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Molecular and Genetic Characterization of RasGRP: A Novel Ras Guanyl Nucleotide Releasing Protein

by

Julius Okudu Ebinu 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Molecular and Genetic Characterization of RasGRP: A Novel Ras Guanyl Nucleotide Releasing Protein, submitted by Julius Okudu Ebinu in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The guanine nucleotide-binding protein Ras is a critical component of signaling pathways that control cell growth and differentiation. The activity of Ras can be regulated in two different ways. Ras is activated by cellular factors that stimulate guanine nucleotide exchange (guanyl nucleotide exchange factors, GEFs), whereas Ras is inactivated by guanosine triphosphatase-activating proteins (GAPs). Four mammalian Ras specific GEFs have been described, Sos1,2 and RasGRF1,2 (Bofini *et al.*, 1992; Bowtell *et al.*, 1992; Shou *et al.*, 1992; Fam *et al.*, 1992;). Productive interaction of the exchange factor with substrate Ras is regulated primarily by recruitment to the plasma membrane. We have isolated a transforming cDNA, rat brain cDNA #7 (rbc7), which encodes a novel protein that is conserved in evolution. Our analysis of rbc7 reveals that it is a 5' and 3' truncated version of a larger normal product, RasGRP. RasGRP is expressed normally in the brain and in lymphoid cells and is nontransforming. RasGRP contains a domain homologous to the catalytic region of the family of Ras-specific exchange factors, the CDC25 box. In addition, RasGRP has two EF hands and a diacylglycerol-binding C1 domain- two motifs, which are unique among GEFs and suggest a novel mechanism of regulation. The biochemical activities of the various domains of RasGRP were investigated. Here I show that the EF hands and the C1 domain of RasGRP bind calcium and diacylglycerol respectively. In addition, the catalytic domain of RasGRP activates Ras *in vitro*. Ectopic expression of RasGRP in rat fibroblasts resulted in an increase in Ras-GTP. Treatment of RasGRP-expressing rat fibroblasts with diacylglcyerol analogs caused the recruitment of RasGRP to the

membrane and a further increase in Ras-GTP levels. These observations led to the hypothesis that RasGRP links calcium and diacylglycerol signals to Ras output in neurons and lymphoid cells.

I also investigated the role of RasGRP in T cell activation. In collaboration with other investigators in Dr. Stone's lab, we demonstrated that a PLC-γ1 inhibitor diminished activation of Ras in response to T cell receptor (TCR) engagement. Furthermore, overexpression of RasGRP in T cells enhanced TCR-Ras-Erk signaling and potentiated interleukin-2 secretion in response to calcium ionophore plus diacylglycerol analogs. Here, I show that in Jurkat T cells, stimulation of the T cell receptor resulted in the preferential association of RasGRP with cellular membranes. Additionally, membranes isolated from TCR-stimulated Jurkat T cells display increased RasGRP and total Ras guanyl nucleotide exchange activity that was significantly reduced by antibodies directed against the catalytic domain of RasGRP. In response to phorbol ester and bryostatin-1 treatment, thymocytes from RasGRP null mice fail to activate CREB, a transcription factor activated in response to TCR stimulation by a protein kinase C-Ras-ERK-RSK-dependent signaling pathway. Altogether, my studies of RasGRP in rat fibroblasts and T cells suggest that RasGRP links signaling pathways that generate diacylglycerol and calcium as second messengers to the Ras signaling pathway.

This thesis is dedicated to my parents,

Augustine and Mary Ebinu,

whose love and support has given me

the strength and determination

to overcome all challenges and obstacles

faced throughout this endeavor.

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LIST OF ABBREVIATIONS AND SYMBOLS

Akt	AKT8 retrovirus proto-oncogene (also referred to as Protein kinase B)
ATP	adenosine triphosphate
c Ras	cellular Ras
C3G	Crk SH3-binding guanyl nucleotide releasing protein
CaIB	Calmodulin binding domain
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CDK	cyclin-dependent protein kinase
CREB	cyclic AMP response element binding protein
Dbl	Diffuse B-cell Lymphoma
DAG	diacylglycerol
dCTP	deoxycytosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetic acid
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
EGTA	ethylene glycol-bis(β-aminoethyl) ether
FAK	focal adhesion kinase
FBS	fetal bovine serum
G proteins	guanyl nucleotide binding proteins
GAP	GTPase activating protein
GEF	guanyl nucleotide exchange factor
GPCR	G-protein coupled receptors
GRF	guanyl nucleotide releasing factor
GRP	guanyl nucleotide releasing protein

GST	glutathione S-transferase
GTP	guanosine triphosphate
H-Ras	Harvey Ras
HA	hemaglutinin
IgG	immunoglobin G
IP_3	inositol triphosphate
IQ	ilimiquinone
IRS-1	insulin receptor substrate-1
JNK	Jun N-terminal kinase
JNKK	Jun N-terminal kinase kinase
K-Ras	Kirsten Ras
kDa	kilodalton
MAPK	mitogen activated protein kinase (also known as ERK)
MAPKK	MAPK kinase (also known as MEK)
MRNA	messenger RNA
N-Ras	Neuronal Ras
NGF	nerve growth factor
NP40	nonylphenoxy polyethoxy ethanol
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	pleckstrin homology
P_i	inorganic phosphate
PI-3K	phosphoinostide 3 kinase
PIP_2	phosphosphatidyl 4,5-bisphosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
RSK	ribosomal S6 kinase
Ras	rat sarcoma
Rbc7	rat brain cDNA #7

Rin	Ras interacting
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulphate
SH2	Src-homology-2
SH3	Src-homology-3
Sos	Son of Sevenless
Tec	Tyrosine kinase expressed in hepatocellular carcinoma
v Ras	viral Ras

CHAPTER 1
INTRODUCTION

Chapter 1

INTRODUCTION

1.1 OVERVIEW

A complex network of signaling pathways, each of which is modulated by a variety of stimuli, regulates the proliferation of cells in multi-cellular organisms. The regulation of cell growth by extracellular factors and the mechanisms by which these signals are integrated to generate a biological response are not clearly understood. However, impressive achievements are being made in defining these cell growth-regulating mechanisms at the molecular level. The greatest challenge remaining is to discover new signaling pathways and to determine how they operate and interact to process multiple and often conflicting signals into a decision on cell proliferation and differentiation or cell death.

When cells divide, they do so in response to intracellular and extracellular stimuli. The proteins that establish when and under what conditions a cell may divide are perturbed in cancer cells. The study of cancer cells has helped to define the numerous genes that regulate cell-growth and division. These cancer cell genes or oncogenes, which arise from normal proto-oncogenes, can transform normal cells into cancer cells. The study of oncogenic and normal cells has provided insights into known programmed

biological processes including cell differentiation, cell survival and senescence (Lowy and Willumsen, 1993).

One such family of oncogenes is the *ras* genes. The protein products of *ras*, collectively referred to as Ras, are present in every cell and are intimately involved in the transmission of signals for cell division from the cell surface to the nucleus (Furth *et al.*, 1987; Birchmeier *et al.*, 1985; Barbacid, 1987). Ras proteins have been implicated in regulating oncogenic, mitogenic and developmental signaling pathways. However, the mechanisms of Ras activation and signaling in response to external stimuli as well as the molecules that participate in the upstream and downstream signaling events in different cell types, are still not fully understood.

1.2 THE DISCOVERY OF RAS PROTEINS

The viral oncogenes of Harvey and Kirsten murine sarcoma viruses (H-MSV, K-MSV) were the first discovered *ras* genes (Harvey, 1964; Kirsten and Mayer, 1967). These acute transforming retroviruses were generated during passage of murine leukemia viruses through laboratory strains of rats. Molecular analysis of these viruses demonstrated that H-MSV and K-MSV contained sequences in their genomes that were absent in the genomes of the parental viruses. These newly acquired sequences appeared to be the result of viral transduction of cellular genes and were shown to be responsible for oncogenic transformation. This observation was confirmed by demonstrating that subgenomic fragments of H-MSV containing only cell-derived sequences were able to transform cells (Chang *et al.*, 1980; De Feo *et al.*, 1981; Ellis *et al.*, 1981).

Using immunological methods, the proteins produced by H-MSV and K-MSV were detected in rats bearing tumors induced by these viruses. These antibodies were found to react with a p21 species produced by H-MSV transformed cells. Further studies mapped the gene for p21 H-MSV to the region of viral DNA known to be of cellular origin. Additionally, p21 proteins were identified in normal, untransformed cells (Langbeheim *et al.*, 1980). These findings led to the conclusion that the p21 gene was the oncogene of H-MSV and K-MSV, as well as other closely related viruses, and that this oncogene was derived from a proto-oncogenic form present in normal cells. The

genes that encoded for the p21 proteins were thereafter referred to as the *ras* genes, from rat sarcoma genes.

Much of the interest in mammalian *ras* genes has been due to the presence of activated *c-ras* in chemically transformed cells and many types of human cancers (Shih *et al.*, 1979; Bos, 1989; Rodenhuis *et al.*, 1992). Early studies that led to this proposition began in 1979 when it was shown that high molecular weight genomic DNA from chemically transformed cells induced morphological transformation of NIH-3T3 cells (Shih *et al.*, 1979; Cooper *et al.*, 1980; Perucho *et al.*, 1981). These results suggested the existence of dominant acting transforming genes in these cells and provided an assay for their identification. Subsequent testing of a number of cell lines and tumor types revealed that several genes were responsible for transformation (Krontis and Cooper, 1981; Perucho *et al.*, 1981). One of these genes identified from the EJ/T24 bladder carcinoma cell line was identified as the human homolog of the H-MSV ras gene (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982). This discovery raised the interesting question of how this gene differed from its normal proto-oncogene counter part, which was apparently unable to transform NIH3T3 cells in this assay. Several groups reported that a single base change accounted for the activation of the EJ/T24 bladder carcinoma H-ras gene (Tabin *et al.*, 1992; Reddy *et al.*, 1982; Taparowsky *et al.*, 1983). Specifically, the twelfth amino acid of the ras p21 gene product was changed from glycine to valine.

The recognition that retroviral oncogenes, such as *v-ras*, are derived from normal cellular genes (*c-ras*) was followed by the demonstration that many of the oncogenes, identified by the NIH 3T3 cell transformation assay, represented cellular genes that had been activated by a point mutation (Barbacid, 1987). Activated *c-ras* genes were identified in several human tumors as well as in experimental tumors induced by chemical and physical agents (Bos, 1989; Rodenhuis *et al.*, 1992). In 10-50% of human tumors, the endogenous *ras* genes are activated by a somatic point mutation leading to the substitution of a single amino acid, usually in position 12 or 61 (Seeburg *et al.*, 1984; Der *et al.*, 1986). These mutations play an important role in the acquisition of transformed phenotypes (Barbacid, 1987). The general consequence of activating mutations in *ras* is the abrogation of normal control of Ras protein function, converting a normally regulated cell protein into one that is constitutively active (Seeburg *et al.*, 1984;

Der *et al.*, 1986). It is apparent that such deregulation of normal Ras function is responsible for the transforming activity of the mutated oncogene products (Bos, 1989).

The cellular genes encoding Ras proteins have been cloned and characterized (Barbacid, 1987). Mammalian cells express three closely related *ras* genes. Two of these are the proto-oncogene homologs of H-MSV and K-MSV oncogenes and are referred to as H-*ras* and K-*ras*, respectively. The K-*ras* gene, however, is able to generate two forms of p21 that differ in their C-terminal sequences (K-*ras*A and K-*ras*B) and are generated by alternative splicing of its fourth exon. The third mammalian *ras* gene has no known retroviral homolog and was initially identified by transfecting DNA from neuroblastoma cells into NIH 3T3 cells (Shimuzu *et al.*, 1983). Subsequent molecular analysis of the transfected gene indicated homology with known *ras* genes, and it was thereafter designated N-*ras* (Hall *et al.*, 1983; Shimizu *et al.*, 1983). Thus, three cellular genes encode Ras: H-*ras*, K-*ras*, and N-*ras*. Ras is expressed in all cell types. At the mRNA level H-*ras* is highest in brain, skin and muscle, K-*ras* in gut and thymus, and N-*ras* in thymus and testes (Leon *et al.*, 1987). Throughout this dissertation, Ras is used to collectively refer to the products of these three genes even though in various cell types one may predominate.

1.3 THE EVOLUTION OF RAS AND RAS SUPERFAMILY PROTEINS

Ras genes have been highly conserved during evolution (Shilo *et al.*, 1981). They have been identified in all eukaryotic species including *Drosophila* (Neurman-Silberberg *et al.*, 1984; Mozer *et al.*, 1985; Schejter *et al.*, 1985), mollusks *Aplysia* (Swanson *et al.*, 1986) and yeast (*S. cerevisiae* RAS1 and RAS2 genes and *S. pombe* SPRAS) (DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984; Fukui *et al.*, 1985). Comparative analysis of the primary structure of the deduced protein products of mammalian *ras* genes showed a high degree of homology. Of particular interest was the ability of *ras* genes to function in heterologous systems. For example, mammalian *ras* genes under the control of appropriate yeast promoters can complement non-viable *ras1*$^-$ and *ras2*$^-$ yeast mutants (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). Additionally, mammalian *ras* oncogenes can induce phenotypic alterations in yeast cells (Clark *et al.*, 1985). Similarly, chimeric yeast-mammalian *ras* genes, and a yeast *ras* gene which carries a deletion in its

long hypervariable region domain, are able to efficiently transform mouse NIH3T3 cells in gene transfer assays (DeFeo-Jones *et al.*, 1983). These examples illustrate the high degree of conservation of *ras* genes during evolution.

In addition to H-, K-, and N-Ras, mammalian cells contain a family of Ras related proteins (Figure 1-1). The closest relatives are the Rap proteins (Rap1A, Rap1B, Rap2A and Rap2B), which are found in the Golgi and endoplasmic reticulum (Beranger *et al.*, 1991a, 1991b; Pizon *et al.*, 1994). Rap1A can antagonize the transforming ability of Ras, a function that led to its isolations as a suppressor, called *Krev-1*, of the *K-ras* oncogene (Kitayama *et al.*, 1989). Also closely related to Ras are the Ral A and Ral B proteins, which are mainly localized in the membranes of endocytic and exocytic vesicles (Feig *et al.*, 1996). Ral appears to regulate the activity of exocytic and endocytic vesicles and has been proposed to be involved in the regulation of endocytosis and/or receptor down-regulation (Yamaguchi *et al.*, 1997; Ikeda *et al.*, 1998).

Less closely related to Ras are several other families in the Ras superfamily. For example, the Rho family embraces small GTP-binding (G) proteins that play dynamic roles in the regulation of the actin cytoskeleton and focal contacts, transcriptional regulation, cell cycle progression, membrane trafficking, and mediating formation of filopodia and lamellipodia (Kjoller and Hall, 1999). The Rho family can be grouped into six different classes consisting of the following members: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42Hs, G25K, TC10), Rnd (RhoE/Rnd3, Rnd1/Rho6, Rnd2/Rho7), RhoD, and TTF (Kjoller and Hall, 1999).

