ISOLATION AND CHARACTERIZATION OF THREE NOVEL DICTYOSTELIUM RAS GENES: EVIDENCE FOR A UNIQUE, DEVELOPMENTALLY REGULATED RAS GENE FAMILY.

By

JULIET MICHELLE DANIEL

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Department of MICROBIOLOGY

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Members of the ras gene superfamily encode low molecular weight (21-23 kDa) GTP-binding proteins that are believed to play a role in signal transduction. In the cellular slime mold Dictyostelium discoideum, two ras genes, (rasD and rasG), and one ras-related gene, (rap1), have been previously isolated and characterized, but preliminary evidence suggested that there were additional ras genes in Dictyostelium. Using the differential hybridization and polymerase chain reaction (PCR) techniques, no additional rap genes were isolated, but three novel ras genes, rasB, rasC, and rasS, were cloned and characterized. The expression of all three genes is developmentally regulated; in the wild type Dictyostelium strain V12M2, maximum levels of the rasB transcript were detected during growth and early development, while maximum levels of the rasC and rasS transcripts were detected during aggregation. Consequently, during early development and aggregation, at least four ras genes (rasB, rasC, rasG, and rasS) are being expressed simultaneously in Dictyostelium. The deduced amino acid sequences of rasB, rasC, and rasS are more related to the Dictyostelium RasD and RasG proteins, and the human H-Ras protein than they are to any of the other Ras-related proteins such as Ral, R-Ras, Rho, and Rab. Like RasD, RasG, and Rap1, the RasB protein possesses a conserved effector binding domain relative to H-Ras. However, RasC and RasS have single amino acid substitutions in their effector binding domains at residues that are believed to be necessary for Ras biological activity (RasC: D38N, and RasS: I36L). RasB, RasC, and RasS also have at least three amino acid differences in their effector-proximal domain, a domain which is believed to be involved in Ras target activation and which also acts in conjunction with the effector domain. In addition, all three proteins have amino acid substitutions in the Y13-259 epitope, a region believed to be involved in mediating Ras:GAP interactions. The RasB and RasC GST-fusion proteins, which have single amino acid substitutions in the Y13-259 epitope, reacted
with the Y13-259 monoclonal antibody but the RasS fusion protein, with three amino acid substitutions in the epitope, did not. Since RasD and RasG have identical Y13-259 epitope, effector, and effector-proximal domain sequences, the atypical variations in the corresponding domains of RasB, RasC, and RasS, suggest that RasB, RasC, and RasS may each interact with different effector and/or GAP molecules than RasD and RasG, and hence have distinct functions. Furthermore, the multiplicity of ras genes in Dictyostelium, coupled with their distinctive gene expression patterns, suggests that the distinct ras gene products may be involved in an intricate network of signal transduction pathways which regulate the Dictyostelium developmental process.
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LIST OF ABBREVIATIONS

aa amino acid(s)
bp base pair(s)
cAMP cyclic adenosine 3', 5' -monophosphate
cDNA complementary deoxyribonucleic acid
dNTP deoxyribonucleoside triphosphate
DNA deoxyribonucleic acid
dATP deoxyadenosine triphosphate
dCTP deoxycytidine triphosphate
dGTP deoxyguanosine triphosphate
dTTP deoxythymidine triphosphate
DNase I deoxyribonuclease I
DTT dithiothreitol
EDTA ethylenediaminetetraacetic acid (disodium salt)
GTP guanosine triphosphate
kb kilobases
kDa kilodaltons
mRNA messenger ribonucleic acid
μg microgram
μl microlitre
μM micromolar
mg milligram
ml millilitre
mM millimolar
M molar
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>M-MLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinopropanesulfonic acid</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene-20-sorbitan monolaurate</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RNase A</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
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Single letter code for amino acids: A, Alanine; R, Arginine; N, Asparagine; D, Aspartic acid; C, Cysteine; Q, Glutamine; E, Glutamic acid; G, Glycine; H, Histidine; I, Isoleucine; M, Methionine; F, Phenylalanine; P, Proline; S, Serine; T, Threonine; W, Tryptophan; Y, Tyrosine; V, Valine.
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I would like to dedicate this thesis to my father Lionel and my late mother June, in appreciation of their constant love, immeasurable support, and faith in me, and for giving me the opportunity to explore new horizons.
CHAPTER 1

GENERAL INTRODUCTION

1.1 Oncogenes

Oncogenes were first described as the virally encoded genes which were capable of inducing tumours in birds and animals, and capable of transforming cells in culture (for review, see Cantley et al., 1991; Storms & Bose, 1989). It was later discovered that these viral genes had a high sequence identity with normal, host, non-transforming genes, and it became evident that the viral oncogenes were acquired from the host through recombination events that occurred between the viral genome and the host genome during viral infection. The strong transforming potential of the viral oncogenes, relative to their normal cellular counterparts (proto-oncogenes), was subsequently determined to be due to either a single point mutation in the coding sequence of the gene or the aberrant overexpression of the gene which was now under the control of a strong viral promoter.

Approximately forty oncogenes have been identified to date and they have been categorized into six groups based on their functional and structural similarities. These oncogenes encode growth factors (eg. sis), growth factor receptors (eg. neu, fms, kit, mas, and erbB), tyrosine kinases (eg. src, abl, fes/fps, and yes), cytoplasmic serine/threonine kinases (eg. raf and mos), GTP-binding proteins (eg. ras), and nuclear transcription factors (eg. myc, myb, fos/jun and erbA) (for a recent review, see Cantley et al., 1991). Interestingly, most of these oncogenes encode proteins that appear to be involved in the signal transduction pathways which influence cell proliferation and differentiation (Cantley et al., 1991; Storms & Bose, 1989).
1.2 Ras Oncogenes

The ras family of oncogenes were first identified as the transforming principles of the Harvey and Kirsten rat sarcoma viruses (reviewed in Barbacid, 1987). They later became the focus of intensive research when it was discovered that they had a high incidence of expression in human tumours; in some cases, eg. pancreatic cancer, activated ras genes were found in as many as 95% of the tumours.

Although there is strong circumstantial evidence for the involvement of ras genes in tumorigenesis, their exact role in this process remains unknown. However, their direct involvement in neoplastic development was recently demonstrated by the induction of pancreatic tumours in transgenic mice bearing an activated ras gene under the control of a pancreas-specific gene promoter. Quaife et al. (1987) found that the temporal expression of the activated ras gene correlated with the onset of aberrant proliferation of the pancreatic cells, and this observation suggested that ras gene activation was an initiating event in tumour development.

1.3 Ras Proteins

The Ras proteins are small (21-23 kDa), monomeric, guanine nucleotide binding proteins that have been found in organisms as diverse as yeasts, plants, slime mold, and mammals (Barbacid, 1987; Valencia et al., 1991). They have been highly conserved throughout evolution and they all display at least 50% identity with the human H-Ras protein which has become the prototype Ras protein; to date, three functional and highly related Ras proteins have been identified in humans (H-Ras1, K-Ras2, and N-Ras), and they are 85% identical to each other at the amino
acid level (Capon et al., 1983; Chang et al., 1982; Der, 1989; McGrath et al., 1983; Taparowsky et al., 1983).

The first 80 amino acids are the most conserved residues in the Ras proteins, and the mammalian Ras proteins are 100% identical to each other over this stretch of amino acids. The Ras proteins of other species display at least 80% identity to the mammalian Ras proteins in this region. The high degree of conservation of the Ras proteins, and their ability to function in heterologous systems (DeFeo-Jones et al., 1985; Haubruck et al., 1989), suggested that the Ras proteins played a fundamental role in normal cellular activities.

1.3.1 Functional Domains And Biochemical Properties

Guanine nucleotide binding

The Ras proteins bind guanine nucleotides, possess an intrinsic GTPase activity, and are associated with the inner surface of the plasma membrane. These properties resemble those of the regulatory, heterotrimeric G-proteins that are involved in transmembrane signal transduction, and so it was postulated that the Ras proteins may play a role in signal transduction (Hurley et al., 1984). Subsequent comparison of the amino acid sequence of Ras with the amino acid sequences of various members of the heterotrimeric G-protein family revealed a high level of sequence identity in certain domains (reviewed in Barbacid, 1987). These conserved domains were co-linear, had specific spacings in all the different GTP-binding proteins, and corresponded to the domains which confer guanine nucleotide binding ability (Dever et al., 1987) (Figure 1).
Figure 1. Schematic representation of the defined structural and functional domains within the Ras proteins. Regions whose functions are understood from crystallographic or mutagenesis studies are highlighted. The asterisks indicate the "hot-spots" for activating mutations, and numbers represent the position of the amino acid residues that define the indicated domain.
The importance of these domains in the GTP-binding ability of the Ras proteins was definitively established by structure-function studies which employed site-directed and random mutagenesis techniques, (reviewed in Der, 1989). The first domain, which encompasses amino acids 10-16 relative to H-Ras, is believed to play a role in the hydrolysis of GTP since mutations in this domain alter the intrinsic GTPase activity (Clanton et al., 1987). Residues in the second GTP-binding domain (amino acids 57-62 relative to H-Ras) are also involved in GTP hydrolysis (Der et al., 1986; Gibbs et al., 1984). Activating mutations in the third GTP-binding domain, residues 116-119, do not affect the intrinsic GTPase activity of H-Ras but they do result in decreased affinity for the guanine nucleotide and an enhanced guanine nucleotide exchange rate (Der et al., 1988; Walter et al., 1986). The fourth putative GTP-binding domain, encompassing amino acids 143-147, is not found in the classical GTP-binding proteins and is unique to the Ras family proteins. It is believed to be involved in forming the pocket for the guanine base (de Vos et al., 1988; McCormick et al., 1985).

The correlation between the GTP-binding properties of Ras and its biological activity was later demonstrated by two laboratories who reported that microinjection of the Ras-specific, Y13-259 monoclonal antibody inhibited GTP-binding, and impeded the development of the transformed phenotype (Kung et al., 1986; Mulcahy et al., 1985). Other studies using H-Ras deletion mutants that lacked the guanine nucleotide binding domains, found that the mutant Ras proteins lacked guanine nucleotide binding and had no transforming ability (Willumsen et al., 1986). The importance of the GTP-binding ability of Ras was further emphasized when it was observed that the naturally occurring, oncogenic, Ras proteins had a reduced GTPase activity and hence were maintained in a GTP-bound state (McGrath et al., 1984; Sweet et al., 1984).
Isoprenylation and membrane localization

The Ras and Ras-related proteins all have a carboxy-terminal cysteine residue that is part of a consensus CAAX, CC, or CXC motif, where C= cysteine, A= any aliphatic amino acid, and X= any amino acid (reviewed in Cox & Der, 1992; Magee & Newman, 1992). These consensus motifs are a prerequisite for post-translational processing and efficient membrane localization of the Ras and Ras-related proteins.

The Ras proteins possess the consensus CAAX motif, and they must undergo a series of post-translational modifications to ensure their precise localization on the inner surface of the plasma membrane. These modifications include the isoprenylation of the cysteine residue, proteolytic cleavage of the AAX sequence, and carboxy-methylation of the now C-terminal cysteine residue (Gutierrez et al., 1989; Hancock et al., 1989). The human H-Ras and N-Ras proteins can be further modified by palmitylation of a cysteine residue that lies upstream of the CAAX motif, and it is believed that this modification provides an additional anchor for Ras attachment to the membrane (Hancock et al., 1989).

The C-terminal "X" residue determines what type of isoprenyl moiety is added to the Ras protein (Cox & Der, 1992; Cox et al., 1992; Finegold et al., 1991). When X= L, I, F, or N, the Ras proteins are isoprenylated with a C-20 geranylgeranyl moiety, when X= S, M, C, or A, they are isoprenylated with a C-15 farnesyl moiety. The various isoprenyl groups are believed to increase protein hydrophobicity and facilitate membrane association of the Ras protein (Cox & Der, 1992). The significance of the effects of different isoprenoid moieties on Ras protein function is unknown but it is speculated that, in addition to generalized membrane targeting, each isoprenoid moiety promotes and specifies the interaction of the prenylated Ras protein with a specific membrane-bound protein or receptor (Cox & Der, 1992).

There are three lines of evidence, however, which suggest that isoprenoid modification alone is not sufficient for efficient membrane association and specific
targetting of the Ras proteins to the plasma membrane. (1) One third of the total cellular isoprenylated protein is localized to the cytosol (Hancock et al., 1989). (2) Addition of a CAAX motif to *S. aureus* protein A results in isoprenylation but not membrane association (Hancock et al., 1991). (3) The Ras-related Rap and Rab proteins are also isoprenylated but they are localized on different membranes (Goud et al., 1990; Mizoguchi et al., 1990). These observations therefore suggest that other factors are required for the specific targetting of the Ras family of proteins to the correct membrane.

The importance of the conserved cysteine residue in the CAAX motif was determined by studies of activated H-Ras proteins which lacked the cysteine residue. In NIH 3T3 cells, these mutant proteins were not post-translationally processed, remained in the cytosol, and were unable to produce the transformed phenotype (Hancock et al., 1989; Willumsen et al., 1984). These findings proved that post-translational modification and the subsequent membrane localization of the Ras proteins were necessary for Ras biological activity. However, it is still not known why the Ras proteins need to be membrane localized. One hypothesis is that membrane localization brings the protein in contact with the appropriate upstream and/or downstream components in a signal transduction pathway (Cox & Der, 1992).

**Effector domain**

One highly conserved region that is found only in members of the Ras protein subfamily, but not in the larger Ras-related protein family, is the putative effector-binding domain. This domain was first identified during a deletion mutagenesis study as a region that was essential for the transforming activity of the viral H-Ras proteins (Willumsen et al., 1986). A hydropathy plot of the H-Ras protein indicated that this region corresponded to a hydrophilic, exposed portion of
the protein, and this observation suggested that this domain was involved in protein-protein interactions. Subsequent studies by Sigal et al. (1986) further defined this domain to encompass residues 32 to 40 of the human H-Ras protein. Amino acid substitutions in this domain reduced Ras biological activity but did not disrupt membrane localization, guanine nucleotide binding, or the intrinsic Ras GTPase activity (Gideon et al., 1992; Schaber et al., 1989; Sigal et al., 1986; Willumsen et al., 1986).

The recent isolation and identification of the GTPase activating protein (GAP) (Trahey & McCormick, 1987) has contributed greatly towards an understanding of the significance of the putative effector domain with respect to the biological properties of Ras. Amino acid substitutions in the effector domain that abolished Ras biological activity resulted in Ras proteins that were not responsive to the stimulatory effects of the GAP molecule, and it was postulated that these effector-mutants were impaired in GAP binding (Adari et al., 1988; Calés et al., 1988). Subsequent studies by Schaber et al. (1989) determined that at least one of these substitutions (Asp38Ala) did indeed diminish GAP binding. These observations indicated that GAP interacted with Ras through the residues of the putative effector domain, and consequently GAP was implicated as the Ras effector protein.

**Effector-proximal region**

Since the members of the Ras protein subfamily (Ras, R-Ras, Ral, and Rap) all have identical effector domains, but only the Ras proteins are able to cause cellular transformation, it was proposed that additional amino acids in the Ras protein sequence were involved in mediating the cellular transformation effects of Ras. Independent studies by Zhang et al. (1990) and Marshall et al. (1991) subsequently demonstrated that amino acids in the two regions which flank the effector domain played a crucial role in mediating the biologically divergent effects of Ras and Rap.
These effector-flanking regions are now postulated to play a role in Ras target activation and/or protein-protein interactions. In this thesis, the effector-proximal domain is defined as that portion of the Ras protein that encompasses amino acid residues 23-31 relative to H-Ras. The crucial residues in this domain are residues 26, 27, 30, and 31. The other effector-flanking region encompasses amino acids 45-54, but only mutations of residues 45 and 48 have been demonstrated to affect Ras biological function (Fujita-Yoshigaki et al., 1991; Marshall, 1993; Marshall et al., 1991).

**Neutralizing epitope**

The rat monoclonal antibody Y13-259 (Furth et al., 1982) has been a widely used reagent for the characterization of Ras proteins, due to its ability to recognize and immunoprecipitate Ras proteins from a variety of organisms (Papageorge et al., 1984; Pawson et al., 1985; Swanson et al., 1986). This reactivity with Ras proteins of diverse origin suggested that the antibody recognized a highly conserved epitope on the Ras protein. The epitope was subsequently localized and found to encompass amino acids 63 to 73 of the human H-Ras protein (Lacal & Aaronson, 1986; Sigal et al., 1986).

Even though this epitope is conserved in all members of the Ras protein subfamily, deletion studies suggest that it is dispensable for the transforming activity of H-Ras (Willumsen et al., 1986). However, two pieces of evidence hint at a functional role for the Y13-259 epitope. First, Kung et al. (1986) found that microinjection of Y13-259 into H-Ras transformed NIH 3T3 cells reversed the transformed phenotype. Second, Mulcahy et al. (1985) discovered that the Y13-259 antibody blocked the serum-induced mitogenic response in untransformed NIH 3T3 cells. Since the Y13-259 epitope is contiguous with the second GTP binding domain (see Figure 1), it is possible that the neutralizing ability of the Y13-259 antibody was
due to steric interference of Ras protein-protein interactions or Ras-guanine nucleotide interactions. In support of this hypothesis is the finding that Y13-259 did not block nucleotide binding but impaired guanine nucleotide exchange on Ras (Hattori *et al*., 1987; Lacal & Aaronson, 1986). The complete functional significance of the conserved Y13-259 epitope therefore remains to be determined.

**Hyper-variable region**

The most divergent region of the Ras proteins is found in the 20 carboxy-terminal residues that lie upstream of the CAAX motif (residues 165 to 185; see Figure 1). Deletion of these residues did not alter the guanine nucleotide binding properties of the viral H-Ras protein, and deletion studies revealed that these residues were dispensable for transformation (Lacal *et al*., 1986b; Willumsen *et al*., 1986). Even though this hypervariable region is dispensable for the transforming ability of the activated H-Ras, it is still possible that it is essential for the normal function of the Ras proteins. Accumulating evidence now suggests that this hypervariable region could function as a membrane targeting sequence (Adamson *et al*., 1992; Chavrier *et al*., 1991; Hancock *et al*., 1991).

1.3.2 Three-Dimensional Structure

The three-dimensional (3-D) crystal structures of the normal H-Ras-GDP, and the activated H-Ras-GTP protein complexes have now been determined (de Vos *et al*., 1988; Pai *et al*., 1989). The protein consists of a six-stranded β-sheet, four α helices, and ten connecting loops. The loops are designated L1 to L10 starting at the N-terminus, and most of them are exposed on the surface of the protein (see Figure 2). Four of the nine loops (L1, L4, L8, and L10) form a pocket for the binding of the guanine nucleotide. Loops L1 and L4 interact with the β and γ phosphates, while
Figure 2. Ribbon cartoon of the three-dimensional structure of the human H-Ras-GTP protein as determined by Pai et al. (1989). The secondary structure loop regions involved in guanine nucleotide binding are labelled L1, L4, L8, and L10, and are highlighted in black. The Y13-259 epitope and the effector domain regions are represented by the checkered and hatched areas respectively. The position and conformation of the bound GTP molecule is indicated. The "hot-spots" for oncogenic, activating mutations (codons 12, 13, & 61) are indicated. This cartoon was modified from an illustration by Han and Sternberg (1991).
loops L8 and L10 interact with the guanine base. The biological functions of the remaining five loops are presently unknown.

Comparison of the 3-D crystal structure of the GTP- and GDP-bound H-Ras protein revealed that the major conformational differences between the inactive (GDP-bound) and the active (GTP-bound) H-Ras protein are confined to loops L2 (effector) and L4 (GTP-binding domain #2 & Y13-259 epitope), loops which are in close proximity to the γ phosphate group (Jurnak et al., 1990; Milburn et al., 1990). Exactly how these conformational changes influence Ras biological activity remains unclear.

Two of the hotspots for activating lesions on the Ras proteins are residues 12 and 13, and they are located in the first GTP-binding domain. The 3-D structure revealed that these two residues are in loop L1, the loop which straddles the β and γ phosphate of the GTP molecule when it is bound to H-Ras (de Vos et al., 1988; Pai et al., 1989). It is therefore speculated that the substitutions which occur in this loop alter the conformation of the loop, affect GTP binding, inhibit GTP hydrolysis, and hence maintain the Ras protein in an active GTP-bound state (Jurnak et al., 1990; Milburn et al., 1990). Likewise, substitutions in the second GTP-binding domain, at residues 59, 61, or 63 (loop L4), are postulated to indirectly affect GTP binding and hydrolysis by inducing conformational changes in the neighbouring L1 loop.

