The Function of Ras Proteins in \textit{Dictyostelium discoideum}

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Abstract

The Ras family of GTP-binding proteins has been implicated in the control of cellular growth, differentiation and motility. They are molecular switches which are active when bound to GTP and inactive when GDP-bound. Dictyostelium cells express five different Ras family members. Ras activation is mediated by guanine nucleotide exchange factors (RasGEFs) which facilitate exchange of GDP for GTP by Ras proteins. The identification and molecular genetic characterisation of novel Dictyostelium RasGEFs is the main subject of this thesis. The discovery of a physiological function for the Dictyostelium RasD protein is also described.

I have identified five partial cDNAs from the Tsukuba Dictyostelium cDNA project which have strong sequence homology to known RasGEFs. To investigate the function of these proteins, Dictyostelium cells with single gef gene disruptions were generated by homologous recombination. Four of the five mutants appeared to grow and develop normally but one mutant, gefB', showed growth and developmental abnormalities. gefB' cells are unable to proliferate in axenic culture and are impaired in phagocytosis and fluid-phase endocytosis. Conversely, gefB' cells show an enhanced rate of migration, moving twice as fast as wild-type cells. The mutant cells have abnormal morphology; they are highly polarised, have many elongated microspikes and have an absence of pinocytic crowns on the cell surface. In addition, gefB' cells show a cell autonomous impairment in multicellular development. Although the phenotype of vegetative gefB' cells is similar to that of rasS' cells, it was not possible to show a direct interaction between RasS and GefB. However, GefB does exhibit the catalytic properties of a RasGEF in vivo, since it is able to complement the temperature sensitive cdc25-5 S.cerevisiae mutant.

The Dictyostelium RasD protein is a small GTP-binding protein closely related to the mammalian Ras proteins Ha-, Ki- and N-Ras, and is maximally expressed during the multicellular stage of Dictyostelium development. Previous work had predicted that RasD was essential for correct differentiation and pattern formation in Dictyostelium aggregates. To further investigate the function of RasD, Dictyostelium cells containing a disrupted rasD gene were generated by homologous recombination. Surprisingly, rasD' cells proliferate, aggregate and develop indistinguishably from wild-type cells. However rasD' slugs exhibit a clear defect in phototaxis and thermotaxis exhibiting an approximately thirty-fold decrease in the efficiency of orientation towards a light or heat source relative to wild-type slugs. RasD is the first signalling protein shown to be necessary for phototaxis in Dictyostelium slugs.
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Abbreviations

Standard S.I. units and single letter amino acid codes are used throughout.

7-TMR seven-span transmembrane receptor
ADP adenosine diphosphate
AEBSF 4-(2-aminoethyl)benzenesulphonyl fluoride
ARF ADP-ribosylation factor
APS ammonium persulphate
ATP adenosine triphosphate
BSA Bovine serum albumin (Fraction V)
bp base pairs
°C degrees Celsius
cAMP cyclic adenosine monophosphate
cAR1 cyclic AMP receptor 1
cAR2 cyclic AMP receptor 2
cAR3 cyclic AMP receptor 3
cAR4 cyclic AMP receptor 4
cDNA complementary deoxyribonucleic acid
CDKI cyclin-dependent kinase inhibitors
cGMP cyclic guanosine monophosphate
CMF conditioned medium factor
dATP deoxyadenosine triphosphate
dCTP deoxycytidine triphosphate
dGTP deoxyguanosine triphosphate
dTTP deoxythymidine triphosphate
DIC differential interference contrast
DIF differentiation inducing factor
DNA deoxyribonucleic acid
dsDNA double stranded deoxyribonucleic acid
DTT dithiothreitol
ECM extracellular matrix
ECL Enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
EST expressed sequence tag
EtOH Ethanol
FGF fibroblast growth factor
FMLP formylated-methione-leucine-phenylalanine tripeptide
GAP GTPase activating protein
GBF G-box factor
GFP green fluorescent protein
GSK3 glycogen synthase kinase 3
GST glutathione-S-transferase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES-buffered saline</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2 Hydroxyethylpiperazine-N'2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>IP₄</td>
<td>inositol tetrakisphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertoni medium</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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<td>MAP</td>
<td>mitogen activated protein</td>
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</tr>
<tr>
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<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulphonid acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-1</td>
<td>neurofibromatosis type 1</td>
</tr>
<tr>
<td>NGF</td>
<td>neural growth factor</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
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<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
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<td>polymerase chain reaction</td>
</tr>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
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<td>PEG</td>
<td>polyethylene glycol</td>
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<td>pleckstrin homology</td>
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</tr>
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</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PSF</td>
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<tr>
<td>pstA</td>
<td>prestalk-A</td>
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<tr>
<td>pstB</td>
<td>prestalk-B</td>
</tr>
<tr>
<td>pstO</td>
<td>prestalk-O</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SAP</td>
<td>stress activated protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard sodium citrate</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>vol.</td>
<td>volume(s)</td>
</tr>
<tr>
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<td>volume for volume</td>
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1 Introduction

1.1 The Ras Superfamily of Small GTPases

Members of the Ras superfamily have been implicated in the regulation of a large number of eukaryotic cellular processes. These include cell proliferation and differentiation, vesicle and macromolecular transport and cell motility and polarity. These proteins have a low molecular weight (between 20 and 35kDa), bind guanine nucleotides with high affinity and hydrolyse bound GTP with a low catalytic rate. The superfamily can be classified by sequence homology into five subfamilies: Ras, Rho, Ran, Rab and Arf.

**Ras:** Mammalian Ras proteins were originally discovered in an activated form as oncogenic agents of certain murine tumour-inducing retroviruses (Ellis et al., 1981). The cellular homologues of Ras were subsequently found to play a fundamental role in the control of cellular proliferation and more recently, requirements for Ras in the control of differentiation and processes requiring cytoskeletal reorganisation have been demonstrated. The Ras subfamily consists of the closely related prototypic Ha-Ras, Ki-Ras and N-Ras; Rap1A and Rap1B; Rap 2A and Rap2B; R-Ras, R-Ras2 and R-Ras 3; RalA and RalB; Rheb; Dex-Ras; Rin and Rit. The function of Ras signalling in the cellular slime mould *Dictyostelium discoideum* is the subject of this thesis and, therefore, a more detailed account of Ras will follow.

**Rho:** The Rho subfamily (Madaule and Axel, 1985) is further subdivided into groups. The Rho, Rac and Cdc42 protein families are the best characterised and have been shown to control phagocytosis, cell motility, adhesion and polarity through modulation of the actin cytoskeleton. Roles for these proteins in transcriptional activation and cellular transformation have also been described (Hall, 1998).

**Rab:** Rab GTPases are key regulators of vesicle transport within the cell (Chavrier and Goud, 1999). GTP-bound Rab proteins can induce the fusion of vesicles with their target membranes. Individual Rab proteins have been assigned to specific vesicle trafficking events e.g. Rab5 is required for the docking and fusion of endocytic vesicles with early endosomes (Bucci et al., 1992).

**Ran:** Ran proteins are predominantly found in the nucleus, unlike the other Ras superfamily members, which are cytoplasmic. They have been shown to regulate macromolecular traffic into
and out of the nucleus (Moore, 1998). Underlying this regulation is the ability of Ran to assemble
and disassemble nuclear transport complexes in response to its nucleotide state. This GTPase has
also been implicated in the regulation of microtubule dynamics during mitosis (Kalab et al., 1999).
Ran proteins do not undergo post-translational lipid modifications, unlike other small GTPases.

**Arf:** The Arf subfamily is the most distantly related of all the Ras subfamily GTPases. The
proteins share sequence and structural homology with other Ras superfamily members but also with
the α-subunits of heterotrimeric G-proteins. Arf proteins are required for the formation of
intracellular transport vesicles and are therefore essential for vesicle trafficking and the maintenance
of organelle integrity (Boman and Kahn, 1995). They also have important roles in signal
transduction. For example, Arf is required for the activation of phospholipase D, a molecule
implicated as an effector of several growth factors (Cockcroft et al., 1994). Arf proteins have
several properties that distinguish them from other GTPases: GTP binding is highly dependent upon
phospholipids; Arf-GTP associates with membranes, whereas Arf-GDP is cytosolic; purified Arf
proteins have no detectable intrinsic GTPase activity; Arf proteins are myristoylated at an aminoterminal glycine residue (unlike Ras, Rho and Rab proteins, which are prenylated at their carboxy
termini) (Kahn, 1995).

### 1.2 Structure and Biochemical Properties of Ras Proteins

#### 1.2.1 Biochemical Properties

Ras proteins bind guanine nucleotides with high affinity (Kd values are between 10⁻¹¹ and 10⁻⁷M).
Since the cytosolic concentrations of GDP and GTP are considerably higher than these dissociation
constants (10⁻³M for GDP and 10⁻⁴M for GTP), Ras proteins are predominantly found bound to
guanine nucleotides *in vivo* (Bourne et al., 1991). A class of proteins can catalyse the replacement
of GDP with GTP. These Guanine-nucleotide Exchange Factors (GEFs) bind to the GTPase and
stimulate nucleotide dissociation (Boguski and McCormick, 1993). The resultant nucleotide-free
Ras can form a stable complex with the GEF. The complex is disrupted by the subsequent
association of guanine nucleotide with the Ras protein; the differential cytosolic concentrations of
GTP and GDP favour GTP-Ras association (Lai et al., 1993). The GTP-bound form of Ras is
considered the biologically active form. It can interact with and stimulate the activity of various
"effector" molecules that include protein kinases, lipid kinases and GEFs for other small GTPases. The period of activation is limited by the intrinsic GTPase activity of Ras which removes the γ-phosphate of GTP, causing the molecule to revert to the inactive, GDP-bound conformation (Barbacid, 1987). The GTP hydrolysis rate varies between Ras proteins, but it is usually slow ($k_{cat,GTP} < 0.03 \text{ min}^{-1}$) (Bourne et al., 1991). This GTP hydrolysis can be stimulated by the action of GTPase Activating Proteins (GAPs). Studies of Rho family GTPases have revealed a third mechanism of GTPase regulation. Guanine-nucleotide Dissociation Inhibitors (GDIs) are proteins that block the dissociation of nucleotide from small these GTPases (Fukumoto et al., 1990; Mackay and Hall, 1998). The mechanism of action is poorly understood and a GDI for Ras has not yet been identified.

The ability of Ras proteins to cycle between a GTP-bound active state and a GDP-bound inactive state has resulted in them being likened to a "molecular switch" (Bourne et al., 1990). Several other biological processes employ this GDP-GTP switch mechanism. For example, heterotrimeric G-proteins are associated with many transmembrane hormone and photo-receptors and are responsible for transduction of signals from these receptors, upon receptor activation (Neer, 1995). The α-subunits of these αβγ-trimers bind guanine nucleotide, and the interaction of the G-protein with an activated receptor results in replacement of GDP with GTP. With GDP bound, the α-subunit is associated with the βγ-dimer. With GTP bound, the α-subunit and the βγ-dimer separate and interact with downstream effector molecules. The intrinsic GTPase activity of the α-subunit limits the period of activation of the G-protein. Another example is the GDP-GTP switch of the E.coli translation elongation factor EF-Tu which regulates association of aminoacyl-tRNA molecules with ribosomes during protein synthesis (Thompson, 1988; Thompson et al., 1986). Only with GTP bound, are EF-Tu molecules able to bind the aminoacyl-tRNA. When an EF-Tu-GTP-tRNA complex forms the aminoacyl-tRNA is brought to the mRNA in the ribosome where the anticodon can associate with the mRNA codon. The association of EF-Tu with the ribosome triggers GTP hydrolysis. The speed of GTP hydrolysis is slow, allowing a kinetic proof-reading step to take place – any mismatched anticodons and codons will usually dissociate before hydrolysis is complete thus rejecting the incorrect aminoacyl-tRNA. Tubulin is also a GTPase; αβ tubulin dimers polymerise to form microtubules when β-tubulin is GTP-bound. GTP hydrolysis is
promoted by inter-dimer interactions within the microtubule. GDP-bound $\alpha\beta$ tubulin dimers then dissociate resulting in depolymerisation of microtubules (Mitchison and Kirschner, 1984).

1.2.2 The Structure of Ras

A highly refined, 1.35Å resolution crystal structure of Ras bound to the non-hydrolysable GTP analogue GppNp was obtained in 1990 (Pai et al., 1990). Ras comprises a single globular domain consisting of a central six stranded, $\beta$-sheet and five associated $\alpha$-helices. The guanine nucleotide is bound by three motifs $^{10}$GXGXXGKS/T, $^{116}$NKXD and $^{146}$SAK (where X is any amino acid and the residue number is prefixed) which are found in the vast majority of guanine nucleotide binding proteins (see Figure 1.1). The $^{10}$GXGXXGKS/T motif forms a tight loop that binds to the phosphate groups of GTP. The main chain NH groups of this region plus the side chains of S17 and K16 are hydrogen bond donors to the oxygen atoms of the GTP phosphate groups and to the Mg$^{2+}$ ion which co-ordinates between the $\beta$ and $\gamma$ phosphate groups of GTP. Previous mutagenesis studies had identified two regions important for Ras signalling: Switch-I (residues 32-40) and Switch-II (residues 57-73) (Moodie et al., 1995; Sigal et al., 1986)(see Figure 1.1). The crystal structure and NMR studies suggest that these regions change conformation in response to changes in the bound nucleotide (Milburn et al., 1990).

1.2.3 Mechanism of GTP Hydrolysis

The crystal structure has suggested a possible mechanism for the intrinsic GTPase activity of Ras. In the crystal, the side chain of Q61 hydrogen bonds with the side chain of E63 and a water molecule which is adjacent to the phosphorus atom of the $\gamma$-phosphate of GTP. In this model the water molecule performs an in-line nucleophilic attack on the $\gamma$-phosphate. Q61 and E63 combine to abstract a proton from the water molecule and stabilise the developing charge on the leaving phosphate group catalysing the reaction. In support of this model, the E63 residue is absolutely conserved in all Ras proteins and the Q61 is conserved in every Ras protein except Rap1, which has T61. Rap1 has a slower intrinsic rate of GTP hydrolysis and if T61 in Rap is mutated to Q61 it increases the hydrolytic rate of Rap to that of Ras (Frech et al., 1990). Mutation of Q61 to E61 increases the rate of intrinsic GTP hydrolysis 20-fold whereas mutation to any other residue drastically reduces the GTPase rate constant of Ras (Der et al., 1986; Frech et al., 1994).
**Figure 1.1** Alignment of human Ha-Ras and Dictyostelium Ras protein sequences

Dashes indicate amino acid residues identical to Ha-Ras and gaps have been inserted to optimise the alignment. The red boxes identify the highly conserved residues required for guanine nucleotide binding whilst the the black box indicates the CAAX motif required for post-translational prenylation. The switch-I and switch-II regions are indicated by * and + respectively.
1.2.4 Mechanism of GEF Action

The crystal structure of Ha-Ras complexed with the catalytic domain of the exchange factor, Sos (Son of Sevenless), has suggested an obvious mechanism of nucleotide exchange (Boriack-Sjodin et al., 1998). The structure of the Ras-Sos complex confirms biochemical and genetic data that suggested that GEFs interact with the same region of Ras as do effectors (Quilliam et al., 1996). Sos disrupts the tight interaction of GTP and Ras in two ways. Firstly, the insertion into Ras of an alpha-helix from Sos results in the displacement of the Switch-I region of Ras, opening up the nucleotide binding site. Secondly, side chains presented by this helix and by a GEF-induced distorted conformation of the Switch-II region of Ras alter the chemical environment of the nucleotide binding site, destabilising the co-ordination of the phosphate groups of the nucleotide and the Mg$^{2+}$ ion so that their binding is no longer favoured. Sos does not impede the binding sites for the base and the ribose of GTP or GDP, so the Ras-Sos complex adopts a structure that allows nucleotide release and rebinding. It seems feasible that GTP can enter and compete for the residues required for co-ordinating the Mg$^{2+}$ ion and $\gamma$-phosphate group, resulting in GEF displacement.

1.2.5 Mechanism of GAP Action

The crystal structures of p120 RasGAP alone and in complex with Rap1-GTP and subsequently of p120 RasGAP in complex with Ras-GDP and AlF$_4^-$ have been solved (Scheffzek et al., 1996; Scheffzek et al., 1997). AlF$_4^-$ was included in the Ras/RasGAP complex because it is presumed to be structurally similar to a hydrolysed $\gamma$-phosphate thus mimicking the transition-state of the reaction. These structures have suggested a mechanism whereby GTPase Activating Proteins (GAPs) stimulate GTP hydrolysis by Ras. An arginine side chain (R789) of RasGAP is supplied into the phosphate binding pocket of Ras forming salt bridges with the conserved, negatively charged residues in the Ras Switch-I region and acts to neutralize developing negative charges in the transition state of the phosphoryl transfer reaction. The Switch-II region of Ras is stabilized by GAP, positioning Q61 of Ras to participate in catalysis more effectively. The interaction with Q61 in Ras is supported by data from mutational studies where mutation of Q61 to any other amino acid prevents GAP activity on Ras (Cales et al., 1988; Vogel et al., 1988).
1.2.6 Dominant Alleles of Ras

Mutational studies and analysis of naturally occurring, oncogenic forms of Ras have revealed activating and inhibitory mutations (Bos et al., 1987; Feig et al., 1987). Amino acid substitutions at position 12 and 61 create constitutively active Ras proteins which are unable to hydrolyse GTP, whilst the mutation of S17 to N17 produces a protein with dominant inhibitory characteristics in vivo (Feig and Cooper, 1988; Jung et al., 1994; Krengel et al., 1990; Sigal et al., 1986). The various Ras crystal structures provide explanations for these phenomena. It is clear that Q61 is essential both for the intrinsic GTPase activity of Ras and that catalysed by GAP. Although G12 is not directly involved in catalysis, it is within one Van Der Waals distance of Q61 and even its mutation to A12 would disturb the arrangements of residues required for correct positioning of Q61 in catalysis. In fact mutation of the residues adjacent to G12 has a similar, activating affect. As previously mentioned S17 is involved in co-ordination of the $\beta$ and $\gamma$ phosphate groups of GTP and N17 Ras mutants have a very low affinity for nucleotide. The N17 Ras can bind RasGEFs with extremely high affinity since the GEF is not displaced by nucleotide following GDP release (Jung et al., 1994). These unproductive Ras/GEF complexes sequester RasGEFs preventing activation of normal cellular Ras, and thus have a dominant inhibitory effect on Ras signalling.

1.2.7 Effector Binding

In addition to its role in the regulation of nucleotide exchange, the conformational flexibility of the Switch-I domain of Ras has important implications for downstream signalling. When Ras is GTP-bound, the residues 32-40, contained within the Switch-I region, adopt a conformation which allows them to bind and activate Ras effectors. This stretch of amino acids forms what is called the "effector loop" and was so defined by screens for mutations affecting Ras transformation activity without altering the affinity of the molecule for nucleotide (Barbacid, 1987). Confirmation that these amino acids bind effectors came with the resolution of the crystal structure of Rap1 complexed with the Ras-binding domain of Raf1 (Nassar et al., 1995). The sequence of the effector loop is highly conserved in all Ras family proteins (see Figure 1.1) and has subsequently become the subject of mutational analyses aimed at delineating the downstream pathways activated by Ras (White et al., 1995). Ras can bind to and activate several molecules and specific mutations in the Switch-I domain can prevent the activation of some of these downstream effectors, whilst leaving
the activation of other effectors intact. These mutants have allowed some of the many functions of Ras to be attributed to specific downstream signalling pathways (Joneson et al., 1996; Rodriguez-Viciana et al., 1997).

1.2.8 Post-Translational Modification

Ras superfamily proteins, with the exception of Ran, Arf, Rin and Rit are post-translationally modified by the addition of one or more lipid moieties to their carboxy termini (Hancock et al., 1989). These lipids partition into lipid bilayers allowing association of the proteins with cellular membranes. For Ras subfamily proteins this interaction is ultimately with the cytosolic face of the plasma membrane. The importance of the post-translational lipid modification has been demonstrated by expression of mutant Ras proteins that cannot be modified. These proteins are localised to the cytosol and have no detectable \textit{in vivo} biological activity (Willumsen et al., 1984).

The initial lipid modification is directed by a consensus sequence at the extreme carboxy terminal of Ras proteins. This sequence is CAAX, where C is cysteine, A is an aliphatic amino acid and X is usually methionine, serine or leucine (see \textbf{Figure 1.1}). The sequence is recognised by a cytosolic prenyl transferase that covalently adds a prenoid lipid moiety to the cysteine residue of the sequence. Lipid addition is followed by proteolytic cleavage of the terminal three amino acids and carboxymethylation of the, now terminal, cysteine (Hancock et al., 1991a). CAAX farnesyl transferase adds a farnesyl moiety to the cysteine residue of the CAAX motif (Seabra et al., 1995a). It recognises CAAX sequences where the terminal X residue is methionine, as found in K-Ras and N-Ras, and with reduced affinity, sequences that end in serine, such as H-Ras. CAAX geranylgeranyl transferase is responsible for the addition of a geranylgeranyl moiety to CAAX sequences ending in leucine (Seabra et al., 1995b). Geranylgeranylated Ras proteins bind with higher avidity to biological membranes than the Ras proteins modified with the shorter farnesyl moiety although the functional significance of this is not yet clear (Hancock et al., 1991b).

Targeting of Ras to the plasma membrane is not direct; proteins are trafficked via the endomembrane system, where the proteins first become membrane associated (Choy et al., 1999). Prenylated CAAX sequences are required for targeting to the endomembrane system but these are not sufficient for plasma membrane targeting of Ras. A second targeting signal is required at the carboxy terminus of the protein (Hancock et al., 1991b). In Ha-Ras and N-Ras, this comprises two
palmitoylated cysteine residues at positions 181 and 184. These palmitoyl lipids are added by a palmitoyltransferase that only recognises Ras proteins that have first been prenylated and carboxymethylated (Liu et al., 1996). The palmitoyl moieties can be removed by a palmitoyl thioesterase (Camp and Hofmann, 1993) and cycles of acylation/deacylation have been shown in vivo although the functional significance of this is not yet clear (Magee et al., 1987). Human Ki-Ras lacks the additional cysteine residues and is not palmitoylated (Hancock et al., 1989). Instead, a series of lysine residues at the carboxy terminus of the protein are sufficient for interaction with the plasma membrane, possibly via an electrostatic interaction between the positively charged lysines and the negatively charged head groups of membrane phospholipids (Hancock et al., 1991b). A recent study has demonstrated that palmitoylation and polybasic domains do not simply promote plasma membrane association, but are necessary for the trafficking of Ras out of the endomembrane system (Choy et al., 1999).

1.3 The Biological function of Ras

The Ha-ras and Ki-ras genes were first identified as the oncogenes carried by the Harvey and Kirsten murine retroviruses (Shih et al., 1979). After these initial studies, it became clear that the viral ras genes are normal cellular genes carrying activating mutations (Ellis et al., 1981). The closely related N-ras was cloned soon afterwards (Taparowsky et al., 1983). The vast majority of the work on mammalian Ras function has centred around understanding the functions of the prototypic Ha-Ras, Ki-Ras and N-Ras. They share many of the same biological properties and it is these properties that will be discussed in this section. The biological functions of the other members of the Ras subfamily are poorly understood and what is known is briefly summarised at the end of this section.

Much of the work on Ras has been aimed at an understanding of its proliferation control function. However, the morphological changes associated with Ras cellular transformation also implicate Ras in the control of cell motility, polarity and morphology. Moreover, Ras signalling pathways are found in all eukaryotes and have been shown to control processes as diverse as differentiation (Kayne and Sternberg, 1995), apoptosis (Kauffmann-Zeh et al., 1997), synaptic transmission (Brambilla et al., 1997), cell cycle arrest and senescence in mammalian cells (Lloyd, 1998), cytoskeletal rearrangements and pinocytosis (Bar-Sagi and Feramisco, 1986), cytokinesis
(Tuxworth et al., 1997), chemotaxis (Insall et al., 1996) and yeast pheromone signalling (Fukui et al., 1986).

1.3.1 Cellular Transformation and Proliferation

Transformation of cells is broadly defined by the ability to grow in the absence of serum and substratum anchorage, combined with a loss of contact inhibition of proliferation. Viral ras (v-ras) genes are capable of transforming immortalised cell lines directly and produce aggressive, highly metastatic tumours (Partin et al., 1988). The ability of Ras proteins to transform cell lines was directly demonstrated by microinjection studies using purified, recombinant Ras proteins in the mid-1980s (Feramisco et al., 1984; Stacey and Kung, 1984). Microinjection of viral Ras protein caused rapid morphological changes including rounding of the cells, membrane ruffling and blebbing and also stimulated cell proliferation. Microinjection of normal cellular Ras proteins failed to produce these effects. The viral Ras protein was only able to transform immortalised cell lines and not primary cells. This observation supported the theory of oncogene co-operation in transformation (Land et al., 1983). In fact, activated Ras proteins have been shown to induce senescence and not proliferation in primary cell cultures (Lloyd, 1998; Ridley et al., 1988; Serrano et al., 1997).

A role for cellular Ras proteins in the control of cell proliferation was demonstrated by microinjection of the anti-Ras Y13-259 blocking antibody into untransformed cells. Serum induced proliferation was blocked by the antibody (Mulcahy et al., 1985). Microinjection of a dominant negative Ras protein has a similar effect (Feig and Cooper, 1988). A similar proliferation block can be seen in whole organisms. Mouse embryos at the two cell stage are prevented from further development by the microinjection of dominant negative Ras expression constructs (Yamauchi et al., 1994). Early Xenopus development is also blocked by inhibiting Ras function. Injection of the anti-Ras Y13-259 blocking antibody prevents the rapid cleavage events that occur immediately after fertilisation of the oocyte (Miron et al., 1990). The budding yeast Saccharomyces cerevisiae also illustrates a role for Ras in control of proliferation. It has two RAS genes (DeFeo-Jones et al., 1983; Powers et al., 1984), RAS1 and RAS2 that share extensive homology with their mammalian counterparts although have extended C-termini. They are redundant such that either can be disrupted without any obvious effect. However, the RAS1/RAS2 double null mutant is incapable of
proliferation or spore germination (Kataoka et al., 1985; Tatchell et al., 1984). Although human Ha-Ras is capable of substituting for the yeast Ras proteins (Kataoka et al., 1985), the signalling pathways downstream of Ras differ; adenylate cyclase is the direct target of the Ras1p and Ras2p proteins (Toda et al., 1985) but it is not an effector of Ras in mammalian cells.

Changes in cell morphology seen during transformation by \textit{v-ras} have not been demonstrated to be due directly to the mutated Ras protein as opposed to an indirect consequence of the transformation process. However, a direct morphological response to Ras in untransformed cells has been demonstrated by microinjection of either normal Ras or oncogenic viral Ras protein. Rapid morphological changes, including ruffling of the cell cortex and an increase in pinocytosis were observed, even in the presence of cycloheximide which prevents \textit{de novo} protein synthesis (Bar-Sagi and Feramisco, 1986). The morphological effects of Ras are thought to mediated by Rho subfamily GTPases (Ridley et al., 1992) and the pinocytotic changes by GTPases of the Rab subfamily (Li et al., 1997).

\textbf{1.3.2 Differentiation}

A role for Ras in control of differentiation and cell fate specification has been identified in several systems. PC12 cells can differentiate in culture in response to neural growth factor (NGF) and adopt a morphology resembling that of sympathetic neurones. Microinjection of viral Ras induces differentiation in the absence of NGF (Bar-Sagi and Feramisco, 1985) whereas addition of the anti-Ras antibody, Y13-259, inhibits NGF induced differentiation (Hagag et al., 1986). More recently, the Ras subfamily GTPase, Rap1 has been implicated in the differentiation of PC12 cells and it now appears that Rap and Ras regulate different aspects of the response to NGF (York et al., 1998). Genetic evidence from \textit{D.melanogaster} and \textit{C.elegans} suggests Ras functions downstream of receptor tyrosine kinases in cell fate specification. The differentiation of the R7 photoreceptor in the ommatidia of the \textit{D.melanogaster} compound eye involves Ras (Fortini et al., 1992). Loss of function Ras mutations prevent differentiation of R7 cells whereas activating Ras mutations cause all photoreceptors to differentiate into R7 cells. Similarly, in \textit{C.elegans} the Ras protein, Let-60, is required for Vulval induction (Han and Sternberg, 1990). Activated Ras induces the formation of multiple vulvae, whereas worms carrying partial loss of function Ras alleles have no vulvae at all (Beitel et al., 1990). Early patterning of the \textit{D.melanogaster} embryo also requires Ras.
Specification of the terminal regions of the syncitial embryo requires the receipt of an extracellular ligand by the receptor tyrosine kinase, Torso, and Ras functions downstream of this molecule (Lu et al., 1993). A role for Ras in the differentiation response of *S. cerevisiae* was demonstrated by expression of an activated Ras mutant which blocked sporulation in response to starvation (Kataoka et al., 1984).

### 1.3.3 Cell Motility and Chemotaxis

Cells transfected with *v-ras* exhibit increased chemotactic and chemokinetic responses suggesting a role for Ras in cell migration (Ochieng et al., 1991; Varani et al., 1986). Direct evidence for an effect of Ras on cell motility was provided using wound healing assays. An important aspect of the wound healing response is an increase in the motility of fibroblasts and endothelial cells that enables rapid closure of the wound. Dominant active Ras was shown to increase the motility of primary endothelial cells in this assay and also to increase the randomness of movement, as if the controls that allow the cells to migrate in a co-ordinated manner in the wound had been disrupted (Sosnowski et al., 1993). Conversely, cells injected with the anti-Ras Y13-259 blocking antibody were almost completely immobile. More specifically, Ras has been implicated in the control of chemotaxis to PDGF (platelet-derived growth factor). Primary fibroblasts transfected with constructs expressing dominant negative Ras displayed impaired chemotaxis towards PDGF, although their general cell motility appeared normal (Kundra et al., 1995). The chemoattractants C5a, FMLP and interleukin-8 activate Ras in neutrophils (Buhl et al., 1994; Knall et al., 1996; Worthen et al., 1994; Zheng et al., 1997) although a direct role for Ras in control of chemotaxis has not been demonstrated. *Dictyostelium* cells lacking the putative RasGEF, Aimless, show a defective chemotactic response to cAMP (Insall et al., 1996).

### 1.3.4 Other Functions for Ras

Genetic analysis of Ras function in mice has not confirmed the expected roles of Ras proteins in proliferation and differentiation. Mice with a disrupted *N-ras* gene develop entirely normally and are fully fertile (Umanoff et al., 1995). Two *Ki-ras* null mice strains have been generated independently and have shown *Ki-ras* to be an essential gene, with mice dying between embryonic days 12 and 15. One mutant has defects in the generation of cells in the haematopoietic lineage...
(Johnson et al., 1997) whilst the other displays impaired cardiac muscle development and widespread neuronal apoptosis (Koera et al., 1997). N-ras/Ki-ras double null mice display more severe phenotypes than the single mutants (Johnson et al., 1997) suggesting at least partial overlap of function. The phenotype of an Ha-ras null mouse has not been published but the phenotype is reported to be outwardly wild-type (Johnson et al., 1997). The potential role for Ras in control of apoptosis identified in Ki-ras null mice has also been demonstrated in cell culture experiments. Withdrawal of serum from Ki-Ras transformed thyroid cells induces apoptotic cell death (Di Jeso et al., 1995). It has subsequently been demonstrated that Ha-Ras can induce or inhibit apoptosis in cultured fibroblasts depending on which effector it activates (Kauffmann-Zeh et al., 1997).

The fission yeast Schizosaccharomyces pombe has a single ras gene which shares greater homology with its counterparts in higher eukaryotes than do the two S.cerevisiae genes (Fukui and Kaziro, 1985). Fission yeast cells lacking Ras1p can proliferate normally but exhibit a defective mating response and a rounded morphology (Fukui et al., 1986). In the mating response, Ras1p is required for the activation of a MAP kinase cascade which stimulates the transcriptional activation of mating genes (Herskowitz, 1995; Hughes, 1995; Nielsen et al., 1992). Ras1p controls cellular morphology through the Scd1 protein, which encodes a putative exchange factor for the Rho family GTPase Cdc42p. The interaction between Ras1p and Scd1p is direct (Chang et al., 1994). Although a similar interaction has been reported in S.cerevisiae, where the Ras-related protein Bud1 is a direct activator of the Scd1 homologue, Cdc24 (Park et al., 1997), no such interaction has been found in mammalian cells. The ability of Ras to activate MAP kinases is an activity shared by Ras in most eukaryotes and will be discussed below.

1.3.5 Other Members of the Ras Subfamily

Activated mutants of R-Ras and R-Ras2 can both induce transformation in some cells (Cox et al., 1994; Graham et al., 1994). An activated mutant of R-Ras3 has a similar effect on cellular transformation and differentiation to Ha-Ras (Quilliam et al., 1999). R-Ras also plays a role in control of apoptosis and can associate with the anti-apoptotic protein Bcl-2 (Fernandez-Sarabia and Bischoff, 1993; Suzuki et al., 1997; Wang et al., 1995). Overexpression of Rap1A can cause cellular transformation in systems in which cAMP stimulates cell proliferation (Altschuler and Ribeiro-Neto, 1998) but more typically acts as an antagonist of Ras signalling by forming
nonproductive complexes with Ras effectors (Kitayama et al., 1989; Wittinghofer and Nassar, 1996; Zhang et al., 1990). Rheb can similarly inhibit Ras-induced transformation (Clark et al., 1997) but both Rheb and Rap1A may have other physiological functions, for example Rap1A contributes to the differentiation of PC12 cells into neurites via activation of B-Raf and has been linked to superoxide generation in phagocytes (Bokoch et al., 1991; Vossler et al., 1997). Most recently, a function for Rap1 in regulating normal morphogenesis in the D.melanogaster eye disk, the ovary and the embryo has been found (Asha et al., 1999). Expression of dominant mutants of Ral in D.melanogaster results in defects in the cell shape changes required for particular morphogenetic movements (Sawamoto et al., 1999). Ral has been shown to act downstream of Ras in a pathway regulating phospholipase D activity (Luo et al., 1998). The neuronally specific Rin and Rit both lack CAAX motifs and Rin binds calmodulin through a C-terminal binding motif (Lee et al., 1996). The gene for Dex-Ras1 is induced by dexamethasone in AT-20 cells (Kemppainen and Behrend, 1998).