Additional Ras-related proteins include R-Ras, and TC21. R-ras is 55% identical to H-Ras (Bourne *et al.*, 1991). Two functions have been attributed to R-Ras. The first role is in the regulation of apoptosis (Fernandez-Sarabia *et al.*, 1993). This was suggested by the finding that R-Ras interacts with Bcl2 in a yeast 2-hybrid screen (Fernandez-Sarabia *et al.*, 1993). The second proposed function of R-Ras is the control of integrin activation (Zhang *et al.*, 1996). Zhang *et al.* (1996) gathered evidence to suggest that R-Ras is directly involved in the activation of integrins, for instance as part of an integrin complex, or involved in mediating signals that are responsible for integrin activation. TC21 shares 55% sequence similarity to Ras and is the only non-Ras GTPase that is found mutated in human tumor cell lines (Chan *et al.*, 1994; Huang *et al.*, 1995; Graham *et al.*, 1994). For example, in an ovarian carcinoma cell line a single point

mutation substituting glutamine for leucine at position 72 was found (Chan *et al.*, 1994). This mutation is equivalent to a mutation in glutamine 61 in Ras, which is frequently found in human tumors. Interestingly, proteins that regulate Ras can also activate TC21 *in vivo*, and inactivate TC21 *in vitro* (Graham *et al.*, 1996). The biological function of TC21 is still unknown. Newly discovered members of the Ras subfamily include Rheb (Yamagata *et al.*, 1994), Rin (Lee *et al.*, 1996), Rit (Lee *et al.*, 1996), and M-Ras/R-Ras3 (Quilliam *et al.*, 1999), Ric (Wes *et al.*, 1996) and kB-Ras (Fenwick *et al.*, 2000), all of which share several common structural features. The biological functions of these GTP-binding proteins are still not known.

1.4 THE STRUCTURE OF RAS PROTEINS

Ras genes encode a globular 21kDa protein containing 188 or 189 amino acids. The four species of Ras (H-Ras, K-RasA, K-RasB, and N-Ras) share many common structural properties. Comparison of the amino acid sequences of these proteins revealed the presence of four distinct domains (Figure1-2). The first region comprises the N-terminal 85 amino acids. These residues are identical and comprise a highly conserved domain. The next 80 amino acids (86-165) are 85% identical and define a second region where the structures of the different proteins diverge from each other. The high degree of conservation between the first 165 amino acids of mammalian Ras proteins suggested a common function for these proteins. The highly conserved domain encompassing amino acids 1-165 was shown to contain the catalytic domain of Ras that is responsible for the GTP hydrolyzing (GTPase) activity of Ras proteins (Temeles *et al.*, 1985; Gibbs *et al.*, 1984; McGrath *et al.*, 1984; Sweet *et al.*, 1984; Manne *et al.*, 1985). A highly variable region (residues 166-185) forms the third domain. This region appears to be the most divergent among all Ras proteins (4% identity). This observation suggested that this region of the different proteins might be associated with distinct functions. The fourth region constitutes the last four amino acids (residues 186-189) which are highly conserved and are important for the post-translational modification that is required for the attachment of Ras to the cell membrane (discussed below).

X-ray crystallographic methods have greatly improved our understanding of the biochemical and cellular activities of Ras proteins (De Vos *et al.*, 1988; Pai *et al.*, 1989).

Indeed, much of our understanding of the mechanism of Ras action has come from biochemical and structural studies. Figure 1-3 depicts the resolved structure of Ras in its GDP and GTP-bound form. The secondary structure of Ras consists of 5 α-helices and a central six-stranded β-sheet, with 5 of the strands being parallel and one being anti-parallel (De Vos *et al.*, 1988; Pai *et al.*, 1989, 1990). The hydrophobic core comprises the 6 strands of β-sheet which are connected by hydrophilic loops and α-helices. Structurally, this "G protein fold" is a variation upon the classical nucleotide-binding fold (Bourne *et al.*, 1991). Available crystal structures of G proteins demonstrated that all members of this group share a common structural core, exemplified by that of Ras. This structural similarity is reflected in significant sequence identity (Bourne *et al.*, 1990, 1991).

In addition to a common fold, three structural elements serve a common and essential purpose in all G proteins. In Ras, these conserved structural regions are crucial for Ras function and are responsible for the ability of Ras to bind to guanine nucleotides. The three defined structural domains are polypeptide loop regions designated L1 (residues10-17), L2 (residues 27-37) and L4 (residues 59-66) (Pai *et al.*, 1989, 1990; Bourne *et al.*, 1991). The primary structure of these three loop regions conform to sequence motifs that are well conserved in and diagnostic of guanine nucleotide binding proteins such as EF-Tu, Ras, and Gα subunits (Bourne *et al.*, 1991).

The first loop region, L1, comprises residues essential for nucleotide binding. This region forms a rigidly conserved structural unit that is common in all G proteins. L1 contains the sequence ^{10} GAGGVGKS ^{17} (single-letter amino-acid code), which fits the general recognition motif GXXXXGK[ST] found in all guanosine and some adenine nucleotide binding proteins (Moller and Amons, 1985). The two conserved glycine residues of this motif (10 and 15 in Ras) facilitate the unusual and highly specialized conformation of L1 and adopt a sterically unfavorable position for all residues but glycine. L1 is rigid in both the crystal structures of G proteins and the solution structure of Ras and does not participate in any of the conformational changes that occur upon GTP hydrolysis (Milburn *et al.*, 1990). The backbone chain of several residues within L1 form hydrogen bonds with the α- and β-phosphates of bound GDP and GTP. In addition, the side chain of lysine at position 16 (K16) forms hydrogen bonds with the β- and γ-phosphates and the hydroxyls of serine at position 17 (S17) help coordinate Mg^{2+} (Pai *et*

al., 1990). The importance of this motif is demonstrated by the fact that substitutions of G12 with other amino acids such as aspartic acid, lysine, valine, or arginine are the most common point mutations found in human tumor cells that carry a *ras* oncogene (Barbacid, 1987). The functional significance of mutations at position 12 involves impaired GTPase activity and reduced affinity of Ras for proteins that negatively regulate Ras activity. A mutation in Ras at position 17 of serine to asparagine, produces a mutant protein (RasS17N) that displays a significant reduction in its affinity for GTP (Feig and Cooper, 1988). One consequence of the abnormally low affinity of the RasS17N mutant for GTP is the formation of stable dead-end complexes with proteins that positively regulate Ras activity.

According to the 3-dimensional structure of Ras, the second and third loop regions, L2 (residues 27-37) and L4 (residues 59-66), are in close contact with the nucleotide. These two loop regions are highly flexible and are essential for Ras to function as a molecular switch. The structures contained in L2 and L4, comprise two switch regions, switch I and II respectively, whose conformation "switches" when GTP replaces GDP in the protein (Milburn *et al.*, 1990; Pai *et al.*, 1990). The switch I region, also known the "effector loop", is located between amino acids Y32–Y38 in c-H-Ras (composed primarily of L2). The effector domain was initially discovered by analysis of the transforming ability of deletion mutants of an activated version of c-H-Ras, v-H-Ras (Sigal *et al.*, 1986; Willumsen *et al.*, 1986). Further studies characterized residues in this domain for their sensitivity to mutation and analyzed point mutations that destroyed transforming ability (Sigal *et al.*, 1986; Stone *et al.*, 1991). The results from molecular and genetic studies of several groups showed that the effector domain is necessary for interacting with and signaling to proteins transmitting downstream signaling events (Nur-e-kamal *et al.*, 1992; Willumsen *et al.*, 1986; Sigal *et al.*, 1986; Stone *et al.*, 1991). Switch II comprises residues 60-76 (composed of helix 2 and loop 4) and has been shown to interact with proteins that positively regulate the activities of Ras (Quilliam *et al.*, 1995). Switch II also contains residues critical for the intrinsic GTPase activity of Ras (discussed in detail below). For example Q61, which is in close contact with the γ-phosphate of GTP (Pai *et al.*, 1990; Prive *et al.*, 1992).

X-ray crystallographic analyses have revealed structural differences between the GTP- and GDP-bound forms of G proteins that must account for their different biological

activities (De Vos *et al.*, 1988; Milburn, 1990; Pai *et al.*, 1989, 1990). Such structural studies of G proteins have shown that GTP-binding and hydrolysis trigger reciprocal conformational changes within the two switch regions, present in the catalytic domain. These localized but relatively large conformational differences allow G proteins to use the binding energy of GTP to stabilize the switch regions and produce a conformation that permits its association with its effector (Milburn *et al.*, 1990; Wittinghofer *et al.*, 1991). The conformational changes observed are caused by two sets of interactions between the γ-phosphate of GTP and Ras. The backbone of T35 in the switch I region forms hydrogen bonds with the γ-phosphate of GTP and the associated Mg^{2+} ion. These interactions give rise to a conformational change in the switch I region whereby the sidechain of T35 flips in orientation between the GTP and GDP-bound Ras structures. In the second set of interactions, the backbone of residues G60 and Q61 form hydrogen bonds with the γ-phosphate of GTP. These interactions are responsible for the conformational change observed in the switch II region.

1.5 BIOCHEMICAL ACTIVITIES OF RAS PROTEINS

1.5.1 GTP HYDROLYSIS

Ras proteins form relatively stable complexes with their substrate GTP, and product GDP (Scolnick *et al.*, 1979; Shih *et al.*, 1980; Tamanoi *et al.*, 1984). Ras proteins bind magnesium complexes of guanine nucleotides with high affinity (K_d approximately 10^{-11} M) and selectivity to ensure saturation by cytoplasmic concentrations of GTP (> 10^{-4} M) or GDP (>10^{-5} M) (Feuerstein *et al.*, 1987; Neal *et al.*, 1988; John *et al.*, 1990). These affinity constants were mainly determined by the dissociation rates of nucleotide because the association rates are nearly diffusion limited. As determined by structural analysis of p21 Ras crystals, in the GDP-bound state, Mg^{2+} coordinates directly with the β-phosphate of GDP and a serine residue at position 17 (S17), and indirectly with other amino acids through four water molecules (Milburn *et al.*, 1990; Pai *et al.*, 1989, 1990). In the GTP-bound state, coordination with two of the water molecules is replaced by direct coordination with the γ-phosphate and a second conserved threonine

residue (T35). Consistent with the highly conserved nature of the Mg^{2+} coordination sites, the cation is essential for guanine nucleotide binding and the GTPase activity for numerous GTP-binding proteins. At physiological concentrations of Mg^{2+} (mM), Mg^{2+} inhibits GDP dissociation when measured *in vitro* from Ras (Hall *et al.*, 1986; John *et al.*, 1993). This is supported further by experiments whereby mutation of the first Mg^{2+} coordination site (S17) in Ras, abolishes the cation's effect on GDP dissociation: a RasS17N or RasS17A mutant displays accelerated rates of GDP release and nucleotide dissociation is no longer inhibited by Mg^{2+} (John *et al.*, 1993; Feig *et al.*, 1988). This indicates that inhibition of GDP dissociation by Mg^{2+} is exerted directly through the cation's coordination within the nucleotide-binding pocket.

The Ras GTPase activity results in the slow hydrolysis of bound GTP leaving the p21 Ras protein complexed with GDP (Feuerstein *et al.*, 1987; Neal *et al.*, 1988; John *et al.*, 1990). Mutations found to particularly affect this intrinsic rate involve residues 12 or 61 which suggest that these amino acids are important for the p21 conformation mediating GTP hydrolysis (Neal *et al.*, 1988). *In vitro*, the spontaneous rate of hydrolysis gives a Ras-GTP a half-life of 2 hours at 25 °C for normal Ras (Sweet *et al.*, 1984), with that of activated versions being 3-9 times longer (Feuerstein *et al.*, 1987; Neal *et al.*, 1988; John *et al.*, 1990). *In vivo*, proteins that negatively regulate Ras can stimulate catalysis by more than 10^5-fold (Gideon *et al.*, 1992).

1.5.1.1 Mechanism of GTP Hydrolysis

The mechanism of GTP hydrolysis has been a matter of considerable debate. Insight into the mechanism of GTP hydrolysis has been inferred from high-resolution X-ray crystallographic analyses of p21 Ras crystallized with a nonhydrolyzable GTP analog (Pai *et al.*, 1990; Prive *et al.*, 1992). Studies employing caged GTP, which photolyzes to GDP after UV exposure and then undergoes hydrolysis *in situ* in the crystal, have provided additional understanding of the mechanism of GTP hydrolysis (Schlichting *et al.*, 1990). Analysis in the crystal structure of the residues comprising the active site revealed that in all the structures of activated G-proteins, a possible catalytic water is found close to the γ-phosphate (Figure 1-4). This water molecule is stabilized by hydrogen bonding both to an oxygen of the γ -phosphate and to the conserved threonine (T35) in L2 (Pai *et al.*, 1990). This water molecule is assumed to be activated to OH^- in

the catalysis. It is generally accepted that after activation, an in-line attack by this hydroxyl on the γ-phosphate leads to a pentacoordinate transition state. The leaving group, GDP, is coordinated to both Mg^{2+} and the ε-amino group of K16. This mechanism of GTP hydrolysis involves the direct transfer of the γ-phosphate from GTP to water, with an inversion of configuration around the γ-phosphate. Such a mechanism requires a catalytic base close to the water molecule. This catalytic base has been exceedingly hard to find. Early studies focused on Q61 in Ras, which was potentially close to the water molecule and in position to polarize and activate the water (Pai *et al.*, 1990). This side chain resides in switch II (L4) and, with a few exceptions (i.e. EF-G, EF-Tu, Rap1A), is conserved in most members of the G protein family. The majority of mutations at this site in Ras reduce its GTPase activity 10-fold, prevents response to RasGAPs and are oncogenic (Der *et al.*, 1986; Vogel *et al.*, 1988). This finding implicates this residue in catalysis (Der *et al.*, 1986; Vogel *et al.*, 1988). The residual GTPase activity suggests that this residue is not the catalytic residue, but is important in stabilizing the transition state (Prive *et al.*, 1992). In a more recent computer modeling study, Schweins *et al.* (1994) proposed an alternative mechanism for GTP hydrolysis. This mechanism involves activation of the catalytic water by the γ-phosphate itself (Schweins *et al.*, 1994, 1995).

1.5.2 POST-TRANSLATIONAL MODIFICATION OF RAS PROTEINS

While the structure of Ras is also conserved throughout many different levels of evolution, for all isomers of Ras protein, plasma membrane localization is essential for their biological function (Gibbs *et al.*, 1989; Bourne *et al.*, 1990). Ras proteins are bound to the inner surface of the plasma membrane by several lipophilic interactions involving post-translational modifications of the C-terminus (Glomset *et al.*, 1990; Kawata *et al.*, 1990; Katayama *et al.*, 1991; Farnsworth *et al.*, 1994). Membrane binding generally requires a C-terminal isoprenyl group, which is added post-translationally by a mechanism that involves recognition of a terminal CAAX motif (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid). In some Ras related proteins, CXC or CC motifs are present instead of CAAX (Hancock *et al.*, 1989). Either internal

palmitoylation or a C-terminal cluster of basic amino acids (Hancock *et al.*, 1990; Cadwallader *et al.*, 1994) often provides a second component of binding energy.