1.3.3 Mechanism Of Activation Of Ras, And Regulators Of Ras

The Ras proteins cycle between an inactive GDP-bound form and an active GTP-bound form, and in the absence of any stimuli, the Ras-GDP complex is more abundant because the GTPase rate is higher than the GDP/GTP exchange rate (Figure 3). However, in the presence of an activating signal, the active Ras-GTP complex accumulates, either through the stimulation of a nucleotide exchange factor or
Figure 3. Proposed model for the regulation of Ras activity. Upon receipt of an activating signal, the exchange factor interacts with the inactive GDP-bound Ras and promotes the exchange of GDP for GTP. The activated Ras now interacts with its effector(s) to transmit the signal which will produce a variety of cellular effects. Stimulation of the intrinsic Ras GTPase by GAP/NF1, inactivates Ras by returning it to the GDP-bound state. The role of GAP/NF1 as an effector is still unresolved.
Activating Signal
e.g. Growth factors

+ Exchange factors
(Sos, CDC25)

GDP

Ras

GTP

Ras

- Activating Signal
e.g. Growth factors

GTPase activating proteins
(GAP, NF1)

INACTIVE

ACTIVE

CELLULAR EFFECTS
DNA synthesis
Gene expression
Cell growth
Cell differentiation

EFFECTOR
inhibition of the GTPase activating protein. The activated Ras-GTP now interacts with its specific effector/target molecule to exert its biological effects. The activated Ras protein is deactivated by the hydrolysis of the bound GTP to GDP. This occurs in response to stimulation of the intrinsic Ras GTPase by a cytoplasmic molecule termed the GTPase activating protein (GAP).

Under normal circumstances, the Ras GDP/GTP cycle is tightly regulated and the Ras proteins are mostly in an inactive GDP-bound state. However, this regulation is lost in the oncogenic Ras proteins. The oncogenic Ras proteins exist predominantly in the GTP-bound state and are constitutively active, exerting their biological effects even in the absence of an activating signal.

Most of the naturally occurring activated ras genes have a single point mutation in either codon 12, 13, or 61. When mutagenesis studies of the H-Ras protein were undertaken to assess the significance of these "hot-spots", it was demonstrated that substitution of glycine-12 with any other amino acid except proline resulted in an activated H-Ras protein (Seeburg et al., 1984). This suggested that glycine-12 played a crucial role in endowing H-Ras with its regulatable biological properties. A similar analysis of codon 61 substitutions revealed that glutamine-61 could be substituted with any of fifteen other amino acids to produce an activated H-Ras protein (Der et al., 1986). It was subsequently determined that oncogenic Ras proteins with substitutions at codon 12, 13, or 61 had a lower affinity for GAP than the normal non-transforming Ras proteins. Hence, the basis for the constitutive activity of the oncogenic Ras proteins appeared to be their inability to be down-regulated by GAP. As a result, they are maintained in an active GTP-bound state (Adari et al., 1988; Der et al., 1986; Gideon et al., 1992; Trahey et al., 1988; Vogel et al., 1988).

Mutation of amino acid residues 59, 63, 116, 117, or 119, also activates the H-Ras protein (Der, 1989; Der et al., 1988; Fasano et al., 1984; Feig & Cooper, 1988; Sigal
et al., 1986b; Walter et al., 1986). Like the naturally occurring oncogenic Ras proteins, experimentally-generated activated Ras with position 59 or 63 substitutions (in GTP-binding domain #2) had decreased rates of GTP hydrolysis, and they were not regulated by GAP. In contrast to the Ras proteins activated by substitutions in the first or second GTP-binding domains, Ras proteins activated by substitutions in the third GTP-binding domain, (at residues 116, 117, or 119), had decreased GTP-binding affinity and increased guanine nucleotide exchange rates. The increased guanine nucleotide exchange rate is believed to be the basis for the transforming potential of these mutants since the physiological concentrations of GTP and GDP are in the ratio of 25:1, and the increased exchange rate would shift the equilibrium of the Ras cycle in favour of the active GTP-bound complex (Der, 1989; Sigal et al., 1986b; Walter et al., 1986).

Through biochemical and genetic approaches, both positive and negative regulators of the Ras proteins have now been identified in a variety of organisms (for review, see Bollag & McCormick, 1991; Downward, 1992). The first protein to be identified as a regulator of Ras activity was the GTPase activating protein or GAP. It was identified as a cytoplasmic factor in Xenopus oocytes that stimulated the intrinsic GTPase of the normal N-Ras-GTP (Trahey & McCormick, 1987). GAP genes have now been cloned from bovine and murine cells, and in S. cerevisiae, two genes which encode GAP-like proteins, IRA1 and IRA2, have been isolated (Bollag & McCormick, 1991). GAP possesses one SH3 and two SH2 domains (Trahey et al., 1988; Vogel et al., 1988), domains that are found in a variety of intracellular signalling molecules and receptor tyrosine kinases, and which are believed to play a pivotal role in establishing and regulating protein-protein interactions with other tyrosine phosphorylated proteins (Pawson & Gish, 1992). The presence of SH2 and SH3 domains in GAP therefore suggested an association of GAP, and possibly Ras, with receptor tyrosine kinases.
The human neurofibromatosis gene, NF1, encodes a protein that has a region of high similarity to the catalytic domains of GAP and the IRA proteins (Ballester et al., 1990; Xu et al., 1990b). This NF1 catalytic domain has been designated as NF1-GRD for NF1-GAP-related domain (Ballester et al., 1990; Xu et al., 1990a). Like GAP, the NF1-GRD binds both normal and oncogenic Ras, but it only stimulates the intrinsic GTPase of normal Ras (Martin et al., 1990; Xu et al., 1990a).

Positive regulators of the Ras proteins stimulate the exchange of GDP for GTP. In this thesis, proteins with this ability will be referred to as 'GDS', GDP dissociation stimulators, or 'GEF', guanine nucleotide exchange factors (reviewed in Bokoch & Der, 1993; Downward, 1992). Exchange factors of mammalian, insect, and yeast origin have been identified but how they bring about guanine nucleotide exchange is still unknown. Since these exchange factors all stimulate the exchange of GDP for GTP, it was surprising to find that they were localized primarily in the cytosol while their targets, the Ras and heterotrimeric G-proteins, were localized on the plasma membrane. This enigma has been recently explained by the discovery of 'adaptor' proteins which are believed to bind the cytosolic exchange proteins and translocate them to the membrane (Clark et al., 1992; Lowenstein et al., 1992) (see section 1.5.2).

1.4 Does GAP Lie Upstream Or Downstream Of Ras?

While there is no doubt that GAP is a negative regulator of Ras activity, there are still conflicting views on whether GAP acts upstream or downstream of Ras in signal transduction. The strongest evidence in support of an upstream regulatory role for GAP was obtained by Downward et al. (1990b) in a study of the activation of T lymphoblasts. In this study, the rapid activation of Ras that occurred upon
stimulation of the T-cell antigen receptor was mediated by protein kinase C through an inhibitory effect on GAP (Downward et al., 1990b).

Most of the evidence which suggests that GAP lies downstream of Ras, favours GAP as an effector of Ras. First, GAP interacts with Ras through its putative effector domain and binds Ras in a GTP-dependent manner (Adari et al., 1988; Calés et al., 1988; Sigal et al., 1986; Vogel et al., 1988). Second, mutations in the Ras effector domain which reduce the transforming potential of Ras (eg. Asp38Ala) prevent the stimulation of the Ras GTPase by GAP, and also appear to prevent GAP binding to Ras (Adari et al., 1988; Calés et al., 1988). Additional evidence for a GAP effector function comes from studies using a S. cerevisiae mutant RAS protein which was locked in the GTP-bound state and was localized in the cytosol. This mutant RAS protein had an increased affinity for GAP, and inhibited the [Val^{12}]H-Ras-stimulated germinal vesicle breakdown in Xenopus oocytes (Gibbs et al., 1989b). The block was overcome by microinjecting purified mammalian GAP into the oocytes. This argued for an effector role for GAP since if the sole function of GAP was to be a negative regulator of Ras, addition of purified GAP should have returned the RAS-GTP protein to the inactive GDP-bound state. The exact role of GAP in the Ras signal transduction pathway thus remains unresolved.

1.5 Ras Physiological Function

The idea that Ras was involved in transmembrane signal transduction was further reinforced when it was observed that a variety of extracellular mitogens triggered the rapid activation of Ras in cultured cell lines (Downward, 1990). Exactly how Ras transduces this signal in eucaryotic cells has, until recently, been an enigma because the activation of Ras produces different effects in different tissues and in
different organisms. In mammalian cells, these diverse effects include the proliferation and differentiation of hematopoietic cells, the activation of T lymphocytes, neuronal differentiation of pheochromocytoma cells, and growth inhibition of epithelial cells (for a recent review see Satoh et al., 1992). These diverse effects all hint at the involvement of the Ras proteins in both proliferative and developmental events. In non-mammalian cells, Ras activation produces effects that include cell cycle progression and cell proliferation in the budding yeast, *S. cerevisiae*, and cell fate determination of specific precursor cells in the fruit fly *Drosophila melanogaster* and in the nematode *Caenorhabditis elegans*. In the last few months however, researchers have discovered that, despite the pleiotropic effects elicited by Ras activation, there are similar upstream and downstream components in the mammalian, *Drosophila*, and *C. elegans* Ras signal transduction pathways (see below).

1.5.1 Ras In *S. cerevisiae*

Two ras genes have been isolated and characterized in the budding yeast *S. cerevisiae*. Like their mammalian counterparts, the yeast Ras proteins (RAS1 & RAS2) bind GTP and GDP, and possess an intrinsic GTPase activity (for review, see Gibbs & Marshall, 1989). Neither gene by itself is essential, but disruption of both genes is a lethal event; cells bearing the disrupted genes are non-viable and appear to be blocked in the G1 to S phase of the cell cycle (Tatchell et al., 1984).

Through genetic and biochemical analyses, it was established that the *S. cerevisiae* RAS proteins are essential regulatory elements in the cAMP metabolic pathway (Gibbs & Marshall, 1989; Toda et al., 1985). In this pathway, the RAS proteins are positive regulators of adenylate cyclase (AC), the protein which catalyzes the synthesis of cAMP from ATP. In yeast, the cycling of the RAS proteins between the inactive GDP-bound form and the active GTP-bound form involves the
positive regulator CDC25 (Broek et al., 1987) and the negative regulators IRA1 and IRA2 (Tanaka et al., 1990). CDC25 catalyzes guanine nucleotide exchange to and from RAS (Broek et al., 1987; Jones et al., 1991), while IRA1 and IRA2 are believed to stimulate the intrinsic GTPase of the yeast RAS proteins since the mammalian NF1-GRD and GAP can replace IRA function in IRA-deficient yeast strains (Ballester et al., 1990; Tanaka et al., 1990; Xu et al., 1990a).

Although the major function of RAS in S. cerevisiae appears to be the regulation of cAMP metabolism, the observation that AC deficient mutants are viable while Ras null mutants are not (Toda et al., 1985) suggests that the yeast RAS proteins are involved in another essential, non-AC mediated, pathway.

1.5.2 Ras Function In C. elegans And D. melanogaster

The first direct evidence linking Ras proteins to signal transduction pathways controlling specific developmental processes came from studies in C. elegans and D. melanogaster. Through epigenetic studies and complementation analysis, it was established that in both organisms, Ras proteins were involved in receptor tyrosine kinase-mediated signal transduction pathways that control cell fate.

In C. elegans, Ras is involved in the inductive signalling pathway for vulval development, and it acts as a switch to control cell fate (reviewed in Greenwald & Broach, 1990). When the switch is on, i.e. Ras in the GTP-bound state, the precursor cells develop into vulval cells, but when the switch is off, i.e. Ras in the GDP-bound state, the precursors develop into hypodermal cells. The pathway originates at the gonadal anchor cell, where an EGF-like protein is produced and acts as an inductive signal for the neighbouring vulval precursor cells (Hill & Sternberg, 1992). The let-23 gene encodes a receptor tyrosine kinase (Aroian et al., 1990) that is expressed on the precursor cells and which is believed to be the receptor for the EGF-like protein. The newly identified Sem-5 protein (Clark et al., 1992) is believed to be an adaptor
molecule that brings an as yet unidentified GEF in close juxtaposition with the membrane-localized receptor and the Ras protein, which is encoded by the let-60 gene (Han & Sternberg, 1990). The "adaptor" role for Sem-5 was postulated because Sem-5 has no catalytic domains but consists solely of SH2 and SH3 domains, domains which play a role in protein-protein interactions. The lin-45 gene encodes a Raf serine/threonine kinase homolog, and it was recently identified as a downstream component in the Ras-stimulated vulval development pathway (Han et al., 1993).

In D. melanogaster, the Ras proteins are involved in determining the fate of the precursor cells in the compound eye during development (reviewed by Rubin, 1991). Ras again acts as a switch in control of cell fate, and determines whether the R7 precursor cell becomes a photoreceptor neuronal cell (Ras on, GTP-bound) or a non-neuronal cone cell (Ras off, GDP-bound). The upstream components of this receptor tyrosine kinase signalling pathway have all been recently identified, but the direct target of Ras action in the R7 photoreceptor cell is still unknown. Activation of the receptor tyrosine kinase, Sev (Bowtell et al., 1988; Hafen et al., 1987), by the transmembrane ligand, Boss (Hart et al., 1990; Kramer et al., 1991), stimulates the guanine nucleotide exchange factor, Sos, which is closely related to the yeast CDC25 protein (Bonfini et al., 1992). The Sem-5 homolog, encoded by the drk gene (Olivier et al., 1993; Simon et al., 1993), is believed to initiate complex formation with the receptor (Sev), the exchange factor (Sos) and Ras. The signal is then relayed from the Ras protein to an as yet unknown target which ultimately produces cell fate determination effects, and results in the development of the R7 photoreceptor neuron.
1.5.3 Ras In Mammalian Signal Transduction Pathways

Several researchers have recently presented evidence that links the activation of tyrosine kinase receptors (e.g., EGF-R) to the activation of Ras in rodent cells (Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). These experiments all demonstrated that the mammalian Ras GEF, Sos, normally existed in the cytosol complexed with the adaptor protein, Grb2. However, upon EGF receptor activation and autophosphorylation, the Grb2/Sos complex translocated to the membrane and formed a multiprotein complex with the phosphorylated receptor through the interaction of the Grb2-SH2 domain with the phospho-tyrosine residues of the receptor (Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). The formation of the multiprotein complex is believed to bring the exchange factor, Sos, in close proximity to the membrane-bound Ras protein so that it can stimulate guanine nucleotide exchange and activate Ras (Gale et al., 1993) (Figure 4). A similar mechanism for Ras activation also appears to be operative in the mammalian L6 cell line, where the insulin receptor appears to be linked to a Ras signalling pathway through multiprotein complex formation with Grb2 and Sos (Skolnik et al., 1993; Skolnik et al., 1993b).

While the downstream components of the Ras signalling pathway are proving more difficult to identify, the experiments of Moodie and co-workers (1993) were the first to provide evidence linking the membrane-bound activated Ras to the cytoplasmic mitogen-activated protein (MAP) kinases. These kinases form a phosphorylation cascade that ultimately results in the phosphorylation of nuclear transcription factors and other cellular proteins that regulate gene expression (Pazin & Williams, 1992; Roberts, 1992). Moodie et al. (1993) demonstrated that oncogenic H-Ras-GTP, or normal H-Ras complexed to a non-hydrolyzable GTP analog, interacted specifically in vitro with rodent Raf-1 kinase, while an effector domain mutant, Ile36Ala, did not react with Raf-1. They therefore postulated that Raf-1 was
the elusive downstream effector of Ras since it fulfilled the criteria for a Ras effector, i.e. it interacted only with the activated Ras-GTP complex and it was unable to bind to a Ras effector mutant (Moodie et al., 1993). Subsequent experiments by independent researchers have confirmed that normal and oncogenic Ras bind to the amino-terminal regulatory region of Raf-1 \textit{in vitro} (Warne et al., 1993; Zhang et al., 1993). These latter experiments also confirmed that the Ras effector mutants were unable to interact with Raf-1 kinase.
Figure 4. A proposed model of the mammalian Ras signalling pathway. Upon binding of the extracellular ligand to the receptor tyrosine kinase, the receptor becomes enzymatically activated and autophosphorylated. This modification of the receptor enables it to bind to, and hence localize, a cytosolic complex consisting of an adaptor protein (e.g. Grb2) and a guanine nucleotide exchange factor (e.g. Sos). This brings the exchange factor in close proximity to the membrane-bound Ras protein such that the GEF can now bind and stimulate the exchange of GDP for GTP on the Ras protein. The activated GTP-bound Ras now transduces the signal to the nuclear apparatus via a kinase cascade that originates with Raf-1 kinase.
DNA synthesis
Gene expression
Cell growth
Cell differentiation
1.6 Ras And Known Second Messenger Systems

Unlike the situation in *S. cerevisiae*, no connection between Ras and adenylate cyclase (AC) activation has been found in mammalian cells, and thus it has been concluded that activated Ras has no effect on cAMP levels in mammalian cells (Beckner *et al.*, 1985; Birchmeier *et al.*, 1985). The search for an alternative signal transduction pathway involving Ras led to the discovery of an association between Ras and the phosphatidyl inositol (PI) pathway. It was observed that H-Ras activation led to increased levels of 1, 2-diacylglycerol (DAG) in NIH 3T3 cells, and this suggested that Ras played a role in the regulation of phospholipase C (PLC) (Lacal *et al.*, 1987; Wilkison *et al.*, 1989; Wolfman & Macara, 1987). PLC hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate the second messengers, inositol 1, 4, 5-trisphosphate (IP3), and DAG, which regulate intracellular calcium stores and activate protein kinase C (PKC) respectively (reviewed in Sternweiss & Smrcka, 1992). However, the reproducible increase in DAG levels in Ras-transformed cells, in the absence of a detectable increase in IP3 levels, casted doubts on the involvement of Ras in the PI pathway (Fleischman *et al.*, 1986; Lacal *et al.*, 1987b). Other researchers subsequently reported that microinjection of antibodies to PIP2 or PLC blocked Ras-stimulated DNA synthesis, and these observations argued that the hydrolysis of PIP2, and hence IP3 and DAG production, were prerequisites for Ras action (Fukami *et al.*, 1988; Smith *et al.*, 1990).

Since Ras activation correlated with increased DAG levels, and PKC is activated by DAG, it was suggested that PKC lies downstream of Ras in the signalling pathway (Morris *et al.*, 1989). This hypothesis was based on the finding that pretreatment of Swiss NIH 3T3 cells with the PKC inhibitor, 12-O-tetradecanoylphorbol-13-acetate (TPA), prevented phosphorylation of the 80 kDa PKC substrate, and blocked Ras-induced DNA synthesis (Morris *et al.*, 1989).
However, not all Ras-induced events in NIH 3T3 cells are mediated through PKC because Ras can morphologically transform cells in the absence of PKC (Lloyd et al., 1989). Furthermore, there is some contradictory evidence which suggests that Ras acts downstream of PKC. In T lymphocytes and PC12 cells, the activation of PKC by phorbol esters stimulates the activation of Ras to the GTP-bound state (Downward et al., 1990b; Nakafuku et al., 1992). PKC may therefore have both upstream and downstream functions in the Ras signalling pathway, or Ras and PKC may be linked in multiple interconnecting pathways.

1.7 Ras-related Proteins

1.7.1 Structure

The Ras proteins are members of a large superfamily (Table 1) where the major sequence conservation between members occurs in the four guanine nucleotide binding domains. Variations in the amino acid sequence outside these four domains make it possible to divide the superfamily into the three main subfamilies of Ras, Rab, and Rho (for review, see Chardin, 1988; Valencia et al., 1991).

The Ras subfamily consists of the Ras, Rap, Ral, and R-Ras proteins. Rap, Ral, and R-Ras are all at least 50% identical to Ras and, with the exception of the Ral proteins, they all have identical residues in the putative effector domain (YDPTIEDSY, residues 32-40 relative to H-Ras). There is, however, one major difference between the Rap proteins and the other members of the subfamily. The Rap proteins possess a threonine residue at position 61 instead of a glutamine. This substitution is an activating lesion in the human H-Ras protein, and if the Rap
### TABLE 1. The Ras Superfamily

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<sup>a</sup>sequences described in this thesis  
<sup>b</sup>also known as Krev1 or smg p21  
<sup>c</sup>also known as Dm rap1  
<sup>d</sup>also known as BUD1

Abbreviations: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum*; Ce, *Caenorhabditis elegans*.

Adapted from Downward (1990), and Kahn (1992).
proteins are activated by the same mechanism as H-Ras, one would expect the Rap proteins to be constitutively active.

The Rho subfamily is comprised of the Rho, Rac, TC10, and CDC42 proteins, which are at least 50% identical to each other but only 30% identical to Ras (Downward, 1992b). The Rho subfamily proteins possess two striking variations relative to the Ras subfamily proteins. They possess a different effector domain motif, (YVPTVFENY), and they all have a glycine-13 to alanine substitution in the first GTP-binding domain.

The Rab subfamily proteins are 40% identical to each other, and share 30% amino acid identity with the Ras proteins. The putative effector domain is not at all conserved in the Rab proteins, and each member of the subfamily has a different effector domain sequence. In addition, the Rab proteins all have a substitution at the equivalent of codon 12 (glycine) relative to H-Ras.