1.4 Ras Signalling Pathways

1.4.1 Activation of Ras

1.4.1.1 Cdc25, the Prototypic RasGEF

The first RasGEF to be identified was Cdc25 of S.cerevisiae. Initial genetic evidence suggested it was a component of the Ras/adenylate cyclase pathway and likely to act as an activator of Ras (Broek et al., 1987; Robinson et al., 1987). It was subsequently demonstrated to be an exchange factor for Ras in vitro (Jones et al., 1991). Cdc25 has since become the prototypic member of the RasGEF family and the temperature sensitive cdc25-5 strain, which suffers a growth arrest at the non-permissive temperature, has been used for the cloning of novel RasGEFs from other species (Goldberg et al., 1993; Martegani et al., 1992). The cdc25-5 strain is also commonly used to determine if a putative GEF has RasGEF activity in vivo (Coccetti et al., 1995; Liu et al., 1993).

Cdc25 is a large protein (1589 amino acids) but the minimal domain required for exchange factor activity is a region of approximately 450 amino acids near the C-terminus of the protein (Lai et al., 1993). This domain is highly conserved in RasGEFs from other species and consists of two distinct regions of homology: one of approximately 200 amino acids, the Cdc25 box, found in both
Ras-specific exchange factors and the more distantly related Bud5 and Lte1 proteins; the other, a smaller segment of 48 amino acids found only in the Ras-specific exchange factors, called a REM (Ras Exchange Motif) box (Lai et al., 1993). Cdc25 also contains a SH3 domain near the N-terminus of the protein which has been shown to bind the C-terminal catalytic region of adenylate cyclase (Mintzer and Field, 1999). Cdc25 is localised to cell membranes and residues 1441-1552 are required for this association (Garreau et al., 1996). Cdc25 can be phosphorylated by a cAMP-dependent protein kinase, which is activated in response to elevated levels of cellular cAMP generated by the Ras effector, adenylate cyclase. Phosphorylation of Cdc25 stimulates the release of the GEF from the plasma membrane reducing its accessibility to membrane-bound Ras (Gross et al., 1992).

1.4.1.2 Tyrosine Kinase Receptors and the RasGEF Sos

The best characterised mechanism of Ras activation is that mediated by tyrosine kinase growth factor receptors (RTKs) via the Son of Sevenless (Sos) family of RasGEFs. Activation of RTKs by their agonists promotes receptor dimerisation, allowing trans-autophosphorylation of the cytoplasmic domains (Ullrich and Schlessinger, 1990). The resultant phosphotyrosine residues act as binding sites for the Src homology 2 (SH2) domains of a number of signalling molecules. Studies of the EGF (epidermal growth factor) and PDGF (platelet derived growth factor) receptors, have identified p120 RasGAP, p85 regulatory subunits of class Iα phosphatidylinositide 3-kinases (PI 3-kinases), phospholipase Cγ, kinases of the Src family and the adapter molecule Grb2 as among the many interacting proteins (Fantl et al., 1992).

A requirement for Grb2 and its homologues in the activation of Ras by RTKs has been firmly established. In addition to its SH2 domain, the Grb2 protein contains two Src homology 3 (SH3) domains, which are responsible for binding a proline-rich region on the RasGEF, Sos (Rozakis-Adcock et al., 1993). The homologues of Grb2 in D. melanogaster and C. elegans, Drk and Sem-5, have been shown genetically to act downstream of RTKs and upstream of Ras in developmental signalling pathways in these organisms (Clark et al., 1992; Simon et al., 1993). Differentiation of the R7 photoreceptor in the D. melanogaster eye requires the receipt of an inductive signal by the Sevenless receptor, which is an RTK (Hafen et al., 1987; Tomlinson et al., 1987). In addition to Ras, Drk and Sos were identified in D. melanogaster screens for enhancers of a weak sev allele
(Simon et al., 1991; Simon et al., 1993). These and other genetic interaction studies implied that Ras, Sos and Drk function downstream of the Sevenless receptor, with Sos and Drk required upstream of Ras. Vulval specification in *C. elegans* also requires the activation of an RTK, the product of the *let-23* gene. As in the fly eye, Sem-5 and Let-60 (Ras) proteins operate downstream of the RTK in this differentiation pathway (Beitel et al., 1990).

The N-terminal region of Sos contains a Dbl homology (DH) and a pleckstrin homology (PH) domain. The central region of Sos contains the RasGEF catalytic domain (Bonfini et al., 1992; Liu et al., 1993) whilst the proline rich, C-terminal region contains the binding sites for the SH3 domains of the adapter molecule Grb2 and its homologues (Chardin et al., 1993). DH domains have been shown to act as GEFs for Rho family GTPases and are often associated with and regulated by PH domains, which have been shown to bind various phospholipids and the $\beta\gamma$-subunits of heterotrimeric G-proteins. The *in vivo* function of these domains has not yet been determined, however functional analysis of human Sos revealed that regulation of the GEF activity is mediated through both the N-terminal and C-terminal domains (Byrne et al., 1996; McCollam et al., 1995). Sos mutants lacking the N-terminal domains behave as potent dominant negative proteins in *D. melanogaster* (Karlovich et al., 1995). Overexpression of the PH domain of human Sos also has a pronounced dominant-negative effect on serum-induced activation of the Ras signalling pathway tissue culture cells (Chen et al., 1997). In addition, inhibition of human Sos RasGEF activity by binding of PI(4,5)P2 to the PH domain has been demonstrated (Jefferson et al., 1998).

A popular model for Grb2-mediated activation of Ras proposes that the interaction of Grb2 and Sos allows recruitment of Sos to the plasma membrane as Grb2 binds phosphorylated tyrosine residues on RTK cytoplasmic tails. This postulated role of Grb2 has led to its description as an "adapter" protein. The membrane recruitment of Sos brings it into close proximity with Ras, allowing Sos to activate Ras by promoting GDP/GTP exchange. There is much evidence supporting this hypothesis. Grb2-Sos complexes can be precipitated from the cytosols of quiescent cells (Buday and Downward, 1993) but become associated with growth factor receptors upon mitogen stimulation, a treatment which also leads to Ras activation. If murine Sos is inappropriately targeted to the plasma membrane by addition of either myristoylation or farnesylation signals, Ras becomes activated constitutively (Aronheim et al., 1994) and this confers
transforming potential to the Sos hybrid (Quilliam et al., 1994). However, there is convincing data conflicting with the proposed role of the Grb2/Drk/Sem-5 adapters in the recruitment model for Ras activation. A Sos fragment lacking the Drk binding site rescues the *D. melanogaster* Sos mutant to the same extent as the full length Sos protein and in *Drk* mutant flies, Sos membrane localisation in response to receptor activation is unaffected (Karlovich et al., 1995). Therefore, at present, the importance of Grb2 and its homologues in Ras activation by RTKs is uncertain.

### 1.4.1.3 Other Methods of Ras Activation

In addition to Sos, two other types of neuron-specific, mammalian RasGEFs have been identified; the closely related Ras-GRF1 (Farnsworth et al., 1995) and Ras-GRF2 (Fam et al., 1997) proteins and RasGRP (Ebinu et al., 1998).

The C-terminal region of both RasGRF1 and RasGRF2 contains the RasGEF domain whilst the N-terminal region contains two PH domains, a DH domain and an IQ-motif domain. The IQ-motif binds to the Ca^{2+} binding protein, calmodulin. RasGRF is activated by Ca^{2+} both *in vivo* and *in vitro* (Farnsworth et al., 1995). Structure/function analysis has demonstrated that the N-terminal region of RasGRF controls the activity of the catalytic domain and that calmodulin binding is required for activation (Baouz et al., 1997). Interestingly Ras-GRF1 activates Ha-Ras, but not N-Ras or Ki-Ras proteins *in vivo* (Jones and Jackson, 1998). There is some evidence that RasGRF1 mediates signals from G-protein coupled receptors rather than RTKs since both serum and lysophosphatidic acid (LPA)-induced Ras activation in RasGRF overexpressing cells can be completely inhibited by pertussis toxin (Zippel et al., 1996). In addition, experiments using rat brain explants suggest that Ras activation by muscarinic receptors requires βγ-subunits of heterotrimeric G-proteins and phosphorylation of RasGRF1 (Mattingly and Macara, 1996). Mice lacking Ras-GRF1 are impaired in the process of memory consolidation. Electrophysiological measurements in the basolateral amygdala reveal that long-term plasticity is abnormal in mutant mice. In contrast, Ras-GRF1 mutants do not reveal major deficits in spatial learning tasks. These results implicate Ras-GRF1-mediated Ras signaling in synaptic events leading to formation of long-term memories and is consistent with its localisation to synaptic junctions (Brambilla et al., 1997; Sturani et al., 1997). Membrane localisation of RasGRF has not been demonstrated to occur or to be necessary for its
activation. If, as in the case with Sos, membrane recruitment is required, a possible mechanism is via binding of βγ subunits of heterotrimeric G-proteins to either of the N-terminal PH domains.

RasGRP has a different domain structure to the RasGRF proteins. In addition to its central RasGEF domain, it contains two, Ca²⁺-binding, EF-hand motifs and a putative diacylglycerol (DAG) binding motif at the C-terminus. A DAG analogue caused sustained activation of GEF activity in vivo. This was associated with partitioning of RasGRP protein into the plasma membrane fraction. Sustained ligand-induced signaling and membrane partitioning were absent when the DAG-binding domain was deleted. RasGRP activated Ras and caused transformation in fibroblasts including MAP kinase activation and changes in cell morphology (Ebinu et al., 1998).

GEFs for other members of the Ras superfamily illustrate other possible methods of GEF activation. A family of mammalian RapGEFs has been identified which directly bind and are activated by cAMP (Kawasaki et al., 1998). The catalytic activity of mammalian RhoGEF, p115 RhoGEF is modulated by heterotrimeric G-protein α-subunits. Activated Gα₁₃ binds tightly to p115 RhoGEF and stimulates its capacity to catalyze nucleotide exchange on Rho proteins. In contrast, activated Gα₁₃ inhibited stimulation by Go₁₃ (Hart et al., 1998; Kozasa et al., 1998). Thus, p115 RhoGEF can directly link heterotrimeric G-protein alpha subunits to activation of Rho proteins. In addition to modulation of GEF activity the, association with heterotrimeric G-protein α-subunits presumably recruits the RhoGEF to the plasma membrane and brings it into close proximity with its target. A similar mechanism may operate for PDZ-RhoGEF which also binds Gα₁₂ and Go₁₃ (Fukuhara et al., 1999). The RacGEF, Vav, is activated by tyrosine phosphorylation by the Lck tyrosine kinase. This activation is inhibited by PI(4,5)P₂ and enhanced by PI(3,4,5)P₃, a product of PI 3-kinase (Han et al., 1998). These methods of GEF regulation may be employed by other, as yet undiscovered, RasGEFs.

Activation of Ras by modulation of p120GAP activity has been suggested by a study of neutrophil chemotaxis. Upon stimulation of these cells by the chemotactic peptide FMLP, a rapid increase in GTP-bound Ras was observed, paralleled by a decrease in the levels of the GAP activity of p120 RasGAP (Zheng et al., 1997). Inhibition of other candidate Ras activation pathways had no effect on the stimulation of Ras, suggesting FMLP may activate Ras via the inhibition of p120GAP.
Signalling by FMLP is mediated by heterotrimeric G-proteins in neutrophils, not tyrosine kinase receptors (RTKs). To date, GAP-mediated Ras activation by RTKs has not been described.

1.4.2 Ras Effectors

1.4.2.1 Raf and the MAP Kinase Cascade

The first *bona fide* Ras effector to be identified was the product of the proto-oncogene, *raf*. Raf is a serine/threonine kinase of approximately 67kDa that binds directly to Ras. The first evidence that Raf was involved in Ras signalling came from studies of the oncogenic, viral Raf protein, *v-raf* was shown to be capable of inducing many of the same phenotypes as *v-ras*, including the differentiation of PC-12 cells and induction of *Xenopus* oocyte maturation. (Fabian et al., 1993; Wood et al., 1993). Additionally, Raf-1 was shown to be required for cell proliferation in response to mitogenic stimuli (Kolch et al., 1991). Genetic evidence from the development of the *D.melanogaster* eye placed Raf downstream of Ras (Dickson et al., 1992) as did genetic evidence from vulva development of *C.elegans* where the Raf homologue is encoded by the *lin-45* gene (Han et al., 1993). Direct binding of GTP-bound Ras to Raf was first demonstrated using the yeast two-hybrid system, whereupon several reports simultaneously demonstrated the interaction by biochemical methods (Koide et al., 1993; Vojtek et al., 1993; Warne et al., 1993).

One important feature of the Ras/Raf interaction is the sub-cellular localisation of the two proteins. Raf is primarily cytosolic in quiescent cells whereas Ras is at the plasma membrane. A fundamental part of the activation of Raf by Ras is the recruitment of Raf to the plasma membrane. The importance of this was demonstrated by targeting Raf to the membrane by fusing a Ras CAAX motif to the C-terminus of the protein. This results in a constitutively active Raf protein (Leevers et al., 1994). However, membrane recruitment is not the sole requirement for Raf activation. Phosphorylation of Raf and binding to 14-3-3 family proteins are also required. In addition, Raf has been shown to dimerise in a Ras-dependent manner (Luo et al., 1996). However, the exact mechanism of Raf activation is far from clear.

Raf is at the head a highly conserved cascade of protein kinases, known as the MAP kinase pathway, that ultimately activate transcription factors regulating genes required for proliferation and differentiation. The cascade consists of three kinases that successively phosphorylate and activate
the next. Starting at the bottom, a mitogen activated protein (MAP) kinase is phosphorylated by a MAP kinase kinase (MAPKK or MEK) which in turn is phosphorylated by a MEK kinase (MAPKKK or MEKK). The MAP kinase pathway has been conserved throughout evolution. In both budding and fission yeast the pathway mediates the pheromone response and is activated by Ras (Herskowitz, 1995). In *D. melanogaster* and *C. elegans* Ras activates a MAP kinase pathway that regulates the differentiation of the photoreceptors and the vulva respectively (Kayne and Sternberg, 1995; Wassarman et al., 1995). Multiple MAP kinase pathways are known to operate within individual cells (Schaeffer and Weber, 1999). In *S. cerevisiae* and mammalian systems five distinguishable MAPK cascades have been identified. The best characterised MAP kinase cascade is headed by Raf (a MEKK) which activates MEK and in turn the MAP kinases Erk1 and Erk2. The Erks translocate from the cytosol to the nucleus when activated by phosphorylation whereupon they can phosphorylate and activate the transcription factor Elk-1, a member of the serum response factor family (Marais et al., 1993). The activation of this MAP kinase cascade by Ras results in transcription of immediate early response genes which are associated with entry into the cell cycle.

Recently the interaction of Ras signalling pathways with the cell cycle machinery has come under scrutiny. Activation of Ras and the MAP kinase pathway has been demonstrated to be essential for the induction of cyclin D by growth factors and for the phosphorylation of the retinoblastoma (Rb) protein that results from cyclin D induction. Cells from mice lacking Rb do not require Ras activity for cell cycle progression (Mittnacht et al., 1997; Peeper et al., 1997). The cyclin dependent kinase inhibitors (CDKIs) are also involved in the mediation of Ras signalling in the cell cycle. Based on the observations that activated Ras induces senescence rather than proliferation in primary cells (Ridley et al., 1988), many studies have been aimed at understanding how Ras induces the cell cycle arrest, and how this control is altered in immortalised cell lines. What is now clear is that activated Ras increases the levels of the CDKIs, p16\textsuperscript{INK}, p21\textsuperscript{CIP} and p27 thus blocking the induction of cyclin D and causing a cell cycle arrest, although the exact interactions vary from cell type to cell type (Lloyd et al., 1997; Serrano et al., 1997). Also, emerging from these studies is the understanding that a common step in the cell immortalisation step is the deletion of CDKIs. For example the commonly used NIH3T3 cells have lost expression of p16 (Lloyd, 1998). This allows the cells to react to a signal from Ras (via the MAP kinase cascade) by entering the cell cycle rather than arresting in G\textsubscript{1}. 

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1.4.2.2 PI 3-Kinase and Downstream Lipid Signalling

The discovery that activated Raf could not elicit all the effects of Ras transformation suggested that Raf was not the only effector of Ras (Thorburn et al., 1994; White et al., 1995). The subsequent generation of Ras proteins with effector loop mutations has allowed the relative contributions of different downstream pathways to Ras-regulated processes to be assessed (Joneson et al., 1996). An activated Ha-Ras protein carrying an effector loop mutation allowing binding and activation of Raf but not PI 3-kinase or RalGDS is not able to transform NIH 3T3 cells. If this protein is expressed in combination with a Ras capable of activating only PI 3-kinase, cell transformation is effected (Rodriguez-Viciana et al., 1997). These studies illustrate how the cooperation of multiple downstream pathways is required for the full biological effects of Ras.

Ras binds directly to and activates the catalytic subunits of the Class-I family of phosphatidylinositol 3-kinase (PI 3-kinases) (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). PI 3-kinases phosphorylate phosphatidylinositol (PI) at the 3' position of the inositol ring and can produce phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3, 4-bisphosphate (PI(3,4)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)-P_3) (Vanhaesebroeck et al., 1997). In resting cells PI(3,4)P_2 and PI(3,4,5)-P_3 are barely detectable. Upon stimulation of cells with a variety of ligands, their concentrations rise sharply, a response mediated by Ras and other signalling pathways. More specifically, a rise in cellular PI(3,4,5)-P_3 levels can be brought about by transfection of cells with constructs expressing activated Ras (Rodriguez-Viciana et al., 1994).

An important cellular target for the lipid products of PI 3-kinases is the PH domain. This domain is present in many signalling molecules and has been shown to bind 3-phosphorylated PIs. One such PH domain containing protein, and the best characterised downstream target of PI 3-kinase is protein kinase B (PKB). PKB binds PI(3,4,5)P_3 via its PH domain but this has no affect on the kinase activity. Instead, this interaction serves to recruit PKB to cellular membranes (Andjelkovic et al., 1997). For activation, PKB requires PI(3,4,5)P_3 binding plus phosphorylation on serine and/or threonine residues (Alessi and Cohen, 1998; Currie et al., 1999). This appears to be mediated by one kinase called phosphoinositide-dependent kinase 1 (PDK1) (Alessi et al., 1997; Balendran et al., 1999). PDK1 also contains a PH domain and PDK1 kinase activity is directly
activated by binding PI(3,4,5)P₃ (Alessi et al., 1997). Hence PI 3-kinase activity results in PKB activation (Galetic et al., 1999; Stephens et al., 1998).

PKB activation is required for Ras-mediated inhibition of apoptosis in a number of cell lines (Kauffmann-Zeh et al., 1997; Khwaja et al., 1997). For example the loss of adherence of epithelial cells to the extracellular matrix results in anoikis (a form of apoptosis) but this can be prevented by Ras-induced activation of PI 3-kinase and PKB but not by a Ras-induced activation of Raf and the MAP kinase pathway (Khwaja et al., 1997). PKB promotes anti-apoptotic cell survival, at least in part, by stimulating the expression of cellular genes via the transcription factors CREB/CBP and NF-κB (Du and Montminy, 1998; Romashkova and Makarov, 1999) and inhibits pro-apoptotic gene expression by phosphorylation and subsequent nuclear exclusion of the forkhead/winged-helix family of transcription factors (Biggs et al., 1999; Brunet et al., 1999). PKB also phosphorylates and inactivates the pro-apoptotic proteins BAD (Datta et al., 1997). Another target of PKB is glycogen synthase kinase 3 (GSK3), a protein required for the insulin-mediated regulation of metabolism and also developmental patterning (van Weeren et al., 1998). Insulin stimulation of cells results in PKB-mediated phosphorylation and inhibition of GSK3, leading to an increase in glycogen and protein synthesis (Cross et al., 1995).

PDK1 has other cellular targets in addition to PKB. The S6 kinase is directly phosphorylated and activated by PDK1 and has been shown to be required for cell cycle progression from G1 to S phase (Lane et al., 1993). Protein kinase C (PKC) isoforms are also controlled by PI 3-kinase through direct activation by PDK1 (Le Good et al., 1998). Interestingly, PKC is a negative regulator of PKB thus it seems that PDK1 can both activate and indirectly inhibit PKB activity (Doornbos et al., 1999).

PI 3-kinase has also been implicated in mediating the cytoskeletal changes induced by activated Ras. Chemical inhibitors of PI 3-kinases interfere with activated Ras-induced membrane ruffling (Rodriguez-Viciana et al., 1997). Conversely, activated PI 3-kinase and activated Ras mutants which specifically activate PI 3-kinase are potent stimulators of membrane ruffling (Rodriguez-Viciana et al., 1997). This effect can be blocked by dominant negative Rac proteins (Ridley et al., 1992) and does not involve PKB (van Weening et al., 1998). There is evidence that binding of PI(3,4,5)P₃ to PH domain of the RacGEF, Vav, in synergy with the tyrosine phosphorylation of Vav by the Lck kinase, results in a stimulation of Vav exchange activity on Rac (Han et al., 1998).
There is also some evidence that the Sos RasGEF may directly activate Rac in response to PI 3-kinase via its Dbl homology domain (Nimnual et al., 1998). These data have implicated PI 3-kinase, operating via regulation of Rac, as a mediator of activated Ras-induced membrane ruffling. However, the increase in pinocytosis associated with expression of activated Ras, although blocked by inhibitors of PI 3-kinase is unaffected by dominant negative Rac proteins (Li et al., 1997). Instead, pinocytosis requires PKB and Rab5 activity (Barbieri et al., 1998; Li et al., 1997). It seems, therefore, that the control of these different cytoskeletal processes bifurcates after PI 3-kinase activation.

PI 3-kinase-γ has recently been demonstrated to directly phosphorylate and activate MAP kinase in vivo (Bondeva et al., 1998). This provides another mechanism for Ras activation of MAP kinase.

1.4.2.3 RasGAPs

p120 RasGAP was the first protein to be identified that interacted only with GTP-bound Ras (Trahey et al., 1988). The existence of a GTPase-activating activity in cells was apparent from work on the maturation of *Xenopus* oocytes. Dominant activated N-Ras but not wild-type N-Ras is capable of inducing oocyte maturation. The wild-type N-Ras protein was shown to be almost exclusively GDP-bound in oocyte extracts, despite the 10-fold excess of GTP over GDP. This was shown to be due to the action of a cytoplasmic protein that stimulated the GTP hydrolysis rate of wild-type Ras, promoting the switch to the GDP-bound form. Importantly, the protein (p120 RasGAP) was shown to be incapable of performing a similar function on dominant activated Ras, resulting in its permanent, activated, GTP-bound state (Trahey and McCormick, 1987). p120 RasGAP was later shown by mutational analysis to bind to the Switch-I region of Ras in a similar manner to Ras effectors (Adari et al., 1988). To date, two other types of GAP protein have been identified in mammalian cells: neurofibromin (the product of the human tumour suppressor gene NF1) (Martin et al., 1990) and the closely related, Gap11 (Cullen et al., 1995) and Gap11P48 (Maekawa et al., 1994).

NF-1 and p120 RasGAP will bind to and stimulate GTP hydrolysis on all of the mammalian Ras proteins including the more divergent R-Ras. They also bind Rap1 but exhibit no RapGAP activity (Frech et al., 1990; Hata et al., 1990). The N-terminal region of p120 RasGAP contains 2 SH2 and SH3 domains plus a PH domain and a C2 phospholipid binding domain. The PH domain of p120
RasGAP is sufficient for \textit{in vitro} binding to $\beta\gamma$ subunits of heterotrimeric G proteins (Xu et al., 1996). The SH2 domains of p120 RasGAP facilitate binding to activated tyrosine kinase receptors, and to the cytosolic proteins p190 RhoGAP and p62dok although the biological significance of these interactions is not fully understood. Less information is known about the interactions of NF-1 with other signalling molecules. NF-1 interacts with tubulin (Gregory et al., 1993) and arachidonic acid inhibits its GAP activity (Han et al., 1991). Gap1\textsuperscript{th} and Gap1\textsuperscript{1848} contain inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P\textsubscript{4}) binding domains. Gap1\textsuperscript{1848} shows GAP activity against both Rap and Ras proteins, and the RasGAP activity is inhibited by phospholipids and is specifically stimulated by Ins(1,3,4,5)P\textsubscript{4}.

Genetic analysis shows that both NF-1 and p120 RasGAP are essential genes in mice. NF-1 knock-out mice die during embryonic development due to cardiac defects and exhibit hyperplasia of the neural crest cells (Brannan et al., 1994). p120 RasGAP deficient mice also die \textit{in utero} with abnormal vasculature and increased neural apoptosis. Since \textit{Ki-ras} null mice exhibit neural apoptosis, this may indicate an effector function for p120 RasGAP. A loss of function mutation in the gene for \textit{D.melanogaster} NF-1 does not cause any phenotype indicative of a role for the molecule in Ras signalling. The NF-1 mutant flies, although reduced in size, have normal early embryonic patterning and ommatidial development (The et al., 1997). Furthermore, the size phenotype is not sensitive to manipulations of Ras signalling, again indicating that NF-1 is not an important mediator or regulator of Ras function in flies. However, heterozygous loss of the \textit{D.melanogaster} p120 RasGAP homologue, \textit{Gap1}, is lethal in the NF-1 mutant, suggesting that some of the roles of NF-1 in flies may be masked in the NF-1 mutants by partial overlap of function (The et al., 1997). However, overexpression of p120 RasGAP in \textit{D.melanogaster} wing imaginal discs downregulates signalling through receptor tyrosine kinases and inhibits wing growth (Feldmann et al., 1999). This is consistent with the attenuation of Ras activity mediated by the GAP activity of p120 RasGAP.

There are several other indications that RasGAPs have a physiological function distinct from their GAP activity. Activated Ras blocks the heterotrimeric G-protein coupled opening of K\textsuperscript{+} channels in atrial cells. This is dependent on p120 RasGAP, in particular the N-terminal, non-catalytic region (Martin et al., 1992; Yatani et al., 1990). p120 RasGAP is required for activated Ras-induced gene expression in cardiac myocytes (Abdellatif and Schneider, 1997). The N-
terminus of RasGAP will induce cytoskeletal changes in cells when overexpressed (McGlade et al., 1993). Transformation of fibroblasts by activated Ras, whose protein product is resistant to GTPase stimulation by NF1, was inhibited in a cell line overexpressing NF1, suggesting inhibition of Ras-dependent growth by a regulatory mechanism that is independent of NF1 RasGAP activity (Johnson et al., 1994). Gap1
\textsuperscript{14b}
 has been implicated in Ras-dependent modulation of Ca\textsuperscript{2+} mobilization by Ins(1,3,4,5)P\textsubscript{4}, suggesting it has a Ras effector function (Loomis-Husselbee et al., 1998).

### 1.4.2.4 Other Putative Ras effectors

Another family of Ras effectors comprises RalGDS and two related exchange factors which can activate the Ras family GTPases, RalA and RalB, in response to Ras activation (Kikuchi et al., 1994; Urano et al., 1996). The function of Ral is unknown, although a role in the regulation of phospholipase D has been suggested (Jiang et al., 1995). Activated Ral interacts with a RacGAP domain-containing protein suggesting the existence of another effector pathway by which Ras can modulate the actin cytoskeleton (Feig et al., 1996).

In addition to Raf, two other serine-threonine kinases have been shown to interact specifically with Ras-GTP. PKC\textsubscript{z}, whose function is not understood, has been shown to associate with Ras after PDGF stimulation and so may play a role in mitogenic stimulation (Diaz-Meco et al., 1991). Ras can also activate MEK kinase (MEKK) which lies at the head of the stress-activated protein kinase (SAP Kinase) pathway at the same level as Raf (Lange-Carter and Johnson, 1994). The SAP Kinase pathway is homologous to the MAP kinase pathway and involves a similar cascade of serine-threonine kinases that eventually activate transcription factors.

The N-terminal portion of AF-6, a protein purified from bovine brain, forms part of a fusion protein caused by the t(6:11) chromosome abnormality associated with some human leukemia. AF-6 is homologous to D.melanogaster Canoe, which is assumed to function downstream from Notch in a developmental signaling pathway. The N-terminal domains of AF-6 and Canoe specifically interact with GTP-bound Ha-Ras via the Ras effector domain (Kuriyama et al., 1996).

The Nore1 protein interacts with Ras in vitro in a GTP-dependent manner, and the interaction requires an intact Ras effector domain. Nore1 becomes associated with Ras \textit{in vivo} following activation of the EGF receptor. It has no significant sequence similarity to known mammalian
proteins and lacks an identifiable catalytic domain, but contains sequence motifs that predict a DAG binding site and SH3 domain binding (Vavvas et al., 1998).

One *D. melanogaster* and two *C. elegans* screens for genetic suppressors of activated Ras, identified the same protein, KSR (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). KSR is a kinase with similarities to conserved regions of Raf kinases, especially within the kinase domain. It appears to inhibit activation of MAP kinase by binding to its direct upstream activator, MEK, and preventing Raf-mediated MEK activation (Denouel-Galy et al., 1998).

Another putative effector of Ras was identified in a *C. elegans* screen for genetic modulators of activated Ras. This protein, Sur-8, shares some homology with the Ras binding domain of *S. cerevisiae* adenylate cyclase and has homologues in other species including human, mouse and *Dictyostelium* (Sieburth et al., 1998). Sur-8 binds to activated Ki-Ras and N-Ras but not Ha-Ras. It positively modulates Ras activity and binds to the effector region of Ras while epistasis experiments suggest it acts at, or above the level of Raf.

A screen for human proteins which can interfere with the Ras signal transduction pathway in the yeast *S. cerevisiae* identified another putative Ras effector, Rin1 (Han and Colicelli, 1995; Han et al., 1997). Rin1 interacts with GTP-bound Ha-Ras via the Ras effector domain but no biological function has yet been ascribed.

The most recently discovered putative Ras effector was detected in a yeast two-hybrid screen using the *C. elegans* Let-60 Ras homologue. The screen identified a cDNA encoding a phosphoinositide-specific phospholipase C (PI-PLC) with a predicted molecular mass of 210kDa, designated PLC210 (Shibatohge et al., 1998). PLC210 possesses two additional functional domains unseen in any known PI-PLCs. One is the C-terminal Ras-binding domain with structural homology to those of RalGDS and AF-6. This domain associates with human Ha-Ras in a GTP-dependent manner *in vitro*. The other functional domain is an N-terminal Cdc25-like domain, which possesses structural homology to RasGEFs. No biological function has yet been ascribed.

1.5 The Biology of *Dictyostelium discoideum*

The following sections discuss the biology and experimental use of *Dictyostelium discoideum*, with specific emphasis placed upon areas relevant to my research.
1.5.1 The Life Cycle of Dictyostelium discoideum

Although lacking vegetative hyphae it is clearly not a true fungus, Dictyostelium has been classified in the Kingdom Mycetae (Raper, 1984). The high A-T base content of the D.discoideum genome (approximately 85%) has made phylogenetic analysis difficult. Based on comparisons of the 18S rRNA, D.discoideum is predicted to have diverged from the eukaryotic evolutionary line to mammals prior to divergence of the yeasts (McCarroll et al., 1983). However, the A-T base content of the DNA encoding 18S rRNA is much higher than that of most other eukaryotes and may therefore not be a reliable measure with which to determine phylogeny. More recently, the protein sequences of several metabolic enzymes were compared with those of other eukaryotes. This analysis predicted that Dictyostelium diverged from the evolutionary line to mammals subsequent to the divergence of the yeasts but prior to that of the worm C.elegans (Loomis and Smith, 1995; Loomis and Smith, 1990).

Dictyostelium discoideum amoebae exist in the upper soil layers of forest floors. They are obligate phagocytes with bacteria as their preferred food. Vegetative amoebae are chemotactic towards the bacterial metabolite folate, which presumably allows them to locate their food. In a non-limiting nutritive environment D.discoideum cells proliferate with a doubling time of approximately 4h. Upon exhaustion of their food supply, the amoebae can adopt two different programmes of differentiation. An asexual programme of differentiation results in the aggregation of up to 100,000 cells. The resultant cell aggregate undergoes a series of morphogenetic changes resulting in the formation of a spore-containing fruiting body. These spores are resistant to environmental stress and germinate into amoebae under favourable environmental conditions. At low cell densities, an alternative, sexual differentiation process can occur. The amoebae, which are haploid, can fuse with cells of an opposite mating type to form diploid giant cells competent for genetic recombination. The giant cell develops into an alternative form of spore, called a macrocyst, which is also highly resistant to environmental stress.

The aggregation events underlying both the sexual and asexual differentiation processes rely on chemotaxis towards cAMP. In the sexual cycle, the giant cell secretes cAMP, which acts as a chemoattractant to other amoebae. Chemotaxis of these amoebae brings them into the proximity of the giant cell, which promptly phagocytoses them. This cannibalistic behaviour allows the diploid
to accumulate sufficient nutritional resources for macrocyst formation, dormancy and subsequent germination. The mechanisms regulating sexual chemotaxis, the development of sexual competency and the stimuli for macrocyst germination are poorly understood. It is not known to what extent the sexual cycle occurs in the wild.

