation, and 2.5 x $10^7$ cells were lysed in 1 ml RBD-LB, incubated on ice for 10 min, and lysates were cleared by centrifugation (13,000 x g, 10 min, 4°C). Lysates were loaded with either 1 mM GTPγS or 1 mM GDP, and activated Ras was measured as described in Figure 7. Bound levels of (A) RasB, (B) RasC, and (C) RasG protein were detected by immunoblot analysis. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel.

Figure 9A shows the ability of the GST-RBD proteins to bind to RasB. Consistent with the results from Figure 7A employing cells over-expressing RasB (G15T), GST-Byr2, GST-Raf1 and GST-RalGDS were able to bind to RasB-GTP in cellular lysates, while the GST-PI3K1 and GST-PI3K2 proteins were not. As shown for RasG, GDP loading of lysates prior to incubation with the GST-RBDs completely abolished the interaction with RasB (Figure 9A).

The pull down performed on the GTPγS loaded lysates shows that it was possible to detect RasC-GTP only when using GST-Byr2, and the amount of RasC bound to GST-Byr2 was reduced when lysates were incubated with GDP prior to incubation with GST-Byr2 (Figure 9B). Consistent with the experiments using cells over-expressing activating mutants (Figure 7B), none of the other RBDs permitted detection of RasC-GTP (Figure 9B).

### 3.3 Optimizing protein load for a RBD pull down assay

The data in Figures 7, 8, and 9 was from experiments that employed 500 µg of total cellular protein and 100 µl of a 50:50 bead:buffer slurry (Materials and Methods). The amount of cellular protein and GST-RBD bead volume was not an issue as the aim of the aforementioned experiments was to determine whether the RBDs were able to bind to activated Ras or not. However, if the beads are saturated with Ras-GTP in the absence of any stimulus, an increase in activated Ras in response to a stimulus will not be detected. To investigate the effect of input cellular protein, lysates from cells loaded with GTPγS were used in a pull down with 50 µl of a 50:50 GST-Byr2 bead slurry. The cell lysates were loaded with GTPγS in order to simulate maximum activation of RasC and RasG. The volume of GST-Byr2 bead slurry was chosen as an amount that would be practical to use in future experiments. The results of the experiment show that the amount of RasC-GTP binding to 50 µl GST-Byr2 bead slurry saturated when 400 µg of

*Dictyostelium* extract protein were added (Figure 10B). RasG-GTP binding to 50 µl GST-Byr2 bead slurry saturated between 400 to 500 µg *Dictyostelium* extract protein, as evidenced by monitoring the unbound fraction of RasG (Figure 10A). Thus, the amount of *Dictyostelium* extract protein used in subsequent experiments was 400 µg.

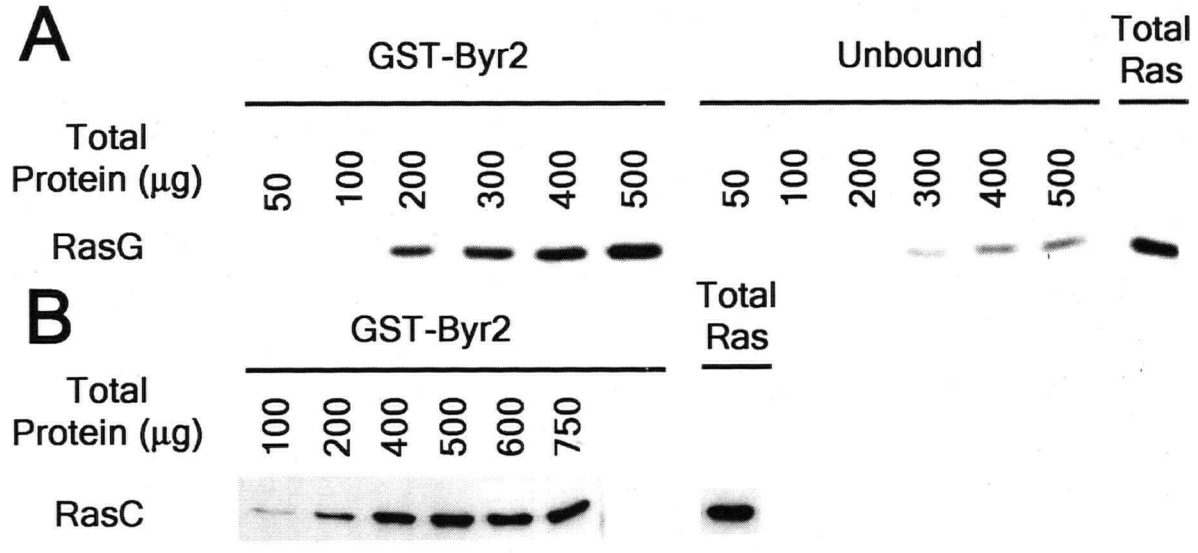


**Figure 10. Effect of input protein on RBD binding assay.** Ax3 cells were loaded with GTPγS, and 2.5 x $10^7$ cells were lysed in 1 ml RBD-LB, incubated on ice for 10 min, and lysates were cleared by centrifugation (13,000 x g, 10 min, 4°C). Total protein was quantified using Bio-Rad DC Protein Assay. Increasing quantities of total protein ranging from 50 to 500 µg were incubated with 50 µl of GST-Byr2 bound to glutathione-sepharose beads, and the mixture was subjected to the RBD binding assay as described in Figure 7. After elution of the bound Ras protein in 50 µl of 1X SDS loading buffer, 10 µl of the samples were fractionated by SDS-PAGE (10 % acrylamide) and bound (A) RasG and (B) RasC protein was detected by immunoblot analysis. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein was loaded on the gel. 10 µl of 6X SDS loading buffer were added to 50 µl of the supernatant (1 mg/ml) from the first centrifugation before the first wash, indicated as 'Unbound,' and approximately 8.3 µg total protein were loaded on the gel. 'GST-Byr2' refers to Ras bound to GST-Byr2, 'Unbound' refers to fraction of Ras not bound to GST-Byr2.

## 3.4 Chemoattractant induced activation of RasC and RasG

Previous evidence had suggested that RasC is involved in regulating signalling events during the early development of *Dictyostelium*. Ablation of the *rasC* gene results in cells that are unable to undergo development, largely due to an inability to activate ACA (Lim et al., 2001). As no other *ras* gene knockout strains characterized at the time this thesis was started showed any defects in early development, it was hypothesized that RasC was activated by cAMP in early developmental cells, and that RasC was the sole Ras protein involved in cAMP stimulated signalling. To test the first part of this hypothesis using the RBD binding assay, wild type Ax3 cells (Table 2) were suspended in non-nutrient buffer and induced to initiate early development by applying exogenous pulses of cAMP (final concentration 50 nM) for 6 h (referred to as pulsed cells henceforth), and then stimulated with 200 nM cAMP (Materials and Methods). These exogenous pulses mimic the developmental program of *Dictyostelium*, thus all strains, even those defective in endogenous signalling, undergo synchronized developmental changes. Aliquots were taken at the indicated time points after the 200 nM stimulation, lysed, and the level of activated Ras was measured from each aliquot using the RBD binding assay. RasC displayed a rapid and transient increase in GTP binding upon cAMP stimulation, with peak activation levels reached within 10 s of stimulation (Figure 11A). A second, less intense peak was observed starting at 80 s and remained on until at least 150 s (Figure 11A). However, the timing and intensity of the second peak was variable and thus its physiological relevance is unclear at this time. This data provided direct evidence that RasC activation is responsive to cAMP signalling events.

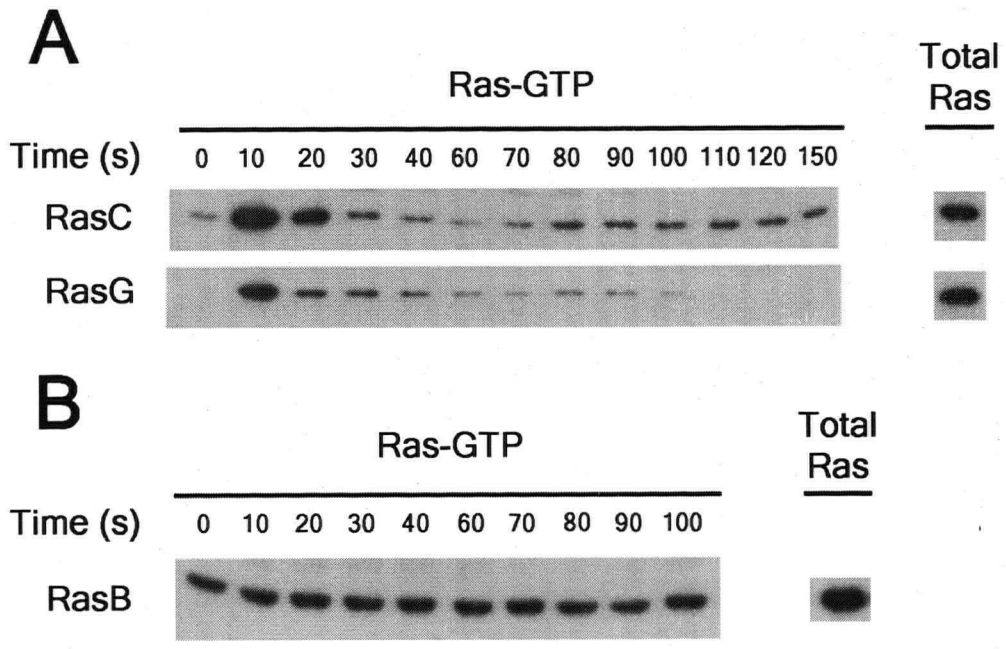


**Figure 11. cAMP stimulated Ras activation.** Pulsed Ax3 cells were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and 400 µl of cells were added to 400 µl 2X RBD-LB before stimulation and at the time points indicated, and incubated on ice for 10 min. Lysates were quantified and 400 µg of protein lysate were added to 50 µl of GST-Byr2 bound to glutathione-sepharose beads. After elution of the bound Ras protein in 50 µl of 1X SDS loading buffer, 10 µl of the samples were fractionated by SDS-PAGE (10 % acrylamide) and bound Ras protein was detected by immunoblot analysis using specific antibodies against (A) RasC and RasG, (C) RasB. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2. The experiment is representative of four separate experiments.

RasG also displayed a similar rapid and transient activation upon cAMP stimulation (Figure 11A). Up to this point, all the evidence had suggested that RasG functioned primarily during the growth stage of *Dictyostelium* cells. Thus it was not expected for RasG to be responsive to cAMP, so this observation was somewhat surprising. Subsequent generation of a *rasC⁻/rasG⁻* double knockout strain corroborated the involvement of RasG in early development (Bolourani et al., 2006; Section 4.2). Activation of RasB was measured as a control as the available evidence suggested that RasB is involved in the control of the cell cycle rather than development (Sutherland et al., 2001). No increase in activated RasB was observed in response to cAMP stimulation (Figure 11B).

### 3.5 Ras activation is dependent on the cAMP receptor complex

cAMP signalling during early development involves a GPCR, cAR1 (encoded by the *carA* gene), that binds cAMP with high affinity, as well as a low affinity GPCR, cAR3 (*carC*) (Soede et al., 1994); a *carA⁻/carC⁻* double knockout is unable to initiate any cAMP induced signalling during aggregation (Insall et al., 1994; Soede et al., 1994). Thus, in order to determine whether the activation of RasC and RasG was dependent on the cAMP receptors, pulsed Ax3 and *carA⁻ /carC⁻* cells (Table 2) were stimulated with cAMP and assayed for activation of Ras. No increase in activated RasC or RasG was observed in pulsed *carA⁻/carC⁻* cells, indicating that all RasC and RasG activation during early development is dependent on cAMP receptors cAR1 and cAR3 (Figure 12A).

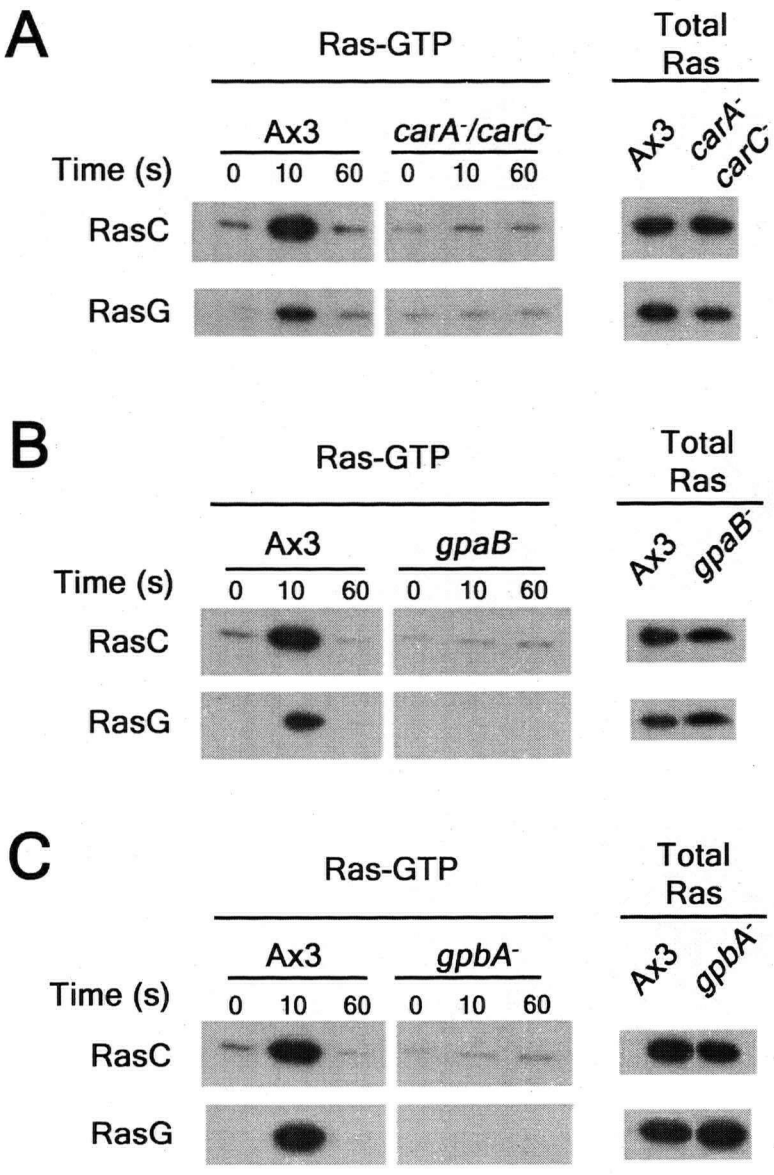


**Figure 12. The cAMP receptor complex is required for Ras activation.** Pulsed Ax3, *carA⁻/carC⁻*, *gpbA⁻*, *gpaB⁻* cells (Table 2), were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2 from (A) *carA⁻/carC⁻*, (B) *gpbA⁻*, and (C) *gpaB⁻* strains. The experiments are representative of three separate experiments.