1.7.2 Function

The Ras-related proteins regulate a diverse array of cellular processes. The Rho and Rac proteins are involved in regulating cytoskeletal processes such as actin stress fibre formation and membrane ruffling (Paterson et al., 1990; Ridley et al., 1992), and the recent experiments of Perona et al. (1993) have now linked the Rho proteins to growth control. Mutant Aplysia Rho proteins with a valine-14 substitution, (equivalent to the oncogenic valine-12 substitution in mammalian cells), were tumorigenic and produced a transformed phenotype in the mammalian NIH 3T3 cell line (Perona et al., 1993).

The mammalian rab genes are related to the yeast YPT1 and SEC4 genes whose protein products are believed to have regulatory roles in membrane trafficking, vesicular transport, and secretion (Balch, 1990). The Rab proteins are therefore postulated to play a similar role in higher eucaryotes since they can complement
mutant *S. cerevisiae* which lack functional YPT1 protein (Segev *et al.*, 1988). The localization of the Rab proteins to exocytic and endocytic cellular compartments (Chavrier *et al.*, 1990), and the inhibition of vesicular transport by GTP-binding mutants of Rab1 and Rab2 (Tisdale *et al.*, 1992), provides additional evidence in support of a role for Rab proteins in cellular transport processes.

While it is believed that the highly related members of the Ras subfamily proteins (H-Ras, K-Ras, & N-Ras) are involved in growth control, little is known about the less related members of the subfamily (R-Ras, Ral, & Rap), none of which appear to possess oncogenic properties. The Rap proteins have been the most characterized and evidence indicates that they may also play a role in growth control since overexpression of the *rap* gene in K-ras transformed NIH 3T3 cells resulted in the suppression of the transformed phenotype (Kitayama *et al.*, 1989). Hence, it is speculated that Ras and Rap are linked in the same signal transduction pathway through their interaction with a common effector molecule (Bokoch, 1993).
1.8 *Dictyostelium discoideum*

1.8.1 Life Cycle

The cellular slime mold *Dictyostelium discoideum* is a simple eucaryotic organism in which a variety of developmental questions can be addressed. Its short life cycle, in conjunction with its synchronous development, make it amenable to experimental analyses aimed at understanding processes such as signal transduction and morphogenesis.

During the vegetative or proliferative stage of its life cycle, *Dictyostelium* exists as solitary amoebae that feed on bacteria. However, upon depletion of the bacterial food source, the amoebae stop proliferating and immediately enter the developmental cycle through a unique and characteristic series of events (reviewed in Loomis, 1982) (Figure 5). At the onset of starvation, a few cells secrete pulses of the chemoattractant cAMP, and establish a cAMP signal relay. Neighbouring amoebae respond to these cAMP pulses by moving toward these "initiator" cells, and then they in turn secrete pulses of cAMP to recruit additional amoebae. Ultimately, a multicellular aggregate containing approximately $10^5$ amoebae is formed. The aggregate rounds up and then elongates to form a migrating slug, or pseudoplasmodium, in which the differentiating cells are spatially segregated so that the anterior 20% of the slug consists of pre-stalk cells while the posterior 80% consists of pre-spore cells. These precursor cells eventually give rise to the terminally differentiated stalk and spore cells of the mature fruiting body.
Figure 5. The Dictyostelium discoideum developmental life cycle. Upon starvation (time, 0 hr), the vegetative Dictyostelium amoebae enter the developmental cycle and proceed to aggregate in response to pulses of cAMP. The aggregate then develops into a pseudoplasmodium (time, 16 hr), in which the amoebae differentiate to form either pre-stalk or pre-spore cells. The entire cycle is completed approximately 24 hr after the onset of starvation, and results in the formation of a fruiting body that consists of a spore sorus supported by a fibrous stalk. Time after the onset of starvation is indicated in hours along with the corresponding developmental stage.
1.8.2 Signal Transduction In Dictyostelium

Cell to cell communication plays a central role in regulating multicellular development in Dictyostelium, and one of the primary extracellular signalling molecules in Dictyostelium is cAMP (for reviews, see Firtel, 1991; Kimmel & Firtel, 1991). Three cAMP-dependent signal transduction pathways have been identified so far in Dictyostelium (see Figure 6), and these three pathways, (chemotaxis, signal relay, and gene expression), are all activated when the bacterial food source is depleted and the developmental program is initiated. In the context of Dictyostelium development, signal relay refers to the synthesis and release of cAMP to the extracellular medium to establish a directional cAMP signal emanating from an initiator, cAMP-producing amoeba.

In Dictyostelium, the cell surface cAMP receptors (cAR) are coupled to heterotrimeric G-proteins, and these receptors mediate the cAMP-dependent responses that are elicited from the Dictyostelium amoebae when the developmental program is initiated. Four distinct cAR genes have been cloned in Dictyostelium and they encode receptors that possess the seven transmembrane spanning domains that are typical of G-protein coupled receptors. In addition, each receptor has a characteristic intracellular carboxy-terminus and a characteristic developmental pattern of expression (Saxe et al., 1988; Saxe et al., 1991). These features therefore suggested that each receptor may be linked to a different G-protein and hence may be involved in distinct signal transduction pathways.

The cAMP receptors had been previously categorized into two classes based on their rates of dissociation of cAMP (fast, RA and slow, RB; reviewed in Janssens & Van Haastert, 1987). Now, with the cloning of four cAR genes, it is not known whether the different classes are encoded by different genes or whether each of the four cARs has a fast and slow dissociating form.
Figure 6. Model of the cAMP-dependent signal transduction pathways in *Dictyostelium discoideum*. Two types of cell surface cAMP receptors are available for binding extracellular cAMP. The RA receptors are coupled to an as yet unidentified G protein which stimulates adenylate cyclase (AC), and activates the signal relay pathway. The RB receptors are coupled to a heterotrimeric G protein (Ga2, β, γ) that is linked to the activation of guanylate cyclase (GC) and phospholipase C (PLC), and the stimulation of the chemotaxis and gene expression pathways. The stimulated cAMP receptors become desensitized (DA) by an adaptation process until the levels of extracellular cAMP are lowered by the action of the phosphodiesterase (PDE). For more details, please see text. This diagram was adapted from a figure in a recent review by Kimmel & Firtel (1991).
PDE

5' AMP $\rightarrow$ cAMP $\rightarrow$ cAMP $\rightarrow$ Extracellular

Chemotaxis

Ca$^{2+}$ $\rightarrow$ IP$_3$ + DAG $\rightarrow$ Developmentally regulated gene expression

Signal-relay
Activation of PKA
Regulated gene expression

GC $\rightarrow$ cGMP $\rightarrow$ Chemotaxis

$\beta_Y$ $\rightarrow$ $\gamma$ $\rightarrow$ PLC

$\alpha_2$ $\rightarrow$ $\alpha_2$ $\rightarrow$ PIP$_2$

$\gamma$ $\rightarrow$ P

$\alpha$ $\rightarrow$ G

ATP $\rightarrow$ cAMP
The fast dissociation receptors ($R^A$) are linked to the activation of adenylate cyclase (AC) and the signal relay pathway (Figure 6). Binding of cAMP to these receptors results in AC activation, the synthesis of cAMP, and the subsequent release of the de novo cAMP into the extracellular medium to establish a cAMP signal relay (Firtel, 1991; Kimmel & Firtel, 1991). The secreted cAMP can bind to cAMP receptors on the same cell which secreted it (positive feedback loop) or it can bind and activate cAMP receptors on neighbouring cells. The autocatalytic activation of the cAMP receptors on the same cell is controlled by an adaptation and deadaptation mechanism which is dependent on the phosphorylation of the receptors; phosphorylation of the receptors desensitizes the cell until the high level of extracellular cAMP has been reduced by natural diffusion or degradation by the cell surface phosphodiesterase (Janssens & Van Haastert, 1987; Vaughan & Devreotes, 1988). The neighbouring amoebae respond to the secreted cAMP by activating their own signal relay pathway and secreting pulses of cAMP so that the signal is relayed outward from the aggregation center and the initial cAMP secreting amoeba.

The chemotaxis pathway is responsible for the movement of the amoebae up the cAMP gradient towards the aggregation center. This pathway operates in parallel with the signal relay pathway and ensures coordinate movement of the amoebae up the cAMP gradient. The slow dissociation receptors ($R^B$) are coupled to this pathway via the $G_\alpha 2$ protein subunit, and binding of cAMP to these receptors results in the activation of phospholipase C (PLC) and guanylate cyclase (GC) (Figure 6). The activation of these enzymes increases the levels of the second messengers IP$_3$ and cGMP, and results in a) the polymerization of actin and myosin, and b) the eventual movement of the cells towards the aggregation center.

Many of the developmentally regulated Dictyostelium genes can be classified according to their pattern of expression and/or mechanism of regulation. One group consists of the cAMP pulsed-induced genes which are expressed in early
development, and are required for aggregation (reviewed by Dottin et al., 1991). A second group of genes are repressed by pulses of cAMP during early development and this group can be further subdivided into two groups. One subgroup is induced upon starvation, reaches maximum expression within 2-3 hrs, and is repressed during aggregation (Mann & Firtel, 1987; Mann et al., 1988). This repression appears to be mediated via PLC and components of the chemotaxis signalling pathway. The other subgroup requires a rise in intracellular cAMP and is dependent on AC activation. The genes in this subgroup therefore appear to be regulated through the signal relay pathway (Kimmel, 1987; Kimmel & Carlisle, 1986).

Many of the genes whose expression is induced by the continuous application of cAMP are expressed post-aggregation (Chung et al., 1981). Since the concentrations of cAMP required to induce post-aggregative gene expression should cause adaptation of the early class of cAMP receptors, the induction of these late genes is believed to involve a class of cAMP receptors that are not sensitive to the same mechanism of adaptation as the cAMP receptors present in early development. Interestingly, one of the Dictyostelium cAMP receptors (cAR1), which is expressed in early development, possesses several clusters of serine residues in its carboxy-terminal tail, and these serines are believed to be the phosphorylation sites that bring about adaptation of the receptor (Dottin et al., 1991). Since the other Dictyostelium cAMP receptors, cAR2, cAR3, and cAR4, lack potential carboxy-terminal phosphorylation sites, and are expressed post-aggregation, it is speculated that they are involved in the regulation of late gene expression since they would not be subject to the same mechanism of adaptation as the cAR1 receptor. Furthermore, since the late genes can be induced by DAG and IP3 (Ginsberg & Kimmel, 1989; Kimmel & Eisen, 1988), the involvement of PLC, and possibly components of the chemotaxis pathway, is implicated in the regulation of late gene expression.
1.8.3 ras Genes In Dictyostelium discoideum

Two ras genes, rasD and rasG (Reymond et al., 1984; Robbins et al., 1989), and one highly ras-related gene, rap1 (Robbins et al., 1990), had been isolated and characterized in Dictyostelium when the work in this thesis was started. These genes all encode proteins that are conserved in the four functional GTP-binding domains, the putative effector domain, and the carboxy-terminal CAAX motif.

The rasD gene was the first Dictyostelium ras gene to be isolated, and it was discovered during the characterization of a collection of genes that were preferentially expressed in the pre-stalk cell population (Reymond et al., 1984). Two mRNA transcripts, 0.9 kb and 1.2 kb, are produced from the rasD gene, and they are maximally expressed during the pseudoplasmodial stage of development. However, studies by Pawson et al. (1985) indicated that the highest levels of Dictyostelium Ras proteins were synthesized during vegetative growth and not during the pseudoplasmodial stage of development as expected from Reymond's data. This discrepancy of low ras gene expression in vegetative cells but high levels of protein synthesis, led to the search for, and subsequent isolation of, a vegetative-specific ras gene. This second ras gene was designated rasG, and it encodes a protein that is 82% identical to the rasD gene product and 68% identical to the human H-Ras protein (Robbins et al., 1989). The rasG gene is maximally expressed during growth and early development (0-4 hr post-starvation) and is transcribed into a 1.2 kb mRNA (Robbins et al., 1989).

The Dictyostelium rap1 gene was isolated as a vegetative cDNA clone that hybridized weakly to a rasD probe (Robbins et al., 1990). The deduced amino acid sequence of rap1 is 76% identical to the human Rap1A protein and in contrast to rasD and rasG, rap1 is expressed throughout development in a biphasic pattern with maximal expression during aggregation (8 hr), and culmination (20 hr) (Robbins et al., 1990). During vegetative growth and early development, a single 1.1 kb rap1
mRNA is detected while in late aggregation and throughout the rest of development, two transcripts (1.0 and 1.3 kb) are detected (Robbins et al., 1990).

In an effort to determine the physiological role of rasD in Dictyostelium, Reymond and co-workers (1986) transformed vegetative Dictyostelium cells with an 'activated' rasD gene that encoded a threonine residue instead of a glycine at position 12. A Dictyostelium transformant, which overexpressed the activated form of the rasD gene, produced an aberrant multi-tipped aggregate that failed to terminally differentiate. Under certain conditions the transformant was deficient in aggregation (Reymond et al., 1986). The Dictyostelium rasD-Thr-12 transformed cells had a reduced number of cAMP receptors, and it is now believed that RasD is involved in the down-regulation of the cAMP receptors (Luderus et al., 1992; Luderus et al., 1988).

In a parallel study in our laboratory, cells transformed with an activated rasG gene, (Gly-12 to Thr-12), also exhibited aberrant aggregation under certain conditions (Thiery et al., 1992). However, since neither rasG nor rasD are expressed maximally during aggregation, it is questionable whether the aberrant aggregation phenotypes of the activated rasD and rasG transformants were due to interference with the function of the endogenous rasD and rasG genes or whether the phenotypes were actually due to interference with other as yet uncharacterized ras gene(s).
1.9 Rationale And Research Objective

During the characterization of the rasG gene by southern blot analysis of Dictyostelium genomic DNA, it was observed that a large number of genomic fragments hybridized to the rasG cDNA probe under low stringency wash conditions (Robbins et al., 1989). Many of these fragments could not be accounted for by the rasD or rap1 genes, and this suggested that there were additional ras or ras-related genes in Dictyostelium.

In order to study ras gene function in Dictyostelium, and to address the issue of Ras and Rap interaction with a common effector, it is important to ascertain how many ras and rap genes exist in Dictyostelium. The goal of this thesis was to determine if there were additional ras or rap genes in Dictyostelium, and to characterize these genes at the molecular level.
CHAPTER 2

METHODS AND MATERIALS

2.1 Materials

All restriction endonucleases and modifying enzymes were purchased from GIBCO BRL (Burlington, Ont., Canada) unless stated otherwise. All other chemical reagents were purchased from either BDH (Vancouver, B.C., Canada), SIGMA Chemical Co. (St. Louis, Mo., USA), or FISHER Scientific Co. (New Jersey, USA). X-ray film was purchased from Kodak, Canada, and the enhanced chemiluminescence (ECL) immunodetection kit was purchased from Amersham Corporation (Oakville, Ont., Canada). $[\alpha^{32}\text{P}]$ dCTP was purchased from ICN Flow Labs (Mississauga, Ont., Canada) and $[^{35}\text{S}]$ dATP was purchased from DuPont NEN Canada Inc. (Mississauga, Ont., Canada).

The *D. discoideum* strains used in this thesis were the wild type strain V12M2, and the axenic mutant strain Ax2. The *E. coli* strains used in this thesis are listed in Table 2 with their respective genotypes.

The 3hr λgt11 library was kindly donated by Dr. P. Devreotes and the 8 hr λgt11 library was donated by Dr. C.-H Siu. The Y13-259 monoclonal antibody was a gift from Dr. N. Auersberg.

Amino acid sequence comparisons and alignments were done using the PALIGN and CLUSTAL programs of PCGENE.
**TABLE 2.**

*E. coli* strains and their respective genotypes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1090</td>
<td>ΔlacU169, proA+, Δlon, araD139, strA, supF, (trpC22::Tn10), (pNC9), (rk-, mK+).</td>
</tr>
<tr>
<td>DH5αF'</td>
<td>F', ø80d lacZΔM15, Δ(lacZYA-argF), U169, endA1, recA1, hsdR17 (rk-, mK+), deoR, thi-1, supE44, λ-, gyrA96, relA1.</td>
</tr>
<tr>
<td>XL1-blue</td>
<td>endA1, hsdR17(rk-, mK+), supE44, thi-1, λ-, recA1, gyrA96, relA1, (lac-), F' [proAB, lacIqZΔM15, Tn10 (tetR)].</td>
</tr>
</tbody>
</table>

2.2 Growth And Development Of Dictyostelium discoideum

To obtain vegetative V12M2 Dictyostelium cells, spores were inoculated into a culture of Enterobacter aerogenes (bacterial food source), and 0.2 ml aliquots containing the spores and bacteria were plated on SM nutrient agar plates (10 g glucose, 10 g neutralized bacteriological peptone (Oxoid), 1 g yeast extract (Oxoid), 1 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 1.55 g NaH\textsubscript{2}PO\textsubscript{4}.H\textsubscript{2}O, 1 g KH\textsubscript{2}PO\textsubscript{4} and 20 g bacto agar (Difco) per liter of deionized H\textsubscript{2}O). The plates were then incubated for 48 hr at 22°C to obtain a confluent lawn of vegetative amoebae. Each plate yielded approximately 2-3 x 10\textsuperscript{8} vegetative cells. The vegetative amoebae were scraped off the plates, resuspended in KK2 buffer (20 mM KH\textsubscript{2}PO\textsubscript{4}, pH 6.0), and harvested by low speed centrifugation (700g) for 5 minutes. The amoebae were then washed free of residual bacteria by repeated resuspension in KK2 buffer and recentrifugation at 700g for 3 minutes.

To obtain vegetative axenic cells, spores of the axenic strain Ax2 were inoculated into HL-5 medium (14.3 g neutralized bacteriological peptone (Oxoid), 7.15 g yeast extract (Oxoid), 0.96 g Na\textsubscript{2}HPO\textsubscript{4}, and 0.486 g KH\textsubscript{2}PO\textsubscript{4} per liter of H\textsubscript{2}O, pH 6.8) supplemented with 1.5 % glucose, and the culture shaken at 200 rpm and incubated at 22°C. Exponentially growing cells were later diluted into fresh HL-5 to give a fresh culture with a final density of 2 x 10\textsuperscript{5} cells per ml. This culture was then incubated at 22°C and allowed to reach a density of 2-4 x 10\textsuperscript{6} cells per ml (approximately 36 hr). Cells were harvested by centrifugation at 700g for 5 minutes, and washed twice by resuspension in KK2 buffer and recentrifugation at 700g for 3 minutes.

To initiate development, 1-2 x 10\textsuperscript{8} washed cells (wild type V12M2 or axenic Ax2) were plated on non-nutrient agar plates containing Bonner's Salts (10 mM NaCl, 10 mM KCl and 2 mM CaCl\textsubscript{2}; Bonner, 1947), incubated at 22°C for various times, and then harvested and washed as before.
2.3 cDNA Library Construction

2.3.1 Total RNA Isolation

Total RNA was isolated from vegetative (0 hr) or pseudoplasmodial (15 hr) stage *Dictyostelium* V12M2 cells by a modification of a previously described protocol (Birnboim, 1988). Approximately $2 \times 10^8$ cells were resuspended in 5 ml RES-I buffer (0.5 M LiCl, 1 M urea, 1% SDS, 0.02 M sodium citrate, and 2.5 mM cyclohexanediamine tetracetate pH 6.8), and placed on ice for five minutes before adding proteinase K to a final concentration of 50 µg/ml. The mixture was then incubated at 50°C for 30 minutes, allowed to cool to room temperature, and then extracted twice with 0.5 ml phenol/chloroform (1:1), and once with 0.5 ml chloroform/isoamyl alcohol (24:1). One tenth volume of 2 M sodium acetate, pH 5.2, and 2 volumes of 95% ethanol were added to the aqueous phase to precipitate the nucleic acids. The precipitated nucleic acid was then collected by centrifugation, washed with 70% ethanol and air dried. The pellet was resuspended in 3 ml RES-I buffer and the RNA re-precipitated overnight at 4°C after the addition of 30 µl 2 M acetic acid and 3 ml of 5 M LiCl/95% ethanol (3:2). The RNA pellet was washed several times with 70% ethanol to remove traces of LiCl, resuspended in diethylpyrocarbonate-treated water, and stored at -70°C.

2.3.2 Poly (A+) RNA Purification

Polyadenylated (poly (A+)) RNA was purified from total RNA by oligo(dT)-cellulose chromatography as previously described (Maniatis *et al.*, 1982), except that the loading buffer contained 0.5 M LiCl instead of 0.5 M NaCl. The RNA was quantitated by ultraviolet (UV) absorption spectrophotometry at a wavelength of 260 nm where 1 A$_{260}$ unit corresponded to a concentration of 40 µg/ml. The efficiency of the oligo(dT)-cellulose purification was tested by northern blot analysis as follows.
Equal amounts of the poly (A+) RNA fraction, poly (A-) RNA fraction, and total RNA were electrophoresed, blotted and fixed onto a nitrocellulose membrane which was then probed with an \([\alpha^{32}\text{P}]\)-labelled \textit{rap1} cDNA. The \textit{rap1} transcript was detected only in the poly (A+) RNA fraction and the total RNA fraction (data not shown).