Ras proteins are post-translationally modified by prenylation, a process whereby a cysteine residue very near to the C-terminus, in the hypervariable (amino acids 165-185), typically has a polyisoprenyl moiety attached to it [either 15-carbon isoprenoid (farnesyl) for Ras or C20 (geranylgeranyl) for some Rap proteins and members of the Rho superfamily]. Prenylation is catalyzed by one of two soluble prenyltransferases that attach, respectively, a farnesyl or geranylgeranyl lipid via a stable thioether linkage to the CAAX cysteine. The substrate specificity for farnesyltransferase versus geranylgeranyl transferase is determined by the residue in the X position of the CAAX motif (Casey and Seabra, 1996). After prenylation, further modification of this sequence involves proteolytic removal of the last three amino acids denoted "AAX", followed by carboxymethylation of the exposed cysteine residue (cysteine 186). Polyisoprenylation has been shown to be essential for the localization of Ras proteins to the plasma membrane (Glomset *et al.*, 1990). In N-Ras and H-Ras, but not K-Ras, a further modification is made in the hypervariable region of the protein just upstream of the CAAX motif whereby one or two other cysteine residues are modified by palmitic acid via a labile thioester linkage (Hancock *et al.*, 1989). K-Ras(B) does not have a cysteine residue that can be palmitoylated, but does have a polybasic domain that fulfills a similar function. Hence, this polybasic sequence upstream of the CAAX motif substitutes for palmitoylated cysteines in acting as a second required plasma membrane targeting signal (Hancock *et al.*, 1990).

1.6 THE REGULATION OF RAS PROTEINS

Ras belongs to a diverse family of GTPases and plays a pivotal role in the signal transduction pathways that control proliferation, differentiation, and metabolism (Barbacid, 1987; Lowy and Willumsen, 1993). Ras is able to fulfill diverse functions through a common molecular switch that cycles between GDP- and GTP-bound forms, which are the inactive and active forms of Ras respectively. In this way the biological activity of Ras is dependent upon the guanine nucleotide-bound state. The intrinsic conversion of GTP- to GDP-bound Ras, which is associated with GTP hydrolysis, is very

slow and in the order of k_{cat}= 0.0028 min^{-1} (Temeles *et al.*, 1985). *In vivo*, this reaction is catalyzed by regulatory proteins, which increase the rate by more than 10^5-fold (Gideon *et al.*, 1992). In a similar fashion, the exchange of GDP for GTP, also know as guanyl nucleotide exchange, is a slow reaction $k_{cat} < 0.03min^{-1}$ (Feuerstein *et al.*, 1987). This can be enhanced *in vivo* by 100-fold by proteins that positively regulate Ras (Jones *et al.*, 1991; Lai *et al.*, 1993). Since the nucleotide exchange rate is slower than the rate of GTP hydrolysis (Feig and Cooper, 1988), guanyl nucleotide exchange is considered to be the rate-limiting step in Ras activation (Haubruck and McCormick, 1991). Thus, tight regulation of the guanine nucleotide bound state is critical for the regulation of normal cellular proliferation and differentiation because constitutively active (GTP bound) Ras proteins can induce cellular transformation (Barbacid, 1987).

Since the intracellular concentration of GTP is much higher than that of GDP, GTP is more than likely bound to the native Ras protein as it is synthesized and acquires its tertiary structure. The spontaneous (intrinsic) rate of GDP dissociation is very low, approximately 10^{-5} moles per second dissociating per mole of complex (John *et al.*, 1990). The Ras that does dissociate from the nucleotide will reassociate very quickly with GTP, which implies that during the normal GTPase cycle of Ras, dissociation of bound GDP leads to activation of the protein. It is worth noting that mutations of residues necessary for guanyl nucleotide binding can affect the intrinsic dissociation rate of Ras. This can lead to a situation whereby the spontaneous loading of GTP exceeds the rate of spontaneous GTP hydrolysis. Moreover, mutations at positions 12, 13, 59, 61, 63, 116, 117, or 119 endow Ras with cell-transforming capability (Barbacid, 1987; Bos, 1989; Bourne *et al.*, 1990). These positions constitute part of the guanine nucleotide-binding pocket (Milburn *et al.*, 1990; De vos *et al.*, 1988; Pai *et al.*, 1989), and these can be mapped to either the phosphate region (positions 12, 13, 59, 61 and 63) or base region (positions 116, 117, 119 and 146). All of the biochemically characterized phosphate mutants display a decrease in intrinsic GTP hydrolysis rate and are insensitive to proteins that negatively regulate Ras. Additionally, all of the base region mutants and some of the phosphate region mutants that have been studied show a decreased affinity for the guanine nucleotide. Either or both of these properties- reduced hydrolysis rates and weakened nucleotide binding- are thought to be the biochemical reasons for cell transformation by oncogenic Ras proteins.

While the small GTPases of the Ras superfamily are involved in regulating many intracellular processes, including cell growth and division, cell morphology and movement, vesicular transport and nuclear events (Bokoch *et al.*, 1993; Lowy and Willumsen, 1993; Macara *et al.*, 1996), the immediate control of these GTPase-mediated events resides in the proteins that regulate their GTP- or GDP-binding status. Two classes of regulatory proteins have been identified: the guanine nucleotide exchange factors (GEFs), whose physiological function is to convert Ras from a GDP- to a GTP-bound state, and the GTPase activating proteins (GAPs), which turn off Ras by activating its intrinsic GTPase activity (McCormick *et al.*, 1994; Boguski *et al.*, 1993).

1.6.1 GUANYL NUCLEOTIDE EXCHANGE FACTORS- POSITIVE REGULATORS OF RAS

In accordance with the GTPase cycle, GTP must replace GDP. Excess GTP can drive the overall reaction in the forward direction, as is the case in the cytoplasm, by greater affinity of the GTPase for GTP than for GDP, or by the intervention of other molecules such as GEFs. In signal transduction pathways via Ras or Ras-like proteins, one of the most essential processes is their activation by GEFs. GEFs act as stimulators of the slow intrinsic dissociation rate of the Ras-GDP nucleotide complexes. Since the cellular concentration of GTP is higher than that of GDP and the affinity of Ras for GTP is slightly higher than that for GDP (Feuerstein *et al.*, 1987; John *et al.*, 1990; Neal *et al.*, 1988), GTP will bind to Ras, thereby activating the Ras protein. Only Ras-GTP complex can bind with high affinity to its downstream effectors and thus transmit a signal.

Genetic studies of Ras function in lower eukaryotes provided the first identification of RasGEFs. For example, in yeast *Saccharomyces cerevisiae* RAS is required for the activation of adenylate cyclase and for maintaining cell viability (Toda *et al.*, 1986). Of particular importance to RAS was the CDC25 gene product, which was genetically mapped as being upstream of RAS and required for RAS activation. CDC25 was subsequently confirmed to possess GEF activity *in vitro* (Jones *et al.*, 1991). The minimum functional CDC25 catalytic domain sequence has been determined to be approximately 450 amino acids (Lai *et al.*, 1993). Other proteins bearing strong homology to CDC25 were subsequently detected in various yeast strains. These included a second yeast protein, SDC25, which was found to share strong sequence identity with

this CDC25 catalytic domain and shown to act as a RasGEF but, unlike CDC25, is not an essential *S. cerevisiae* gene product (Boy-Marcotte *et al.*, 1989; Crechet *et al.*, 1990; Damak *et al.*, 1991). Other examples include Ste6, which appears to be the CDC25 homolog in fission yeast *S. pombe* and Lte1, which also exhibits CDC25 homology, but its function as a GEF is presently unclear (Hughes *et al.*, 1990).

Mammalian homologs of CDC25 were subsequently isolated either by genetic complementation of a CDC25-defective yeast strain with a mammalian cDNA expression library (Lai *et al.*, 1993) or by using degenerate PCR primers to conserved regions of yeast CDC25 sequences (Shou *et al.*, 1992;Wei *et al.*, 1992). Related mouse, rat and human genes were isolated that encoded proteins with exchange activity for Ras, but not for Ras-related proteins. One example was RasGRF1 (CDC25Mm), a mouse RasGEF which was found to be expressed in a brain specific manner. However, since Ras proteins are ubiquitous, the exclusive expression of mammalian CDC25 Mm in the brain suggested the existence of other RasGEFs.

The isolation of additional mammalian RasGEFs was prompted by genetic studies of the Ras signal transduction pathway in fruit flies. Developmental studies on *D. melanogaster* had indicated that formation of the R7 photoreceptor cell during compound eye development required a pathway involving *Ras* and the *Sevenless* receptor protein tyrosine kinase (Han *et al.*, 1992). Further genetic analysis identified a gene product, *Son of Sevenless* (d*Sos*), that was downstream of *Sevenless* but upstream of Ras (Simon *et al.*, 1991). d*Sos* contains a domain sharing approximately 30% identity with yeast and mammalian CDC25 catalytic domains (Simon *et al.*, 1991). There was little homology outside of the catalytic domains between d*Sos* and RasGRF1/CDC25Mm, suggesting differential regulation. Using low stringency hybridization with the *Drosophila* cDNA, d*Sos* homologs were independently cloned from mouse and human cDNA libraries, designated Sos1 and 2 (~67% identity). Unlike RasGRF1/CDC25Mm, Sos mRNA and proteins are expressed in most tissues (Rogge *et al.*, 1991; Bonfini *et al.*, 1992; Bowtell *et al.*, 1992; Chardin *et al.*, 1993).

RasGEF proteins are of considerable length and contain several conserved regions representing conserved structural domains (Boguski and McCormick, 1993). RasGEFs are characterized by the presence of a conserved catalytic region first identified in the prototype exchange factor in yeast CDC25 (Figure 1-5A). All GEFs that act on members

of the Ras subfamily share this region, known as the CDC25 box, and their activity is specific toward Ras, Ral, or Rap. An additional upstream sequence called the Ras exchange motif (REM) is characteristic of GEFs which act on members of the Ras superfamily (Lai *et al.*, 1993). In addition to their catalytic domain, GEFs carry modules such as src-homolgy-3 (SH3) and pleckstrin homology (PH) domains connecting this class of proteins to upstream partners of the signaling pathway (Pawson *et al.*, 1995). A family of GEFs carrying a common catalytic sequence but different regulatory parts could potentially activate each family of G protein (Boguski and McCormick, 1993).

To date, several genes have been isolated from different organisms encoding proteins that have a GEF activity specific for Ras. The products of the yeast *CDC25* and *Drosophila Sos* genes are the prototypes of this class of enzyme (Broek *et al.*, 1987; Robinson *et al.*, 1987; Simon *et al.*, 1991; Bofini *et al.*, 1992). These RasGEFs can be grouped into three different protein families- *Son of Sevenless* (Sos) type proteins, guanyl-nucleotide releasing factors (GRFs), and guanyl-nucleotide dissociation stimulators (GDSs) (Boguski and McCormick, 1993). This classification is based on the relatedness, distribution and specificity of their interactions with Ras proteins.

1.6.1.1 *Son of Sevenless (Sos)* Family of Exchange Factors

One of the best-studied RasGEFs in mammalian cells is Sos (Chardin *et al.*, 1993; Bowtell *et al.*, 1992; Downward *et al.*, 1996). Sos was originally identified in *Drosophila melonogaster* (dSos), where it was placed genetically between a receptor protein tyrosine kinase (*Sev*) and Ras (Bonfini *et al.*, 1992; Simon *et al.*, 1991). Mammalian cells contain two closely related and ubiquitously expressed Sos genes, *Sos1* and *Sos2* (Rogge *et al.*, 1991; Bonfini *et al.*, 1992; Bowtell *et al.*, 1992; Chardin *et al.*, 1993). C3G a 120-130 kDa protein was originally identified as a ubiquitously expressed guanyl nucleotide exchange factor for Ras (Tanaka *et al.*, 1994). This discovery was based on the sequence homology of its C-terminal domain with CDC25 and Sos as well as on studies which showed that C3G could complement CDC25 function in yeast (Tanaka *et al.*, 1994; Broek *et al.*, 1987; Bonfini *et al.*, 1992; Cen *et al.*, 1992). Recent evidence, however, has shown that C3G is not a Ras exchanger (Gotoh *et al.*, 1995). C3G has been shown to have GEF activity for a Ras-like protein Rap1 *in vitro*, as well as a Ras-related protein R-Ras (Gotoh *et al.*, 1995; Gotoh *et al.*, 1997). This indicates the

importance of conducting both *in vivo* and *in vitro* experiments to demonstrate that a RasGEF homologous protein is a true Ras activator.

The Structure and Function of Sos proteins

The encoded 140-150 kDa gene products of *Sos* consist of several defined structural domains each mediating a distinct function (Figure 1-5A). The N-terminus of Sos contains a Dbl homology (DH) domain. The DH domain is a segment of approximately 180 amino acids that is known to catalyze guanine nucleotide exchange on the Rho and Rac family of small GTP binding proteins (Quilliam *et al.*, 1995, Hall, 1998). Until recently, the function of the DH domain in Sos was unknown. In a recent report, NimNual *et al.* (1998) demonstrate that Sos can activate Rac through its DH domain.

The N-terminus of Sos also contains a pleckstrin homology (PH) domain. PH domains are approximately 100 amino acids in length and were originally identified as an internal repeat present at the N and C termini of pleckstrin, a 47 kDa protein which is the major protein kinase-C substrate in activated platelets (Tyers *et al.*, 1998; Haslam *et al.*, 1993). This domain has been proposed to be analogous to the previously characterized SH2 and SH3 domains in that it mediates protein-protein interactions. For instance, some PH domains including that of Sos can bind to the Gβγ-subunit of heterotrimeric G proteins *in vitro* and *in vivo* (Touhara *et al.*, 1994; Luttrel *et al.*, 1995). The binding interactions mediated by PH domains allow the formation of numerous types of specific, transient, signal-dependent and, usually membrane localized protein complexes important in cellular responses to a variety of stimuli.

Sos also contains a central domain commonly referred to as the CDC25 box, which is highly conserved among different Ras exchange factors and mediates the catalytic activity of Sos (Boguski and McCormick, 1993). This domain permits the mammalian Sos1and Sos2 proteins to act on their substrates which include all three Ras proteins: H-, K- and N-Ras (Chardin *et al.*, 1993). The C-terminus of Sos is characterized by the presence of multiple proline-rich SH3 binding sites which mediate the interaction with adaptor molecules such as Grb2 (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Lowenstein *et al.*, 1992; Rozakis-Adacock *et al.*, 1993). This proline-rich region also allows Sos to associate directly with

a range of adaptor proteins including Shc, CrkII and Nck (Okada *et al.*, 1996). Adaptor proteins typically have no enzymatic activity, but contain protein-protein interacting motifs, which display sequence similarity with pp60src, known as src homology (SH) domains (Pawson, 1992). Such proteins can serve as adaptors (Koch *et al.*, 1991) by linking proteins with phospho-tyrosine and proline-rich motifs.

The Regulation of Sos Function

Sos proteins have been established as the link between receptor tyrosine kinase, non-receptor tyrosine kinase coupled receptor, and heterotrimeric G protein coupled receptor stimulation and Ras activation (Li *et al.*, 1993; Faure *et al.*, 1994; Shou *et al.*, 1995; Zippel *et al.*, 1996). In *Caenorhabditis elegans*, an adapter protein (Sem-5) was shown to lie genetically between a receptor protein tyrosine kinase (RTK) and Sos (Clark *et al.*, 1992). In addition, a mammalian Sem-5 homolog, Grb2, was shown to bind to the C-terminus of mammalian Sos (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993). Whereas Sos is largely cytoplasmic and functionally inactive in quiescent cells, activation of RTK by growth factors such as EGF has been shown to lead to the Grb2-dependent binding of a Sos-complex to the activated receptor, and a concomitant increase in Ras-GTP (Buday and Downward, 1993; Gale *et al.*, 1993). Furthermore, the addition of a membrane-targeting signal to Sos renders the protein constitutively active *in vivo*, leading to Ras-dependent focal transformation of established rodent fibroblasts (Aronheim *et al.*, 1994; Quilliam *et al.*, 1994). The membrane-targeted protein did not require the Sos C-terminal Grb2-binding site, which suggested that the membrane-targeting signal substituted for this function. These results led to the widely accepted model in which activation of Ras by Sos depended entirely upon the C-terminal Grb2 binding site (Migliaccio *et al.*, 1997).

