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Department of Microbiology

The University of British Columbia
Vancouver, Canada

Date May 10, 1998

DE-6 (2/88)
ABSTRACT

*Dictyostelium discoideum* contains at least 5 ras genes. It had been previously demonstrated that the expression of an activated *rasG* gene in *D. discoideum* resulted in cells which were unable to aggregate and a subset of which had a large, flattened morphology (Khosla et al., 1996. Mol. and Cell. Biol. 16: 4156-62). To investigate the role of the *rasB*, *rasC*, and *rasS* genes, an activating mutation (a glycine to threonine substitution at position 12) was made in each of the genes. The mutated genes were then expressed under the control of the inducible discoidin promoter. Overexpression of the *rasC*-G12T and *rasS*-G12T genes produced no evident abnormalities in cell morphology, growth, or development. Thus, *rasC* and *rasS* are not functionally equivalent to *rasG*, although no clues as to their roles in the cell were revealed. Overexpression of the *rasB*-G12T gene resulted in the appearance of morphologically aberrant amoebae: a large proportion of the cells were large and flat with an increase in cell surface projections known as crowns. These cells also became multinucleate and failed to divide by traction-mediated cytofission when grown on a surface. The response of these cells to nutrient stimulation and to azide were normal, as was their motility. Growth and development of the cells was unaffected by overexpression of *rasB*-G12T. These data suggest that the defects observed in the *rasB*-G12T cells are due to altered regulation of cell division processes since other actin and myosin-dependent processes are unimpaired.

The growth and developmental defects caused by overexpression of *rasG*-G12T were not seen in the *rasB*-G12T cells. Comparison of
the morphological phenotypes of \textit{rasB-G12T} and \textit{rasG-G12T} cells revealed that the \textit{rasB-G12T} cells were, on average, larger and more highly multinucleate than the \textit{rasG-G12T} cells. The predominant actin-based cell surface structure seen on each of the two was also different: \textit{rasB-G12T} cells frequently possessed crowns, while \textit{rasG-G12T} cells more commonly displayed filopodia. Thus, it appears that \textit{rasB} and \textit{rasG} have at least some distinct roles in the cell, although they may overlap in some cellular functions.
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The effect of mutated rasB-G12T, rasC-G12T, and rasS-G12T genes on cell morphology
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How does RasB differ from RasG?

Effect on cell growth and development.

Effect on morphology.

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BS</td>
<td>Bonner's salts buffer</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DIF</td>
<td>Differentiation Inducing Factor</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemilluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid (disodium salt)</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>kb</td>
<td>kilobases</td>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>MOPS</td>
<td>N-morpholinopropanesulfonic acid</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene-20-sorbitan monolaurate</td>
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Finally, I thank my parents for their unwavering love and support and for believing in me. You too, Brendan.
To my parents, for everything.
CHAPTER 1

INTRODUCTION

"Difference is not and cannot be thought in itself, so long as it is subject to the requirements of representation... difference in itself appears to exclude any relation between different and different which would allow it to be thought. It seems that it can become thinkable only when tamed - in other words, when subject to the four iron collars of representation: identity in the concept, opposition in the predicate, analogy in judgement and resemblance in perception. Every other difference, every difference which is not rooted in this way, is an unbounded, uncoordinated, and inorganic difference; too large or too small, not only to be thought but to exist. Ceasing to be thought, difference is dissipated in non-being."

Gilles Deleuze, *Différence and Repetition*

"Différance is a structure and a movement that cannot be conceived on the basis of the opposition presence/absence. *Différance* is the systematic play of differences, of traces of differences, of the spacing by which elements relate to one another. This spacing is the production, simultaneously active and passive, of intervals without which the 'full' terms could not signify, could not function."

Jacques Derrida, *Positions*

The primary objective of this thesis was to analyze the effects of expressing an activated form of the *rasB*, *rasC*, and *rasS* genes in *Dictyostelium discoideum* with particular reference to differences seen between expression of these genes and expression of an activated *rasG* gene. In the Introduction, I will review the literature on the *ras* gene superfamily, particularly the *ras* subfamily. I will place special emphasis on the roles of *ras* and rho-related proteins in regulating cell morphology. I will then discuss *D. discoideum,*
focusing on the cytoskeleton and regulation of cell morphology, then reviewing what is known about \textit{ras} and \textit{ras}-related genes in this organism.

\textbf{The \textit{ras} gene superfamily}

The \textit{ras} superfamily of genes is composed of three major groups: the \textit{ras}, \textit{rho}, and \textit{rab} subfamilies. Membership in a group is based upon amino acid identity and, in some cases, on the protein function (Valencia \textit{et al.}, 1991; Kahn and Der, 1992). All members of the superfamily encode small, monomeric guanine nucleotide binding proteins which closely resemble the heterotrimeric G proteins (reviewed in Downward, 1990; Valencia \textit{et al.}, 1991; Downward, 1992; Marshall, 1993; Hall, 1992, McCormick, 1994; Zerial and Huber, 1995). The \textit{ras} sub-family includes the proto-oncogenes \textit{K-ras}, \textit{H-ras}, and \textit{N-ras}, which share 85\% amino acid sequence identity (Valencia \textit{et al.}, 1991; Bollag and McCormick, 1991), as well as the genes encoding the closely related (sharing \textasciitilde55\% identity with H-Ras) R-Ras and TC-21 proteins (Cox \textit{et al.}, 1994; Graham \textit{et al.}, 1994), and the \textit{rap} genes (which show approximately 50\% sequence identity with H-Ras) (Bokoch, 1993; Noda, 1993). The proteins encoded by the \textit{ras} sub-family of genes have also been highly conserved throughout evolution. The presence of the same sub-families in divergent species suggests some degree of conserved function (reviewed in Valencia \textit{et al.}, 1991). Functional equivalence of the proteins has been confirmed experimentally in some cases; for example, the mammalian \textit{H-ras} has been shown to be able to complement \textit{ras}-
strains of \textit{S. pombe} and \textit{S. cerevisiae} (Nadin-Davis \textit{et al.}, 1986; Kataoka \textit{et al.}, 1985; DeFeo-Jones \textit{et al.}, 1985).

Members of the \textit{rab} sub-family encode proteins sharing approximately 30\% amino acid homology with the Ras proteins (Rothman and Orci, 1992). Proteins in this sub-family have been shown to be involved in vesicle transport between organelles and in the production of synaptic vesicles (Rothman and Orci, 1992; Sudhof, 1995).

The \textit{rho} gene sub-family encodes Rho, Rac, and Cdc42 proteins, all of which share \textasciitilde 30\% amino acid identity with the Ras proteins (Nobes and Hall, 1994). Rho, Rac, and Cdc42 are capable of inducing, respectively: the formation of actin stress fibers and focal adhesions; membrane ruffles and lamellipodia; and filopodia (Ridley and Hall, 1992; Ridley \textit{et al.}, 1992; Kozma \textit{et al.}, 1995; Nobes and Hall, 1995).

\textbf{Biological roles of Ras proteins}

Since their original identification in the Harvey and Kirsten rat sarcoma viruses (reviewed in Barbacid, 1987), more than 50 members of the \textit{ras} superfamily have been found in a wide range of eukaryotes (Barbacid, 1987, Hall and Zerial, 1995). As the number of known \textit{ras} genes has increased, so has the diversity of functions attached to these proteins. Ras proteins have been found to play roles in transformation, cell proliferation, and differentiation.

Originally identified due to their transforming ability, \textit{ras} genes have since been found to be mutated in up to 80\% of certain types of human tumors (reviewed by Bos, 1989). The most frequent of these
mutations are at codons 12, 13, and 61 and cause the protein to exist in a constitutively active, GTP bound, form. In vitro, activated Ras transforms cells and bypasses the requirements for serum and anchorage dependent growth (Hall et al., 1984; Paterson et al., 1987).

Studies on a variety of mammalian cell types have provided extensive evidence for the role of Ras proteins in cell proliferation. Injection of H-Ras into NIH 3T3 cells produces a transformed morphology and stimulates quiescent cells to enter S-phase of the cell cycle (Feramisco et al., 1984; Stacey et al., 1984). The mitogenic response of fibroblast NIH 3T3 cells to serum-derived growth factor is blocked by the injection of the Ras specific monoclonal antibody Y13 259 (Mulcahy et al., 1985). Numerous studies have shown that growth stimulation of various cell lines with serum or peptide growth factors such as PDGF and EGF increases the level of active Ras-GTP (Satoh et al., 1990; Filmus et al., 1994; Quincoces and Leon, 1995; Winston et al., 1996).

In some cases, a role for Ras proteins in differentiation and development has also been shown. PC12 pheocytomroma cells develop into neural type cells upon treatment with NGF in a process which is dependent upon Ras (Bar-Sagi and Feramisco, 1985; Satoh et al., 1987). Also, transfection with ras can induce differentiation of 3T3-L1 fibroblasts into adipocytes (Benito et al., 1991). In C. elegans, the Ras homolog let60 is involved in vulva development (Breitel et al., 1990; Wassarman et al., 1995; Kayne and Sternberg, 1995). In D. melanogaster, activation of Ras disrupts normal cell fate specification in the compound eye. When Ras is microinjected into Drosophila
embryos, it disrupts the terminal cell fates of posterior cells (Simon et al, 1991; Lu et al, 1993).

**Regulators and effectors of Ras**

All Ras family proteins cycle between an active GTP-bound state and an inactive GDP-bound state (Fig. 1). The binding and hydrolysis of GTP by Ras is regulated by other cellular proteins. The switch from the GDP-bound to the GTP-bound state is mediated by guanine nucleotide exchange factors (GEFs) which stimulate the dissociation of GDP from Ras. These proteins may also affect the translocation of Ras to the plasma membrane (reviewed in Feig et al., 1992; Boguski and McCormick, 1993). The exchange factors are linked to cell surface receptors via adaptor proteins such as GRB2 and Shc (reviewed in Schlessinger, 1994; Pawson, 1995). Several Ras exchange factors have been identified, possibly linking Ras to different receptors (Boguski and McCormick, 1993).

Ras signaling terminates when GTP is hydrolyzed to GDP. The weak endogenous GTPase activity of Ras is greatly enhanced by GTPase activating proteins (GAPs) (Boguski and McCormick, 1993). At present, five mammalian GAPs, including the prototype p120GAP, have been described (Boguski and McCormick, 1993; Wittinghofer et al., 1995). Numerous GEFs and GAPs which regulate other Ras superfamily proteins have been identified (Boguski and McCormick, 1993), suggesting that the regulatory mechanisms controlling these
Figure 1. The Ras signaling pathway.
proteins are similar to that of Ras. In addition to regulating Ras, some evidence suggests that GAPs may also function as downstream effectors of Ras (Marshall, 1996).

A number of downstream effectors which interact with GTP-bound Ras and have been proposed to transmit the signal have been identified. Raf is the best characterized of these; others include Ral guanine nucleotide dissociation stimulator (Ral-GDS) and phosphatidylinositol-3-OH kinase (PI 3-kinase) (reviewed in Katz and McCormick, 1997).

Raf, a cytoplasmic serine/threonine kinase, was the first mammalian effector identified for Ras (Marshall, 1995). Ras interaction with Raf leads to activation of the MAP kinase cascade which is believed to convey growth and differentiation signals to the nucleus resulting in specific patterns of gene expression (Hill et al., 1993; Marias et al., 1993; Cowley et al., 1994).