2.3.3 cDNA Synthesis And Purification

The \(\lambda\) Librarian cDNA library construction system (Invitrogen) was used to make \textit{Dictyostelium} 0 hr and 15 hr cDNAs from 10 \(\mu\text{g}\) of poly (A+) RNA, according to the manufacturer's protocols. Excess unreacted EcoRI/NotI linkers were removed as follows. A Sepharose Cl-4B (Pharmacia) chromatography column was made in a silanized 1 ml glass pipette. The column was pre-equilibrated with TNE buffer, pH 7.5 (10 mM Tris, 100 mM NaCl, 1 mM EDTA), and preadsorbed with 500 \(\mu\text{l}\) of a 10 mg/ml yeast tRNA (Sigma) solution to block non-specific binding sites. The reaction mixture (50 \(\mu\text{l}\)), containing radiolabelled cDNA, unreacted linkers, and T4 kinase, was then loaded onto the column and the cDNA eluted with TNE. Twenty 200 \(\mu\text{l}\) fractions were collected and analysed for radioactivity by Cerenkov counting for \(^{32}\text{P}\) on a scintillation counter (Beckman). The seven fractions which constituted the first peak of radioactivity were presumed to contain the radiolabelled cDNA, and these fractions were subsequently pooled. The cDNA was precipitated with 2 volumes of 95% ethanol, washed with 80% ethanol, and then resuspended in 5 \(\mu\text{l}\) \(\text{H}_2\text{O}\).
2.3.4 Lambda ZapII Recombinant Phage Construction

The Lambda ZapII vector arms (Stratagene) were prepared and ligated to the Dictyostelium cDNAs according to the manufacturer's protocols. Ligation mixes containing the recombinant λZapII phage DNA were packaged using the GIGAPACK II Gold Packaging Extracts (Stratagene) as instructed by the manufacturer.

The resulting cDNA libraries had titres of $7.3 \times 10^5$ pfu/ml (0 hr) and $1.33 \times 10^6$ pfu/ml (15 hr). These were subsequently amplified as previously described (Maniatis et al., 1982) to yield 17 ml of the vegetative library (0 hr) at a titre of $9 \times 10^{10}$ pfu/ml, and 37 ml of the pseudoplasmodial library (15 hr) at a titre of $3.4 \times 10^{11}$ pfu/ml. The libraries were stored at 4°C with 0.3% chloroform.

2.4 cDNA Probes And Hybridization Procedures

Recombinant plasmids containing the desired cDNA fragments were digested with the appropriate restriction endonucleases to release the cDNA inserts. The cDNA inserts were then separated by electrophoresis on an agarose-TAE gel (1x TAE is 40 mM Tris-acetate, 1 mM EDTA). An agarose gel slice containing the cDNA insert was excised from the gel and the cDNA purified using the Geneclean II Kit (Bio 101 Inc.). Approximately 100 ng of the purified cDNA insert was labelled by the random oligonucleotide primer method (Feinberg & Vogelstein, 1983) using [$\alpha$-32P]dCTP (ICN). Filters were prehybridized for 3-6 hr at 42°C and hybridized overnight at 37°C or 42°C in a solution containing 30% formamide, 5x SSC (1x SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 5x Denhardt's (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 50 mM sodium phosphate,
0.5% sodium dodecyl sulfate (SDS) and 250 μg/ml of sheared, denatured, salmon sperm DNA.

2.5 Screening Of cDNA Libraries

Two cDNA libraries were employed in the search for full-length cDNAs; a λgt11 cDNA library prepared from mRNA of Dictyostelium cells harvested 3 hr after starvation, and a λZap cDNA library constructed using vegetative phase (0 hr) mRNA. Approximately 250,000 recombinant plaques of each library were screened according to standard protocols (Maniatis et al., 1982).

2.6 DNA Sequencing

PCR products and cDNAs from the λgt11 phage were subcloned into the pTZ18R vector, while cDNAs from the 0 hr λZap phage were rescued according to the Stratagene in vivo excision protocols. Single stranded DNA was isolated as previously described (Kristensen et al., 1987), while double-stranded DNA was isolated with the Nucleobond® AX-20 kit (Macherey-Nagel). The DNA was sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using either M13 reverse or universal primers, or internal primers specific to a portion of the already sequenced gene (Table 3). The sequencing reaction mixtures were electrophoresed through a 0.6% polyacrylamide/urea gel which was then dried and used to expose Kodak X-ray film overnight. The deduced nucleotide sequences were analyzed for the presence of open reading frames using the DNA strider program (© Christian Mark).
### TABLE 3.

Nucleotide sequences of the various oligonucleotide primers used for sequencing the *rasB*, *rasC*, and *rasS* genes. The universal one-letter amino acid code is indicated above each codon or part thereof.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Codon</th>
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</thead>
<tbody>
<tr>
<td>rasB</td>
<td>JD-1</td>
<td>Q D R M A S Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTG GTC TCT CAT AGC ACT G</td>
</tr>
<tr>
<td></td>
<td>JD-2</td>
<td>N L R E V T E G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C CTT CGT GAA GTT ACT GAA GG</td>
</tr>
<tr>
<td>rasC</td>
<td>JD-8</td>
<td>E R F T T V A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C ACG GAA TGT TGT AAC AGC</td>
</tr>
<tr>
<td></td>
<td>JD-7</td>
<td>L D T A G Q E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A GAT ACA GCC GGT CAA G</td>
</tr>
<tr>
<td>rasS</td>
<td>JD-4</td>
<td>L F G E G T R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G AAA TCC CTC ACC TGT ACG</td>
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<tr>
<td></td>
<td>JD-3</td>
<td>F S A V R D Q</td>
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<tr>
<td></td>
<td></td>
<td>C AGT GCG GTT AGG GAT C</td>
</tr>
<tr>
<td>ras</td>
<td>JD-5</td>
<td>E Q G Q T D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC TTG ACC TGC AGT ATC A G T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G T</td>
</tr>
</tbody>
</table>
2.7 Isolation Of Bacteriophage DNA

A small scale liquid culture (5 ml) of the bacteriophage clone of interest was grown and harvested as described by Maniatis et al. (1982). The phage-containing supernatant was then treated for 30-60 minutes at 37°C with RNaseA and DNaseI, both at a final concentration of 1 μg/ml. Cellular debris was removed by centrifugation of the lysate at 7000 rpm, and an equal volume of a 20% polyethylene glycol, 2.5 M NaCl solution was added to the supernatant to precipitate the DNA. The pellet was dissolved in 500 μl TE pH 8.0 (10 mM Tris-Cl, 1 mM EDTA) and this solution was extracted twice with an equal volume of phenol/chloroform (1:1). Thirty-five μl of a premixed cocktail containing proteinase K (3 mg/ml), SDS (5.7%) and EDTA (71 mM pH 8) were added to the aqueous phase and the mixture was incubated at 65°C for 45 minutes. The solution was then phenol/chloroform extracted, and an equal volume of isopropanol added to the aqueous layer to precipitate the DNA overnight at -20°C. The DNA pellet was finally dissolved in 50 μl deionized H2O and stored at -20°C.

The isolated phage DNA was digested with the EcoRI restriction endonuclease for 4-6 hr at 37°C, and an aliquot of the digestion mix was run on a 0.7% agarose-TBE gel to determine if the cDNA insert had been released during the digestion. When the digest was successful, as indicated by the presence of a cDNA insert on the ethidium bromide-stained gel, 2 volumes of 95% ethanol were added to the remainder of the digestion mix to precipitate the DNA. The DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 20 μl deionized H2O. 10 μl of this was then used in a ligation reaction with dephosphorylated, EcoRI-cut pTZ18R vector DNA (Maniatis et al., 1982).
2.8 Southern and Northern Blot Analyses

2.8.1 Southern Blot Analysis

Nuclei from vegetative Dictyostelium cells were isolated as previously described (Cocucci & Sussman, 1970), lysed with a solution of 3.42% sucrose, 10 mM Tris-Cl pH 7.4, 10 mM NaCl, 1 mM MgCl₂ and 3 mM CaCl₂, and the genomic DNA extracted by standard protocols (Maniatis et al., 1982). Genomic DNA, (10 μg), was digested with either EcoRI alone, BglII alone or a combination of EcoRI and BglIII restriction endonucleases for 12-16 hrs, and the DNA separated on a 0.7% agarose-TBE gel (1x TBE is 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA). Following electrophoresis, genomic fragments on the ethidium bromide-stained gel were detected by ultraviolet irradiation, and the gel photographed before treating as follows. The gel was first soaked in a solution of 1.5 M NaCl, 0.5 M NaOH for 30 minutes to denature the double-stranded DNA, and then neutralized for 30 minutes in a solution of 1.5 M NaCl, 0.5 M Tris pH 8.0. The gel was then briefly soaked in 2x SSC before the DNA was transferred and fixed onto a nitrocellulose membrane (MSI) as previously described (Maniatis et al., 1982). The membrane was prehybridized and hybridized as described in section 2.4, and then washed twice for 10 minutes in 2x SSC, 0.1% SDS at room temperature, and twice for 15 minutes in 2x SSC, 0.1% SDS at 50°C to provide low stringency conditions. Hybridization of the radiolabelled probe to genomic fragments was detected by overnight exposure of X-ray film to the membranes. For high stringency conditions, the membranes were washed twice for 15 minutes in a solution of 0.1x SSC, 0.1% SDS at 60°C, unless stated otherwise.
2.8.2 Northern Blot Analysis

*Dictyostelium* cells were harvested at various times during the developmental life cycle of the wild type strain V12M2, or the axenic strain Ax2, and total RNA was extracted by the acid guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). Total RNA (20 µg) was denatured for 10 minutes at 65°C in a solution containing 50% formamide, 17.5% formaldehyde, 20 mM MOPS pH 7.0, 5 mM sodium acetate and 0.5 mM EDTA pH 8.0. The RNA was fractionated on a 1.25% agarose-formaldehyde gel and assessed for equal loading by ethidium bromide staining of ribosomal bands. The gel was then rinsed for 10 minutes with deionized water before transferring and fixing the RNA onto a nitrocellulose membrane (Maniatis *et al.*, 1982). The membrane filters were prehybridized and hybridized as described previously except that 30 µg/ml of polyadenylic acid was included in the prehybridization and hybridization reactions to minimize non-specific hybridization of the probe due to the high AT content of *Dictyostelium* DNA. After hybridization, the filters were washed twice for 15 minutes in 2x SSC, 0.1% SDS at room temperature, and twice for 15 minutes in 1x SSC, 0.1% SDS at 60°C before they were used to expose X-ray film overnight.

2.8.3 Reprobing Blots

Southern blots were stripped of bound radiolabelled probe by washing for 30 minutes in 50 mM NaOH. The membranes were then rinsed with a solution of 10 mM Tris-Cl pH 7.5 and 50 mM NaCl, prehybridized as before and hybridized with the appropriate probe. Northern blots were stripped by washing for 20 minutes in a solution of 30% formamide, 0.1x SSC, 0.1% SDS at 65°C. The membranes were then prehybridized as before and hybridized with the appropriate probe.
2.9 Oligonucleotide Synthesis And Purification

Oligonucleotides were synthesised by the Oligonucleotide Synthesis Lab., UBC, using an automated DNA Synthesiser 380B (Applied Biosystems). The crude dried oligonucleotide was dissolved in 1.5 ml of 0.5 M ammonium acetate and purified using a C-18 Sep-Pak (Waters, Millipore), as previously described (Zoller & Smith, 1984).

2.10 Polymerase Chain Reaction

The primer extension reaction was used to generate single stranded cDNA from total RNA extracted at six stages (0, 8, 12, 14, 15, & 18 hr post-starvation) during the development of the Dictyostelium wild type strain, V12M2. Each primer extension reaction contained 1 μg of total RNA as template, 0.1 μg of poly-dT primer (5' TCT CCG AAT TCT AGA TTT TTT TTT TTT TTT TTT TTT 3'), 80 mM Tris-HCl pH 8.4, 80 mM KCl, 8 mM MgCl₂, 5 mM DTT, 0.8 mM of each dATP, dCTP, dGTP, dTTP and 200 units M-MLV reverse transcriptase (BRL) in a 25 μl volume. The reaction was incubated at 48°C for 30 minutes and then an additional 100 units of M-MLV reverse transcriptase were added and the reaction incubated for a further 30 minutes.

Degenerate oligonucleotide primers with a PstI adaptor sequence were designed to amplify additional ras or ras-related Dictyostelium genes. Primer JD-11, (5' AGCTGCA GGT G(T)G(C)T(A) GG(C,A)T GGT GTT GG 3'), was derived from the sequence encoding the first of the highly conserved GTP-binding domains (GGGGVG, residues 10-15 of RasG; Robbins et al., 1989). Primer JD-6, (5' AGCTGCAG CAA AAT(C) CAT(C) TTT(C) A(G)TT(C,A) GAT(C) G 3'), was designed to prime from a sequence specific to the Ras proteins (QNHFVDE, residues 25-31 of
RasG; Robbins et al., 1989). Primer JD-12, (5' AGCTGCA GTG CAA TTT(C) GTT(C) C(A)A(C)A(T,C) GG 3'), was derived from the amino acid sequence VQFVQG (residues 21-26 of Rap1; Robbins et al., 1990), which is an amino acid sequence unique to Rap proteins. Primer JD-13, (5' AGCTGCA GAC(A) ATC G(A,T)C(G)A(C) TTT G(A)TT ACC 3'), was designed to prime from the sequence corresponding to the third GTP-binding domain, GNKCDL (residues 115-120 of RasG; Robbins et al., 1989). This primer was used as the downstream 3' primer in all amplifications along with either JD-11, JD-6, or JD-12 as the 5' primer.

Each amplification reaction consisted of 0.5 μl cDNA from the 25 μl extension reaction, 100 pmole of each of the two primers, 50 mM Tris-HCl pH 8.0, 0.05% Tween-20, 0.05% NP40, 1.5 mM MgCl₂, 200 μM dNTPs (dATP, dCTP, dGTP, dTTP) and 1.25 units of Taq DNA polymerase (Promega) in a final volume of 50 μl. The amplification protocol was two cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 2 minutes and extension at 72°C for 3.5 minutes, followed by thirty cycles of denaturation (94°C, 30 sec), annealing (45°C, 100 sec), and extension (72°C, 3.5 minutes), and ending with a final extension of 7 minutes at 72°C. The amplifications were performed using an Ericomp Twin Block Thermocycler.

A 10 μl aliquot of the reaction mixture was electrophoresed on a 2% agarose-TBE gel to determine if a PCR product of the predicted size had been amplified, and the DNA from the remaining 40 μl was precipitated with 2 volumes of 95% ethanol and 0.5 volumes of 7 M ammonium acetate. The precipitated PCR products were digested with PstI and ligated to the PstI-cut pTZ18R vector. 5 μl of a 20 μl ligation mixture were then used to transform competent DH5αF' cells. Colony blots of transformants were probed with the Dictyostelium rasG-c3 cDNA insert (Robbins et al., 1989) and washed at low stringency (2x SSC, 0.1% SDS at 50°C) for 30 minutes, unless stated otherwise. Under these conditions, the Dictyostelium rasG cDNA probe crosshybridizes to both rap1 and rasD, and it should therefore detect other
genes of the ras superfamily. Positive clones were characterized either by dideoxy chain-termination sequencing (Sanger et al., 1977) or restriction endonuclease digestion, or both.

2.11 Construction Of pGEX-Ras Bacterial Expression Vectors

The pGEX series of plasmids (Smith & Johnson, 1988), designed for the expression of foreign DNA sequences as glutathione S-transferase (GST) fusion proteins, were used for the construction of the Dictyostelium Ras-expression vectors. Restriction fragments containing the full-length cDNAs of rasB, rasC, or rasS were cloned in frame into the appropriate pGEX vector as follows. A 750 bp NlaIII fragment containing the entire coding sequence of the rasB 13-1 clone was isolated and the ends chewed back with T4 DNA polymerase as previously described (Maniatis et al., 1982). This fragment was then digested with EcoRI and the 650 bp fragment generated from this digest was forced-cloned into the SmaI/EcoRI cut pGEX-3X vector. A 600 bp EcoRI fragment from the rasC 12-1 cDNA clone was subcloned into the EcoRI site of pGEX-1N, while a 600 bp EcoRI fragment from the rasS 14-1 cDNA clone was subcloned into the EcoRI site of pGEX-3X. The structures of the recombinant plasmids were confirmed by restriction endonuclease mapping and DNA sequencing of the fusion junctions (data not shown).

The GST fusion proteins were produced in E. coli strain DH5αF' cells or Y1090 cells growing in the rich nutrient media, TYP-0.5% glycerol (16 g bacto-tryptone, 16 g yeast extract, 5 g NaCl, 2.5 g K2HPO4, per liter of H2O). The bacterial cells were pelleted by centrifugation in a Sorvall G-3 rotor at 6000 rpm for 15 minutes and resuspended in 1/100 volume of PBS. The bacteria were lysed by, a) treatment with lysozyme at a concentration of 1 mg/ml, and b) passage through a french pressure
cell. The lysate was centrifuged at 9000 rpm for 20 minutes and the fusion proteins purified from the supernatant by glutathione-agarose chromatography as previously described (Smith & Johnson, 1988). When this procedure yielded low amounts of the recombinant RasC and RasS fusion proteins, the bacterial lysate pellet was solubilized with 1.5% N-laurylsarcosine (sarkosyl) (Grieco et al., 1992), and the solubilized proteins purified from the supernatant by incubation of the supernatant with glutathione-agarose beads.

2.12 Western Blot Analysis

Approximately 1 μg of the purified GST-Ras fusion proteins was electrophoresed on duplicate 12% SDS-polyacrylamide gels (Maniatis et al., 1982). One gel was stained with Coomassie blue to verify equal loading of the protein samples, while the other gel was used to transfer the proteins by electroblotting onto a Hybond-C nitrocellulose membrane (Amersham) as previously described (Maniatis et al., 1982). The membrane was then blocked overnight at 4°C in 1x TBS containing 5% non-fat dry milk and 1% Tween-20 (Fisher). The membrane was then processed with the ECL immunodetection system (Amersham) as instructed by the manufacturer. The primary antibody (the ras-specific monoclonal antibody Y13-259) was diluted 1:1000 in a 0.5% milk-TBS solution while the secondary antibody (sheep anti-rat) was diluted 1:10,000. Immunoreactive proteins were detected by addition of the ECL reagent and exposure of X-ray film to the membranes for 1-5 minutes.
CHAPTER 3

RESULTS

3.1 Search For Additional \textit{ras} Genes Using The PCR Technique

3.1.1 Introduction

The Polymerase Chain Reaction or PCR is a rapid and powerful technique which facilitates the \textit{in vitro} amplification of a specific DNA sequence from a complex mixture of sequences (for review, see White \textit{et al.}, 1989). The specificity of the technique relies upon the use of two sequence-specific oligonucleotide primers which flank the sequence of interest at its 5' and 3' ends, and which are oriented with their 3' ends towards each other. The \textit{in vitro} amplification of the desired DNA sequence is achieved by repeated cycles of heat denaturation of the double-stranded DNA, annealing of the oligonucleotides primers to the DNA at a low temperature, and DNA synthesis by extension of the annealed primers with a thermostable DNA polymerase. The DNA sequence is amplified exponentially since the extension product of each primer serves as a template for the other primer and effectively doubles the amount of target DNA sequence in the reaction after each cycle.

During the characterization of the \textit{Dictyostelium rasG} gene, a large number of genomic fragments were detected when a \textit{Dictyostelium} genomic southern blot was probed with a \textit{rasG} cDNA under low stringency wash conditions (Robbins \textit{et al.}, 1989). This suggested that there were additional \textit{ras} or \textit{ras}-related genes in \textit{Dictyostelium}. The strong evolutionary conservation of the amino acid sequences of the Ras superfamily proteins made it possible to design degenerate
oligonucleotide primers that would direct the amplification of a spectrum of ras or ras-related sequences from Dictyostelium using the PCR technique. Figure 7 is a schematic representation of the primary structure of a Ras protein, and summarizes the strategy used to amplify novel ras and ras-related genes from Dictyostelium.