The asexual developmental life cycle has been more extensively characterised. Cells at the prospective centre of an aggregation field respond to depletion of their food supply by secreting cAMP, which is perceived by nearby cells. Reception of the cAMP signal causes cells to synthesise and secrete their own cAMP, and move chemotactically towards the initial source. The secondary release of cAMP serves to stimulate cells further from the aggregation centre, which in turn become competent to signal and perform chemotaxis. This process, termed signal relay, results in spiral or concentric circular wave patterns of cAMP centred on the aggregation foci, as can be visualised by isotopic dilution experiments (Tomchik and Devreotes, 1981). Rapidly moving cells are more elongated than the slow moving cells, and appear brighter when visualised by dark field microscopy. This has allowed the cAMP waves and cell behaviour to be correlated. During the ascending phase of the cAMP wave, cells migrate towards the aggregation centre whereas during the peak and descending phase of the wave, the cells become static.

As the cell density increases closer to the aggregation centre, the cells form streams. Cells within a stream adhere tightly to one another in a head to tail fashion. After 8h of starvation, the streaming amoebae converge to form a loose aggregate containing essentially identical cells. The loose aggregate gradually becomes more compact, forming the tight mound, by about 10h of starvation. At this stage the cells begin their differentiation into distinct cell types. Two hours later, a tip forms in the centre of the mound which extends further to form a structure called the first finger. By this stage cells have differentiated into two classes: the prestalk cells and prespore cells, precursors of the stalk and spore cells of the final fruiting body. In the dark, or if the aggregates are exposed to a lateral source of light, the finger collapses onto the substratum, giving rise to the pseudoplasmodium, a phototactic slug-like structure. In conditions of overhead light, many of the aggregates will culminate to form fruiting bodies without going through this slug phase of development. Two hours after pseudoplasmodium formation, the final differentiation stages are initiated. The pseudoplasmodium rears up and a stalk tube begins to form, pushing the cells which are destined to become spores above the substratum. The stalk tube is constructed from
approximately twenty percent of the cells in the initial aggregate. The stalk cells die during culmination by a process resembling apoptosis (Olie et al., 1998) and do not contribute to the next generation of amoebae. The remaining cells differentiate into a mass of spores as the stalk elevates them off the substrate, giving rise to a fully mature fruiting body approximately 24h after the onset of starvation.

1.5.2 *D.discoideum* as an Experimental System

During the last thirty years, the utility of *Dictyostelium discoideum* as an experimental model has developed profoundly. The first major development was the generation of axenic cell lines and the culture conditions allowing these to proliferate (Watts and Ashworth, 1970). Wild isolates of *D. discoideum* cannot grow in conventional liquid growth medium, since they have very little pinocytic activity (Clarke and Kayman, 1987). They can be propagated solely using a bacterial food source, which until recently has precluded their transformation with DNA. Mutagenesis of these wild isolates and selection for strains capable of growth in a peptone-based liquid medium yielded the axenic strains. The most commonly used axenic strains are AX2 and AX3, which were derived independently by serial passage of the wild isolate NC4 (Loomis, 1971; Raper, 1935; Watts and Ashworth, 1970). These cell lines and their derivatives carry mutations allowing them to macropinocytose and consequently proliferate in liquid culture. The development of transformation techniques for *D. discoideum* in the 1980's was made simple by the use of these axenic strains. Effective selection of transformants was permitted by the addition of antibiotic to the culture medium (Nellen et al., 1984). Selection for transformants on bacterial lawns has also been achieved recently, but required extensive optimisation since the bacteria act as a sink for the antibiotics (Wetterauer et al., 1996). Subsequently, tagged mutagenesis screens (Kuspa and Loomis, 1992) and targeted gene disruption (De Lozanne and Spudich, 1987) have become widely used in the investigation of gene function and the speed with which these methods can be applied outpaces all other eukaryotic models except the yeasts. The haploid genome of *Dictyostelium* further facilitates the rapid screening and analysis of recombinants, although being haploid prevents the recovery of recombinants with essential genes deleted, hindering the potential of these mutagenesis tools. The use of the sexual cycle, for mapping and combining mutations, has been considered by some researchers (Wallace and Raper, 1979). However, the poor frequency of germination obtained in
sexual crosses and the fact that NC4 and its axenic derivatives appear unable to form functional macrocysts has impaired the development of what might be a very useful technique. In the last few years, Dictyostelium research has been aided by the initiation of a number of sequencing projects. Two, which are approaching completion, have aimed to sequence entire cDNA libraries obtained from several stages of the life cycle (Morio et al., 1998). The other programmes are attempting to sequence the entire D.discoideum genome and are an international collaboration between the University of Cologne, Germany, the Institute of Molecular Biotechnology in Jena, Germany, the Baylor College of Medicine in Houston, USA, the Pasteur Institute in Paris, France, and the Sanger Centre in Hinxton, England. These resources have already considerably enhanced the experimental utility of the organism.

Arguably the most generally applicable area of Dictyostelium research has been the investigation of the organisation and function of the actin cytoskeleton. The organism is considered a good model system since its phagocytosis and motility resemble those of mammalian leukocytes, and it has become clear that the molecular events underlying these processes are conserved. Disruption of genes encoding specific actin-associated proteins has been very informative about the functions of these proteins and some of these proteins have been subsequently identified and assigned similar functions in higher eukaryotic cells (de Hostos et al., 1993; Niewohner et al., 1997). Another large focus of Dictyostelium research is the study of chemotaxis. The parallels between neutrophils and Dictyostelium are great. The cells have similar chemoattractant receptors, similar downstream effects in response to chemoattractant stimulation and very similar crawling motility (Devreotes and Zigmond, 1988).

1.5.3 Cell Behaviour During Early Development

As their food supply becomes exhausted, Dictyostelium amoebae initiate the expression of early developmental markers, such as the, discoidin-1 family of lectins and a set of lysosomal enzymes (Clarke and Gomer, 1995). This early starvation response is paralleled by a decrease in the velocity of the amoebae to approximately 5μm/min (Varnum et al., 1986). During the first 2h of starvation there is also an increase in the number of filopods and phagocytic cups visible on the cell surface correlating with an approximately four fold increase in the phagocytic rate (De Chastellier and Ryter, 1977). From 2h of starvation onwards, the expression of cAR1, cAMP phosphodiesterase
and adenylate cyclase begins and after 3.5h, cells begin to secrete pulses of cAMP. Initially, the pulses of cAMP are unsynchronised but by 4h the pulses have synchronised within the population and occur with an interval of approximately 10min. By the time of aggregation, this interval has reduced to 2-3 minutes (Devreotes et al., 1987). After 6h of starvation, cell velocity gradually increases to about 15μm/min as the cells begin chemotaxing efficiently towards the aggregation centre (Varnum et al., 1986). Around this time, the amoebae commence expression of the cell surface adhesion molecule contact sites A (csA), which facilitates the formation of cell contacts in streams and the mound (Faix et al., 1992). Also during the period from 2h to 6h of starvation, the number of cell surface filopods increases dramatically whilst phagocytic cups disappear and there is a decrease in the phagocytic rate (De Chastellier and Ryter, 1977).

1.5.4 Starvation Response and Early Signalling Events

The switch from growth to development involves changes in gene expression. Many of the genes expressed during growth are downregulated and others are expressed specifically during the multicellular stages. Most of the components of the aggregation system, for example the cARl receptor, the extracellular phosphodiesterase and adenylate cyclase are not expressed during growth but expression begins soon after the onset of starvation. It is important that all cells within the starving population should induce the aggregation stage genes together lest some cells be unable to aggregate at the appropriate time. It is also important that aggregation does not begin before enough cells have reached the appropriate stage. The result of too few cells aggregating into a mound would be a smaller final culminant that presumably would be less able to distribute spores. The mechanism by which Dictyostelium cells ensure synchrony of the starving population involves the secretion of at least two autocrine factors that are used to assess population size and to induce the aggregation genes.

Pre-Starvation Factor (PSF) is secreted constantly by growing Dictyostelium cells, whatever the density of the population. It can therefore be used to assess the population density since it accumulates in proportion to cell number. PSF activity is first detectable in a population of cells in log phase growth approximately four generations before the start of stationary phase, although medium taken from cells growing at lower densities does contain PSF (Clarke et al., 1988). PSF induces early developmental gene expression, most notably expression of the genes encoding the
discoidin-1 family of lectins, but also of other genes including lysosomal enzymes (Clarke et al., 1987). The pre-starvation response mediated by PSF is not strictly dependent on its concentration. Instead it is governed by the presence of bacteria, with high bacterial densities inhibiting PSF activity as judged by discoidin-1γ induction; (Clarke et al., 1987; Rathi et al., 1991). Hence, PSF secretion appears to be a mechanism of preparing the population for development as the nutrient supply becomes short.

PSF secretion ceases as soon as the food supply is exhausted and is replaced by a second starvation factor, Conditioned Medium Factor (CMF). In contrast to PSF, CMF has been purified and the gene encoding it cloned (Jain et al., 1992). Medium taken from cells starving at high density is able to induce the development of cells at low density, hence the name CMF (Mehdy and Firtel, 1985). CMF is an entirely novel glycoprotein with no homology to known proteins. It is secreted at a constant rate throughout at least the first 10h of development. Interestingly, it is also produced in growth phase cells but then sequestered until the onset of starvation at which time the slow secretion begins (Gomer et al., 1991). There is convincing data that CMF is required for multicellular development. An antisense RNA construct blocks development by preventing aggregation (Yuen et al., 1995). The proposed role of CMF is to allow cells to sense the density and size of a starving population and thereby allow the population to synchronise its development so that aggregation will only occur with the number of cells sufficient to form an effective fruiting body (usually 1x10^5 cells) (Gomer, 1997; Jain et al., 1997; Yuen et al., 1995). CMF has many of the same properties as PSF. Both induce expression of the dislγ gene and other early developmental genes such as those encoding the cAMP receptor, cAR1, and phosphodiesterase that are required for aggregation.

1.5.5 cAMP Signalling

1.5.5.1 cAMP Receptors

The cAMP receptors that perceive the extracellular cAMP pulses belong to the seven transmembrane spanning (7-TM) G-protein coupled receptor family and are termed cARs. Of these the expression patterns of cAR1 and cAR3 overlap and they share partial functional overlap. cAR1 is expressed and functions predominantly during aggregation and disruption of the gene blocks the
process (Sun and Devreotes, 1991). Development can still proceed, however, if extracellular cAMP pulses are provided. cAR3 provides residual cAMP signalling in the cAR1 null cells but appears to activate the cAMP relay through activation of adenylate cyclase only very poorly. A double mutant cannot aggregate even when provided with extracellular cAMP pulses (Insall et al., 1994a). Recently cAR3 has been shown to mediate the activation of Glycogen Synthase Kinase-3 (GSK-3) by extracellular cAMP and hence play a role in regulating cell type patterning during the initial stages of multicellular development (Plyte et al., 1999). cAR2 is expressed only after cells have aggregated and, then, preferentially in prestalk cells. cAR2 null cells proceed normally through early development, but arrest at the tight mound stage. Although prestalk-specific genes are expressed normally in these aggregates, they exhibit an enhanced expression of prespore-specific genes. cAR2 may be required for cAMP-directed sorting of prestalk cells during pattern formation within the mound (Saxe III et al., 1993). cAR4 is initially expressed during tip elongation and becomes maximally expressed in the anterior of pseudoplasmodia. Although cAR4 null cells progress unperturbed through early development, they exhibit major patterning aberrations as the anteroposterior axis becomes established. Prestalk gene expression is significantly reduced in cAR4 null aggregates, whereas prespore-specific markers are overexpressed and detected in zones normally restricted to prestalk cells. Patterning defects are similarly apparent in terminally differentiated fruiting bodies (Louis et al., 1994). Hence cAMP signalling is important at every stage of the development of Dictyostelium.

**1.5.5.2 Heterotrimeric G-proteins**

The heterotrimeric G-proteins which mediate signalling through 7-TM receptors consist of three associated subunits, α, β, and γ (Neer, 1995). It is the Gα subunit which can bind and hydrolyse GTP. Activation of the 7-TM receptor by ligand results in the exchange of GDP for GTP by the Gα subunit and subsequent dissociation of the G-protein into free Gα and Gβγ subunits which are both capable of eliciting cellular responses (Neer, 1995). So far, eight Gα subunits (Gα1 to Gα8) have been cloned from Dictyostelium which share 35-50% amino acid identity with each other and their mammalian counterparts (Pupillo et al., 1989; Wu et al., 1994). The Dictyostelium Gα subunits cannot be divided into subfamilies such as the G1, Gi and Gq subfamilies of mammalian cells. Gα1, Gα2, Gα3, Gα4, Gα7, and Gα8 are expressed throughout growth and development.
whereas G\(\alpha_3\) is expressed in the multicellular stages of development and G\(\alpha_4\) expression is restricted to vegetative growth. With the exception of G\(\alpha_6\), *Dictyostelium* null mutants of all the G\(\alpha\) subunits have been generated (Brandon et al., 1997; Hadwiger et al., 1994; Kumagai et al., 1991; Wu et al., 1994). Only G\(\alpha_2\), G\(\alpha_3\) and G\(\alpha_4\) null mutants exhibit striking phenotypes. g\(\alpha_2\) cells are unable to aggregate or differentiate upon starvation and show no cAMP-induced stimulation of adenylate cyclase, guanylate cyclase, PLC or actin polymerisation. However, chemotaxis to folate and folate activation of guanylate cyclase is normal in vegetative g\(\alpha_2\) cells (Bominaar et al., 1994; Kumagai et al., 1991). It is likely that G\(\alpha_2\) is coupled to cAR1 and cAR3 and mediates cAMP-dependent events in aggregating cells. Similarly, ablation of G\(\alpha_3\) prevents aggregation and subsequent multicellular development. g\(\alpha_3\) cells fail to express cAR1, adenylate cyclase and cAMP phosphodiesterase upon starvation. However, expression of these genes can be restored by starving the g\(\alpha_3\) cells in the presence of exogenous cAMP pulses. It therefore appears that G\(\alpha_3\) is required for initiating the expression of components of the transmembrane cAMP signaling system but is not essential for cAMP-mediated signaling events. (Brandon and Podgorski, 1997). In contrast, g\(\alpha_4\) cells are defective in folate chemotaxis but show normal chemotaxis to cAMP and normal cAMP-mediated adenylate cyclase and guanylate cyclase activation (Hadwiger et al., 1994). The g\(\alpha_4\) cells develop normally to the mound stage but show abnormal late development and a low spore count. *Dictyostelium* has only one G\(\beta\) subunit which shares 90% identity with those of other species. Loss of G\(\beta\) following gene disruption blocks chemotaxis, aggregation and most of the intracellular responses to extracellular cAMP (Lilly et al., 1993; Wu et al., 1995).

1.5.5.3 Cellular Responses to Extracellular cAMP

Exposure of cells to cAMP results in three main intracellular responses – rises in the second messengers cAMP, cGMP and Ca\(^{2+}\). cAMP synthesis results from the activation of adenylate cyclase and serves several functions. Secreted cAMP propagates the cAMP chemotactic signal, whilst intracellular cAMP activates Protein Kinase-A (PKA). Adenylate cyclase is activated by the free \(\beta\gamma\) subunits of the heterotrimeric G-proteins which are released upon cAMP receptor activation (Wu et al., 1995). Activation requires a cytosolic protein, CRAC (Cytosolic Regulator of Adenylate
Cyclase), that translocates to the plasma membrane following cAR1 activation although its mechanism of action is unknown (Insall et al., 1994b; Lilly and Devreotes, 1994).

cAMP stimulation of cells also results in synthesis of cGMP, mediated by Go2 activation of guanylate cyclase. The rise in cGMP has several functions including regulation of myosin function during chemotaxis and migration (Liu and Newell, 1988). A "streamer" cell line that carries a mutation in an intracellular cGMP phosphodiesterase is unable to break down the cGMP that is synthesised in response to extracellular cAMP and consequently displays increased association of myosin with the cytoskeleton. The myosin heavy-chain remains phosphorylated for longer and the cells remain polarised for extended periods (Liu and Newell, 1991). Further evidence for the function of cGMP in the regulation of the cytoskeleton during chemotaxis is provided by a several chemically mutagenised cell lines defective in chemotaxis to both cAMP and the growth phase chemoattractant, folate. Two of the mutant cell lines, Kl-8 and Kl-10, are defective in cGMP synthesis. Kl-8 synthesises very little cGMP and Kl-10 fails to elevate cGMP in response to either chemoattractant (Kuwayama et al., 1993; Liu et al., 1993). The response to each of these chemoattractants is mediated by different Go subunits downstream of the receptors suggesting convergence upon a single cGMP signalling pathway is required for chemotaxis (Kesbeke et al., 1990).

A rise in intracellular Ca2+ is also seen in response to extracellular cAMP. The function of Ca2+ is a source of debate and many studies provide contradictory evidence. The Ca2+ rise would seem likely to be responsible for the concomitant rise in inositol trisphosphate (IP3) levels since the only phospholipase C (PLC) identified thus far in Dictyostelium belongs to the PLC8 family which is partially activated by Ca2+ in mammalian cells (Singer et al., 1997). Although the Ca2+ influx is independent of G-protein activity (Milne and Coukell, 1991), the IP3 rise (and the cAMP and cGMP rises) are mediated by G-proteins and is prevented when Go2 or Gbeta are ablated. Several studies have indicated a potential role for Ca2+ in stalk cell differentiation. Ca2+ levels appear to rise preferentially in the prestalk cell area of the mound and slug (Cubitt et al., 1995; Saran et al., 1994).

1.5.5.4 Adaptation of the cAMP Response

Chemotaxis of cells following a pulse of cAMP is not continuous. The cells migrate towards the aggregation centre for only one or two minutes immediately following the cAMP pulse and move
randomly or remain static for the remaining five or six minutes before the next pulse. This results in the visible waves of movement seen during aggregation. This short period of movement is due to adaptation of the cAMP signalling pathway to the cAMP signal. In this period no further cAMP is secreted from the adapted cells and the extracellular concentration of cAMP is lowered by the action of a secreted phosphodiesterase. The phosphodiesterase is an essential component of the aggregation system since it allows the cAMP concentration to drop to the point where the cells can respond to the next pulse. Loss of the phosphodiesterase prevents aggregation (Sucgang et al., 1997). A prolonged stimulation of cells with cAMP results in a reduction of both adenylate cyclase and guanylate cyclase activities. The kinetics of adenylate cyclase adaptation parallel the increase in phosphorylation of the intracellular loop of the cAR1 receptor. This correlation suggested that phosphorylation may be the mechanism of adaptation, however mutation of the phosphorylated residues to ones that cannot be phosphorylated has no effect on the adaptation response (Kim et al., 1997). Adaptation may therefore not occur by modification of cAR1 but by modulating the activity of downstream signalling components such as CRAC. Indeed, membranes from adapted cells show a reduced ability to bind CRAC (Lilly and Devreotes, 1995).

The cAR1 and cAR3 receptors have an higher affinity for cAMP than cAR2 (Johnson et al., 1992). In the developing mound the levels of cAMP are higher than the nanomolar concentrations that direct aggregation with the consequence that the cAR1 and cAR3 receptors will be constantly in an adapted state. The lower affinity of the cAR2 receptor may allow the chemotaxis of pstA cells to the anterior tip to proceed despite the adaptation of the other receptors.

1.5.6 Differentiation

1.5.6.1 Pattern Formation and Cell Fate Specification

The *Dictyostelium* fruiting body consists of approximately 20% stalk cells and 80% spores. The cells that aggregate into the mound are essentially identical. However, within 12-14 hours these identical cells have differentiated into several different cell types that will eventually become mature spores and stalk cells. The choice of cell type for the initially, identical cells may be governed by the stage of the cell cycle at the time of differentiation. The *Dictyostelium* cell cycle appears to have little or no G₁-phase (Weijer et al., 1984a). Cells in S-phase or early in G₂-phase at
the time of differentiation have a greater tendency to adopt a prestalk fate, whereas cells late in G2-phase will tend to adopt a prespore fate (Gomer and Firtel, 1987; Weijer et al., 1984b).

Prespore cells, and prestalk cells, show a scattered distribution in the early aggregate. Cell sorting begins when a concentration gradient of cAMP develops in the mound with the greatest concentration at the top. A population of prestalk cells then moves to the top of the aggregate to form a tip, due to their increased chemotactic motility to cAMP (Early et al., 1995; Williams et al., 1989). These prestalk cells migrate towards the tip preferentially, ahead of the prespore cells, with resultant sorting of the two cell types (Williams et al., 1989). If the gradient of cAMP is reversed, by soaking the substratum in high concentrations of cAMP, the prestalk cells migrate to the base of the slug rather than to the tip (Traynor et al., 1992). cAMP is released from the tip of the mound in waves which result in concentric circular and spiral waves of cell movement that can be seen in the developing mound by use of dark-field microscopy (Siegert and Weijer, 1995). The tip secretes cAMP throughout development and its importance as an organising centre for morphogenesis is clear. For example, tips from slugs and fruiting bodies can direct the movement of amoebae or disaggregated slug cells (Rubin, 1976; Rubin and Robertson, 1975; Sternfeld and David, 1981). The tip of the aggregate elongates forming a standing slug, which falls over and migrates under conditions of low light, high humidity and high ionic strength. The prestalk cells from the tip of the aggregate form the anterior one fifth of the slug, which stain with vital dyes such as neutral red (Sternfeld and David, 1982), while most of the posterior four fifths of cells are prespore. The tip of the slug is proposed to emit a signal which inhibits the formation of secondary tips (Durston, 1976; Sternfeld and David, 1981). At the slug stage differentiation is regulative and a cell's fate is reversible (Abe et al., 1994; Harwood et al., 1991; Rubin and Robertson, 1975; Sternfeld and David, 1981; Sternfeld and David, 1982). Around 10% of scattered cells in the prespore region of the slug have some prestalk cell characteristics. These are called the Anterior-Like Cells (ALCs) (Sternfeld and David, 1981; Sternfeld and David, 1982). If the tip of a slug is removed, these ALCs move forward to form a new tip (Sternfeld and David, 1981).

The prestalk cells of the slug are heterogeneous, as shown by analysis of the regulated expression of two prestalk-specific genes, \textit{ecmA} and \textit{ecmB}. By transforming \textit{Dictyostelium} with reporter genes driven by promoter sequences from these genes, a variety of distinct prestalk cell populations was discovered (Jermyn et al., 1989; Williams et al., 1989). EcmA is an extracellular...
matrix protein which is secreted into the slime sheath of the slug and the stalk tube of the culminant (McRobbie et al., 1988). The \textit{ecmA} gene is expressed by scattered cells in early aggregates but later the \textit{ecmA}-expressing cells move to the apex of the mound to form a tip. \textit{ecmA} is highly enriched in the anterior 10\% of slug cells which are called the prestalk-A cells (Early et al., 1995; Early et al., 1993; Jermyn et al., 1989; Jermyn and Williams, 1991; Williams et al., 1987). The rear half of the prestalk zone of the slug contains cells that express \textit{ecmA}, but at a much lower level than the extreme tip, driven by a distinct part of the \textit{ecmA} promoter (Early et al., 1995; Early et al., 1993; Jermyn et al., 1989; Jermyn and Williams, 1991). These cells have been named prestalk-O cells. A further subset of prestalk cells express a matrix protein highly related to \textit{ecmA}, called \textit{ecmB} (Gaskell et al., 1992; Jermyn et al., 1989). The \textit{ecmB}-expressing cells, termed prestalk-B cells, arise in a scattered fashion in the early aggregate but become concentrated at the base of the mound as the tip forms (Williams et al., 1989). In the slug there is a band of prestalk-B cells situated in the front half of the prespore zone tightly opposed to the substratum (Jermyn et al., 1996). A second population of \textit{ecmB}-expressing cells, known as prestalk-AB cells, arise later in development. These cells form a cone-shaped stalk tube primordium within the prestalk-A/O region of the slug, and initiate stalk tube formation during fruiting body formation (Ceccarelli et al., 1991; Gaskell et al., 1992; Jermyn et al., 1989; Jermyn and Williams, 1991; Sternfeld, 1992). ALCs are heterogeneous with respect to \textit{ecmA} and \textit{ecmB} expression, expressing \textit{ecmA} and/or \textit{ecmB} to different degrees (Gaskell et al., 1992). Interestingly, all reporter constructs that are expressed in prestalk-O cells are also expressed in ALCs (Early et al., 1993). ALCs have been implicated in the maintenance of cell-type proportioning due to their ability to transdifferentiate between prestalk and prespore cells (Abe et al., 1994). The extreme posterior of the slug is enriched in ALCs which are periodically lost during slug migration. This group of cells is termed the “rearguard”.

At culmination, the slug stands on end and prestalk cells in the tip migrate down through the developing prespore mass whilst secreting cellulose in a process similar to gastrulation of embryos. The prestalk-AB cells, which form the stalk tube primordium, migrate first, followed by the prestalk-A cells and some of prestalk-O cells (Early et al., 1993; Jermyn and Williams, 1991; Sternfeld, 1992). Entry of these cells into the developing stalk tube results in the induction of \textit{ecmB} expression. In this manner the cellulose-encased stalk elongates, lifting the spore mass away from the substratum. Two populations of ALCs migrate in opposite directions through the spore mass to
Figure 1.2  Cell-type differentiation and patterning during Dictyostelium development
A) The Tipped Mound.
Early aggregates consist of 80% scattered prespore cells and 20% prestalk cells. The prestalk-A
cells, defined by high levels of ecmA mRNA expression, migrate to the top of the aggregate to form a
tip whereas the ecmB expressing prestalk-B cells migrate to the bottom of the aggregate.
B) The Slug.
The anterior one fifth (tip) of the slug consists of prestalk-A cells and prestalk-O cells. Prestalk- O
cells express lower levels of ecmA than the prestalk-A cells in the extreme tip. In addition a cone of
cells is present which expresses ecmA and ecmB. These prestalk-AB cells initiate stalk tube
formation at culmination. The posterior four fifths of the slug contain prespore cells, prestalk-B cells
and a population of anterior-like cells (ALCs). The prestalk-B cells are tightly apposed to the
substratum within the anterior portion of the prespore zone. The prestalk-like ALCs express ecmA
and/or ecmB and are scattered throughout the prespore region. A population of ALCs at the rear of
the slug are called the "Rearguard" and are shed during migration.
C) The Fruiting body
At culmination, prestalk-A cells, and later some prestalk-O cells, move from the tip down into the
stalk tube and induce stalk-specific ecmB expression. ALCs move to surround the developing spore
mass, forming the upper and lower cups and part of the basal disc which anchors the culminant to the
substratum. The prestalk-B cells contribute to the basal disc and the lower cup whereas, the
remaining prestalk-O cells contribute to the tip and upper cup. The origin of the cells in each part of
the fruting body and the regions expressing ecmA and ecmB are indicated.
form the basal disc (which anchors the fruiting body to the substratum) and the upper and lower cups, which cradle the top and bottom of the spore mass (Dormann et al., 1996; Jermyn et al., 1996; Jermyn and Williams, 1991; Sternfeld and David, 1982). The prestalk-B cells and rearguard cells also form part of the basal disc and lower cup (Jermyn et al., 1996) whilst some prestalk-O cells also become part of the upper cup (Early et al., 1993). Once cells enter the stalk tube, they begin to terminally differentiate into vacuolated stalk cells by a process resembling apoptosis (Dormann et al., 1998) and the surrounding prespore cells differentiate into mature spores. A schematic illustration of cell type patterning and gene expression during development is shown in Figure 1.2.

1.5.6.2 Developmental Signalling Pathways

The rise in cAMP level that occurs during mound formation produces a switch in gene expression. Aggregation specific genes such as cAR1 and Go2 that are induced by starvation factors and by pulses of cAMP are downregulated whilst post-aggregative and eventually cell-type specific gene expression is upregulated. One of the first genes to be expressed in response to the rising cAMP levels is the transcription factor G-box factor (GBF), named because of its ability to bind to G-box regulatory sequences within the promoters of some post-aggregative and cell-type specific genes (Schnitzler et al., 1994). Disruption of the gbf gene does not prevent mound formation but does prevent further development and post-aggregative gene expression is lost. gbf mounds then disaggregate, thereby reducing the levels of cAMP and presumably allowing the adapted cAR1 and cAR3 signalling pathways to function once again. As a consequence aggregation reoccurs. The cycle of aggregation and disaggregation then continues until the cells eventually die (Schnitzler et al., 1994). Expression of GBF alone is not sufficient to induce the post-aggregative gene expression; cAMP is also required.

The serine/threonine kinase, Protein kinase A (PKA), is a crucial component of the signalling pathways that control multiple aspects of development. In Dictyostelium, PKA is a heterodimer composed of a single catalytic subunit associated with a single regulatory subunit (De Gunzburg et al., 1984). PKA is activated by intracellular cAMP, which binds to the regulatory subunit and causes it to dissociate from the catalytic subunit (De Gunzburg and Veron, 1982). The catalytic subunit now translocates to the nucleus where in mammalian cells it can phosphorylation transcription factors. The downstream targets of PKA in Dictyostelium have not yet been identified.
and no homologues of the most significant mammalian targets have been identified. Disruption of the gene encoding the PKA catalytic subunit demonstrates that it is required early in development for the relay of cAMP during aggregation (Mann et al., 1992). It does not function directly in the relay process but is required for expression of the adenylate cyclase, ACA (Mann et al., 1997). Cells expressing dominant inhibitory forms of the regulatory subunit also fail to aggregate (Schulkes and Schaap, 1995) and when mixed with wild-type cells fail to induce post-aggregative gene expression suggesting that PKA lies upstream of GBF (Harwood et al., 1992). PKA acts as an inhibitor of stalk cell differentiation and as an activator of spore cell differentiation. Overexpression of the catalytic subunit in prespore cells results in a dramatic increase in spore number with spores being formed precociously in development (Hopper et al., 1993). The use of a prestalk cell promoter to express the catalytic subunit blocks development at the mound stage. The prestalk cells still migrate to the top of the mound in these cell lines but no tip is formed (Hopper et al., 1993). Loss of the regulatory subunit has a similar effect as overexpression of the catalytic subunit and causes precocious spore production (Simon et al., 1992). In fact, the mutant will differentiate into spores in low density monolayer culture in response to cAMP, in contrast to wild-type cells which require the cell-permeant PKA agonist 8-Br-cAMP for induction of spore differentiation (Simon et al., 1992).

Mutations in an intracellular histidine kinase, RdeA, or a phosphodiesterase, RegA, cause accelerated terminal differentiation due to permanently elevated levels of intracellular cAMP (Chang et al., 1998; Thomason et al., 1998). When activated, RdeA phosphorylates, and is likely to activate, RegA (Thomason et al., 1999). Thus activation of RdeA would be expected to inhibit culmination and terminal differentiation.

A second protein involved in the promotion of spore cell differentiation is glycogen synthase kinase-3 (GSK-3). In D. melanogaster and Xenopus GSK-3 regulates the choice of cells between two developmental fates during embryogenesis and plays a similar role in the choice between the spore and stalk fate in Dictyostelium. A gskA null cell line develops with a dramatically increased number of stalk cells and a concomitant decrease in spore number (Harwood et al., 1995). GSK-3 represses prestalk cell specific gene expression in response to extracellular cAMP and appears to operate downstream of the cAR3 receptor (Plyte et al., 1999).
Late in the morphogenetic process when PKA appears to be acting to promote spore cell differentiation a cell permeant chlorinated alkyl phenone molecule known as Differentiation Inducing Factor (DIF) acts as an antagonist of PKA and promotes stalk cell differentiation (Berks and Kay, 1990). DIF synthesis can be stimulated by extracellular cAMP and DIF is present in aggregates and slugs but not vegetative cells (Brookman et al., 1982). Levels of DIF are controlled by the enzyme DIFase, synthesis of which is induced by DIF (Insall et al., 1992). DIF induces the transcription of prestalk-specific (Berks and Kay, 1990; Jermyn et al., 1987; Williams et al., 1987) and inhibits prespore-specific gene expression (Berks and Kay, 1990; Early and Williams, 1988). The mechanism of action of DIF is not clear but one theory suggests that it may function as a mitochondrial uncoupler in stalk cells (Shaulsky and Loomis, 1995). Uncoupling would lead to a rise in intracellular Ca\(^{2+}\) levels (since the active processes that maintain the usual low concentrations would run out of an ATP) and presumably apoptosis. Prestalk cells have been demonstrated to have higher intracellular Ca\(^{2+}\) levels than prespore cells and a rise in Ca\(^{2+}\) has also been shown to promote stalk cell differentiation (Cubitt et al., 1995; Kubohara and Okamoto, 1994). In support of this theory, DIF will raise Ca\(^{2+}\) levels and induce apoptosis in mammalian tissue culture cells (Kubohara et al., 1995b; Kubohara et al., 1995a). However, the high concentrations of DIF required to elicit this response in mammalian cells brings into question the physiological significance of the data.

A second more recently identified promoter of stalk cell differentiation is a STAT (Signal Transducer and Activator of Transcription) (Kawata et al., 1997). STATs are SH2 domain containing proteins first identified as components of interferon signalling pathways (Liu et al., 1998). The *Dictyostelium* STAT appears to be regulated by tyrosine phosphorylation in the same manner as mammalian STATs and can be seen to translocate to the nucleus in prestalk cells during the mound stage, at the time of initial differentiation into pre-stalk and pre-spore cells (Araki et al., 1998). *STATa* null cells show little or no terminal stalk cell differentiation. Paradoxically, *STATa* appears to be the *in vivo* repressor of *ecmB*, since *STATa* mutants express *ecmB* throughout the tip of the slug instead of being restricted to the normal small core (Mohanty et al., 1999).