Since the identification of Raf, it has become apparent that additional effector pathways downstream of Ras exist (White et al., 1995; Joneson et al., 1996). Activation of Raf is not sufficient for efficient transformation by Ras and, in fact, it has been demonstrated that inactivation of the PI 3-kinase pathway inhibits transformation (Rodriguez-Viciana et al., 1996). Direct biochemical evidence for an interaction between Ras and PI 3-kinase was provided by experiments designed to identify proteins in cell extracts which bound to Ras in a GTP-dependent manner (Rodriguez-Viciana et al., 1994). PI 3-kinase interacts specifically with the GTP-bound form of Ras and is activated both in vitro and in vivo as a result of this interaction (Rodriguez-Viciana et al., 1994; Kodaki et al., 1994).
Activation of PI 3-kinase has also been shown to have effects on
the cytoskeleton, specifically inducing membrane ruffling in response
to PDGF and insulin through a pathway believed to involve Rac
(Kotani et al., 1994; Wennstrom et al., 1994; Nobes and Hall, 1995).
Since other experiments have demonstrated that Rac and Cdc42 are
downstream of Ras (Nobes and Hall, 1995), it may be that PI 3-
kinase provides the link between Ras and Rac, thus mediating the
effects of Ras on the actin cytoskeleton.

An exchange factor for the Ras-related protein Ral, Ral-GDS, has
also been shown to interact with Ras in experiments using the yeast
two-hybrid system (Hofer et al., 1994; Spaargaren and Brischoff,
1994: Kikuchi et al., 1994). Ras activation leads to activation of Ral
via Ral-GDS and two related proteins (Urano et al., 1996). The role of
Ral is currently unclear although some evidence points to a possible
function in the regulation of phospholipase D (Urano et al., 1996).

**Action of Ras superfamily proteins on cell morphology**

The cytoskeleton is required for the structural integrity and shape of
the cell and consists of a dense network made up of multiple protein
components. These proteins combine to form a matrix which is both
rigid and capable of resisting mechanical stress. The matrix must
also be capable of rapid disassembly and reassembly as the cell
undergoes processes such as cellular division, developmentally
regulated changes in cell shape, cell movement, and chemotaxis.
Extensive cytoskeletal changes also accompany cell transformation
and occur during the treatment of cells with growth factors (Wang et al., 1993; Heidemann, 1993).

Three primary structures make up the cytoskeleton: microtubules, intermediate filaments, and microfilaments. Microtubules are composed primarily of tubulin; intermediate filaments are made up of a mixture of proteins; and microfilaments are made up of actin. It is the actin cytoskeleton which is of the greatest interest here since it is known to be subject to modification by Ras-related proteins.

The actin cytoskeleton consists of monomeric actin subunits (G-actin) which are assembled into filaments (F-actin) which are in turn bound together in a matrix by a variety of actin crosslinking proteins (reviewed in Matsudaira, 1991). Numerous actin associated proteins regulate the extent of actin polymerization by preventing the addition of further subunits of G-actin to the existing actin filament, by sequestering G-actin subunits so as to prevent their assembly, and by severing actin fibers (Luna and Condeelis, 1990; Button et al., 1995).

Mammalian cells contain several structures which contain a variety of actin filaments (reviewed in Matsudaira, 1991). Stress fibers are composed of long bundles of actin filaments that traverse the cell and are linked to the extracellular matrix via integrins and focal adhesion complexes. Focal adhesions are involved in cell adhesion to the substratum and consist of membrane protein complexes linked to stress fibers. Lamellipodia are broad but thinly spread actin-containing regions observed at the periphery of adherent cells. Membrane ruffles are similar to lamellipodia and are made up of actin cross-linked in wavy, curtain-like structures on the cell surface.
and cell periphery. Filopodia are narrow spike-like projections which are proposed to have a sensory function.

In addition to regulation by actin-associated proteins, the actin cytoskeleton is acted upon by force-generating myosin molecules. Myosin II, the conventional force-generating myosin, forms filaments which bind to actin fibers and generate contractile forces (reviewed in Spudich, 1994; Ruppel and Spudich, 1995). Myosin is involved in processes such as muscle-driven movement, the maintenance and alteration of cell morphology, and cell motility. In *D. discoideum*, myosin II is required for a normal cell morphology. Cells in which the myosin II gene is disrupted become large, flat, and multinucleate (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). Many unconventional myosins have also been isolated (classes I and III-VIII) (Titus *et al*., 1994; Bement *et al*., 1994); the roles of such unconventional myosins have not yet been ascertained. A typical mammalian cell is estimated to have at least 11 myosins (1 or 2 members from each class) and it has been speculated that each myosin family member may mediate a different actin-based process (Bement *et al*., 1994).

**Effects of the Ras superfamily on the cytoskeleton**

Structures such as membrane ruffles, stress fibers, and focal adhesions are produced in response to growth factors and appear to involve members of both the Ras and Rho sub-families. Microinjection of activated H-Ras into fibroblasts results in a refractile, transformed morphology and an increase in membrane
ruffling and blebbing (Feramisco et al., 1984; Bar-Sagi and Feramisco, 1986). Microinjection of activated K-Ras into Swiss 3T3 cells also causes an increase in membrane ruffling and a decrease in stress fiber formation (Yoshida et al., 1992).

Similar studies have identified roles for Rho sub-family members in the regulation of the cytoskeleton. Microinjection of an activated RhoA protein into serum starved Swiss 3T3 cells causes rapid stress fiber and focal adhesion formation (Ridley and Hall, 1992). The same effects are seen when cells are treated with either serum or lypophosphatidic acid (LPA), suggesting that RhoA may mediate some of the effects of growth factors on the cytoskeleton. Specifically blocking RhoA function in vivo by microinjection of a dominant negative form of RhoA or by treatment of cells with the Rho-specific inhibitor, C3 transferase, blocked the effects of either serum or LPA (Ridley and Hall, 1992).

When activated Rac1 is microinjected into Swiss 3T3 cells, actin filament accumulation at their plasma membrane is stimulated, resulting in the formation of lamellipodia and membrane ruffles (Ridley et al., 1992). Induction of membrane ruffling by growth factors or an activated H-Ras protein can be blocked by a dominant negative Rac1 protein, leading to the conclusion that Rac1 is required for growth factor induced membrane ruffling. A delayed response to Rac1 activation is the formation of actin stress fibers. The previously described results of blocking Rho activity with C3 transferase suggest that growth factors act through Rac1 to stimulate both a Rho-dependent response (stress fiber formation) as well as a Rho-independent response (membrane ruffling).
The human Cdc42 protein has also been shown to regulate actin structures. Microinjection of human Cdc42 into Swiss 3T3 cells results in the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). A similar effect is observed when cells are treated with the growth factor bradykinin. This effect is blocked when cells are treated with a dominant negative form of Cdc42, suggesting that Cdc42 is required for formation of filopodia. Microinjection of cells with Cdc42 also causes stress fiber formation and membrane ruffling, indicating that Rac and RhoA have also been activated. Specific inactivation of the endogenous Rac and Rho proteins with a dominant negative Rac1 protein and C3 transferase blocks the formation of these structures, suggesting that Cdc42 acts in part through Rac and Rho.

Figure 2 summarizes the relationship between Rac, Rho, and Cdc42. A similarly complex pathway linking multiple Ras-related proteins exists in S. cerevisiae. In this system, bud site selection depends on the GTPase cycle of the Rap1 homolog RSR1/BUD1 which is, in turn, linked to the GTPase cycle of Cdc42. A series of Rho proteins have been proposed to function downstream of Cdc42 and may act on the cytoskeleton. The protein Cdc24 forms a potential link between RSR1/BUD1 and Cdc42 since it both binds to RSR1/BUD1 and acts as an exchange factor for Cdc42 (Chant and Stowers, 1995).
Intrinsic spatial signals for budding Mating pheromone

Figure 2. GTP cascades controlling cell morphology and bud formation.

A) The pathway that controls cytoskeletal polarization during vegetative division or mating of *S. cerevisiae*.

B) The proposed pathway that controls the formation of filopodia, lamellipodia, and stress fibers in mammals.

Continuous arrows indicate physical contact, broken arrows indicate indirect interactions. This figure is modified from Chant and Stowers, 1995.
**Dictyostelium discoideum life cycle**

*D. discoideum* is a simple eukaryote with a distinctive life cycle in which cell division and cell differentiation remain largely distinct (Loomis, 1982; Firtel *et al.*, 1989; Mann *et al.*, 1994). *Dictyostelium* live as individual amoebae which feed on bacteria but which, when starved, aggregate and begin a differentiation process (reviewed in Gross, 1994; Kay, 1994; Firtel, 1995; Fig. 3). Approximately 3 hours after the onset of starvation, certain cells randomly initiate a cAMP-mediated relay by producing and secreting cAMP. Nearby cells respond by moving toward the originator cell and by themselves beginning to produce pulses of cAMP. As a result of this chemotaxis and signal relay, an aggregate of ~$10^4$ cells is formed. The aggregate becomes compact and forms a tip which eventually elongates to form a finger-like structure. At this point, the initial differentiation of two functionally distinct cell types (prestalk and prespore) begins. The finger falls over and continues to elongate, forming a migrating pseudoplasmodium or slug. The slug tip (~15% of the cell total) is made up of prestalk cells, while the rear 85% of the slug consists primarily of prespore cells. When the slug reaches an area of suitable temperature and light conditions, development continues with the formation of a mature fruiting body containing a spore head suspended above the substratum by a column of vacuolated stalk cells.
Figure 3. The life cycle of *D. discoideum*.

Black areas represent prestalk and stalk cells while clear areas represent prespore and spore cells. The time of development is shown in hours.
Signal transduction events during the life cycle of *D. discoideum*

Vegetative *Dictyostelium* cells feed on bacteria which they locate by sensing and chemotaxing towards the bacterial metabolites folate and pterin, then take up by phagocytosis (Gross, 1994; Firtel, 1995). The response to folate and pterin is not well understood, but it is believed to involve folic acid receptors (Hadwiger *et al.*, 1994) coupled to a heterotrimeric G protein subunit, Gα4 (Hadwiger *et al.*, 1994; Wu *et al.*, 1995; Burdine and Clarke, 1995). Axenic strains of *Dictyostelium* take up nutrients by pinocytosis. Two autocrine factors, prestarvation factor (PSF), and conditioned medium factor (CMF) act as indicators for the availability of nutrients and for cell density, respectively (Clarke *et al.*, 1987; Clarke *et al.*, 1988; Gomer *et al.*, 1991; Yuen *et al.*, 1995).

Soon after the onset of starvation, amoebae develop all the components making up the cAMP-mediated signaling system involved in the process of aggregation and early gene expression (reviewed in Gross, 1994; Firtel, 1995; Williams, 1995; Chen *et al.*, 1996; Loomis, 1996). The response to cAMP during early development includes: 1) the activation of adenylate cyclase leading to secretion of cAMP and propagation of the chemotactic signal relay, 2) activation of guanylate cyclase and phospholipase C (PLC) which are involved in chemotaxis, and 3) expression of a number of cAMP pulse-induced genes, the products of which are required for aggregation. The cAMP signal relay machinery includes a serpentine cAMP receptor, cAR1, (Saxe *et al.*, 1996), a heterotrimeric G protein,
Ga2 (Kumagai et al., 1989; Kumagai et al., 1991; Kimmel and Firtel, 1991), an aggregation specific adenylate cyclase, ACA (Pitt et al., 1992), a cytosolic regulator of adenylate cyclase, CRAC (Insall et al., 1994), cAMP specific phosphodiesterase, PDE (Franke et al., 1991), and its inhibitor, PDI. At least two other components are also required; the MAP kinase, ERK2 (Segall et al., 1995), and cAMP-dependent protein kinase A, PKA (Simon et al., 1989; Harwood et al., 1992). Cells overexpressing an activated form of rasG are unable to aggregate (Khosla et al., 1996) and both ERK2 and a GEF are required for aggregation (Segall et al., 1995; Insall et al., 1996), suggesting that RasG may be involved in carrying the signal from the cAMP receptor to adenylate cyclase.