PH domains are frequently found in signal transduction proteins, and there is a considerable amount of evidence indicating that PH domains are critical for the biological and enzymatic activity of these proteins (Buchsbaum *et al.*, 1996; Lemmon *et al.*, 1996; Pawson, 1995; Whitehead *et al.*, 1997). Recent data show that PH domains can directly control enzymatic activity of adjacent kinases or nucleotide exchange domains - not only by targeting to the correct subcellular location but also by a direct allosteric activation via ligand binding to the PH domain (Salim *et al.*, 1996). For example, a PH

domain immediately preceded by a DH domain can regulate the nucleotide exchange activity of the DH domain on the Dbl protein family. In this case, a structural hallmark of such regulation is the presence of a DH-PH domain pair which can be found in Sos. This DH-PH module has been shown to regulate the ability of Sos to activate Rac (NimNual *et al.*, 1998). The PH domain of Sos has also been implicated in regulating the ability of Sos to activate Ras (Karlovich *et al.*, 1995; McCollam *et al.*, 1995; Wang *et al.*, 1995) and is required for the ability of Sos to function as a Ras activator (Byrne *et al.*, 1996). Deletion of the DH-PH pair of Sos causes a loss of function *in vivo* ((Karlovich *et al.*, 1995; Byrne *et al.*, 1996; McCollam *et al.*, 1995). This finding highlights the importance of the DH and PH domains in regulating Sos function.

Several studies have shown that serpentine receptors can activate Ras by releasing the βγ-subunit of heterotrimeric complexes (Van Biesen *et al.*, 1995). In the case of proteins such as Sos, which contains a PH domain, extracellular agents that signal through G protein coupled receptors are known to mediate regulatory interactions of the βγ-subunits of G proteins with these signaling molecules (Harlan *et al.*, 1994; Inglese *et al.*, 1995). For example, binding of Gβγ-subunit to the PH domain of Sos would localize Sos to the plasma membrane in a manner dependent on the activation of G protein coupled receptors (Chen *et al.*, 1997). Most PH domains are also known to bind to phosphatidyl inositol phosphates, such as phosphatidyl inositol (4,5)-bisphosphate (PIP_2) and phosphatidyl inositol (3,4,5)-triphosphate (PIP_3) (Harlan *et al.*, 1994), or their inositol phosphate head groups. The PH domains in this case serve as regulated membrane-anchoring modules. For instance, the PH domain of Sos has been shown to be crucial for the ligand-dependent membrane targeting of Sos (Chen *et al.*, 1997). In either case, the PH domains enable the βγ-subunits of G proteins in combination with PIP_2 or related lipids, to interact with and regulate the guanine nucleotide exchange capability of the Sos class of exchangers (Chen *et al.*, 1997; McCollam *et al.*, 1995; Jefferson *et al.*, 1998).

In most cell types, the predominant mechanism by which Ras proteins are activated following receptor tyrosine kinase stimulation involves an increase in the rate of Sos-mediated guanine nucleotide exchange on Ras (Budy and Downward, 1993; Egan *et al.*, 1993; Li *et al.*, 1993). This increase does not reflect an enhancement of the catalytic activity of Sos, as demonstrated by the finding that the GEF activity of Sos is not altered

by growth factor stimulation (Buday and Downward, 1993; Gale *et al.*, 1993). Instead, the activation of Ras appears to be achieved through the growth factor-dependent recruitment and association of Sos-Grb2 complexes with activated RTKs or tyrosine phosphorylated Shc proteins (Gale *et al.*, 1993). The translocation of Sos to the membrane and the subsequent protein-protein interactions are mediated by Grb2 (Gale *et al.*, 1993). The SH3 domains of Grb2 bind to the proline-rich sequences of Sos and the SH2 domains bind to the phosphotyrosine residues of RTKs (Pawson and Gish, 1992). The translocation of Sos presumably increases the local concentration of Sos in the plasma membrane where Ras is located, an event considered sufficient to induce Ras activation (Aronheim *et al.*, 1994). Several lines of evidence, however, suggest that this Grb2-mediated membrane-targeting model of Sos activation may require at least some modification. Studies in flies have shown that the C terminus of *Drosophila* Sos is dispensable for *Sev*-dependent Sos function (Karlovich *et al.*, 1995). Furthermore, a premature termination mutant of mammalian Sos1, which lacks the Grb2 binding site, has been shown to be more active *in vivo* than full-length Sos1 (Wang *et al.*, 1995). In addition, the N terminus of mammalian Sos1 has recently been shown to interfere with EGF-dependent signaling in mammalian cells (Byrne *et al.*, 1996).

The preceding studies suggest that the N terminus of Sos may contribute significantly to Sos function. Two potential motifs in the N terminus are DH and PH domains. Dbl is a protein shown to have GEF activity for the human homolog of Cdc42 and RhoA, which are members of the Rho GTPase family (Cerione *et al.*, 1996; Hart *et al.*, 1991). Domains with homology to the catalytic region of Dbl have been identified in a variety of signaling molecules (Cerione *et al.*, 1996). In most of these proteins, including Sos, the function of the DH region is not well characterized. PH domains are also present in many signaling molecules (Shaw, 1996). In most well studied examples, they contribute to membrane association and have functional importance (Li *et al.*, 1995; Paterson, 1995; Pitcher *et al.*, 1995; Vihinen *et al.*, 1995; Yenush *et al.*, 1996). In a recent report, the functional significance of the N terminus of Sos is studied by mutational analyses of the DH and PH domains in Sos. This group demonstrated that membrane targeting of Sos depends on both the DH and PH domains for full biological activity (Qian *et al.*, 1998). They also showed that the Sos N-terminus is required for

stable association between Sos and an activated EGF receptor. These observations suggest a role for DH and PH domains that is independent of membrane association.

Sos also appears to be regulated by the growth factor-induced phosphorylation of serine residues within its C-terminal domain (Cherniack *et al.*, 1994; Corbalan-Garcia *et al.*, 1996; Rozakis-Adcock *et al.*, 1993). In this case, the phosphorylation of Sos is mediated by ERK mitogen-activated protein (MAP) kinase and results in the dissociation of the Grb2-Sos complex (Cherniack, 1995; Corbalan-Garcia, 1996; Waters, 1995b). In addition to ERK, p90Rsk-2 can phosphorylate Sos (Douville *et al.*, 1997). While the phosphorylation-dependent disassembly of the Grb2-Sos complex might contribute to the down modulation of Sos activity (Waters *et al.*, 1995a, 1995b), the physiological significance of Sos phosphorylation remains to be determined. Lastly, binding of PIP_2 to the PH domain of Sos, inhibits its activity *in vitro* (Jefferson *et al.*, 1998).

1.6.1.2 The Ras Guanyl nucleotide Releasing Factor (RasGRF) Family of Exchange Factors

GEFs displaying sequence similarity with the prototype exchange factor in yeast, CDC25, and which act on all three isoforms of Ras (Leonardsen *et al.*, 1996), include p140 RasGRF1 (CDC25Mm) and RasGRF2 (Shou *et al.*, 1992; Fam *et al.*, 1997). RasGRF2 displays 80% amino acid sequence identity with RasGRF1 (Fam *et al.*, 1997). While early studies reported ubiquitous expression of Sos RNA (Bowtell *et al.*, 1992), RasGRF expression was reported to be restricted mostly to brain tissue (Shou *et al.*, 1992). However, a variety of RasGRF cDNA and protein forms have been reported in various cellular settings (Cen *et al.*, 1992; Schweighoffer *et al.*, 1993; Wei *et al.*, 1994, Ferrari *et al.*, 1994) suggesting the possibility of alternative splicing in these loci. RasGRF1 is predominantly expressed in the brain with particular localization to the synaptic junction (Sturani *et al.*, 1997; Shou *et al.*, 1992), whereas RasGRF2 displays a much wider tissue expression profile (Fam *et al.*, 1997). RasGRF1 gene encodes for several protein products which vary in size from 70-140 kDa. The C-terminal amino acids of each protein are identical and sequential N-terminal extensions account for the larger sized protein products (Cen *et al.*, 1992).

The Structure and Regulation of RasGRF proteins

The structure and regulation of RasGRF are complex. Structural analysis of RasGRF reveals the presence of two PH domains, an ilimaquinone (IQ) motif, a DH domain and a cdc25 box (Figure 1-5A). In contrast to the Sos family of exchangers, RasGRF1 and RasGRF2 contain no proline-rich SH3-binding domains and thus do not respond to tyrosine-kinase induced signals (Cen *et al.*, 1993; Shou *et al.*, 1992, 1995; Farnsworth *et al.*, 1995).

RasGRF1 is activated by calcium elevation, serum or lysophosphatidic acid (LPA) treatment and M1 muscarinic receptor activation (Shou *et al.*, 1995; Farnsworth *et al.*, 1995). The activation of RasGRFs by these stimuli is primarily due to the fact that RasGRFs contain unique sequences at their N-termini: a second PH domain, a putative coiled-coil and a calmodulin-binding IQ domain (Chen *et al.*, 1993; Farnsworth *et al.*, 1995). The PH domains target RasGRFs to the membrane (Buchsbaum *et al.*, 1996). This is due to their ability to bind lipids (Harlan *et al.*, 1994) as well as to βγ-subunit of heterotrimeric G proteins (Touhara *et al.*, 1994). Coiled-coils consist of two or three amphipathic helices in parallel. They can bind to coiled-coils on other proteins, to form either homo- or heterodimers (Lupas *et al.*, 1991). The so called "IQ" motif is a 25-30 amino acid stretch of tandem isoleucine and glutamine residues and is present in a number of proteins including myosin heavy chain (Cheney *et al.*, 1992). IQ motifs in these proteins mediate calcium-dependent binding to calmodulin and calmodulin-related proteins. The IQ domain of RasGRF enables RasGRF to bind to and become activated by Ca^{2+}/calmodulin (Buchsbaum *et al.*, 1996). The IQ motif of RasGRF binds Ca^{2+}/calmodulin in response to Ca^{2+} elevation either by ionophore treatment or K^{+}-induced membrane depolarization (Farnsworth *et al.*, 1995; Fam *et al.*, 1997).

Ca^{2+}/calmodulin binding results in the stimulation of RasGRF exchange activity (Farnsworth *et al.*, 1995; Fam *et al.*, 1997). This calmodulin-binding motif has also been shown to mediate RasGRF2 activation by calcium which results in the translocation of RasGRF2 to the cell periphery (Buchsbaum *et al.*, 1996; Rosen *et al.*, 1996; Fam *et al.*, 1997). Mutation of the IQ motif in order to abolish calmodulin binding results in the loss of Ca^{2+}-induced activation of RasGRF (Sturani *et al.*, 1997). However, the calcium-induced activation of RasGRF also requires the presence of the PH2 and DH domains (Freshney *et al.*, 1997). Thus, Ca^{2+}-activation of RasGRF may require the Ca^{2+}-

dependent binding of calmodulin to the IQ motif and a cooperative/allosteric effect mediated by the PH and DH domains.

RasGRF may also be directly phosphorylated and activated, by as yet an unknown S/T kinase, by stimulation of muscarinic receptors, through the activation of G-protein βγ subunits (Mattingly *et al.*, 1996). Phosphorylation of RasGRF is closely associated with an increase in its exchange activity toward Ras (Mattingly *et al.*, 1996). RasGRF also contains a DH domain near its N-terminus that is distinct from the domains that regulate guanine nucleotide exchange on Ras. As described above, DH domains are known to catalyze guanine nucleotide exchange on the Rho and the Rac family of small GTP binding proteins. In RasGRF1, the DH is necessary for its normal Ca^{2+}-dependent function (Freshney *et al.*, 1997). Recently, the DH domains of RasGRF1 (Innocenti *et al.*, 2000) and RasGRF2 (Fan *et al.*, 1998) were shown display Rac-specific GEF activity.

Unlike Sos, RasGRF1 does not need to be translocated in the cell to access Ras. Instead, it is located in the particulate fraction of cells and this localization appears to require the N-terminal region of the protein (Buchsbaum *et al.*, 1996). Deletion of the N-terminal PH1 domain of RasGRF1 results in the removal of the protein from the particulate fraction. However, when this PH1 domain is fused to the catalytic domain of the protein, this construct is still unable to localize to the membrane (Buchsbaum *et al.*, 1996). Intriguingly, the PH1 domain is also required for serum activation. Since pertussis toxin treatment inhibits serum-induced activation and PH domains are known to bind the βγ subunits of heterotrimeric G proteins (Neer *et al.*, 1995), it is feasible that serum activation of GRFs involves G_i activation and βγ subunit release. Although RasGRF2 displays high sequence homology with RasGRF1, this exchange factor is not membrane localized and requires Ca^{2+} elevation for its translocation to the plasma membrane (Cen *et al.*, 1992).

More recently, RasGRF1 was shown to activate the highly related apoptosis controlling p23 R-ras (Gotoh *et al.*, 1997). The question of whether RasGRF is a specific exchange factor for either p21 or p23 Ras as well as whether it can activate interchangeably both Ras proteins remains to be determined. Nevertheless, it demonstrates selectivity in response to prevailing cellular conditions. Functionally, RasGRF knock-out mice are impaired for memory consolidation processes (Brambilla *et al.*, 1997). This has led to the proposal that RasGRF1 plays a role in long-term memory

processes (Brambilla *et al.*, 1997). In fact, full length RasGRF1 is thought to couple synaptic activity to the molecular mechanisms that consolidate changes in synaptic strength within specific neuronal circuits (Brambilla *et al.*, 1997; Finkbeiner and Dalva, 1998).

1.6.3 Small GTP-binding GDP-Dissociation Stimulator (SmgGDS) Family of Exchange Factors

Smg (small GTP-binding protein) GDS a 55kDA protein was initially purified from bovine brain as the regulator of a Ras-related protein, Rap1B (Kaibuchi *et al.*, 1991). SmgGDS displays only weak homology to the CDC25 catalytic domain, and consists of 11 Armadillo repeats similar to those found in proteins located at adherens junctions e.g. catenins (Peifer *et al.*, 1994). Although SmgGDS was originally described as a GEF for Rap proteins (Kaibuchi *et al.*, 1991), it was subsequently shown to promote nucleotide exchange on different sub-groups of the Ras superfamily including p21 K Ras-B (and not H- or N-Ras), Rho and Rac proteins. It is worth noting that all of these proteins share a polybasic region in their C-terminal hypervariable domains (Hiroaka *et al.*, 1992, Orita *et al.*, 1993; Takai *et al.*, 1993).