Ammonia produced by the catabolism of cellular proteins also inhibits stalk cell differentiation and so blocks the culmination of slugs (Gross et al., 1983). Ammonia has been demonstrated to antagonise the effects of DIF and inhibit culmination; this may be due to its elevating the intracellular pH of prestalk cells, as stalk cell maturation can be induced by lowering the
intracellular pH (Gross et al., 1983; Inouye, 1988; Schindler and Sussmann, 1977). Ammonia also antagonises intracellular cAMP synthesis and could consequently affect PKA activity (Schindler and Sussman, 1979).

1.5.7 Phototaxis and Thermotaxis

*Dictyostelium discoideum* cells form migrating slugs after aggregation presumably to seek out an optimal environment for the fruiting body. This is achieved by phototaxis and thermotaxis, in which the slugs move with great sensitivity towards sources of light and heat. When provided with a lateral light source of as little as $10^4$ lux white light the slug migrates toward the source of light (Francis, 1964; Poff and Butler, 1974). The phototaxis is not direct but at an angle to the light source. Slugs exhibit bi-directional phototaxis, either turning away from or toward the light in order to maintain this preferred angle of migration (Fisher and Williams, 1981). In wild-type slugs the two angles either side of the source are normally so close to the direct route to the source that the bi-directional movement cannot be distinguished form simple unidirectional tactic movement. In phototaxis mutants the bi-directional nature of phototaxis becomes clear (Fisher and Williams, 1981). Mutants that orient poorly to the light source migrate at large angles either side of the source and exhibit obvious wide turns as they try to orient correctly. At temperatures around which the amoebae were grown, the slugs migrate towards warmth (Bonner et al., 1950). The slugs are exquisitely sensitive to shallow temperature gradients, being able to respond to as little as $0.04^\circ$C/cm (Poff and Skokut, 1977). The accuracy of orientation in this response declines at higher and lower temperatures until a point is reached when the slugs switch to negative thermotaxis. The transition temperatures seem to be under the control of the same signalling pathway as those employed for phototaxis, since mutants exhibiting more extreme bi-directional phototaxis always have altered transition temperatures in phototaxis (Fisher and Williams, 1982).

The slug responds to vertical beams of light only if they are directed at the slug tip (Francis, 1964; Poff and Loomis, 1973). This strongly suggests that the slug tip is the organising centre for tactic movements. Further evidence for this is supplied by tip transplant experiments between slugs of wild-type and phototaxis mutants which showed that phototaxis was normal only with wild-type tips and was defective when the tips were from a phototaxis mutants (Poff and Loomis, 1973; Raper, 1940). It is thought that light or temperature induce lateral differences in signalling across
the tip resulting in a change of tip position and thus direction of slug movement (Fisher et al., 1984). Two mechanisms for this change of tip position have been proposed. The first involves cell speed differentials on either side of the slug tip resulting in a turn. The other mechanism requires a wholesale rearrangement of cells in the tip such that it points in a different direction. This second mechanism is supported by analysis of the motility of individual cells in 2D slugs (Bonner, 1998). In these slugs there appears to be no speed differentials in the tip cells during slug turning, instead there is wholesale rearrangement of cells in the tip. However, these observations are of spontaneous slug turning and may not represent the mechanism of tactic slug movement.

Two candidate diffusible factors have been implicated in control of slug tactic movement. NH$_3$ is produced constitutively by the slug as a by-product of catabolism (Gregg et al., 1954). It has been shown to be a slug repellent when applied exogenously (Feit and Sollitto, 1987; Kosugi and Inouye, 1989). Cell speed has been shown to increase in response to NH$_3$ (Bonner et al., 1988; Bonner et al., 1989; Bonner et al., 1986). Scroll waves of cAMP control cell movement in the tip and NH$_3$ antagonises cAMP signalling (Siegert and Weijer, 1989). Taken together these data make a strong case for the involvement of NH$_3$ in tactic movement and also support the differential speed hypothesis of slug turning. However, an average slug secretes 2-20fmol NH$_3$ per second (Bonner et al., 1988). Even if all of this NH$_3$ were produced on one side of the slug tip the secretion rate would only maintain a steady-state gradient across the tip that is 100-fold shallower than the minimum slugs can detect (Kosugi and Inouye, 1989). This is due to the rapid diffusion rate of NH$_3$ – it takes only 15 milliseconds to diffuse 100µm (Fisher, 1991). It seems likely that lateral NH$_3$ gradients leading to slug turning are only significant when diffusion of the gas is restricted by the close proximity of a barrier. The second candidate molecule is Slug Turning Factor (STF). STF is a small (<500Da) non-volatile, molecule able to repel slugs, impair slug tactic movements and whose secretion by the slug is stimulated by light (Fisher et al., 1981). It has been demonstrated that disorientation of slugs at high cell densities is entirely due to non-volatile, diffusible molecules with no contribution from NH$_3$ (Fisher, 1991). Aside from the debate about the diffusible molecules involved, the signalling pathways involved in slug tactic behaviour are as yet unidentified. Several reports, based on pharmacological data, have implicated cAMP (Darcy and Fisher, 1990), cGMP (Darcy et al., 1994), Ca$^{2+}$ (Dohrmann et al., 1984), IP$_3$ and G-proteins (Darcy and Fisher, 1989) but, as yet, no coherent pattern has emerged.
Many photo- and thermotaxis mutants exist, but these were generated by chemical mutagenesis and at present the genes involved have not been identified (Darcy et al., 1994). However, many of these mutants show defects in cGMP signalling. Recently, three genes required for tactic responses have been discovered by targeted gene disruption. These are the genes for, the actin binding proteins ABP120 (Fisher et al., 1997; Wallraff and Wallraff, 1997) and GRP125 (Stocker et al., 1999) and for the mitochondrial large subunit rRNA (McMahon et al., 1996). The exact role for these molecules in slug tactic movement is far from clear.

1.5.8 Endocytosis

Phagocytosis in *Dictyostelium*, as in other species, is driven by the actin cytoskeleton, as demonstrated by several experimental approaches. Phagocytosis is sensitive to cytochalasin-A, an inhibitor of actin polymerisation (Temesvari et al., 1996). Phagocytic cups become coated by F-actin and associated proteins as they form (Maniak et al., 1995). Genetic ablation of some of these actin-associated proteins, notably talin, myosin-IB and coronin, strongly impairs phagocytosis (Jung and Hammer III, 1990; Maniak et al., 1995; Niewohner et al., 1997). Talin is believed to couple the cytoskeleton to the membrane of the phagocytic cup. Members of the myosin-I family are likely to contribute the dynamic properties of the actin cytoskeleton during particle uptake. The accumulation of F-actin and its associated proteins at the sites of phagocytosis occurs after particle attachment. These molecules drive the formation of a cup-like vessel around the external particle, which eventually closes, withdrawing the particle into the cell. As the mature phagosome is encapsulated, F-actin and coronin dissociate (Maniak et al., 1995). At all stages of particle uptake, the process appears to be reversible. Redistribution of actin and its associated proteins to other active areas of the cell cortex can result in the cessation of cup progression and complete dissociation of the external particle. One candidate molecule required for the perception of external particles by a *Dictyostelium* cell is a heterotrimeric G-protein. Cells lacking Gβ, and consequently all heterotrimeric G-protein function, are impaired in phagocytosis to a similar extent to coronin-null cells implying a role for a whole receptor/G-protein pathway in the regulation of phagocytosis (Peracino et al., 1998). This proposed role for the heterotrimeric G-proteins in particle uptake is supported by the finding that Gβγ-subunits are localised to the phagosomes of macrophages (Desjardins et al., 1994).
Fluid phase endocytosis in axenic *Dictyostelium* cells is thought to occur primarily by macropinocytosis (Hacker et al., 1997). This is a process which strongly resembles phagocytosis, both morphologically and in its dependence on the actin cytoskeleton (Hacker et al., 1997). The sites of macropinocytosis in *Dictyostelium* are crowns, which are circular ruffles of the plasma membrane of a similar size to the phagocytic cups which engulf bacteria. Crowns are cup-like structures which envelop and internalise aliquots of extracellular fluid. The time course of crown extension and retraction is very similar to that of phagocytic cups and macropinocytosis has a similar cytochalasin-A sensitivity to phagocytosis (Hacker et al., 1997). As with phagocytic cups, F-actin and its associated proteins coat the cytoplasmic face of crowns as they internalise their contents. The myosin I double null and coronin null cells are defective in macropinocytosis to the same extent as they are in phagocytosis (Hacker et al., 1997; Novak et al., 1995). The *Dictyostelium* class I PI 3-kinase molecules have been implicated in the regulation of macropinocytosis. *Dictyostelium* cells lacking the two class I PI 3-kinase genes have a defect in fluid uptake, in addition to other problems in actin-dependent processes. The PI 3-kinase-null cells may also have a defect in phagocytosis, however the two groups which independently performed this work gave conflicting reports of the phagocytic activity of these mutants (Buczynski et al., 1997; Zhou et al., 1998).

Recently, roles for the *Dictyostelium* Rap1 homologue and for a novel Rho subfamily GTPase, RacC, in endocytosis have been identified (Seastone et al., 1998; Seastone et al., 1999). Overexpression of the wild-type RacC protein caused an increase in the rate of phagocytosis and a concomitant decrease in the rate of fluid-phase endocytosis. Expression of an inhibitory RacC mutant protein had the opposite effect, increasing the rate of fluid-phase endocytosis at the expense of phagocytosis. Overexpression the wild-type and dominant active Rap1 protein had the same effect as wild-type RasC whereas a dominant negative Rap1 protein had no obvious effect on endocytosis. The effects of Rap1 required phospholipase C activity whereas the RacC-mediated effects required activation of diacylglycerol-binding proteins.
1.6 Ras Signalling in Dictyostelium.

1.6.1 Dictyostelium ras Genes

Dictyostelium expresses at least six ras subfamily genes at different times during through the life cycle: rasB, rasC, rasD, rasG, rasS and rapl. rasD was the first ras gene identified and the initial characterisation of its expression profile indicated that the gene was expressed during vegetative growth, was downregulated as the cells experienced starvation and finally upregulated during the multicellular stage of development (Reymond et al., 1984). However, this expression profile was demonstrated to be incorrect several years later. rasD expression is restricted to the multicellular stage of development being first detectable around the tipped mound stage and maximal during the slug stage (Robbins et al., 1989). Although rasD expression initially occurs in all cell types, it eventually becomes enriched in prestalk cells (Jermyn and Wiliams, 1995). The original studies of rasD expression presumably failed to distinguish rasD mRNA from that of rasG, which is expressed maximally during vegetative growth and is undetectable by the onset of aggregation (Robbins et al., 1989). rasB, rasC, rasS and rapl are expressed throughout the life cycle of Dictyostelium (Daniel et al., 1994; Daniel et al., 1993; Robbins et al., 1990).

RasD and RasG are most similar to the prototypic human H-Ras, 62% and 69% identical at the amino acid level respectively, and both have only 6 conservative changes in the first 80 residues relative to H-Ras - the region thought to contain all residues important in effector interaction. RasD and RasG are 82% identical with only 2 conservative changes in first 80 residues. RasB, RasC and RasS are more distantly related to the prototypic Ras with the predicted protein sequences of these molecules being respectively, 52%, 56% and 54% identical to H-Ras. Interestingly, the effector region of human Ha-Ras (amino acids 32-40) is identical in all Dictyostelium Ras proteins except RasS and RasC. Both contain a single effector domain substitution, D38N in RasC and the conservative substitution, I36L in RasS. The significance of this is unclear but may confer highly specialised functions to these proteins mediated by interaction with effectors distinct from those of the other Ras proteins. The Dictyostelium Rap1 homologue shares 87% amino acid identity with the human Rap1 protein. Figure 1.1 shows an alignment between the five Dictyostelium Ras proteins and human H-Ras.
1.6.2 Functional Analysis of the *Dictyostelium ras* Genes

At present, only *rasD*, *rasG*, *rasS* and *rapl* have been functionally analysed.

1.6.2.1 *rasD*

Although RasD is the most extensively researched *Dictyostelium* Ras protein, its biological function has remained unclear. *Dictyostelium* cells overexpressing an activated RasD protein (G12T), although unable to form normal aggregation streams, are able to aggregate upon starvation but arrest development after forming multi-tipped mounds (Louis et al., 1997; Reymond et al., 1986). These structures express enhanced levels of the prestalk cell specific genes, *ecmA* and *tagB*, and very low levels of the prespore cell specific gene, *cotC*, relative to wild-type aggregates but contain no mature spore or stalk cells. The mutant cells are able to differentiate into stalk and spore cells in low density monolayers although severely impaired relative to wild-type, suggesting that inhibition of mature stalk and spore formation is at least in part due to cell-cell interactions in the aggregates. When mixtures of mutant and wild-type cells are allowed to develop, mutant cells appear at the periphery of the mounds and are excluded from all subsequent developmental structures. Chemotaxis is also slightly impaired in the mutant cells explaining the lack of normal aggregation streams. This may be due to a downregulation of the number of cAMP receptors (Luderus et al., 1992; Luderus et al., 1988), in addition to an increased desensitisation of guanylate cyclase in response to cAMP pulses (Van Haastert et al., 1987). Abnormal changes in phosphatidylinositol levels have also been observed (Europe-Finner et al., 1988; Van der Kaay et al., 1990). Experiments to ablate *rasD* using an antisense cDNA driven by a *rasD* promoter fragment produced no transformants (Reymond et al., 1985). As a result of these data, it was suggested that RasD controlled cell fate specification and was essential for growth and development (Esch and Firtel, 1991).

The generation and characterisation of a *rasD* cell line is described in Chapter 5 of this thesis.

1.6.2.2 *rasG*

*Dictyostelium* cells with a disrupted *rasG* gene have also been generated in our laboratory. The mutant cells have several defects in actin-dependent processes, notably cytokinesis and cell movement (Tuxworth et al., 1997). Cell motility is impaired in the *rasG* cells, with the cells
migrating at approximately half the rate of the parental strain. The cells also have a noticeable loss of polarity, appearing flat and round when viewed by phase contrast microscopy. In the rasG cells, cytokinesis begins normally with the formation of a cleavage furrow but the daughter cells ultimately fail to separate, remaining attached via a thin cytoplasmic bridge. The cells eventually divide by an alternative process, termed traction mediated cytofission, where the cells pull themselves apart using traction generated against the substrate (Fukui et al., 1990). Since traction-mediated cytofission is impossible in suspension culture the rasG cells, like several other mutants with defective actin function, cannot divide under these conditions becoming large and multinucleate. Contrary to predictions, RasG does not appear to be required for cell proliferation since the doubling time of cells in tissue culture dishes is normal.

Low level expression of a RasG protein containing the activating mutation, G12T, from the rasG promoter prevents the formation of aggregation streams but does not impair subsequent development of Dictyostelium cells (Thiery et al., 1992), whereas high level expression from the dis1γ promoter inhibits both aggregation and multicellular development (Khosla et al., 1996). These effects appear to be due to a defect in the cAMP relay such that cells are unable to respond to the cAMP pulses required for the initiation of development. The overexpression of wild-type RasG or a dominant negative S17N RasG protein has no apparent effect on these processes (Khosla et al., 1996).

1.6.2.3 rasS

Dictyostelium cells with a disrupted rasS gene have been independently generated, in our laboratory, by J Chubb and me. Disruption of the rasS gene has multiple effects. Mutant amoebae are unable to proliferate in liquid culture medium and this correlates with impaired phagocytosis and fluid-phase endocytosis. Vegetative amoebae are highly polarised, with prominent pseudopods and elongated microspikes and migrate three times faster than wild-type cells. 3D analysis of the movement of the mutant cells indicates their rapid movement is due to differences in pseudopodium behaviour; there are fewer pseudopods per cell, but they have an increased volume and turn over more rapidly. Although the early starvation responses of the rasS cells are apparently normal, the cells display aberrant multicellular development forming very small aggregation territories and
small mounds which are delayed in tip formation. They are, however, able to form morphologically
normal fruiting bodies.

1.6.2.4 rap1

Overexpression of wild-type Rap1 protein in Dictyostelium cells results in a flattened
morphology and an increase in peripheral F-actin (Rebstein et al., 1993). This behaviour is similar
to the flattening of fibroblasts overexpressing the mammalian Rap1 protein. Overexpression of the
activated G12V Rap1 protein produced similar results to that of wild-type Rap1 overexpression
whereas an S17N dominant inhibitory Rap1 mutant resulted in an increase in cell polarity (Rebstein
et al., 1997). Recently, overexpression of the wild-type or activated G12V Rap1 proteins has been
shown to increase the rate of phagocytosis and impair fluid-phase endocytosis in phospholipase C
dependent manner (Seastone et al., 1999). Overexpression of wild-type Rap1 can also modify the
developmental phenotype induced by an activated RasD protein (Louis et al., 1997). This is
reminiscent of studies in mammalian systems, where Rap1 can antagonise the transforming activity
of activated Ras proteins (Kitayama et al., 1989) and argues for a conservation of Rap1 function.
Our lab has repeatedly attempted to disrupt the Dictyostelium rap1 gene, without success,
suggesting it may be essential (R.Insall and J.Chubb personal communication).

1.6.3 Ras Signalling Pathways

Only a small number of putative Ras effectors and signalling pathway components have been
identified in Dictyostelium. For the most part, direct biochemical interaction of theses molecules
with Ras signalling pathway components has not been demonstrated and their presumed role in Ras
signalling has been inferred from sequence and, in some instances, genetic interaction. At present
no tyrosine kinase receptors have been identified in Dictyostelium and all transmembrane receptor-
mediated signalling so far characterised is via G-protein couple serpentine receptors.

The Aimless protein is a putative RasGEF, containing a domain with a high level of homology
to the S.cerevisiae Cdc25 catalytic domain (Insall et al., 1996). The aleA gene was identified in a
screen for aggregation defective mutants of Dictyostelium. aleA null cells are impaired in cAMP
induced activation of adenylate cyclase and perform chemotaxis very weakly to cAMP. To date, no
GEF activity has been demonstrated towards any of *Dictyostelium* Ras protein by the Aimless protein. No other *Dictyostelium* Ras GEFs have yet been published.

The ubiquitous MAP kinase pathway seems to be present in *Dictyostelium* although not all the components have been identified. Homologues of the mammalian MAP kinases ERK1 and ERK2 are present (Gaskins et al., 1994; Segall et al., 1995). Antisense mutagenesis from a derepressible promoter indicates that ERK1 may essential for vegetative growth. Overexpression of ERK1 results in abnormal morphogenesis starting at the slug stage (Gaskins et al., 1994). *erk2* null cells have a phenotype similar to the *aleA* null mutant exhibiting defective chemotaxis to folate and cAMP (Gaskins et al., 1996; Segall et al., 1995). Although required for activation of adenyl cyclase, ERK2 is not essential for aggregation-stage, cAMP pulse-induced gene expression, or for the expression of postaggregative genes, which are induced at the onset of mound formation in response to cAMP in wild-type cells. Experiments using a temperature-sensitive ERK2 mutant have shown that ERK2 is essential for proper morphogenesis and for the induction and maintenance of prespore but not prestalk gene expression (Gaskins et al., 1996). The mutant cells also exhibit an inability to repolarise when exposed to high concentrations of the chemoattractant, suggesting an impairment in the adaptation response to high chemoattractant concentrations (Wang et al., 1998).

There is evidence that RasD and/or RasG are involved in modulating the activity of ERK2 downstream of chemoattractant receptors. Exposure of cells to the growth phase chemoattractant, folate, or to cAMP leads to a rapid increase in the activity of the *Dictyostelium* ERK2 homologue. Phosphorylation of ERK2 occurs within 10 seconds if exposure to the chemoattractant and remains so for 1-2 minutes (Aubry et al., 1997; Kosaka et al., 1998; Maeda and Firtel, 1997). Cells lacking RasG or overexpressing a dominant negative RasD protein show an increased activation of ERK2 in response to chemoattractant whereas, cells expressing an activated RasD protein show a reduced and delayed activation of ERK2. This suggests that in contrast to mammalian systems Ras is a negative regulator of MAP kinase. In support of this, *aleA* null cells which lack a putative RasGEF also show a potentiation of ERK2 activation when exposed to cAMP (Aubry et al., 1997). Kinases corresponding to the MEK and Raf levels of the MAP kinase cascade have also been identified. Cells lacking the *Dictyostelium* MEK homologue, MEK1, also have defects in chemotaxis, characterised by the formation of small multicellular aggregates (Ma et al., 1997). Cells are able to produce cAMP waves that move through the aggregation domains. However, these cells are unable to
to activate guanylate cyclase, a known regulator of chemotaxis in *Dictyostelium*. Analysis of a temperature-sensitive MEK1 mutant suggests that MEK1 activity is required throughout aggregation at the time of guanylate cyclase activation, but is not essential for proper morphogenesis during the later multicellular stages. Significantly, the activation of the MAP kinase ERK2, which is essential for chemoattractant activation of adenylate cyclase, is not affected in mekl null strains, indicating that MEK1 does not regulate ERK2 and suggesting that at least two independent MAP kinase cascades control *Dictyostelium* aggregation. *Dictyostelium* cells lacking the MEK kinase homologue, MEKK\(\alpha\), aggregate normally but display abnormally fast development, completing fruiting body morphogenesis after 18h of starvation, rather than the 24h characteristic of wild-type cells (Chung et al., 1998). They also exhibit abnormal cell-type patterning with an increase in one of the prestalk compartments (pstO), a concomitant reduction in the prespore domain, and a loss of the sharp compartment boundaries, resulting in overlapping prestalk and prespore domains. Sequence analysis of MEKK\(\alpha\) does not reveal an obvious Ras binding domain as seen in mammalian Raf.

The Ras effector PI 3-kinase is also represented in *Dictyostelium*. Four *Dictyostelium* PI 3-kinase genes have been cloned (Zhou et al., 1995). The PIK1, PIK2 and PIK3 proteins resemble mammalian class-I PI 3-kinases, which have been shown to bind and be activated by Ras. Sequence homology suggests that all three have a binding site for Ras at their N-termini and recently the binding of the putative Ras binding site of PIK1 to activated mutants of RasD and RasG has been demonstrated in the yeast 2-hybrid system (Lee et al., 1999). However there is no obvious binding site for the adapter molecules that associate with class-I PI 3-kinases in other systems and mediate the interaction of PI 3-kinases with activated tyrosine kinase receptors. The PIK5 protein resembles the *S.cerevisiae* Vps34 protein, which is required for the trafficking of nascent proteins from the Golgi to the yeast vacuole. Null mutants of each of the three Class I PI 3-kinases have no perceptible phenotypes and attempts to disrupt either PIK1 or PIK2 together with PIK3 failed. However PIK1/PIK2 double null mutants have strong cytoskeletal defects (Buczynski et al., 1997; Zhou et al., 1998). They cannot grow in suspension culture, due to defects in both macropinocytosis and cytokinesis. The cells orient to chemoattractant faster than wild-type cells and exhibit an aberrant developmental morphology.
One likely target for lipid products of the PI 3-kinases is the Dictyostelium PKB homologue. The phenotypes of cells lacking PKB are distinct from those displayed by the PIK1/2 double mutant; PKB-null cells exhibit defective cell polarity and chemotaxis to cAMP (Meili et al., 1999). However, cAMP-induced stimulation of PKB kinase activity is strongly impaired in the PIK1/2 mutants. These data suggest that, as in mammalian cells, the Dictyostelium PI 3-kinases are involved in PKB activation.

A yeast two-hybrid screen using activated murine Ha-Ras identified two putative effectors for Dictyostelium Ras proteins (Lee et al., 1997; Lee et al., 1999). One of the proteins, RIP3, binds specifically to the activated RasG protein. RIP3 null cells exhibits a phenotype almost identical to that of the Erk2 and Aimless mutants suggesting all three molecules may be components of the same signalling pathway (Lee et al., 1999). The RIP3 protein has no obvious catalytic activity but contains a region of homology to a mammalian protein previously identified in a screen for suppressors of activated Ras in S. cerevisiae (Colicelli et al., 1991). The other protein, RasGAP1, is related to the mammalian IQGAP proteins, which are potential effectors of Rho subfamily GTPases (Faix and Dittrich, 1996; Lee et al., 1997). IQGAPs bind activated Rac and CDC42 but not Ras or Rho proteins, can interact with F-actin and exhibit no GAP activity (Kuroda et al., 1996). Similarly, RasGAP1 binds activated Dictyostelium Rac1A without modulating its intrinsic GTPase activity. RasGAP1 binds to only Dictyostelium RasD and RasB in the yeast 2-hybrid system although this interaction takes place outwith the Rac-binding GAP homology domain (Lee et al., 1997; Lee et al., 1999). Two independently generated RasGAP1 null mutants exhibit different phenotypes. One mutant exhibits normal development but amoebae show increased cell motility and extensive perturbation of the actin cytoskeleton (Faix et al., 1998). The other mutant has a defect in cytokinesis with amoebae consequently become multinucleate when grown in suspension culture. Multicellular development is normal until the mid-slug stage, after which, cell type patterning becomes aberrant. No fruiting-body is formed and the final structure contains no stalk cells and very few spores (Lee et al., 1997).

These candidate Ras signalling pathway members give rise to phenotypes, when their function is removed, which differ from those of the single published Ras mutant. It is therefore likely that the generation of null mutants for the remaining Dictyostelium ras genes will reveal these and possibly other functions for Ras in Dictyostelium.
1.7 Aims of this Thesis

The aims of the work described in this thesis were two fold:

1) to identify a physiological function for the RasD protein

2) to identify and functionally analyse novel Dictyostelium RasGEFs in an attempt to determine factors controlling specific aspects of the multiple and distinct phenotypes of the \textit{rasS} and \textit{rasG} null mutants

A molecular genetic approach was taken in both cases. Gene disruption mutants were generated and the phenotypes of the recombinants examined to determine which cellular processes were affected.
2 Materials and Methods

Unless otherwise stated all chemicals were obtained from The Sigma Chemical Company, all restriction and DNA modifying enzymes (and appropriate buffers) were from New England Biolabs, all DNA oligonucleotides from were from MWG Biotech, all polypropylene and polycarbonate tubes and tissue culture plates were from Falcon and all microcentrifuge tubes were from Eppendorf. Recipes for all media and reagents used are listed in section 2.4.

The cell-type specific reporter constructs, act15-lacZ, ecmA0-lacZ, ecmO-lacZ, ecmB-lacZ and pspA-lacZ and the DNA probes for dis1γ, ecmA, ecmB, pspA, spiA and IG7 were a kind gift from Dr A Harwood. The ESTs from the Tsukuba Dictyostelium cDNA (Morio et al., 1998) project which were used as the basis for the gef gene disruption vectors were a gift from Dr Morio. Yeast two-hybrid vectors for human RasGRF and Ha-Ras and the temperature sensitive cdc25-5 S.cerevisiae mutant (including all vectors required for its complementation) of were a kind gift from Dr D Broek.

2.1 Molecular Biology

2.1.1 Bacterial Strains and Growth

The E.coli strain, XL1-blue (Stratagene) was used for all subcloning and preparation of plasmid DNA and for propagation of the λZAPII vector (Stratagene). The E.coli strain SOLR (Stratagene) was used during the excision of the pBluescript phagemid (Stratagene) from the λZAPII vector. The E.coli strain Y1090 was used as the host for propagation of the λgt11 vector.

Bacteria were grown in LB medium or on LB agar plates at 37°C unless otherwise stated. Selection was obtained, where appropriate, by addition of ampicillin (100µg/ml), tetracyclin (20µg/ml) or Kanamycin (50µg/ml).

2.1.2 Preparation of Competent Bacteria and Transformation

Subcloning grade competent cells were generated using the CaCl₂ method exactly as described in (Sambrook et al., 1989). Transformation was performed by addition of 10µl ligation reaction or 1µl supercoiled plasmid to 100µl chemically competent E.coli in a 1.5ml tube. The mixture was incubated on ice for 30min, heat shocked at 42°C for 1min then incubated on ice for a further 2min.
After addition of 1ml LB, the mixture was incubated for 1h at 37°C on a rotary shaker at 225rpm before plating on LB plates containing an appropriate-antibiotic.

Electro-competent cells were prepared exactly as described in (Harwood, 1996). To transform the cells, 50µl bacteria were added to a chilled 0.1cm electroporation cuvette (Flowgen) with 2µl of DNA in ddH2O. The mixture was agitated and left on ice for 1min before electroporating at 1.8kV, 25µF, 200Ω in a BioRad GenePulseII electroporator. 1ml LB was immediately added and the mixture incubated for 1h at 37°C before plating on LB plates containing an appropriate antibiotic.

2.1.3 Plasmid DNA Preparation

Small-scale preparation of plasmid DNA was carried out by boiling lysis. The bacteria from a 2ml overnight culture were pelleted and resuspended in 0.4 ml STET supplemented with 100µg/ml lysozyme. After a 5min incubation at 25°C, the mixtures were boiled for exactly one minute then centrifuged at 13,000rpm in a microcentrifuge (Eppendorf) for 10min. The fluffy precipitate was removed with a toothpick and 0.6ml isopropanol and 120µl 7.5M ammonium acetate added to the supernatant. After a further 10min centrifugation at 13,000rpm, the resultant DNA precipitate was washed in 70% ethanol then resuspended in 100µl TE.

Large-scale preparation of DNA for transformation of Dictyostelium cells, S.cerevisiae cells and for DNA sequencing was carried out using the Qiagen Maxi Kit (Qiagen) according to the manufacturer’s instructions. The DNA concentrations of the final preparations were estimated by measuring the absorbance at 260nm using a spectrophotometer (Amersham Pharmacia Biotech) and applying the rule that a 50µg/ml solution of double stranded DNA has an A260 of 1 unit.

2.1.4 Restriction Enzyme Digestion and Enzymatic Modification of DNA

All restriction enzyme digests and enzymatic modifications of DNA were performed under the conditions recommended by New England Biolabs. For routine sub-cloning operations, 1-5µg DNA was digested in 40µl of the appropriate buffer for 1h. T4 DNA polymerase or the Klenow fragment of DNA polymerase-I were used to blunt DNA fragments with overhanging ends where appropriate. For large scale digestion of plasmid DNA used for transfection of Dictyostelium cells, 100µg DNA was digested in 400µl of the appropriate buffer for 4-16h. In each case RNA was digested by the addition of RNaseA to 10µg/ml.
Where necessary restriction enzymes and buffer were removed from the DNA by extraction with phenol/chloroform/isoamyl alcohol (24:23:1) and precipitation with 2vol. ethanol and 0.1vol. 3M sodium acetate pH5.2. After a final wash in 70% ethanol, the DNA pellet was resuspended in an appropriate volume of TE. Plasmid DNA for transfection of Dictyostelium cells was resuspended at 1mg/ml whilst recombinant genomic DNA fragments were resuspended in 50µl.

2.1.5 Agarose Gel Electrophoresis and Gel Purification of DNA Fragments

Intact plasmid DNA or restriction fragments of DNA were separated for sub-cloning or analytical purposes by electrophoresis through agarose gel. Gels were cast by dissolving 0.7-2% general purpose agarose (Gibco) in TAE. 0.1µg/ml ethidium bromide was included in gels for visualisation of DNA under UV light. DNA samples were loaded in 10% v/v DNA loading buffer and a 1kb DNA ladder (New England Biolabs) run in parallel. Electrophoresis of samples was at 90V in Hybaid horizontal gel apparatus. DNA was visualised by UV trans-illumination, images captured by CCD camera and printed on thermal paper (Sony).

Specific DNA fragments for sub-cloning purposes were purified from the gel following electrophoresis using the QiaExII kit (Qiagen) used according to the manufacturer’s instructions.

2.1.6 Ligation of DNA Fragments

For routine sub-cloning operations, ligations were performed using 40units of T4 DNA ligase, 50ng vector DNA and 150ng insert DNA in a PEG containing ligase buffer (Gibco). The total reaction volume was 10µl. The ligations were allowed to proceed for 1h at 25°C before transformation into the appropriate competent E.coli strain.

Ligation of oligonucleotides was performed in a similar manner except that 1µg of oligo DNA and 50ng vector DNA was used. The complementary oligos were annealed by boiling for 5 minutes followed by slow cooling to room temperature prior to ligation.

2.1.7 Polymerase Chain Reaction

Polymerase chain reaction (PCR) to amplify specific DNA fragments was carried out using a MJ Research thermal cycler. 50µl reactions were set up in thin-walled 200µl tubes (MWGBiotech) as follows: forward and reverse primers each at 0.1µM final, dATP, dCTP, dTTP and dGTP.
(Promega) each at 100μM final, 1 unit of Taq polymerase (Promega) in ThermoPolymerase buffer (New England Biolabs). Templates were 100ng Dictyostelium genomic DNA, 3μl of λ phage stock or 1ng plasmid DNA. After a 5min incubation at 94°C, cycles of {94°C for 30s, 50°C for 30s, 60°C for 1min} were used for DNA amplification. A final extension step at 60°C for 10min concluded the reaction. 15 cycles were used for preparative mutagenesis reactions to reduce the frequency of errors whereas 35 cycles were used for analytical purposes.