After aggregation, higher levels of cAMP accumulate and pulse-induced gene expression is repressed while postaggregative stage gene expression (including the transcription factor GBF (G-box binding factor)) and differentiation into prestalk and prespore cells are triggered (reviewed in Gross, 1994; Williams, 1995; Loomis, 1996). cAMP promotes the development of both prestalk and prespore cells at this point, but at later stages inhibits stalk development while continuing to promote spore cell development. Several distinct cAMP pathways may also come into operation since four developmentally regulated cAMP receptors and 8 heterotrimeric Ga protein subunits have been identified. Protein kinase A is essential for normal development and has been shown to act directly to induce prespore cell type specific gene expression and subsequent spore cell formation (Maeda et al., 1992; Hopper et al., 1993 and 1995; Hopper and Williams, 1994; Mann et al., 1994) as well as
promoting stalk cell maturation during fruiting body formation (Maeda, 1988; Simon et al., 1989; Harwood et al., 1992; Kubohara et al., 1993; Inouye and Gross, 1993). The substrates for phosphorylation by PKA during Dictyostelium development have not yet been identified. Glycogen synthase kinase 3 (GSK-3)(Harwood et al., 1995) and ERK2 (Gaskins et al., 1996) also play an important role in prespore differentiation, however, the signalling pathways by means of which they exert their effects are presently unknown.

Differentiation inducing factor (DIF) promotes prestalk and suppresses prespore cell differentiation (Kopachik et al., 1983; Williams et al., 1987). DIF is believed to act via a cytosolic binding protein, STAT (signal transducer and activator of transcription) (Insall and Kaye, 1990; Kawata et al., 1997). Finally, ammonia and Ca^{2+} also influence morphogenesis and cell type differentiation by as yet unknown mechanisms (reviewed in Gross, 1994).

**ras and ras-related genes in D. discoideum**

*D. discoideum* contains at least 6 highly related *ras* superfamily genes (Reymond et al., 1984; Robbins et al., 1989 and 1990; Daniel et al., 1993 a and b) (Table 1). *rasG* is expressed maximally during growth and early development and shares 68% amino acid identity with H-Ras (Robbins et al., 1989). *rasD* is expressed primarily during post-aggregative development and shares 65% amino acid identity with H-Ras (Reymond et al., 1984; Esch and Firtel, 1991). *rasB*, *rasC*, and *rasS* are less conserved, with amino acid homologies to the
canonical H-ras of 59%, 56%, and 54% respectively (Daniel et al., 1993 a and b). Each of these three proteins contains significant amino acid differences in the effector-proximal domain, suggesting that each may interact with a different effector. mRNAs for rasB and rasC are constitutively expressed, while rasS mRNA is expressed specifically during early aggregation (Fig. 4). The unique patterns of expression further point towards possibly divergent roles. A homolog of the human rap1A gene product, rap1, has also been identified in D. discoideum (Robbins et al., 1990). The amino acid sequence of the protein is 76% identical to Rap1A and only 56% identical to the other D. discoideum ras subfamily members (Daniel et al., 1993 a and b).

The Ras proteins have not been definitively linked to any specific growth or developmental signalling pathway in D. discoideum. Overexpression of an activated form of RasD (G12T) results in an aberrant multitipped aggregate and a block in further development (Reymond et al., 1986; Louis et al., 1995). Prestalk gene expression in these cells is enhanced while prespore gene expression is virtually abolished (Louis et al., 1997), leading to the hypothesis that RasD may play a role in cell type determination. A rasD null mutant strain, however, shows a normal pattern of development (Williams et al., 1997).

Overexpression of an activated form of RasG (G12T) results in cells which are unable to aggregate and which display marked morphological abnormalities. Many of these cells have a large, flattened appearance and nuclear staining has revealed a defect in
### Table 1. Ras proteins in *D. discoideum*

A) Percentage amino acid sequence identity between *Dictyostelium* Ras proteins and H-Ras. Numbers above the diagonal represent identity over the full length protein, numbers below the diagonal represent identity over the first 80 N-terminal amino acids. This table is adapted from one in Daniel, 1993.

B) Comparison of the primary structures of the effector proximal region of *Dictyostelium* Ras proteins and H-Ras.
Figure 4. The expression of ras genes in *Dictyostelium discoideum*. Time after onset of development is indicated in hours. High levels of expression, as detected by Northern blot analysis, are indicated by higher bars and moderate levels by lower bars. This figure was adapted from Daniel, 1993.
cytokinesis with an average of 1.7 nuclei per cell in the rasG-G12T
transformant compared to 1.2 nuclei per cell in the wild type Ax2
cells (Khosla et al., 1996). Overexpression of the wild type RasG also
results in a cytokinesis defect, although development is normal. A
rasG null strain is able to differentiate normally although these cells
grow extremely slowly and have a severe defect in cytokinesis.
Actin distribution is also altered and these cells show an increase in
the number of filopodia (Tuxworth et al., 1997).

Eight rho and 5 rab genes have been isolated from D. discoideum
(Bush et al., 1993 a and b; Vithalani et al., 1995). Disruption of one
of the rho-related genes, racE, results in cells which complete normal
development but are unable to grow in suspension and have a defect
in cytokinesis (Larochelle et al., 1996).

Ras regulatory proteins have also been identified in D. discoideum.
The gapA and Rga/DGAP1 genes encode two homologs of the
mammalian Ras-GAP (Adachi et al., 1994; Faix and Dittrich, 1996; Lee
et al., 1997). Which of the multiple D. discoideum Ras proteins these
GAPs interact with is currently unknown, but it is interesting to note
that mutations in both genes produce defects in cytokinesis. Finally,
the aimless gene, which encodes the D. discoideum homolog of
RasGEFs, has been identified (Insall et al., 1996). aimless null
mutants are severely impaired in both chemotaxis and activation of
adenylyl cyclase, suggesting a possible role for Ras in the processing
of chemotactic signals through G-protein-coupled receptors.
Cell morphology of *D. discoideum*

*D. discoideum* has been used extensively as an experimental model for analysis of the function of actin associated proteins and of myosin using both gene targeting and gene replacement techniques (Patterson *et al.*, 1991). Several myosin I genes have been disrupted (Jung and Hammer, 1990; Jung *et al.*, 1993; Peterson *et al.*, 1995) resulting in only subtle changes in motility in the mutants. Disruption of either the heavy or light chain of myosin II, however, produces cells which are enlarged, flattened, and multinucleate (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). These mutant strains are unable to cap surface proteins, have reduced cortical tension, and no longer contract and detach from the substratum when treated with azide (Pasternak *et al.*, 1989). These cells are motile, however, and are able to aggregate and continue development up to the mound stage (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Springer *et al.*, 1994). Together, these defects suggest a role for myosin II in cytokinesis, receptor capping, control of cell morphology, and morphogenesis.

Myosin II is structurally and functionally similar to non-muscle myosins in other organisms (reviewed in Tan *et al.*, 1992; Spudich, 1994). The protein is a hexamer composed of two heavy chain subunits, two essential light chains, and two regulatory light chains which combine in a structure with two globular heads and a helical coiled-coil tail. The hexamers assemble into polar filaments which contract in a manner analogous to muscle myosin filaments.
Both myosin heavy chain and regulatory light chains are subject to phosphorylation (reviewed in Tan et al., 1992; Hammer III, 1994). Three myosin heavy chain kinases have so far been identified; one of these is a member of the protein kinase C family (Maruta et al., 1983; Cote and Bukiejko, 1987; Ravid and Spudich, 1989; Ravid and Spudich, 1992). Phosphorylation of the myosin heavy chain prevents assembly of filaments capable of generating force. Amino acid substitutions which mimic the phosphorylated state of the myosin II heavy chain prevent the assembly of myosin onto the actin cytoskeleton, while disruption of three sites of phosphorylation results in overassembly of myosin II on the actin cytoskeleton in vivo (Egelhof et al., 1993). The myosin light chain is phosphorylated by the myosin light chain kinase which causes an increase in actin activated ATPase activity of myosin II in vitro (Griffith et al., 1987) but does not regulate the assembly/disassembly of myosin II (Tan et al., 1992).

When D. discoideum amoebae respond to a pulse of cAMP, there is a rapid accumulation of F-actin into the cytoskeleton (peaking 5 s after the cAMP stimulus), followed by phosphorylation of the myosin II heavy chain and association of myosin II with the actin cytoskeleton (25-30 s after the cAMP stimulus) (McRobbie and Newell, 1983; McRobbie and Newell, 1984; Berlot et al., 1985; Berlot et al., 1987). The myosin light chain is also phosphorylated in response to cAMP (Berlot et al., 1985). It has been shown that the transient increase in cGMP which follows a cAMP stimulus is important for the regulation of the myosin II responses (Liu and Newell, 1991; Liu et al., 1993; Liu and Newell, 1994). In strains with
a defective cGMP-specific phosphodiesterase, cAMP stimulation results in persistent elevated levels of cGMP. In these strains, there is a prolonged association of myosin II with the cytoskeleton and a delay in myosin II light and heavy chain phosphorylation. Although it has been proposed that guanylate cyclase is regulated via Ca\(^{2+}\) influx (Newell and Liu, 1992), the pathways mediating myosin phosphorylation (and myosin kinases) in response to cAMP are not yet fully understood.

Actin represents approximately eight percent of all cellular protein in *D. discoideum*. Seventeen to twenty actin genes are present in the genome, and most are transcribed and translated (Romans and Firtel, 1985; Romans *et al.*, 1985). Although transcription of individual actin genes is developmentally regulated, the resulting proteins are essentially identical in primary sequence (Vandekerrckhove and Weber, 1980), suggesting that this multiplicity of genes may regulate actin levels, rather than interactions between actin and other proteins at various developmental stages.

Tyrosine phosphorylation of actin has been associated with shape changes. When *Dictyostelium* amoebae are transferred from starvation buffer to growth medium (Schweiger *et al.*, 1992; Howard *et al.*, 1993), or when oxidative phosphorylation is inhibited (Jungbluth *et al.*, 1994), the cells retract all pseudopods, round up, and become immobile. In both cases, the time course of the cell shape changes correspond to the appearance of a phosphotyrosine epitope on actin (Schweiger *et al.*, 1992; Howard *et al.*, 1993; Jungbluth *et al.*, 1994). Disruption of the tyrosine phosphatase PTP1, but not PTP2, caused a more rapid and more prolonged
phosphorylation of actin and an acceleration of cell rounding when starved cells were returned to growth medium (Howard et al., 1993; Howard et al., 1994). Overexpression of PTP1 decreased the amplitude and duration of actin phosphorylation and also diminished the cell rounding response (Howard et al., 1993). Aside from the possible involvement of actin phosphorylation, however, this cell rounding response is not well understood.