The broad specificity of SmgGDS is surprising as Ras and Rho proteins are only 35% identical in sequence, whereas K-Ras and H-Ras are 85% identical. The discovery that SmgGDS works efficiently only on isoprenylated proteins (Mizouno *et al.*, 1991) provided insight into the reason for its broad specificity and also suggested that SmgGDS recognizes a feature of the hypervariable C terminus that includes this modification. While Ras proteins receive 15-carbon farnesyl moieties and Rho/Rac proteins receive 20-carbon moieties, the question of how GDS distinguishes farnesylated K-Ras and geranylgeranylated Rho from farnesylated H-Ras remains to be answered.

SmgGDS has no sequence similarity to any other Ras GEF. GDS displays weak homology to the catalytic regions of Cdc25, and the degree of homology is less than that of Sos or RasGRFs. Having little in common with other classes of GEFS, the unique properties of SmgGDS suggested that SmgGDS represented a distinct class of Ras regulator. Additionally, *in vivo* studies have yet to establish a physiological role for this exchange factor. However, it has been shown that SmgGDS cDNAs can cooperate with K-rasB to induce cell transformation and expression from the c-fos promoter (Fujioka *et*

al., 1992). In a recent report, mice lacking SmgGDS died of heart failure shortly after birth as a result of enhanced apoptosis of cardiomyocytes triggered by cardiovascular overload (Takakura *et al.*, 2000). Takakura and coworkers (2000) also provided evidence suggesting that SmgGDS is involved in anti-apoptotic cell survival signaling through K-Ras.

1.6.4 Mechanism of RasGEF action

RasGEFs exhibit a modest preference for binding GDP-bound forms of Ras (Haney *et al.*, 1994; Lai *et al.*, 1993). Thus GEFs, which affect the nucleotide binding status of Ras, preferentially bind their respective substrates rather than their products. The high affinities for substrates likely reflect structural differences between the two-nucleotide bound forms of Ras (Milburn *et al.*, 1990). Significantly, the switch I and II regions of H-Ras, which are known to have altered structures when bound to either GDP or GTP, fall within the regions implicated in the interactions with GEFs (Schlichting *et al.*, 1990, Boriack-Sjodin *et al.*, 1998).

While guanyl nucleotide exchange is a crucial step in the GTPase cycle of small GTPases, the mechanism of exchange has only recently began to be understood. The elusive nature of the mechanism of GEF action has largely been due to the fact that, for different GTPases, GEFs appear divergent in primary sequence, tertiary structure, as well as subcellular localization. However, despite the molecular diversity a common mechanism for GDP/GTP exchange can be envisioned wherein GEFs activate their respective GTPases by stimulating the exchange of GTP for GDP in a multi-step mechanism (Mistou *et al.*, 1992). This involves the formation of binary and ternary complexes between the GTPase (i.e. Ras), guanine nucleotide and the exchange factor (Mistou *et al.*, 1992).

According to a model proposed by Mistou *et al.* (1992), GEFs bind to the GDP bound form of the GTPase and cause a transient disruption of Mg^{2+} coordination in the nucleotide-binding pocket (Figure 1-6). By interacting extensively with Ras, GEFs displace the residues that coordinate the Mg^{2+} ion and the phosphate groups of the nucleotide, partly occluding the Mg^{2+}-binding site (Lai *et al.*, 1993). Mutational experiments on Ras led to the proposal that GEFs interact with the switch-II region of Ras thereby disrupting the interactions with the nucleotide and the Mg^{2+} ion (Muegge *et*

al., 1996). This results in the stabilizing of a nucleotide-free (and cation-free) conformation and triggers the dissociation of GDP (Orita *et al.*, 1993; Quilliam *et al.*, 1995). Since the GTP concentration in cells is higher than that of GDP (>30 fold), when GEFs yield this GEF-apo-Ras reaction intermediate, GTP then binds to nucleotide-free Ras and the formation of the active GTP-bound GTPase is favored (Quilliam *et al.*, 1995; Jacquet *et al.*, 1995). Hence, GEFs increase the dissociation rate of guanyl nucleotides, permitting GTP, which is more abundant in the cell, to bind Ras, thereby accelerating guanyl nucleotide exchange. Although the GEF-Ras complex is stable in the absence of guanine nucleotide, it is quite short lived *in vitro* because GTP immediately enters the empty guanine nucleotide-binding site (Mistou *et al.*, 1992). After guanine nucleotide exchange, the binding of GTP to Ras prompts the dissociation of the exchange factor from the transient GEF-Ras-GTP complex and Mg^{2+} coordination is restored to complete the conformational switch to the active GTP-bound state (Mistou *et al.*, 1992). Both steps are reversible, with either GDP or GTP as the ligand. This mechanism, in which the GTPase passes through a transient GEF-bound apo state has also been documented biochemically for other G proteins such as EF-Tu and G protein α chains, and is inferred from genetic studies with yeast Ras proteins and EF-Tu (Sprang, 1997). It is also consistent with the proposed mechanisms of CDC25 (*S. cerevisiae*) and SDC25 (*S. cerevisiae*), the exchange factors of the Ras homolog RAS2 in yeast *S. cerevisiae* (Powers *et al.*, 1989).

Recently, the three dimensional structure of Ras and Sos was determined allowing for a better understanding of the exchange mechanism (Boriack-Sjodin *et al.*, 1998). The crystals were obtained with a truncated form of Sos consisting of two distinct α-helical structural domains corresponding, respectively, to the catalytic core and the N-terminal sequence. Residues 62 to 75 in the switch II region of H-Ras were found to interact with Sos, as were residues 103 and 105 in the α-helix 3-loop 7 region (Crechet *et al.*, 1996; Leonardsen *et al.*, 1996; Mosteller *et al.*, 1994; Quilliam *et al.*, 1994; Segal *et al.*, 1993, 1995; Verroti *et al.*, 1992). The effector loop (switch I region) of Ras was also implicated in direct interactions with GEFs (Boriack-Sjodin, 1998; Leonardsen *et al.*, 1996; Mistou *et al.*, 1992; White *et al.*, 1995). The switch I, II and α3-L7 regions of H Ras are found adjacent to each other on the surface of the molecule (Krengel *et al.*, 1990). The recently described crystal structure of H-Ras complexed with Sos

demonstrates that each of these three regions is indeed at the interface of the Ras-Sos complex (Boriack-Sjodin *et al.*, 1998). In addition, Boriack-Sjodin *et al.* (1998) provided evidence which indicates that Sos disrupts the binding site of Ras for the phosphate moiety of the nucleoside and the associated Mg^{2+} ion. Most notably was the major conformational change that Ras undergoes in the switch I region when binding to Sos. Although all GEFs of the CDC25 family are likely to share the same general features of the mechanism of nucleotide release, sequence similarity is low outside the catalytic domain, which means that detailed comparisons of their interactions and regulation modes might provide additional insight into Ras activation.

1.6.2 GTPASE ACTIVATING PROTEINS -NEGATIVE REGULATORS OF RAS

Stimulation of the intrinsic GTPase activity of GTP-binding proteins by GAPs is a basic principle of GTP-binding protein downregulation. Numerous vital processes including protein synthesis, growth control and differentiation are controlled enzymatically by the conversion of GTP to GDP and inorganic phosphate (P_i)(Bourne *et al.*, 1990). GTP hydrolysis by GTP-binding proteins is intrinsically very slow but can be accelerated by orders of magnitude upon interaction with GAPs, which are specific for their respective GTP-binding proteins (Gideon *et al.*, 1992; Boguski et al, 1993). The GTPase reaction of Ras is of particular importance because 25-30% of human tumors contain defective versions of Ras which have point mutations in either of two positions, 12 or 61. These oncogenic Ras mutations alter amino acids important for guanine nucleotide coordination and result in reduced GTPase activity (Barbacid *et al.*, 1987), reduced sensitivity to RasGAPs and/or enhanced nucleotide exchange (Lowy and Willumsen, 1993). Consequently, such mutations contribute to uncontrolled cell growth and proliferation (Lowy and Willumsen, 1993). The importance of the GTPase reaction is evident from the diseases associated with mutations in either Ras proteins themselves or GAPs. For example, certain Ras mutants are oncoproteins (Lowy and Willumsen, 1993) and loss of functions due to mutations in the GAP neurofibromin (NF1) are responsible for the disease phenotype in type 1 neurofibramatosis patients (Guttman *et al.*, 1992, 1993).

GAPs act to accelerate the intrinsic GTPase activity of Ras by up to 10^5-fold (Gideon *et al.*, 1992). This activity of GAPs has been mapped to a catalytic domain in the protein (Marshall *et al.*, 1989). All GAPs typically have this common structurally conserved region called the GAP related domain (GRD). The GRD is the catalytic unit of the protein which is responsible for stimulating the weak intrinsic GTPase activity of Ras proteins (Gibbs and Marshall, 1989; Wang *et al.*, 1991) and promoting the formation of the inactive GDP-bound state of Ras. In addition to their role as Ras regulators, GAPs have been speculated to play an effector role in Ras signaling (Pronk and Bos, 1994). Such speculation has led to the investigation of the primary structures of the mammalian RasGAPs and has revealed these proteins to contain multiple structural domains, which distinguish them from one another and which could be involved in their differential regulation or activity.

The GAPs represented the first cellular proteins that negatively regulated Ras activity (Trahey and McCormick, 1987). At present, several RasGAPs have been cloned, these include p120GAP, GAP1m, GAP1IP4BP, GAPIII, p98GAP, p135 SynGAP and neurofibromin (NF1) (Vogel *et al.*, 1988; Maekawa *et al.*, 1994; Cullen *et al.*, 1995; Baba *et al.*, 1995; Yamamoto *et al.*, 1995; Marchuk *et al.*, 1991; Chen *et al.*, 1998). These GAPs can be divided into four distinct groups: p120GAP, SynGAP, GAP1 and NF1 (Figure 1-5). GAPs have been identified in mammalian cells as well as in lower eukaryotes. For example, yeast *S. cerevisiae* possess two RasGAPs, termed *IRA1* and *IRA2*, which specifically act on Ras (Tanaka *et al.*, 1989,1990). Disruption of either *IRA1* or *IRA2* genes leads to a phenotype which resembles that seen in cells containing constitutively active Ras. In particular, *ira1*$^-$ or *ira2*$^-$ strains are highly sensitive to heat shock and cannot survive after heat shock treatment (Tanaka *et al.*, 1989; 1990). However, when RasGAP molecules from mammalian cells are introduced and ectopically expressed in these *ira*$^-$ cells, heat shock resistance is acquired (Xu *et al.*, 1990; Wang *et al.*, 1991; Martin *et al.*, 1990). Hence, mammalian GAPs can replace both of these genes (Ballester *et al.*, 1989; Tanaka *et al.*, 1989). However, human Ras proteins are not substrates for the IRA proteins (Adari *et al.*, 1988; Tanaka *et al.*, 1991). Altogether, this demonstrated that RasGAPs are structurally as well as functionally conserved throughout eukaryotic evolution. Accordingly, RasGAPs have been identified in *D. melanogaster* (Gaul *et al.*, 1992) and in *C. elegans* (Hajnal *et al.*, 1997; Hayashizaki *et al.*, 1998).

1.6.2.1 The Mammalian Families of RasGAPs

p120GAP

p120 GAP was the first GAP to be identified, purified and characterized (Trahey and McCormick, 1987; Trahey *et al.*, 1988; Vogel *et al.*, 1988). p120 exists in two forms: a 120-kDa form (Trahey *et al.*, 1988; Vogel *et al.*, 1988) and an alternately spliced 100-kDa form (Halenbeck *et al.*, 1990: Trahey *et al.*, 1988). While p120 is ubiquitously expressed in mammalian tissues, p100 has only been identified in placenta (Halenbeck *et al.*, 1990: Trahey *et al.*, 1988). p100 lacks the N-terminal hydrophobic portion of the full-length protein.

p120RasGAP is a widely expressed modular protein composed of a C-terminal GAP domain that specifically recognizes GTP-bound Ras, a central PH domain and two N-terminal SH2 domains that flank a single SH3 domain (Figure 1-5; Pawson, 1995). The presence of SH2 domains suggests that p120GAP regulates Ras proteins downstream of protein tyrosine kinases. RasGAP is indeed phosphorylated by a variety of activated PTKs, and associates through its SH2 domains with autophosphorylated receptors and two intracellular phospho-tyrosine containing proteins, p62 and p190 (Carpino *et al.*, 1997; Molloy *et al.*, 1989; Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauzkas *et al.*, 1990; Moran *et al.*, 1990). One of these proteins, p190 RhoGAP, contains a GAP specific for Rho-related GTPases, which direct the formation of actin stress fibres and focal adhesion (Settleman *et al.*, 1992; Ridley *et al.*, 1992). The SH2 domains of p120GAP also interact with other phosphotyrosine proteins including p62, and its SH3 domain is believed to play an effector role in Ras-induced processes (Ellis *et al.*, 1990; Duchesne *et al.*, 1993; Carpino *et al.*, 1997). The ability of p120GAP to bind several proteins through its various domains suggests that it might be involved in several biological processes (Marshall, 1991). p120 GAP also contains a C2 domain, known to interact with negatively charged phospholipids and believed to be important for the plasma membrane association of the protein in response to intracellular Ca^{2+} elevation (Clark *et al.*, 1991; Gawler *et al.*, 1995).

Neurofibromin (NF1)

A second GAP to be identified was the NF1 Tumor suppressor gene. NF1 encodes a 280-kDa-gene product called, neurofibromin (Marchuk *et al.*, 1991). Within the predicted protein sequence of NF1 is the highly conserved catalytic domain of GAP, the GRD. NF1 has even more extensive sequence homology with the *S. cerevisiae* IRA proteins than with mammalian GAP (Figure 1-5).

NF1 is inactivated in neurofibromatosis type 1 disease and is responsible for von Recklinghausen's neurofibromatosis. Neurofibramatosis type 1, or von Recklinghausen neurofibramatosis is one of the most common genetic disorders predisposing to cancer. The identification of the gene responsible for NF1 disease, and the subsequent realization that the encoded protein functions as a Ras-specific GAP, has implicated abnormal regulation of Ras in the pathogenesis of NF1 disease (Declue *et al.*, 1992). An important clue to the function of the NF1 gene was obtained when the encoded protein, neurofibromin, was found to contain a segment related to the catalytic segment of mammalian p120GAP (Xu *et al.*, 1990; Buchberg *et al.*, 1990). Additionally, it was found to share sequence similarity to the IRA1 and IRA2 negative regulators of the Ras/cAMP pathway in *S. cerevisiae* (Buchberg *et al.*, 1990). The attractive suggestion that neurofibromin might be a second mammalian RasGAP, indicated that some tumor suppressors might be regulators of dominant oncogenes. Further analysis of the encoded protein confirmed NF1 as a RasGAP. Three lines of evidence supported this contention. First, baculovirus or *Escherichia coli*-produced proteins representing the GAP-related domain of neurofibromin (NF1-GRD), stimulated the GTPase of wild-type N-Ras, but was inactive towards oncogenic (RasG12D and RasG12V) Ras mutants (Xu *et al.*, 1990; Martin *et al.*, 1990). Second, the NF1-GRD rescued the heat shock sensitive phenotype of yeast *ira*$^-$ strains (Martin *et al.*, 1990; Xu *et al.*, 1990; Ballester *et al.*, 1990). Lastly, the finding that neurofibromin deficient malignant neurofibrosarcoma lines contain 30-50% of Ras in the GTP-bound form, as opposed to less than 10% in most normal cells, was also consistent with a role for neurofibromin as a RasGAP (Basu *et al.*, 1992; De clue *et al.*, 1992).