Results

3.1.2 Amplification of ras-subfamily sequences from Dictyostelium: sequence analysis

The oligonucleotide primer JD-11 was derived from the amino acid sequence of the first of the highly conserved GTP-binding domains, while primer JD-13 was derived from the amino acid sequence of the third GTP-binding domain. These domains are conserved in all members of the Ras protein superfamily identified to date. Therefore, when these primers were used together in a PCR amplification, it was anticipated that a wide spectrum of ras family gene sequences would be amplified. The 320 bp PCR products generated from reactions using 0 hr, 8 hr, or 12 hr cDNAs as template were pooled and cloned into the vector pTZ18R. Thirty-six clones were detected by hybridization with the rasG cDNA probe under low stringency conditions, and all thirty-six clones were sequenced using the M13 reverse primer (data not shown). Eleven of these clones were identified as ras gene fragments (9 rasD, 1 rasG, and 1 which represented a novel gene) by sequence comparison with the previously described Dictyostelium ras genes. Three other clones were likewise identified as rap1 gene fragments. Two clones each contained two different PCR fragments in a tandem arrangement, and it was established that these fragments were derived from the rasG and rap1 genes. This tandem
Figure 7. PCR Strategy for the amplification of *Dictyostelium ras* gene family sequences. Schematic representation of the primary structure of a prototype Ras protein with shaded boxes highlighting regions to which complementary, degenerate oligonucleotide primers were designed. Oligonucleotide primer pairs used in each amplification are indicated. QNHFVDE (residues 25-31 of H-Ras) is a conserved Ras-specific sequence, and VQFVQG (residues 23-28 of Rap1) is a conserved Rap-specific sequence.
N
GGGGVG
QNHFVDE
VQFVQG
JD-11
JD-6
JD-12
ras - specific
JD-13
GD-13
ras - specific
JD-12
JD-11
ras family
JD-13
arrangement of PCR fragments in these two clones probably occurred during the ligation reaction.

Nine other clones had open reading frames which predicted amino acid sequences that were different from the previously isolated Dictyostelium ras genes. A search of the SwissProt database of PCGENE revealed that the deduced amino acid sequences of these clones were related to the Rab protein subfamily. Two of these nine clones (11-2 and 11-3) had nucleotide sequences that were identical to a portion of the previously characterized Dictyostelium sas1 gene (Saxe & Kimmel, 1990). The other seven clones with rab-related sequences represented previously uncharacterized Dictyostelium genes. Table 4 summarizes the results of this analysis.

The origins of the remaining eleven clones could not be established. Five of these clones had short open reading frames with little homology to protein sequences in the database, and two attempts at sequencing the remaining six clones with the M13 reverse primer were unsuccessful.
### TABLE 4.

**Summary of the analysis of PCR-amplified ras sequences from Dictyostelium**

<table>
<thead>
<tr>
<th>Primers</th>
<th># Clones analysed by the indicated methods and their inferred sequence identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD-12, JD-13</td>
<td>Total #</td>
</tr>
<tr>
<td>JD-11, JD-13</td>
<td>8</td>
</tr>
<tr>
<td>JD-11, JD-13</td>
<td>36</td>
</tr>
<tr>
<td>JD-6, JD-13</td>
<td>90</td>
</tr>
</tbody>
</table>

* identified in this thesis.
3.1.3 Preliminary characterization of the rab-related clones

Four of the rab subfamily clones (11-4, 11-12, 11-13, and 11-14) were identical to each other and encoded products that were 94% identical to a portion of the human Rab1 protein sequence (Touchot et al., 1987; Figure 8a). These clones are derived from the recently isolated Dictyostelium rab1A gene, which encodes a protein that is 85% identical to the human Rab1 protein (Bush et al., 1993). Two other clones, (11-9 and 11-16), encoded products that were not identical to each other, but which were at least 83% identical to a portion of the human Rab1 protein (Figure 8b). Clone 11-3Y encoded an amino acid sequence that was 52% identical to a portion of the S. pombe YPT1 protein (Miyake & Yamamoto, 1990; Figure 8c). This clone appears to be derived from the recently identified Dictyostelium rabC gene (Bush et al., 1993) which is a rab subfamily gene with no mammalian or yeast homologs.

When the largest rab-related cDNA (11-9) was used as a probe in a southern blot analysis of Dictyostelium genomic DNA, multiple genomic fragments hybridized to the probe under low stringency wash conditions (1x SSC, 0.1% SDS at 50°C, Figure 9, panel A). When the blot was further washed in 1x SSC, 0.1% SDS at 65°C, a single fragment of approximately 19 kb was detected in the BglIII digested genomic DNA lane, and a single fragment of approximately 8 kb was detected in the EcoRI and EcoRI/BglIII digested DNA lanes (Figure 9, panel B).
Figure 8. Alignment of the deduced amino acid sequences of the three PCR-amplified rab family gene fragments with the corresponding region of the human Rab1 (Touchot et al., 1987) or the S. pombe YPT1 protein sequences (Miyake & Yamamoto, 1990). (A) The amino acid sequence of the representative Dictyostelium 11-4 clone is approximately 94% identical to the human Rab1 protein sequence over this stretch of amino acids. (B) The predicted amino acid sequence of the Dictyostelium 11-16 clone was only 83% identical over the same region. Clone 11-9 encoded a longer amino acid sequence than clone 11-16 and this sequence was also 83% identical to Rab1. (C) The Dictyostelium 11-3 amino acid sequence was approximately 52% identical to the S. pombe YPT1 protein over this stretch of amino acids. Dashes indicate amino acid residues that are identical to those in the human Rab1, or the S. pombe YPT1 proteins.
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAB1</td>
<td>RAB1</td>
<td>YPT1</td>
</tr>
<tr>
<td></td>
<td>IGDSGVGKSCLLLRFADDTYTESYISTIGDFKIRTIELDGKTIKLQIWDTA</td>
<td>IGDSGVGKSCLLLRFADDTYTESYISTIGDFKIRTIELDGKTIKLQIWDTAGQERF</td>
<td>AGQERFRTITSSYRGAHGIIVVYDVTQESFNVKQWLQIDRYASENVNLVGNKCDLT</td>
</tr>
<tr>
<td>11-4</td>
<td>A-GG------------------------------N----I---------</td>
<td>A-GG------------SW-DTH---------K-LN-----------</td>
<td>A-GG-------------M-------------NRF-D-----T-----C------K---------</td>
</tr>
<tr>
<td></td>
<td>RAB1</td>
<td>RAB1</td>
<td>YPT1</td>
</tr>
<tr>
<td>11-9</td>
<td>RTITSSYRGAHGIIVVYDVTQESFNVKQWLQIDRYASENVNLVGNKCDLT</td>
<td>RTITSSYRGAHGIIVVYDVTDSFNNVKQWLQIDRYAVEGVNLVGNKSDM</td>
<td>AGQERFRTITSSYRGAHGIIVVYDVTQESFNVKQWLQIDRYASENVNLVGNKCDLT</td>
</tr>
<tr>
<td></td>
<td>11-9</td>
<td>11-16</td>
<td>11-3Y</td>
</tr>
<tr>
<td></td>
<td>A-GG--------MK-------------------T--------C--------K---------</td>
<td>A-GG--------M--------NRF-D------T------C--------K---------</td>
<td>A-GG--------MK-------------------T--------C--------K---------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65-125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aa 17-68</td>
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<td>aa 17-68</td>
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</table>
Figure 9. Southern blot analysis of Dictyostelium genomic DNA using a rab-gene fragment (11-9) as a probe. Genomic DNA (10 μg) from the wild type strain V12M2 was digested with either EcoRI (lane 1), BglII (lane 2), or a combination of EcoRI and BglII (lane 3). The digested DNA was fractionated on a 0.7% agarose gel and then transferred and fixed to a nitrocellulose membrane. The filter was probed with an [α-32P]-labelled 11-9 cDNA fragment, and washed with (A) 1x SSC, 0.1% SDS at 50°C or (B) 1x SSC, 0.1% SDS at 65°C. Molecular size standards (kb) are indicated.
3.1.4 Specific amplification of ras subfamily gene sequences in Dictyostelium: restriction pattern analysis

To determine if there were additional as yet uncharacterized ras subfamily genes in Dictyostelium, the degenerate primers JD-6 (ras-unique) and JD-13 were used in four separate reactions with cDNA samples derived from 0 hr, 8 hr, 12 hr, and 14 hr Dictyostelium mRNAs as the templates. Primer JD-6 was derived from the amino acid sequence of the effector-proximal region which has a characteristic motif in the Ras subfamily proteins (QNHFVDE, residues 25-31 of RasG; Robbins et al., 1989). The 290 bp PCR products generated from these reactions were cloned into the pTZ18R vector, and the recombinant vector was then used to transform competent DH5αF' cells. The bacterial transformants were then screened with rasG or rasD cDNA probes.

Ninety positive clones, representative of the four reactions, were first analysed by restriction endonuclease digestion. Restriction endonucleases which distinguish rasD (NdeI, PstI) from rasG (AccI) were used for the analysis. The PstI digest (Figure 10A) was expected to generate single restriction fragments of either 100 bp or 190 bp, or two fragments, one of 100 bp and another of 190 bp, from clones containing rasD-derived PCR fragments. Clones containing rasG-derived PCR fragments were not expected to be cut by PstI and were therefore expected to generate the uncut 290 bp PCR fragments. The AccI digest (Figure 10C) was expected to generate single fragments of either 170 bp or 120 bp from rasG-derived clones. No fragments were expected from rasD-derived clones when they were digested with AccI. The PstI/NdeI double digest (Figure 10B) was expected to release three 90 bp fragments from rasD-derived clones or a 290 bp fragment from rasG-derived clones.

Those clones which generated unexpected restriction fragments (see Figure 10) were further analysed by sequencing, and it was discovered that their unusual restriction patterns were due to either an AccI site in the degenerate JD-6 primer
sequence, or due to ligation of two different gene fragments. For example, an AccI digest of clone #65 released a fragment whose size was between the expected fragment sizes of 120 bp and 170 bp. The novel size AccI fragment suggested that clone #65 may have represented a novel gene. However, sequencing revealed that this clone was identical to a portion of the \textit{rasG} gene. The unexpected, approximately 150 bp fragment was in fact due to an AccI site in the sequence of the JD-6 primer. An example of a clone containing two different gene fragments was clone #69 which also gave an unexpected restriction fragment with the AccI digest. Sequence analysis using the M13 reverse primer revealed that clone #69 contained a portion of the \textit{rasG} gene and a portion of a previously unidentified \textit{ras}-related gene. These fragments were probably ligated in tandem to each other during the ligation reaction with the vector pTZ18R.

In summary, of the ninety clones analysed, 63 were characterized as \textit{rasG}-derived, 25 were characterized as \textit{rasD}-derived, and the remaining 2 clones represented the novel \textit{ras} gene that was also amplified with primers JD-11 and JD-13 (Table 4). This novel gene sequence was designated \textit{rasB}, and its characterization will be described in more detail in section 3.3.
Figure 10. Restriction endonuclease digest analysis of clones containing products from a ras-specific PCR. Approximately 1 µg of plasmid DNA from ninety clones was digested with the following restriction endonucleases before electrophoresis on a 1.5% agarose-TBE gel. Molecular sizes are indicated in bp. Clones #64-80 were derived from 0 hr mRNA (expect rasG fragments). Clones #38-51 and #59-63 were derived from 8 hr mRNA (expect rasG and rasD fragments). Clones #1-37 and #52-58 were derived from 12 hr mRNA (expect rasD fragments). Clones #81-90 were derived from 15 hr mRNA (expect rasD fragments). Clones which gave unexpected restriction fragments were further analysed by sequencing.

Panel (A), PstI digest. Clones which were further analysed by sequencing were # 1, 5, 6, 10, 12, 27, 28, 32, 56, and 69.

Panel (B), PstI and NdeI double digest. Clones which were further analysed by sequencing were # 9, 20, 21, 28, 30, 50, 57, 59, 60, 63, and 69.

Panel (C), Accl digest. Clones which were further analysed by sequencing were # 1, 5, 6, 7, 8, 9, 11, 21, 25, 27, 28, 30, 32, 56, 65, 68, 69, 73, and 75.
3.2 Search For Additional rap Genes Using The PCR technique

3.2.1 Introduction

To date, four rap genes (rap1A, rap1B, rap2A, and rap2B) have been isolated and characterized in humans, and they encode proteins that are 55% identical to the Ras proteins (reviewed in Bokoch, 1993). Rap1A and Rap1B are 95% identical to each other while Rap2A and Rap2B are 91% identical to each other. The highest level of identity between any of the Rap1 proteins and any of the Rap2 proteins is only 70%, with most of the variation occurring in the C-terminal third of the protein.

Interest in the rap genes and their encoded products stems from the following observations. First, the Rap proteins possess an effector domain sequence that is identical to that of the Ras proteins (Pizon et al., 1988; Pizon et al., 1988b) and so it was postulated that the Ras and Rap proteins may interact with a common effector molecule. Secondly, the Rap proteins possess a threonine residue instead of a glutamine at position 61 in the second of the highly conserved GTP-binding domains. This is an interesting characteristic because in the H-Ras protein, any substitution at position 61 is an activating mutation and results in a protein with transforming potential (Der et al., 1986). Interest in the rap genes later intensified when it was observed that the transformed phenotype of cells expressing an activated K-ras gene was reverted when these cells were transfected with a rap1 cDNA (Kitayama et al., 1989). This latter observation suggested that Ras and Rap were linked in the same signal transduction pathway since Rap could suppress the biological activity of an activated Ras protein. If Ras and Rap do indeed interact with the same downstream effector molecule, then the activating lesion at residue 61 in the Rap proteins would result in a constitutively active Rap protein that could
compete with Ras for the common effector molecule. In this scenario, Rap would act as an antagonist of Ras protein function.

One rap gene has been isolated and characterized from Dictyostelium and it encodes a product that is 76% identical to the human Rap1A protein (Robbins et al., 1990). Since a Dictyostelium genomic southern blot probed with the Dictyostelium rap1 cDNA revealed several genomic fragments under low stringency wash conditions, (Figure 11), I wanted to determine if these genomic fragments represented additional rap genes. To address this question, I opted for a PCR approach.

Results

3.2.2 Amplification of rap gene sequences from Dictyostelium: sequence analysis

To determine if there were additional rap genes in Dictyostelium, a rap-unique oligonucleotide primer was designed to amplify rap gene sequences. The sequence of this degenerate primer, JD-12, was derived from the amino acid residues of the effector-proximal domain that are specific to the rap proteins (residues 21-26 of Dictyostelium rap1; Robbins et al., 1990). This primer was used in conjunction with the downstream 3' primer, JD-13, which was designed to be complementary to the third GTP-binding domain that is conserved in all proteins of the Ras superfamily. These primers were used in five separate PCR amplifications as follows. cDNA derived from the mRNA of vegetative (0 hr), aggregative (8 hr), and tipped aggregate (12 hr) stage Dictyostelium was first used in three separate PCR amplifications with the degenerate primers JD-11 and JD-13. A wide spectrum of ras superfamily gene sequences was expected to be amplified in this reaction. A 10 µl aliquot of this reaction was then run on a low-melting agarose gel, and an agarose
Figure 11. Southern blot analysis of Dictyostelium genomic DNA with a rap1 probe. Genomic DNA (10 μg) from the wild type strain V12M2 was digested with either EcoRI (lane 1), BglII (lane 2), or EcoRI and BglII (lane 3). The digested DNA was fractionated on a 0.7% agarose gel and then transferred and fixed to a nitrocellulose membrane. The filter was probed with an [α32P]-labelled rap1 cDNA and washed under low stringency conditions (2x SSC, 0.1% SDS at 50°C). Molecular size standards (kb) are indicated.
plug containing the 320 bp PCR products was removed from the gel. This agarose plug was then used as a source of DNA in subsequent PCR amplifications with the rap-unique primer, JD-12, and the downstream primer JD-13. cDNAs derived from mRNA of pseudoplasmodial (14 hr) or culminating (18 hr) stage *Dictyostelium* were used in two direct PCRs with primers JD-12 and JD-13. All five reactions (0, 8, 12, 14, 18 hr) yielded PCR products of the expected size (~290 bp) (data not shown). The reaction mixes were pooled, digested with the PstI restriction endonuclease, and cloned into the PstI-cut dephosphorylated vector, pTZ18R. Thirty clones that hybridized with either the rasG or rap1 cDNA probes were analyzed by sequencing. All thirty clones possessed nucleotide sequences that were identical to the corresponding region of the previously isolated *Dictyostelium rap1* gene (data not shown).

*Dictyostelium* 14 and 18 hr cDNAs were also used in two PCR reactions with the degenerate primers JD-11 and JD-13. The 320 bp PCR products (data not shown) were subcloned and approximately 200 clones were screened with a rap1 cDNA probe under medium stringency conditions (1x SSC, 0.1% SDS at 65°C). Only 8 clones hybridized with the rap1 probe. Sequencing revealed that 4 of these were also identical to the corresponding region of the previously identified rap1 gene. The remaining four clones did not grow during the preparation for single-stranded DNA isolation and so they were not further analysed.

3.2.3 Amplification of rap gene sequences from *Dictyostelium*: restriction pattern analysis

An additional fifteen clones that were derived from the products of the JD-12, JD-13 PCR amplification, and that also hybridized to the rasG probe under low stringency conditions, were analysed by restriction endonuclease digestions (Figure 12). Plasmid DNA from these clones was digested with restriction endonucleases
which were single cutters in the *rap1* cDNA, and cut in either the conserved (eg. PvuII, RsaI) or unconserved regions of the *rap1* DNA sequence (eg. NsiI, BglII).

If the PCR products were derived from the previously identified *rap1* gene, the PstI restriction endonuclease would not digest the 290 bp product since there is no PstI site in the *rap1* gene. A 290 bp fragment was therefore expected from the PstI digest. The PstI/NsiI double digest was expected to generate a 200 bp fragment and a 90 bp fragment if the clones were derived from *rap1*, while the PstI/BglII double digest was expected to generate fragments of 140 bp, 80 bp and 70 bp. If the PCR products were derived from *rap1*, the RsaI digest was expected to generate two different patterns depending on the orientation of the PCR product in the vector; some clones were expected to generate an 85 bp fragment and a 56 bp fragment while other clones were expected to generate an 85 bp fragment and a 215 bp fragment. The PvuII digest was expected to generate a 350 bp and a 280 bp fragment or a 420 bp and a 210 bp fragment depending on the orientation of the PCR fragment in the vector. Fourteen of the fifteen clones generated restriction patterns consistent with the restriction map of the *Dictyostelium rap1* gene (Figure 12), and therefore do not represent additional *Dictyostelium rap* genes. Insufficient DNA was isolated from the remaining clone to allow restriction pattern analysis. These results are summarized in Table 4.
Figure 12. Restriction endonuclease digest analysis of clones containing products from a rap-specific PCR. Approximately 1 μg of plasmid DNA from fourteen clones (lanes 1-14) was digested with the following restriction endonucleases and then electrophoresed on a 1.5% agarose-TBE gel. (A) PstI, (B) PstI and BglII, (C) PstI and NsiI, (D) PvuII, and (E) RsaI. Expected sizes: PstI, 290 bp; PstI/BglII, 140, 80, & 70 bp; PstI/NsiI, 200 & 90 bp; PvuII, 350 & 280 bp or 420 & 210 bp; RsaI, 85 & 56 bp or 85 & 215 bp. The molecular sizes are indicated in bp. All fourteen clones generated the same size restriction fragments as would be expected from a rap1 cDNA digested with the same enzymes.
3.3 Isolation And Characterization Of The rasB Gene Of Dictyostelium discoideum

3.3.1 Introduction

As stated in section 3.1.2, one novel ras subfamily DNA sequence was amplified when primers, JD-11 (GTP #1) and JD-13 (GTP #3), or primers JD-6 (ras-specific) and JD-13, were used in two separate PCR experiments. The nucleotide sequence of this putative ras subfamily gene was distinct from the previously identified Dictyostelium rasD and rasG gene sequences, and encoded a ras-related product that was 82% and 84% identical to residues 10 to 59 of the RasD and RasG proteins respectively. In this section, the isolation of full-length rasB cDNAs, and characterization of the rasB gene are described.

Results

3.3.2 Isolation of full-length rasB cDNAs and analysis of the RasB amino acid sequence

The novel, ras-related, PCR-amplified fragment was used to screen an 8 hr λgt11 cDNA library with high stringency washes in 1x SSC, 0.1% SDS at 65°C. However, none of the isolated positive phage clones possessed full-length cDNAs, and all of the cDNAs appeared to be missing the 3' portion of the coding sequence since they all lacked a termination codon. The clone possessing the longest open reading frame encoded a Ras-related amino acid sequence which, when compared to the RasD and RasG amino acid sequences, indicated that it encoded the N-terminal 80% of a potentially novel Ras protein. A 322 bp PstI/NsiI fragment of this clone (encoding amino acids 58 to 152 relative to RasG) was therefore used to screen the 0
hr λZap cDNA library with washes in 0.1x SSC, 0.1% SDS at 60°C, in an attempt to isolate a clone which possessed the 3' coding sequence and a termination codon.

Three independent phage clones were isolated and the phagemids obtained by \textit{in vivo} excision from the λZap phage according to the manufacturer's protocols (Stratagene). The cDNAs from these three clones, 12-1, 13-1, and 18-1, were sequenced and found to have open reading frames that were identical to each other, and predicted the sequence of a 197 amino acid Ras-related protein. The M13 reverse primer was used in conjunction with the \textit{rasB} specific primers, JD-1 and JD-2 (Table 3), to sequence the cDNAs. The nucleotide sequence common to all three cDNAs, and the deduced amino acid sequence of \textit{rasB} are depicted in Figure 13.