Linkages between cAMP binding to its receptor and subsequent cytoskeletal rearrangements during both growth and development are being elucidated through a combination of biochemical and molecular genetic approaches (reviewed in Noegel and Luna, 1995). In the resting cell, G- and F-actin are present in approximately equal amounts. Within a few seconds of cAMP stimulation, F-actin increases by 50-60% and becomes incorporated into cytoskeletal structures (McRobbie and Newell, 1985; Hall et al., 1988). This peak of actin assembly is rapidly followed by a decrease to almost the initial level by 20 seconds. Then, starting at about 30 seconds post-stimulation, a longer-lasting phase of polymerization occurs that can persist for several minutes. These changes in actin assembly correlate with the retraction and extension of pseudopods, respectively (Futrelle et al., 1982; Hall et al., 1988).

A wide range of actin-associated proteins are involved in the regulation of such complicated responses (reviewed in Noegel and Luna, 1995). In Dictyostelium, the major proteins responsible for sequestering G-actin appear to be profilin I and profilin II. In addition to its G-actin binding activity (which reduces the rate and extent of actin polymerization in vitro), profilin (I and II) has been
shown to bind PIP2, to potentiate ADP/ATP exchange on actin monomers, and to promote the efficient addition of ATP-actin to the rapidly assembling ends of filaments. *Dictyostelium* mutants lacking both profilins show altered growth in shake suspension, decreased motility, and defects in cytokinesis and development.

Due to the presence of monomer-sequestering proteins, localization of actin assembly in the cell is controlled by proteins that regulate elongation at the growing "plus" ends of actin filaments (reviewed in Noegel and Luna, 1995). Such localized actin assembly is required for pseudopod formation during chemotaxis and for regulation of other three-dimensional actin based structures such as filopodia and membrane ruffles. Sites of actin filament assembly in the cell are regulated by proteins that promote the nucleation of new actin filaments and by proteins that control the number of accessible growing ends on existing filaments through regulated capping and/or filament severing activities (reviewed in Noegel and Luna, 1995). Ponticulin, hisactophilin, talin, cap32/34, protovillin, and severin appear to be the major proteins performing these roles in *Dictyostelium*. Actin binding, capping, and severing activities are inhibited by PIP2 and other negatively charged phospholipids (Yin et al., 1990; Eddy et al., 1993). Three-dimensional cortical networks are stabilized by actin crosslinking and bundling proteins, these stabilizing proteins include the highly conserved proteins, filamin (ABP-240), gelation factor (ABP-120), α-actinin, fodrin (ABP-220, spectrin), EF-1a (ABP-50), p30a, and comitin (reviewed in Noegel and Luna, 1995). The precise roles and regulation of these proteins are not yet clear.
Most of the actin-associated proteins already mentioned are structurally and/or functionally similar to cognate proteins described in vertebrates. F-actin binding proteins which are, so far, unique to Dictyostelium include hisactophilin, coronin, coactosin, cortexillin I and II, and p30b (Faix et al., 1996; reviewed in Noegel and Luna, 1995).

A number of actin-associated proteins have been eliminated by gene disruption techniques with little or no effect on cell morphology or motility (reviewed in Noegel and Luna, 1995), however profilin I/II double mutants and mutants lacking two actin filament crosslinking proteins (α-actinin and gelation factor) display impaired development. The profilin double mutants as well as cortexillin double mutants and cells lacking coronin all show impaired cytokinesis, similar to the myosin II null mutants. Although the signal transduction pathways involved in these cytoskeletal defects is not yet known, it is interesting to note that both overexpression of Gα1 and the expression of a calmodulin antisense RNA also produce large multinucleate cells (Kumagai et al., 1991; Liu et al., 1992).
CHAPTER 2

MATERIALS AND METHODS

Materials

G418 (Geneticin), restriction endonucleases and modifying enzymes were purchased from GIBCO BRL (Canada). Sequenase was purchased from United States Biochemical. FITC-phalloidin, streptomycin, ampicillin, and folate were purchased from Sigma (USA). Radiolabelled $[^{32}\text{P}]d\text{ATP}$ was purchased from ICN Flow labs (Canada), $[^{35}\text{S}]d\text{ATP}$ was purchased from Dupont NEN Canada Inc. X-ray film was purchased from Kodak (Canada), filters were purchased from Millipore (USA), the enhanced chemiluminescence kit for Western Blot analysis was purchased from Amersham (Canada), bacteriological peptone and yeast extract were purchased from Oxoid (UK). All other chemicals were purchased from Fisher Scientific Co. (Canada) or BDH (Canada). Hoescht 33258 dye was a gift from Dr. R.E.W. Hancock's laboratory (UBC). Oligonucleotides were synthesized by the NAPS unit (UBC).

Y13 259 antibody was a gift from Dr. Mike Gold (UBC). Goat anti-rat IgG antibody conjugated to horseradish peroxidase and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Amersham (USA).

The *E. coli* strains DH5αF', XL1MRF', and RZ1032 were used for bacterial transformations. The genotype of DH5αF' is: F'*/ermD* A1 hsdR17(rk-mk+) supE44 thi-1 recA1 gyrA1(Nal') relAI D(lacZYA-
argF)U169 deoR (80dlacD(lacZ)M15) (Raleigh et al., 1989). The genotype of XL1MRF' is: endA1 hsdR17(rK-mK+) supE44 thi-1 I-recA1 gyrA96 relA1(lac-) F[proAB lacIqZ M15 Tn10(tetR)] (Stratagene). The genotype of RZ1032 is: HfrKL16PO/45[lysA(61-62)] dut1 ung1 thi-1 relA1Zbd-279::Tn10supE44 (Kunkel et al., 1987). The pVEII vector was donated by Wolfgang Nellen and modified to remove the discoidin ATG translation start site and add the Bluescript polylinker by Meenal Khosla (Rebstein et al., 1993).

**D. discoideum growth and differentiation**

The parental axenic strain Ax2 strain of *D. discoideum* that was used in all experiments was grown axenically in HL5 medium (Watts and Ashworth, 1970) (14.3 g neutralized bacteriological peptone, 7.15 g yeast extract, 0.96 g Na2HPO4, and 0.486 g KH2PO4 per litre of water) with gyratory shaking at 150 rpm at 22°C or on SM nutrient agar (10 g glucose, 10 g neutralized bacteriological peptone, 1 g yeast extract, 1 g MgSO4·7H2O, 1.55 g NH2PO4·H2O, 1 g KHPO4 and 20 g Bacto-agar per litre of water) in association with *E. aerogenes*. Cell numbers were determined in duplicate with a hemocytometer. The transformed Ax2 strains were maintained in HL5 medium in the presence of 10 µg/mL G418 (Geneticin).

*D. discoideum* development on filters was initiated as previously described (Khosla et al., 1990). Exponentially growing cells were harvested at a density of between 1X10^6 and 2X10^6 cells/mL by centrifugation at 700Xg for 2 minutes then washed twice in Bonner's salt (10 mM NaCl, 10 mM KCl, and 2 mM CaCl2) (Bonner, 1947).
2.5X10^7 cells were then plated on a 4.0 cm diameter nitrocellulose filter (pore size = 0.45 μm) resting on a Bonner's salt (BS) saturated pad in a 60 mm petri dish. The filters were incubated at 22°C in a moist chamber.

*D. discoideum* development following growth on bacteria was accomplished by pipetting 1-5 mL of *Dictyostelium* cells in HL5 medium at a density of 2X10^6 cells/mL onto a freshly inoculated plate of *E. aerogenes* on a SM nutrient agar plate. Plates were incubated at 22°C and after the *D. discoideum* cells had consumed the bacteria (usually 4 days), development ensued in the region depleted of bacteria.

**Induction of the discoidin promoter**

To maintain strains containing genes under the control of the discoidin *dis1a* promoter in a suppressed state, 1 mM folate was added to the HL5 medium. To induce maximal expression from the discoidin promoter, cells were grown for 24 hours in HL5 in the absence of folate and then transferred to conditioned medium. Conditioned medium contains a pre-starvation factor (PSF) (Clarke *et al.*, 1987; Clarke *et al.*, 1988) which induces expression from the discoidin promoter (Rathi *et al.*, 1991). Conditioned HL5 medium was prepared by growing Ax2 cells to a density of 1X10^6 cells/mL, removing the cells by centrifugation, and filtering the medium through a 0.2 μm pore size nitrocellulose filter. In some experiments, induction was achieved by growing cells to a density of between 1X10^6 and 2X10^6 cells/mL in HL5 in the absence of folate.
Transformation of *D. discoideum*

*D. discoideum* cells were transformed by the calcium phosphate precipitation technique (Nellen *et al.*, 1984) in Bis-Tris HL5 (Egelhof *et al.*, 1989). A total of 8X10^6 exponential phase Ax2 cells were incubated with 15 μg of vector DNA in the form of a calcium phosphate DNA precipitate for 4 hours, then the cells were given a 2 minute osmotic shock, as described previously (Early and Williams, 1987). Transformants were selected in HL5 medium containing 30 μg/mL G418, 50 μg/mL streptomycin, 50 μg/mL ampicillin, and 1 mM folate. Colonies were visible after 10-14 days. Individual colonies were picked using a pipette and transferred initially to 24-well plates and subsequently to 100 mm plates. Once cell growth was well established, stable transformants were maintained in shake suspension in HL5 medium containing 10 μg/mL G418, 50μg/mL streptomycin, 50μg/mL ampicillin, and 1 mM folate.

**Analysis of cell morphology**

To examine cell morphology, cells were plated at 3X10^3 cells/cm² on a glass coverslip in a 60 mm petri dish and incubated for 24 hours either in the presence of 5 mL of conditioned HL5 medium or in HL5 medium containing 1 mM folate to induce or repress the discoidin promoter, respectively. To evaluate the ability of cells to undergo nutrient stimulus induced cell rounding, the cells were then starved for 8 hours in BS buffer and then exposed to 5 mL of HL5 medium.
Cells were photographed on in inverted microscope under 250X magnification before HL5 stimulation and then 5 and 10 minutes afterwards.

To determine the effect of azide on cell substratum adherence, an adherent monolayer of cells was exposed to 2mM sodium azide (NaN₃) in HL5 medium for 3 minutes with swirling at 60 rpm, in a modification of a previously described procedure (Pasternak et al., 1989; Springer et al., 1994). The plates were gently rinsed 3 times to remove floating cells. The coverslip was photographed before and after azide treatment.

**Flow cytometric analysis**

Cells were grown in either conditioned medium or in HL5 medium containing 1 mM folate in shake suspension and analyzed directly in medium using a flow cytometer (Becton-Dickinson) running Lysis II software. Ten thousand events were analyzed for forward and side light scatter for each sample. Mean values were determined for forward scatter events above a cutoff point of 200.

**Nuclear staining**

Cells were plated at 3X10³ cells/cm² on a glass coverslip in a 60 mm petri dish and incubated for 24 hours either in the presence of 5 mL of conditioned HL5 medium or in HL5 medium containing 1 mM folate to induce or repress the discoidin promoter, respectively. To determine nuclear number, the adherent cells were washed, fixed
with 3.7% formaldehyde in BS for 10 minutes, then washed 3 times with BS. Cells were then permeabilized for 5 minutes in -20°C acetone, rehydrated in BS, overlaid with 0.0005% Hoescht dye #33258 for 5 minutes, then washed with BS. The glass coverslips were briefly dried before mounting on glass slides using 50% glycerol as mounting solution and viewed with a Zeiss Axiophot microscope equipped with epifluorescence. All photographs were taken using Kodak TMAX400 film.