There are two general types of signalling pathways that transmit signals induced by cAMP: those that involve the heterotrimeric G-protein associated with the cAMP receptor, and those that do not (reviewed in Manahan et al., 2004). The *Dictyostelium* genome contains a single gene that encodes the Gβ protein, *gpbA*, component of the heterotrimeric G-protein, so all G-protein mediated events are dependent on Gβ (Wu et al., 1995). To determine the involvement of Gβ in Ras activation, pulsed Ax3 and *gpbA*$^-$ cells (Table 2) were stimulated with cAMP and assayed for activation of Ras. The increase in RasC or RasG activation observed in Ax3 cells in response to cAMP stimulation was not seen in the *gpbA*$^-$ mutant (Figure 12C). This indicated that the Gβ protein is required for cAMP dependent RasC and RasG activation.

There are at least 11 genes that encode for G alpha proteins, but only one, *gpaB* (Gα2 protein), is believed to mediate the stimulatory pathways associated with cAR1 during aggregation (Kumagai et al., 1991). To test whether Gα2 is necessary for cAMP stimulated activation of RasC and RasG, pulsed Ax3 and *gpaB*$^-$ cells (Table 2) were stimulated with cAMP and assayed for activated Ras. No increase in activation of RasC and RasG was observed in cells lacking the *gpaB* gene (Figure 12B). The results of these experiments demonstrate that during aggregation, RasC and RasG activation are dependent on the cAMP stimulus signalling through the cAMP receptor and its associated G protein complex Gα2βγ.

### 3.6 Ras activation is independent of ACA, CRAC, and PiaA

Two cytosolic proteins have been shown to be required for the activation of ACA (encoded by the *acaA* gene) downstream of the cAMP receptor: CRAC, or the 'Cytosolic Regulator of Adenylyl Cyclase' (*dagA*) and Pianissimo (*piaA*) (Chen et al., 1997; Lilly and Devreotes, 1994). Their involvement in Ras activation was tested by stimulating pulsed Ax3, *acaA*$^-$, *dagA*$^-$, and

*piaA*⁻ cells (Table 2), and assaying for activated RasC and RasG. None of the strains exhibited a defect in the activation of RasC and RasG (Figure 13). These data demonstrated that ACA, CRAC, and PiaA are not involved in cAMP simulated activation of RasC and RasG. Taken together with the previous experiments (Figure 11, 12), these results demonstrate that RasC and RasG activation lie downstream of the cAMP receptor and its associated G protein. Previous results have indicated that RasC is required for ACA activation (Lim et al., 2001). The cAMP dependent activation of RasG clearly depends on cAR1 (Figure 12A), and the apparent involvement of RasG in regulating the TORC2 complex (Lee et al., 2005), which is also required for ACA activation, also places RasG activation upstream of ACA and its regulators. It should be noted that the loss of CRAC and PiaA resulted in an increased level of activated RasC at 60 s relative to wild type (Figure 13A, 13B), and the loss of ACA resulted in an increased level of activated RasG at 60 s, relative to wild type (Figure 13C). The reason for this is not clear, but as cAMP is an important intracellular second messenger, it is possible that the defect in cAMP stimulated ACA activation demonstrated by these strains (Chen et al., 1997; Lilly and Devreotes, 1994; Pitt et al., 1992) results in a deregulation of Ras activation (Section 4.5).

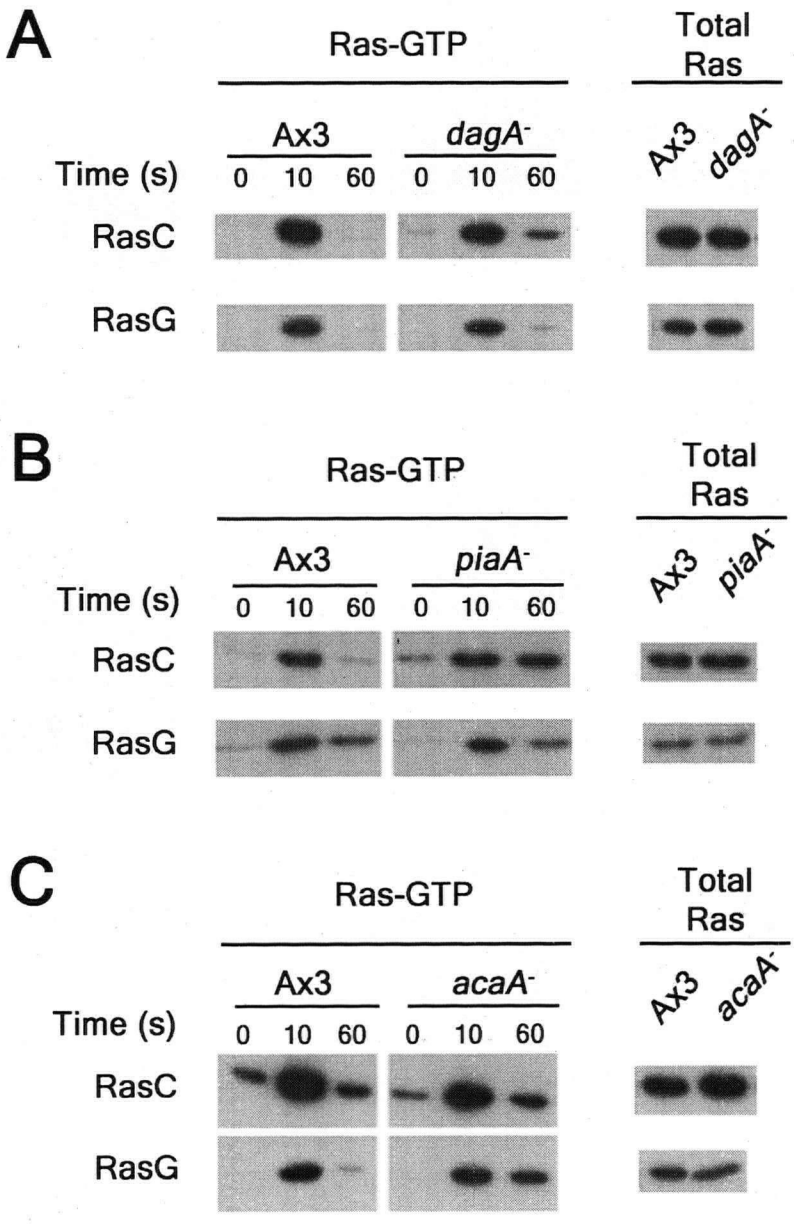


**Figure 13. Ras activation is upstream of ACA and its cytosolic regulators.** Pulsed Ax3, *dagA*$^-$, *piaA*$^-$, and *acaA*$^-$ cells (Table 2), were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2 from (A) *dagA*$^-$, (B) *piaA*$^-$, (C) *acaA*$^-$ strains. The experiments are representative of three separate experiments.

### 3.7 RasGEFA mediates activation of RasC

Insall et al. identified RasGEFA as a component involved in G-protein mediated cAMP stimulated signaling events (Insall et al., 1996). Strains with a disrupted *rasC* gene have similar phenotypes to those of the *gefA*$^-$ mutant, and hence it was speculated that RasGEFA might mediate RasC activation (Lim et al., 2001). However, expression of activated RasC in *gefA*$^-$ cells fails to rescue the phenotypic defects of the *gefA*$^-$ strain (C.J. Lim, unpublished observations). This was not entirely unexpected as the expression of activated RasC in *rasC*$^-$ cells does not fully rescue the defect in multicellular development, and appears to be detrimental to development when expressed in wild type cells (Lim, 2002). Thus, the relationship between RasC and RasGEFA remained unclear. The RBD binding assay allowed for an alternative strategy to test this relationship. Pulsed Ax3 and *gefA*$^-$ cells (Table 2) were stimulated with cAMP, and the levels of activated Ras were measured at various time points post stimulation. In cells lacking RasGEFA, no activation of RasC was observed, indicating that RasGEFA is required for all of the cAMP stimulated RasC activation (Figure 14). In contrast, there was no reduction of RasG activation upon cAMP stimulation in *gefA*$^-$ cells compared to wild type cells, indicating that RasGEFA is not involved in RasG activation (Figure 14).

This result led to a collaborative effort with A. Kortholt from the Van Haastert lab, who had established an *in vitro* fluorescence nucleotide exchange assay to identify substrates for different *Dictyostelium* RasGEFs. This assay involves loading the Ras protein with a fluorescent analogue of GDP, 2'-, 3'-bis(O)-(N-methylanthraniloyl)-guanosine diphosphate, mGDP, and incubating Ras-mGDP in the presence of a RasGEF (Lenzen et al., 1995). mGDP displays a higher level of fluorescence in the hydrophobic environment of the GTP binding pocket relative to the aqueous environment of the buffer solution, so RasGEF stimulated nucleotide release can be monitored as

a decay in fluorescence over time. Nucleotide exchange in the absence of a RasGEF was measured to determine the intrinsic nucleotide exchange activity of the Ras protein, and used as a background control. The rate of fluorescence decay can be fitted onto a single exponential curve to derive the observed rate constant, $k_{obs}$. As summarized in Table 4, results from this assay confirmed that RasGEFA mediates nucleotide release specifically from RasC, and not any of the other Ras proteins tested (Kae et al., 2007). It bears pointing out that the loss of *gefA* appeared to coincide with a prolonged RasG activation (Section 4.5).

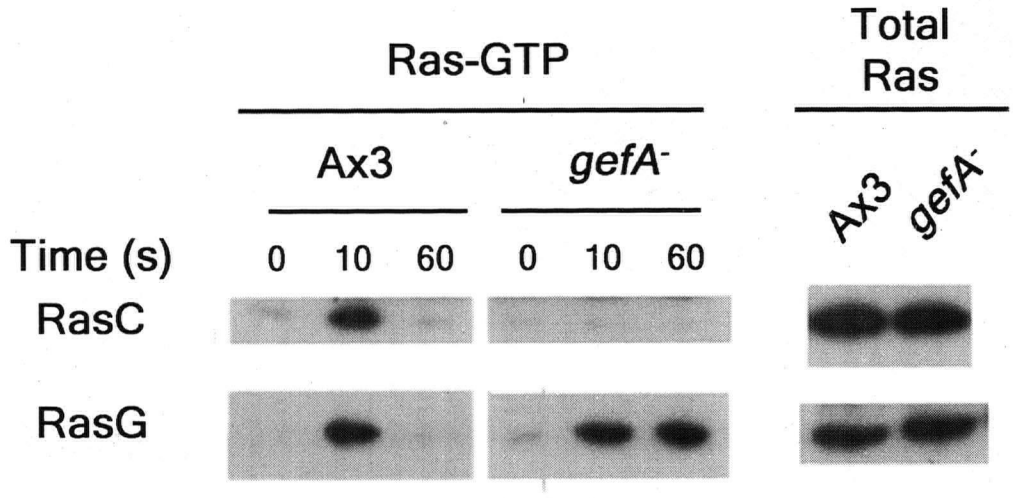


**Figure 14. *gefA*⁻ mutants are deficient in RasC activation**. Pulsed Ax3 and *gefA*⁻ cells (Table 2), were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2. The experiment is representative of three separate experiments.

**Table 4. Observed rate constant, $k_{obs}$, of RasGEF stimulated nucleotide exchange.**

| | **Intrinsic** / x $10^{-4}$ $s^{-1}$ | **+ve Control** / x $10^{-4}$ $s^{-1}$ | **Fold Increase** +ve/ Intrinsic | **RasGEFA** / x $10^{-4}$ $s^{-1}$ | **Fold Increase** RasGEFA/ Intrinsic | **RasGEFR** / x $10^{-4}$ $s^{-1}$ | **Fold Increase** RasGEFR/ Intrinsic | **GbpD** / x $10^{-4}$ $s^{-1}$ | **Fold Increase** GbpD/ Intrinsic |
|---|---|---|---|---|---|---|---|---|---|
| RasB | 1.2 | 2.3 | 1.9 | 1.1 | 0.9 | 1.2 | 1.0 | 1.1 | 0.9 |
| RasC | 1.2 | 2.7 | 2.3 | 5.70 | 4.8 | 1.1 | 0.9 | 1.4 | 1.2 |
| RasD | 2.9 | 8.0 | 2.8 | 2.7 | 0.9 | 2.6 | 0.9 | 3.1 | 1.1 |
| RasG | 0.4 | 6.0 | 15 | 0.5 | 1.2 | 2.7 | 6.8 | 0.3 | 0.8 |
| RasS | 1.1 | 2.4 | 2.2 | 1.1 | 1.0 | 1.2 | 1.1 | 1.1 | 1.0 |
| Rap1 | 0.4 | 2.8 | 7.0 | 0.4 | 1.0 | 0.3 | 0.8 | 2.7 | 6.8 |

The *in vitro* nucleotide exchange assay employs a fluorescent analogue of GDP, mGDP. mGDP has a higher level of fluorescence in the hydrophobic GTP binding pocket of Ras, so nucleotide release can be observed as a decrease in fluorescence as mGDP is released into the aqueous environment of the surrounding buffer. The observed rate of decay can be fitted onto a single exponential curve to derive the observed rate constant, $k_{obs}$. 'Intrinsic' refers to the $k_{obs}$ in the absence of a RasGEF, and serves as a background control. '+ve Control' refers to the $k_{obs}$ when the Ras protein was incubated with the positive control: mammalian RasGRF1 for RasB, RasC, RasD, RasG, RasS, and mammalian C3G for Rap1. 'Fold increase' is the ratio of the $k_{obs}$ for the indicated RasGEF-Ras reaction over the $k_{obs}$ for the intrinsic value. The data from this table was summarized from Kortholt et al. (2006) and Kae et al. (2007), and the analysis was performed by A. Kortholt.