2.1.8 Oligonucleotides for Analytical PCR

The following primers were used to confirm that the gefB cDNA represented a single contiguous region of the genome:

gefB-F2 GTTAACATGTGAATCAAATACCG
gefB-F3 CCTCAACCTCAATTACAACAAAG
gefB-R3 CAGGTTTTGACCAAGCTTGATTC
gefB-F4 CTACCCAAACAATTATTTCCC
gefB-R4 GAAGGGGAATAATTGTTGGG
gefB-F5 CCACACTATTCCTCAATCTCAG
gefB-R5 GGCGAGATTGAGGAATAGTGTGG
gefB-R6 GTTTTTGTGAATCTTAATTCGG
gefB-F7 CCATCGATTTCATCACCAAG
gefB-R7 CTTGGTGATGAAATCGATGG
gefB-F9 GAGCACATGTGAAACAC

2.1.9 DNA Sequencing

For routine sequence determination, DNA sequencing reactions were carried out using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer’s instructions. Sequence data was determined from these reactions by Oswel, University of Southampton using an ABI 373A DNA Sequencer (Perkin Elmer). For high quality long-range sequence determination (>800bp), 20μg of plasmid DNA was sent to MWG Biotech for analysis. Sequences were analysed using DNASTrider, DNASTar and MACAW computer software for the Apple PowerMac.

2.1.10 cDNA Library Screening

A λZAPII cDNA library (Stratagene) provided by Dr A. Harwood and a λgt11 cDNA library (Clontech) were screened.
XL1-blue (λZAPII host) and Y1090 (λgt11 host) plating bacteria were prepared exactly according to Sambrook et al 1989. Both phage libraries were prepared for screening as follows: LB agar plates were poured (300ml in a 250mm square bioassay dish (Nunc)) and allowed to set and dry. The bacteriophage (2x10^5 pfu/plate) were incubated with 2ml of plating bacteria for 30min, mixed with 50ml top agarose at 50°C then quickly poured onto the LB agar plates prewarmed to 37°C. After the top agarose had set, plates were inverted and incubated for 8-12h at 37°C until discreet plaques could be seen in the bacterial lawn, at which point the plates were transferred to 4°C for 30min. At this stage 200mm squares of Hybond N* membrane (Amersham Pharmacia Biotech) were placed carefully onto the top agarose to adsorb the λphage, and the orientations of the filters marked by making holes through the membranes into the agar. The membrane was lifted after 1min, then a second (duplicate) lift was carried out for 3min. λ phage DNA was denatured and fixed to the membrane by placing the filter, DNA side up, onto a pad of 3MM paper (Whatman) soaked in 1.5M NaCl, 0.5M NaOH for 7min. Filters were then neutralised by placing on a pad soaked in 1.5M NaCl, 0.5M Tris-HCl pH7.2, 1mM EDTA for 5min. Membranes were now screened with radiolabelled probes in an identical fashion to that employed for Southern blotting (2.1.13).

Positive plaques were picked by aligning the black spots on the autoradiograph with the library plate and excising the plaque and surrounding area of the agar with the open end of a 1ml micropipette tip (Gilson). At this stage λ phage particles from the agar plugs were not clonal so second and third rounds of screening were needed. These were carried out on 90mm plates (with 3ml top agarose and 200μl host bacteria) using serial dilutions of the λ phage isolated in the primary screen and resulted in the isolation of positive, clonal λphage.

The cDNAs from positive ZAPII clones were excised according to the manufacturer’s instructions. This yields the cDNA cloned in the EcoRI site of pBluescriptSK(-). In order to recover the cDNAs from positive λgt11 clones, λ phage DNA was first prepared using the Lambda DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. From the 20μg of DNA obtained, 5μg was digested with EcoRI. The cDNA insert gel purified away from the rest of the vector and ligated into the EcoRI site of pBluescriptKS(+).
2.1.11 DNA Constructs

2.1.11.1 Gene Disruption Plasmids

gefB: To obtain more gefB sequence a λgt11 cDNA library (Clontech) was screened (see 2.1.9). One round of screening was performed using the cDNA insert from clone SSK260 from the Tsukuba Dictyostelium cDNA project (excised by NotI/SalI digestion and agarose gel purification) as a probe. The inserts from positive clones were amplified by PCR using a λgt11 forward primer (GGTGCGAGCAGACTCCTGGAGCCCG) or a λgt11 reverse primer (TTGACACCAGACCAACTGTAGTAATG) in combination with a gefB reverse primer (CAGGGTTTGACCAAGCTTGATTC). PCR products were cloned into the EcoRV site of pZERO (Invitrogen) according to the manufacturers instructions and the DNA sequences determined. The longest clone (pATW68) contained an additional 700bp of gefB sequence.

Clone SSK260 from the Tsukuba Dictyostelium cDNA project was digested with BstZ117 and a blasticidin resistance cassette (Sutoh, 1993) with EcoRV ends, excised from pRHI148, was inserted to create plasmid, pATW61. The more 5' gefB sequence was excised EcoRI/BstZ117 from pATW68 and ligated into the EcoRI and SmaI of pATW61. The resultant plasmid, (pATW71), was digested with EcoRI and NotI prior to electroporation.

gefC: Clone SLB595 from the Tsukuba Dictyostelium cDNA project was digested with BglII and a Blasticidin resistance cassette with BamHI ends, excised from pRHI103, was inserted. This plasmid, pATW69, was digested with NotI and SalI prior to electroporation.

gefD: To obtain more gefD sequence a λgt11 cDNA library (Clontech) was screened (see 2.1.9). One round of screening was performed using the cDNA insert from clone SSH128 from the Tsukuba Dictyostelium cDNA project (excised by NotI/SalI digestion and agarose gel purification) as a probe. The inserts from positive clones were amplified by PCR using a λgt11 forward primer (GGTGCGAGCAGACTCCTGGAGCCCG) or a λgt11 reverse primer (TTGACACCAGACCAACTGTAGTAATG) in combination with a gefD reverse primer (CCACCAACGATTGCATTGCAGC). PCR products were cloned into the EcoRV site of pZERO (Invitrogen) according to the manufacturers instructions and the DNA sequences determined. The longest clone (pATW65) contained an additional 500bp of gefB sequence.
Clone SSH128 from the Tsukuba Dictyostelium cDNA project was digested with HincII and a Blasticidin resistance cassette with EcoRV ends, excised from pRHI148, was inserted to create plasmid, pATW60. The more 5' gefD sequence was excised EcoRI/BspHI from pATW65 (after blunting of the BspHI site with T4 DNA polymerase) and ligated into the EcoRI and Smal of pATW60. The resultant plasmid (pATW70), was digested with EcoRI and NotI prior to electroporation.

**gefE:** Clone SLA704 from the Tsukuba Dictyostelium cDNA project was digested with BstZ17 and a Blasticidin resistance cassette with EcoRV ends, excised from pRHI148, was inserted. This plasmid, pATW57, was digested with NotI and SalI prior to electroporation.

**gefG:** Clone SLD476 from the Tsukuba Dictyostelium cDNA project was digested with Clal, blunted with T4 polymerase and a Blasticidin resistance cassette with EcoRV ends, excised from pRHI148, was inserted. This plasmid, pATW72, was digested with NotI and SalI prior to electroporation.

**rasD:** A 2.0kb, EcoRI/Bcll fragment of rasD genomic DNA cloned into the EcoRI and BamHI sites of pBluescriptKS(+)) was digested with PstI, blunted with T4 polymerase and BamHI linkers inserted giving rise to a vector, containing a BamHI at the 5' end of the rasD gene. This BamHI site was used to insert a Blasticidin resistance cassette with BamHI ends, excised from pRHI103. This plasmid, pATW1, was digested with EcoRI and SpeI prior to electroporation.

### 2.1.11.2 Assembly of the gefB cDNA

Two partial cDNA clones were isolated from a λZAPII cDNA library (Stratagene) which encompassed the entire gefB ORF. pATW99 contained the 3' end and pATW116 contained the 5' end. To assemble the full-length cDNA, the 3' end was excised BsrGI/EcoRV from pATW99 and cloned into the BsrGI and Smal of pATW116 creating pATW117.

### 2.1.11.3 Expression Plasmids

**gefB:** The complete gefB cDNA was excised from pATW117 by EcoRV/Xbal digestion and ligated into the KpnI and HindIII sites of pDXA (Manstein et al., 1995) after blunting all ends with T4 DNA polymerase. This plasmid, pATW118, puts the gefB cDNA under the control of the strong constitutive act15 promoter. pATW118 was linearised by Scal digestion prior to electroporation.
**rasD**: An *EcoRI/XbaI* fragment of the *rasD* genomic clone containing the complete ORF with putative transcriptional promoter and terminator sequences was ligated into *EcoRI* and *XbaI* sites of pBluescriptKS(+) . This plasmid, pATW8, was digested with *XbaI* and a neomycin resistance cassette with *XbaI* ends, excised from pDNEO, was inserted.

The resultant plasmid, pATW9, was linearised by *ScaI* digestion prior to electroporation.

### 2.1.11.4 Yeast Two-Hybrid Vectors

**gefB**: pATW99 was digested *BstBI/NotI* and a double stranded oligonucleotide inserted (CGAATGATTCTAGAGATCT and GGCCAGATCTCTAGAATCATT) to create pATW112. This resulted in the creation of an *XbaI* before the catalytic domain of *gefB*. The modified GEF domain was excised from pATW112 by *XbaI/XhoI* digestion and inserted into the *SpeI/SalI* sites of pGADGH (Clontech) to create pATW113. This vector expresses a fusion of the yeast Gal4 activation domain and the GefB catalytic domain using the strong constitutive *ADH1* promoter.

**aleA**: The catalytic domain of *aimless* was excised by *NdeI/EcoRI* digestion of the cDNA (obtained from Dr R Insall) and inserted into the *BamHI* site of pGADGH after blunting of all DNA ends with Klenow DNA polymerase. This plasmid, pATW24, expresses a fusion of the yeast Gal4 activation domain and the aimless catalytic domain.

**rasG**: A *rasG* cDNA with the S17N mutation was obtained from Dr G Weeks. This was amplified by PCR to remove the CAAX prenylation signal and add *NcoI* sites at each end using the following pair of primers: CCAGGATCCCTAAGCTTTTATGGTCTCTTC and CCAGAATTCAATGACAGAATACAAATTAG. The PCR product was digested with *NcoI* and ligated into the *NcoI* site of pAS2 (Durfee et al., 1993). The resultant plasmid pATW122 expresses a fusion of the yeast Gal4 DNA binding domain and the RasG protein using the strong constitutive *ADH1* promoter.

**rasS**: A pAS2 based vector expressing the Gal4 DNA binding domain fused with a dominant negative (N17) RasS protein was provided by J Chubb. This fusion had the CAAX prenylation signal removed.
2.1.11.5 Yeast Complementation Vector

pATW99 was digested with BstBl/NotI and a double stranded oligonucleotide inserted (GGCCTGCAGATGTT and CGAACATCTGCA) to create pATW123. This resulted in a PstI and in-frame ATG translational initiation codon being added before the catalytic domain of gefB. The modified GEF domain was excised from pATW123 by PstI/Sall digestion and inserted into the PstI/Xhol sites of pAD4 (Park et al., 1994) to create pATW124. This vector expresses the transgene using the strong constitutive ADH1 promoter.

2.1.12 Preparation of Dictyostelium Genomic DNA

Approximately $10^9$ Dictyostelium cells were harvested from shaking axenic culture or SM agar/Klebsiella plates, washed and shaken in KK2 at 5x10^7/ml for at least 6h. Starved cells were pelleted and lysed to release intact nuclei by adding 40ml of ice-cold Cell Lysis Buffer and mixing. Nuclei were pelleted at 4000g for 10min at 4°C, washed with a further 40ml lysis buffer and resuspended in 0.3ml lysis buffer. 10ml Digestion Buffer, containing 100µg/ml Proteinase K, was added and the mixture incubated at 56°C for at least 4h. Genomic DNA was extracted with 10ml phenol pH7.5. The aqueous layer was further extracted with 5ml phenol/chloroform/isoamyl alcohol (24:23:1), precipitated with 2vol. ethanol and 0.1vol. 3M sodium acetate pH5.2 and spooled out on a plastic loop. After washing with 70% ethanol, the DNA was dissolved in TE and the concentration estimated by measuring the absorbance at 260nm on a spectrophotometer (Amersham Pharmacia Biotech).

2.1.13 Southern Analysis of Dictyostelium DNA

Genomic DNA was digested with appropriate restriction enzymes (see 2.1.4) then separated on a 0.8% agarose gel. Restriction digests were as follows: gefB DNA with Bcll, gefC DNA with BsaBl/HindIII, gefD DNA with BsaBl/BsrGI, gefE DNA with EcoRI, gefG DNA with BsrGI and rasD DNA with EcoRI/Bcll. The DNA in the gel was transferred onto Hybond N⁺ (Amersham) by capillary blotting using 0.4M NaOH as the transfer solution. The membrane was prehybridised for 1h at 65°C in bottles in a rotating oven (Hybaid) with 20ml of Church Hybridisation Buffer. Denatured radiolabelled probe (see 2.1.16) was added and allowed to hybridise for 4-16h. The membrane was then washed 3 times with Church Wash Buffer changing every 20min. The
membranes were then sealed in plastic bags and exposed to XOMAT film (Kodak). Where necessary, membranes were stripped with boiling 0.5% SDS.

2.1.14 Preparation of Dictyostelium RNA

5x10³ Dictyostelium cells were placed on dry ice and lysed immediately into 500μl RNA Extraction Buffer. 500μl phenol pH7.5 was added, the mixture vortexed vigorously for 15min then centrifuged at 13,000rpm for 5min. The upper phase was removed and added to 1ml of ethanol. This mixture was centrifuged at 13,000rpm for 5min, the supernatant aspirated and the RNA pellet dissolved in DEPC-treated ddH₂O. The RNA solution was stored -80°C. The RNA concentration was estimated by determining the absorbance of the solution at 260nm and assuming that a solution of 40μg/ml RNA has an A₂₆₀ of 1 unit.

2.1.15 Northern Analysis of Dictyostelium RNA

RNA was prepared for electrophoresis in 1x MOPS buffer, 0.2M formaldehyde, 50% v/v formamide and 10% v/v sterile RNA loading dye, denatured at 60°C for 15min and cooled on ice. 10μg RNA per lane was loaded on a 1% agarose gel containing 1xMOPS buffer, 0.65M formaldehde and 0.01μg/ml ethidium bromide. The gel was run in 1xMOPS buffer at 105V for 4h and visualised under UV light before equilibrating in 10xSSC and blotting onto Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech) by capillary transfer in 10xSSC. The RNA was subsequently UV-crosslinked to the membrane using a Stratalinker (Stratagene). Membranes were prehybridised at 42°C for 2h in bottles in a rotating oven (Hybaid) with 20ml RNA Hybridisation Buffer. Denatured radiolabelled probe (see 2.1.16) was added and allowed to hybridise for 4-16h. The membranes were then washed with 2xSSC/0.5% SDS at room temperature, followed by four 15min washes at 67°C. After one final wash with 0.5xSSC/0.5% SDS at 67°C the membranes were sealed in plastic bags and exposed to XOMAT film (Kodak).

2.1.16 Radiolabelled Probes

Radiolabelled probes for Northern blotting, Southern blotting and λ phage library screening were generated by a random priming reaction. The reaction mixture consisted of 50ng of agarose gel purified DNA, 10μg bovine serum albumin, 3units Klenow polymerase, 50μCi α-³²P-dATP
(specific activity of 6000Ci/mmol) in 50μl of OLB. The DNA and water are initially heated to 95°C for 5min before addition of the other components, on ice. The labelling reaction was incubated for 1h at 37°C then the probe purified using a G50 spin column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The mixture was again heated to 95°C for 5min, then added to the hybridisation buffer.

**gefB**: A 671 bp probe, amplified from the 3' end of the *gefB* cDNA using the primers CAGGTTTTGACCAAGCTTGATTC and GTTAACATGTGAATCAAATACCG was used for both Southern/Northern analysis and first round screening of the λZAPII cDNA library. A 500bp *EcoRI/PstI* fragment from the extreme 5' end of pATW99 (see 2.1.10.2) was used for the second round of screening of the λZAPII cDNA library.

**gefC**: A *BsaBI/HindIII* fragment of clone SLB595 from the Tsukuba *Dictyostelium* cDNA project was used as probe in Southern analysis.

**gefD**: A 571 bp probe, amplified from the *gefD* cDNA using the primers GACTCTGTACCACAGGTACCACC and CCACCAACGATTGCATTGGCAGC was used for Southern analysis.

**gefE**: A 633bp probe, amplified from the *gefE* cDNA using the primers CCTTTACCCAGGGCCCGCCAACAC and GTTGTAGTATTGGTTGTTGC was used for Southern analysis.

**gefG**: A *BsrGI* fragment of clone SLD476 from the Tsukuba *Dictyostelium* cDNA project was used as probe in Southern analysis.

**rasD**: A *Clal/Bcll* fragment of *rasD* genomic DNA was used as probe in Southern analysis.

**Other probes**: To generate probes for *ecmA, ecmB, pspA, spiA, IG7 and dis1γ*, entire plasmids containing the cDNAs of these genes were used as the template for the radiolabelling reaction.

### 2.1.17 Yeast Transformation and Two-Hybrid Assay

*S.cerevisiae* strains Y190 or LV25-5 (Broek et al., 1987) (a strain carrying a temperature sensitive *CDC25* allele) were maintained at 30°C in YPD medium or on YPD agar plates. Leu, Trp, Leu- Trp and Leu-Trp-His drop-out media were obtained as powders from Bio101. Both liquid drop-out media and agar plates were prepared according to the manufacturers instructions. Yeast transformations were performed according to the TRAFO protocol (Agatep et al., 1998) and the
Yeast two-hybrid assays performed according to (Geitz et al., 1997) and (Parchaliuk et al., 1999). Both growth of transformants on Leu', Trp', His'-synthetic agar plates containing 25mM 3-aminotriazol (3-AT) and expression of β-galactosidase were taken as indicating a positive two-hybrid interaction.

2.2 Cell Biology

2.2.1 Dictyostelium Strains, Cell Culture and Development

*Dictyostelium discoideum* strains AX2, AX3 are the parents of all the mutants described in this thesis.

Vegetative cells were maintained at 22°C on SM agar plates in association with *Klebsiella aerogenes*, or axenically in HL5 medium (Sussman and Sussman, 1967) supplemented with appropriate antibiotic selection either in 90mm tissue culture plates or shaking in flasks at 150rpm.

Growth rates of cells in axenic medium and bacterial suspension were calculated from semi-log plots of cell proliferation against time. The gradient of the best-fit straight line through points taken in the exponential phase of growth was determined and taken as the mean growth rate. For axenic growth, 3x10^5 *Dictyostelium* cells were added to HL5 in 90mm dishes or 100ml flasks shaken at 150rpm. For growth rates in bacterial suspension, the method described by (Witke et al., 1992) was used. A suspension of *E.coli* strain B/r at a density of 10^10 cells/ml KK2 buffer was inoculated with 3x10^5 *Dictyostelium* cells and shaken at 150rpm. Cells were counted using a haemacytometer.

Development was initiated by plating vegetative cells at a density of 3x10^6/ml on KK2 agar plates or on 47mm 0.2μm nitrocellulose filters (Millipore) on top of 3 layers of 3MM paper (Whatman) soaked in KK2. These were incubated in a humidified atmosphere in overhead light. In most cases bacterially grown cells were used. To obtain large quantities of cells, SM agar plates were inoculated with 10^5-10^6 *Dictyostelium* cells plus 500μl of a suspension of *Klebsiella aerogenes* in KK2. Cells were harvested into KK2 prior to detectable clearance of the bacterial lawn and pellet at 2000g for 2min. Several more KK2 washes were performed until the cells were free of bacteria. In the case of cells transformed with lacZ reporter constructs, which need constant selection with 20μg/ml G418, exponentially growing axenic cells were used. Aggregates were
photographed using a dissecting microscope (Wild M8 with MPS11 camera) and Kodak Ektachrome 160T 35mm slide film.

To assess development in the presence of exogenous NH₃, vegetative cells were plated at a density of 3x10⁶/ml on nitrocellulose filters (as above) and allowed to develop to tipped-mounds. The filters were then transferred on to 3 layers of 3MM paper (Whatman) soaked in 25mM MES(pH6.3) containing various concentrations of NH₄Cl.

2.2.2 Transfection of Dictyostelium Cells

Electroporation was used for generating gene knockout strains and for the reintroduction of gefB and rasD genes into the null cell lines since it results in a high percentage of transformants containing only a single copy of introduced vector.

Transformation was performed by a modification of the method of (Howard et al., 1988). 2x10⁷ exponentially growing cells were pelleted at 2000g for 2min resuspended in 400µl ice cold KK2/50mM sucrose, mixed with 25µg of linearised DNA and electroporated in a 0.2cm cuvette (Biorad) in a BioRad Gene pulser at 1.1V, 3µF with a 5Ω resistance in series. After 10 minutes incubation on ice 1mM MgCl₂/1mM CaCl₂ was added and the cells placed at 22⁰C for 15min before addition of 10ml HL5 supplemented with heat killed E.coli B/r. 10µg/ml Blasticidin-S (ICN) or 10µg/ml G418 (Calbiochem) was added 16h after electroporation. After 7 days antibiotic selection, transformants were cloned on lawns of Klebsiella aerogenes on SM agar plates.

CaPO₄-mediated transfection was used to introduce lacZ reporter constructs since it generates high copy number random integrations of plasmid DNA into the Dictyostelium genome. 10ml of axenically growing cells (10⁴/ml) were plated onto 90mm tissue culture dishes and allowed to attach for 30min. The axenic medium was then replaced with HEPES-HL5 medium and cells were left to equilibrate for a minimum of 2h. A DNA precipitate was obtained by the addition of 38µl of 2M CaCl₂ to 600µl HBS containing 12µg plasmid DNA whilst vortexing vigorously. The mixture was left for 30min at 22⁰C before being added dropwise to the plate of cells and incubated for at least 4h. Cells were then osmotically shocked with 2ml of 15% glycerol in HBS for 2min. The glycerol/HBS was then replaced with HL5 supplemented with heat killed E.coli B/r and incubated for 16h. Transformants were selected by the addition of 50µg/ml G418. After 7 days selection transformant colonies were picked with a 2µl micropipette (Gilson) and cloned out by serial
dilution on lawns of *Klebsiella aerogenes* on SM agar plates. Clones were picked into HL5 and stable transformants were maintained at 20μg/ml G418.

Sterile heat-killed bacteria were made as follows: a 500ml stationary culture of *E.coli* B/r was pelleted at 5000g for 5min and washed three times in 500ml KK2. Cells were resuspended in 50ml KK2, heat-killed at 80°C for 20min and stored in aliquots at -20°C for use. 20μl was used for each transfection.

### 2.2.3 Pinocytosis Assays

This assay was modified from that designed by Dr M Maniak (Hacker et al., 1997; Maniak et al., 1995). Bacterially grown cells were pelleted at 2000g for 2min, washed free of bacteria with KK2 and plated at 5x10⁶ cells/plate in HL5 on 90mm tissue culture plates for 24h. Cells were harvested by trituration, pelleted then resuspended at 5x10⁶ cells/ml in 10ml HL5 containing 2mg/ml TRITC-dextran (MW 70000) and shaken at 150rpm. 1ml of culture was removed at regular intervals and 100μl of Trypan Blue added to quench extracellular fluorescence. Cells were then washed once, resuspended in 1ml KK2 and intracellular fluorescence measured by Fluorimeter (Kontron SFM25). Excitation was at 544nm and emission read at 574nm. For each cell line, the assay was performed on 3 separate occasions using the same batch of TRITC-dextran. Results are presented in terms of relative fluorescence with the data points from each separate experiment and their mean value displayed on the same graph.

### 2.2.4 Phagocytosis Assays

Phagocytosis of bacteria was performed as described previously (Witke et al., 1992). *Dictyostelium* cells were inoculated at a density of 10⁶ cells/ml into a 20ml suspension of *E.coli* strain B/r at 10⁶ cells/ml in KK2. 1ml of culture was removed at regular intervals and the optical density at 600nm measured. Each assay was performed in triplicate, the mean and SD plotted against time and an estimate of phagocytic rate calculated from the best fit straight line.

Phagocytosis of TRITC-labelled yeast was performed in a similar manner to the pinocytosis assay described in 2.2.3 except that cells were assayed at 2x10⁶ in 10ml of HL5 containing TRITC-dextran labelled yeast at 10⁶ cells/ml (a gift from Dr M Maniak) (Niewohner et al., 1997).
2.2.5 Stalk and Spore Assays

Stalk and spore monolayer assays were performed as described previously in (Harwood et al., 1995). To induce stalk cell formation, cells were washed in Stalk Medium and plated at a density of 1.5x10^4 cells/cm^2 in 30mm tissue culture plates containing Stalk Medium supplemented with 5mM cAMP. After 20h, the medium was removed, the cells washed three times and replaced with fresh medium supplemented with 100nM DIF alone or 100nM DIF plus 5mM cAMP. To induce spore cell formation, cells were washed in Spore Medium and plated at a density of 1.5x10^4 cells/cm^2 in 30mm tissue culture plates containing Spore Medium supplemented with 15mM 8-bromo-cAMP or 5mM cAMP. The numbers of vacuolated stalk cells and phase bright spore cells were counted using a phase-contrast microscope (Zeiss) after 48h and expressed as a percentage of the total cell count. Each experiment was performed three times, counting a minimum of 500 cells from five separate dishes on each occasion.

In vivo spore cell formation was assessed following differentiation of 2x10^7 cells at 3x10^6/cm^2 on Millipore filters soaked in KK2. The number of spores formed during differentiation was determined by haemacytometer after harvesting of mature fruiting bodies with 10ml of KK2 containing 0.5% Triton X-100. The total number of spores formed during the development of 1:1 mixtures of mutant and wild-type amoebae was determined in a similar manner. To determine the relative contribution of each cell type to the spore mass in these chimeras, spores were plated at serial dilutions onto SM agar in association with Klebsiella aerogenes. Cells from plaques formed in the bacterial lawns were transferred to HL5 containing 10μg/ml blasticidin, the resistant clones (which represent the spores from mutant amoebae) counted and expressed as a percentage of the total number of clones assayed. The assay was repeated 3 times; at least 200 colonies were assayed each time. The viability of in vivo derived spores was determined by resuspending them in 10mM EDTA (pH 7.5), heating for 30min at 37°C and plating at serial dilutions onto SM agar in association with Klebsiella aerogenes. The percentage viability was calculated by comparing the number of colonies formed to the number of phase-bright spores plated.

In all in vivo spore assays, 2x10^7 vegetative cells were subject to the same assay conditions. In each case they yielded no phase bright spores or colonies on SM plates.
2.2.6 β-Galactosidase Staining of Aggregates

Aggregates were developed on nitrocellulose filters or on KK2 agar and fixed for 15min in Z-buffer containing 1% glutaraldehyde. Samples were washed twice in Z-buffer then incubated in X-Gal Staining Solution at 37°C until sufficient staining was achieved (Dingermann et al., 1989). Stained structures were photographed at 50x magnification on the filters using a dissecting microscope (Wild M8 with MPS11 camera). Alternatively, structures were mounted onto glass slides and photographed at high magnification (200x) on a Zeiss Axioskop fluorescence microscope with MC100 camera using Kodak Ektachrome 160T 35mm slide film.

2.2.7 Fluorescence Microscopy

Cells from bacterial lawns or axenic culture were harvested, washed and seeded onto glass coverslips. Fixation was carried out with 1% glutaraldehyde, 0.1% Triton X-100 in KK2 for 10min. Autofluorescence was quenched using 5mg/ml NaBH₄ for 10min. Actin filaments were stained using Texas Red-conjugated phalloidin (Molecular Probes). Cells were observed using a scanning confocal microscope (MRC1024; BioRad, Hercules, CA).

2.2.8 Scanning Electron Microscopy

The protocol described by (Condeelis et al., 1987) was used with modifications. Cells were fixed on glass coverslips using 1% osmium tetroxide in KK2 for 5s followed by 2% glutaraldehyde in KK2 for 2h. The fix was removed and the samples were progressively dehydrated through an ethanol series of 30-100% ethanol then taken into hexamethyl disilane. After air-drying, the cells were sputter coated in gold, and viewed on a Jeol JSM5410 scanning electron microscope.

2.2.9 Motility Analysis

The mean speed of bacterially grown cells was estimated using time-lapse recordings of digital phase-contrast images of the cells. Cells were washed free of bacteria, plated in KK2 onto 90mm tissue culture plates and allowed to adhere for 15min. Phase-contrast images of the cells were obtained using an Axiovert 100 inverted microscope (Zeiss). Using a CCD camera and a Scion frame grabber attached to an Apple PowerMac computer running NIH Image 1.62 software, 40 frames were captured at 20s intervals. Cell centroids were determined by eye and the mean
centroid displacement between each frame was calculated by the software in terms of pixels. A graticule slide was used to calibrate pixel dimensions and hence the series of images was used to gain an estimate of mean cell speed.

2.2.10 Phototaxis and Thermotaxis Assays

All phototaxis and thermotaxis assays were performed by Dr P Fisher.

Qualitative phototaxis tests were performed by using sterile spatula-style toothpicks to transfer cells to charcoal agar plates from the edges of colonies growing on *Klebsiella aerogenes* lawns. Phototaxis was scored after 48h incubation at 21°C with a lateral light source. For quantitative phototaxis experiments, washed amoebae were inoculated onto the centres of charcoal agarose plates at various densities and incubated with a lateral light source for 48h at 21°C. For quantitative thermotaxis experiments, washed amoebae were inoculated onto the centres of water agarose plates (~ 2.4 x 10^6 cells/cm^2) and incubated for 72h in darkness on a heat bar producing a 0.2°C/cm temperature gradient at the agarose surface. Arbitrary temperature units correspond to a temperature range of 14°C (T1) to 28°C (T8), as measured at the centre of plates in separate calibration experiments. Slug trails were transferred to PVC disks, stained with Coomassie Blue, and digitised. Slug orientation behaviour was analysed using directional statistics.
2.2.11 Statistical Analysis of Physiological Data

A statistical analysis of the apparent physiological differences between mutant amoebae and the parental strain was performed using two-sided t-tests. The t statistic was calculated using the formula:

\[ t = \frac{X_1 - X_2}{\left[S^2 \left(\frac{n_1 + n_2}{2}\right)\right]^{0.5}} \]

Where:

\[ S^2 = \frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2} \]

Symbols:
- \( X \) = Sample mean
- \( s \) = Sample standard deviation
- \( n \) = Sample size
- \( S \) = Common standard deviation

The P-value was determined from a table of the critical values of the t statistic for \( n_1 + n_2 - 2 \) degrees of freedom. The significance level was set at 0.05

2.3 Biochemistry

2.3.1 SDS PAGE and Western Blotting

For SDS-PAGE, cells were pelleted at 2000g for 2min and resuspended in 1x Laemmli Buffer. After boiling for 5min the cellular debris was pelleted at 15000g for 5min. 10^6 cell equivalents per lane and molecular weight markers (7708S New England Biolabs) were loaded on 10% resolving/4% stacking polyacrylamide gel (37.5:1 acrylamide:bisacrylamide, Protogel, National Diagnostics Corporation), and run at 150V for 1.5h using standard procedures (Sambrook et al., 1989). Some gels were then stained with Coomassie Blue overnight and destained over several hours with 4-5 changes of Coomassie Destain to visualise total protein.

For Western blotting gels were equilibrated in Western Transfer Buffer and protein electrophoretically transferred to Hybond C-extra nitrocellulose membrane (Amersham Pharmacia Biotech) by semi-dry blotting in a BioRad transfer cell according to the manufacturer's instructions. The membrane was rinsed in PBS and proteins were visualised using 2% PonceauS in 1% acetic
acid to ensure even transfer and loading. Membranes were blocked for 1h at room temperature or overnight at 4°C in 10% dried skinned milk, 0.1% Tween-20. After five washes in PBS/0.1% Tween-20 (PBST) membranes were incubated in primary antibody diluted in PBST for 1-2h at room temperature washed four times in PBST and incubated in secondary antibody diluted in PBST for a further hour. After further washing with PBST the membrane was sealed in Saranwrap (Dow Chemical); the secondary antibody was detected using enhanced chemiluminescence (ECL, Pierce) according to the manufacturer’s instructions and exposure to Biomax (Kodak) film. To strip membranes for reprobing, they were incubated at room temperature for 10min in 0.1M glycine pH 2.5 followed by neutralisation in 1M Tris pH 8.0.