The discovery of the second mammalian RasGAP was of particular interest also because then the only known protein to interact with the effector domain of Ras was p120GAP (Adari *et al.*, 1988). Since Ras can transmit both mitogenic and

differentiation-inducing signals (Noda *et al.*, 1985), depending on the context in which the signal is generated, and because NF1 was shown to interact with the effector domain of Ras (Martin *et al.*, 1990; De Clue *et al.*, 1991; Marshall *et al.*, 1993), it was proposed that p120GAP and NF1 might play a role as effectors of different Ras responses (Bollag *et al.*, 1991).

p135 SynGAP

Another RasGAP, p135SynGAP, was recently cloned and analysis of its primary amino acid sequence revealed the presence of several structural domains including a C2 domain (Figure 1-5; Chen *et al.*, 1998). SynGAP is predominantly found in the brain where it is exclusively localized in synapses in hippocampal neurons in a protein complex with NMDA receptors. p135 is phosphorylated in response to Ca^{2+} elevation by CaM kinase II (Chen *et al.*, 1998). Phosphorylation of SynGAP reduces the its ability to stimulate Ras GTPase activity. Due to its distribution and co-complexing with NMDA receptors, it has been suggested that this GAP (and therefore also Ras) may play a role in synaptic plasticity in the brain.

GAP1

The initial member of the GAP1 family, GAP1, was identified in *Drosophila* and characterized as a negative regulator of the *Sevenless* signal-transduction cascade (Gaul *et al.*, 1992). The recognized members of this family now include proteins which are in essence identical to GAP1, namely murine GAPIII a bovine brain R-Ras GAP (Baba *et al.*, 1995), GAP1IP4BP, p98GAP and mammalian GAP1m (Gaul *et al.*, 1992; Maekawa *et al.*, 1993, 1994; Cullen *et al.*, 1995). These GAPs are structurally similar proteins and constitute a family of functionally related proteins. GAP1m proteins are predominantly expressed in the brain, but GAPIII is also found in the spleen and lung, while GAP1m is enriched in the placenta and kidney (Maekawa *et al.*, 1994)

Structurally, members of the Gap1m family contain three functional domains, an N-terminal calcium-dependent phospholipid-binding domain, GRD, and a C-terminal extended PH/Btk domain (Figure 1-5). All members of this family possess the highly conserved RasGAP domain which contains the RasGAP consensus sequence and a number of invariant residues that are crucial for RasGAP activity (Scheffzek *et al.*, 1997).

The N-terminal calcium-dependent phospholipid-binding domain is comprised of two non-identical C2 domains denoted C2A and C2B (Nalefski *et al.*, 1996). By analogy with synaptogamins, the C2A and C2B proteins may be responsible for Ca^{2+}-dependent and Ca^{2+}-independent phospholipid binding respectively. In support of this, the N-terminal C2A domain has been shown to bind phospholipids in a Ca^{2+}-dependent manner (Fukuda and Mikoshiba, 1996; Fukuda *et al.*, 1997) while the role of the C2B domain remains unclear.

The PH/Btk domain of GAPIII has high homology with cytoplasmic tyrosine kinases such as T-cell-specific tyrosine kinase (itk) (Siliciano *et al.*, 1992) and Bruton's tyrosine kinase (Btk) (Rawlings *et al.*, 1993), mutations of which are responsible for human X-linked aggammabulinemia (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). The PH/Btk domain consists of two subdomains, PH domain and Btk motif. The PH domain is approximately 100 residues in size and was described earlier in p120GAP, and Sos (Musacchio *et al.*, 1993). This domain is extended in Btk kinase family proteins by a region called Tec homology (TH) domain, which consist of a conserved 27 amino acid stretch designated Btk motif and a proline-rich region (Vihinene *et al.*, 1994). Although the physiological relevance of any of these domains for Gap1 *in vivo* function has not been established, recent data suggests that under basal conditions GAP1 is localized to the membrane through its extended PH domain in a less active form; upon elevation of Ca^{2+} and IP4 concentrations, the PH domain binds to IP4 thereby stimulating catalytic activity, while at the same time, the C2A domain takes over the role of membrane tether (Chapman *et al.*, 1994; Rawlings *et al.*, 1993). This model is supported by the observation that Gap1 appears to be constitutively associated with the plasma membrane.

IQGAP

IQGAP was isolated as a cDNA clone which displayed extensive homology to the GRD sequence of RasGAPs (Weissbach *et al.*, 1994). IQGAP is most closely related to the *S. pombe* RasGAP-like protein Sar1. The deduced 1657 amino-acid protein product harbors 4 tandem IQ motifs, implicated in the binding of calmodulin (Figure 1-5; Cheney *et al.*, 1992). These observations suggested that IQGAP1 represented a novel member of the RasGAP family and that it might integrate Ras and Ca^{2+}/ Calmodulin mediated processes. p190 IQGAP1 was found to be expressed at high levels in placenta, lung and

kidney. Lower levels of mRNA expression were detected in heart, liver, skeletal muscle and pancreas and no mRNA was detected in the brain (Weissbach *et al.*, 1994). The restricted expression of IQGAP1 suggested that it might function in Ras pathways that are specific to these particular cell types (Wiesbach *et al.*, 1994). IQGAP2, a liver-specific GAP, has recently been discovered (Brill *et al.*, 1996) and found to display 61% homology with IQGAP2.

1.6.2.2 Mechanism of GAP Function

The domain structures of mammalian RasGAPs vary, presumably determining the functional specificity of each RasGAP. GAP1m bear the C2 domain and PH/Btk domain. p120GAP has a PH domain, a C2 domain, two SH2 domains and a SH3 domain. In contrast to p120GAP, GAP1 members do not possess SH2 or SH3 domains. NF1, which appears to be most closely related to the yeast RasGAPs IRA1 and IRA2, does not contain SH2, SH3, or PH domains. Hence, the mechanism and/or regulation of the Ras signal transduction pathway appears to be different for different RasGAPs.

Two models have been proposed as to the mechanism of GAP function (Hall *et al.*, 1990; Boguski and McCormick, 1993). The first model predicts that p120GAP serves as an effector of Ras. Several lines of investigation led to the early proposals that p120GAP might not only inactivate Ras, but also couple it to downstream effectors (McCormick, 1989; Hall *et al.*, 1990; Cales *et al.*, 1988). First, p120GAP binds Ras via its effector domain (Adari *et al.*, 1988). Second, p120GAP binds oncogenic Ras, but fails to stimulate its GTPase activity (Vogel *et al.*, 1988). Third, effector mutations that prevent signaling also blocked p120GAP binding (Trahey *et al.*, 1987; Cales *et al.*, 1988). The discovery that GAP only binds to the GTP form of Ras (Vogel *et al.*, 1988) and that mutant forms of Ras which lack effector function fail to interact properly with GAP, led to the hypothesis that GAP may be a target of Ras action (Adari *et al.*, 1988; Cales *et al.*, 1988). Subsequent reports by Yatani *et al.* (1990) that GAP and Ras cooperate in the inhibition of coupling between muscarinic receptors and strial potassium channels provided additional evidence in support of this hypothesis. Recent evidence from studies in *Xenopus* oocytes, argues further in favor of this model and suggests that p120GAP influences downstream signaling of Ras (Duchesne *et al.*, 1993; Pomerance *et al.*, 1996). In this model, Ras-GTP binds to and forms a complex with the cytosolic protein

p120GAP. This association is believed to generate a mitogenic signal (Hall *et al.*, 1990). The duration of the association of this complex as well as the signal generated is limited by the ability of GAP to stimulate GTP hydrolysis. GTP hydrolysis and the subsequent release of p120GAP from Ras-GDP, terminate downstream signaling.

Even before other downstream molecules emerged as possible targets of Ras, suggestions that GAPs were the primary Ras targets had already been made unlikely by findings that p120GAP could suppress NIH3T3 cell transformation by over-expressed wild-type Ras (Zhang *et al.*, 1990). Experiments with effector mutants that differentiate between GAP-binding and other effector molecules binding to Ras, also argued against models in which GAPs played major effector roles (Stone *et al.*, 1993). Perhaps the best evidence that p120GAP may have additional function rests in the identification of several motifs within the non-catalytic segment of p120GAP that are widely shared amongst signaling proteins. These motifs include: a calcium/phospholipid-binding (CALB) domain, implicated in Ca^{2+} inducible membrane translocation (Clark *et al.*, 1991), a PH domain, which is also present in RasGEFs and appears to mediate binding to G-protein βγ-dimers and phosphoinositol-4-5-bisphosphate (Touhara *et al.*, 1994; Harlan *et al.*, 1994), a single SH3 domain, which directs interaction with proline-rich sequences of other proteins, and 2 SH2 motifs, responsible for association with activated PTKs or other phosphotyrosine-containing proteins. Studies of these domains have provided indirect evidence for an "effector-like" function of this non-catalytic segment. For instance, microinjection of an antibody against the SH3 domain of p120GAP blocked Ras-mediated germinal vesicle breakdown in *Xenopus* oocytes, and SH3 domain peptides had the same effect (Duchesne *et al.*, 1993). In the same way, the non-catalytic segment of p120GAP inhibited coupling of muscarinic acetylcholine receptors to heterotrimeric G proteins that regulate K^+ channels in atrial membranes (Martin *et al.*, 1992), and blocked fibroblast transformation by muscarinic m1 receptors (Xu *et al.*, 1994). While full-length p120GAP only blocked receptor coupling in the presence of activated Ras, it was proposed that Ras might induce a conformational change in p120GAP, making the N-terminal segment available for further interactions (Martin *et al.*, 1992). Additionally, the N-terminal region of p120GAP has been implicated in cooperating with Ras in the activation of the *fos* promoter (Medema *et al.*, 1992), and in the regulation of cytoskeletal structure and cell adhesion in growth factor stimulated cells (McGlade *et al.*, 1993).

These results are also related to the finding that the N-terminal segment of p120GAP binds to two phosphotryosine proteins called p62 and p190 (Settelmen *et al.*, 1992; Wong *et al.*, 1992, Ellis *et al.*, 1990). Since p190 is a RhoGAP, it was proposed that its association with p120GAP might serve to link Ras and Rho-mediated signaling (Settlemen *et al.*, 1992b). Because Rac and Rho-like proteins regulate membrane ruffling, focal adhesions, and actin stress fibers (Ridley *et al.*, 1992a, 1992b), signals form p120GAP to p190 were thought to be responsible for cytoskeletal changes in growth factor stimulated cells (Nobes *et al.*, 1994).

The second model proposes that the primary function of p120GAP is to down-regulate Ras activity. Reports by Zhang *et al.* (1990) provided the first direct evidence that GAP can inhibit Ras function in mammalian cells. In this model, Ras-GTP binds to p120GAP which in turn stimulates the intrinsic GTPase activity of Ras and promotes the formation of the inactive GDP-bound state of Ras. GAPs are, therefore, considered to be solely upstream regulators of Ras protein, capable of down-regulating Ras and keeping it inactivated in the GDP-bound form. Consequently, the period for which Ras is activated is determined by the net rate of Ras activation by GEFs and inactivation by GAPs (Lowy and Willumsen, 1993).

1.6.2.3 The Mechanism of GAP-Mediated GTPase Stimulation

The molecular mechanism underlying GAP mediated GTPase stimulation in Ras proteins remains debatable. One hypothesis (isomerization hypothesis) favors a model whereby Ras itself possesses all the features necessary for efficient GTP hydrolysis, but exists in a GTPase impaired conformation. In this model, GAPs lower the energy barrier, and act catalytically (by catalyzing a rate-limiting isomerization reaction) to drive the GTPase into an enzymatically competent conformation (Neal *et al.*, 1988; Moore *et al.*, 1993; Nixon *et al.*, 1995; Neal *et al.*, 1990; Rensland *et al.*, 1991). However, conflicting evidence for such an isomerization reaction catalyzed by GAP has been presented by experiments using fluorescently labeled GTP analogues designed to test such a model (Schlichting *et al.*, 1990). The second hypothesis (arginine finger hypothesis) postulates the active participation of GAPs in the process of GTP hydrolysis, most likely by supplying a catalytic residue to the active site for GTP hydrolysis on Ras and thereby stabilizing the transition state of the reaction (Rensland *et al.*, 1991; Mittal *et al.*, 1996;

Scheffzek *et al.*, 1997). This model is based on the structure of very efficient enzymes, such as adenylate kinase, where a number of positively charged residues are involved in catalysis (Muller *et al.*, 1992).

1.7 SIGNAL PATHWAYS LEADING TO RAS ACTIVATION

Association of ligands with several classes of cell surface receptors results in guanine-nucleotide exchange factor-mediated conversion of Ras from the inactive GDP-bound to the active GTP-bound state. Active Ras-GTP then interacts with a number of effector molecules to elicit downstream signaling. The best understood and characterized pathway for the activation of Ras is the receptor protein tyrosine kinase-mediated pathway.

1.7.1 PROTEIN TYROSINE KINASE SIGNALING PATHWAY

Extracellular ligands have been shown to trigger the activation of Ras in mammalian cells (Buday and Downward, 1993). Ligands such as EGF, PDGF and insulin are known to bind to and activate receptor tyrosine kinases (RTKs). Activation of RTKs results in the dimerization and the autophosphorylation of specific tyrosine residues near the C terminus (Ullrich and Schlessinger, 1990). Tyrosine phosphorylated receptors recruit proteins containing SH2 domains, such as Grb2 and Shc, as well as tyrosine kinases such as Src to the plasma membrane (Kypta *et al.*, 1990; Pelicci *et al.*, 1992; Gale *et al.*, 1993).

Two proteins mediate growth factor receptor-induced activation of Ras: Grb2 and Sos (Figure 1-7). Grb2 is a cytoplasmic protein that consists of two SH3 domains and one SH2 domain (Lowenstein *et al.*, 1992). The presence of these binding motifs allows Grb2 to serve as a cytoplasmic sensor that detects ligand-induced tyrosine-phosphorylation of the EGF receptor (Lowenstein *et al.*, 1992). Through the SH2 domain of GRB2, the phosphorylated EGF receptor binds to and recruits the cytosolic Grb2/Sos complex to the plasma membrane where Ras is localized (Buday and Downward, 1993; Rozakis-Adock *et al.*, 1993). Translocation of Sos to the plasma membrane allows it to interact with and activate Ras (Buday and Downward, 1993). Upon activation, Ras

undergoes a conformational change that allows it to associate with specific effectors that transmit signals (Jurnak *et al.*, 1990; Moodie *et al.*, 1995).

There are several mechanisms for the association of the Grb2/Sos complex with different growth factor receptors. One case is the EGF receptor, whereby the Grb2/SH2 domain binds directly to a receptor phosphotyrosine-containing motif. Other receptor and non-receptor tyrosine kinases, however, do not interact directly with Grb2, but employ various adaptors to bind to the Grb2/Sos complex. An example of such an adaptor is Shc. The SH2 domain of Grb2 binds to a phosphotyrosine-containing motif on Shc, and the binding of Shc to activated growth factor receptors through its SH2 domain results in receptor association of the Grb2/Sos complex. Another example of such an adaptor molecule is the SH2 containing protein tyrosine phosphatase (SHP-2) or Syp, a protein-tyrosine phosphatase which is associated with the PDGF receptor through its SH2 domain (Feng *et al.*, 1993). Tyrosine phosphorylation of Syp results in the recruitment of the Grb2/Sos complex to the receptor and subsequent activation of Ras. Other examples of SH2/SH3-containing adaptor molecules include Crk (Mayer *et al.*, 1988) and Nck (Lehmann *et al.*, 1990) which associate with Sos and other guanyl nucleotide exchange factors, allowing for the differential regulation of multiple pathways that lead to Ras activation.