### 3.8 RasGEFR is required for maximal cAMP stimulated activation of RasG

Secko et al. (2004) used a proteomics based approach to search for proteins involved in the RasG signalling pathway. This screen involved looking for proteins that underwent changes in their state of phosphorylation, as assayed with antibodies specific for phosphorylated serine, threonine and tyrosine residues, in response to the expression of activated RasG (G12T). One signalling protein identified was RasGEFR, which displayed a decrease in threonine phosphorylation when RasG (G12T) was expressed. As it has been shown in mammalian systems that RasGEF phosphorylation can lead to either positive or negative regulation of RasGEF activity (Kesavapany et al., 2004; Yang et al., 2003), it was speculated that RasGEFR either mediates RasG activation, and that this activation is itself regulated by RasG, or RasGEFR functions downstream of RasG signalling, activating a different Ras GTPase. The availability of a *gefR*$^-$ strain allowed a test of the first possibility. Pulsed Ax3 and *gefR*$^-$ cells (Table 2) were stimulated with cAMP, and assayed for activated Ras. There was a partial reduction in RasG activation in *gefR*$^-$ cells, suggesting that RasGEFR is partially responsible for the activation of RasG (Figure 15). Since the reduction in RasG activation was only partial, it would appear that there are at least two RasGEFs that mediate cAMP stimulated RasG activation. This conclusion is consistent with the observation that *gefR*$^-$ cells display no apparent defect in early development (Secko et al., 2004), although the phenotypic examination of the *gefR*$^-$ cells was limited to the ability of the mutant cells to complete multicellular development (D.M. Secko, unpublished observations). There was no defect in RasC activation in *gefR*$^-$ cells (Figure 15); however, the level of RasC-GTP in *gefR*$^-$ cells at 60 s was elevated compared to wild type cells (Section 4.5). The *in vitro* nucleotide exchange assay performed by A. Kortholt demonstrated that RasGEFR mediates GDP release specifically from RasG (summarized in Table 4), confirming the *in vivo*

activation data (Figure 15) and indicating that the observed decrease in RasG activation is likely due to the specific loss of RasGEFR activity on RasG (Kae et al., 2007).

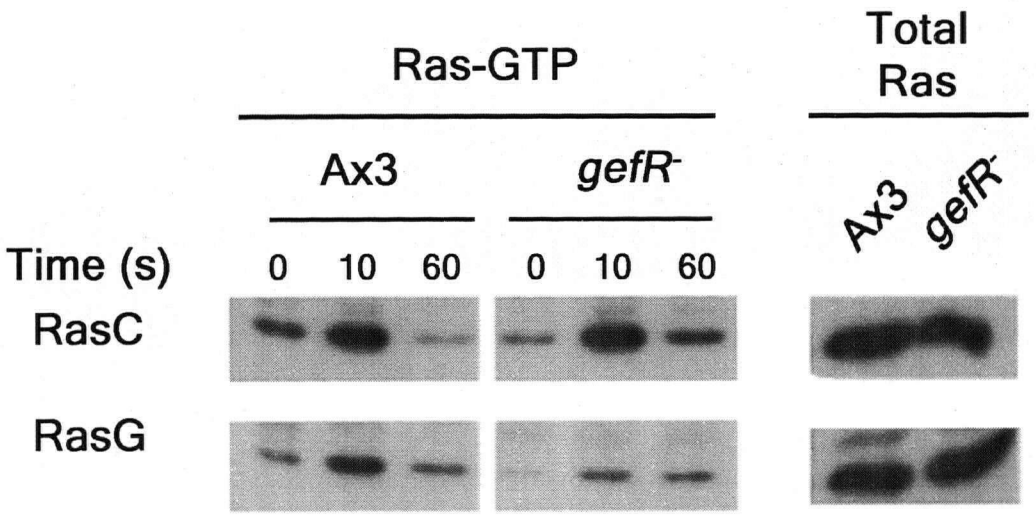


**Figure 15. Loss of RasGEFR reduces activation of RasG during development.** Pulsed Ax3 and *gefR*⁻ cells (Table 2), were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2. The experiment is representative of four separate experiments.

In light of the above observations, the developmental phenotype of *gefR*⁻ cells was scrutinized in more detail. When *Dictyostelium* cells are placed on plastic dishes under non-nutrient buffer, wild type cells aggregate via a process called streaming, and this behaviour initiates after ~10h in starvation buffer, as was the case for Ax3 cells (Figure 16). Similar to Ax3 cells, *gefR*⁻ cells also formed aggregation stream in about 10 h (Figure 16), unlike *rasG*⁻ cells, which exhibit a delay of about 4 h relative to wild type cells (Bolourani et al., 2006). It's been shown that ACA activity is required for cells to be able to form streams (Kriebel et al., 2003), so the observation that *gefR*⁻ cells were able to form streams suggests that RasGEFR is not required for ACA activity. It appears that the streams formed by *gefR*⁻ cells are less robust than the streams formed by wild type cells, (Figure 16), a phenotype similar to *rasG*⁻ cells (Bolourani et al., 2006). As RasG is involved in regulating chemotaxis and cell motility (Bolourani et al., 2006), the difference in streaming pattern displayed by *gefR*⁻ cells would suggest that RasGEFR may be involved in one of these processes.

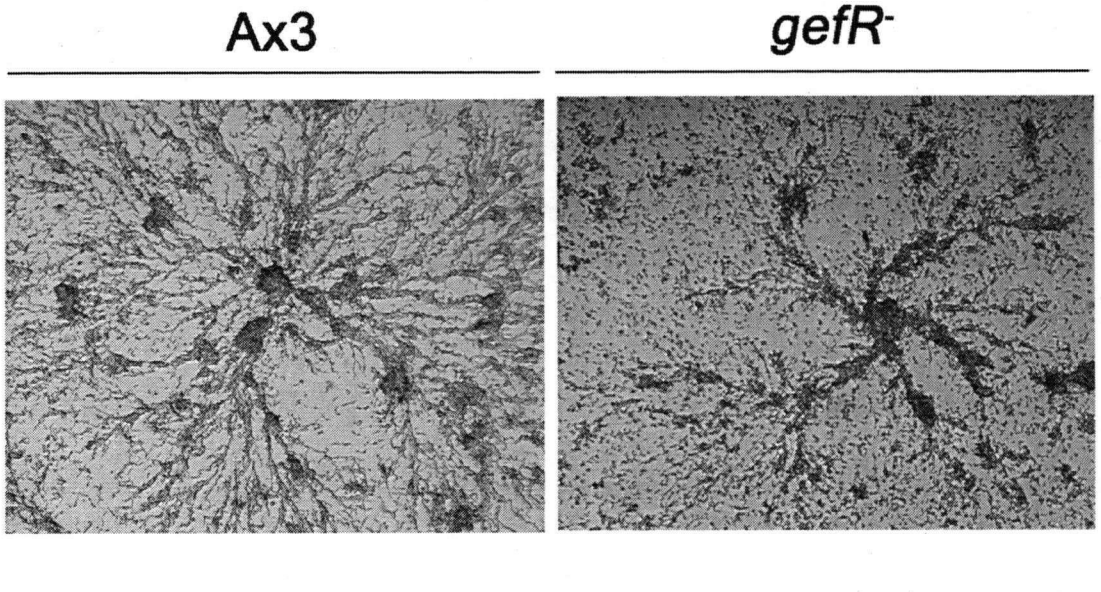


**Figure 16. Streaming behaviour of Ax3 and *gefR*⁻ cells during aggregation.** Cells were seeded as a monolayer at a density of 5 x $10^5$ cells/$cm^2$ and submerged under Bonner's salts solution. Images were photographed at 10 hours after the initiation of starvation.

cAMP stimulation of aggregation stage cells results in the rapid activation of PKB, and loss of PKB has been associated with defects in cell motility and polarity (Meili et al., 1999). It has been shown that PKB activation is dramatically reduced in *rasG*$^-$ cells relative to wild type cells (Bolourani et al., 2006). cAMP stimulated activation of *Dictyostelium* PKB requires the phosphorylation of two threonine residues (T278 and T435) on PKB. An immunoblot analysis using an anti-phospho-threonine antibody detects a number of threonine phosphorylated proteins in *Dictyostelium* cell lysates, including a ~51 kDa band that corresponds to PKB. An anti-*Dictyostelium* PKB specific antibody has confirmed that the band corresponds to PKB (Lim et al., 2001). Mutation of the threonine residues to non-phosphorylatable alanines results in loss of both *in vivo* phosphorylation of PKB and *in vitro* protein kinase activity, demonstrating that the phosphorylation state of PKB can be used as a direct measure of PKB activation (Lim, 2002). In order to determine the involvement of RasGEFR in the RasG mediated activation of PKB, pulsed Ax3 and *gefR*$^-$ cells were incubated in the presence of 1 mM caffeine for 30 min and stimulated with 15 μM cAMP, as described in Materials and Methods. Samples were collected at the time points indicated, and activation of PKB was assayed for by immunoblot analysis using a phospho-threonine specific antibody. Phosphorylation of PKB was rapid and transient in both Ax3 and *gefR*$^-$ cells, peaking at 10 s post-stimulation and returning to basal levels by 30 s (Figure 17A). Thus, a loss of RasGEFR did not affect PKB phosphorylation. As defects in either the PI3K or TORC2 signalling pathways results in a loss of PKB activation (Lee et al., 2005; Meili et al., 1999), the result from Figure 17A implies that RasGEFR is not involved in the regulation of either of these pathways.

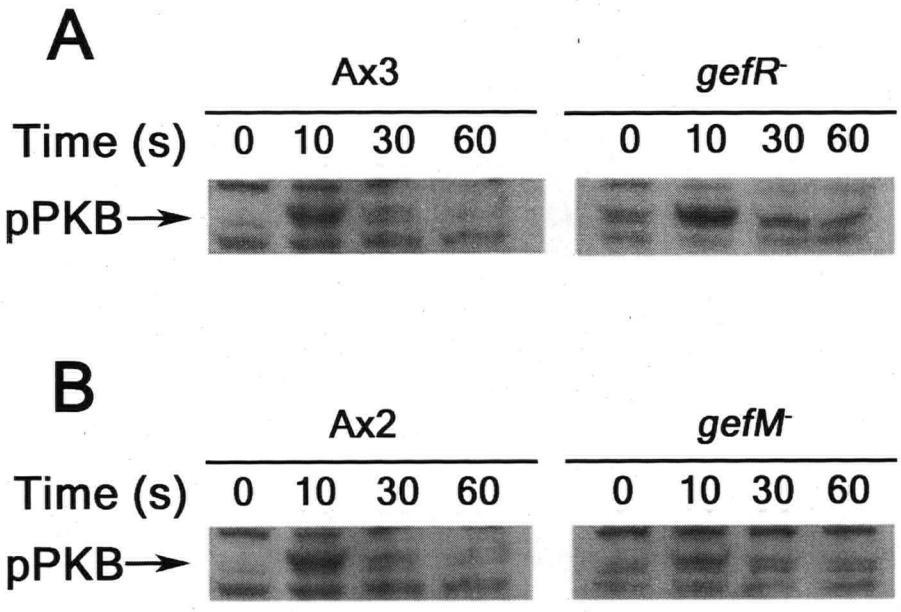


**Figure 17. cAMP induced stimulation of PKB phosphorylation.** (A) Pulsed Ax3 and *gefR*⁻, (B) Ax2 and *gefM*⁻ cells (Table 2) were harvested by centrifugation (500 x g, 5 min), washed two times with $KK_2$, resuspended at 5 x $10^7$ cells/ ml in $KK_2$ supplemented with 1 mM caffeine, and incubated with shaking for 30 min. 100 µl of cell suspension was added to 20 µl 6X SDS loading buffer before stimulating cells with 15 µM cAMP (final concentration) and at the time points indicated after stimulation. Samples were resolved by SDS-PAGE, and phosphorylated PKB was detected by immunoblot analysis. The band corresponding to phosphorylated PKB is indicated as 'pPKB.'

### 3.9 RasGEFM is required for maximal cAMP stimulated activation of RasG

Using a bioinformatic approach to search the *Dictyostelium* genome for regulators of Ras, Arigoni et al. (2005) identified a RasGEF, RasGEFM. When the *gefM* gene was ablated, the strain displayed defects in early development. *gefM*⁻ cells have chemotaxis and polarity defects, and development arrests after forming small, flat aggregates (Arigoni et al., 2005). Given the involvement of RasGEFM in early developmental processes, it was postulated that RasGEFM may regulate RasG activation. Pulsed wild type Ax2 and *gefM*⁻ cells (Table 2) were stimulated with cAMP and assayed for activated RasC and RasG. cAMP stimulation of Ax2 cells resulted

in a similar rapid increase in RasG activation when compared to Ax3 cells (compare Figures 11 and 18). However, unlike in Ax3, RasG activation in Ax2 did not decrease to near basal levels at 60 s (compare Figures 11 and 18). This difference in the RasG activation profile observed may be attributed to differences between the wild type Ax2 and Ax3 strains. Ax3 was initially generated by mutagenesis of a non-axenic strain (Loomis, 1971), whereas Ax2 was generated from a non-axenic strain without using mutagenesis (Watts and Ashworth, 1970), and the different methods used in generating these strains is evidenced by the fact that Ax3 contains a ~755 kb duplication of Chromosome 2 (Eichinger et al., 2005; Loomis et al., 1995). While it has been proposed that the duplication has no severe consequences on either growth or development (Loomis et al., 1995), it has been shown that Ax2 aggregates earlier than Ax3, and that culmination proceeds directly from 'finger' to 'fruiting body' (Figure 3), bypassing the motile slug stage present in Ax3 (Kellerman and McNally, 1999). Whether or not these differences are related to the observed difference in RasG activation is unclear at this time. Compared to the activation in the parental Ax2 strain, there was a partial reduction in RasG activation when *gefM*⁻ cells were stimulated with cAMP (Figure 18), suggesting that RasGEFM is involved in mediating RasG activation along with RasGEFR. However, the decrease in RasG activation in *gefM*⁻ cells is modest relative to Ax2, and since there is no *in vitro* data to confirm that RasG acts as a substrate for RasGEFM, it is possible that the decrease in activated RasG is due to some unspecified secondary effect such as gene expression of a regulator of RasG. There was no dramatic difference in cAMP stimulated RasC activation between Ax2 and Ax3 strains (Figures 11 and 18), and while the activation of RasC was not affected in *gefM*⁻ cells, the level of RasC-GTP at 60 s was higher in *gefM*⁻ cells than Ax2 (Figure 18, Section 4.5).