Figure 14 shows the amino acid sequence encoded by \textit{rasB}, aligned with the human H-Ras protein sequence, and the \textit{Dictyostelium} RasD, RasG, and Rap1 protein sequences (Capon \textit{et al.}, 1983; Reymond \textit{et al.}, 1984; Robbins \textit{et al.}, 1990; Robbins \textit{et al.}, 1989). The predicted amino acid sequence of RasB is 71% and 68% identical to RasG and RasD respectively, but only 52% identical to \textit{Dictyostelium} Rap1 (Table 5, above the diagonal). In addition, RasB is less related to the human H-Ras protein (59% identity) than RasD and RasG (65% and 68% identity respectively; Table 5, above the diagonal). However, RasB possesses the four GTP-binding domains that are characteristic of the Ras proteins and other GTP-binding proteins, and the carboxy-terminal CAAX motif that is a prerequisite for post-translational modification and membrane attachment of the Ras proteins (Gutierrez \textit{et al.}, 1989; Hancock \textit{et al.}, 1991). RasB is also totally conserved in the Ras-specific effector domain (Sigal \textit{et al.}, 1986).
Figure 13. Nucleotide and predicted amino acid sequences of the *Dictyostelium rasB* gene. Numbers indicate the nucleotide and amino acid positions in the sequence starting with the ATG initiation codon. The asterisk indicates the protein synthesis termination codon. Flanking untranslated nucleotide sequences common to the clones sequenced are shown. The *rasB* sequence has been deposited in the EMBL/Genbank database, accession number M96622.
Figure 14. Alignment of the predicted amino acid sequence of RasB with the human H-Ras protein sequence (Capon et al., 1983) and the Dictyostelium RasD (Reymond et al., 1984), RasG (Robbins et al., 1989), and Rap1 (Robbins et al., 1990) protein sequences. Numbers indicate amino acid positions in each protein. Dashes indicate amino acids identical to those in H-Ras, and gaps have been inserted to optimize alignment. The asterisks (***') indicate the effector domain residues (Sigal et al., 1986), the open circles (ooo') the Y13-259 epitope residues (Furth et al., 1982; Sigal et al., 1986), and the triangles (aaa') the effector-proximal domain residues (Marshall et al., 1991; Zhang et al., 1990). Boxed residues represent the conserved GTP-binding domains and the C-terminal CAAX motif.
TABLE 5.

Percentage identities between the *Dictyostelium* Ras proteins, *Dictyostelium* Rap1, and the human H-Ras protein sequences.\(^a\)

<table>
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<tr>
<th></th>
<th>RasD</th>
<th>RasG</th>
<th>RasB</th>
<th>RasC</th>
<th>RasS</th>
<th>HRas</th>
<th>Rap1</th>
</tr>
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<td>-</td>
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<td>69</td>
<td>-</td>
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<td>52</td>
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<td>81</td>
<td>75</td>
<td>76</td>
<td>-</td>
<td>52</td>
</tr>
<tr>
<td>Rap1</td>
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<td>63</td>
<td>61</td>
<td>55</td>
<td>56</td>
<td>61</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Numbers represent percent identity between two sequences. Numbers above the diagonal represent identity over the full-length protein, while numbers below the diagonal represent identity over the first 80 N-terminal amino acids. Percentages were generated using the PALIGN program of PCGENE.
Outside of the conserved GTP-binding domains and membrane localization motif, there are some interesting variations in the amino acid sequence of RasB relative to the human H-Ras protein sequence, and the Dictyostelium RasD and RasG protein sequences. In the effector-proximal domain, (residues 23-31, relative to H-Ras), a domain which is believed to play a role in Ras target activation, there are three conservative amino acid substitutions in RasB; Leu-23 to Phe, Val-29 to Ile and Asp-31 to Glu. There is also a glutamic acid to aspartic acid substitution at residue 62. This residue is one of the residues that defines the second conserved GTP-binding domain. Such a substitution has not been seen in any of the naturally occurring Ras proteins characterized to date; they all possess a glutamic acid residue at position 62. RasB also has one conservative substitution (Glu-63 to Asp) in the recognition site for the Ras-specific monoclonal antibody Y13-259 (residues 63-73, relative to H-Ras; Furth et al., 1982; Sigal et al., 1986). This residue is one of six residues in the Y13-259 epitope of H-Ras that directly interacts with the antibody (Sigal et al., 1986). The substitution of glutamic acid-63 has never been observed in naturally occurring Ras proteins, but it has been reported that substitution of this residue using in vitro mutagenesis resulted in an activated H-Ras protein with transforming potential (Fasano et al., 1984). Whether or not this conservative substitution of residue 63 in RasB influences the biological activity of RasB remains to be determined.

Another interesting characteristic of RasB is that it is larger than any of the previously isolated Dictyostelium Ras proteins, 197 versus 186 (RasD) and 189 (RasG) amino acids (Figure 14). Since these additional residues are not located in the core of the protein but are found at the N-terminus (3 additional amino acids) and C-terminus (5 additional amino acids) of the protein, they probably will not alter the intrinsic GTP-binding and GTPase activities of the protein relative to the other Dictyostelium Ras proteins. However, these extra residues at the N- and C-
termini of the RasB protein may play significant roles in protein-protein interactions, or determine the extent to which the protein protrudes from the membrane.

3.3.3 Expression of rasB during Dictyostelium growth and development

When a rasB cDNA probe was used in a northern blot analysis of mRNA from developing Dictyostelium V12M2 cells, a single transcript of approximately 950 bp was detected at all time points (Figure 15A). The rasB mRNA levels were highest during growth (0 hr) and early development (4 hr and 8 hr) and then decreased significantly during the post-aggregative stages of development (12 hr). There was a slight burst of expression during the pseudoplasmodial stage of development (16 hr) but the level of expression then decreased again.

Previous studies with the Dictyostelium rasG-c3 cDNA had indicated that the rasG developmental pattern of gene expression in the axenic strain Ax2 was different from its pattern of expression in the wild type strain V12M2 (Robbins thesis). To determine if this was also the case for rasB, a northern blot of mRNA from developing Ax2 cells was probed with the rasB cDNA. The rasB gene expression profile was significantly different from that seen in V12M2. In Ax2, there was little expression of rasB in vegetative cells, but 4 hr post-starvation, maximum levels of the rasB transcript were detected. The rasB mRNA levels then gradually decreased throughout development but as with the wild type cells, the mRNA was still detectable during culmination (20 hr post-starvation; Figure 15B).
Figure 15. Northern blot analysis of rasB gene expression during Dictyostelium development. After the onset of development, total RNA was extracted from V12M2 (Panel A) or Ax2 (Panel B) cells at each of the indicated times, and 20 μg of RNA from each sample were fractionated on a 1.25% agarose-formaldehyde gel, and transferred and fixed to a nitrocellulose membrane. The filters were probed with an [α32P]-labelled full-length rasB 12-1 cDNA, and washed in a solution containing 1x SSC and 0.1% SDS at 60 °C (Panel A, V12M2) or 0.1 xSSC and 0.1% SDS at 60°C (Panel B, Ax2).
3.3.4 Analysis of a *Dictyostelium* genomic southern blot with a *rasB* probe

To determine whether *rasB* is a single copy gene, a *Dictyostelium* genomic Southern blot was probed with a *rasB* cDNA fragment under high stringency wash conditions (0.1x SSC, 0.1% SDS at 60°C). The probe hybridized to a single fragment in each digest, indicating that *rasB* is a single copy gene (Figure 16). The fragments that were detected produced a pattern that was unique to *rasB* (~ 6.0 kb and 20 kb) and different from the pattern detected with the *rasG* (~ 8.0 kb) and *rasD* (~ 3.0 kb, 6.0 kb and 8.0 kb) probes (data not shown). Under low stringency conditions, multiple fragments were detected, providing additional evidence for an extensive gene family.
Figure 16. Southern blot analysis of Dictyostelium genomic DNA with a rasB probe. Genomic DNA (10 μg) from the wild type strain V12M2 was digested with either EcoRI (lane 1), BglII (lane 2), or a combination of EcoRI and BglII (lane 3). The digested DNA was fractionated on a 0.7% agarose gel and then transferred to a nitrocellulose membrane. The filter was probed with an [α-32P]-labelled full-length rasB 12-1 cDNA, and washed under (A) low stringency conditions (1x SSC, 0.1% SDS at 50°C), or (B) high stringency conditions (0.1x SSC, 0.1% SDS at 60°C). Molecular size standards (kb) are indicated.
3.4 Isolation And Characterization Of The rasC Gene From Dictyostelium discoideum

3.4.1 Introduction

During an oligonucleotide screen for rab-related genes in Dictyostelium, Dr. J. Bush, in the laboratory of Dr. James Cardelli (LSUMC, Louisiana), isolated a ras-related cDNA from a Dictyostelium 4 hr λgt11 cDNA library. The nucleotide sequence of this cDNA clone, G-36, differed from the nucleotide sequences of rasB, rasD, and rasG, but it encoded the C-terminal 70% of a Ras-related protein. As part of a collaboration with Dr. Cardelli, the G-36 clone was sent to me for further characterization. This section details the isolation of a full-length rasC cDNA, and characterization of the rasC gene.

Results

3.4.2 Isolation of full-length rasC cDNAs and analysis of the RasC primary structure

The G-36 cDNA insert was amplified from the 4 hr λgt11 phage DNA using the PCR technique and λgt11 specific primers. The 500 bp G-36 PCR product was then digested with EcoRI to remove the 5' and 3' flanking λgt11 phage DNA which would hybridize to phage sequences in subsequent screenings of λgt11 cDNA libraries. This approximately 450 bp fragment was then used to screen the 3 hr λgt11 cDNA library under high stringency wash conditions (0.1x SSC, 0.1% SDS at 65 °C for 30 minutes), and several positive cDNA phage clones were recovered. The cDNA inserts were isolated from the positive clones and subcloned into the vector pTZ18R. Sequencing revealed that two independent clones possessed cDNAs with
identical open reading frames, and both cDNAs encoded the sequence of a 189 amino acid Ras protein. One clone (12-1) was sequenced on both strands while the second clone (14-1) was sequenced on one strand to confirm the nucleotide sequence of the gene. The M13 reverse and universal primers were used in conjunction with rasC-specific primers (JD-7 and JD-8) (Table 3) to sequence the cDNAs. Outside of the open reading frame, the two cDNAs had a very short region of overlap. This new gene was designated rasC, and the nucleotide sequence common to both full-length cDNAs, and the derived amino acid sequence of the open reading frame, are depicted in Figure 17.

Figure 18 depicts the alignment of the deduced amino acid sequence of RasC with the human H-Ras protein sequence and the Dictyostelium RasD, RasG, and Rap1 protein sequences (Capon et al., 1983; Reymond et al., 1986; Robbins et al., 1990; Robbins et al., 1989). This alignment highlights the strong conservation of the amino acid sequence of the Dictyostelium RasC protein with respect to the four functional GTP-binding domains and the carboxy-terminal CAAX motif. RasC is more diverged from H-Ras, RasG, and RasD than RasB, having only 56% identity with H-Ras and 65% identity with RasD and RasG (Table 5, above the diagonal). In addition, RasC is only 52% identical to the Dictyostelium Rap1 protein, and since it lacks the Rap-characteristic effector-proximal sequence (residues 23-31 relative to Rap1), and the threonine-61 residue, RasC is not a Rap protein.

There are several striking variations in the RasC amino acid sequence that are worth noting. The most significant is the single amino acid substitution in the putative effector domain. The negatively charged aspartic acid residue has been substituted with a polar but uncharged asparagine residue (D38N) (Figure 18). This is an interesting variation because the residues of this domain, (residues 32-40 relative to H-Ras, YDPTIEDSY), are identical in all members of the Ras subfamily proteins identified to date, with the exception of the Ral proteins which have three
Figure 17. Nucleotide and predicted amino acid sequences of the *Dictyostelium* rasC gene. Numbers indicate the nucleotide and amino acid positions in the sequence starting with the ATG initiation codon. The asterisk indicates the protein synthesis termination codon. Flanking untranslated nucleotide sequences common to the clones sequenced are shown. The rasC sequence has been deposited in the EMBL/Genbank database, accession number Z18926.
Figure 18. Alignment of the predicted amino acid sequence of RasC with the human H-Ras protein sequence (Capon et al., 1983) and the Dictyostelium RasD (Reymond et al., 1984), RasG (Robbins et al., 1989), and Rap1 (Robbins et al., 1990) protein sequences. Numbers indicate amino acid positions in each protein. Dashes indicate amino acids identical to those in H-Ras, and gaps have been inserted to optimize alignment. The asterisks (***), indicate the effector domain residues (Sigal et al., 1986), the open circles (ooo) the Y13-259 epitope residues (Furth et al., 1982; Sigal et al., 1986), and the triangles (△△△) the effector-proximal domain residues (Marshall et al., 1991; Zhang et al., 1990). Boxed residues represent the conserved GTP-binding domains and the C-terminal CAAX motif.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRas</td>
<td>MT EYKLVVV GAGGVGKS ALTIQLI QNFVDEYDPTIEDSYRKQVVIDGETCLLD</td>
</tr>
<tr>
<td>RasC</td>
<td>-S KLL---I- -D------ ------T--Q-IA------N------N--E-VYM--</td>
</tr>
<tr>
<td>RasD</td>
<td>-- ------I- -G------- ------I--------------S--D------</td>
</tr>
<tr>
<td>RasG</td>
<td>-- ------I- -G------- ------I--------------T--E------</td>
</tr>
<tr>
<td>Rap1</td>
<td>-PLR-F-I--L -S------ ----V-FV-GI--EK----------EV-SNQ-M-E</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>RasD</td>
<td>-- ------ ------Q-----YS-TSRS-YDE-ASF-----L----K-R--L</td>
</tr>
<tr>
<td>RasG</td>
<td>-- ------ ------Q-----YS-TSRS-DE-ASF-----L----K-R--L</td>
</tr>
<tr>
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</tr>
<tr>
<td>RasG</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>RasG</td>
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</tr>
<tr>
<td>Rap1</td>
<td>RKNPVGPPSKAK-K -A-L</td>
</tr>
</tbody>
</table>
changes (Chardin & Tavitian, 1986). Interestingly, aspartic acid-38 has been conserved in the Ral proteins. Previous studies aimed at characterizing this domain have indicated that aspartic acid-38 is necessary for Ras biological activity (Adari et al., 1988; Calès et al., 1988; Schaber et al., 1989; Sigal et al., 1986).

The second notable difference between RasC and the other Dictyostelium Ras proteins occurs in the effector-proximal domain (Marshall et al., 1991; Zhang et al., 1990). RasD and RasG have identical residues in this domain and only differ from H-Ras by the conservative substitution of valine-29 with an isoleucine residue (Figure 18). In RasC however, the non-polar isoleucine-24 residue has been replaced with a polar but uncharged threonine, the positively charged histidine-27 residue replaced with a polar but uncharged glutamine residue, the non-polar valine-29 residue replaced with a non-polar isoleucine residue, and the negatively charged aspartic acid-30 residue replaced with a non-polar alanine residue (Figure 18).

Finally, in the binding site for the monoclonal antibody Y13-259, there is a single conservative substitution of methionine-72 with isoleucine.

3.4.3 Expression of rasC during Dictyostelium growth and development

When a rasC cDNA probe was used in a northern blot analysis of mRNA isolated from Dictyostelium cells at different stages during the development of the wild type strain V12M2, a single transcript of approximately 850 bp was detected (Figure 19A). Maximum expression of the rasC gene occurred during early aggregation (4 hr after starvation) with the mRNA level decreasing as the aggregates progressed to the tipped aggregate stage (12 hr after starvation). There was then a second peak of mRNA expression at the pseudoplasmodial stage of development (16 hr after starvation). A second larger transcript of approximately 1.2 kb was first detected during the second peak of expression of the 850 bp mRNA, and this larger transcript was still evident at 20 hr post-starvation.
Figure 19. Northern blot analysis of rasC gene expression during Dictyostelium development. After the onset of development, total RNA was extracted from V12M2 (Panel A) or Ax2 (Panel B) cells at each of the times indicated (in hours) and 20 μg of RNA from each sample were fractionated on a 1.25% agarose-formaldehyde gel. The gels were assessed for equal loading by ethidium bromide staining of the ribosomal bands before transferring the RNA to nitrocellulose membranes. The membranes were probed with an [α^{32}P]-labelled rasC PCR fragment, washed in a solution containing 0.1x SSC and 0.1% SDS at 60°C, and used to expose X-ray film overnight.
Northern blot analysis of mRNA from developing Ax2 cells, using a rasC probe, indicated that the pattern of expression of rasC in Ax2 was similar to that observed in the wild type strain V12M2 (Figure 19B). The mRNA levels were again maximal during aggregation, and during the later stages of development of the axenic strain, two transcripts were again detected by the rasC probe. In Ax2 however, the larger rasC transcript was first detected 12 hr post-starvation. The level of expression of the two transcripts increased gradually until the pseudoplasmodial stage of development (16 hr post-starvation) and then remained constant during culmination.

3.4.4 Southern blot analysis of Dictyostelium genomic DNA using a rasC probe

When a Dictyostelium genomic southern blot was probed with a rasC cDNA fragment under low stringency wash conditions, the rasC probe hybridized to multiple genomic fragments. At least five of these detected fragments do not correspond to the genomic fragments detected by the Dictyostelium rasD, rasG, rasB, or rap1 gene probes, and this suggested that there may be other uncharacterized ras subfamily genes in Dictyostelium. Under high stringency wash conditions, however, only a single fragment from each genomic DNA digest hybridized to the rasC probe, suggesting that rasC is a single copy gene (Figure 20). The genomic digest pattern of restriction fragments was unique to rasC (~ 18 kb & 20 kb), and different from the pattern of fragments detected with the rasD (~ 3.0 kb, 6.0 kb & 8.0 kb), rasG (~ 8.0 kb), and rasB (~ 6.0 kb & 20 kb) probes.
Figure 20. Southern blot analysis of *Dictyostelium* genomic DNA with a rasC probe. Genomic DNA (10 μg) from the wild type strain V12M2 was digested with either EcoRI (lane 1), BglII (lane 2), or a combination of EcoRI and BglII (lane 3). The digested DNA was fractionated on a 0.7% agarose gel and then transferred and fixed to a nitrocellulose membrane. The filter was probed with an [α^32P]-labelled rasC PCR fragment and washed under (A) low stringency conditions (1x SSC, 0.1% SDS at 50°C) or (B) high stringency conditions (0.1x SSC, 0.1% SDS at 60°C). Molecular size standards (kb) are indicated.
3.5. **Isolation And Characterization Of A *Dictyostelium* Aggregation-Specific ras Gene, rasS**

3.5.1 **Introduction**

During the search for full-length rasG cDNAs, six clones which hybridized to the rasG probe under low stringency wash conditions were isolated from a 3 hr λgt11 cDNA library (Robbins unpublished data). One of these six clones was further characterized and determined to be equivalent to the previously isolated rasD gene (data not shown). Another clone, S3, was successfully subcloned into pTZ18R, and when sequenced, was found to have an open reading frame that predicted a novel Ras-related protein. No open reading frames could be found for the remaining four clones and so these clones were not further analysed. This section describes the isolation and subsequent characterization of this novel ras-related gene, which has been designated rasS.

**Results**

3.5.2 **Isolation of a full-length rasS cDNA and analysis of the RasS primary structure**

The S3 clone had a partial cDNA that lacked an ATG initiation codon preceeded by the characteristic AT-rich sequences of *Dictyostelium* genes (Loomis, 1982). The cDNA insert from this clone was therefore isolated and used to re-screen the 3 hr λgt11 cDNA library under high stringency conditions (0.1x SSC, 0.1% SDS at 60°C for 30 minutes) in an attempt to isolate a full-length cDNA. Of the six positive clones that were analysed, only one (S3-8) had a full-length cDNA (as indicated by the presence of an ATG initiation codon preceeded by an AT-rich nucleotide
sequence), and this clone was sequenced on both strands. The other five clones had partial cDNAs whose sequences overlapped the full-length clone to varying degrees and confirmed the sequence of the new gene, rasS. The nucleotide and derived amino acid sequences of rasS are depicted in Figure 21.

The alignment of the deduced amino acid sequence of rasS with the human H-Ras protein sequence, and the Dictyostelium RasD, RasG, and Rap1 protein sequences (Capon et al., 1983; Reymond et al., 1984; Robbins et al., 1990; Robbins et al., 1989) is depicted in Figure 22. Like the other Dictyostelium ras genes, rasS is predicted to encode a protein which is highly conserved in the four GTP-binding domains, and the C-terminal CAAX motif. However, the conservation of amino acid residues diminished outside of these regions and this was reflected in the low level of identity of RasS with the other Dictyostelium Ras proteins. RasS is only 61% identical to RasD, 60% identical to RasG, 51% identical to Rap1, and 54% identical to the human H-Ras (Table 5, above the diagonal). This level of identity with H-Ras is lower than that of any of the other Dictyostelium Ras proteins with H-Ras, and it is only slightly higher than the level of identity between RasS and Rap1. If only the first 80 N-terminal amino acids are considered in the comparison, then the amino acid identity increases disproportionately and RasS is clearly more related to the Ras proteins than to the Rap proteins (74% identity with RasG vs. 56% identity with Rap1, Table 5, below the diagonal).