**Actin distribution**

To observe F-actin distribution, cells adherent to a glass coverslip were fixed, washed, permeabilized, and rehydrated as described for nuclear staining. Cells were then overlaid with 0.2 mM FITC-phalloidin in BS for 10 minutes to stain F-actin. Cells were washed in BS, dried briefly and mounted on glass slides using 50% glycerol as the mounting medium. Cells were viewed with a Zeiss Axiophot microscope equipped with epifluorescence. All photographs were taken using Kodak TMAX 400 film.

**Scanning electron microscopy**

A total of $2 \times 10^3$ cells were plated on 12 mm round glass coverslips in 24 well plates and incubated for 24 hours in the presence of either 2 mL of conditioned medium or 2 mL of HL5 medium containing 1 mM folate to induce or repress, respectively, the discoidin promoter. The cells were fixed in 2.5% glutaraldehyde buffered with 100 mM
sodium cacodylate, pH 7.4. The cells were treated for 30 minutes with 1% OsO₄ + 2% tannic acid, then for 30 minutes with 1% OsO₄ in 100 mM cacodylate buffer, pH 7.4. The attached cells were dehydrated in a graded ethanol series and critical point dried with liquid carbon dioxide. The coverslips were mounted on specimen stubs, sputter coated with gold, and viewed with a Cambridge 250T scanning electron microscope.

**Motility assay**

Random motility was measured by plating approximately 1X10⁶ cells in a volume of 1 mL on 2% agar plates containing PDF/MES. The average maximal distance (determined as the field of view furthest from the origin of migration with five cells visible) migrated by the cells from the marked point of origin was measured after 24 hours using a stage micrometer.

**Plasmid DNA preparation**

Plasmid DNA was isolated from *E. coli* strains DH5αF', RZ1032, and XL1MRF' cells using an alkaline lysis miniprep procedure (Maniatis *et al.*, 1989) or a PEG-precipitation large scale procedure (Maniatis *et al.*, 1989). Competent *E. coli* cells of each strain were prepared using the rubidium chloride technique and transformation of competent cells was performed as described in Maniatis *et al.*, 1989. Transformed *E. coli* cells were selected on LB ampicillin plates (10 g
Bacto-tryptone, 5 g Bacto yeast extract, 10 g NaCl, 15 g Bacto-agar, pH 7.0, plus 60 μg/mL ampicillin).

**Sequencing**

Single stranded DNA was produced by infection of transformed DH5αF' cells with M13 KO7 helper phage, followed by PEG precipitation, isolation on glass filters, and elution into TE (Maniatis *et al.*, 1989). Double stranded DNA was sequenced directly from samples isolated by the alkaline lysis procedure. Sequencing reactions were performed according to the manufacturer's (United States Biochemical) protocol except that the Sequenase reaction buffer was added after DNA denaturation. DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) with modified T7 DNA polymerase.

**Site directed mutagenesis and vector constructions**

Missense mutations of the *rasB*, *rasC*, and *rasS* genes were created as follows. The pTZ19R-*rasB*, pTZ19R-*rasC*, and pTZ19R-*rasS* vectors (Daniel *et al.*, 1993 a and b) were transformed into *E. coli* strain RZ1032 (Kunkel *et al.*, 1987). Uracil-containing single-stranded phagemid DNA was isolated after infection with the helper phage, M13KO7 (Virria *et al.*, 1987) and was used as the template for oligonucleotide-directed mutagenesis reactions (Kunkel et al., 1987). To create the mutations, the following mutagenic oligonucleotides were used:
**rasB** G12T:

5'-P-GTT GTT ATG GGT GGT ACC GGT GTT GGT AAG AGT GC-3'

**rasC** G12T:

5'-P-GC CGATTT ACC ACC GGT ATC ACC AAC GAT AAC-3'

**rasS** G12T:

5'-P-GTA TTA GTT GGA CCA ACC GGT GTT GGT AAA TCA TG-3'

In each case, the substituted bases are underlined. The mutated genes were transformed into *E. coli* strain XL1MRF' (Stratagene). The pVEII (ΔATG+Bluescript polylinker) vector was modified by removing the portion of the polylinker upstream of the site into which the *ras* genes were to be inserted in order to minimize the length of the sequence between the *discoidin* promoter and the transcription start site of each of the genes. The pVEII (ΔATG+Bluescript polylinker) vector was cleaved with KpnI and EcoRV, treated with T4 DNA polymerase to generate blunt ends, and ligated. To insert the mutated genes into the modified pVEII vector, each of the mutated genes was released from pTZ19R using EcoRI and inserted into the EcoRI site of the modified pVEII vector (Fig. 5). The junctions of the constructs were confirmed by restriction digests and by double stranded DNA sequencing.
Figure 5. Vector construction.

The mutated rasB, rasC, and rasS genes were cloned under the control of the inducible discoidin promoter in the modified pVEII vector as described in the text.
**Electrophoresis and immunoblotting**

SDS-PAGE and immunoblotting techniques were performed as described previously (Robbins, 1991). For Western blot analysis of transformants expressing genes under the control of the discoidin promoter, cells were inoculated at a density of 5X10^5 cells/mL and grown for 24 hours in shake suspension in either conditioned medium or HL5 containing 1 mM folate. Cells were lysed in 1% SDS. Protein concentration was estimated by UV absorbance (Harlow and Lane, 1988). About 20 μg of protein from each transformant was mixed with an equal volume of 2X loading dye (20% glycerol, 10% B-mercaptoethanol, 4.6% SDS, 125 mM Tris-HCl, pH 6.8), boiled for 5 minutes, then electrophoresed on a 12% SDS-polyacrylamide gel.

After electrophoresis, the protein was transferred to a nylon membrane for 16 hours at 30V (Towbin et al., 1979). Prestained molecular weight markers (BioRad) were used to estimate protein sizes. The membrane was stained with Ponceau S (Harlow and Lane, 1988) to confirm that equal amounts of protein had been loaded and transferred in all lanes. The membrane was blocked at room temperature with 1X TBS (8 g NaCl, 0.2 g KCl, 3 g Tris-HCl per litre water, pH 7.4), 5% skim milk powder, and 0.1% Tween-20 for at least 1 hour. The membrane was then washed 3 times for a total of 30 minutes with TBS and 0.1% Tween-20. RasB protein was detected by incubation with the general Ras antibody Y13 259 at a 1:20 dilution in TBS containing 0.5% skim milk powder and 0.1% Tween-20. Incubation was performed at room temperature for 1 hour. The membranes were then washed three times for a total of 30 minutes.
with PBS plus 0.1% Tween-20. The binding of the Y13 259 antibody was detected using a secondary goat anti-rat antibody. The signal generated by ECL was recorded on X-ray film.

**RNA isolation and Northern analysis**

Total RNA was extracted from *Dictyostelium* cells using the guanidinium isothiocyanate method (Chomczynski and Sacci, 1987). RNA (20 mg) suspended in 50% formamide, 40 mM 3-(N-morpholino) propanesulfonic acid (pH7.0), 10 mM sodium acetate, 1 mM EDTA, 6% formaldehyde was size-fractionated on a 1.25% formaldehyde gel. Equal RNA loading was assessed by ethidium bromide staining of ribosomal bands. The RNA was transferred and fixed to a nitrocellulose membrane (Maniatis *et al.*, 1989), then prehybridized for 3-6 hours at 42°C in a solution containing 50% formamide, 5X SSC, 5X Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 50 mM sodium phosphate, 0.5% SDS, 250 mg/mL sheared, denatured salmon sperm DNA and 60 mg/mL polyA RNA. The filter was then hybridized overnight at 42°C in the same solution with either *rasC* or *rasS* specific cDNA that had been radiolabelled by the random primer method using [α-32P]-dCTP. The filter was washed twice under low stringency conditions (2X SSC, 0.1% SDS at room temperature for 20 minutes) and then twice under high stringency conditions (0.1% SSC, 0.1% SDS at 65°C for 20 minutes). The Northern blot was exposed to X-ray film for the time indicated in the figure legend to detect the hybridized probe.
**Preparation of cDNA probes**

Recombinant plasmids were digested with the appropriate restriction endonucleases to release the cDNA fragments. The insert was then separated from the vector fragment by electrophoresis on a 0.7% agarose TBE gel (Maniatis *et al.*, 1989). A gel slice containing the cDNA fragment was excised from the gel and purified using the GeneClean II Kit Bio 101 Inc. (USA). Approximately 100 ng of the purified cDNA fragment was labelled by the random oligonucleotide primer method (Feinberg and Vogelstein, 1983) using $[^\alpha-\text{32P}]d\text{ATP}$. The radiolabelled probes were separated from unincorporated nucleotides by a Sephadex G-25 spin column (Maniatis *et al.*, 1989).
CHAPTER 3

RESULTS

The effect of overexpression of rasB-G12T, rasC-G12T, and rasS-G12T on D. discoideum

Introduction

Of the five ras genes in Dictyostelium, only two, rasD and rasG, have been characterized beyond the level of patterns of expression during growth and development. Dictyostelium transformants overexpressing an activated rasG-G12T grow slowly, are unable to aggregate, are multinucleate, and contain populations of cells with a flattened, spread morphology (Khosla et al., 1996; Rebstein et al., 1996). Overexpression of a rasD gene containing the activating G12T mutation produces cells which develop normally on filters up to the aggregation stage, but then instead of forming a single tip, the cell mass generates several tips and development ceases to proceed (Reymond et al., 1986). rasD is expressed specifically between 12 and 16 hours of development while rasG is expressed during growth and the first four hours after the onset of starvation, a period which overlaps the highest levels of expression of rasB and rasC, and immediately precedes the expression of rasS mRNA at 4-8 hours of development (see Fig. 4). In order to begin to address the question of whether the numerous Dictyostelium ras proteins are functionally equivalent, activating mutations (G12T) were created in the rasB,
rasC, and rasS genes. The mutated genes were transformed into cells under the control of the discoidin promoter (Blusch et al., 1992). This promoter was also used in the studies of overexpression of various forms of rasG and is expressed in vegetative cells. It is repressed by folate and induced by conditioned medium.

**Overexpression of the mutated rasB-G12T, rasC-G12T, and rasS-G12T genes**

EcoR1 fragments containing the mutated rasB-G12T, rasC-G12T, and rasS-G12T genes were cloned into the EcoRI site downstream of the folate-repressible discoidin (dis1g) promoter in the vector pVEII(ΔATG+Bluescript polylinker). The vector was introduced into D. discoideum using a calcium phosphate precipitate. Transformants were selected in the presence of 10 μg/ml G418 to select for presence of the vector and 1 mM folate to repress expression from the discoidin promoter.

Twelve G418 resistant clones were isolated from the rasB transformation and RasB protein levels under inducing conditions (24 hour growth in suspension in HL5 medium without folate, followed by 24 hour growth on plates in conditioned medium) were determined by Western blot analysis using the Y13 259 monoclonal antibody to human Ras. RasB runs at the same molecular weight as RasG on SDS-PAGE and the affinity of Y13 259 for RasB is approximately half that for RasG (Daniel, 1993). Several transformants showed considerably higher levels of Y13 259 reactive protein, of the correct molecular size, than those in the
parental Ax2 cells (data not shown). Two transformants were selected for a more careful analysis of protein levels and the one with clear overexpression of rasB selected for further work (Fig. 6). All twelve transformants were also screened for morphological phenotype and the transformant selected by Western blot analysis showed the highest proportion of cells with an aberrant morphology.