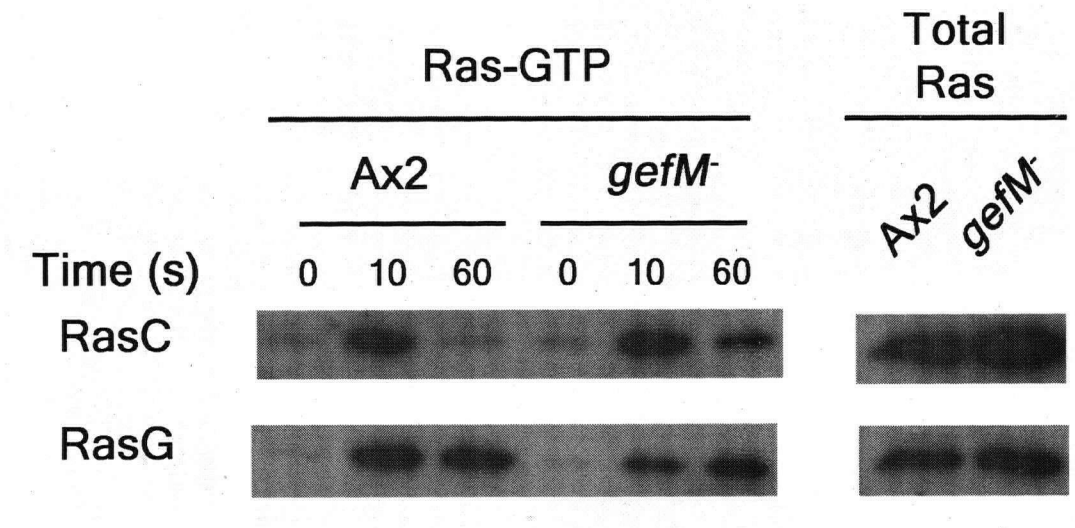


**Figure 18. Loss of RasGEFM reduces activation of RasG during development.** Pulsed Ax3 and *gefM*⁻ cells were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2. The experiment is representative of three separate experiments.

The polarity defect observed in chemotaxing *gefM*⁻ cells (Arigoni et al., 2005) suggested that there may be an effect on either the PI3K or TORC2 pathways. PKB phosphorylation is an effective way to determine the involvement of RasGEFM in these pathways as both PI3K and TORC2 lead to PKB activation (Lee et al., 2005; Meili et al., 1999). Pulsed Ax3 and *gefM*⁻ cells were incubated in the presence of 1 mM caffeine for 30 min, stimulated with 15 µM cAMP, and samples were collected at the time points indicated. Samples were resolved using SDS-PAGE, transferred to a PVDF membrane, and probed for phosphorylated PKB using a polyclonal anti-phospho-threonine antibody. cAMP mediated PKB activation in *gefM*⁻ cells was reduced

relative to Ax2 (Figure 17B), demonstrating that RasGEFM is involved in PKB phosphorylation. While the amounts of protein loaded onto the gel were normalized by observing Coomassie blue staining of the gels following electrophoresis (data not shown), there was no way to measure the level of total PKB protein in these blots, so it is possible that the decrease in phosphorylated PKB could be a reflection of a decrease in total PKB. Assuming that the level of total PKB is not affected by the loss of RasGEFM, these data support the idea that RasGEFM regulates RasG mediation of the pathways leading to PKB phosphorylation.

### 3.10 RasGEFC and RasGEFD are not involved in cAMP stimulated activation of Ras

The availability of other *gef* gene strains (Wilkins et al., 2005) provided an opportunity to search for other RasGEF proteins involved in the cAMP mediated RasC and RasG signalling pathways. cAMP stimulated RasC and RasG activation was normal in *gefC*$^-$ and *gefD*$^-$ strains (Table 2, Figures 19A and 19B), indicating that RasGEFC and RasGEFD are not involved in cAMP stimulated RasC or RasG activation during early development, however, the activation of RasC and RasG was prolonged in *gefC*$^-$ cells (Section 4.5). It cannot be ruled out that RasGEFC and RasGEFD regulate the activation of either RasC or RasG in response to other stimuli.

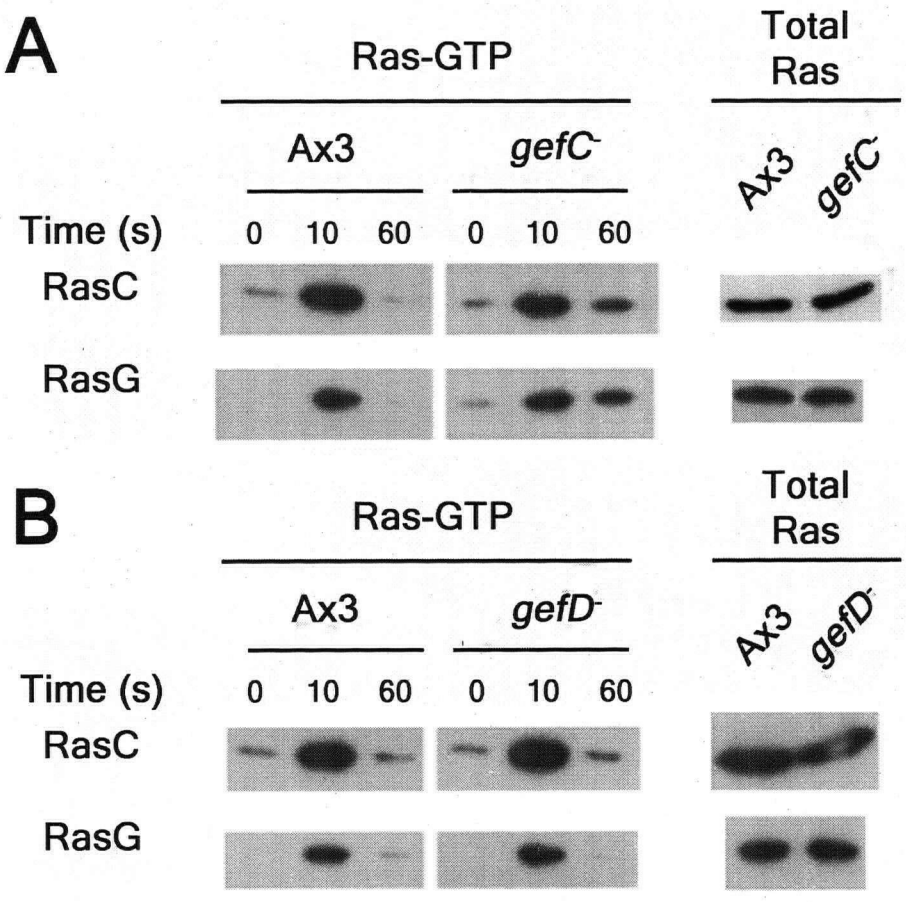


**Figure 19. Loss of RasGEFC or RasGEFD does not affect Ras activation during aggregation.** Pulsed Ax3, (A) *gefC*$^-$ and (B) *gefD*$^-$ cells (Table 2), were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to Ras bound to GST-Byr2 and 'Total Ras' refers to total Ras present in cell lysates. The experiments are representative of three separate experiments.

### 3.11 Over-expression of GbpD leads to an increase in activated Rap1, RasC

GbpD was isolated in a bioinformatic search for cGMP binding proteins, and along with a cyclic nucleotide binding domain, GbpD was found to contain a putative CDC25 domain (Goldberg et al., 2002). Over-expression of GbpD protein results in cells that are flat, exhibit greatly increased adhesion, and extend many lateral pseudopodia (Bosgraaf et al., 2005), a phenotype that bears a striking resemblance to cells that over-express activated Rap1 (Rebstein et al., 1997), suggesting a possible role for GbpD as a regulator of Rap1 activation. To investigate the role of GbpD in mediating Rap1 activation, the steady-state levels of activated Rap1 was measured in vegetative Ax3, *gbpD*$^-$ and GbpD over-expressing (Ax3*::act15-gbpD*$^{OE}$) cells (Table 2). While there was no difference in activated Rap1 levels between Ax3 and *gbpD*$^-$ cells, Ax3*::act15-gbpD*$^{OE}$ cells displayed an increase in activated Rap1 (Figure 20A). As a control, activated RasC and RasG levels were also measured. The low level of activated RasC in Ax3 and *gbpD*$^-$ cells (Figure 20A) could be a reflection of the importance of RasC during early development over vegetative stage events. This is mirrored by the mRNA expression pattern of the *rasC* gene, which is lower in vegetative cells, increasing to maximal expression during early development (Lim et al., 2001). While only a trace amount of RasC-GTP was seen in Ax3 and *gbpD*$^-$ cells, there was a modest increase in RasC-GTP observed in Ax3*::act15-gbpD*$^{OE}$ cells (Figure 20A). While these results suggest that GbpD affects both RasC and Rap1 activation, the modest increase in activated RasC in Ax3*::act15-gbpD*$^{OE}$ cells could be an indirect effect of over-expressing *gbpD*. Pulsed *gbpD*$^-$ cells stimulated with cAMP displayed no defects in RasC activation (Figure 20B), showing that GbpD is not necessary for cAMP stimulated activation of RasC during early development. The activated levels of RasG-GTP remained similar between the three strains tested (Figure 20A), suggesting that GbpD is not involved in mediating RasG

activation. Consistent with the evidence that suggests GbpD is a RapGEF, not a RasGEF, an *in vitro* nucleotide exchange assay demonstrated that GbpD exhibits specific activity for Rap1 (Table 4), and not RasC or RasG (Kortholt et al., 2006).

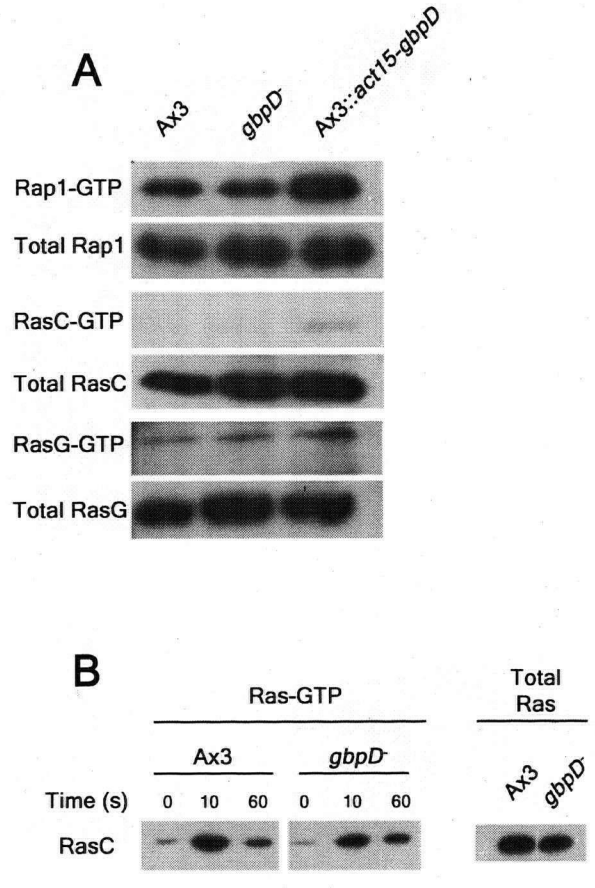


**Figure 20. Activation of Ras proteins by GbpD *in vivo*.** (A) Exponentially growing Ax3, *gbpD*$^-$, and Ax3::*act15-gbpD* cells (Table 2) were harvested by centrifugation (500 x g, 5 min). 2.5 x $10^7$ cells were lysed in 1 ml RBD-LB, incubated on ice for 10 min, and lysates were cleared by centrifugation (13,000 x g, 10 min, 4°C). Activated Ras was measured as described in Figure 7. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. Representative of three separate experiments. (B) Pulsed Ax3 and *gbpD*$^-$ cells, were harvested by centrifugation (500 x g, 5 min), washed 2 times with $KK_2$, and resuspended in $KK_2$ at 5 x $10^7$ cells/ml. Cells were stimulated with 200 nM cAMP, and activated Ras was measured as described in Figure 11. 10 µl of 6X SDS loading buffer were added to 50 µl of *Dictyostelium* lysates (1 mg/ml), indicated as 'Total Ras,' and approximately 8.3 µg total protein were loaded on the gel. 'Ras-GTP' refers to RasC bound to GST-Byr2. The experiment is representative of three separate experiments.

# 4 DISCUSSION

## 4.1 Implications of the RBD Binding Assay as a tool for studying Ras activation

Previous data had implicated a role for RasC as a major regulator of the cAMP signalling pathway during *Dictyostelium* early development. Phenotypic characterization of a *rasC* knockout strain revealed that RasC was required for the optimal activation of ACA and PKB in response to cAMP (Lim et al., 2001), and it was hypothesized that RasC was activated by cAMP and mediated at least some of the signalling pathways downstream of the cAMP signal. In order to test the hypothesis, an assay was needed that could be used to measure levels of activated Ras.

Much of the early work done in studying the Ras GTP cycle was performed with the use of radioactivity (Satoh et al., 1988). This involved labelling cells with $^{32}P$, immunoprecipitating Ras from cell lysates, and resolving GTP from GDP on thin layer chromatography (TLC). However, there are a number of problems associated with using this method. First and foremost is the requirement of millicurie levels of $^{32}P$. This requires extensive safety procedures and equipment, potentially excluding some labs from performing this assay. Another problem is the fact that the cells require incubation in phosphate free medium for several hours. This has been shown to lead to a decrease in intracellular ATP, potentially affecting normal physiological processes (Atkinson, 1968). Finally, incubating cells in the presence of $^{32}P$ can lead to cell cycle arrest, or even apoptosis (Yeargin and Haas, 1995), making this assay problematic when studying Ras activation in growing or developing cells.

Another method described measured Ras-GTP enzymatically (Scheele et al., 1995). Much like the radionucleotide based assay, this method begins by immunoprecipitating total Ras from cell lysates. The bound nucleotides are released from Ras, and subsequent enzymatic reactions employing NDP Kinase and Luciferase are used to quantify GTP and GDP. This assay

is useful for its ability to measure absolute levels of GTP and GDP bound to Ras to femtomolar accuracy, and it has been used to measure Ras-GTP in human tissues *in situ* (Guha et al., 1996). However, this assay has not been widely used, as evidenced by the fact that it has been cited only 59 times since 1995 (Web of Science), perhaps due to the seemingly labour intensive nature of this assay. For example, measurement of GDP requires conversion of cellular GDP to [$^{32}$P]GTP using NDP kinase, and then resolving the samples by TLC. Measurement of GTP involves converting the cellular GTP to ATP using NDP kinase, and then converting ATP to AMP and light, which is measured in a photon counting luminometer, using Luciferase (Scheele et al., 1995).