Recipes

2.3.2 Cell Biology

HL5 axenic medium
1.43% peptone (Oxoid, L34)
0.72% yeast extract (Oxoid L21)
3.6mM Na2HPO4, 3mM KH2PO4
30% glucose,
0.5mg/ml vitamin B12
1mg/ml folic acid pH9.0
Final pH 6.4

HEPES-HL5
20mM HEPES pH 7.05
0.5% yeast extract (Oxoid L21)
1% peptone (Oxoid L34)
1% glucose

HBS
270mM NaCl
10mM KCl
12mM Na2HPO4
40mM HEPES pH7.05
0.2% glucose

KK2
15.5mM KH2PO4
3.8mM K2HPO4
Final pH 6.2

KK2 Agar
KK2
1.5% Bacto-agar (Difco)
LB
1% Bacto-tryptone (Difco)
0.5% Bacto-yeast extract (Difco)
17mM NaCl
Final pH 7.0

LB Agar
L-broth
1.5% Bacto-agar (Difco)

LB Top Agarose
L-broth
0.7% agarose (Boehringer Mannheim)

PBS
137mM NaCl
2.68mM KCl
7.98mM Na₂HPO₄
1.47mM KH₂PO₄
Final pH 7.2

Slide Mountant
PBS
140mM NaCl
0.04% Na azide
25% w/v polyvinyl alcohol
2.5% DABCO anti-fading agent
25% v/v glycerol
centrifuge at 5500g, 15 min, 4°C

SM Agar
1% glucose
% peptone (Oxoid L34)
0.1% yeast extract (Oxoid L21)
2% agar (Difco)
4mM MgSO₄
4mM KH₂PO₄
6mM K₂HPO₄

Spore Medium
10mM MES pH 6.2
20mM NaCl,
20mM KCl
1mM MgCl₂
1mM CaCl₂
200μg/ml streptomycin, 20μg/ml tetracycline

Stalk Medium
10mM MES pH 6.2
2mM NaCl
10mM KCl,
1mM CaCl₂
<table>
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<th><strong>X-gal Staining Solution</strong></th>
<th><strong>Z-buffer</strong></th>
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<td></td>
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<td></td>
<td>5mM K₃Fe(CN)₆</td>
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<tr>
<td></td>
<td>0.1% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase</td>
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<th><strong>YPD yeast medium</strong></th>
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<tr>
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<td>1.8% Bacto-agar</td>
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**2.3.3 Molecular Biology**

<table>
<thead>
<tr>
<th><strong>Church Hybridisation Buffer</strong></th>
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<tbody>
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<table>
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<th><strong>50x Denhardt’s Solution</strong></th>
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<tr>
<td>1% Ficoll</td>
<td>0.5% xylene cyanol</td>
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<tr>
<td>1% Polyvinylpyrrolidine</td>
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<th><strong>Genomic DNA Digestion Buffer</strong></th>
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<tr>
<td>0.32M sucrose</td>
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<td>0.02% sodium azide</td>
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<td>1% Triton X-100</td>
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<tr>
<td>50mM Tris-HCl pH 7.5</td>
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85
0.01% gelatin

**MOPS RNA Gel Buffer**
- 20mM MOPS pH 7.0
- 5mM Na Acetate
- 1mM EDTA

**OLB (5x)**
- 250mM Tris-Cl pH 8
- 25mM MgCl₂
- 5mM β-mercaptoethanol
- 2mM each dGTP, dCTP, dTTP
- 1M Hepes pH 6.6
- 1mg/ml Random hexadeoxyribonucleotides (Pharmacia)

**RNA Extraction Buffer**
- 100mM Tris-HCl, pH 7.5
- 200mM NaCl,
- 20mM EDTA
- 1% SDS

**RNA Hybridisation Buffer**
- 43% v/v formamide
- 5x SSC
- 10x Denhardt’s solution
- 10mM Na₂HPO₄/NaH₂PO₄ pH 6.8
- 200mg/ml boiled, sheared herring sperm DNA
- 0.5% SDS

**RNA Gel Loading Buffer**
- 50% v/v glycerol
- 1mM EDTA
- 0.4% w/v bromophenol blue
- 0.4% w/v xylene cyanol

**SSC**
- 150mM NaCl
- 15mM Na₃citrate

**STET**
- 50mM Tris-HCl pH 8
- 50mM EDTA
- 8% sucrose
- 5% Triton-X 100

**TAE**
- 40mM Tris-acetate
- 1mM EDTA

**TE**
- 10mM Tris-HCl pH 7.5
- 1mM EDTA
2.3.4 Biochemistry

CLAP 1000x
5mg/ml Chymostatin
10mg/ml Leupeptin
5mg/ml Antipain
5mg/ml Pepstatin A
dissolved in DMSO

Coomassie Stain
0.25% Coomassie Brilliant Blue
10% acetic acid
45% methanol

Coomassie Destain
25% methanol
16% acetic acid

Laemmli Buffer
10% glycerol
100mM DTT
2% SDS
50mM Tris-HCl pH6.8
0.1% bromophenol blue

Western Transfer Buffer
48mM Tris-Cl pH8.0
96mM glycine
20% v/v methanol

SDSPAGE 4% Stacking Gel
55mM Tris-Cl pH6.8
0.2% w/v SDS
0.05% v/v TEMED
0.1% w/v APS
4% acrylamide

SDSPAGE 10% separating gel
500mM Tris-Cl pH8.8
0.2% w/v SDS
0.05% v/v TEMED
0.1% w/v APS
10% acrylamide
3 Identification and Molecular Genetic Analysis of Five Novel RasGEFs from *Dictyostelium discoideum*

3.1 Introduction

*Dictyostelium* cells lacking RasG are unable to proliferate in shaking axenic culture and exhibit a number of defects associated with cytoskeletal disfunction (Tuxworth et al., 1997). These include impairment of cytokinesis and cell motility and a loss of cell polarity. *rasS* cells have increased motility and a highly polarised cell morphology. They are also are unable to grow in shaking axenic culture and recently this has been found to correlate with an inability to perform pinocytosis (J Chubb and A Wilkins, in press). The phenotype of the only published *Dictyostelium* RasGEF mutant, *aimless*, is unrelated to those of the *rasG* and *rasS* mutants. *aimless* null cells have no apparent defects in growth or cell morphology but are defective in chemotaxis and activation of adenylate cyclase in response to exogenous cAMP, resulting in a failure to aggregate upon starvation (Insall et al., 1996). It therefore, seemed likely that additional RasGEFs must exist which regulate the Ras activity required for correct cytoskeletal function. The aim of the work in this chapter was, firstly, to identify novel *Dictyostelium* RasGEFs from the Tsukuba cDNA sequencing project databases (Morio et al., 1998). Secondly, using targeted gene disruption, it was hoped to generate RasGEF mutant cell lines, which had some or all of the phenotypic properties of the *rasG* and *rasS* mutants. In this way it was hoped to begin the identification of the upstream elements of Ras signalling pathways controlling specific aspects of Ras function in *Dictyostelium*. The aim of this chapter was not to fully characterise any RasGEFs identified but to determine quickly which, if any, RasGEFs were involved in the Ras signalling processes identified in the *rasG* and *rasS* mutants.

3.2 Results

3.2.1 Identification of Five Novel RasGEFs

The Tsukuba, *Dictyostelium* cDNA sequencing project databases (Morio et al., 1998) were used to identify novel RasGEFs from *Dictyostelium*. The tBLAST-n search engine identified five cDNA clones with homology to the GEF domain of the putative RasGEF, *aimless* (Insall et al., 1996). The partial cDNA clones were obtained from Dr T Morio and the full DNA sequence of each clone was determined. The genes represented by clones SSK260, SLB595, SSH128,
Figure 3.1 Alignment of the protein sequence of the catalytic domain of the human RasGEF, Sos, against the predicted protein sequences of five partial cDNAs from *D. discoideum*. The Cdc25 box homology region found in all Ras subfamily GEFS is boxed in black and the REM box homology region found only in Ras-specific GEFS is boxed in red. Residues conserved in more than 50% of the sequences are coloured red while gaps are inserted to aid alignment.
SLA704 and SLD476 were named gefB, gefC, gefD, gefE and gefG respectively. The predicted protein sequences of these clones suggest that they will function as RasGEFs. All clones contain a Cdc25 homology domain at the C-terminus - this region of homology is found in the catalytic domains of known GEFs for Ras and Ral subfamily proteins. Also, with the exception of gefD, the predicted protein sequences all clones possess a region of sequence homology known as a REM box found only in Ras-specific GEFs (Lai et al., 1993). Figure 3.1 shows an alignment of the predicted coding sequences of these cDNA clones against the catalytic domain of the human RasGEF, Sos. Although the partial gefD cDNA is too short to determine if it contains a REM box homology region, the predicted protein sequence of the putative GefD catalytic domain is more similar to RasGEFs than to GEFs for other small GTPases and is therefore likely to function as a RasGEF in vivo.

Two further gef genes, gefA (clone SSD492) and gefF (clone FCBL15), were identified in the Tsukuba, Dictyostelium cDNA sequencing project databases by Dr Robert Insall. The molecular genetic analysis of the gefA and gefF genes is not discussed in this chapter although a summary of the unpublished findings of Dr Robert Insall is presented in 4.3.1.

3.2.2 Generation of gef Null Mutants

To determine the biological function of these putative RasGEFs, the generation of five separate Dictyostelium mutants, each with a single, targeted RasGEF gene disruption, was attempted. In each case gene-replacement vectors were generated in which the RasGEF homology region of the cDNA was disrupted by insertion of a Blasticidin resistance cassette (Sutoh, 1993). The partial cDNA clones of gefB and gefD were too small, 0.9kb and 0.7kb respectively, to facilitate vector construction so longer cDNA clones were obtained by screening a λgt11 phage library (an additional 0.7kb and 0.5kb of cDNA sequence were obtained for gefB and gefD respectively). The construction of all of these vectors is precisely detailed in section 2.1.11.1. The gene disruption constructs were transfected into AX3 Dictyostelium cells, and transformants cloned out following seven days of Blasticidin selection. Dictyostelium transformants were screened by Southern blotting. For each gene-replacement vector, ten transformants were examined and four gefB, five gefC, three gefD, nine gefE and five gefG cell lines were identified in which homologous recombination had replaced the endogenous gef gene with a single copy of the disrupted version. Schematic diagrams of the
A) Schematic representation of the strategy employed to disrupt the gefB gene. A 1.6kb fragment of gefB cDNA disrupted with a 1.3kb blasticidin resistance cassette was constructed. The 2.9kb construct was used to disrupt the gefB gene by homologous recombination. A probe from the gefB cDNA was used to screen recombinants by Southern blotting. The sizes of the expected hybridising fragments from AX3 cells and gefB disruptants are indicated.

B) Southern blot of gefB mutant and parental AX3 DNA. Genomic DNA from gefB' and AX3 cells was digested with BclI, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled fragment of gefB DNA generated by PCR (see 2.1.16). Bands representing the wild type (2.3kb) and disrupted (3.6kb) gefB genes are indicated.

C) AX3 and gefB' cells were harvested at the indicated times of development (in hours) and total RNA was prepared. 20μg of RNA was separated on an agarose gel and transferred to a nylon membrane. The membrane was cut horizontally and simultaneously hybridised to a radiolabelled fragment of gefB DNA generated by PCR (see 2.1.16) and a radiolabelled IG7 probe used as a loading control.
**Figure 3.3** Disruption of the *gefC* gene

A) Schematic representation of the strategy employed to disrupt the *gefC* gene. A 1.5kb fragment of *gefC* cDNA disrupted with a 1.3kb blasticidin resistance cassette was constructed. The 2.8kb construct was used to disrupt the *gefC* gene by homologous recombination. A probe from the *gefC* cDNA was used to screen recombinants by Southern blotting; the sizes of the expected hybridising fragments from AX3 cells and *gefC* disruptants are indicated.

B) Southern blot of *gefC* mutant and parental AX3 DNA. Genomic DNA from *gefC* and AX3 cells was digested with *BsaBI* and *HindIII*, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled *BsaBI/HindIII* fragment of *gefC* cDNA (see 2.1.16). Bands representing the wild type (1.0 kb) and disrupted (2.3kb) *gefC* genes are indicated.
A) Schematic representation of the strategy employed to disrupt the gefD gene. A 1.2kb fragment of gefD cDNA disrupted with a 1.3kb blasticidin resistance cassette was constructed. The 2.5kb construct was used to disrupt the gefD gene by homologous recombination. A probe from the gefD cDNA was used to screen recombinants by Southern blotting; the sizes of the expected hybridising fragments from AX3 cells and gefD disruptants are indicated.

B) Southern blot of gefD mutant and parental AX3 DNA. Genomic DNA from gefD- and AX3 cells was digested with BsaBI and BsrGI, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled fragment of gefD DNA generated by PCR (see 2.1.16). Bands representing the wild-type (0.7 kb) and disrupted (2.0kb) gefD genes are indicated. The central lane of the blot represents a transformant in which the gene disruption construct has integrated randomly into the genome.
Figure 3.5 Disruption of the gefE gene
A) Schematic representation of the strategy employed to disrupt the gefE gene. A 1.2kb fragment of gefE cDNA disrupted with a 1.3kb blasticidin resistance cassette was constructed. The 2.5kb construct was used to disrupt the gefE gene by homologous recombination. A probe from the gefE cDNA was used to screen recombinants by Southern blotting; the sizes of the expected hybridising fragments from AX3 cells and gefD disruptants are indicated.

B) Southern blot of gefE mutant and parental AX3 DNA. Genomic DNA from gefE and AX3 cells was digested with EcoRI, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled fragment of gefE DNA generated by PCR (see 2.1.16). Bands representing the wild-type (5.0 kb) and disrupted (4.0kb and 2.3kb) gefE genes are indicated.
Figure 3.6 Disruption of the gefG gene

A) Schematic representation of the strategy employed to disrupt the gefG gene. A 1.5kb fragment of gefG cDNA disrupted with a 1.3kb blasticidin resistance cassette was constructed. The 2.8kb construct was used to disrupt the gefG gene by homologous recombination. A probe from the gefG cDNA was used to screen recombinants by Southern blotting; the sizes of the expected hybridising fragments from AX3 cells and gefG disruptants are indicated.

B) Southern blot of gefG mutant and parental AX3 DNA. Genomic DNA from gefG' and AX3 cells was digested with BsrGI, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled BsaBl/HindIII fragment of gefG cDNA (see 2.1.16). Bands representing the wild type (1.1 kb) and disrupted (2.4kb) gefG genes are indicated.
gene disruption strategies employed and Southern blots of single \textit{gef} gene disruptants are shown in Figure 3.2, 3.3, 3.4, 3.5 and 3.6.

To confirm that the \textit{gef} gene-disruptant cell lines no longer expressed mRNA from the disrupted gene, total RNA was extracted from cells at various times during development and Northern blots were prepared. In the case of \textit{gefC}, \textit{gefD}, \textit{gefE} and \textit{gefG} the radiolabelled probes cross-reacted so extensively with the largest \textit{Dictyostelium} rRNA band that it was impossible to identify the \textit{gef} mRNA or to determine if it were absent in the RasGEF disruptant cells. However, under the same conditions of stringency a Northern blot probed for \textit{gefB} mRNA was obtained (Figure 3.2C). The approximately 5kb \textit{gefB} mRNA is expressed throughout growth and development with maximal expression between 8h and 12h of development, whilst no \textit{gefB} mRNA is detectable in the \textit{gefB} disruptant. Since all of the other \textit{gef} disruptants were generated using a similar scheme to that employed to generate the \textit{gefB} cells and since the Southern blots indicate only one copy of each gene is present in the genome, it is likely that the targeted \textit{gef} genes in these cell lines will not express functional mRNA. Due to time constraints I decided to assume that gene disruption had resulted in ablation of mRNA expression in all the mutants. However, the slim possibility still remains that they express functional RasGEF mRNA.

3.2.3 Proliferation of AX3 and \textit{gef} Null Mutant Cells in Shaking Axenic

\textit{Dictyostelium} cells lacking RasG or RasS are unable to proliferate in shaking axenic culture. \textit{rasG} cells have a cytokinesis defect and become large and multinucleate (Tuxworth et al., 1997) whereas \textit{rasS} cells are unable to perform pinocytosis (J Chubb and A Wilkins, unpublished data). To determine if any of the \textit{gef} mutants shared this phenotype, the proliferation rates in shaking axenic culture of each \textit{gef} mutant relative to the AX3 parent were assessed (Figure 3.7). The mean doubling time of the \textit{gefC}, \textit{gefD'}, \textit{gefE} and \textit{gefG'} cells was similar to the parental AX3 cells - approximately 8h. However, \textit{gefB'} cells were unable to proliferate. When viewed under a haemacytometer, \textit{gefB'} cells kept in axenic culture for several days did not exhibit the huge increase in size seen in shaking, axenic \textit{rasG'} cells (data not shown). As a consequence of this defect \textit{gefB'} cells have to be maintained on bacterial lawns where they are able to proliferate.
Figure 3.7 Proliferation of AX3 and gef null cells in shaking axenic culture
AX3 and gef null mutant cells were seeded into HL5 medium at 3x10^5 cells/ml and the cell number determined at various time intervals using a haemacytometer. The mean and range of two separate cultures are displayed.
Figure 3.8 Morphology of AX3 and gef null cells
Vegetative AX3 and gef null cells were placed in axenic medium in tissue culture dishes for 24h and visualised by phase contrast microscopy. The white bar represents 20μm.
Figure 3.9 Development of AX3 and gef null cells
AX3 and gef null cells were developed at $3 \times 10^6$ cells/cm$^2$ on nitrocellulose filters for 24h. The white bar represents 2mm.
3.2.4 Morphology of AX3 and gef Null Mutant Cells

*Dictyostelium* cells lacking RasG or RasS have aberrant cellular morphology on surfaces. *rasG* cells appear phase dark, flattened and non-polar (Tuxworth et al., 1997) whereas *rasS* cells are highly polarised (J Chubb and A Wilkins, in press). The cellular morphology of the RasGEF mutants from axenic culture was examined by phase contrast microscopy (Figure 3.8). *gefC*, *gefD*, *gefE* and *gefG* had a phase bright, polar morphology similar to the parental AX3. However, *gefB* cells had a strikingly flattened, polarised morphology similar to that of *rasS* cells.

3.2.5 Development of AX3 and gef Null Mutant Cells

The development of the *gef* mutant cells on nitrocellulose filters was assessed. *gefC*, *gefD*, *gefE* and *gefG* cells exhibited development that was temporally and morphologically indistinguishable from the AX3 parent (Figure 3.9). However, *gefB* cells were delayed in aggregation, formed extremely small aggregates and formed aberrant final structures. *gefB* cells took 36h to complete development as opposed to the 24h taken by the other cell lines. This phenotype is not exhibited by either *rasG* or *rasS* cells.

3.3 Discussion

3.3.1 Summary of Findings

I have identified five partial cDNAs with strong sequence homology with known RasGEFs. *Dictyostelium* cell lines were generated each with a single *gef* gene disrupted by homologous gene replacement. Cells with single disruptions in *gefC*, *gefD*, *gefE* and *gefG* have no apparent cellular or developmental abnormalities. However, *gefB* cells are unable to proliferate in shaking axenic culture, exhibit a highly polar morphology and show delayed and aberrant development. The cell morphology and proliferation defect of *gefB* cells is very similar to that of the *rasS* mutant. Further characterisation of the *gefB* mutant and a possible link between GefB and RasS are the subject of Chapter 4. Two further *gef* genes, *gefA* (clone SSD492) and *gefF* (clone FCBL15), were identified in the Tsukuba, *Dictyostelium* cDNA sequencing project databases by Dr Robert Insall. A *gefA* mutant has been generated which has no apparent phenotype but repeated attempts to generate a *gefF* mutant have failed (Dr Robert Insall personal communication).
3.3.2 Seven *gef* Nulls but Only Two have Phenotypes!

Although the *aleA* (Insall et al., 1996) and *gefB* mutants have clear phenotypes the remaining *gef* null mutants, *gefA*, *gefC*, *gefD*, *gefE* and *gefG*, have no obvious phenotypes. There are a number of explanations for this. Firstly, partial overlap of function or functional redundancy amongst the *Dictyostelium* RasGEFs may allow the cell to compensate for the loss of a single *gef* gene. The generation of strains containing various multiple *gef* gene disruptions may help to address this question. Secondly, the RasGEFs may have highly specialised roles, unable to be compensated for by other RasGEFs, but the phenotypes resulting from *gef* gene disruption may be subtle or not apparent under the laboratory conditions used. Investigation of this will take some time, requiring detailed examination of growth and development under a variety of different laboratory conditions. A good starting point would be to determine the mRNA expression profile of the remaining *gef* genes and hence determine at which stage of the *Dictyostelium* life cycle the RasGEF may be active.

3.3.3 Unresolved Question

I was unable to demonstrate by Northern blotting that the apparent disruption of *gefC*, *gefD*, *gefE* and *gefG* had resulted in ablation of functional mRNA expression from these genes. The cross reactivity of the radiolabelled probes for these genes with the large rRNA band made this impossible. The use of polyA+ RNA would have circumvented this problem. However, Northern blotting demonstrated that the *gefB* disruptants did not express *gefB* mRNA. This demonstrates that, in principle, the insertion of a Blasticidin resistance cassette, containing the strong *act8* transcriptional termination sequence, into the middle of the GEF homology region of the genes would be capable of ablating gene function. Although Southern blotting had produced apparently correct patterns of restriction fragments for each RasGEF mutant, it was still possible that some aberrant recombination event had occurred which had not resulted in disruption of the endogenous gene. To confirm the disruption of the endogenous *gef* genes, PCR was performed on genomic DNA using RasGEF-specific primer-sets that anneal to the RasGEF coding sequence either side of the spot where the Blasticidin resistance cassette should disrupt the gene. PCR using these primer-sets should only amplify a product if the undisrupted gene is present in the genome. For each RasGEF mutant a gene specific primer-set only amplified a product from AX3 DNA or from the other different RasGEF mutant cell lines (data
It therefore seems unlikely that \textit{gefC}, \textit{gefD}, \textit{gefE} and \textit{gefG} disruptants express functional \textit{gefC}, \textit{gefD}, \textit{gefE} and \textit{gefG} mRNA respectively.
4 Characterisation of gefB- Dictyostelium Mutants

4.1 Introduction

Chapter 3 describes the identification of five novel Dictyostelium RasGEF genes. Disruption of only one of these, gefB, resulted in a phenotype similar to that of the previously characterised rasS mutant. This chapter describes the cloning of the full length gefB cDNA and a more extensive characterisation of the phenotype of gefB cells. It also describes an attempt to demonstrate that GefB functions as a RasGEF in vivo and that GefB is a GEF for RasS.

4.2 Results

4.2.1 Cloning of the gefB cDNA

Two separate rounds of screening of a λZAPII cDNA library yielded two overlapping cDNA fragments which together encoded a single contiguous open reading frame. The probes used to clone these partial cDNAs and the exact details of the assembly of the full length cDNA are described in 2.1.16 and 2.1.11.2 respectively. Figure 4.1A schematically illustrates the relative positions of the cloned cDNA fragments and the probes used for their isolation. The predicted open reading frame of the gefB cDNA encodes a protein of 1529 amino acids with a mass of 174kDa (Figure 4.1B). The C-terminal region of this protein contains the putative RasGEF catalytic domain whilst the remainder of the protein has no obvious sequence homology with known proteins. The protein sequence is characterised by stretches of repeated glutamine and asparagine residues. Although this is a feature of many Dictyostelium proteins, its functional significance has not been determined.

During the construction of λ phage cDNA libraries it is possible that more than one cDNA species can be ligated together in tandem into the λ phage vector. It was therefore possible that the putative gefB cDNA did not represent the entire gefB gene but instead was a fusion of the 3' end of gefB with the 5' end of another gene. To exclude this possibility PCR was performed on the gefB cDNA and AX3 genomic DNA using overlapping sets of oligonucleotide primers spanning the entire length of the cDNA (Figure 4.2). With the exception of one primer set, PCR products of the expected size were amplified from both the gefB cDNA and genomic DNA. The primer set used at the extreme 5' end of gefB also produced PCR products from both the cDNA and genomic DNA. In this case, although the PCR product amplified from the
**Figure 4.1 Cloning of the gefB cDNA**

A) Schematic scale diagram of the gefB cDNA showing (i) the complete 4911bp gefB cDNA with unique EcoRI and BsrGI restriction sites; (ii) the probe used for the first round phage library screening; (iii) the 3' cDNA fragment isolated from the first round of phage library screening; (iv) the probe used for the second round of phage library screening; (v) the 5' cDNA fragment isolated from the second round of phage library screening. The BsrGI site was used to assemble the full length gefB cDNA.

B) The gefB cDNA sequence and predicted translation. The putative catalytic domain is coloured red.

---

**A:**

![Diagram of gefB cDNA with restriction sites and probes](image)