Like RasC, RasS has a single amino acid substitution in the effector domain; isoleucine-36 is replaced with a leucine residue. Interestingly, substitution of Ile-36 with Leu has been demonstrated to decrease the biological activity of Ras (Sigal et al., 1986; Stone et al., 1988). There are also several amino acid substitutions in the effector-proximal domain of RasS: Leu23Phe, Gln25Ala, Asn26Gln, and His27Lys.
Figure 21. Nucleotide and predicted amino acid sequences of the Dictyostelium rasS gene. Numbers indicate the nucleotide and amino acid positions in the sequence starting with the ATG initiation codon. The asterisk indicates the protein synthesis termination codon. Flanking untranslated nucleotide sequences common to the clones sequenced are shown. The rasS sequence has been deposited in the EMBL/Genbank database, accession number Z14134.
Figure 22. Alignment of the predicted amino acid sequence of RasS with the human H-Ras protein sequence (Capon et al., 1983) and the Dictyostelium RasD (Reymond et al., 1984), RasG (Robbins et al., 1989), and Rap1 (Robbins et al., 1990) protein sequences. Numbers indicate amino acid positions in each protein. Dashes indicate amino acids identical to those in H-Ras, and gaps have been inserted to optimize alignment. The asterisks (***); indicate the effector domain residues (Sigal et al., 1986), the open circles (ooo) the Y13-259 epitope residues (Furth et al., 1982; Sigal et al., 1986), and the triangles (△△△) the effector-proximal domain residues (Marshall et al., 1991; Zhang et al., 1990). Boxed residues represent the conserved GTP-binding domains and the C-terminal CAAX motif.
Interestingly, in the RasS protein, there are three amino acid substitutions in the binding site for the ras-specific monoclonal antibody Y13-259. Two of these substitutions are at residues that are believed to interact directly with the antibody, (E63D and M67V), and immunoblot analysis of the RasS fusion protein revealed that these changes resulted in a recombinant protein which did not react with the Y13-259 antibody (see section 3.6).

Another interesting feature of the RasS protein is its carboxy-terminus. In addition to being five and seven residues longer than RasG and RasD respectively, the RasS C-terminal hypervariable region is very hydrophilic, consisting of serine, glutamine and asparagine residues, and four lysine residues in a row (Figure 22).

3.5.3 Expression of rasS during Dictyostelium growth and development

Using the rasS cDNA as a probe, a single transcript of approximately 800 bp was detected on a northern blot of mRNA isolated from developing wild type, Dictyostelium V12M2 cells (Figure 23A). This transcript was highly aggregation-specific; mRNA was only detected between 4 hr and 8 hr of development when the amoebae were primarily aggregating to form a multicellular mass.

Northern blot analysis of mRNA from developing Ax2 cells, using rasS as a probe, revealed that the rasS gene was expressed throughout development, unlike the aggregation-specific expression seen in the wild type strain V12M2 (Figure 23B). However, the mRNA levels were again maximal during aggregation (4 hr and 8 hr).
Figure 23. Northern blot analysis of rasS gene expression during Dictyostelium development. After the onset of differentiation, total RNA was extracted from V12M2 (Panel A) or Ax2 (Panel B) cells at each of the times indicated (in hours) and 20 μg RNA aliquots of each sample were fractionated on a 1.25% agarose-formaldehyde gel. The gels were assessed for equal loading by ethidium bromide staining of the ribosomal bands before transferring and fixing the RNA to nitrocellulose membranes. The filters were probed with an [α^{32}P]-labelled rasS-S3 cDNA, washed in a solution containing 0.1x SSC and 0.1% SDS at 60°C, and used to expose X-ray film overnight.
3.5.4 Southern blot analysis of *Dictyostelium* genomic DNA using a rasS probe

When a *Dictyostelium* genomic Southern blot was probed with a *rasS* cDNA fragment under low stringency wash conditions, few fragments were detected in any one digest (Figure 24). The low number of fragments detected by *rasS*, compared with the large number of fragments detected when *rasB*, *rasC*, or *rasG* were used as probes, was not surprising since *rasS* is the most diverged of the *Dictyostelium* ras genes. Under high stringency wash conditions, only a single hybridizing fragment was detected with *rasS*, suggesting that *rasS* is a single copy gene (Figure 24).
Figure 24. Southern blot analysis of Dictyostelium genomic DNA with a rasS probe. Genomic DNA (10 µg) from the wild type strain V12M2 was digested with either EcoRI (lane 1), BglII (lane 2), or a combination of EcoRI and BglII (lane 3). The digested DNA was fractionated on a 0.7% agarose gel and then transferred and fixed to a nitrocellulose membrane. The filter was probed with an $\alpha^{32P}$-labelled rasS-S3 cDNA and washed under (A) low stringency conditions (1x SSC, 0.1% SDS at 50°C) or (B) high stringency conditions (0.1x SSC, 0.1% SDS at 60°C). Molecular size standards (kb) are indicated.
3.6 Characterization Of The *Dictyostelium* Ras Proteins With The Y13-259 Monoclonal Antibody

3.6.1 Introduction

With the exception of the *S. cerevisiae* RAS proteins, which interact with Y13-259 despite one amino acid substitution in the epitope (Papageorge et al., 1984), the Ras proteins of diverse origin that have been identified to date possess identical amino acid residues in the region defined as the Y13-259 epitope. However, the more divergent Ras-related proteins R-Ras, Ral, and Rap have their own unique motifs in this region (Figure 25). The Rap proteins do not interact with Y13-259 (4 out of 6 amino acids that directly interact with the antibody are different) but it is not known whether R-Ras or Ral do interact with the antibody.

When the *Dictyostelium* proteins are compared in the Y13-259 epitope region with the human H-Ras, R-Ras, Ral, and Rap protein sequences, the distinctions between the *Dictyostelium* Ras proteins and the human Ras proteins become evident (Figure 25). Both RasD and RasG are identical to H-Ras in the Y13-259 epitope (Figure 25). However, RasB and RasC each have a single amino acid substitution in the epitope while RasS has three amino acid substitutions. To determine whether these differences in the Y13-259 binding sites of RasB, RasC, and RasS would affect their interaction with the monoclonal antibody, western blot analysis was carried out on recombinant Ras-GST fusion proteins using Y13-259 as the primary antibody.
**Figure 25.** Alignment of the Y13-259 epitope residues of representative members of the human Ras subfamily with the equivalent residues of the *Dictyostelium* Ras subfamily proteins. The human Ras proteins are represented by the canonical H-Ras (Capon *et al.*, 1983), R-Ras proteins by R-Ras (Lowe *et al.*, 1987), Ral proteins by RalA (Chardin & Tavitian, 1989), and the Rap proteins by Rap1 (Pizon *et al.*, 1988). Dashes indicate conserved residues relative to the H-Ras sequence.

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Results

3.6.2 Immunoreactivity of the Dictyostelium Ras fusion proteins with Y13-259

Of the Dictyostelium Ras proteins, RasD and RasG are the most highly related to the canonical H-Ras protein, and they are the most related to each other. They both possess intact Y13-259 binding sites relative to H-Ras and both were equally immunoreactive with the Y13-259 antibody (Figure 26). Although in Figure 26 RasG bound slightly less antibody than RasD, the Coomassie blue staining indicated that there was actually less RasG fusion protein.

In the RasB protein, there is a single conservative substitution in the Y13-259 binding site; Glu-63 to Asp. This residue is one of the six residues that are believed to directly interact with Y13-259 (Sigal et al., 1986), and this single change was sufficient to weaken the interaction of RasB with Y13-259 at least 4-fold relative to the interaction of RasD and RasG (Figure 26).

In the Y13-259 binding site of the RasC protein, there is a single conservative methionine to isoleucine substitution at residue 72. Surprisingly, this single amino acid change at a residue that has not been shown to directly interact with Y13-259, resulted in a protein that was less immunoreactive than either RasD or RasG (Figure 26). From this analysis, it therefore appears that methionine-72 plays a role in the interaction of the Dictyostelium RasC protein with Y13-259.

In the RasS protein, there are three amino acid substitutions in the Y13-259 epitope (Glu-63 to Asp, Tyr-64 to Phe, and Met-67 to Val), and two of these are at residues that are believed to interact directly with Y13-259 (Glu-63 and Met-67) (Sigal et al., 1986). As anticipated, these three amino acid substitutions in the RasS Y13-259 binding site resulted in a recombinant fusion protein which exhibited no reaction with Y13-259 (Figure 26). Likewise, the Dictyostelium Rap1 protein, which has five substitutions in the Y13-259 epitope, did not react with Y13-259 (Figure 26).
Figure 26. Western blot analysis of *Dictyostelium* Ras-GST fusion proteins with the monoclonal antibody Y13-259. Equal amounts (~1 μg) of *Dictyostelium* Ras-GST fusion proteins were electrophoresed on a 12% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane. The membrane was probed with the monoclonal antibody Y13-259 using the ECL immunodetection system (Amersham). The upper panel depicts the immunoreactivity of the different fusion proteins with Y13-259, as detected by ECL while the lower panel depicts the Coomassie-stained gel. The multiple bands of protein seen in the RasS lane are probably degradation products of the fusion protein.
CHAPTER 4

GENERAL DISCUSSION

The isolation and characterization of three additional ras genes, as described in this thesis, now substantiates the preliminary evidence of Robbins et al. (1989) which suggested that there were additional ras genes in Dictyostelium apart from rasD and rasG. Each gene was first identified by a different technique; rasB was identified as the result of a PCR, rasC was identified by oligonucleotide screening of a cDNA library, and rasS was identified by low stringency hybridization screening of a cDNA library using a rasG cDNA probe.

In this study, the PCR technique was used for the in vitro amplification of novel, Dictyostelium ras superfamily DNA sequences. Gene fragments were amplified from the already characterized Dictyostelium rasG, rasD, rap1, and sasl genes (Reymond et al., 1984; Robbins et al., 1990; Robbins et al., 1989; Saxe & Kimmel, 1990), and from four previously unidentified genes. In addition to the highly ras-related gene, rasB, whose characterization was described in this thesis (section 3.3), three genes of the Dictyostelium rab subfamily were identified.

Preliminary southern blot analysis of Dictyostelium genomic DNA, using the largest amplified rab-related cDNA as probe, suggested that there might be an extensive rab gene subfamily in Dictyostelium. This hypothesis has now been substantiated by Bush and co-workers who recently isolated and characterized five rab genes (rab1A, rab1B, rabA, rabB, and rabC) from Dictyostelium (Bush et al., 1993). Two of the rab-related sequences amplified in my PCR analysis have identical nucleotide sequences to portions of two of these genes. The nucleotide sequences of clones 11-4, 11-12, 11-13, and 11-14 are identical to a portion of the gene designated rab1A, while the nucleotide sequence of clone 11-3Y is identical to a portion of the
gene designated \textit{rabC} (data not shown). The third \textit{rab}-related sequence amplified in my PCR analysis appears to be derived from a previously unidentified \textit{Dictyostelium} \textit{rab} gene. The failure to detect the remaining three \textit{Dictyostelium} \textit{rab} genes (\textit{rab1B}, \textit{rabA}, and \textit{rabB}) in my PCR analysis is surprising because they all had the same number of mismatches with the PCR primers as the \textit{rab1A} and \textit{rabC} genes which were amplified. It is therefore likely that the \textit{rab1B}, \textit{rabA}, and \textit{rabB} gene sequences were amplified but subcloned at a low frequency, and hence they were not among the collection of clones screened.

In addition to the three \textit{rab} genes amplified by PCR, one novel \textit{ras} gene was amplified and this was designated \textit{rasB}. Considering the high level of expression of \textit{rasB} during vegetative growth and early development (see Chapter 3, section 3.3), the low frequency with which the \textit{rasB} gene was detected by PCR, relative to \textit{rasG}, is surprising (see Table 3). There is a single mismatch between each of the PCR primers JD-6 and JD-13, and the nucleotide sequence of the corresponding region of \textit{rasB}, and this may have contributed to a lower frequency of amplification. Alternatively, \textit{rasB} may have been amplified as much as \textit{rasG} but may have subcloned at a lower frequency.

No \textit{rasC} or \textit{rasS} gene fragments were detected by the PCR approach. There is one mismatch between the JD-13 primer sequence and the corresponding nucleotide sequences of \textit{rasC} and \textit{rasS}, and this was probably the determining factor in the PCR amplification. Both \textit{rasC} and \textit{rasS} have one mismatch with JD-13 at the third nucleotide from the 3' end of the primer, and this mismatch probably prevented the formation of a stable primer-gene complex. Since this was the downstream primer used in all the PCR amplifications, and since \textit{rasC} and \textit{rasS} also had mismatches with primers JD-6 and JD-11, this might explain why no \textit{rasC} or \textit{rasS} gene fragments were detected.
No rho, ral, or R-ras genes were detected in my PCR analysis. The failure to amplify rho gene sequences is consistent with the fact that the Rho proteins are not as well conserved in the region corresponding to the degenerate primer, JD-11 (GTP-binding domain #1), as are the Ras and Rab proteins (Santos & Nebreda, 1989). However, it was surprising that the ras-related genes, R-ras and ral, were not detected since the mammalian R-Ras and Ral proteins are as well conserved as are the Rab proteins in the 1st and 3rd GTP-binding domains. In fact, relative to the Ras superfamily consensus sequence of the first GTP-binding domain, GXGGVG, both the R-Ras and Ral proteins are more conserved in this domain than the Rab proteins (GXGGVG vs. GXGVG). The failure to amplify the Dictyostelium homologs of the ral and R-ras genes in a PCR therefore suggests that either a) there may be no Dictyostelium homologs to R-ras or ral, b) the Dictyostelium R-ras and ral genes may be expressed at very low levels compared to the ras, rap, and rab genes, c) the Dictyostelium ral and R-ras genes have diverged from their mammalian counterparts in the regions corresponding to the PCR primers, or d) insufficient clones were screened in the analysis.

All 34 of the clones derived from a rap-specific PCR had nucleotide sequences that were identical to a portion of the previously identified Dictyostelium rap1 gene. Restriction endonuclease digest analysis of an additional fourteen clones indicated that they were also derived from the previously identified Dictyostelium rap1 gene (Table 4). Although the results of this analysis suggest that there are no additional rap genes in Dictyostelium, the possibility that there are additional rap genes in Dictyostelium can not be totally excluded. As mentioned earlier, a single mismatch with the oligonucleotide primer at its 3' end may be sufficient to hinder amplification even if there is a perfect match with the rest of the primer. Furthermore, if a hypothetical Dictyostelium rap gene was sufficiently diverged from the Dictyostelium rap1 gene at the nucleotide level in the effector-proximal
domain, (primer JD-12), then the gene would not be amplified in the PCR. It is also possible that like the rab1A, rabA, rabB, rasC, and rasS genes, the hypothetical rap gene was amplified but subcloned at such a low frequency that it was not represented among the clones screened or sequenced.

The RasB, RasC, and RasS proteins are all more related to the Ras subfamily proteins than they are to any other subfamily (Rab or Rho), and within the subfamily, they are more related to the Ras proteins than they are to the R-Ras, Ral, and Rap proteins. However, despite their conservation of the four GTP-binding domains and the C-terminal CAAX motif, RasB, RasC, and RasS are less related to the human H-Ras protein than are RasD and RasG to H-Ras (59%, 56%, & 54% vs. 65% & 68% respectively, Table 5).

The most highly conserved region of the Ras proteins encompasses the first 80 amino acids, and it has sometimes been referred to as the catalytic domain. This region can be used as a more stringent measure to determine whether or not a novel ras-related protein is a Ras subfamily member. When the Dictyostelium Ras proteins are compared to the mammalian Ras proteins over these 80 residues, their relatedness to the Ras subfamily of proteins becomes more significant (Table 5, below the diagonal). RasD and RasG are 98% identical to each other in this domain and are very highly conserved relative to the human H-Ras (93%). This suggests that RasD and RasG may be functionally equivalent to the mammalian Ras proteins which are 100% identical to each other in this domain. Since RasG is expressed during growth and early development while RasD is expressed primarily during the pseudoplasmodial stage of development, RasD and RasG may perform the same function but at different times. Over this same stretch of amino acids (residues 1-80), RasB is 84% identical to RasD, 85% identical to RasG, and 81% identical to the human H-Ras. RasB may therefore have a slightly different function from RasD and RasG. RasC and RasS are more diverged from H-Ras than RasD and RasG (75%
and 76% identity respectively vs. 93% identity) but they are more related to H-Ras than they are to any other member of the subfamily. For example, RasC is 75% identical to H-Ras but only 55%, 63% and 56% identical to Rap, R-Ras, and Ral respectively. Since RasB, RasC, and RasS are more related to H-Ras (81%, 75%, 76%) than the Ras subfamily proteins, Rap1 (61%), R-Ras (70%), and RalA (63%), RasB, RasC, and RasS can be classified as Ras subfamily proteins.

The conservation of the carboxy-terminal CAAX motif that specifies the post-translational modification and membrane localization of the Ras proteins suggests that RasB, RasC, and RasS are likely to undergo the Ras-characteristic post-translational modifications that include prenylation of the carboxy-terminal cysteine residue, proteolytic cleavage of the AAX sequence, and carboxyl-methylation of the now terminal cysteine residue. In the human H-Ras, K-Ras, and N-Ras proteins, 'X' is either serine or methionine, and this specifies that the Ras protein be farnesylated (reviewed in Cox & Der, 1992; Magee & Newman, 1992). However, the terminal 'X' residue in all of the Dictyostelium Ras proteins is a leucine, and since in yeast and mammalian cells this leucine in the last position of a Ras or Ras-related protein specifies a C-20 geranylgeranyl modification of the cysteine residue in the CAAX motif (Cox & Der, 1992; Finegold et al., 1991), one might predict that the Dictyostelium Ras proteins would also be modified with a geranylgeranyl moiety.

The human H-Ras and N-Ras proteins are further modified by palmitylation of a cysteine residue that lies upstream of the CAAX motif, and it is believed that the palmityl moiety provides an additional membrane anchor for the proteins (Hancock et al., 1989). The absence of an additional cysteine residue upstream of the CAAX motif in the Dictyostelium Ras proteins would suggest that they are not further modified by palmitylation. However, the previous studies of Weeks and co-workers (1987) are inconsistent with this finding. They reported that a Y13-259 immunoprecipitable p23 Dictyostelium protein, which was assumed to be a Ras
protein, appeared to be palmitylated. Since all the *Dictyostelium* Ras proteins identified to date lack the cysteine residue that is considered to be the palmitylation signal, the discrepancy with the immunoprecipitation studies needs to be investigated. It is possible that the palmitylation signal for post-translational modification of the Ras proteins is different in *Dictyostelium*, or the palmitate used in the labelling was degraded and the radioactivity incorporated into prenyl groups. It is also possible that there are additional unidentified Ras protein(s) in *Dictyostelium* which do possess a cysteine residue upstream of the CAAX motif and which do become palmitylated.

Interestingly, the *Dictyostelium* Ras proteins resemble the human K-Ras proteins in the C-terminal hypervariable region in that they possess several basic lysine residues. This polybasic region is postulated to provide an additional membrane anchor for the K-Ras proteins since substitution of the lysine residues of K-Ras with neutral glutamine residues resulted in a cytosol-localized K-Ras protein (Hancock *et al.*, 1991). It is believed that this additional anchoring is achieved by the binding of the polybasic stretch of amino acids to the negatively charged phospholipids (Hancock *et al.*, 1991). This hypothesis is supported by the observation that a K-Ras protein, possessing a polybasic stretch of six arginines instead of six lysines, can still be membrane localized and produce transformed foci in NIH 3T3 cells (Hancock *et al.*, 1991). If the *Dictyostelium* Ras proteins undergo a similar process of membrane anchoring, then the polybasic tail would provide the same function as the palmitate moiety and obviate the need for the upstream cysteine residue and its subsequent palmitylation.

As mentioned in the introduction, three distinct regions of the human Ras proteins have been implicated in the binding of the putative effector molecule: they are the effector domain (residues 32-40 relative to H-Ras), the effector-proximal domain (residues 23-31 relative to H-Ras), and the Y13-259 monoclonal antibody
binding site (residues 63-73 relative to H-Ras). Utilizing information based on structure-function studies of these three domains, a model of Ras activation of an effector other than GAP has been recently proposed (Marshall, 1993). In this model, GAP functions solely as a negative regulator and interacts with Ras through the effector domain and the Y13-259 epitope, whereas the putative effector interacts with Ras through the effector and the effector-proximal domains (Marshall, 1993). It was therefore interesting to note that several of the atypical amino acid substitutions in RasB, RasC, and RasS were located in these three biologically significant domains.