The method devised by Taylor and Shalloway (1996) is advantageous because it does not require the incorporation of any radioactivity, and it is not as labour intensive as the previous methods described (Figure 5). The GST-RBD fusion can be easily expressed and purified from bacteria, and the glutathione-sepharose resin is commercially available from a large number of manufacturers. Furthermore, pan-Ras antibodies that will recognize most Ras isoforms are also available commercially. The pan-Ras antibody from Calbiochem (Cat # OP40), for example, reacts with *Dictyostelium* RasB, -D, -G, and –S. On the other hand, if the objective of the study involves the assay of a specific Ras protein, isoform specific antibodies are required. In specialized cell types that may express only one isoform of Ras, this is not usually a concern; however, *Dictyostelium* expresses a number of *ras* genes at any one time during the course of its life cycle. For example, vegetatively growing cells express at least RasB, RasC, RasG, RasS, and Rap1 (Daniel et al., 1995). While the pan-Ras antibody can be used to determine whether any Ras proteins are activated, only isoform specific antibodies can be used to determine which specific Ras proteins are activated.

The use of a RBD has been extended to visualize Ras activation in living cells (reviewed in Walker and Lockyer, 2004). This has been accomplished by tagging the RBD to a fluorescent tag such as green fluorescent protein (GFP), and the resulting probe can be used to provide both temporal and spatial information about Ras activation. However, since many RBDs can bind to multiple Ras proteins, identifying the specific activated Ras species *in vivo* may be virtually impossible. For example, Sasaki et al. (2004) observed that GFP-Raf1 (RBD) localizes to the membrane that is spatially closest to the cAMP gradient (the leading edge) in chemotaxing *Dictyostelium* cells. PI3K1 localizes to the same area in chemotaxing cells when placed in a spatial gradient of cAMP (Funamoto et al., 2002). Since RasG is involved in regulating PI3K1 activity (Bolourani et al., 2006), and Raf1 (RBD) does not interact well with RasC (Kae et al., 2004), Funamoto et al. concluded that the GFP-Raf1 (RBD) probe was detecting only activated RasG. What this analysis cannot rule out is the possibility that another Ras protein that can bind Raf1 (RBD) is also activated by cAMP. One method of visualizing the activation of a specific Ras protein *in vivo* employs a FRET based probe that incorporates both the Ras protein and the Raf1-RBD as one fusion protein, flanked by YFP and CFP at the N- and C-termini, respectively (Mochizuki et al., 2001). This probe is capable of observing isoform specific Ras activation because only the intramolecular interaction elicits the FRET response, while binding of endogenous Ras will not. One problem with this probe is the possibility that the proximity of the RBD may competitively interfere with RasGAP function (Mochizuki et al., 2001), thereby affecting the extent of the activation observed. Another possible way to monitor activation of a specific Ras is to identify a RBD that will interact with only one Ras protein. The RBD from *Dictyostelium* Phg2 shows the most potential as it has been shown via a yeast two-hybrid assay that Phg2 binds only to Rap1 (Gebbie et al., 2004; G. Weeks, unpublished observations).

Whether Phg2 is able to interact with any of the other Ras proteins is not yet known, but the possibility of a Rap1 specific probe remains promising.

During the RBD binding assay, the protein content in the *Dictyostelium* cell lysates must be quantified to ensure equal input of total protein. As a result there will be a period of time during which cell lysates are left on ice, and during this time Ras-GTP will be susceptible to RasGAP activity. This is only a problem until lysates are incubated with GST-RBD, which acts as a competitive inhibitor to RasGAP activity, since they both bind to Ras at the same site, provided that the amount of GST-RBD is far greater than the amount of cellular RasGAPs. In order to avoid GAP activity, one must work quickly and leave cell lysates in ice at all times. The *DC* Protein assay used in this thesis is fast, and it can be carried out in less than 30 min. Another potential limitation is the question of the linearity of the RBD binding assay, i.e. does a band that is twice the intensity indicate that there is twice the amount of Ras-GTP? The data from Figure 10 suggests that this assay is not linear as increasing the total protein content from 200 to 400 μg results in bands representing RasC-GTP and RasG-GTP that appear to be more than 2 times in intensity. This is an issue when the results show a reduced activation of Ras, as is the case for RasG activation in *gefM*$^-$ and *gefR*$^-$ cells (Figures 15 and 18). While it is easy to speculate that the approximately half reduction of RasG activation observed in both strains means that RasGEFM and RasGEFR are the only two RasGEFs that mediate RasG activation in response to cAMP, given the non-linearity of the assay, this can be a dangerous assumption to make. Finally, when the signal of the activated Ras detected is low relative to the background control, interpretation of the results can be potentially difficult. As small variations of Ras-GTP can occur due to experimental error while handling the samples, small differences observed in Ras-

GTP may not be significant. Thus, in the cases when the differences in Ras-GTP are small, large number of repetitions must be performed in order to ensure the validity of the results.

Structural determinants of the specificity of the RBD-Ras interaction were analyzed by screening H-Ras proteins harbouring mutations in the Switch 1 region (Akasaka et al., 1996). It was discovered that a H-Ras (D38N) mutant lost the ability to bind to Raf1 while still retaining the ability to bind to Byr2. An alignment of RBD sequences does not provide much insight into how this specificity is determined. A structure of a Byr2-Ras complex has revealed that K101 of Byr2 interacts with D38 of H-Ras, most likely by forming a salt bridge between the two residues (Scheffzek et al., 2001). Thus, it is curious that substituting the negatively charged aspartate residue with a positively charged asparagine doesn't abolish the binding interaction. Furthermore, substituting D38 with the negatively charged but longer side chain of glutamate does abolish the Byr2-Ras interaction (Scheffzek et al., 2001), whereas H-Ras with a position 38 substitution to an alanine binds to Byr2 with similar affinity as H-Ras (D38N) (Ohnishi et al., 1998), demonstrating that the structure of Ras residue 38 is more important than its charge. The corresponding residue of Raf1 to Byr2 (K101) is a Lysine, suggesting that the factors that determine specificity are very subtle. As expressing H-Ras (D38N) rescued the sporulation defect of a *S. pombe* strain bearing a *ras1* gene ablation (Akasaka et al., 1996), it appears that the mutation does not severely affect the overall structure of Ras.

## 4.2 cAMP and Ras signalling

The main objective of this thesis project was to develop an assay that allowed for the measurement of activated Ras, and using this assay, determine the involvement and regulation of RasC in the cAMP signalling pathway. Figure 11A demonstrates that cAMP stimulation of

pulsed cells led to a rapid increase in activated RasC, peaking at 10 s after stimulation, substantiating the hypothesis that RasC is involved in regulating cAMP stimulated signalling events. The activation of RasC was transient, with a return of RasC-GTP to basal levels by 40 s post stimulation. A subsequent second burst of activation was seen at 80 s and remained on until at least 150 s, although the magnitude of this second peak was significantly lower than the initial peak at 10 s (Figure 11A). Whether this observed second peak is physiologically relevant is not known at this time, but one intriguing consequence of this second peak is the regulation of the cytoskeleton during chemotaxis. Both actin and myosin II filaments rapidly localize to the cell cortex upon cAMP stimulation, followed by a depolymerization and return of actin and myosin II to the cytosol by about 90 to 100 s There is evidence that RasC may be involved in the depolymerization of actin and myosin II as chemotaxing *rasC*$^-$ cells have defects in the depolymerization events (Wessels et al., 2004; Section 4.6).

The observation that activated RasG levels increased upon cAMP stimulation was initially surprising, because there was limited evidence to suggest that RasG was involved in early development. For example, expression of activated RasG (G12T) results in cells that are unable to aggregate, and while this was initially believed to be an indicator of a role for RasG in the onset of development, over-expression of wild type RasG or a dominant negative RasG (S17N) results in no defects in development (Khosla et al., 1996). Therefore, the block in aggregation caused by expressing RasG (G12T) was explained as the result of RasG (G12T) interfering with other Ras signalling pathways. Another link between RasG and development was suggested when RIP3 was shown to bind specifically to RasG, and ablation of *ripA* produced cells that did not aggregate or develop (Lee et al., 1999). While RIP3 has been shown to bind specifically to RasG, the phenotypes of *ripA*$^-$ and *rasG*$^-$ cells were so dissimilar it was

deemed unlikely that RasG signalled through RIP3 (Lee et al., 1999; Tuxworth et al., 1997). The finding that RasG is activated by cAMP shed new light on the connection between RasG and RIP3.

## 4.3 The involvement of GPCR in Ras activation

*Dictyostelium discoideum* possesses four *car* genes that encode for cAMP receptors, named *carA* to *carD*, but only one, *carA*, is highly expressed during early development (6 h post starvation) (Chen et al., 1996). Another, *carC*, is expressed during mid-development (9 h post starvation), and can somewhat compensate for the loss of *carA* (Soede et al., 1994). *carB* and *carD* are not expressed until late development, and a *carA*$^-$/*carC*$^-$ double knockout is incapable of initiating cAMP stimulated signalling events (Soede et al., 1994). RasC and RasG activation was abolished in the *car* double knockout strain (Figure 12A), demonstrating that Ras activation during aggregation in response to cAMP requires cAR1 and cAR3 receptors.

While the majority of the stimulatory pathways downstream of cAR1 are regulated by the activation of the associated heterotrimeric G-protein, Gα2βγ, there are a number of responses that function in response to cAMP but independently of Gα2βγ. The cAMP mediated activation of the MAP kinase ERK2 has been shown to be partly independent of Gα2βγ (Briscoe et al., 2001; Maeda et al., 1996), suggesting the possibility that a Ras protein may be involved in mediating some G-protein independent responses given that MAP kinase cascades are typically downstream of Ras proteins in mammals (Arbabi and Maier, 2002). However, the relationship between Ras and ERK2 in *Dictyostelium* has been somewhat controversial. As measured by an *in vitro* kinase assay, cAMP stimulated activation of ERK2 peaks one min post-stimulation, and returns to basal levels by three min post-stimulation (Knetsch et al., 1996). Expression of an

activated RasD (G12T) in wild type cells results in an approximately 4-fold increase in the basal level of cAMP stimulated ERK2 activity, with a subsequent decline in ERK2 activation to levels comparable to wild type by 3 min post-stimulation. It was postulated that activated RasD (G12T) stimulates the activation of ERK2, hence the elevated basal level, but does not affect the subsequent adaptation of ERK2, leading to ERK2 inactivation. Aubry et al. (1997) observed the opposite effect. Expression of activated RasD (Q61L) in wild type cells results in a significant reduction in cAMP stimulated ERK2 activation, as measured by an in-gel kinase assay. Furthermore, the basal level of activated ERK2 was the same as that seen in wild type cells, and the kinetics of ERK2 activation mirrored that of wild type cells. The observation that cAMP stimulated RasC and RasG activation is ablated in *gpaB*$^-$ or *gpbA*$^-$ cells (Figure 12B and 12C) demonstrates that RasC and RasG activation in response to cAMP is linked to G$\alpha$2$\beta\gamma$, suggesting that RasC and RasG are not involved in the G-protein independent activation of ERK2.

Upon agonist binding to the cAMP receptor, both G$\alpha$2 and the G$\beta\gamma$ dimer participate in distinct signalling pathways, and a combination of genetic and *in vitro* data has suggested that G$\alpha$2 is involved in the pathway leading to guanylyl cyclase activation, and G$\beta\gamma$ is responsible for PI3K and adenylyl cyclase activation (Okaichi et al., 1992; Roelofs et al., 2001; Wu et al., 1995). Activated RasC and RasG are also thought to participate in discrete pathways, with RasG involved in regulating the chemotactic response and cell polarity, which includes the activation of guanylyl cyclase and PI3K, respectively, and RasC in adenylyl cyclase activation (Bolourani et al., 2006). Thus, it can be speculated that G$\alpha$2-GTP mediates the activation of RasG in the pathway leading to GC activation, whereas the G$\beta\gamma$ dimer mediates the activation of both RasC and RasG for the activation of ACA and PI3K, respectively. A model of dual Ras protein

activation from one GPCR has been proposed in HEK293 cells (Schmitt and Stork, 2002). Stimulation of the β2-adrenergic receptor with isoproterenol leads to the activation of Ras and Rap1. Furthermore, through the use of dominant negative mutants it was demonstrated that Ras activation was dependent on Gβγ, and Rap1 activation was dependent on Gα (Schmitt and Stork, 2002). This model of G-protein dependent Ras activation provides a mechanism for how two distinct signalling pathways originating from one stimulus can be activated and regulated.

## 4.4 Role of RasGEFs in Ras activation

The *Dictyostelium* genome encodes 15 Ras subfamily proteins (Weeks et al., 2005), a number that is considerably larger than that found in worms and yeast, which contain between four and ten (Weeks et al., 2005). In addition, there are more *gef* genes than *ras* genes encoded by the *Dictyostelium* genome (Wilkins et al., 2005), suggesting that at least some Ras proteins are activated by more than one RasGEF. Consistent with this idea is the fact that of the ten *gef* gene knockouts that have been described, four *gef* gene knockout strains have no distinct phenotypes (Wilkins et al., 2005).

The isolation of a *gefA*$^-$ strain that failed to aggregate was the first indication of a Ras protein being involved in cAMP signalling during *Dictyostelium* development (Insall et al., 1996). The subsequent isolation of a *rasC*$^-$ mutant with a similar phenotype suggested the possibility that RasC functioned downstream of RasGEFA (Lim et al., 2001). The pattern of Ras activation in the *gefA*$^-$ mutant provided convincing evidence that RasGEFA is necessary for the activation of RasC in response to cAMP, but not required for RasG activation (Figure 14). This has been verified by the use of an *in vitro* nucleotide exchange assay (Table 4). RasGEFA

catalyzed the removal of GDP from RasC, but not from other *Dictyostelium* Ras proteins, confirming that RasGEFA is specific for RasC.