As described above, several mechanisms exist for the positive regulation of Ras signaling by Sos. There are also mechanisms by which Sos may down-regulate Ras signaling. It has been postulated that phosphorylation of the C-terminal region of Sos by ERK constitutes a negative feedback loop that participates in the downregulation of Ras activation. Phosphorylation of Sos reduces its ability to interact with the growth factor receptor (Porfiri *et al.*, 1996).

Activation of Ras can also be mediated by non-receptor tyrosine kinases. In contrast to receptor tyrosine kinases, these intracellular proteins are associated with the inner leaflet of the plasma membrane by virtue of lipids covalently added post-translationally. The prototype of this group is *src*. Other members include oncogenes initially derived from transforming retroviruses- *abl, yes, fgr,* and *fes*. (Brown and Cooper, 1996).

The src subfamily of protein-tyrosine kinases consists of nine closely related proteins- Src, Yes, Fgr, Lck, Fyn, Lyn, Hck, Blk, and Yrk (Brown and Cooper, 1996).

These 60kDa proteins are highly homologous throughout their entire amino acid sequences. Post-translational processing such as myristoylation and palmitoylation, in addition to membrane association is conserved for all these proteins. It has been shown that such modification is required for both the membrane association and transforming activity, implicating the plasma membrane as the functional site of these protein kinases (Kypta *et al.*, 1990). In addition to the catalytic domain, the src subfamily of protein-tyrosine kinases also possesses two other domains that are critical to their function- the SH2 and SH3 domains.

In response to binding of extracellular ligands to cell surface receptors, the Src SH2 domain binds to the PDGF receptor which mediates the activation of src in response to mitogen stimulation (Kypta *et al.*, 1990). $pp60^{src}$ activation is due to the phosphorylation of a tyrosine residue at position 416 by a distinct protein kinase Csk (C-terminal Src kinase) and the dephosphorylation of a tyrosine residue at position 527 by some protein tyrosine phosphatase (Nada *et al.*, 1991; Brown and Cooper, 1996). Activation of src allows it to interact with other cellular proteins, through its SH2 and SH3 domain, and phosphorylate substrates some of which lead to the activation of Ras. Examples of src substrates include phospholipase Cγ, RasGAP, Syp, Shc and the regulatory subunit of PI-3 kinase (Brown and Cooper, 1996). Thus, activation of protein tyrosine kinases such as src provides another mechanism for modulating the Ras signaling cascade, although details of these mechanisms are still unclear.

1.7.2 G-PROTEIN COUPLED RECEPTOR SYSTEM AND RAS SIGNALING

In mammalian cells, hormones that bind to G-protein-coupled receptors can also stimulate Ras signaling to the extracellular-signal regulated kinase (ERK) (Crespo *et al.*, 1994; Mattingly *et al.*, 1994). However, much less is known about the mechanism(s) of activation of the ERK pathway by G-protein coupled receptors. Recent evidence has implicated Ras and Raf in the stimulation of ERK by GPCRs (Figure 1-7; Della Rocca *et al.*, 1997).

G-proteins are membrane-associated proteins that transduce signals from hormone or sensory receptors to various effector systems, such as adenylate cyclase (Hamm and Gilchrist, 1996). Receptor molecules and guanine nucleotides regulate the interaction

between G-proteins with effector enzymes. Activation of G-proteins is associated with GDP-release and GTP-binding, upon which G-proteins interact with specific effectors. Subsequent GTP hydrolysis results in the inactivation of G-proteins and the termination of their interaction with effectors molecules.

Gα proteins are 40 kDa and bind guanine nucleotides which allow them to transmit signals by interacting with target effectors such as adenylate cyclase or PLC-β. The β and γ subunits are 37 and 8 kDa respectively, and form a stable complex with the α subunit. In the resting state, the α subunit is tightly bound to GDP and complexed with the βγ subunits and a cell surface receptor. Ligand binding to a G protein coupled receptor causes an increase in the rate of dissociation of bound GDP and association of GTP with the α-subunit. The activated GTP-bound α-subunit dissociates from βγ-receptor complex and interacts with effectors such as adenylate cyclase or PLC- β (Clapham and Neer, 1993; 1997). These effector molecules mediate processes that result in cAMP synthesis or PIP_2 hydrolysis.

The metabolism of inositol lipids such as PIP_2 in the membrane as a result of G protein signaling yields two products that act as second messengers: inositol triphosphate and diacylglycerol (Berridge, 1993). IP_3 causes the immobilization of intracellular stores of calcium, which acts as a second messenger. Diacylglycerol activates a serine/threonine kinase, protein kinase C, by increasing its affinity for calcium ion (Kreutter *et al.*, 1985). Activation of PKC is associated with an apparent translocation of PKC to the membrane in a calcium-dependent manner (Wolf *et al.*, 1985). Once bound to the membrane, PKC, an enzyme that plays a critical role in the mitogenic response of fibroblasts, is believed to phosphorylate a number of proteins (Kolch *et al.*, 1993). Although the critical substrates of phosphorylation by PKC that result in cell proliferation have not been conclusively identified, they include growth factor receptors and nuclear proto-oncogene products (Jaken *et al.*, 1996). For example, PKC can phosphorylate and activate Raf-1 directly (Sozeri *et al.*, 1992; Kolch *et al.*, 1993).

It was originally thought that G_α was the sole activator of effectors (Clapham and Neer, 1993). However, recent evidence suggests that $G_{\beta\gamma}$ is an important independent activator of various effectors of G protein actions (Faure *et al.*, 1994; Crespo *et al.*, 1994; Della Rocca *et al.*, 1997). The discovery that mitogens that use GPCRs activate the ERK kinase through Ras-dependent and independent pathways stimulated investigation as to

which subunits, α or βγ regulate the Ras-Raf-MEK-ERK cascade. There is now evidence to suggest that both α-subunits and βγ dimers regulate the ERK cascade (Crespo *et al.*, 1994; Della Rocca *et al.*, 1997). In addition, the ability of βγ to activate ERK has been shown to be Ras-dependent (Van Biesen *et al.*, 1995; Faure *et al.*, 1994; Della Rocca *et al.*, 1997). Recent reports have also revealed that signaling from PI3-Kγ to ERK appears to require a tyrosine kinase, Shc, Grb2, Sos, Ras and Raf (Lopez-Ilasaca *et al.*, 1997). These findings indicate that PI3-Kγ mediates $G_{\beta\gamma}$-dependent activation of the Ras-ERK cascade. Several studies have also shown that βγ dimers may assemble a signaling complex at the membrane to activate Ras and other effector pathways by using PH domains to direct protein-protein interactions (Harlan *et al.*, 1994; Inglese *et al.*, 1995). PH domains mediate protein-protein interactions between cytoplasmic and plasma membrane proteins- in an analogous fashion to the better characterized SH2 and SH3 domains (Musacchio *et al.*, 1993). More specifically, PH domains including those found in RasGRF1 have been shown to bind to the βγ complex of trimeric G proteins *in vitro* (Touhara *et al.*, 1994). These observations imply that there are multiple mechanisms by which heterotrimeric G-proteins in association with RasGEFs can activate Ras. Furthermore, they suggest a common protein-protein interaction theme that localizes important signaling molecules to their appropriate membrane compartments.

Ras proteins are involved in a diverse system that transmits mitogenic signals from the cell surface to the nucleus. As described above, Ras proteins mediate growth responses to receptor tyrosine kinases and are also activated by heterotrimeric G protein-coupled receptors. Thus, it is evident that multiple pathways can be used by receptors to stimulate Ras. It is also becoming clear that the regulation of cell growth by Ras involves a complex network of interacting pathways. The work described in this thesis adds a new level of complexity to this signaling system and suggests the need for revision of some of these previously established ideas.

1.8 RAS EFFECTOR SIGNALING PATHWAYS

Ras is known to interact with various molecules to induce mitogenesis or differentiation. Ras proteins transmit intracellular signals when bound to GTP. Just as numerous upstream signal mechanisms can contribute to Ras activation, Ras-GTP can apparently regulate downstream effector pathways and modulate diverse biological processes.

Ras proteins mediate their actions through binding of effector proteins which depends on Ras being in the GTP-bound form. The region involved in the interactions of Ras with its effectors is the effector loop (residues 30-38) and the switch II region (residues 60-76). Adenylate cyclase, in budding yeast *Saccharomyces cerevisiae*, was the first discovered Ras effector (Gibbs *et al.*, 1989). Both yeast RAS and mammalian H-Ras were shown to activate *S. cerevisiae* adenylate cyclase directly but were not able to activate adenylate cyclase from *Schizosaccharomyces pombe*, *Dictylstelium* or *Drosophila*. This provided clues which indicated that Ras might activate various effectors in different organisms. This notion was confirmed by both genetic studies in *Drosophila* as well as biochemical studies in mammalian cells. Additionally, several studies indicated that Ras regulated multiple signaling pathways in the same organism. While a number of putative Ras effectors have been identified, the direct effect of Ras is not understood in most cases. Besides, although some of the biochemical activities of these putative effectors are generally known, little is known abut the role these molecules play in mediating Ras function.

1.8.1 RAF-DEPENDENT RAS FUNCTION

When Ras is activated it binds to and recruits specific effectors to the plasma membrane. The best-characterized Ras-activated mitogenic pathway is that mediated by the Raf family of serine/threonine protein kinases- A-Raf, B-Raf and Raf-1 (Figure 1-8; Avruch *et al.*, 1994; Rapp, 1991; Van Aelst *et al.*, 1993). Ras proteins are associated with the plasma membrane and are possibly localized with specific receptors in caveolae microdomains (Song *et al.*, 1996; Mineo *et al.*, 1996). In quiescent cells, Ras is in an inactive GDP-bound state. The accumulation of Ras-GTP complexes in response to

receptor activation by ligand recruits Raf to the plasma membrane (Vojtek *et al.*, 1993; Koide *et al.*, 1993; Warne *et al.*, 1993; Leevers *et al.*, 1994). Activated GTP-bound Ras binds cytoplasmic Raf-1 through two distinct amino-terminal regions of Raf: Ras interacting domain (RID/RBS1) residues 51-131 (Fabian *et al.*, 1994; Brtva *et al.*, 1995) and Raf-CRD (Brtva *et al.*, 1995). These observations that Ras interacts with two domains of Raf suggested that Ras might promote more than just membrane translocation of Raf and instead may also facilitate the subsequent events that lead to Raf-1 activation. Although the mechanism by which Raf becomes activated is still poorly understood, other components necessary for Raf activation include 14-3-3 proteins, phospholipids, and serine/threonine and tyrosine kinases (Morrison *et al.*, 1997). Hence, the connection between Ras and Raf alone requires multicomplex formation to complete Raf activation. Once activated, Raf causes subsequent stimulation of a cascade of protein kinases that include mitogen activated protein kinase kinase (MEK) or Erk kinase and mitogen activated protein kinase or extracellular-signal regulated kinase (ERK) (Crews *et al.*, 1992; Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). MEK is a dual specific protein kinase and activates ERK by phosphorylating threonine and tyrosine residues. Upon activation, ERK phosphorylates distinct cytosolic protein kinases including p90RSK and p70-S6Kinase (Sturgill *et al.*, 1988) or Erk translocates to the nucleus and thus directly contributes to the transduction of proliferative signals by activating transcriptional control elements such as c-Fos (Gonzalez *et al.*, 1991). ERK can also activate phospholipase A_2 and transcription factors such as Elk-1 that induce gene expression from Ras-responsive elements (Marais *et al.*, 1993; Lin *et al.*, 1993). Thus, activation of Ras leads to the triggering of signaling pathways such as Raf-MEK-ERK-pp90rsk that results in the regulation of genes involved in cell proliferation and differentiation.

1.8.2 RAF-INDEPENDENT RAS FUNCTION

Genetic studies in the budding yeast *Saccharomyces cerevisiae* and later the fission yeast *Schizosaccharomyces pombe*, provided the earliest observations that Ras is likely to act through additional proteins besides Raf. Budding yeast that lack functional Ras are not viable, but yeast lacking adenylate cyclase, an effector of Ras in this

organism, were often capable of forming slow growing microcolonies (Toda *et al.*, 1987). This finding indicated that in *S. cerevisiae*, Ras might have an essential function other than the activation of adenylate cyclase. Genetic studies in *S. pombe* identified at least two distinct downstream effector-mediated signaling pathways that facilitate full Ras function (Marcus *et al.*, 1995).

In *S. pombe*, Ras directly interacts with two effectors: Byr2, a MAPK kinase kinase (MEKK/Raf homolog) (Van Aelst *et al.*, 1993; Wang *et al.*, 1991), and Scd1, a GEF for the Rho family of protein Cdc42 (Chang *et al.*, 1994). Byr2 is a member of a kinase cascade that includes Byr1 (Mek) and Spk1 (ERK) which regulate agglutination, conjugation and sporulation. Scd1 regulates the pathway involving Cdc42 and Shk1 (PAK homolog) and controls cell morphology (Marcus *et al.*, 1995). In mammalian cells, several clues suggested that Ras might regulate multiple effector proteins. First, activated Raf induces only a subset of the events mediated by activated Ras. For instance, activated Ras activates three distinct MAPK cascades (ERK, JNK, p38), whereas Raf causes direct activation only of ERK (Minden *et al.*, 1995; Olson *et al.*, 1995). Second, activated Raf cannot promote all functions of Ras, such as the transformation of some epithelial cells (Oldham *et al.*, 1996). In cardiac myocytes, expression of oncogenic Ras induces both genetic and morphological changes (Thorburn *et al.*, 1994). Activated Raf-1 induces ERK activity and new gene expression but not the cytoskeletal reorganization associated with Ras function (Thorburn *et al.*, 1994). Third, studies with effector mutants, which discriminate between effectors, suggest that multiple effector-mediated pathways are important for establishing and maintaining the transformed state (White *et al.*, 1995; Khosravi-Far *et al.*, 1996).

The presence of several putative effectors of Ras suggests that this GTPase may be involved in other less characterized pathways and thereby play a role in regulating a variety of biological processes. It is not surprising to find that in addition to controlling Raf and MEK kinases, Ras may also directly regulate a number of other important proteins (Figure 1-8). Several candidate Ras effectors have been identified. These include p120RasGAP (Yatani *et al.*, 1990), GEFs for the small GTPase Ral (RalGDS, RGL, RLF/RGL2) (Feig *et al.*, 1996), AF6/Canoe (Van Aelst *et al.*, 1994; Kuriyama *et al.*, 1996), RIN1 (Han *et al.*, 1995) and phosphatidylinositol 3-kinase (PI3-K) (Rodriguez-Vicinana *et al.*, 1994). While these candidate effectors comprise a very

functionally diverse and structurally distinct group of proteins, they all display preferential affinity for Ras-GTP. They all interact with residues in Ras that correspond to the switch I, which contains the effector domain, and switch II. In particular, the Ras effector domain is essential for all putative effector interactions.