Because Y13 259 recognizes RasC poorly and RasS not at all (Daniel, 1993), and because specific antibodies for these proteins were not available, expression of the mutated genes from these transformations was measured at the level of transcription. Six G418 resistant clones were isolated from each transformation, cells were grown under inducing conditions and levels of rasC and rasS specific mRNA were determined by Northern blot analysis. The levels of rasC transcripts were much higher in all of the clones than in the parental Ax2 cells (Fig. 7). The level of rasS mRNA was higher than in Ax2 in 4 of the clones. All 6 of the G418 resistant clones from each transformation were subjected to morphological, growth, and developmental analysis.

The effect of mutated rasB-G12T, rasC-G12T, and rasS-G12T genes on cell growth and development

To determine the effects of overexpression of the mutated rasB-G12T, rasC-G12T, and rasS G12T genes on vegetative growth of
Figure 6. Expression of RasB protein containing a G12T substitution. Cells were transformed with pVEII rasB-G12T and selected for growth in G418 then incubated under inducing (conditioned medium, rasB CM) conditions for 24 hours. Ax2 cells were also grown in conditioned medium. Cells were lysed in 1% SDS and 20 μg of total protein was separated by SDS-PAGE, transferred to nitrocellulose, and probed with Y13 259 antibody. The molecular masses of the size markers are indicated in kDa. The two lanes are taken from the same gel.
Figure 7. Expression of rasC and rasS mRNA.

Six different clones of cells (lanes 2-7) transformed with either the rasC-G12T (A) or rasS-G12T (B) gene and selected for G418 resistance were incubated in conditioned medium for 24 hours. Cells were harvested and total RNA was prepared. 20 μg of RNA was separated on 1.25% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to a probe specific for the rasC (A) or rasS (B) gene. Lane 1 is the parental Ax2 strain.
Table 2. Growth rates of rasB-G12T, rasC-G12T, and rasS-G12T cells. Generation time, t_{gen}, of transformants overexpressing RasB-G12T, RasC-G12T, and RasS-G12T under inducing conditions. Times are indicated in hours and are the average of 6 independent lines for each transformant.

<table>
<thead>
<tr>
<th></th>
<th>t_{gen}</th>
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<tbody>
<tr>
<td>AX2</td>
<td>8.0 +/- 0.5</td>
</tr>
<tr>
<td>rasB</td>
<td>7.5 +/- 0.3</td>
</tr>
<tr>
<td>rasC</td>
<td>8.0 +/- 0.4</td>
</tr>
<tr>
<td>rasS</td>
<td>8.2 +/- 0.4</td>
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</table>
Dictyostelium amoebae, transformants were grown under inducing (conditioned medium) or repressing (HL5 + 1 mM folate) conditions in shake suspension from a starting density of 5X10^4 cells/mL and samples were counted every eight hours to determine growth rates. The rasB-G12T clones grew slightly slower than the parental Ax2 cells, and no significant differences were seen in the growth rates of the rasC-G12T and rasS-G12T transformants. (Table 2). Development of all transformants was examined by growing cells in HL5 medium in the absence of folate to a density of between 1X10^6 and 2X10^6 cells/mL, plating the cells on a Millipore filter, and incubating the cells understarvation conditions to induce differentiation as described in Methods section. All transformants developed normally (data not shown). High density samples of each of the transformants were also incubated on non-nutrient agar; again, development was normal in each case.


The effects of overexpression of the mutated rasB-G12T, rasC-G12T, and rasS-G12T genes on cell morphology in vegetative Dictyostelium were examined by plating cells on glass coverslips and incubating them for 24 hours in conditioned medium. The cells were then observed directly in an inverted microscope. None of the rasC-G12T or rasS-G12T transformants showed any gross morphological changes (cells from a representative clone of each are shown in Fig. 8), however, several of the rasB-G12T clones showed a marked
increase in the number of cells with a large, flattened morphology. The number of larger cells in the clones was estimated visually. Cells were divided into three types: normal, cells clearly larger than wild type (called 2-3X), and cells which were dramatically larger than wild type (called >3X). The rasB-G12T transformants showed a much higher proportion of 2-3X and >3X cells (Fig. 8, B).

Activation of the rasB gene results in phenotypic changes distinct from those caused by activation of the rasG gene

Introduction

In the previous section, I showed that the expression of the activated rasB-G12T induced morphological changes in Dictyostelium amoebae but, in contrast to rasG-G12T, had no effect on cell growth and development. To distinguish the two genes and to gain some insight into the mechanisms by which the different phenotypes are produced, further analyses of the morphology of the rasB-G12T and rasG-G12T clones was carried out. In an attempt to determine the cytoskeletal changes underlying the observed morphological phenotype, a number of functional assays were performed.

Because no developmental or morphological changes were detected in the rasC-G12T or rasS-G12T transformants, no further analysis of those clones was carried out.
Figure 8. Morphology of vegetative \( rasB \)-G12T, \( rasC \)-G12T, and \( rasS \)-G12T cells.

\( rasB \)-G12T, \( rasC \)-G12T, \( rasS \)-G12T, and the parental Ax2 cells were plated at \( 3 \times 10^3 \) cells/cm\(^2\) on glass coverslips, incubated 24 hours in conditioned medium and photographed as described in Methods.
Table 3  The proportion of large cells.

The size of the cells was estimated visually from photomicrographs and categorized into 3 classes. Numbers are the average of three independent trials with at least 200 cells counted in each trial.
Comparison of cell morphology in cells overexpressing the \textit{rasB}-G12T and \textit{rasG}-G12T genes

The flattened, spread morphology observed for the \textit{rasB}-G12T transformants was similar to that previously observed for cells overexpressing the activated RasG-G12T protein (Rebstein \textit{et al.}, 1996). In the case of the \textit{rasB}-G12T transformant, however, the proportion of large cells was greater (45\% for \textit{rasB}-G12T vs. 30\% for \textit{rasG}-G12T) (Rebstein \textit{et al.}, 1996). Also, the \textit{rasB}-G12T cells showed an increase in the number of extremely large cells (>10 times the size of an average Ax2 cell) (Meenal Khosla, unpublished observation).

To more closely observe the morphology of these two transformants, cells were grown on glass coverslips under inducing conditions, fixed, and examined by scanning electron microscopy (SEM). Both transformants showed a heterogeneous population in terms of cell morphology, but, again, the proportion of large, flattened cells as well as the sheer size of the largest cells in the \textit{rasB}-G12T cells was greater (Fig. 9). SEM also revealed differences in the predominant actin-based structures visible on each cell type under inducing conditions. While \textit{rasG}-G12T cells possessed numerous long filopodia (Fig. 9, E), the \textit{rasB}-G12T cells displayed more crowns, actin-based structures of unknown function which were first described by de Hostos \textit{et al.} (1991) as being a site of coronin (an actin binding protein) localization. Crowns are typical of growth phase cells.
Figure 9. Scanning electron microscopy of vegetative cells.

*RasB*-G12T (a,b,c), *rasG*-G12T (d,e,f), and Ax2 (g,h) cells were grown in HL4 medium containing 1 mM folate (a,d) or in conditioned medium (b,c,e,f,g,h) fixed, dehydrated, critical point dried, sputter coated with gold, then viewed with a Cambridge 259T scanning electron microscope.
Figure 10. Forward and side light scatter analysis of vegetative cells.

rasB-G12T (A,B), rasG-G12T (C,D), and Ax2 (E,F) cells were grown for 24 hours in shake suspension in conditioned medium (A,C,E) or in HL5 containing 1 mM folate (B,D,F) then analyzed for forward and side light scatter as described in methods. 10,000 cells were counted for each. The mean forward light scatter (x-axis value) for Ax2 cells was 452.38, for rasB-G12T cells was 509.10 (113% of Ax2 mean), and for rasG-G12T cells was 443.90 (98.1% of Ax2 mean).
Counting the number of cells with filopodia in three fields of 100-200 cells each revealed that filopodia were present on 12% of parental Ax2 cells, but in the rasB-G12T transformant 66% of cells were seen to have crowns. An intermediate number, 21%, of cells in the rasG-G12T population displayed these structures.

Analysis of forward light scatter of rasB-G12T and rasG-G12T cells growing in suspension under inducing conditions showed a significant increase in the mean of the forward light scatter for rasB-G12T (increased by 14% compared to Ax2 cells) but not rasG-G12T cells (Fig. 10). This indicates that although both types of cells show a large, spread morphology when grown on surfaces, only the rasB-G12T cells actually had an increased cell volume. It should be noted that the shaking growth conditions used in these experiments did not produce as many large cells in the rasB-G12T transformant as does growth on a solid surface (20% large cells in suspension vs. 45% large cells on a surface), but because cells could not be removed intact from surfaces, suspension cultures had to be used in this experiment. It seems likely that the increase in cell volume observed for rasB-G12T cells grown on a surface would be greater than for cells grown in suspension, however, this could not be measured and so remains uncertain.
The localization of F-actin in cells expressing RasB-G12T and RasG-G12T

To determine whether the observed morphological changes correlated with alterations in the actin cytoskeleton, cells were stained and viewed (as described in the Methods section) to visualize the distribution of F-actin. rasB-G12T (Fig.11, a) cells exhibited pronounced staining of the crowns while rasG-G12T (Fig. 11, b) cells displayed the increased peripheral staining noted previously by others (Rebstein, 1996). The parental Ax2 cells show a characteristic punctate F-actin staining (Fig. 11, c).

Determination of the number of nuclei in transformed cells

The abnormal morphology of cells overexpressing the activated rasB-G12T and rasG-G12T genes was similar to that observed in a number of other mutants, most notably of myosin II null mutants, which are impaired in their ability to undergo cytokinesis (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Haugwitz et al., 1994). The number of nuclei in the rasB-G12T and rasG-G12T transformants was determined by staining with Hoescht dye and viewing by fluorescence microscopy as described in the Methods section (Fig. 12A). In both cases the number of nuclei was found to be increased relative to Ax2 (Fig. 12B). The increase was, however, much greater in the rasB-G12T cells (2.45 nuclei per cell) than in the rasG-G12T cells (1.70 nuclei per cell). Most Ax2 cells were mononucleate, but some contained two nuclei resulting in an average
Figure 11. Localization of F-actin.

Transformants overexpressing RasB-G12T (A) or RasG-G12T (B) as well as the parental Ax2 (C) cells were grown on coverslips in conditioned medium for 24 hours, fixed, stained with FITC-phalloidin and viewed in a microscope equipped with epi-fluorescence.
Figure 12. Nuclear staining of rasB-G12T and rasG-G12T transformants.

Ax2 (a,b), rasB-G12T (c,d) and rasG-G12T (e,f) cells were grown on coverslips in conditioned medium for 24 hours then fixed and stained with Hoescht dye as described in the Materials and Methods. Cells were photographed using fluorescence (A,C,E) or phase contrast (B,D,F) microscopy. The average number of nuclei per cell was determined as the mean from three independent experiments. Nuclei in 100-200 cells were counted for each experiment. The mean number of nuclei for Ax2 cells was 1.04 (+/- 0.11), for rasB-G12T cells was 2.45 (+/- 0.22) and for rasG-G12T cells was 1.70 (+/- 0.15).
of 1.04 nuclei per cell. All nuclei, whether in mononucleate or highly multinucleate cells, appeared to be the same size, thus, there was no indication of polyploidy. To examine the possibility that the large cells arose through cell fusion rather than through failure of cytokinesis, cells were plated at a range of densities (from 2 cells/cm² to 10⁴ cells/cm²) in conditioned medium and the proportion of large cells after 24 hours calculated from photomicrographs (data not shown). Even at the lowest cell density, where cell-cell contact is unlikely to occur, the proportion of large cells was consistently high. Cell fusion, therefore, is not likely to be the cause of the large cells.