A proteomic study has shown that RasGEFR is one of the proteins that could be identified as exhibiting an alteration in tyrosine phosphorylation in response to the expression of activated RasG (Secko et al., 2004). This suggested a possibility that RasGEF could mediate activation of RasG as there are examples of RasGEF regulation by phosphorylation. Stimulation of rat forebrain slices with the vasodilator forskolin leads to phosphorylation of RasGRF1, and this phosphorylation is required for maximal RasGEF activity (Yang et al., 2003). Conversely, it has been shown in CHO cells that RasGRF2 is phosphorylated by the cyclin dependent kinase, Cdk5, and this phosphorylation serves to downregulate the RacGEF activity of RasGRF2 (Kesavapany et al., 2004). However, there was no direct evidence to suggest that RasGEFR mediated RasG activation. The finding of a reduction in the magnitude of RasG activation in response to cAMP in a *gefR*$^-$ mutant (Figure 15) provided evidence that RasGEFR is responsible for mediating at least some of the RasG activation. This conclusion was confirmed by an *in vitro* nucleotide exchange assay which showed that RasGEFR was specific for RasG (Table 4; Kae et al., 2007). A closer examination of the *gefR*$^-$ phenotype revealed that *gefR*$^-$ cells do not have defects in the activation of PKB (Figure 17A), demonstrating that RasGEFR is not responsible for RasG mediated activation of either PI3K or the TORC2 complex. An examination of the streaming characteristics of *gefR*$^-$ cells during aggregation revealed that, unlike *rasG*$^-$ cells, there was no delay in the onset of aggregation (Figure 16). However, the streams formed by *gefR*$^-$ cells appeared to involve fewer cells and thus appeared less dense when compared to streams formed by wild type cells (Figure 16). It has been shown that ACA activity is required for stream formation (Kriebel et al., 2003), so the fact that *gefR*$^-$ cells are able to stream would

suggest that ACA activity is not abolished in this strain. Instead it can be speculated that the less robust streams formed by *gefR*$^-$ cells is due to a defect in either motility or chemotactic efficiency.

Since RasG activation is not completely abolished in the *gefR*$^-$ strain, it would appear that there is at least one other RasGEF responsible for the activation of RasG upon cAMP stimulation. One candidate RasGEF is RasGEFM. cAMP stimulation of *gefM*$^-$ cells resulted in a moderate reduction of RasG activation, relative to wild type (Figure 18), demonstrating that RasGEFM, like RasGEFR, is required for maximal activation of RasG. It has been shown in splenic B cells that both RasGRP1 and RasGRP3 contribute to maximal activation of Ras, detected using a pan-Ras antibody, and loss of either RasGEF results in a decrease in activation (Coughlin et al., 2005). Furthermore, RasGRP1 and RasGRP3 appear to mediate different pathways as *rasgrp1*$^{-/-}$ and *rasgrp3*$^{-/-}$ knockout mice exhibit different phenotypes. A model involving the regulation of RasG activation by two RasGEFs can provide a means to regulate two independent pathways mediated through RasG. However, without supporting *in vitro* evidence, it is impossible to conclude that RasGEFM regulates RasG activation.

RasG has been linked to the cAMP mediated activation of PKB (Section 1.13). The observed reduction in the activation of PKB in *gefM*$^-$ cells suggests that RasGEFM mediates the RasG dependent activation of PKB (Figure 17B), and the reduced PKB activation is consistent with the defects in cell polarity and motility seen in both *gefM*$^-$ and *rasG*$^-$ cells (Arigoni et al., 2005; Bolourani et al., 2006). However, at this point the evidence linking RasGEFM and RasG is circumstantial, and it cannot be ruled out that the reduced activation of both RasG and PKB could be due to some secondary effect such as a change in gene expression, or the result of an indirect effect on a separate signalling pathway. We do know that RasGEFC and RasGEFD are

not involved in the activation of RasG in response to cAMP, as neither the *gefC*$^-$ nor *gefD*$^-$ mutants were defective in RasG activation (Figure 19).

GbpD was isolated in a search for cGMP binding proteins identified by bioinformatic analysis and was found to contain a domain with homology to CDC25p (Goldberg et al., 2002). A *gbpD*$^-$ strain revealed an involvement in early developmental signalling, as *gbpD*$^-$ cells chemotax faster due to a hyper-polarized cell shape, a phenotype that is similar to *rasC*$^-$ cells (Lim et al., 2001), and to cells that over-express a dominant negative Rap1 (S17N) (Jeon et al., 2007). Over-expression of GbpD, on the other hand, leads to cells that are more adherent when placed on a solid surface, and a loss of polarity when chemotaxing (Bosgraaf et al., 2005). Assaying for activated Ras revealed that over-expression of GbpD results in an increase in activated Rap1 and RasC, but not RasG (Figure 20A). However, an *in vitro* nucleotide release assay demonstrated that GbpD can catalyze activation of Rap1, and not RasC (Table 4), indicating that the *in vivo* increase in activated RasC-GTP is not likely a direct effect (Kortholt et al., 2006). In support of these observations, cAMP stimulated RasC activation in a *gbpD*$^-$ strain is largely unaffected (Figure 20B). It has been shown that cAMP stimulation of aggregation stage cells results in transient activation of Rap1 (Jeon et al., 2007). Furthermore, expression of a dominant negative Rap1 (S17N) results in cells that hyper-polarize during chemotaxis, and expression of an activated Rap1 (G12T) abolishes cell polarity. However, use of dominant negative and activating Rap1 mutants could interfere with other Ras signalling pathways (Feig, 1999), including those of RasC and RasG, so the physiological effects of these Rap1 mutants may be the result of perturbing the signalling of another Ras protein. How Rap1 integrates with the Ras signalling pathway, if at all, is unclear at this time, but there is evidence to suggest that

cAMP stimulated activation of Rap1 lies downstream of either RasC or RasG (P. Bolourani, unpublished observations).

Initial experiments have shown a high degree of RasGEF-Ras specificity in *Dictyostelium*. These findings seem to be in contrast to the situation in mammalian cells where overlapping specificities have been reported. For example, SOS exhibits *in vitro* exchange activity on H-, K-, N-, M-, and R-Ras2 (Nielsen et al., 2001; Ohba et al., 2000; Porfiri et al., 1994), and RasGRF1 has been shown to activate H-, K-, N-, M-, R-, and R-Ras2 (Nielsen et al., 2001; Ohba et al., 2000). In an organism like *Dictyostelium*, activation of Ras pathways by specific interactions with a RasGEF would be an efficient mechanism to ensure high fidelity as there are normally multiple Ras and RasGEF proteins present. During aggregation there are 18 *gef* genes and at least 7 *ras* genes expressed (Chisholm et al., 2006; Daniel et al., 1995; Wilkins et al., 2005). This mechanism may not be as important in metazoans, as many RasGEFs have tissue or cell specific expression, bypassing the need for tight substrate specificity. For example, RasGRP1 is the only RasGRP family member to be expressed in T cells and mediates all of the DAG stimulated Ras activation (Dower et al., 2000), while RasGRF1 is predominantly expressed in brain tissue (Guerrero et al., 1996).

Little is known regarding the Ras structural determinants that might be involved in conferring RasGEF specificity. Analysis of a SOS-Ras crystal structure has revealed that 3 regions of Ras are involved in the RasGEF-Ras interaction; the Switch 1 region (residues 21-40), the Switch 2 region (57-75) and the helix α3 region (95-111) (Boriack-Sjodin et al., 1998). RasC and RasG exhibit a number of differences in the Switch 1 (4 out of 19 residues), Switch 2 (2 out of 19), and helix α3 regions (6 out of 17) (Figure 2), providing a variety of possible determinants of specificity. However, RasGEFR is capable of activating RasG but is without

activity for RasD (Table 4), despite the fact that RasD and RasG are highly related (89% identity), including identical Switch 1, Switch 2, helix α3 regions (Figure 2). The observation that the CDC25 domain of mammalian RasGRF1, used as a positive control, was able to activate all the *Dictyostelium* Ras proteins indiscriminately in an *in vitro* nucleotide exchange assay (Table 4) would indicate that the factors that determine the RasGEF-Ras interaction specificity are subtle, and involves other residues outside of the Switch 1, Switch 2, and helix α3 regions. The region of SOS neighbouring the Ras-SOS interface is surrounded by three residues that have been proposed to be involved in mediating RasGEF specificity. When residues R826, T935, and E1002 of SOS are mutated to alanines there is no effect on the binding and activation of Ras by SOS (Hall et al., 2001). However, a mutation of yeast CDC25p in the residue equivalent to R826 of SOS disrupts the ability of CDC25p to bind to Ras (Park et al., 1994), and a mutation in RasGRF1 in the residue equivalent to T935 of SOS disrupts the catalytic activity on Ras (Vanoni et al., 1999). Alignment of the protein sequences of RasGEFA and RasGEFR (Figure 21) reveals that at the position corresponding to R826 of SOS, RasGEFA has a negatively charged aspartate residue, whereas RasGEFR has a positively charged asparagine residue, and at the position corresponding to E1002 of SOS, RasGEFA has a leucine residue, whereas RasGEFR has a gap in its alignment. RasGEFA has a threonine residue and RasGEFR has a serine residue at the position corresponding to T935 of SOS (Figure 21). If the structures of RasGEFA and RasGEFR are analogous to the structure of SOS, it's possible that the differences in the residues at position 826 and 1002, relative to SOS, contribute to the specificity of the RasGEF-Ras interaction observed. There are also a number of differences in other residues that have been implicated in the SOS-Ras interaction when comparing the alignment of the RasGEFA and RasGEFR sequences (Figure 21), providing a number of possible residues that may be involved

in mediating specificity. A detailed mutational analysis will be needed to determine which of these residues, or any other residues, are the important determinants that confer specificity for the RasGEF-Ras interaction.

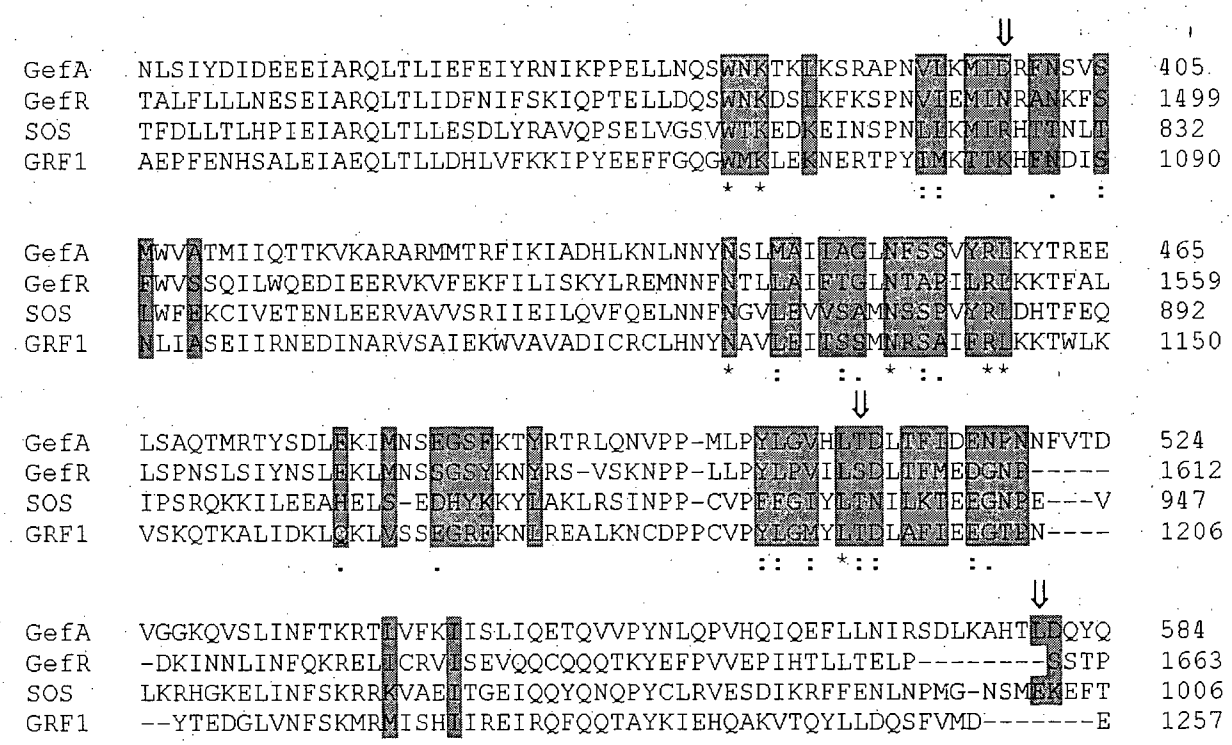


**Figure 21. Alignment of RasGEF protein sequences.** Gapped alignment of protein sequences of the CDC25 catalytic domains of the *Dictyostelium* RasGEFA and RasGEFR, and mammalian SOS and RasGRF1 (labelled GRF1). Alignment was performed using CLUSTAL W multiple sequence alignment program, version 1.83. Numbers indicate position of amino acids in protein and dashes indicate gaps in alignment. Shaded residues represent those that are involved in the RasGEF-Ras interaction, as deduced from the SOS-Ras crystal structure (Boriack-Sjodin et al., 1998; Hall et al., 2001). Residues marked on the bottom with (*) are conserved residues, those marked with (:) are 'strongly' conserved as defined by CLUSTAL W, and residues marked with (.) are 'weakly' conserved as defined by CLUSTAL W. Residues marked on top with a (⇓) represent the residues corresponding to R826, T935, and E1002 of SOS.

An *in vitro* study employing purified Ras proteins demonstrated that Ras prenylation may play a role in contributing to RasGEF substrate specificity in mammalian cells (Porfiri et al., 1994). The proteins were expressed in Sf9 insect cells so that the Ras proteins underwent the correct post-translational modification that is absent when expressed in bacteria. The report demonstrates that SOS does not exhibit *in vitro* exchange activity on unprenylated H-Ras or K-Ras4B, and this specificity is limited to prenylation as changes to the palmitoylation sites of H-Ras have no effect. On the other hand, CDC25 is able to mediate nucleotide exchange on either form of Ras, indicating that prenylation does not determine specificity for all RasGEFs. Both RasC and RasG are predicted to undergo the same post-translational modification, a geranylgeranylation (Moores et al., 1991), suggesting that prenylation does not account for the difference in specificity towards RasGEFA and RasGEFR. Furthermore, the results from an *in vitro* nucleotide exchange assay shown in Table 4 employed a C-terminally truncated form of RasC (Kae et al., 2007), supporting the notion that regions of Ras other than the C-terminus are important in determining substrate specificity for RasGEFA and RasGEFR. However, it cannot be ruled out that post-translational modification is a determinant for substrate specificity for other *Dictyostelium* RasGEFs.