**B:**

```plaintext
---

**gefB cDNA sequence and predicted translation:**

```
---

**Predicted translation:**

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---

**Legend:**

```
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Figure 4.2 PCR analysis of gefB
A) Schematic diagram of the strategy used for PCR analysis of the gefB cDNA and genomic locus. The central thick and thin black lines represent the coding and non-coding regions of the gefB cDNA respectively. The relative positions of oligonucleotide primers (red arrows) and the sizes of the PCR products expected to be amplified by each primer-set are indicated. The sequences of all these gefB primers are detailed in section 1.1.8.
B) Agarose gel of the amplified DNA products obtained from PCR analysis of the gefB cDNA and genomic locus. The templates for PCR were 1) the gefB cDNA; 2) AX3 genomic DNA; 3) water as a negative control. The primer-sets used for each reaction are printed above the lanes.
cDNA was of the predicted size, the PCR product amplified from genomic DNA was approximately 150bp larger. This may represent an intron present at the 5' end of gefB. From these results it seemed likely that the cloned cDNA represented a continuous stretch of genomic DNA sequence and as such was the full length gefB cDNA. This has subsequently been confirmed by analysis of overlapping clones from the Dictyostelium genome sequencing project (data not shown).

4.2.2 The Proliferation Defect of gefB' Cells

The preliminary experiments performed in Chapter 3 indicated that gefB' cells were unable to proliferate in shaking axenic culture. To confirm that this phenotype is a general property of cells lacking gefB and not caused by a secondary mutation specific to the clone previously analysed, the growth of four other independently generated gefB' clones was examined. In all cases the gefB' cells were unable to proliferate in shaking axenic culture. Figure 4.3A shows the proliferation of two of these gefB' cell lines in shaking axenic culture relative to that of AX3 cells. The AX3 cells proliferated with a doubling time of approximately 8h whereas even after 100h the gefB' cell lines failed to proliferate. In addition, all clones in which the gefB gene replacement construct had integrated randomly into the genome, without disrupting the endogenous gefB gene, have no obvious proliferation defect (data not shown). It therefore seems likely that the proliferation defect is a function of the disruption of gefB. However, to demonstrate completely that gefB is required for proliferation in shaking axenic culture, the gefB gene must be reintroduced into gefB' cells and result in correction of the proliferation defect. Several attempts were made to transfect gefB' cells with a gefB cDNA under the control of the constitutive act15 promoter (see 1.1.1.3 for details of construct used). To date it has not been possible to transform gefB' cells.

Several Dictyostelium mutants exhibit conditional proliferation defects - although severely impaired in their ability to proliferate in shaking axenic culture, they are capable of normal proliferation when attached to surfaces. Two examples of this phenomenon are rasG' cells which are defective in cytokinesis (Tuxworth et al., 1997) and cells lacking two myosin-I genes, which are severely impaired in pinocytosis (Novak et al., 1995). The proliferation defect of gefB' cells in axenic culture is not conditional – gefB' cells are unable to proliferate in axenic culture even when attached to the surface of a tissue culture dish (Figure 4.3B).
Figure 4.3 Proliferation of AX3 and gefB' cells in axenic culture and bacterial suspension
A) Cell Proliferation in Axenic Suspension Culture. AX3 and gefB' cells were seeded at approximately 2x10^5/ml into flasks of HL5 medium and shaken at 150rpm. At various times the cell number was determined using a haemacytometer.
B) Cell Proliferation in Axenic Medium whilst Attached to a Substratum. As in A), except that cells were plated in 90mm tissue culture dishes.
C) Cell Proliferation in Bacterial Suspension. AX3 and gefB' cells were seeded at approximately 2x10^5/ml into flasks containing a suspension of E.coli B/r in KK2 and shaken at 150rpm. At various times the cell number was determined using a haemacytometer.
D) Cell Proliferation in Bacterial Suspension in Axenic Medium. As in C), except that cells were seeded into a suspension of heat-killed E.coli B/r in HL5 axenic medium.
In all cases the the mean ± SD of three independent cultures was plotted against time on a semi-log graph.
Although unable to proliferate in axenic culture, gefB' cells can proliferate when shaken in a dense bacterial suspension, albeit at a reduced rate relative to AX3 cells. Two independent gefB' cell lines had doubling times of 6h and 7h whereas AX3 cells doubled every 4h (Figure 4.3C).

gefB' cells were capable of proliferation in a suspension of bacteria but not when cultured in axenic medium. The osmolarity of HL5 axenic medium is five-fold higher than that of the KK2 buffer used for the bacterial suspension experiments (Hacker et al., 1997). It was therefore possible that the inability of gefB' cells to proliferate when added to axenic medium was as a consequence of osmotic shock. To address this, the ability of gefB' cells to proliferate in axenic medium containing a dense suspension of heat-killed bacteria was assessed. As in the previous set of experiments the two independent gefB' cell lines had doubling times of 6h and 7h whereas AX3 cells doubled every 4h (Figure 4.3D). It therefore seems likely that the proliferation defect of gefB' cells in axenic medium was not a consequence of the change in osmolarity experienced by the cells when transferred to axenic medium.

4.2.3 gefB' Cells are Defective in Both Phagocytosis and Pinocytosis

Only cells capable of efficient pinocytosis can proliferate in liquid medium. A number of Dictyostelium mutants which have impaired axenic growth show a corresponding impairment in pinocytosis (Hacker et al., 1997). To investigate the fluid phase endocytosis of gefB' cells, their ability to take up TRITC-dextran in axenic culture was assessed (Figure 4.4A). AX3 cells rapidly take up TRITC-dextran reaching a maximum after approximately 125min, whereas both gefB' cell lines analysed showed almost no accumulation of the marker during this time. It therefore seems that gefB' cells are incapable of normal fluid phase endocytosis in axenic medium. This strong pinocytosis defect is consistent with the impaired proliferation of gefB' cells in axenic medium.

Since gefB' cells showed a reduced proliferation rate in bacterial suspension, it was possible that they were also impaired in phagocytosis. Accordingly, the ability of gefB' cells to phagocytose live bacteria was assessed (Figure 4.4B). The two gefB' cell lines reduced the optical density of a bacterial suspension at least 50% slower than AX3 cells. gefB' cells were also severely impaired in phagocytosis of, the much larger, yeast cells – AX3 cells rapidly take up the yeast cells reaching a maximum after approximately 150min, whereas both gefB' cell lines analysed showed almost no accumulation of yeast during this time (Figure 4.4C).
Figure 4.4 Pinocytosis and phagocytosis of AX3 and gefB' cells

A) Pinocytosis. AX3 and gefB' cells were seeded at approximately 5x10^6/ml into flasks of HL5 medium containing 2mg/ml TRITC-dextran and shaken at 150rpm. 1ml of culture was removed at various times and the relative fluid uptake determined using a fluorimeter. The graph shows the data points from three separate experiments, the best-fit curve being fitted to their mean value.

B) Phagocytosis of Bacteria. AX3 and gefB' cells were seeded at approximately 1x10^6/ml into flasks containing a suspension of E.coli B/r in KK2 and shaken at 150rpm. 1ml of culture was removed at various times and the optical density at 600nm determined using a spectrophotometer. The decrease in the density of the bacterial culture was taken as a measure of phagocytosis. The graph shows the mean ± SD of three independent cultures.

C) Phagocytosis of S.cerevisiae. AX3 and gefB' cells were seeded at approximately 2x10^6/ml into flasks of HL5 medium containing a suspension of TRITC-dextran labelled yeast (10^6 cells/ml) and shaken at 150rpm. 1ml of culture was removed at various times and the relative uptake of yeast determined using a fluorimeter. The graph shows the data points from three separate experiments, the best-fit curve being fitted to their mean value.
Therefore, the reduced proliferation rate of gefB' cells in bacterial suspension correlates with an impairment in phagocytosis. It should be noted that the concentration of bacteria used for these phagocytosis experiments is at least ten-fold less than that used for the proliferation assays in 4.2.2.

4.2.4 gefB' Cells have a Highly Polarised Morphology

As mentioned in 3.2.4, gefB' cells have a more polar morphology than the parental AX3 cells. To examine this more closely, Scanning Electron Microscopy was performed on gefB' and AX3 cells (Figure 4.5). AX3 cells have a rounded morphology with some membrane ruffles but few filopods. Circular ruffles or crowns were prominent on the majority of the cells, usually on the dorsal surface. These crowns are the sites of macropinosome formation, the vesicles responsible for fluid phase uptake by axenic cells (de Hostos et al., 1991). In contrast gefB' cells were strikingly more flattened and polarised. There is an increased number of membrane protrusions in the gefB' cells and, although some of these are retraction fibres, many are protruded in the apparent direction of movement suggesting that they are filopods. None of the gefB' cells examined possessed the dorsal circular ruffles typical of normal crowns. The absence of normal crowns correlates well with the severe, fluid phase endocytosis defect of gefB' cells. The morphology of gefB' cells is similar to that of rasS' cells although the polarity of gefB' cells is more pronounced. It is also similar to normal Dictyostelium cells which have developed to an aggregation competent state (De Chastellier and Ryter, 1977).

4.2.5 Development of gefB' Cells

When spotted onto a lawn of bacteria, AX3 cells phagocytose the bacteria, clearing a plaque in the lawn which increases in size as the Dictyostelium cells proliferate. Roughly two zones are visible in the plaque; a zone on the periphery where the bacterial lawn has thinned but is not completely cleared called the “feeding front” and a central zone in which the bacteria have been cleared and the Dictyostelium cells are in various stages of multicellular development. When a similar amount of gefB' cells was spotted onto a bacterial lawn the plaque formed was strikingly different from that of AX3 cells (Figure 4.6A). The plaques of gefB' cells were two or three times larger than those of AX3 cells. Although the bacterial lawn was thinned, gefB' plaques were never fully cleared of bacteria. In addition, although some loose aggregates of cells appeared, the gefB' plaques contained no fruiting bodies or even partially developed structures.
Figure 4.5 Scanning electron micrographs of AX3 and gefB\(^{-}\) cells
Vegetative AX3 and gefB\(^{-}\) cells were prepared for Scanning Electron Microscopy as described in 2.2.8. The white arrow indicates the dorsal, circular ruffles known as "crowns" which are the site of macropinosome formation. Although present on the vast majority of AX3 cells, these structures were never seen on gefB\(^{-}\) cells. The white bar indicates 5\(\mu\)m.
Figure 4.6 Development of AX3 and gefB\textsuperscript{-} cells

A) \(10^3\) vegetative AX3 and gefB\textsuperscript{-} cells were spotted onto lawns of \textit{K.aerogenes} in a 5\(\mu\)l drop of KK2. After seven days incubation at 22\(^\circ\)C the resultant plaques were photographed.

B) Vegetative AX3 (i) and gefB\textsuperscript{-} cells (ii-v) were plated at a density of \(3 \times 10^6\) cells/cm\(^2\) on nitrocellulose filters. Cells were initially photographed after 24h, (i, ii). Due to the small size and delayed development of gefB\textsuperscript{-} aggregates, higher magnification pictures were taken at 24h, (iii, iv), and 36h, (v). The white bar represents 1mm.
The size of plaques formed by gefB' cells is strikingly similar to that of plaques formed by rasS cells however, the multicellular development of rasS cells on bacterial lawns is apparently normal.

As mentioned in 3.2.5 the development of gefB' cells on nitrocellulose filters was also aberrant. AX3 cells completed multicellular development within 24h forming spore containing fruiting bodies (Figure 4.6Bi). After 24h, gefB' cells produced very small aggregates with aberrant structure (Figure 4.6Bii-iv). These structures did not contain mature, detergent-resistant spores (data not shown). After 36h, the gefB' aggregates appeared more like mature fruiting bodies although twisted and small (Figure 4.6Bv). They now contained mature, detergent-resistant spores with a similar viability to AX3 spores (data not shown). The ordered aggregation of cells into mounds and the subsequent tipped mound and slug stages of multicellular development were not apparent during the development of gefB' cells. Moreover, aggregation streams are never formed by gefB' cells when developed on non-nutrient agar (data not shown).

Several mutants exist which are unable to initiate development but when starved in shaking suspension and given pulses of exogenous cAMP they are subsequently able to complete multicellular development (Pitt et al., 1993; Soede et al., 1994). gefB' cells were shaken in KK2 with pulses of 50nM cAMP given every 6min. After 8h, cells were removed and transferred to nitrocellulose filters to assess multicellular development. After 24h the extent of development was similar to gefB' cells which were plated directly onto filters and not pulsed with cAMP (data not shown).

4.2.6 The Developmental Defect of gefB' Cells is Cell Autonomous

To investigate further the nature of the developmental defect of gefB' cells, mutant cells were mixed in a 1:1 ratio with AX3. Although able to develop morphologically normally, the mixed population took 4h longer to complete development. This was most striking after 14h of development when the homogeneous AX3 aggregates had reached the slug stage whereas the mixed population had only formed tight mounds (Figure 4.7A). To determine if the presence of AX3 cells had stimulated development of the gefB' cells, the number of Triton X-100 resistant spores produced by homogeneous and 1:1 mixed populations of AX3 and gefB' cells during development was determined using a haemacytometer (Figure 4.7B). The proportion of spores in the mixed aggregates derived from the gefB' cells, was determined by their resistance.
Figure 4.7 Development of mixed populations of AX3 and gefB<sup>+</sup> cells
A) 2x10<sup>7</sup> vegetative AX3 cells (i), gefB<sup>+</sup> cells (ii) or a 1:1 mix of AX3 and gefB<sup>+</sup> cells (iii) were developed at a density of 3x10<sup>6</sup>cells/cm<sup>2</sup> on nitrocellulose filters for 36h. Pictures were taken after 14h of development. The white bar represents 1mm.
B) 2x10<sup>7</sup> vegetative AX3 cells, gefB<sup>+</sup> cells or a 1:1 mix of AX3 and gefB<sup>+</sup> cells were allowed to develop at a density of 3x10<sup>6</sup>cells/cm<sup>2</sup> on nitrocellulose filters for 36h. Culminants were then washed with 1% Triton X100 and the number of phase bright spores determined using a haemacytometer. Spore numbers are presented as a mean (±SD) percentage of the number of amoebae plated. To determine the number of spores which were derived from gefB<sup>+</sup> cells, serial dilutions of spores were plated onto bacterial lawns and the blasticidin resistance of the resultant colonies determined. The number of blasticidin resistant spores are presented as a mean (±SD) percentage of the total spore number.
to blasticidin. The percentage of spores produced relative to the number of plated amoebae was 68±5% for homogeneous aggregates of AX3 cells, 9.0±2% for homogeneous aggregates of gefB' cells and 38±4% for 1:1 mixtures of AX3 and gefB' cells. If the developmental defect of gefB' cells was not corrected by the presence of AX3 cells then, from these figures, the percentage of the spores in the mixed populations derived from gefB' cells should be approximately 13%. In fact, in the chimeric aggregates the percentage of spores which were derived from gefB' cells was 11±2%. From these data, it appears that the developmental defect of gefB' cells is unaffected by synergistic development with AX3 cells and is, therefore, cell autonomous.

4.2.7 gefB' Cells Have Increased Motility

As previously mentioned, the plaque morphologies of gefB' and rasS' cells on bacterial lawns are strikingly similar. In the case of rasS' cells, this correlates with a three-fold increase in the speed of vegetative cells. To determine whether this was also true of gefB' cells, bacterially grown cells were transferred to tissue culture plates and time lapse images collected to allow analysis of cell speed. AX3 cells move at a rate of 6±1.6μm/min whereas gefB' cells move at 13.3±2.1μm/min. Although not as fast as rasS' cells, the speed of gefB' cells is still significantly greater (two sided t-test, P<0.001) than that of AX3 cells. The increased speed of vegetative gefB' cells coupled with their reduced phagocytic rate provides an explanation for the large diffuse plaques formed by gefB' cells on bacterial lawns. The mutant cells are highly motile, thus can move faster across the bacterial lawn, causing an increase in colony diameter. Furthermore, the gefB' cells only partially clear the bacterial lawn as they move out, a phenotype consistent with the impaired phagocytosis observed in the mutant line.

4.2.8 Expression of Early Developmental Genes

Aggregating Dictyostelium cells are characterised by a highly polarised morphology, a large number of filopods and a dramatic decrease in the rate of phagocytosis correlating with a virtual disappearance of phagocytic cups (De Chastellier and Ryter, 1977; Takeuchi et al., 1983). They are also highly motile, moving approximately three times faster than vegetative cells (Varnum et al., 1986). These are all properties of gefB' cells. It therefore seemed possible that the gefB' cells behave in this fashion because they are prematurely advanced into development to an aggregation-competent state. If this were the case the gefB' cells would probably be expressing
Figure 4.8 ACA and cAR1 expression during early development of AX3 and gefB<sup>+</sup> cells

A) AX3 and gefB<sup>+</sup> cells were starved in suspension, samples were taken at the indicated times (hours), separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. Western Blots were cut horizontally and the appropriate halves probed with polyclonal antisera against the Dictyostelium ACA and cAR1 proteins.

B) As in A), except that cells starved in suspension were given pulses of 50nM cAMP every 6min.
**Figure 4.9** *disIγ* expression during early development of AX3 and *gefB*<sup>-</sup> cells

AX3 and *gefB*<sup>-</sup> cells were developed on nitrocellulose filters at a density of 3x10<sup>6</sup>/cm<sup>2</sup>. At the indicated times (hours), cells were removed and total mRNA was extracted, separated on a 1% agarose gel and transferred to a nylon membrane. The membrane was cut horizontally and the appropriate halves probed with radiolabelled DNA probes for *disIγ* and the loading control IG7.
early developmental or aggregation stage genes under growth conditions. One of the earliest genes expressed during Dictyostelium development is that encoding the lectin discoidin-
y. dis1γ expression is first detectable after two hours of starvation and expression is maximal after approximately five hours, thereafter its expression is repressed by the pulses of extracellular cAMP which constitute the aggregation stimulus (Vauti et al., 1990). AX3 cells show the expected induction and repression of dis1γ during development whereas dis1γ expression is barely detectable throughout the early development of gefB' cells (Figure 4.8). The absence of dis1γ expression in gefB' cells suggested that either the cells could not initiate the developmental program or that they were advanced in development to a stage where dis1γ expression was repressed. The repression of dis1γ expression normally requires the cells to be secreting pulses of cAMP. Two proteins whose expression is induced by starvation and which are essential for cAMP signalling during aggregation are the cAMP receptor, cAR1 and the adenylyl cyclase, ACA. Accordingly, the ability of bacterially grown AX3 and gefB' cells to express cAR1 and ACA when starved in shaking suspension with or without the addition of cAMP pulses was determined (Figure 4.9). These experiments were performed by Dr C Parent in the lab of Dr P Devreotes. They have, at the time of writing, been performed only once and therefore the data must be treated as preliminary. Cells were starved in KK2 to determine if they were able to initiate development and express ACA and cAR1 normally (Figure 4.9A). The timing of development and gene expression using this method is highly variable so to obtain more reproducible data on developmental timing and gene expression cells were starved in KK2 and given pulses of 50nM cAMP every 6min (Figure 4.9B). It is possible that cells can respond normally to exogenous cAMP stimulation but are defective in some aspect of cAMP production. Pulsing allows analysis of the response of cells to cAMP without the extra variable of the cells intrinsic ability to make cAMP. Bacterially grown AX3 cells took more than 8h to induce expression of both cAR1 and ACA upon starvation and this time was reduced to 6h when cells given pulses of cAMP. The gefB' cells, however, showed expression of both proteins after 5h of starvation in KK2 but the proteins were only barely detectable when starvation was accompanied by pulses of exogenous cAMP. These results are currently hard to interpret and the experiments are being repeated with several different gefB' clones to confirm this data. However, it appears that gefB' cells are able to induce the expression of cAR1 and ACA much more rapidly than AX3 cells when starved. Strangely, it appears as if pulses of cAMP serve to inhibit the expression of cAR1 and ACA, suggesting fundamental problems in the functioning of the cAMP signalling system in gefB' cells. One thing that is clear from this...
preliminary data is that gefB' cells do not express the aggregation stage markers cAR1 and ACA during vegetative growth (time=0). Therefore, although they do not express disIγ during early development, this is unlikely to be due to the cells being advanced in development to an aggregation competent state where disIγ expression would be repressed by cAMP. The endocytosis and cell migration phenotypes of the gefB' cells are, therefore, unlikely to be a secondary consequence of precocious development.

4.2.9 Interaction of GefB with RasS

The phenotypes of vegetative gefB' and rasS' cells are strikingly similar. Both have defects in pinocytosis and phagocytosis correlating with a lack of cell proliferation in axenic medium. They also both have enlarged plaque sizes on bacterial lawns and this correlates with increased cell motility. These phenotypic similarities suggest that GeFB may function as a physiological activator of RasS.

The most direct demonstration that GefB can act as a GEF for RasS would be to show that recombinant GefB can catalyse guanine nucleotide exchange on recombinant RasS in vitro. Although a bacterially expressed GST-RasS fusion protein was successfully generated by J.Chubb, I was unable to express soluble, recombinant GefB protein in bacteria either as a GST fusion protein or with a nickel-binding six-histidine tag fused to the N-terminus (data not shown).

Another possible way to demonstrate an interaction between GefB and RasS would be to employ the yeast two-hybrid system (Durfee et al., 1993). The two proteins of interest are expressed as fusions with either the DNA binding domain (DNA-BD) or the transcriptional activation domain (AD) of the S.cerevisiae transcription factor Gal4. The S. cerevisiae strain, Y190, has been developed such that two reporter genes, HIS3 and lacZ are under the control of the GAL1 UAS. If the two fusion proteins interact, the two separate elements of Gal4 are brought into close proximity and allow transcription of the two reporter genes. Expression of lacZ can be visualised by a simple colour reaction. The expression of HIS3 allows rapid growth on His' plates containing 3-aminotriazole (3-AT). The strong interaction of dominant negative Ha-Ras with the catalytic domain of RasGRF has successfully been demonstrated using this system (Mosteller et al., 1995). Four two-hybrid vectors were constructed which express the following fusion proteins; dominant negative (N17) RasS fused to the Gal4 DNA-BD, dominant negative (N17) RasG fused to the Gal4 DNA-BD, the GefB GEF domain (residue 1078 to 1463)
fused to the Gal4 AD, the aimless GEF domain (residue 170-590) fused to the Gal4 AD (see 2.1.1.4 for full details). The dominant negative Ha-Ras/Gal4 DNA-BD and the catalytic domain of RasGRF/Gal4 AD constructs were used as positive controls (Mosteller et al., 1995) whilst the empty plasmids pGADGH (Clontech) and pAS2 (Durfee et al., 1993) as negative controls. Plasmids to be tested were cotransfected into Y190 and transformants selected on both Leu'Tryp' or Leu'Tryp'His' + 3AT synthetic medium agar plates (Figure 4.10). The Dictyostelium rasS and rasG constructs showed no interaction with any of the RasGEF constructs. Similarly, the aimless and gefB constructs both showed no interaction with the Ha-Ras construct. As expected, a strong interaction between the Ha-ras and the rasGRF constructs was observed. Although these data suggests that GefB and RasS are unable to interact, it is not clear that the transformed yeast were expressing the fusion proteins. Western blots of lysates from these transformants probed with antibodies (Clontech) specific for either the Gal4 DNA-BD or Gal4 AD did not reveal any protein expression, even in the positive control cells (data not shown). The possibility exists that the expression levels of the fusion proteins were too low for an interaction to be observed. Unfortunately, the time constraints on this work prevented resolution of this problem.

4.2.10 GefB Functions as a RasGEF in S.cerevisiae

Although sequence homology predicts that GefB will function as a RasGEF, the inability to produce recombinant GefB protein precluded an direct demonstration of this in vitro. The temperature sensitive cdc25-5 S.cerevisiae mutant has previously proved useful for assessing the catalytic activity of putative RasGEFs. Expression of the catalytic domains of the murine RasGEFs, Sos and RasGRF, will complement the yeast mutant, allowing growth at the non-permissive temperature (37°C) (Liu et al., 1993; Martegani et al., 1992). However, expression of the S.cerevisiae BUD5 gene, which encodes a GEF for the Rap-related Bud1 protein, is unable to complement the cdc25-5 mutant (Powers et al., 1991). In an attempt to demonstrate that gefB encodes a functional RasGEF, the putative catalytic domain (residues 1078 to 1463) was transfected into the temperature sensitive cdc25-5 S.cerevisiae mutant under the control of the strong ADH promoter (see 1.1.1.5 for precise details of plasmid construction). Transformants were selected by growth on Leu' synthetic medium agar plates. Transformation with a plasmid expressing the putative catalytic domain of GefB allows growth of the cdc25-5 S.cerevisiae mutant at the non-permissive temperature in a similar manner to a plasmid
expressing the Cdc25 catalytic domain (Broek et al., 1987), whereas transformation with the pAD4 vector (Park et al., 1994) alone does not (Figure 4.11). This strongly suggests that GefB functions as a RasGEF in vivo.
Figure 4.10 Yeast two-hybrid interactions

*S. cerevisiae* strain Y190 was cotransformed with vectors pAS2 and pGADGH expressing various Ras and GEF proteins as fusions with the GAL4 DNA binding domain and the GAL4 transcriptional activation domain respectively. In each case a dominant negative Ras allele or a putative GEF catalytic domain was used. Transformants were selected on Leu'/Trp' synthetic medium agar plates (A) to confirm the presence of both plasmids and on Leu'/Trp'/His' synthetic medium agar plates plus 25mM 3-aminotriazole (3-AT) (B) to select for interacting proteins. Positively interacting clones were further screened for the expression of β-Galactosidase (C). The construction of the RasS, RasG, GEFB and aimless vectors are described in 2.1.11.4 and the Ha-Ras and RasGRF vectors, used as the positive control, are described in (Mosteller et al, 1995). The specific Ras and GEF combinations tested are shown on the diagram. Each clone displayed is representative of the entire population of transformed cells from which it was selected - on Leu'/Trp'/His' + 3-AT synthetic medium agar plates, the vast majority of Ha-Ras/RasGRF expressing clones tested positive for β-Galactosidase activity whereas yeast expressing the other Ras/GEF combinations exhibited no growth on Leu'/Trp'/His' + 3-AT synthetic medium agar plates.
Figure 4.11 Complementation of a temperature sensitive cdc25 S. cerevisiae mutant using the putative catalytic domain of GEFB.
The temperature sensitive cdc25 S. cerevisiae strain, LV25-5, was transformed with plasmids expressing either the catalytic domain of Cdc25 or the putative catalytic domain of GEFB. An empty pAD4 vector transformation was used as a negative control. See 2.1.11.5 for details of the GEFB expression construct. Three clones from each transformation were patched onto two separate Leu' synthetic medium agar plates and incubated at either 30°C or 37°C. Colony growth was assessed after five days.
4.3 Discussion

4.3.1 Summary of Findings

The full-length gefB cDNA was cloned and is predicted to encode a protein of 1529 amino acids with a mass of 174kDa. The C-terminal region of this protein contains the putative RasGEF catalytic domain whilst the remainder of the protein has no obvious sequence homology with known proteins.

Analysis of several independent clones confirmed that gefB' cells are unable to proliferate in shaking axenic culture. This defect is also exhibited by gefB' cells grown in axenic medium when attached to tissue culture dishes. However, gefB' cells are able to proliferate in a suspension of bacteria, although at a reduced rate. These proliferation defects correlate with profound defects in phagocytosis and pinocytosis. gefB' cells have a highly polarised morphology, with an increased number of filopods and an absence of the circular membrane ruffles called “crowns”, which are the sites of macropinosome formation. Vegetative gefB' cells also move twice as fast as the parental AX3 cells. The characteristics of vegetative gefB' cells are similar to those of wild-type cells developed to an aggregation-competent state (De Chastellier and Ryter, 1977). However, analysis of the expression of the early developmental genes dis-Iy, carA and acaA shows that gefB' cells are not in a state of precocious development. Therefore the endocytosis and cell migration phenotypes in the gefB' cells do not appear to be a secondary consequence of precocious development.

gefB' cells are also impaired in multicellular development. Although unable to form normal aggregation streams, gefB' cells form small aggregates with aberrant morphology upon starvation. Development of gefB' cells takes 36h instead the normal 24h and the distinct morphological stages of normal multicellular development (namely the tipped mound, first finger and slug stages) are not apparent in developing gefB' cells. Synergistic development with AX3 cells does not stimulate the development of gefB' cells suggesting that the developmental defect is cell autonomous. When spotted onto a lawn of bacteria the plaques formed by gefB' cells were two or three times larger than those of AX3 cells. Although the bacterial lawn was thinned, gefB' plaques were never fully cleared of bacteria and contained no fruiting bodies or even partially developed structures.
Vegetative gefB' cells have a similar phenotype to rasS cells. It therefore seemed possible that GefB was a physiological activator of RasS. However, gefB did not show an interaction with rasS, rasG or human Ha-ras in the yeast two-hybrid system.

Expression of the putative catalytic domain of GefB was able to complement the temperature sensitive cdc25-5 S.cerevisiae mutant, demonstrating that GefB can function as a RasGEF in vivo.

4.3.2 Endocytosis and Motility

4.3.2.1 Comparison with Known Mutants

The phenotypes of vegetative gefB' and rasS' cells are extremely similar yet both are distinct from all previously reported Dictyostelium endocytosis mutants which can be divided into broadly three categories. The first category of mutants have defects in only one aspect of endocytosis and exhibit no obvious changes in cell speed. Talin null cells are an example of such a mutant. The mutant amoebae are strongly impaired in phagocytosis but not in fluid-phase endocytosis or cell motility (Niewohner et al., 1997). This is apparently caused by a defect in cell adhesion to particles. Similarly, disruption of the clathrin heavy chain gene (chcA) in Dictyostelium cells results in a severe defect in fluid-phase endocytosis but not in phagocytosis or cell speed (O'Halloran and Anderson, 1992; Wessels et al., 2000). A second category of endocytosis mutants have defects in fluid-phase endocytosis, phagocytosis and in other actin dependent processes such as cell movement. Coronin mutant cells and myosin I double mutant cells are example of this type of mutant. Both mutants are impaired in fluid-phase endocytosis, phagocytosis and cell motility (de Hostos et al., 1991; Hacker et al., 1997; Maniak et al., 1995; Novak et al., 1995; Titus et al., 1993). A third category of endocytosis mutants show impairment in some actin dependent processes but this is balanced by stimulation of other actin dependent processes. Dictyostelium cells lacking two class I PI 3-kinase genes have a defect in fluid-phase endocytosis but conversely have an increased cell speed (Buczynski et al., 1997; Zhou et al., 1998). The PI 3-kinase null cells may also have a defect in phagocytosis, however, the two groups which independently performed this work gave conflicting reports of the phagocytic activity of these mutants. Overexpression of the wild-type RacC protein causes an increase in the rate of phagocytosis and a concomitant decrease in the rate of fluid-phase endocytosis (Seastone et al., 1998). Expression of an inhibitory RacC
mutant protein has the opposite effect, increasing the rate of fluid-phase endocytosis at the expense of phagocytosis. Although not defective in endocytosis, Dictyostelium cells lacking the IQGAP related RasGAP1 show an increase in cell speed similar to that of gefB' and rasS' cells (Faix et al., 1998).

Two of the three classes of endocytosis mutant discussed above seem to have defects in the mechanics of actin cytoskeletal reorganisation. The actin binding protein, talin, seems to be involved in the mechanics of cell adhesion to particles and surfaces. Coronin and the myosin I proteins colocalise with actin in sites of actin remodelling and the loss of these proteins results in a general impairment in the remodelling of actin. The third class of mutants seem to be defective in controlling the balance between different actin dependent processes rather than in the underlying mechanics of actin remodelling. The proteins involved are signalling molecules rather than actin binding proteins and one actin dependent process seems to be stimulated whilst another is impaired. It is to this third class of mutants that gefB' and rasS' cells seem most similar, in that they show impaired fluid-phase endocytosis and phagocytosis but a concomitant increase in cell speed. Indeed, one explanation for the phenotypes of vegetative gefB' and rasS' cells is that these proteins regulate the balance between cell motility and endocytosis (discussed in 4.3.2.2). There is also the possibility that the RasGAP1 protein is a downstream component of a GefB/RasS pathway controlling cell speed.

4.3.2.2 Competition between Endocytosis and Cell Motility

The cortical projections involved in endocytosis and cell migration in Dictyostelium, phagocytic cups, crowns and pseudopods, share many similarities. They have similar rates of projection from the cell body, approximately 10μm per minute (Hacker et al., 1997; Maniak et al., 1995). They are all actin-rich structures and have similar sensitivities to cytochalasin-A, which inhibit actin polymerisation (Hacker et al., 1997; Hall et al., 1988; Maniak et al., 1995). In addition to actin, many of the proteins that regulate the movement and assembly of polymerised actin filaments are shared between phagocytosis, pinocytosis and cell migration. These proteins include members of the myosin I family of molecular motors (de Hostos et al., 1991; Novak et al., 1995; Titus et al., 1993; Wessels et al., 1996) and the actin-binding protein, coronin, which is related to the β-subunits of the heterotrimeric G proteins (de Hostos et al., 1991). Both coronin and members of the myosin I protein family colocalise with F-actin at the regions of the cell actively involved in endocytosis and migration. Coronin, for example, is
recruited to the leading edges of pseudopods and coats the cytoplasmic face of crowns and phagocytic cups as they internalise their contents (Maniak et al., 1995).

There is considerable evidence to suggest that the processes of endocytosis and cell migration are in competition with each other. The leading edges of cells are known to compete with each other (Segall and Gerisch, 1989). Observations by Maniak and co-workers (Maniak et al., 1995) suggest competition arises between pseudopods and phagocytic cups as they are projected and that the existence of both structures in the same cell at any one time can only be short-lived. It is likely that competition for the recruitment of coronin, myosin I and other cytosolic proteins occurs between the different cellular protrusions. Indeed, leading edges have been observed to recruit coronin from regressing phagocytic cups, and extending phagocytic cups can acquire coronin from retracting pseudopods. A cell is therefore limited in the extent to which it can phagocytose and migrate efficiently at the same time. The corollary of this is that a treatment causing a cell to be highly motile would inhibit phagocytosis and/or pinocytosis. This has been observed in several situations. Phagocytic cups are almost completely absent in rapidly moving, aggregation-competent wild-type cells and this correlates with a reduced rates of phagocytosis (Maniak et al., 1995). Dictyostelium cells lacking the two class I PI 3-kinase genes are highly motile and are impaired in pinocytosis (Buczynski et al., 1997; Zhou et al., 1998). Similarly, cells lacking the rasS or the gefB gene are fast moving, and are strongly impaired in both pinocytosis and phagocytosis. Conversely, any treatment stimulating endocytosis would be expected to impair cell migration. This is observed in Dictyostelium cells overexpressing the RacC GTPase. These cells show a loss of F-actin from pseudopods, whilst the rate of phagocytosis is three times that of wild-type cells (Seastone et al., 1998). An analogous response is seen in Dictyostelium cells cultured axenically. Over a period of 24h after inoculation into axenic medium, amoebae increase their pinocytic capacity, whilst their motility becomes impaired, suggesting that a decrease in cell locomotion is necessary for the efficient pinocytosis (Clarke and Kayman, 1987). This inverse relationship between the rates of endocytosis and locomotion in a cell together with the molecular similarities between the F-actin structures mediating these different processes suggest the existence of a shared regulatory pathway. Such a regulatory pathway would allow a bias towards either endocytosis or locomotion when required. It is likely that GefB and RasS proteins are involved in this process.
4.3.3 Development

The development of *gefB* cells is both delayed and weak compared to the parental AX3 cells. The lack of normal aggregation streams and the small size of the aggregates formed by *gefB* cells could suggest that the cells have a defect in chemotaxis to cAMP (discussed in 4.3.3.1). However, *gefB* cells also fail to express the *disIγ* gene correctly upon starvation suggesting further problems with the initiation of the developmental program (discussed in 4.3.3.2). Although *gefB* cells are severely impaired in multicellular development the nature of the defect is far from clear. The data from synergy experiments (4.2.6) suggest that *gefB* cells are unable to respond correctly to the differentiation signals of wild-type cells. As discussed in 5.2.7, *Dictyostelium* cells can be induced to differentiate into either stalk or spore cells in low density monolayers. This allows an analysis the intrinsic ability of cells to respond to defined differentiation stimuli, without the added complexity of cell contact and regulative cell-cell interactions (Berks and Kay, 1990; Harwood et al., 1995; Kay, 1989). Had time permitted it would have been informative to use this approach to further investigate the differentiation of *gefB* cells. It seems likely that the results would show *gefB* cells to be intrinsically impaired in their ability to respond correctly to specific differentiation signals.

4.3.3.1 Chemotaxis

The weak aggregation of the *gefB* cells during development may suggest that the cells are defective in chemotaxis to cAMP. Before assessing the chemotactic ability of cells it is first necessary to determine whether the cellular proteins required for cAMP chemotaxis are appropriately expressed upon starvation. Without this knowledge it is not possible to make valid comparisons between the chemotactic ability of *gefB* and AX3 cells. The preliminary results generated by Dr C Parent suggest that *gefB* cells are able to express at least two of the proteins, ACA and cAR1, required for cAMP chemotaxis upon starvation, (4.2.8). However, the developmental timing of cells starved in buffer alone is highly variable and whilst it serves to demonstrate that they are capable of initiating development it does not allow a reliable comparison of the timing of early gene expression. In contrast, pulsing cells with cAMP during starvation serves to synchronise early development in a population of cells and consequently induces the expression of developmental genes in a temporally reproducible fashion. It was therefore surprising that pulsing *gefB* cells with cAMP during starvation almost completely blocked ACA and cAR1 expression. Although further work is required to confirm this result, it
may indicate a defect in the cAMP signalling pathways of gefB cells. Furthermore, it will be difficult to synchronise the developmental timing and gene expression of gefB and AX3 cells, and subsequently compare the cAMP chemotaxis of these cell lines.

In addition to an analysis of early gene expression it will be important to analyse cAMP production and ACA activation in response to cAMP or G-protein activation in gefB cells. Several aggregation defective mutants exist, which express all the components the cAMP relay but which are unable to activate ACA in response to cAMP receptor stimulation or G-protein activation. aleA', piaA', dagA' cells which lack the aimless, pianissimo and CRAC proteins respectively are examples of this type of mutant (Chen et al., 1997; Insall et al., 1994b; Insall et al., 1996). gefB cells may fall into this category.

Uncoupling of the Developmental Program

In several respects, vegetative gefB cells behave like aggregation-competent amoebae (De Chastellier and Ryter, 1977). They are highly polarised, highly motile, and they have a reduced rate of endocytosis. It was therefore surprising to find that the mutant does not express early developmental or aggregation stage marker genes. A possible explanation for this phenotype is that there are independent rather than sequential developmental switches required for the progression of cells to an aggregation-competent state. Loss of one of these control switches, perhaps involving GefB, might give rise to cells that manifest a specific behaviour of starved amoebae, such as rapid migration, without any of the others. GefB may be required to control the balance between endocytosis and motility (discussed in 4.3.2.2) in the context of the early developmental program, favouring endocytic feeding during vegetative growth and motility during early development. GefB may control a developmental check-point which allows the cell to only increase motility once it has utilised all available food and has begun starvation. The loss of GefB may result in an inappropriate increase in motility and concomitant decrease in endocytosis in the absence of starvation. There are at least two secreted factors, CMF and PSF, which control the response of cells to starvation and GefB may be involved in the induction of, or response to, one of these factors. A more detailed investigation of the physiological state of vegetative gefB cells is required to address this question.