The response of cells to azide treatment

Wild type Dictyostelium cells round up and detach from the substratum when treated with azide, but this response is abrogated in myosin II null cells (Patterson and Spudich, 1995). It has been previously found that rasG-G12T cells are also somewhat impaired in this response, with 34% of the cells remaining adherent after treatment vs. 2% of Ax2 cells (Rebstein, 1996). To characterize the similar but distinct changes in the cytoskeleton observed in rasB-G12T cells, their response to azide was studied. It was observed that 8.1% of rasB-G12T cells, as compared to 4.3% of Ax2 cells in this set of experiments, remained attached to the substratum after treatment with azide (Fig. 13). Myosin function and the particular signalling pathways involved in activating myosin function in this response therefore appear to be normal in the rasB-G12T cells.
Figure 13. The effect of treating cells with azide.
Vegetative rasB-G12T (a,b) cells were grown on coverslips in conditioned medium for 24 hours (a), then treated with 2 mM sodium azide in HL5 for 3 minutes and washed 3 times to remove detached cells before photographing again (b).
Analysis of morphology after HL5 stimulation

When Dictyostelium cells are transferred from starvation to growth conditions they rapidly round up and detach from the substratum (Schweiger et al., 1992; Howard et al., 1993). This response was shown to be inhibited in rasG-G12T cells as 59% of rasG-G12T cells failed to respond to nutrient stimulation (Rebstein, 1996). The capacity of rasB-G12T cells to respond to HL5 medium stimulation was examined and found to be normal with only 4.5% of rasB-G12T cells (and 1.3 % of AX2 cells) failing to detach from the surface and become round and refractile (Fig. 14). Large cells were not evident after HL5 stimulation, possibly due to their increase fragility.

Random motility

Since the rasG-G12T cells displayed reduced random motility (Khosla et al., 1996), the distance migrated by rasB-G12T cells on PDF-MES plates was measured as described in the Methods section. The random motility of the rasB-G12T cells grown under inducing (CM) and non-inducing (+F) conditions was compared to Ax2 cells and found to be indistinguishable (Fig. 15).
Figure 14. Effect of HL5 stimulation on rasB-G12T and Ax2 cells.

RasB-G12T (a,b) and Ax2 cells (c,d) were plated at 3X10^3 cells/cm^2 on glass coverslips, incubated 24 hours in conditioned medium, then starved for 8 hours in BS buffer (a,c). HL5 medium was re-introduced to the cells for 20 minutes before photographing the cells again (b,d). The proportion of cells rounding in response to HL5 stimulation was found to be 98.7% in the case of Ax2 and 95.5% in the case of rasB-G12T. 100 to 200 cells were counted and the average of three independent determinations shown.
Figure 15. Random motility of rasB-G12T cells relative to Ax2. rasB-G12T cells were grown in either inducing (conditioned medium, CM) or non-inducing conditions (HL5 containing 1 mM folate, +F). Random motility was measured as described in Methods. The height of the bar represents the average of the distance migrated relative to Ax2 as determined from three independent experiments.
CHAPTER 4

GENERAL DISCUSSION

Effect of overexpression of \textit{rasB-G12T, rasC-G12T, and rasS-G12T}

Previously, it has been shown that the overexpression of an activated \textit{rasG} gene in \textit{D. discoideum} results in defects in growth and aggregation, impaired cytokinesis, and alterations in cell morphology (Khosla \textit{et al.}, 1996; Rebstein, 1996). Cells overexpressing an activated \textit{rasC} or \textit{rasS} gene shared none of these traits and, in fact, appeared phenotypically normal for all characteristics examined in the work carried out here. Cells overexpressing an activated \textit{rasB} gene showed no growth or developmental defects, however, they displayed morphological aberrations distinct from those seen with \textit{rasG}, and had a more severe defect in cytokinesis. Thus it appeared that \textit{rasG, rasC, rasS}, and \textit{rasB} were not identical in function. Since the \textit{rasC-G12T} and \textit{rasS-G12T} transformants were identical to the parental Ax2 in all assays performed, it is impossible to know what functions the endogenous forms of these proteins may have in the cell. The failure of overexpression of these genes to cause a distinguishable phenotype could be due to several causes. First, the assays used might be too crude to detect a relatively subtle phenotypic effect. Second, the nature of the phenotype resulting from overexpression of these genes might be one which was not
assayed for. Third, expression from the discoidin promoter might not permit expression of the activated genes at times in development when their specific effectors were present. This is especially likely to be true in the case of rasS since the endogenous gene is specifically expressed only during late aggregation as opposed to the expression during growth and early aggregation seen with the discoidin promoter. Fourth, if the effects of these genes are relatively subtle and if duplication of function, at least in part, between various Dictyostelium ras genes exists, the other endogenous Ras proteins might be able to compensate for the inhibitory or stimulatory effects of the overexpressed proteins. Fifth, although other ras genes are not known to be regulated at the level of translation, the possibility cannot be ruled out that despite high levels of rasC and rasS mRNA in these transformants, the corresponding proteins are not expressed. Specific antibodies to RasC and RasS would allow the latter possibility to be ruled out. Expression of RasS-G12T off its own or another aggregation-specific promoter could address the third possibility. Finally, disruption of these genes might provide some clues as to their roles.

**How does RasB differ from RasG?**

RasB clearly has a different set of functions than RasG, although the functions may be overlapping. The work described in this thesis identifies distinct phenotypic effects for different Dictyostelium ras genes, although the mechanisms by which these effects are produced
remain highly speculative. Four distinct defects can be seen in transformants overexpressing the activated \( \text{rasB} \) or \( \text{rasG} \) genes:

1) impaired growth and failure to aggregate
2) aberrant morphology
3) cytokinesis defect
4) defect in traction-mediated cytofission

Of these, the first is specific to the \( \text{rasG-G12T} \) mutant, the second and third are common to both, and the last is most obvious in the \( \text{rasB-G12T} \) mutant, although the \( \text{rasG-G12T} \) cells are also mildly impaired. In mammals, Ras acts through several distinct downstream pathways: recent work has also shown that the effects of RasG are mediated by more than one downstream effector (Zhang, 1998) since the growth and aggregation defect is separable from the morphological and cytokinesis defects. This line of demarcation also separates the \( \text{rasG-G12T} \) and the \( \text{rasB-G12T} \) transformants, however, it is unlikely that identical effector interactions are responsible for the morphological and cytokinesis defects observed in the two mutants since the effects of \( \text{rasB-G12T} \) are both quantitatively and qualitatively different from those of \( \text{rasG-G12T} \).

**Effect on cell growth and development**

As noted above, no changes in either cell growth or development were observed in the \( \text{rasB-G12T} \) transformant. The effect of the activated RasG-G12T protein on growth and aggregation is believed to be due to disruption of the cAMP relay (Khosla et al., 1996). Overexpression of activated \( \text{rasG-G12T} \) also inhibits activation of
ERK2, a homolog of mammalian MAP kinase, which is required for aggregation (Khosaka et al., 1998). Overexpression of wild type rasG has no effect on aggregation, suggesting that it is the GTP-bound form of the protein which interferes with aggregation and that the regulatory capacity of this pathway is sufficient to compensate for increased expression of wild type RasG but not constitutively active RasG. Consistent with this idea is the finding that cells in which the rasG gene has been disrupted aggregate normally (Tuxworth et al., 1997).

**Effect on cell morphology**

Activated Ras has previously been shown to affect mammalian cell morphology, causing increased membrane ruffling and a loss of stress fibers (Stacey and Kung, 1984; Feramisco et al., 1984; Bar-Sagi et al., 1986; Lloyd et al., 1989). Ras proteins have also been shown to regulate cell morphology in yeasts. In *Schizosaccharomyces pombe*, Ras1 is required for normal cell shape, in a process involving the Rho-like protein CDC42sp (Chang et al., 1994), while in *Saccharomyces cerevisiae* RAS2 is involved in pseudohyphal growth, a process characterized by unipolar budding and an altered cell morphology (Gimeno et al., 1992).

Aberrant cell morphology is associated with both the rasB-G12T and rasG-G12T transformants although the nature of the morphological change differed. Among the effects attributed to the action of RasG on the cytoskeleton are the altered response of rasG-T12T cells to azide and refeeding with HL5 as well as their decreased
motility and increased peripheral F-actin staining. RasB had no effect on either the azide or refeeding responses, the motility of rasB-G12T cells was indistinguishable from wild type, and actin staining was localized primarily to the cell surface projections known as crowns. Also, while overexpression of rasG-G12T produces an increase in the number of filopodia, overexpression of rasB-G12T resulted in an increase in crowns. Only the rasB-G12T transformant showed an increase in cell volume: this is most likely a reflection of the more severe defect in cytokinesis rather than an effect linked directly to changes in the cytoskeleton. Cells in which rasG is disrupted also show a wide range of defects in the regulation of the actin cytoskeleton, including an increase in filopodia, as do cells overexpressing wild type RasG (Rebstein, 1996; Tuxworth et al., 1997).

The mechanism by which RasG and RasB cause these cytoskeletal effects is unclear. One possible explanation is that RasB and RasG act to disrupt one or several signal transduction pathways that directly regulate the cytoskeleton. In mammalian cells, Ras has been shown to be upstream of Rac and Rho in pathways directly regulating actin assembly and disassembly (Ridley and Hall, 1992). Recent in vitro and in vivo analyses suggest a close relationship between the Rho signalling pathway and activation of the actin-membrane cross-linking ability of ezrin/radixin/moesin proteins (Tsukita et al., 1997). Several rac and rho genes have been isolated from D. discoideum (Bush et al., 1993 a and b), and it is possible that there is a similar network of Ras superfamily proteins regulating D. discoideum cell morphology.
A second way in which RasB and RasG may exert their effects on the cytoskeleton is by disrupting the transduction of a signal controlling gene expression which consequently results in an altered cell morphology. Activated Ras has been shown to regulate mammalian gene expression, for example the AP-1 transcription factor is activated by UV irradiation through a Ras-dependent pathway (Engelberg et al., 1994). Since reduced expression of numerous actin-associated genes has been observed in transformed mammalian cells (Button et al., 1995; Janmey and Chaponnier, 1995), this type of regulatory mechanism would not seem unreasonable. Rho, Rac, and Cdc42 have also been shown to transduce signals that activate transcription (Minden et al., 1995; Coso et al., 1995; Hill, 1995), so a similar network in *D. discoideum* might regulate the actin cytoskeleton in this manner.

These two possibilities are not mutually exclusive and, given the difficulty of measuring the function of specific actin-associated proteins and their duplication of function, the former mechanism is difficult to test with any precision. The latter possibility might be more easily addressed by analyzing expression levels of various actin-associated proteins in rasB-G12T and rasG-G12T cells.