It remains to be seen how *Dictyostelium* RasGEFs direct Ras activation *in vivo*. In mammalian Ras pathways, a large number of RasGEFs associate with adaptor proteins that mediate their cellular localization and facilitate Ras activation. A model has been proposed in HEK293 epithelial kidney cell lines that involves a direct interaction of a GPCR and a cAMP regulated RasGEF, CNrasGEF. Pak et al. (2002) showed that CNrasGEF binds directly to the GPCR β1-AR, and in conjunction with binding to cAMP, CNrasGEF activates Ras directly upon agonist binding to β1-AR. This model provides the first evidence of Ras activation directly from

a GPCR. While a key feature of most RasGEFs is the inclusion of other signalling domains that provide clues in elucidating Ras pathways, many *Dictyostelium* RasGEFs contain very few signalling motifs outside of the catalytic domain (Wilkins et al., 2005). CNrasGEF, for example, contains a PDZ domain which is necessary for its interaction with β1-AR and a cNMP binding domain which binds cAMP and serves as a way to regulate its activity (Pak et al., 2002). In contrast, RasGEFM and RasGEFR contain no other signalling domains outside of the catalytic domain (Wilkins et al., 2005), and the only other signalling motif that can be found on RasGEFA is a LisH domain (Wilkins et al., 2005), a poorly characterized motif that has been postulated to be a possible protein oligomerization domain (Kim et al., 2004). The LisH domain has also been suggested to regulate microtubule dynamics (Emes and Ponting, 2001).

The function of the microtubule cytoskeleton in chemotaxis is unclear, but it has been observed that *Dictyostelium* cells exclude the microtubule cytoskeleton from the leading pseudopod during chemotaxis to cAMP (Rubino et al., 1984), and that the microtubule organizing centre localizes to the rear of cells when chemotaxing to folate (Sameshima et al., 1988). This type of distribution has also been observed in dHL-60 cells, a neutrophil-like cell line, where disruption of the microtubule cytoskeleton using the microtubule depolymerising drug, nocodazole, increases cell polarity (Xu et al., 2005). Furthermore, treating cells with nocodazole results in a decrease in C.I. of cells chemotaxing towards fMLP, while increasing the migration speed ~50 % (Xu et al., 2005). RasGRF1 has been shown to associate with microtubules *in vitro*, and RasGRF1 transfected into COS-7 cells localizes to microtubules *in vivo* (Forlani et al., 2006). As this interaction has no effect on microtubule dynamics, it has been suggested that the association serves as an anchor for RasGRF1 (Forlani et al., 2006). Even though the domain involved in RasGRF1 localization shows no homology to the LisH domain

present in RasGEFA, it could be possible that RasGEFA demonstrates a similar localization to the microtubule network.

## 4.5 Ras activation and the adaptation response

The stimulatory signalling events initiated by cAMP during early development in *Dictyostelium* have been studied for many years (reviewed in Manahan et al., 2004). To respond to cyclic pulses of a stimulant, however, there must be a period when the signalling components are deactivated, during which time the cells remain unresponsive to the signal and cells return to a basal state. In *Dictyostelium*, this process is referred to as the adaptation response, and the mechanism for how this proceeds remains largely unresolved (Brzostowski et al., 2004).

The loss of ACA activity in *acaA*$^-$, *dagA*$^-$, *and piaA*$^-$ strains appeared to have an effect on the deactivation of Ras. RasC activation was prolonged in *dagA*$^-$, *and piaA*$^-$ cells (Figure 13A, 13B), whereas RasG activation was prolonged in *acaA*$^-$ (Figure 13C). It is possible that the loss of ACA activity has an effect on the adaptation response, and one possible regulator of the adaptation response is PKA, an important transducer of the intracellular cAMP signal. cAMP stimulation of wild type cells results in a rapid activation of the MAP kinase, ERK2 (Maeda et al., 1996), and it has been postulated that ERK2 regulates the level of intracellular cAMP levels through the regulation of phophodiesterases (Maeda et al., 2004). Cells with an ablation of the gene encoding ERK2 do not aggregate (Segall et al., 1995), presumably due to the elevated action of cAMP phosphodiesterases, and the aggregation defect can be rescued by expressing constitutively active PKA (Aubry et al., 1997), placing it genetically downstream of ERK2. However, ERK2 activation is prolonged in *pka*$^-$ cells (Aubry et al., 1997), suggesting that

intracellular cAMP is important for the adaptation response, and the prolonged activation of RasC and RasG observed (Figure 13) may be due to a defect in this adaptation response.

In some of the studies on Ras activation reported in this thesis, loss of a RasGEF was associated with prolonged activation of those Ras proteins that were still activated. In wild type cells, Ras activation was rapid, peaking by 10 s, and returning to near basal levels by 40 s (Figure 11). *gefA*⁻ cells exhibited no decrease in RasG activation by 60 s (Figure 14), *gefM*⁻ and *gefR*⁻ cells exhibited a level of RasC activation at 60 s that was greater than basal levels (Figures 15 and 18), and *gefC*⁻ cells exhibited a greater level of both activated RasC and RasG by 60 s compared to basal levels (Figure 19A). Given the general model of the GTP dependent activation cycle of Ras proteins (Figure 1), this extended peak of Ras-GTP might reflect reduced activity of a negative regulator of Ras, such as a RasGAP. Nine genes have been annotated in the *Dictyostelium* genome that encode for proteins with putative RasGAP domains (Chisholm et al., 2006), but none have been characterized thus far. Two proteins with RasGAP domains, DGAP1 and GAPA, have been identified as IQGAPs, and the evidence suggests that these two proteins act on Rho family GTPases, and not Ras (Adachi et al., 1997; Faix et al., 1998). Whether the 9 putative RasGAPs show specificity towards individual Ras proteins is unknown at this time.

One candidate negative regulator of Ras activation in cAMP stimulated events is the G alpha protein, Gα9 (*gpaI*), which has been implicated in the negative regulation of events downstream of cAR1 (Brzostowski et al., 2004). While the majority of cAR1 is coupled to Gα2βγ (Kumagai et al., 1991), Brzostowski et al. (2004) have demonstrated that a small but significant population of cAR1 has also been shown to be coupled to Gα9βγ. Dissociation of /cAR1 with its heterotrimeric G-protein reduces its affinity for cAMP, and cAMP binding is

inhibited by about 75 % in membranes from wild type cells when the heterotrimeric G-proteins are activated *in vitro* using GTPγS. In contrast, membranes from *gpaB*$^-$ cells display only ~15 % inhibition when stimulated with GTPγS, indicating that the majority of cAR1 is coupled to Gα2. Ectopic expression of *gpaI* in *gpaB*$^-$ cells is able to partially restore the GTPγS stimulated inhibition of cAMP binding to membranes from *gpaB*$^-$ cells to ~35 %, indicating that Gα9 is also able to couple to cAR1 (Brzostowski et al., 2004). Gene ablation studies have revealed that a *gpaI*$^-$ strain exhibits extended peaks of cAMP stimulated cAMP, cGMP and PI(3,4,5)$P_3$ accumulation (Brzostowski et al., 2004). It is striking that the initial activation kinetics are largely unaffected, with the *gpaI*$^-$ cells often displaying an equal or a small increase in activation relative to wild type cells. However, the adaptation response, defined as the return to basal levels, is significantly delayed. Expression of a constitutively activated Gα9 (Q196L) results in a faster adaptation response. As the loss of Gα9 affects multiple activation pathways, i.e. the PI3K pathways, ACA activation, and cGMP accumulation, it would suggest that Gα9 acts as a general negative regulator of cAMP signalling, functioning to turn off all pathways mediated by cAR1. How Gα9 mediates this regulation is unknown as there is a dearth of information regarding the components of the adaptation response, but it is conceivable that Gα9 functions as a negative regulator of both RasC and RasG activation given the involvement of Gα9 in multiple signalling pathways (Brzostowski et al., 2004).

There are some examples of GAPs functioning downstream of GPCR in mammalian cells. Gα12-GTP interacts with GAP1$^m$ via a tandem PH/BH (Btk homology motif) domain, and this interaction has been shown to stimulate GAP activity in a cell free *in vitro* assay (Jiang et al., 1998). Additionally, while EGF stimulation of MEG-01 human leukemia cells stimulates Ras activation, concomitant over-expression and activation of Gα12 with the agonist U46619

reduces this activation, demonstrating that activated Gα12 potentiates $GAP1^m$ activity *in vivo* as well. On the other hand, the $G\alpha_0$ protein regulates GAP activity in a different manner. In HEK293 cells, Rap1GAP co-immunoprecipitates with $G\alpha_0$-GDP, but not the GTP bound isoform (Jordan et al., 1999). Over-expression of $G\alpha_0$ results in an increase in activated Rap1, whereas over-expression of a constitutively activate $G\alpha_0$ (Q205L) diminishes activated Rap1, suggesting that $G\alpha_0$-GDP regulates Rap1 activity by sequestering Rap1GAP. If the adaptation response in *Dictyostelium* is indeed mediated by GAP regulation, the available evidence suggests that Gα9 lies upstream of the putative RasGAPs for RasC and RasG.

### 4.6 Regulation of the cytoskeleton during aggregation by Ras

Chemotaxing *Dictyostelium* cells, like all motile cells, undergo orchestrated chemoattractant induced changes to the cytoskeleton (Franca-Koh et al., 2006). After an initial "cringe" response in which cells round up and cease motion, the cytoskeleton polarizes such that F-actin assembles at the leading edge, which is the site of new pseudopod formation, and myosin II assembles at the rear and sides of the cell to provide cortical tension and to retract the trailing uropod. These two responses have been reported to be regulated by PI3K activity and cGMP, respectively (Bosgraaf et al., 2002; Chen et al., 2003), and there is an increasing body of evidence to suggest that RasG is involved in both responses. It has been well documented that mammalian Ras-GTP binds to PI3K and stimulates $PI(3,4,5)P_3$ formation (Kodaki et al., 1994; Rubio et al., 1997). Similarly, RasG-GTP has been shown to bind to *Dictyostelium* PI3K1 and PI3K2 in a yeast two hybrid assay (Funamoto et al., 2002), and a RBD binding assay (Figure 7C). Although it has yet to be determined whether this binding stimulates PI3K activity, a PI3K1 isoform that harbours a mutation in the RBD is unable to generate $PI(3,4,5)P_3$ at the

leading edge in response to cAMP when expressed in a *pi3k1⁻/pi3k2⁻* double knockout. Furthermore, constitutively localizing PI3K1 to the membrane via a myristoylation motif does not rescue this defect, suggesting that binding of Ras to PI3K does not function to merely anchor PI3K to the membrane (Funamoto et al., 2002). These data have been interpreted as indicating that RasG is required for PI3K stimulated $PI(3,4,5)P_3$ formation, and consequently for actin polymerization.

The evidence for RasG mediated regulation of myosin II polymerization is equally compelling. Chemotaxing cells rapidly polymerize myosin II to the cortex at the rear and sides of the cell when challenged with cAMP, and this response is defective in a *sgcA⁻/gca⁻* double knockout strain, cells that are unable to synthesize cGMP (Bosgraaf et al., 2002). These cells are motile, but they display a loss in polarity during chemotaxis, and have a severe defect in chemotactic efficiency as measured by the C.I. *rasG⁻* cells display a similar phenotype in that they are also unable to properly polarize during chemotaxis, yet still retaining the ability to locomote (Bolourani et al., 2006). While *rasG⁻* cells display a significant reduction in C.I., the defect is not as severe as that of the *sgcA⁻/gca⁻* strain. The difference in C.I. between *rasG⁻* and *sgcA⁻/gca⁻* cells can be accounted for by the fact that while both receptor and GTPγS stimulated cGMP accumulation is significantly reduced in *rasG⁻* cells, an appreciable level of cGMP is still synthesized (Bolourani et al., 2006, and P. Bolourani, unpublished observations). cGMP synthesis is completely abolished in *rasC⁻/rasG⁻* double null cells; and these cells have the same dramatic defect in C.I. as *sgcA⁻/gca⁻* cells. These data suggest that the inefficient chemotactic response of *rasG⁻* cells may be in part a consequence of defects in mobilizing both the actin and the myosin cytoskeleton.

RasC, on the other hand, appears to antagonize the pathways leading to actin and myosin polymerization. In a study to examine the chemotactic properties of the *rasC*⁻ cells, it was found that both the actin and myosin II cytoskeletons polymerize and mobilize to the cell cortex in response to cAMP much like wild type cells (Wessels et al., 2004). While wild type cells subsequently depolymerise and reorganize actin and myosin II away from the cortex to the cytosol, both remain in the cortex of *rasC*⁻ cells, leading to a hyper-polarized cell morphology. The depolymerization of actin and myosin II occur at approximately 80 to 90 s after the cAMP stimulus, which coincides with the second, more gradual peak of activated RasC (Figure 11), so it is possible that RasC is involved in the depolymerization of both actin and myosin II filaments during chemotaxis. Vegetative *rasC*⁻ cells display polymerized actin staining at several sites around the membrane, whereas wild type cells typically show a single focus of polymerized actin (Lim et al., 2005), supporting a negative regulator role for RasC. It has been shown in HCT116 human cancer cell lines that K-Ras regulates depolymerization of the cytoskeleton (Pollock et al., 2005). HCT116 cells have one mutant K-Ras (G13D) allele, the equivalent to H-Ras (G12T), and these cells are devoid of stress fibres and focal adhesions when placed on glass coverslips. Disruption of the mutant K-Ras allele restores stress fibre and focal adhesions, suggesting that the absence of stress fibres and focal adhesions in HCT116 cells is a result of the presence of activated K-Ras. Furthermore, despite the absence of stress fibres, HCT116 cells display an elevated level of activated Rho, which is involved in the formation of stress fibres, and treatment of HCT116 cells with Rho inhibitors results in the reformation of stress fibres, suggesting that K-Ras mediates its effects on the cytoskeleton by negative regulation of Rho activity, but not by the activation of Rho (Pollock et al., 2005). Whether RasC functions through mediating the activity of a Rho family GTPase remains to be seen, but as Ras has been well

documented to mediate cytoskeletal rearrangement upstream of small GTPase cascades in other cells, this notion is not out of the realm of possibility.