4.3.4 The Role of GefB during Growth and Development

It is clear that the loss of GefB has a profound effect on the morphology, motility, endocytic rate and multicellular development of Dictyostelium cells. However, at present, it is impossible to determine whether GefB plays a direct role in controlling these processes or if it has a more indirect function, perhaps controlling expression of other proteins which are themselves the regulators of these cellular processes. An elegant way to address this question would be to construct a gene encoding a temperature sensitive GefB protein (tsGefB) and substitute this for the endogenous gefB gene in Dictyostelium cells. This cell line would have functional GefB protein when cultured at one temperature (the permissive temperature) whilst at another higher temperature (the non-permissive temperature) the protein would become inactive. This approach would allow a direct investigation of the role of GefB in cellular processes. For example, a tsGefB cell line would be able to perform pinocytosis and phagocytosis normally at the permissive temperature. If when shifted to the higher, non-permissive temperature there was an immediate impairment of endocytosis this would suggest a direct role for GefB in the control of endocytosis. Conversely, if upon shifting to the non-permissive temperature, the endocytic rate declined slowly over a period of several hours, this would suggest a more indirect role for GefB in endocytosis. This approach could similarly be used to determine if GefB was required for correct multicellular development. Cells could be starved for various lengths of time at the permissive temperature then shifted to the non-permissive temperature to inactivate GefB. In this way the requirement for GefB at various stages of development could be assessed.

Temperature sensitive mutant proteins have been used extensively in S.cerevisiae to determine protein function. A relevant example of this is the temperature sensitive cdc25-5 mutant (Hartwell et al., 1973). Mutational analysis of the Cdc25 RasGEF protein has identified several mutations which make the protein temperature sensitive such that it is active at 30°C and inactive at 37°C (Camus et al., 1995; Petitjean et al., 1990). The specific amino acid residues identified by mutational analysis of Cdc25 are conserved in GefB. The temperature shift required to inactivate the yeast Cdc25 protein is outwith the physiological range of Dictyostelium cells; temperatures above 28°C cause growth arrest (Loomis and Wheeler, 1980). However, there is some evidence that Dictyostelium proteins with similar mutations to those in temperature sensitive S.cerevisiae proteins will function in a temperature sensitive manner in Dictyostelium cells but that a reduced temperature is needed for protein inactivation. For
example, *erk2* null *Dictyostelium* cells expressing a tsERK2 protein develop normally at 20°C but at 25°C behave like *erk2* null cells and are unable to aggregate (Gaskins et al., 1996). Using temperature shift experiments, it was shown that ERK2 is not essential for aggregation-stage, cAMP pulse-induced gene expression, or for the expression of postaggregative genes, which are induced at the onset of mound formation in response to cAMP in wild-type cells. However, ERK2 is required for the activation of adenylate cyclase. It was also shown that ERK2 is essential for proper morphogenesis and for the induction and maintenance of prespore but not prestalk gene expression. Another example of the use of a temperature sensitive mutant protein in *Dictyostelium* is the MEK1 protein (Ma et al., 1997). The mutant protein contains similar mutations to those found in a temperature sensitive *S.cerevisiae* MEK homologue. The *Dictyostelium* tsMEK1 protein is active at 15°C but inactive at 22°C. The tsMEK1 protein in was expressed in *Dictyostelium mekl* null cells and temperature shift experiments that MEK1 activity is required throughout aggregation for guanylate cyclase activation, but is not essential for proper morphogenesis during the later multicellular stages.

A temperature sensitive GefB protein could also be used to investigate the cellular effect of a dominant active GefB protein. Targeting of GEFs to the plasma membrane brings them into proximity with Ras and results in constitutive Ras activation (Aronheim et al., 1994; Quilliam et al., 1994). Construction of a membrane targeted tsGefB by addition of the CAAX prenylation motif from a *Dictyostelium* Ras protein would allow the real-time effects of GefB activity to be assessed.

4.3.5 Is GefB a Physiological Activator of RasS?

The phenotypes of *gefB* and *rasS* cells are extremely similar although *rasS* cells do not have an obvious developmental defect. Although it was not possible to demonstrate a direct interaction between the two proteins in the yeast two-hybrid system it is still likely that these proteins interact somehow in the cellular context. One way in which this might occur is if GefB was a downstream component of a RasS pathway. This way, the two proteins would not show an interaction in the yeast two-hybrid system whilst still being part of the same signalling pathway. In this scenario, GefB could relay signals from RasS required for endocytosis and motility control and at the same time be a component of other signalling pathways involved in control of the developmental program. Alternatively, GefB may be a direct physiological
activator of RasS, signalling through RasS during vegetative growth but through another Ras during development.

A clearer understanding of the signalling pathways in which GefB is involved will require an investigation of the specificity of GefB. A direct, **in vitro** demonstration of the specificity of GefB may be impossible due to the difficulty in obtaining soluble recombinant protein. However, there are several alternative approaches that could be employed. An epitope tagged version of the GefB catalytic domain and a dominant negative Ras protein fused to GST could be coexpressed in *Dictyostelium* cells. The GST-Ras fusion protein could be purified from cell lysates using glutathione-sepharose and the binding of the GefB catalytic domain assessed by Western blotting using an antibody to the epitope tag. Overexpression of dominant negative Ras proteins alone in *Dictyostelium* cells may also indicate which Ras proteins interact with GefB; the dominant negative Ras proteins which specifically interact with GefB should bind and sequester the endogenous GefB protein resulting in a phenotype similar to that of *gefB* cells. The generation of null mutants of the remaining ras genes would also greatly facilitate the identification of Ras pathways controlled by GefB.

**4.3.6 Other Experiments to Investigate the Function of GefB**

In the short term, a number of experiments would help define the cellular role of GefB. Firstly, it would be informative to determine the subcellular localisation of GefB. Since GefB is implicated in the control of cell motility and endocytosis it may be localised at sites of cortical actin remodelling such as pseudopods, phagocytic cups or macropinocytic crowns. Secondly structure/function analysis would help determine which regions of the protein are important for GefB function. For example, would overexpression of the N-terminal portion of GefB alone have a dominant negative effect by preventing activation of the endogenous GefB protein? Similarly, would overexpression of the catalytic domain alone or a plasma membrane targeted catalytic domain be sufficient to rescue the phenotype of *gefB* cells?

In the longer term a number of experiments would help to discover the signalling pathways in which GefB is involved. Firstly, it will be important to make single and multiple null mutants of all the *Dictyostelium ras* genes and compare the phenotypes with those of GefB. Similarly, the specificity of GefB for the different Ras proteins must be determined. In addition, a yeast two-hybrid screen using the N-terminal region of GefB as “bait” may identify the upstream activating elements of GefB signalling pathways.
In the future it will become increasingly more important to determine the effect of gene disruption on the entire protein complement of the cell. At present, broadly two ways exist to determine the gene or protein complement of one cell type relative to another. Firstly, some form of subtractive hybridisation of the mRNA species can be performed to isolate genes expressed in one cell line but not another. More recently, 2D electrophoresis has been used to identify proteins expressed in one cell line but not another. The identity of these proteins can be determined by mass spectroscopy. Either of these approaches would help define the precise proteomic differences between wild-type cells and gefB cells and therefore help differentiate between problems of signalling and gene expression in gefB cells.
5 Dictyostelium RasD is Required for Normal Phototaxis, but not Differentiation or Pattern Formation

5.1 Introduction

Dictyostelium RasD is a small GTP-binding protein closely related to the mammalian Ras proteins Ha-, Ki- and N-Ras, and is maximally expressed during the multicellular stage of Dictyostelium development (Reymond et al., 1985). The functional studies of RasD published to date have been limited to overexpression of a dominant activated molecule, attempts to generate rasD mutants having failed (Reymond et al., 1986; Reymond et al., 1985). Cells transfected with a rasD gene containing an activating G12T mutation arrest development after forming multi-tipped mounds. These structures express enhanced levels of the prestalk cell specific genes and very low levels of prespore cell specific genes relative to wild-type aggregates but contain no mature spore or stalk cells (Louis et al., 1997). From these data it was inferred that RasD was essential and that it was required for cell-type differentiation and patterning specification (Esch and Firtel, 1991; Esch et al., 1992).

A number of problems with the experimental approach used to investigate RasD function call into question the physiological significance of these previous data. One problem concerns the expression level of the activated RasD protein. The published phenotype of cells expressing the dominant active G12T RasD protein is dependent upon the expression level of the mutant RasD protein (Louis et al., 1997). The mutant cells showed an approximately ten-fold overexpression of the mutant protein. When the expression level was reduced by half, which still represents a five-fold over-expression, no obvious phenotype was observed (Louis et al., 1997). This contrasts strongly with low level expression of an activated RasG protein, which prevents the formation of aggregation streams (Thiery et al., 1992). In this case the RasG protein is expressed at a level no higher than the endogenous RasG protein. In addition, high level overexpression of a dominant negative RasD protein has no obvious effect on development (Dr R Firtel, personal communication) although it has been demonstrated to have a clear biochemical effect in cells (Aubry et al., 1997). Another major problem concerns the expression profile of the activated rasD transgene. Expression of the activated rasD allele was achieved using a fragment of 5' genomic DNA sequence thought to contain all the necessary cis acting elements required for correct expression of rasD (Reymond et al., 1985). Although the endogenous rasD gene cannot be detected until 12h of development (Robbins et al., 1989) this
construct drives transcription in vegetative cells (Reymond et al., 1985). Thus, not only was the activated \textit{rasD} gene over-expressed, it was also temporally mis-expressed. This may have resulted in the activation of non-physiological effectors and given rise to artefactual biological effects. With this in mind, ablation of RasD function by disruption of the \textit{rasD} gene appeared likely to provide greater insight into the physiological functions of the RasD protein.

This chapter describes the generation and characterisation of a \textit{rasD} cell line and the discovery of a possible physiological function for RasD.

5.2 Results

5.2.1 Disruption of the \textit{Dictyostelium rasD} Gene

\textit{Dictyostelium} cells containing a disrupted \textit{rasD} gene were generated by homologous recombination using the strategy shown in Figure 5.1A. A construct was made containing 2.0kb of \textit{rasD} genomic DNA (Reymond et al., 1984) with a blasticidin resistance cassette inserted into the 5' end of the \textit{rasD} coding sequence (see 2.1.11.1). The construct was transfected into AX2 cells, and transformants were cloned following seven days of blasticidin selection. Out of seven independent clones examined by Southern blotting (see 2.1.13), six were found to contain a simple disruption in \textit{rasD} (Figure 5.1B). All clones were indistinguishable in growth and colony morphology and therefore one representative \textit{rasD} clone was used for all subsequent work. The wild-type \textit{rasD} gene was transfected into this clone, using a G418 resistance cassette (see 2.1.11.3), as a control for non-specific effects of transformation. This cell line will be referred to as \textit{rasD}°°.

5.2.2 Determination of RasD Protein Levels

In order to determine RasD protein levels, an antibody specific for RasD was generated by Dr G Weeks. Western blot analysis of bacterially expressed \textit{Dictyostelium} Ras-GST fusion proteins (data not shown) revealed that the antibody exhibited the highest activity against the RasD protein, but still had residual activity against RasG, the closest cellular homologue of RasD, despite extensive cross absorption. Nonetheless, the antibody was sufficiently specific to allow assay of RasD expression.

Western blot analysis of extracts from \textit{rasD} and parental cells at various stages of development using the RasD antibody detected two proteins of slightly different mobility, each with a different expression pattern (Figure 5.2A i and ii). In light of the cross reactivity of the
Figure 5.1. Disruption of the rasD gene
A) Schematic representation of the strategy employed to disrupt the rasD gene. A 2.0kb fragment of rasD genomic DNA with a 1.3kb blasticidin resistance cassette inserted into the unique PstI site at the 5′ end of the rasD coding sequence was constructed. The 3.3kb construct was used to disrupt the rasD gene by homologous recombination. A probe from the rasD coding sequence was used to screen recombinants by Southern blotting; the sizes of the expected hybridising fragments from wild-type cells and rasD disruptants are indicated.
B) Southern blot of rasD− and parental AX2 DNA. Genomic DNA from rasD− and AX2 cells was digested with EcoRI and BclI, separated on a 0.8% agarose gel, transferred to a nylon membrane and probed with a radiolabelled fragment of DNA from the rasD genomic locus. Bands representing the wild type (2.0kb) and disrupted (3.3kb) rasD genes are indicated.
Figure 5.2 Expression of RasD and RasG proteins during Dictyostelium development
A) 20μg of cell extract protein, isolated from AX2 (i) and rasD' (ii) cells at the indicated hours of development, was separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-RasD antibody, as described in Materials and Methods. The rasD' blot was then stripped and immunoblotted with anti-RasG antibody (iii), which confirmed the upper band as RasG.
B) 20μg of cell extract protein, isolated from AX2, rasD' and rasD' rasD cells at 14 hours of development was immunoblotted as above.
antibody with RasG, the blot was stripped and reprobed with the highly specific RasG antibody (Khosla et al., 1994) (Figure 5.2Aiii). This clearly identified the lower mobility band as RasG. Since the higher mobility band showed the same expression profile as rasD mRNA and was not detected during development of rasD' cells, it was concluded that this represents the RasD protein. Analysis of extracts from AX2, rasD and rasD'' cells after 14 hours of development confirmed the complete loss of RasD protein from rasD' cells, and the restoration of expression upon reintroduction of the rasD gene (Figure 5.2B). Since the antibody shows much less activity towards RasG than RasD (approximately five fold)(Dr G. Weeks, personal communication), the strong signal in the Western blot shows that the RasG protein was present in vegetative cells at a considerably higher level than was RasD protein at any stage of development. Similarly, Figure 5.2Aiii shows that, even in developed cells, the amount of RasG present was at least as high as that of RasD. These Western blots were produced by Dr G Weeks.

5.2.3 RasD is Not Required for Normal Cell Proliferation

Although RasG is highly expressed in vegetative cells it has been shown not to be required for cell proliferation (Tuxworth et al., 1997). The RasD protein was not detectable in growing cells but it was still possible that the protein was present and played a role in control of cell proliferation. To address this question, the growth rate of rasD' and wild-type cells in shaking axenic culture was assessed (Figure 5.3). The mean doubling time of rasD' cells and AX2 cells was approximately 11h for both cell types. RasD is therefore not required for normal cell proliferation.

5.2.4 Development of rasD' Cells is Morphologically and Temporally Normal

It has previously been demonstrated that the expression of an activated RasD protein in wild-type Dictyostelium cells has a profound effect. Although aggregation proceeded normally, abnormal multi-tipped aggregates were formed and further development was blocked (Reymond et al., 1986). These results suggested that rasD played an important role in the multicellular development of Dictyostelium. It was, therefore, surprising that rasD' cells have no obvious developmental abnormalities. The mutant cells aggregate normally upon starvation forming streams after 8h, loose aggregates at 9h, tipped mounds at 11h, first fingers and slugs between
Figure 5.3. Proliferation of AX2 and rasD' cells in shaking axenic culture
AX2 and rasD' cells were seeded into HL5 medium at 2x10^5 cells/ml and the cell number determined at various time intervals using a haemacytometer. The mean ± SD of three separate cultures are displayed.
Figure 5.4 Development of rasD" and AX2 cells on non-nutrient agar

Vegetative rasD" and AX2 cells were plated at a density of 3x10⁶ cells/cm² on KK2 agar plates and development observed. rasD" cells form aggregation streams after 8h (A), slugs after 14h (C) and mature fruiting bodies after 22h (E) which are morphologically similar to those of AX2 cells (B, D and F). The developmental timing of both cell types is also comparable. The scale bar represents 0.2mm.
12h and 14h, preculminants at 16h and mature fruiting bodies at 20h. All structures were morphologically indistinguishable from wild-type (Figure 5.4).

5.2.5 rasD' Cells Exhibit Normal Developmental Gene Expression

It has been shown that the over-expression of an activated rasD gene leads to a marked increase in prestalk cell specific gene expression and a decrease in the expression of prespore cell specific genes during development relative to wild-type (Louis et al., 1997). Therefore, the expression of several cell type specific genes was investigated during the development of rasD' cells to determine if the ablation of rasD had an effect on cell type differentiation. Accordingly, rasD' and wild-type cells were allowed to develop on nitrocellulose filters and total RNA isolated at various intervals for Northern analysis. One Northern blot was cut and simultaneously probed for the prestalk specific genes ecmA and ecmB (Jermyn and Williams, 1991) and the prespore specific gene pspA (Early et al., 1988)(Figure 5.5A). The blot was stripped and reprobed with an IG7 probe. IG7 mRNA is expressed at the same level throughout development and was used as a control for loading (Dr K Jermyn, personal communication). A separate Northern blot was cut and simultaneously probed for the prespore specific gene spiA (Richardson and Loomis, 1992) and the IG7 probe (Figure 5.5B). Unexpectedly, there was no significant difference in the timing or expression level of the four genes during development of rasD' cells. This result is dramatically different from the gross changes seen in cells expressing an activated rasD gene, and indicates that RasD is not required for normal proportioning of prestalk and prespore cells in the developing aggregate.

5.2.6 rasD' Aggregates Exhibit Normal Pattern Formation

Previous work had suggested a role for RasD in pattern formation during development (Esch and Firtel, 1991), in particular in the spatial regulation of gene expression. In order to test this hypothesis, both rasD' cells and the parental strain were transfected with lacZ reporter constructs under the control of different prestalk- and prespore-specific promoters. Cells were allowed to develop to either the slug or preculminant stage, then histochemically stained for β-galactosidase activity (see 2.2.7). The prestalk specific ecmAO promoter (Jermyn and Williams, 1991) drives expression of lacZ in a subset of prestalk cells which occupy the anterior fifth of the slug and the basal disc, stalk, tip, and upper and lower cups of the culminant. The ecmO fragment of the ecmAO promoter causes expression in the posterior portion of the prestalk
Figure 5.5 Expression of cell-type specific mRNA during development AX2 and rasD\textsuperscript{−} cells were harvested at the indicated hours of development and total RNA was prepared. 20\(\mu\)g of RNA from each sample was separated on agarose gels and transferred to nylon membranes. Membranes were cut into strips and simultaneously hybridized with radiolabelled A) ecmA, ecmB, pspA and IG7 probes, or B) spiA and IG7 probes.
Figure 5.6 Spatial expression of cell-type specific lacZ reporter constructs during development

rasD\(^{-}\) and parental AX2 cells were transformed with various lacZ reporter constructs, developed on KK2 agar to either the slug (15 hours) or mid-culminant (18 hours) stage, and fixed and stained as described in Materials and Methods. The black bar represents 0.2mm

(A-D): ecmA0-lacZ marker in parental (A,C) and rasD\(^{-}\) (B,D) aggregates

(E,H): ecmB-lacZ marker in parental (E,) and rasD\(^{-}\) (G,) aggregates

(I+J): ecm0-lacZ marker in parental (I) and rasD\(^{-}\) (J) first fingers

(0-R): 50:50 mixes of act15-lacZ marker in rasD\(^{-}\) cells with unlabelled parental (O,P) or rasD\(^{-}\) cells (Q,R).
region in the slug tip and in scattered ALCs in the prespore region of the slug (Early et al., 1993). The ecmB promoter is expressed in a cone of cells in the tip of the slug and in the culminant shows a similar expression pattern to ecmA except that there is no staining of the tip/papilla outside the stalk tube (Jermyn and Williams, 1991). The prespore specific promoter pspA is active in the posterior four-fifths of the slug and in the spore mass of the culminant (Dingermann et al., 1989). For each of these reporter constructs, patterning in rasD' aggregates was not appreciably different from that of the parental strain (Figure 5.6A-N).

One powerful way of identifying otherwise hidden developmental defects is to mix cells of the mutant and parental strains to produce chimeric aggregates, and observe whether the two cell types behave differently. Accordingly, rasD cells constitutively expressing lacZ from the actl5 promoter were mixed with either rasD' cells or the parental strain in a 1:4 ratio. Again, cells were developed to either the slug or preculminant stage, then stained for β-galactosidase activity. In both mixtures, blue staining could be seen randomly distributed throughout the slugs and preculminants (Figure 5.6O-R). Identical staining patterns were also obtained when these experiments were repeated with AX2 cells expressing act15lacZ in place of rasD' cells (data not shown). Similarly, prolonged slugging toward a lateral light source had no effect on the staining patterns obtained in these chimeric slugs (data not shown).

These data suggest that RasD is not essential for the correct spatial regulation of gene expression or for establishing and maintaining the position of cells within the aggregate.

5.2.7 Differentiation of rasD' Cells into Stalk and Spore Cells is Normal

Dictyostelium cells can be induced to differentiate into either stalk or spore cells in low density monolayers, allowing an analysis the ability of cells to respond to defined stimuli, without the added complexity of cell contact and regulative cell-cell interactions (Berks and Kay, 1990; Harwood et al., 1995; Kay, 1989). To assess any intrinsic differences in response to differentiation stimuli between wild-type and rasD' cells, which would be otherwise masked by cell-cell interactions in aggregates, in vitro monolayer experiments were performed.

To induce stalk cell formation amoebae were incubated for 24h in 5mM cAMP to render them competent to respond to DIF, and then incubated for 24h with 0.1μM DIF, which induces terminal stalk cell differentiation. In the second 24h some cells were incubated with a further 5mM cAMP in combination with DIF. In wild-type cells this represses stalk cell differentiation in monolayers. There was no significant difference between the mean number of stalk cells
A:

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<td>67.1 ± 4.2</td>
<td>17.7 ± 3.1</td>
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B:

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<th>% Blasticidin resistant spores</th>
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<td>rasD'</td>
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Figure 5.7 Stalk and spore cell formation by AX2 and rasD' cells

A) Stalk and spore cell formation in low cell density monolayers
To induce stalk cell formation, cells were plated at a density of 1.5x10⁴ cells/cm² in tissue culture plates containing stalk medium plus 5mM cAMP. After 20h, the medium was supplemented with 100nM DIF alone or 100nM DIF plus 5mM cAMP. Spore cells were induced by plating 1.5x10⁴ cells/cm² in tissue culture plates containing spore medium supplemented with 15mM 8-bromo-cAMP or 5mM cAMP. The numbers of stalk cells and spore cells were counted using a phase-contrast microscope after 48h and expressed as a percentage of the total cell count. Each experiment was performed three times, counting a minimum of 500 cells from five separate dishes on each occasion. All data are expressed as the mean ± SD

B) Spore production and viability during normal development
AX2 and rasD cells alone or mixtures of equal numbers of AX2 and rasD' cells were allowed to develop at 3x10⁴ cells/cm² on nitrocellulose filters. Spores were collected after 48h and counted following treatment with 1% Triton X-100. Numbers of spores produced are expressed as a percentage of the number of initial amoebae. Each experiment was performed four times with at least three replicates. Data are expressed as the mean ± SD.
To determine from which cell type the spores were derived, spores from the above experiments were plated on bacterial lawns and the percentage of blasticidin resistant plaques determined. Data are expressed as the mean ± SD.
The viability of spores was assessed by suspension in buffer containing 10mM EDTA, heating for 30min at 37°C and plating at various dilutions onto SM agar in association with Klebsiella aerogenes. The percentage viability was calculated by comparing the colony forming units to the number of phase-bright spores plated. Data are expressed as the mean ± SD.
formed in each condition by wild-type and rasD' cells (two-sided t-test: P>0.8 with 5mM cAMP) (Figure 5.7A).

Spore cells were induced by incubating amoebae with 15mM 8-bromo-cAMP or 5mM cAMP in low-density culture for 48h. 8-Br-cAMP is a membrane-permeable analogue of cAMP which acts intracellularly to activate PKA and induce terminal spore differentiation. cAMP alone is unable to induce terminal spore cell differentiation in wild-type cells. rasD' cells, like wild-type cells only formed spores in the presence of 8-Br-cAMP, cAMP alone was not sufficient. Again, there was no significant difference between the mean number of spores cells formed in by rasD' and wild-type cells (two-sided t-test: P>0.8 with 15mM 8-bromo-cAMP) (Figure 5.7A).

To examine the ability of rasD' cells to differentiate in the context of multicellular aggregates, the production and viability of spores produced during normal development was assessed. Accordingly, equal numbers of wild-type and rasD' cells were allowed to develop on nitrocellulose filters, and the number of Triton X-100 resistant spores produced was determined using a haemacytometer. In an attempt to reveal any differences in the response of wild-type and rasD' cells to differentiation signals, these experiments were repeated with a 1:1 mixture of wild-type and rasD' cells. To determine from which cell type the spores were derived, spores from the above experiments were plated on bacterial lawns and the percentage of blastcidin resistant plaques determined. In each case, wild-type and rasD' cells behaved similarly; there was no difference between the mean number of mature spores produced by wild-type and rasD' culminants, and wild-type and rasD' cells contributed equally to the spore mass in chimeric fruiting bodies (Figure 5.7B). In addition, there was no significant difference between the mean number of viable spores produced by wild-type and rasD' culminants (two-sided t-test: P>0.6) (Figure 5.7B).

Taken together these data suggest that RasD is not essential for the signalling processes involved in cell differentiation.

5.2.8 rasD' Aggregates are Impaired in Phototaxis and Thermotaxis

RasD is expressed maximally in Dictyostelium slugs but deletion of the rasD gene caused no appreciable changes in developmental timing, aggregate morphology, pattern formation, and cell type specification. Dictyostelium cells form slugs after aggregation in order to seek out an optimal environment for the fruiting body. This is achieved by phototaxis and thermotaxis.
Figure 5.8 Phototaxis and thermotaxis of wild-type and rasD· cells
A) Representative Slug Paths of Qualitative Phototaxis Tests on Charcoal Agar. The light source was toward the right of the figure. The parental strain, rasD· strain, and two examples of the rasD· strain rescued with a genomic rasD construct are shown. Amoebae were inoculated onto plates and incubated for 48h in the presence of a lateral light source. Slug trails were transferred to PVC disks, stained with Coomassie Blue, and digitised. The black bar represents 1cm.
B) Accuracy of Phototaxis. Variation of phototactic efficiency with initial cell density. Parent (squares), rasD· cells (triangles), rasG· cells (circles) and rasD· cells rescued with a genomic rasD construct (diamonds) were plated at different densities, and slug trails were observed as in (A). Data were digitised and analyzed according to (Fisher et al. 1981). The inset panel shows the rasD· data plotted on a narrower scale to show slight phototaxis.
C) Accuracy of Thermotaxis. Variation of thermotactic efficiency with mean temperature. Parent (squares), rasD· cells (triangles), rasG· cells (circles) and rasD· cells rescued with a genomic rasD construct (diamonds) were inoculated at 2.4 x 10⁶ cells/cm² onto water agarose plates and incubated in darkness on a heat bar producing 0.2°C/cm temperature gradient at the agarose surface and slug trails were observed as in (A). Data were digitised and analyzed according to (Fisher et al. 1981). Arbitrary temperature units correspond to a temperature range of 14°C (T1) to 28°C (T8), as measured at the centre of the plates in separate calibration experiments.
Accuracy of thermotaxis (k)

Accuracy of phototaxis (k)

Temperature/arbitrary units

Cell density x 10^6 cells/cm^2
which the slugs move with great sensitivity towards sources of light (Fisher and Williams, 1981) and heat (Smith et al., 1982). The tip of the slug, which is composed almost entirely of prestalk cells, is thought to be the region of the slug responsible for the tactic responses. Since the \textit{rasD} gene becomes enriched in prestalk cells it was possible that RasD was involved in these processes. To address this question, Dr P Fisher performed both qualitative and quantitative phototaxis and thermotaxis assays on \textit{rasD} slugs.

When slugs from wild-type cells are kept in the presence of lateral light, they move nearly directly towards the light source (Fisher et al., 1981). The \textit{rasD} slugs were clearly less able to orient correctly (figure 5.8A). To verify that this phenotype was caused by loss of RasD, and not an incidental consequence of transformation or selection, \textit{rasD} \textit{matD} cells were examined. Several different, \textit{rasD} \textit{matD} transformants were examined, all of which exhibited normal phototaxis (figure 5.8A). As a further control \textit{rasD} strains which had been disrupted, using different selectable markers were examined; loss of RasD caused defective phototaxis in every case (data not shown).

A quantitative measurement of phototaxis (Fisher et al., 1981) shows that \textit{rasD} slugs are weakly phototactic. The concentration parameter $\kappa$ describes the accuracy of orientation, with zero reflecting no phototaxis and infinity indicating perfect orientation along the gradient. For the parental strain, $\kappa$ varied from 100 to 500, depending on the cell density (figure 5.8B). The \textit{rasD} slugs were again substantially less effective, but were still positively phototactic, with a $\kappa$ between 5 and 10 (figure 5.8B). Again, the \textit{rasD} \textit{matD} cells and also slugs containing a deletion in the related \textit{rasG} gene, exhibited similar phototaxis to the wild-type.

Almost all reported phototaxis mutants also exhibit impaired thermotaxis and the \textit{rasD} strain is no exception (figure 5.8B). While thermotaxis of \textit{rasD} slugs was clearly defective, thermotaxis of the \textit{rasG} and \textit{rasD} \textit{matD} slugs was similar to that of the parental strain.

5.2.9 \textit{rasD} Aggregates Exhibit a Normal Response to Ammonia

\textit{Dictyostelium} aggregates produce NH$_3$ during development and this has been shown at high concentrations to repel motile slugs and inhibit culmination (Kosugi et al., 1989). NH$_3$ also has effects on individual cell speed and slug tactic movements NH$_3$ (Bonner et al., 1988; Bonner et al., 1989; Bonner et al., 1986). A physiological role for NH$_3$ in phototaxis is as yet unproven but not impossible. If NH$_3$ is involved in phototactic signalling it is possible that phototaxis mutants might show a different response to exogenously added NH$_3$ during development than wild-type. To examine development of wild-type and \textit{rasD} cells in the presence of NH$_3$. 

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Figure 5.9 Development of AX2 and *rasD* cells in the presence of NH₄Cl.

AX2 and *rasD* cells were developed at 3x10⁶ cells/ml on nitrocellulose filters. When the aggregates reached the tipped-mound stage of morphogenesis the filters were transferred onto filter paper soaked in various concentrations of NH₄Cl/20mM MES pH6.1. Development was then allowed to proceed for a further 12 hours. The cell type and the NH₄Cl concentration is shown in the top right of each image.
vegetative cells were allowed to develop to the tipped-mound stage on nitrocellulose filters. The filters were then transferred on to 3MM paper soaked in 25mM MES(pH6.3) containing various concentrations of NH$_4$Cl. Both wild-type and rasD cells were able to culminate successfully in the presence of up to 100mM NH$_4$Cl and were inhibited to the same extent at 300mM NH$_4$Cl (Figure 5.9) in keeping with previous reports (Hopper et al., 1993). This crude experiment demonstrates that NH$_3$ has no appreciable, gross affect on the development of rasD cells when compared to wild-type. Obviously, it does not exclude the possibility that NH$_3$ has a physiological role in phototaxis or that rasD cells are defective in their response to NH$_3$ as a phototactic signal.

5.3 Discussion

5.3.1 Summary of Findings

Dictyostelium cells containing a disrupted rasD gene were generated by homologous recombination. It was predicted that RasD would be essential for correct differentiation and pattern formation. It was therefore surprising that rasD cells proliferate, aggregate and develop indistinguishably from the parental cells. However rasD slugs exhibit a clear defect in phototaxis and thermotaxis; approximately a thirty-fold decrease in the efficiency of orientation towards a light or heat source relative to wild-type slugs.

Further work by Dr P. Fisher has shown that out of the five gef null mutants discussed in Chapter 3, gefE slugs alone have defects in phototaxis and thermotaxis with similar magnitude to those of rasD mutants (data not shown).

5.3.2 The Lack of a Strong Developmental Phenotype

RasD is clearly not essential for development in Dictyostelium. This result does not exclude the possibility that RasD is normally involved in the control of differentiation and patterning and several plausible explanations for this phenomenon are discussed below (see 5.3.2.1 and 5.3.2.2).

5.3.2.1 Functional Redundancy

RasD might play a major role in Dictyostelium development, but its loss could be compensated for by the modulated activity of other Ras proteins in the cell. Indeed, there are at least three other Ras proteins present in the cell during multicellular development - RasB, RasC
and RasG (Daniel et al., 1994; Daniel et al., 1993; Robbins et al., 1989). RasG would be a particularly appropriate candidate, sharing 100% amino acid identity in the effector and effector proximal regions and 82% identity over its entire length, and although rasG mRNA levels decline early in development (Khosla et al., 1990), a substantial quantity of protein is present throughout development (see Figure 5.2A).

Although there may be a level of redundancy in the Dictyostelium Ras proteins it would appear to be far from absolute. Recently, the binding of Ras proteins to the three identified Ras binding proteins, RasGAP1, PIK1 and RIP3 was assessed using the yeast two-hybrid system (Lee et al., 1999). It was found that only RasD and RasB bind RasGAP1, RasG alone binds to RIP3 and that only RasG and RasD bind PIK1. RasC and RasS showed no interaction with any of these Ras binding proteins.

The generation of cell lines with multiple ras gene disruptions would go some way to addressing the question of functional redundancy. A rasS'/rasD' has been generated in our laboratory. The phenotype was similar to that of the single rasS' mutant, with no apparent synergistic effect on development (J Chubb, personal communication). To date, it has not been possible to generate a rasG'/rasD' cell line (J Chubb, personal communication).

5.3.2.2 No Role for RasD in Control of Dictyostelium Differentiation and Morphogenesis?

Alternatively, RasD might not be involved in the differentiation process but instead, might have a highly specific role in the signal transduction pathways common to phototaxis and thermotaxis in the slug stage. If this is correct then the results of previous experiments involving overexpression of the dominant activated G12T RasD protein must be artefactual. A number of lines of evidence support this assertion. Constitutive high level expression of the dominant inhibitory S17N RasD protein in cells has no obvious phenotypic effect (Dr R Firtel, personal communication). This mutant should theoretically bind to and sequester all RasGEFs capable of activating RasD. Although an obvious phenotypic effect is not apparent in cells expressing the S17N RasD protein, it has been demonstrated to have a biochemical effect on ERK2 regulation opposite to that of the G12T activated RasD protein (Aubry et al., 1997). As discussed previously, the published phenotype of dominant activated G12T RasD expressing cells includes impairment of aggregation streams and arrest of development after formation of aberrant, multi-tipped mounds. This phenotype is only observed with cells expressing approximately ten-fold more mutant RasD protein than endogenous RasD protein (Louis et al.,
An approximate five-fold over-expression of G12T RasD does not result in an observable phenotype (Louis et al., 1997). Constitutive high level expression of dominant activated RasD from the strong act15 promoter results in an even more severe phenotype than that of the published mutant – cells are unable to aggregate or initiate development (Dr R Firtel and Dr R Insall, personal communication). These data are surprisingly similar to those obtained from expressing an activated RasG protein. Low level expression of a G12T RasG protein prevents the formation of aggregation streams but does not impair subsequent development of Dictyostelium cells (Thiery et al., 1992), whereas higher level expression inhibits both aggregation and multicellular development (Khosla et al., 1996). Expression of the activated rasD allele was achieved using a fragment of 5' genomic DNA sequence thought to contain all the necessary cis acting elements required for correctly regulated expression of rasD (Reymond et al., 1985). Although the endogenous rasD gene cannot be detected until 12h of development (Robbins et al., 1989) this construct drives transcription in vegetative cells (Reymond et al., 1985). Thus the activated rasD gene is clearly expressed in cells at a time when the endogenous rasD gene is not. Taken together these data suggest that the phenotypic effects of G12T RasD expression are caused by interference with processes not normally regulated by RasD. The high level over-expression and temporal mis-expression of the G12T RasD protein may have resulted in the inappropriate activation of effectors for other Ras proteins such as those controlled by RasG during the initiation of development.

5.3.3 Phototaxis

If RasD plays specific role in the signal transduction pathways common to phototaxis and thermotaxis in the slug stage, what could it be? The behaviour of the rasD' cells in chimeric aggregates suggests that they have no obvious signalling defects. rasD' cells are found scattered randomly throughout these aggregates including all areas of the tip even after prolonged periods of phototactic slugging. It is therefore difficult to imagine that the rasD' cells are unable to respond correctly to light induced signals in the slug tip. One possible explanation that would fit the data is that the rasD' cells can respond correctly to the light-induced chemical signals required for slug turning but instead are unable to generate these specific chemical signals in response to light. Thus, in chimeric aggregates rasD' cells would sense and respond to slug turning signals produced by wild-type cells in a normal fashion but in homogeneous rasD' slugs the cells would not generate the required chemical signals for slug turning in response to light.
and therefore would exhibit impaired phototaxis. A candidate molecule involved in slug turning and induced by light is STF (Fisher and Williams, 1981). It would be very informative to measure the ability of rasD' slugs to secrete STF in response to light.

5.3.4 Future Experiments

A number of experiments would help to determine the role of RasD in tactic slug movements. Measurement of the phototactic ability of slugs composed of cells expressing S17N RasD or low levels of G12T RasD may confirm the role of RasD in tactic slug responses. To address the question of functional redundancy amongst the Ras proteins, the ability of other Dictyostelium Ras proteins, expressed at physiological levels using a rasD promoter, to rescue the phototaxis defect of the rasD' cells could be assessed. The nature of the phototactic defect could be investigated by measuring the phototactic ability of chimeric slugs comprised of wild-type and rasD' cells. Null mutants of two of the putative Dictyostelium Ras effectors, PIK1/PIK2 double null and RasGAP1 null cells, are able to form slugs. Measurement of the phototactic ability of slugs of these mutants may identify the signalling pathway through which RasD controls slug tactic movement. An in vivo analysis of cell motility in the tips of wild-type and rasD' slugs may reveal differences in the response of cells to factors such as light, ammonia or STF.
References


Appendix

Strain Designations

AW1  rasD null in AX2 background
AW2  rasD null expressing ecmAO-lacZ
AW3  rasD null expressing ecmO-lacZ
AW4  rasD null expressing ecmB-lacZ
AW5  rasD null expressing pspA-lacZ
AW6  rasD null expressing act15-lacZ
AW7  AX2 expressing ecmAO-lacZ
AW8  AX2 expressing ecmO-lacZ
AW9  AX2 expressing ecmB-lacZ
AW10 AX2 expressing pspA-lacZ
AW11 AX2 expressing act15-lacZ
AW12 gefB null in AX3 background
AW13 gefC null in AX3 background
AW14 gefD null in AX3 background
AW15 gefE null in AX3 background
AW16 gefG null in AX3 background