**Effect on cytokinesis**

Cytokinesis, the division of one cell into two daughter cells, is a complex process and one which is poorly understood. It is subject to precise control with respect to both timing (coordination with other events in the cell cycle) and spatial partitioning of the cell. In
general, mitosis comprises karyokinesis followed by cytokinesis; however, the two processes are dissociable. In a large number of eukaryotes karyokinesis is not followed by cytokinesis resulting in the formation of syncytia. The best characterized example of this is *Drosophila* embryogenesis which begins with thirteen mitotic divisions that occur without cytokinesis (Sullivan and Theurkauf, 1995). In yeast, mutants in four cell division cycle genes, *cdc3*, *cdc10*, *cdc11*, and *cdc12* cause a disruption of cytokinesis, giving rise to multinucleate cells with abnormal bud growth (Hartwell, 1971). The related gene, *peanut*, in *Drosophila* is also required for cytokinesis (Neufeld and Rubin, 1994). Cytokinesis can also be experimentally inhibited or delayed from the events of karyokinesis. Cytochalasin B, phalloidin, and metabolic inhibitors such as sodium vanadate have all been shown to suppress cytokinesis suggesting that actin and ATP are required for this process (reviewed in Ghosh and Paweletz, 1993). No cell type is known in which cytokinesis takes place under normal conditions without prior karyokinesis, however the delayed cytokinesis in *Drosophila* embryonic development and a number of similar cases has been interpreted to indicate that cytokinesis requires a special signal distinct from those regulating other aspects of cell cycle progression (Ghosh and Paweletz, 1993).

Cytokinesis can be separated into four steps (reviewed in Glotzer, 1997). In the first step, the position of the cleavage plane is established in a manner dependent on the orientation of the mitotic spindle. The nature of the signalling from the spindle is unknown, but directs the second step, the assembly of actin into the contractile
ring which is followed by the localization of myosin II which is the major force generator for contraction. The contraction of the cleavage furrow is the third step. It should be noted, however, that the contractile ring seems to have no resting state; it begins contracting as soon as it is formed. Thus, it is not clear whether there is any regulation of the contraction (Mabuchi, 1986). During this stage, myosin is thought to slide actin filaments against each other to draw the contractile ring tighter. As the constriction grows smaller, the actin filaments of the contractile ring are disassembled. Finally, in the fourth step, the cell membrane fuses to complete the separation of the two daughter cells. This aspect of cytokinesis remains virtually uncharacterized. A number of Dictyostelium mutants with defects in cytokinesis have been described. Cells with a disruption of the myosin II heavy chain, and of the actin-associated proteins profilin I and II, coronin, and cortexillin I and II all have severe defects in cytokinesis (Knecht and Loomis, 1987; de Hostos et al., 1993; Haugwitz et al., 1994; Faix et al., 1996).

Recently, a number of Ras-related proteins have been associated with defects in cytokinesis in Dictyostelium (Fig. 16). These include, in addition to RasB and RasG, two homologs of mammalian Ras-GAP (GapA and RgaA/DGAP1) and RacE. Cells overexpressing RgaA/DGAP1 as well as cells in which the gapA gene has been disrupted become large and multinucleate (Adachi et al., 1997; Lee et al., 1997). The observation that RgaA/DGAP1 may exist in
Figure 16. *D. discoideum* Ras, Ras-related, and Ras-regulatory protein effects on cytokinesis.

Possible interactions between RasB, RasG, RacE, GapA, and RgaA/DGAP1 are indicated with dashed lines. Solid lines designate the known interaction of RgaA/DGAP1 with actin as well as the role of all these proteins in regulating, whether positively or negatively, cytokinesis (see text). Although a single arrow is used to represent the final stage of cytokinesis, multiple activities are involved: it is not known whether RasB, RasG, RacE, and RgaA/DGAP1 affect the same or different events.
association with actin (Faix and Dittrich, 1996) provides a possibly direct link between Ras signalling pathways and the cytoskeleton. Both GAPA and RgaA/DGAP1 contain calmodulin-binding IQ motifs in a position similar to those in the human IQGAP1 (Adachi et al., 1997; Lee et al., 1997). This is intriguing since IQGAP1 has been found to bind directly to activated Rac and Cdc42 as well as binding and cross-linking actin filaments thus serving as a direct molecular link between GTPases and the actin cytoskeleton (Bashour et al., 1997). Ras proteins in other organisms have not previously been associated with cytokinesis, but given the similarities observed in the reorganization of the actin cytoskeleton by RasG-G12T and RasB-G12T and by Rac, Rho and CDC42, it is interesting to note that this latter group of proteins have also recently been found to be involved in cytokinesis. Studies in sea urchin eggs and Xenopus laevis revealed that Rho works as a switch between the nuclear and cytoplasmic divisions and is required to induce and maintain the contractile ring (reviewed in Narumiya et al., 1997). Cytokinesis is also impaired in both a human cell line expressing a constitutively active form of CDC42 (Dutartre et al., 1996) and in Dictyostelium cells in which the racE gene has been disrupted (Larochelle et al., 1996). Rho-type GTPases appear to regulate these cytoskeletal events through cytoplasmic rather than nuclear targets (Vojtek et al., 1995) although in the case of RacE, no downstream effectors have yet been identified. Although the effects of these proteins on the actin cytoskeleton has been the focus of most studies on their function, it is possible that their effects on cytokinesis may be mediated in other ways. For instance, the activation of myosin II required for
constriction of the contractile ring could be regulated through the signal from the small GTPase, Rho, since it has been shown that phosphorylation of the myosin II light chain, which activates the ATPase of myosin II, is regulated by effectors of Rho, Rho kinase and myosin phosphatase (Kimura et al., 1996; Amano et al., 1996).

Given these findings in other organisms, it is perhaps not surprising that Dictyostelium cells lacking racE expression also become large and multinucleate. These cells seem to have a defect specifically in cytokinesis since they are otherwise normal, being able to complete the normal developmental program and having a normal organization of actin and myosin. The cytokinesis defect of the racE null cells could be rescued by either wild type or constitutively active RacE, but not by inactive RacE. This suggests that while GTP-bound RacE is required for cytokinesis, cycling between the GDP- and GTP-bound forms of the protein is not. It has also been found that, in racE mutants, a contractile ring forms but contraction is not completed, indicating that the defect in these cells occurs late in cytokinesis (Larochelle et al., 1996).

Video microscopy of rasG null cells has also shown that cells undergo normal cleavage furrow formation but fail to separate completely. Both gapA null mutants and cells overexpressing rgaA/DGAP1 also appear to have defects late in cytokinesis, most likely in the final stages of furrow contraction, furrow disassembly, or membrane fusion (Lee et al., 1997; Adachi et al., 1997). The fact that cytokinesis fails at the same stage in the rasG, gapA and rgaA/DGAP1 mutants hints at the possibility that RasG might be the target for one of these GAPs or, alternatively, that GapA or
RgaA/DGAP1 is the target of RasG. Although mammalian IQGAPS interact not with Ras, but with members of the Rho family of GTPases, another GAPA-related protein, Sarl/Gap1 from *S. pombe*, has been shown both genetically (Imai *et al.*, 1991; Wang *et al.*, 1992) and biochemically (Hart *et al.*, 1996) to activate yeast Ras. Alternatively, these GAPs may act on one of the many *Dictyostelium* Rac or Rho proteins (perhaps RacE) which potentially mediate the effects of RasG (and RasB) on the cytoskeleton.

The more severe defect in cytokinesis observed in rasB-G12T cells compared to rasG-G12T cells may suggest that they interact with different GAP homologs or otherwise act in parallel pathways with greater or lesser degrees of inhibition of cytokinesis. Alternatively, this difference may be explained by the defect in traction-mediated cytofission observed in the rasB mutant, particularly since the number of large, multinucleate cells present in suspension cultures of the two mutants is similar. The point at which cytokinesis is blocked in the rasB-G12T mutant is not known at present; video microscopy of these cells might provide some useful information in this regard.

**Effect on traction-mediated cytofission**

Among all the *Dictyostelium* mutants with defects in cytokinesis, only the rasB-G12T and coronin null mutants are more multinucleate when grown on a surface than when grown in suspension. Most *Dictyostelium* cells that are defective in cytokinesis in suspension culture are able to divide by a process known as traction-mediated cytofission when grown on a solid surface. *rasG*
null cells, for instance, grow poorly and become highly multinucleate when grown in suspension. Within 15 minutes of being spread on a surface, most of the large cells have divided and become mono- or dinucleate (Tuxworth et al., 1997). In contrast, the proportion of large multinucleate cells is greatly increased when rasB-G12T and coronin null cells are grown on a surface, indicating that traction-mediated cytofission, as well as cytokinesis, is impaired in these cells. Since little is known about the signalling mechanisms involved in traction-mediated cytofission, little can be hypothesized at the moment about how RasB and coronin might interfere with this process. It appears, however, that there is no significant difference between cytokinesis and traction mediated cytofission in terms of cytoskeletal dynamics and force generation and that there is substantial overlap between the cytoskeletal mechanisms of amoeboid movement and cell division (Fukui, 1993). In fact, it has been reported that myosin II cells despite being unable to undergo cytokinesis in suspension, are able to undergo a form of division on surfaces which is linked to mitosis and is, therefore, distinct from traction-mediated cytofission (Neujahr et al., 1997). This "attachment assisted mitotic cleavage" follows a sequence of shape changes similar to those observed in wild-type Dictyostelium cells undergoing mitosis but, like traction mediated cytofission, is anchorage dependent since migration of the daughter cells is instrumental in disrupting the the thin thread of cytoplasm that remains of the cleavage furrow (Neujahr et al., 1997). Interestingly, the actin, microtubule, and nuclear rearrangements which accompany this anchorage dependent, mitosis-linked process closely
resemble those seen during the cellularization of the *Drosophila* syncytial blastoderm. Within this conceptual framework, cells which have reduced motility would also be expected to be impaired in traction mediated cytofission (and in "attachment assisted mitotic cleavage"). Coronin has been implicated in speeding up disassembly of actin networks involved in cell motility and phagocytosis (Gerisch *et al.*, 1995) so the failure of coronin null cells to undergo traction-mediated cytofission might be explained simply by their reduced motility. This cannot, however, account for the inability of *rasB-G12T* cells to divide by traction-mediated cytofission, since their motility is normal.

An alternative hypothesis can be proposed which is based on the finding that coronin contains a WD motif, a sequence frequently found in proteins involved in multiprotein complexes. Potentially, a coronin complex involving RasB-GTP is involved in actin filament disassembly required for cells to crawl apart from each other in traction-mediated cytofission. If transduction of the signal requires cycling of RasB between the GTP and GDP bound form, the similar phenotype for *coronin* null cells and cells overexpressing an activated *rasB* gene could be understood. The finding that, in wild type cells, coronin is localized to the distal edges of cells undergoing traction-mediated cell division (de Hostos *et al.*, 1993) lends some support to the idea that it may be involved in actin assembly/disassembly at the leading edges of cells undergoing traction-mediated cytofission. Cells overexpressing an activated *rasG-G12T* show some impairment of traction-mediated cell division (although the number of nuclei per cell does not increase upon
transfer of cells from suspension to growth on a surface) but the rasG null mutant displays normal traction-mediated cytofission. These results may indicate that, while the defect in traction-mediated cytofission is specifically a function of RasB, RasG may interact with the signal transduction components involved with a lower affinity such that high levels of activated RasG in the cell causes a slight disruption of the pathway. To gain a greater understanding of the role of Ras proteins in traction-mediated cytofission, it would be useful to know the subcellular localization of RasB and RasG in cells undergoing this process. It would also be informative to examine the effect of overexpression of a wild type RasB and of a disruption of the rasB gene on traction-mediated cell division. A search for proteins which specifically interact with RasB and RasG, via immunoprecipitation or the yeast two hybrid system, would provide additional information to aid in elucidating the varied roles of these two proteins.
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