## 4.7 An emerging model of Ras signalling during aggregation

The work in this thesis project demonstrated that cAMP stimulation of *Dictyostelium* cells during the aggregation stage resulted in the activation of RasC and RasG (Figure 11), and that this activation is downstream of the cAMP receptors, cAR1 and cAR3, as well as its associated heterotrimeric G-protein, $G\alpha 2\beta\gamma$ (Figure 12). Furthermore, it appears that all cAMP mediated RasC activation is mediated by RasGEFA (Figure 14), whereas RasG activation is mediated by at least 2 GEFs, RasGEFR and RasGEFM (Figure15, 18). Closer examination of the phenotypes of *gefR*$^-$ and *gefM*$^-$ cells has revealed a possible role for RasGEFR in chemotaxis (Figure 16) and RasGEFM in regulating cell polarity and motility through the activation of the pathway leading to PKB phosphorylation (Figure17B).

How the *Dictyostelium* RasGEFs are regulated is unknown at this time, but some speculations can be made based on models of analogous systems. The most common way to regulate RasGEF mediated activation is to regulate the localization of the RasGEF, usually via the action of adaptor proteins. In the case of RasGEFA, the LisH domain may function to localize RasGEFA in the proximity of RasC when stimulated with cAMP. Since no obvious motifs exist on RasGEFM and RasGEFR, the model would depend on the existence of novel binding sites for protein-protein interaction domains that would be analogous of SH2 or SH3 domains. RasGEFR undergoes a decrease in threonine phosphorylation in response to the expression of activated RasG. Given that RasGEFR exhibits *in vivo* (Figure 15) and *in vitro* (Table 4) activity on RasG, the phosphorylation of RasGEFR could be a positive regulator of its

RasGEF activity, and the subsequent activation of RasG provides feedback inhibition on the activity of RasGEFR. In other cells, activation of signalling pathways downstream of Ras has been shown to induce negative feedback inhibition of Ras activation. Expression of activated Raf1 in BJ fibroblasts results in an increase in ERK activation when treated with tamoxifen, but in a decrease in Ras-GTP levels, demonstrating that activation of Ras pathways can lead to inhibition of Ras (Courtois-Cox et al., 2006). Whether this inhibition of Ras is through the activation of a RasGAP, or inhibition of a RasGEF is unknown.

A picture of the cAMP stimulated Ras signalling pathways is beginning to emerge (Figure 22). The current evidence suggests that all Gα2βγ dependent signalling is mediated by the concerted functions of RasC and RasG. RasG appears to regulate at least three pathways: 1) activation of GC (Bolourani et al., 2006), 2) activation of PI3K (Bolourani et al., 2006; Funamoto et al., 2002), and 3) regulation of the TORC2 complex via an interaction with RIP3 (Lee et al., 2005). Taken together, the predominant role of RasG is in chemotaxis and in regulating cell motility and polarity. The observation that RasG may be activated at the leading edge of chemotaxing cells is significant as it had been shown that GC, PI3K, and RIP3 translocate to the leading edge when placed in a cAMP gradient (Funamoto et al., 2002; Lee et al., 2005; Sasaki et al., 2004; Veltman et al., 2005), reflecting RasG interaction with its downstream effectors. The streaming pattern of *gefR*$^-$ cells (Figure 16) suggests that there may be defects in cell motility or chemotaxis as the streams formed by *gefR*$^-$ cells appear less dense when compared to streams formed by wild type cells. A quantitative analysis of the chemotactic behaviour of *gefR*$^-$ cells could provide more insight into the role of RasGEFR in this process. Since RasGEFM is required for maximal PKB phosphorylation (Figure 17B), it is possible that RasGEFM activates RasG in a pathway that targets either PI3K or TORC2.

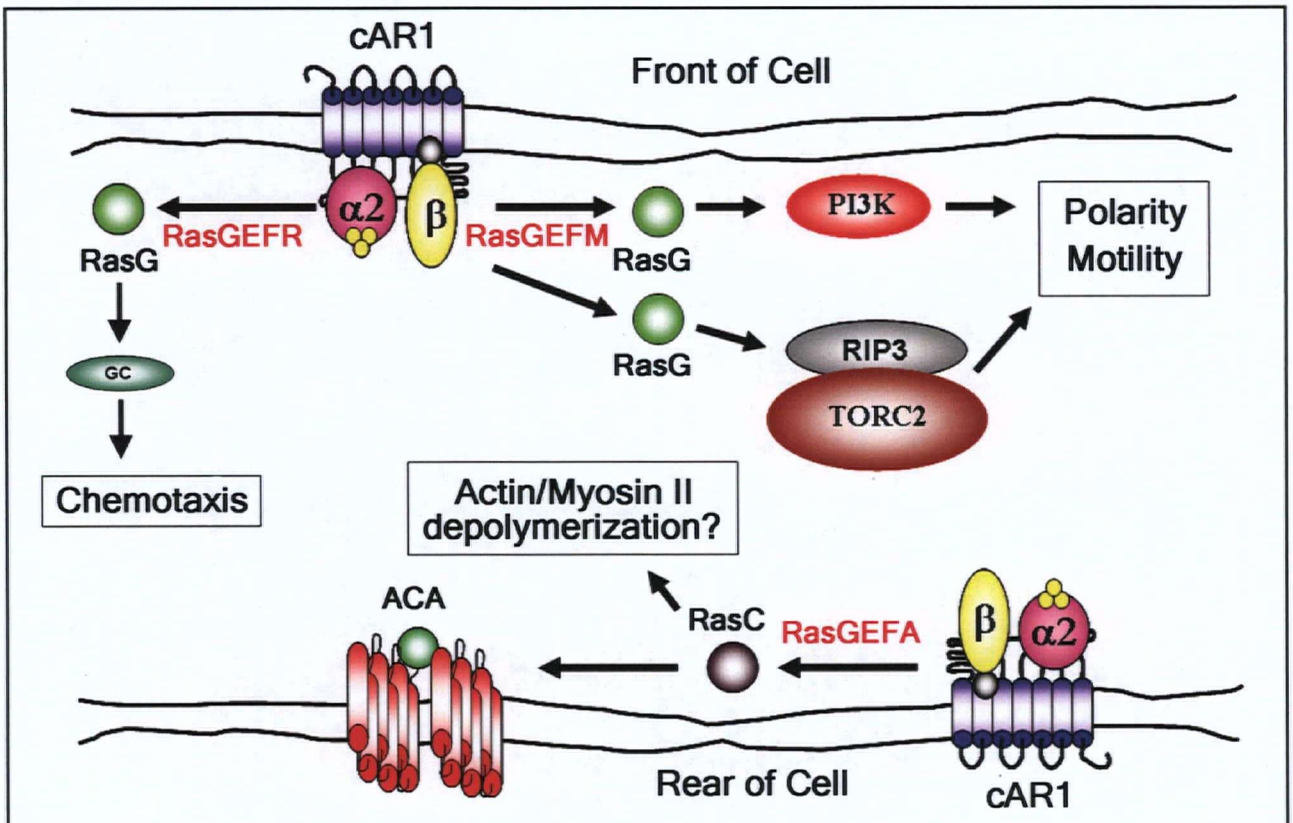


**Figure 22. Proposed model for the involvement of RasC and RasG in cAMP stimulated events during *Dictyostelium* aggregation.** cAMP stimulation leads to a number of responses, but for simplicity have been separated into 3 functions: chemotaxis, cell polarity/motility, and activation of ACA. Activation of RasC and RasG are downstream of the GPCR, cAR1, and its associated G-protein, Gα2βγ. Activation of RasG occurs at the front of chemotaxing cells (Sasaki et al., 2004), and is mediated by at least two RasGEFs: The role of RasGEFR is not clear, but is speculated to mediate RasG activation in the pathway regulating chemotaxis, and RasGEFM regulates RasG activation in the pathway regulating cell polarity and motility. On the other hand, RasC activation regulates the pathway leading to ACA activation, and speculated to be activated at the rear of chemotaxing cells based on the observation that ACA is localized at the rear of cells (Kriebel et al., 2003). Some unclear relationships exist that cannot be addressed with this model. For example, it is clear that RasG signals to PI3K, and that PI3K and its effector protein CRAC are required for ACA activation, so it is not clear why the loss of RasG results in only a modest decrease in ACA activation (Bolourani et al., 2006). The role of RasC in regulating depolymerization of the actin and myosin II cytoskeletons is unresolved at this time, and thus its role in these processes has been marked with a question mark.

The main role of RasC is in the activation of ACA. RasC in turn is activated by RasGEFA, but how RasC activation leads to ACA activation is not clear at this point. *In vivo* localization studies indicate that ACA activation occurs at the rear of the cell (Kriebel et al., 2003), so the stimulatory cAMP signal must transduce from the front of the cell to the back. An intriguing possibility is that RasC is activated at the rear of the cell, placing it in proximity to ACA, and thereby providing a model for RasC mediated activation. However, unlike yeast ACA, *Dictyostelium* ACA does not have a RBD, so there must be at least one other regulatory molecule that transduces the cAMP signal from activated RasC to ACA. It should be noted that there is no evidence of where in the cell RasC is activated. However, given that the microtubule organizing centre is localized towards the rear of chemotaxing *Dictyostelium* cells (Rubino et al., 1984; Sameshima et al., 1988), and that there may be a possible association of RasGEFA to the microtubule cytoskeleton via its LisH domain (Emes and Ponting, 2001; Wilkins et al., 2005), the idea that RasC is activated at the rear is not out of the realm of possibility.

## 4.8 Thesis synopsis and future directions

The work presented in this thesis has made an impact in the field of Ras signalling in general, and in *Dictyostelium* Ras research specifically. This work demonstrated for the first time that chemoattractant signalling in aggregating *Dictyostelium* cells involves two Ras proteins, RasC and RasG. During this process RasC is activated by RasGEFA in the pathway leading to ACA activation. RasG, on the other hand, appears to be regulated by two RasGEFs, RasGEFM and RasGEFR, with RasGEFM involved in the pathway leading to PKB phosphorylation, and RasGEFR potentially involved in chemotaxis. The initial published work (Kae et al., 2004) has stimulated an interest in the use and adaptation of the assay, leading to a

growing number of collaborative efforts, with the results of some of these partnerships summarized in this thesis.

As the number of components involved in the Ras signalling pathways in *Dictyostelium* increases, so does the complexity in regards to how everything fits together. The RasG-PI3K pathway, for example, is particularly confusing. All the evidence suggests that RasG is involved in mediating the PI3K and TORC2 pathways. Both of these pathways are required for ACA activation, and *rasG*$^-$ cells are only modestly diminished in their ability to activate ACA (Bolourani et al., 2006). However, activation of ACA is far more affected by a loss of RasC with *rasC*$^-$ cells being unable to aggregate due to loss of ACA activation (Lim et al., 2001). It is not known how ACA is activated, so it is not clear how the cAMP signal is transmitted from either RasC or RasG to ACA, but it is presumed that there are at least two pathways.

At this time, the exact nature of the involvement of RasGEFM in RasG activation is not clear. The data presented in this thesis (Figure 17B and 18) provide preliminary evidence, but *in vitro* nucleotide release activity is needed to confirm that RasGEFM is an activator of RasG. Furthermore, obtaining a *Dictyostelium* PKB specific antibody will determine whether the levels of total PKB are equal between Ax2 and *gefM*$^-$ cells (Section 3.9), and this will assign a definitive role for RasGEFM in the signalling pathway leading to PKB phosphorylation/activation.

Identification of a RasC interacting protein would go a long way to shed some light on the RasC signalling pathway. Along with its role in ACA activation, RasC also appears to be involved in cytoskeletal rearrangement during chemotaxis. In particular, RasC appears to be required for the depolymerization of both the actin and myosin II cytoskeletons. While RasC exhibits a second peak of activation when stimulated with cAMP (Figure 11) that coincides with

the timing of the depolymerization of actin and myosin II, it is possible that the improper depolymerisation of actin and myosin II in chemotaxing *rasC*⁻ cells could be a result of the inability to activate ACA, as cAMP is an important second messenger involved in mediating the activation of cAMP binding proteins such as PKA (Zhang et al., 2003). A yeast two-hybrid screen recently undertaken by the Weeks lab will hopefully shed some light on this subject by finding which components transmit the cAMP stimulated signal downstream of RasC.

The role of negative regulators of Ras is an interesting avenue of study. The genetic data suggests that Gα9 is the key negative regulator for cAMP stimulated events (Section 4.5). Thus, one would expect that Ras activation in a *gpaI*⁻ strain would be sustained for a longer period of time. Finding the specific RasGAPs for RasC and RasG, on the other hand, requires some more effort. A method involving brute force would be to knock out all of the *gap* genes and determine the effect of their loss on Ras activation. A more directed approach could involve screening for RasGAPs that interact with activated RasC and/or RasG using a yeast two-hybrid assay first. Any *gap* genes that show a positive interaction can then be targeted for knockout, and its role in the negative regulation of Ras can be studied via the RBD binding assay, as well as phenotypic effects of the gene knockout.

The use of GFP-RBD probes can provide important information about the *in vivo* localization of activated Ras. However, the relative promiscuity of the Ras-RBD interaction makes it difficult to be able to differentiate between the different Ras isoforms. One solution to this problem is to use isoform specific RBDs. Thus far, the RBD of the protein kinase Phg2 has proven to be specific for Rap1 when tested for its ability to bind to 5 members of the *Dictyostelium* Ras subfamily (Gebbie et al., 2004; G. Weeks, unpublished observations)). While the *Dictyostelium* genome encodes for at least 10 more Ras proteins, it remains promising that

the RBD of Phg2 can be used as a Rap1 specific probe. Another method of obtaining isoform specific RBDs is to generate them using mutagenesis. This would involve randomly mutagenizing RBDs and screen for those that bind to only one Ras protein via a yeast two-hybrid screen. A similar type of strategy has been used to generate more efficient GFP probes, and fluorescent probes of different colours (Zhang et al., 2002).

The tool developed by Taylor and Shalloway just over 10 years ago has grown tremendously in its use and applications. As there is still much more to learn about the regulation and localization of Ras, I foresee no end to how this assay can be further modified, improved, and applied. And given the relatively 'research friendly' nature of *Dictyostelium discoideum* as a model organism, and its large arsenal of Ras proteins, RasGEFs and RasGAPs, the social amoeba will play a large role in elucidating the complexity of Ras signalling pathways.

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