The recent experiments of Self et al. (1993) have demonstrated that the amino acids of the effector-proximal domain are indeed necessary for Ras transforming activity, and hence, Ras interaction with its putative effector molecule. They showed that RhoA chimeras containing amino acids 23-46 (effector and effector-proximal domains) of H-Ras were capable of morphologically transforming NIH 3T3 cells, while RhoA chimeras possessing amino acids 30-40 (effector domain only) were unable to transform NIH 3T3 cells (Self et al., 1993). In the effector-proximal domain of the Dictyostelium Ras proteins, there are three amino acid substitutions in RasB, and four amino acid substitutions in both RasC and RasS relative to H-Ras, as compared to a single conservative substitution in both RasD and RasG (Val29Ile) (Figure 27). Four residues within this domain (residues 26, 27, 30, and 31) have been demonstrated to contribute to the biologically opposing effects of Rap1 and H-Ras (Marshall et al., 1991). RasB and RasC each have substitutions at one of these residues (RasB: Asp30Glu, and RasC: His27Gln), while RasS has substitutions at two of the four residues (Asn26Gln, and His27Lys). Since this effector-proximal domain is postulated to play a role in Ras target activation and protein-protein interactions (Marshall, 1993; Marshall et al., 1991; Sigal et al., 1986), the substitutions in this domain in RasB, RasC, and RasS will probably determine how, and to what extent, RasB, RasC, and RasS will activate their targets. In addition, these atypical
Figure 27. Alignment of the human H-Ras protein sequence with the Dictyostelium Ras subfamily protein sequences. Numbers indicate amino acid positions in each protein. Dashes indicate amino acids identical to those in H-Ras, and gaps have been inserted to optimize alignment. The asterisks (***), the open circles (ooo), the black dots (••), the triangles (△△△), and the boxed residues represent the conserved effector domain residues (Sigal et al., 1986), the Y13-259 epitope residues (Furth et al., 1982; Sigal et al., 1986), and the effector-proximal domain residues (Marshall et al., 1991; Zhang et al., 1990). Boxed residues represent the conserved GTP-binding domains and the C-terminal CAAX motif.
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<tr>
<td>RasD</td>
<td>-- -----A-----Q------YS-TSRS-YDE-ASF-----L-----K-R--L 111</td>
</tr>
<tr>
<td>RasG</td>
<td>-- -----D-----Q------YS-TSRS--DE-ASF-----L-----K-R-- 111</td>
</tr>
<tr>
<td>RasB</td>
<td>-- -----D-----Q------YDVTSTRT--E-NVV-----I----N-K--I 114</td>
</tr>
<tr>
<td>RasC</td>
<td>-- -----I-S-R----I-YS-ISRA------AVTTF------L----LSTY-I 112</td>
</tr>
<tr>
<td>RasS</td>
<td>-Y ------ DF--V------ ----YS-TYLQ--KE--RLHNHLK--L-S--F 111</td>
</tr>
<tr>
<td>Rap1</td>
<td>----T- QFT----L--KN-Q--V--YS-ISNST-NELPDL----L----CE---- 113</td>
</tr>
<tr>
<td>HRas</td>
<td>VLV GNKCD L AA RTVESRQAQDLARSGYIP YI ETSAK TRQGVEDAFYTLVREIR 164</td>
</tr>
<tr>
<td>RasD</td>
<td>I-- ----A-- DHE-Q-SVNEG-E--KD-SLS FH -S-- S-IN--E--S------- 164</td>
</tr>
<tr>
<td>RasG</td>
<td>IV----- ESD-Q-TTGEK--K-F-S-- FL ----- I-VN--E--S------- 165</td>
</tr>
<tr>
<td>RasB</td>
<td>-- ----E-TEGEGSE--K-FSV- FL ---- K-LN-DEC-FEV----K 168</td>
</tr>
<tr>
<td>RasC</td>
<td>-II ----A-- PDKD-K-PPMEGKE--K-F-A-- FL ---- S-VN--E--F-------K 167</td>
</tr>
<tr>
<td>RasS</td>
<td>----- NEY-E-STAEGEE--KKNCK FL ----- E-IN-SES--E-----VK 165</td>
</tr>
<tr>
<td>Rap1</td>
<td>----- ----- HDQ-VISTE-GEE--KF-DCYFL A--- NKVN--QI--N-I-Q-N 168</td>
</tr>
<tr>
<td>HRas</td>
<td>QHKLRKLNNPPDESFGCMSCK CVLS 189</td>
</tr>
<tr>
<td>RasD</td>
<td>KE-KGDQSSGKAQKKKQ -LIL 186</td>
</tr>
<tr>
<td>RasG</td>
<td>KD-KGDSK-EKGGKRRPLKA -T-L 189</td>
</tr>
<tr>
<td>RasB</td>
<td>KS-KEPGRSSKDKK-GILKFKGGD -LIL 197</td>
</tr>
<tr>
<td>RasC</td>
<td>RWQNPQ--EEMLPKKRG -IL 189</td>
</tr>
<tr>
<td>RasS</td>
<td>KARQSNQHNSQEQQNTDQFIKUKKS -N-L 194</td>
</tr>
<tr>
<td>Rap1</td>
<td>RKNPVGPPSKB-K -A-L 186</td>
</tr>
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</table>
variations in the effector-proximal domains of all three proteins suggest that RasB, RasC, and RasS will each interact with a different effector molecule. The fact that RasD and RasG possess identical effector-proximal domain residues suggests that they will interact with the same effector molecule and have similar mechanisms of target activation. This supports the notion that RasD and RasG perform identical functions in Dictyostelium but at different times during the developmental life cycle.

Several researchers have suggested that residues 45 and 48 be included in the definition of the effector domain since amino acid substitutions at either of these two residues impaired H-Ras biological activity (Fujita-Yoshigaki et al., 1991; Marshall et al., 1991). However, the substitutions did not affect GAP binding or GAP stimulated GTP hydrolysis, and so it was proposed that valine-45 and glycine-48 were target activating residues (Marshall, 1993; Marshall et al., 1991). Interestingly, none of the Dictyostelium Ras proteins are conserved at these two residues (Figure 27). In all five Dictyostelium Ras proteins, the non-polar valine-45 has been substituted with polar but uncharged amino acids, while in RasG, RasB, and RasC, glycine-48 has been substituted with glutamic acid. If the amino acids at these positions (45 and 48) act in conjunction with the effector-proximal domain to play a role in Ras target activation, then the amino acids in these positions in each of the Dictyostelium Ras proteins may specify the interactions of each Ras with its own distinct effector. Interestingly, these two sites are the only positions in the first 80 amino acids of RasD and RasG where these two proteins differ from each other. The significance of this difference remains to be determined but one possibility is that these variations may specify interactions with different effectors or they may reflect a subtle difference in the mechanism of target activation by RasD and RasG.

The effector domain region is one of two regions of the human H-Ras protein which undergoes a dramatic conformational change depending on the guanine
nucleotide state of the protein (Marshall, 1993; Stouten et al., 1993). These conformational changes in the effector or switch I region are believed to be sensed by interacting proteins (e.g., downstream effectors, regulatory proteins, etc.), and thus the effector domain is implicated as an important component in the molecular mechanism which underlies signal transmission from Ras to the downstream elements in the Ras signalling pathway.

Like RasD, RasG, and Rap1, RasB has an effector domain that is identical in sequence to the effector domain of the human H-Ras protein (Figure 27). If the model proposed by Marshall (1993) is correct, the effector domain of RasB may allow RasB to interact with the same effector as RasD and RasG, but the differences in the effector-flanking regions may prevent activation of the effector and transmission of the signal.

RasC and RasS are the first Ras subfamily proteins identified to date that have single amino acid substitutions in the effector domain. The Ral proteins have three amino acid substitutions in this region, YEPTKADSY vs. YDPTIEDSY, but the functional significance of these substitutions has not been determined. In RasC, aspartic acid-38 has been substituted with an asparagine residue while in RasS, isoleucine-36 has been replaced with a leucine residue. Several independent studies have demonstrated that both of these residues are crucial for Ras transforming activity (Adari et al., 1988; Calés et al., 1988; Gideon et al., 1992; Schaber et al., 1989; Sigal et al., 1986). In fact, Ras[Asn38] mutants are transformation defective and have a wild-type affinity for GAP, but their intrinsic GTPase is not stimulated by GAP. Furthermore, Marshall & Hettich (1993b) recently demonstrated that the NF1-GRD, which also possesses GAP activity, had a reduced affinity for the Ras[Asn38] effector mutant, and was unable to stimulate its intrinsic GTPase. Likewise, a Ras[Leu36] effector mutant was found to have diminished biological activity (Stone et al., 1988). The significance of these substitutions in RasC and RasS remains to be determined,
but I would speculate that the single amino acid substitutions in the effector domains of RasC and RasS most likely specify interactions with their own specific GAPs and/or effector molecules.

The Y13-259 epitope region of H-Ras is the other region (switch II) which undergoes major conformational changes upon binding GTP (Marshall, 1993; Stouten et al., 1993). Although evidence suggests that this region is not directly involved in effector binding, it does appear to be essential for the regulation of the nucleotide state of H-Ras by GAP (Marshall, 1993). Since the amino acid substitutions in this domain in RasB, RasC, and RasS affected the immunoreactivity of the Y13-259 antibody with the RasB, RasC, and RasS recombinant proteins to varying extents, one can speculate that these amino acid variations will also be sensed by other interacting proteins (e.g., GAP). If the recently proposed model for Ras interactions holds true (Marshall, 1993), then the variations in the effector, effector-proximal, and Y13-259 domains of RasB, RasC, and RasS will coordinately specify the interactions of RasB, RasC, and RasS with their own particular GAPs and effector molecules.

Outside of the effector, effector-proximal, and Y13-259 domains, there are several unusual differences in the RasB, RasC, and RasS protein sequences. First, in RasB, glutamic acid-62 has been substituted with an aspartic acid residue. This conservative substitution is unusual because glutamic acid-62 is one of the residues that defines the second GTP-binding domain (DTAGQE, residues 57-62 relative to H-Ras), and in all of the Ras and Ras-related proteins isolated and characterized to date, this residue has been strictly conserved with one exception. The S. cerevisiae RSR1 protein, which is related to the Rap proteins, has an alanine residue at position 62 (Bender & Pringle, 1989) but the significance of this variation in RSR1 has not been investigated. There is no evidence to suggest that amino acid substitutions at position 62 are oncogenic or activating lesions, but since this residue is contiguous
with the Y13-259 epitope and the switch II region, this substitution in RasB could have significant implications on the RasB:GAP interaction.

The RasB, RasC, and RasS proteins have intriguing C-terminal hypervariable regions (see Figure 27). Of the *Dictyostelium* Ras proteins, RasB and RasS possess the longest hypervariable regions, and RasC the shortest. Both RasB and RasS are five amino acids longer than RasG, and seven amino acids longer than RasD at the C-terminus. The RasB hypervariable tail is the most polybasic, containing eight lysine residues. The RasS tail is polar and relatively basic as well but it consists mainly of glutamine, serine, asparagine and lysine residues (Figure 27). Assuming that the polybasic hypervariable tail provides the same function in the *Dictyostelium* Ras proteins as it does in the mammalian Ras proteins, i.e. to serve as an additional membrane anchor, then these additional residues in RasB and RasS may enhance the binding of RasB and RasS to the membrane.

The hypervariable region is also believed to play a role in the membrane targeting of the Ras and Ras-related proteins (Adamson *et al.*, 1992; Chavrier *et al.*, 1991; Cox & Der, 1992; Hancock *et al.*, 1991), and it is tempting to speculate that these additional residues may play a role in targeting the RasB and RasS proteins to different membranes. It is also possible that the long hypervariable regions of RasB and RasS play a role in facilitating protein-protein interactions with cellular molecules such as membrane receptors and regulatory proteins. The evidence for the involvement of the hypervariable region in such a role comes from studies by Shirataki *et al.* (1991) who demonstrated that a geranylgeranylated peptide corresponding to the hypervariable region of Rap1B could interact with an exchange factor (smgGDS) and inhibit the GEF stimulation of Rap1B GDP/GTP exchange.

The short hypervvariable tail of RasC is not as polar or lysine rich as the hypervariable regions of the other *Dictyostelium* Ras proteins, suggesting that in
contrast to RasB and RasS, the RasC protein will be loosely bound to the membrane. The functional significance, if any, of this difference thus remains to be determined.

In the deletion mutagenesis studies performed by Willumsen et al. (1986), one of the conserved but dispensable domains for transforming activity corresponded to residues 93-108 (relative to H-Ras) and encompassed the amino acids REQI (residues 98-101). In the human H-Ras protein and the Dictyostelium RasB, RasD, RasG, and RasC proteins, residues 98-101 are identical (REQI) (Figure 27), and in the Ras-related proteins R-Ras, Ral, and Rap, glutamine-100 and isoleucine-101 have been totally conserved. It was therefore intriguing to note that in RasS, amino acids 98-101 were replaced with the amino acids HNHL. Although this region was not necessary for the transforming activity of the H-Ras protein, deletion of these residues and neighbouring residues resulted in a protein with only 50% GDP binding ability when compared to the wild type protein (Willumsen et al., 1986). Therefore, the substitution of amino acids 98-101 in RasS may be significant, and the possibility can not be excluded that these residues do play a role in the overall function of the Ras proteins.

Like the previously characterized Dictyostelium ras genes, rasB, rasC, and rasS are all developmentally regulated, and they each have a distinct pattern of gene expression (Figure 28). As can be seen from the schematic representation of ras gene expression in Dictyostelium, at any given time during development, there are at least two ras genes being expressed. In addition, it is worth noting that during aggregation (4-8 hr post-starvation), four ras genes (rasB, rasC, rasG, and rasS) are being expressed at reasonably high levels. This would suggest that during aggregation, the products of each of these four genes are required for distinct functions.
Figure 28. Schematic representation of the developmental gene expression patterns of the *Dictyostelium* Ras proteins. The height of the shaded boxes are not drawn to scale but are an approximation of the relative levels of transcripts detected by northern blot analysis. Time after the onset of development is indicated in hours.
The cumulative levels of ras gene expression throughout Dictyostelium development correlate well with the previous observations of Pawson et al. (1985) who reported that there were two stages during Dictyostelium development when p23 Ras protein levels were high. The absolute levels of the Y13-259 immunoprecipitable Ras proteins were maximal during growth and early development (0-4 hr post-starvation), diminished gradually during the next 6 hr of development, remained relatively constant for the next 6 hr, and then declined to less than 10% of the vegetative levels at the end of development (Pawson et al., 1985). It was also observed that the rate of p23 Ras synthesis was high during vegetative growth and early development (0-4 hr), low during aggregation (8-10 hr), and then rose transiently during the pseudoplasmodial stage of development (12-16 hr). The fact that there appeared to be very little Ras protein synthesis prior to tip formation, (8-10 hr post starvation), is consistent with the finding in this thesis that the RasS fusion protein is not immunoreactive with the Y13-259 antibody. Therefore, in the analysis of Y13-259 immunoprecipitable Ras proteins from Dictyostelium, the RasB, RasC, RasD, and RasG proteins were most likely in the pool of precipitated Ras protein, while the RasS protein was not. Since RasS would not contribute to the value for the absolute levels of Y13-259 immunoprecipitable Ras proteins in Dictyostelium, this would explain the apparent low rate of Ras p23 synthesis during aggregation.

In the Dictyostelium wild type strain V12M2, rasS gene expression is tightly regulated; the gene is expressed only after development has been initiated and for a very short period of time (4-8 hr post-starvation). The fact that rasS is specifically expressed 4-8 hr post-starvation, in conjunction with the unique features of its amino acid sequence, suggests that rasS has a very specific role during aggregation. It is therefore tempting to speculate that rasS may be involved in one or more of the three aggregation-associated cAMP-dependent signal transduction pathways that
have been characterized so far in *Dictyostelium* (Figure 6; Firtel, 1991). One priority of future studies therefore will be to determine whether rasS is crucial for *Dictyostelium* development, and whether it is involved in a cAMP-dependent signal transduction pathway. Gene knockout studies using strategies such as homologous recombination and antisense transformation should enable us to determine the effects of the loss of rasS gene function on *Dictyostelium* development. If rasS is crucial for *Dictyostelium* development, site directed mutagenesis studies can be employed to determine which of the atypical amino acid residues of RasS, if any, are important for its characteristic biological function. It would also be interesting to study the effects of rasS overexpression on *Dictyostelium* development.

The discovery of these three additional ras genes, rasB, rasC, and rasS, in *Dictyostelium* adds to the complexity of the ras gene subfamily in this lower eucaryote. This family now has six members, rasB, rasC, rasS, rasD (Reymond et al., 1984), rasG (Robbins et al., 1989), and rap1 (Robbins et al., 1990), and it may be even more extensive since *Dictyostelium* genomic southern blots hint at the existence of additional unidentified ras genes. The level of complexity of the ras subfamily seen in *Dictyostelium* has been observed only in human cells, where the ras subfamily consists of the three highly related genes, H-ras (Capon et al., 1983), K-ras (McGrath et al., 1983), and N-ras (Taparowsky et al., 1983), the R-ras genes (Drivas et al., 1990; Lowe et al., 1987), the ral genes (Chardin & Tavitian, 1986; Chardin & Tavitian, 1989), and the rap genes (Ohmstede et al., 1990; Pizon et al., 1988; Pizon et al., 1988b). Unfortunately, the significance of this complex Ras gene family in humans has not been addressed.

Comparison of the amino acid sequences of the members of the human Ras subfamily reveals two interesting primary structure characteristics. First, the effector domain sequence, residues 32 to 40 of the canonical H-Ras protein, YDPTIEDSY, is
identical in all members of the subfamily with the exception of the Ral proteins which have the modified sequence, YEPTKADSY (Chardin & Tavitian, 1986; Chardin & Tavitian, 1989; Sigal et al., 1986). Secondly, there appear to be unique signature motifs in the effector-proximal domain, (residues 23-31 relative to the canonical H-Ras protein), and the Y13-259 epitope sequences of the R-Ras, Ral, and Rap proteins (Figure 29). These unique motifs seem to be characteristic of the subgroups (R-Ras, Ral, & Rap) of the Ras subfamily since they are identical in the homologous proteins of Drosophila (R-Ras and Rap; Mozer et al., 1985; Schejter & Shilo, 1985), mouse (R-Ras; Lowe et al., 1987), apes (Ral; Chardin & Tavitian, 1986) and the electric ray (Rap and Ral; Ngsee et al., 1991). Since RasB, RasC, and RasS differ from R-Ras, Ral, and Rap in both the effector-proximal domain and the Y13-259 epitope (Figure 29), one can conclude that RasB, RasC, and RasS are not the Dictyostelium homologs of R-Ras, Rap, or Ral. The weakly hybridizing fragments detected on the Dictyostelium genomic southern blot probed with a rasB cDNA (Figure 16) may therefore correspond to the Dictyostelium homologs of R-Ras or Ral. These conclusions thus raise the possibility that if RasB, RasC, and RasS represent novel subgroups of the Ras subfamily, there may be unidentified human or mammalian homologs of RasB, RasC, and RasS.

Since there is now a total of six ras genes in Dictyostelium, and no additional rap genes were identified in this thesis, the role of the Rap protein in Dictyostelium development, and hence the proposed Ras:Rap antagonism/competition model, need to be assessed. If the competition model is valid, it is conceivable that the single Dictyostelium rap1 gene product could fulfill all Rap functions in Dictyostelium since rap1 is expressed throughout development. However, the possibility does exist that the ability of Rap to bind Ras-GAP and inhibit Ras function is purely coincidental and secondary to Rap's true function. One interesting hypothesis is that Rap plays a role in regulating cytoskeletal processes and cell
Figure 29. Alignment of, A) the effector-proximal domain residues and B) the Y13-259 epitope residues of representative members of the human and Dictyostelium Ras subfamily proteins. The human Ras proteins are represented by the canonical H-Ras (Capon et al., 1983), R-Ras proteins by R-Ras (Lowe et al., 1987), Ral proteins by RalA (Chardin & Tavitian, 1989), and the Rap proteins by Rap1 (Pizon et al., 1988). Dashes indicate conserved residues relative to the canonical H-Ras sequence.
morphology events (Bokoch, 1993). It is therefore worth recalling that the original selection for revertant NIH 3T3 cells harbouring the viral K-ras and rap1A genes was based on their altered cell morphology. The revertant NIH 3T3 cells displayed an increased attachment to plastic and exhibited a contact-inhibited growth pattern (Noda et al., 1989), hence the description as "flat revertants". There is now preliminary data from our laboratory which suggests that Rap may be involved in cell morphology events in _Dictyostelium_. Transformation of _Dictyostelium_ cells with the rap1 gene resulted in _Dictyostelium_ cells that 1) were 2-3 fold larger than untransformed cells, 2) adhered tightly to plastic, and 3) had a "flat" morphology (Patrick Rebstein, unpublished data).

Why does _Dictyostelium discoideum_, a lower eucaryote, need so many ras genes? Are all of these genes necessary for the normal development of _Dictyostelium_? What are the functions of the _Dictyostelium_ Ras proteins? Is there cross-talk between these proteins? What are the effector molecules in the _Dictyostelium_ Ras signalling pathways? These are just a few of the questions that have been raised as a result of this thesis. Hopefully, future studies in _Dictyostelium_ will determine the functions of this diverse collection of Ras proteins, and this knowledge should contribute to a better understanding of the pleiotropic effects produced by Ras in higher eucaryotes.
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