1.8.2.1 MEKK-dependent pathway

Another effector pathway involved in coupling Ras to nuclear events is that involving MEK kinase (MEKK), a serine/threonine kinase originally thought to function like Raf in activating MAPK (Lange-Carter *et al.*, 1993). There is a large body of evidence to suggest that Ras can indeed regulate MEKK1 and its downstream target JNK, a MAPK family member (Derijard *et al.*, 1994). The signaling pathways that lead to MAPK or JNK activation are biochemically and functionally distinct, although cross talk between these two pathways may exist (Minden and Karin, 1997).

Whereas Raf is crucial for ERK activation in response to Ras (Dent *et al.*, 1992; Kyriakis *et al.*, 1992), Raf does not play any direct role in the activation of the JNK pathway (Minden *et al.*, 1994). Instead, another MAPKKK, MEKK1, leads to the activation of JNK (Minden *et al.*, 1994; Yan *et al.*, 1994). MEKK1 is a 196-kDa protein kinase which activates the ERK and JNK pathways. MEKK1 is a mammalian homolog of Byr2, a Ras-regulated protein kinase which is activated in response to mating pheromones in *S. pombe*. MEKK1 functions upstream in a kinase pathway involving stress-activated protein kinases and can be stimulated in a Ras-dependent manner (Lange-Carter *et al.*, 1994). Evidence for its role as a Ras effector comes from observations that MEKK1 bound directly to GST-RasG12V in a GTP-dependent manner via its C-terminal kinase domain *in vitro* (Russell *et al.*, 1995). This binding was blocked by a Ras effector peptide. Whether MEKK1 is a true effector of Ras remains to be tested. However, since over-expression of MEKK1 causes apoptosis (Johnson *et al.*, 1996; Cardone *et al.*, 1997), it seems unlikely that it would be an important positive effector for Ras transforming activity. MEKK1 has also been shown to bind to GTP-complexed Cdc42 and Rac1 *in vitro*. In addition, kinase-dead MEKK1 can block Cdc42/Rac activation of JNK (Fanger *et al.*, 1997). Hence, MEKK1 may serve as an effector of Cdc42 and Rac as well. It is still not understood how these GTP binding proteins can activate the JNK pathway. However, it has been proposed that the Rac-responsive serine/threonine kinase PAK, or

one of its close relatives, acts between Rac and MEKK1 (Brown *et al.*, 1996; Martin *et al.*, 1995; Manser *et al.*, 1994, 1995; Teo *et al.*, 1995). Furthermore, MEKK1 displays nuclear localization and an association with vesicular-like structures in the cytoplasm (Fanger *et al.*, 1997). The relevance of this subcellular localization however, and the functional interaction with plasma membrane associated Ras protein is not clear.

The mechanism by which MEKK1 activity is regulated in response to Ras activation or other stimuli such as EGF or TNFα is not known, nor is it understood whether such stimuli actually stimulate MEKK1 activity. Interestingly, MEKK1 is constitutively active when isolated from non-stimulated cells (Xu *et al.*, 1996). In addition, expression of wild type MEKK1 is sufficient for JNK activation (Xu *et al.*, 1996). These findings suggest that MEKK by itself may be constitutively active. They also raise the possibility that extracellular stimuli such as growth factors or cytokines could stimulate events which cause the sequestration of a MEKK inhibitor in the cell, rather than triggering its activity directly (Xu *et al.*, 1996). While MEKK was initially identified as a kinase that phosphorylates MEK, it is known that MEKK1 is an inefficient activator of the ERK pathway (Lange-Carter *et al.*, 1993; Minden *et al.*, 1994). MEKK1 functions as a MAPKKK in the JNK pathway (Minden *et al.*, 1994). The effects of MEKK on JNK activity are mediated by the JNK activating kinase JNKK1, also known as SEK1 or MKK4 (Lin *et al.*, 1995; Sanchez *et al.*, 1994; Derijard *et al.*, 1995). JNKK was shown to be activated by MEKK1 through phosphorylation at serine and threonine residues (Lin *et al.*, 1995). Once activated, JNKK1 directly phosphorylates JNK on threonine and tyrosine residues, the conserved MAP kinase activation sites (Yan *et al.*, 1994; Lin *et al.*, 1995). Hence, JNKK functions as the MAPKK in the JNK pathway. In summary, the kinase cascade involving MEKK-JNKK-JNK is stimulated upon Ras activation and is responsible for Jun-mediated gene expression from the AP-1 binding sites (Derijard *et al.*, 1994).

The JNK/SAPKs were discovered as a cyclohexamide-activated proline directed kinase and as an activity that bound to and phosphorylated the N-terminal sites of c-Jun following exposure of cells to UV light (Kyriakis *et al.*, 1990; Hibi *et al.*, 1993). Subsequent cDNA cloning identified three genes which encode the 46- and 54-kDa isoforms of JNK/SAPK (Kyriakis *et al.*, 1994; Derijard *et al.*, 1994). The JNK family of MAP kinases is activated by a variety of different types of cellular stresses as well as

extracellular stimuli. For example, UV, antibiotics, cytokines and other environmental stresses and, to a lesser extent, growth factors such as EGF and NGF have been found to activate JNKs (Kyriakis *et al.*, 1996; Dong *et al.*, 1998). JNKs are involved in a wide variety of biological processes including cytokine biosynthesis, cell transformation, stress responses, and apoptosis (Kyriakis *et al.*, 1996; Dong *et al.*, 1998). Phenotypic analyses of JNK knock-out mice indicated that JNK1 and JNK2 are required for apoptosis in distinct regions of the brain, and JNK1 is also required for proliferation and differentiation of thymocytes (Dong *et al.*, 1998; Kuan *et al.*, 1999).

In addition to the JNK/SAPK, Ras also activates the p38/HOG MAPK cascades, both of which in turn activate Elk1, c-Jun and ATF2 nuclear transcription factors (Derijard *et al.*, 1994). These define parallel pathways that are stimulated independent of Raf-1 activation (Minden *et al.*, 1994; Olson *et al.*, 1995). While ERK activation is associated with growth stimulatory responses, JNK and p38 activation are associated with stress responses that result in apoptosis (Xia *et al.*, 1995; Verheij *et al.*, 1996; Chen *et al.*, 1996). MEKK1 activates both JNK and p38 and causes apoptosis (Johnson *et al.*, 1996). In various cell types such as PC12 cells and U937 leukemia cells, JNK activation is associated with apoptosis (Xia *et al.*, 1995; Verheij *et al.*, 1996; Chen *et al.*, 1996). Interestingly, inhibition of JNK activation impairs Ras transformation indicating a growth-promoting role for the JNK cascade (Clark *et al.*, 1997). Since Jun function is also required for Ras transformation (Granger-Schnarr *et al.*, 1992; Clark *et al.*, 1997), JNK activation may lead to different cellular consequences that depend on the coordinate activation of other pathways. As proposed by Xia *et al.* (1995), JNK activation by stress stimuli alone may cause apoptosis, whereas JNK activation under other conditions may synergize to enhance proliferative effects of activated MAPKs (Xia *et al.*, 1995). Alternatively, the consequences of JNK activation may be cell type specific and in fibroblasts serve a protective function and thereby contribute to cell transformation.

1.8.2.2 p120GAP-Dependent Pathway

As described earlier, activation of the Ras-ERK cascade, with its concomitant activation of transcription factors, is essential for cell proliferation. The Ras GTPase-activating protein, p120GAP, in addition to negatively regulating Ras function may impinge on the Rho family via its association with p190, a GAP for Rho family members

(Settleman *et al.*, 1992). Thus, p120GAP may serve as an effector that facilitates Ras regulation of Rho family protein function. Whether the association of a p120GAP-p190RhoGAP complex with activated Ras leads to up-regulation or down-regulation of Rho family protein function has not been established. However, activation of members of the Rho family of GTPases is likely to contribute significantly to the Ras-transformed phenotype (Zohn *et al.*, 1998).

1.8.2.3 RalGDS-Dependent Pathway

Ras also interacts with and stimulates the activity of at least three different isoforms of RalGDS (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994). RalGDS (Ral GDP-dissociation stimulator) is a guanine nucleotide exchange factor for the Ras-like GTPases RalA and RalB (Albright *et al.*, 1993; Spaargaren and Bischoff, 1994; Kikuchi *et al.*, 1994; Hofer *et al.*, 1994). RalGDS directly associates with the effector domain of Ras in a GTP-dependent manner (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994). In mammalian cells, Ras and RalGDS are also known to associate in response to EGF stimulation (Kikuchi *et al.*, 1996). By co-transfecting Ras in COS cells expressing RalGDS and RalA, Urano *et al.* (1996) observed increased levels of RalA-GTP (Urano *et al.*, 1996). These experiments showed that Ras can activate RalGDS in COS cells. Both R-Ras and Rap1A, which bind RalGDS *in vitro*, failed to stimulate RalGDS exchange activity demonstrating the specificity of RalGDS activation. This group also showed that expression of an activated Ral mutant, (Ral 72L), synergized with sub-optimal amounts of either RasG12V or v-Raf to transform NIH3T3 cells and that a dominant negative of Ral blocked transformation. Expression of Ral72L alone failed to activate ERK2 and did not enhance ERK2 activity when co-expressed with Ras. These findings indicate that activation of Ral via RalGDS constitutes a Ras-dependent signal transduction pathway which is distinct from, and cooperates with, the Ras-ERK pathway.

Recently, two proteins that are related to RalGDS and interact directly with Ras-GTP and not with an effector loop mutant were discovered. Rgl (RalGDS-like) and Rlf (RalGDS-like factor) were discovered in yeast two-hybrid screens (Kikuchi *et al.*, 1994; Wolthius *et al.*, 1996). These proteins share a conserved C-terminal Ras-binding domain as well as an amino-terminal domain and the three conserved regions in the catalytic domain of Cdc25 and mSos1. In Cos7 cells, active Ras interacts with and stimulates the

ability of RalGDS, Rgl and Rlf to activated epitope-tagged Ral, showing that Ras can indeed activate RalGEFs (Urano *et al.*, 1996; Murai *et al.*, 1997; Wolthius *et al.*, 1997).

1.8.2.4 PI3-K-Dependent Pathway

PI3-K is a lipid kinase activated by a number of growth factors and cytokines. PI3-K catalyzes the phosphorylation of the inositol 3' hydroxyl group of phosphatidyl inositol (4,5)-bisphosphate (PI[4,5]P_2) to give phosphatidyl inositol (3,4,5)-triphosphate (PI[3,4,5]P_3). An isoform of PI3-K, the activity of which is regulated by RTKs, is composed of a p85α regulatory subunit and a p110α catalytic subunit (Kapeller *et al.*, 1994). Ras binds directly, in a GTP-dependent manner, to the p110 α catalytic subunit of PI3-K both *in vitro* and *in vivo* (Rodriguez-Viciana *et al.*, 1994, 1997). This interaction required GTP and was not observed with a Ras effector loop mutant (Rodriguez-Viciana *et al.*, 1994). The expression of a dominant negative mutant of Ras (RasS17N) in COS cells inhibited PDGF- and NGF-induced increases in 3' phosphorylated phosphoinositides (Rodriguez-Viciana *et al.*, 1994, 1996). Additionally, the level of PIP_3 in these cells was greatly increased by co-expression of Ras and PI3-K. Furthermore, using an *in vitro* reconstitution system, Ras-GTP was shown to increase the lipid kinase activity of PI3-K directly (Kodaki *et al.*, 1994; Rodriguez-Viciania *et al.*, 1994, 1996). These results provided further evidence to support the finding that Ras can regulate PI3-K activity *in vivo*.

Ras interacts with and stimulates the activity of at least four different isoforms of the PI3-K catalytic p110 subunit. Activation of PI3-K by a variety of extracellular stimuli leads to the accumulation of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3). One target of PI3-K is the serine/threonine protein kinase Akt/Protein Kinase B (Marte *et al.*, 1997). Binding of Akt/PKB via its PH domain to PIP_3, localizes Akt/PKB to the plasma membrane and leads to a partial activation of its kinase activity (Hemmings *et al.*, 1997). Akt/PKB activity is further increased by phosphorylation on two residues by two different kinases, one of which, phosphoinositide-dependent kinase (PDK1), is itself a lipid regulated kinase (Stephens *et al.*, 1998). Activation of PKB/Akt has been strongly correlated with protection from apoptosis (Del Peso *et al.*, 1997; Datta *et al.*, 1997). The events downstream of Akt/PKB are the subject of intense research. Recently, Akt has been found to phosphorylate and

inactivate the pro-apoptotic protein BAD (Del Peso *et al.*, 1997; Datta *et al.*, 1997), the protein kinase Raf (Zimmermann and Moelling, 1999) and is believed to have other targets.

Activation of PI3-K, via direct interaction between Ras and the catalytic subunit of the protein, is necessary for actin cytoskeletal rearrangements associated with the transformed phenotype (Rodriguez-Viciana *et al.*, 1997). PI3-K has been shown to indirectly control the activity of the Ras-related Rho family protein Rac and the protein serine/threonine kinase p70S6K (Hawkins *et al.*, 1995; Ma *et al.*, 1998; Rodriguez-Viciana *et al.*, 1997; Reif *et al.*, 1997). These results indicate that PI3-K might provide a link between Ras and the Rho GTPases and that PI3-K functions upstream of Rac, possibly by generating 3' phosphorylated phosphoinositides which activate Rac GEFs (Hawkins *et al.*, 1995). Other targets of the products of PI3-K include the PH domains of Vav, Sos, RasGRF1 and PKC (Han *et al.*, 1998; Nimnual *et al.*, 1998; Klarlund *et al.*, 1997).

Interestingly, PI3-K has also been implicated as an upstream activator of Ras function. This is supported by several observations. First, phosphorylation of PDGFR at sites that are involved in p85 binding is required for the activation of Ras by PDGF (Fantl *et al.*, 1992). Second, an activated form of p110 caused a small elevation in the level of activated Ras-GTP *in vivo* (Hu *et al.*, 1995). The apparently contradictory roles of PI3-K may be explained by assuming that PI3-K isoforms may act as either an upstream activator or a downstream mediator of Ras function, depending on the specific extracellular stimulus or cell type.

1.8.2.5 Multiple Candidate Effectors of Ras

Other putative effectors of Ras include Rin (Han and Colicelli, 1995; Han *et al.*, 1997), KSR (Kornfeld *et al.*, 1995; Sundaram *et al.*, 1995; Therrien *et al.*, 1995) and AF6/Canoe (Kuriyama *et al.*, 1996; Matsuo *et al.*, 1997). Rin1 (Ras-interacting) was discovered originally in a screen for human cDNAs that were capable of suppressing the phenotype associated with constitutive activation of the Ras pathway in yeast *S. cerevisiae* (Colicelli *et al.*, 1991; Han and Colicelli, 1995). Rin1 was also shown to bind preferentially GTP-bound Ras at the effector region, and compete with Raf for Ras binding and localize to the plasma membrane (Han and Colicelli, 1995). Rin1 was

subsequently shown to interact with Abl and Bcr/Abl *in vitro* and *in vivo* through a domain distinct from the Ras binding domain (Han *et al.*, 1997; Afar *et al.*, 1997). Rin1 was shown to enhance the transforming activity of Bcr/Abl and rescue several transformation-defective mutants of Bcr/Abl (Afar *et al.*, 1997). The aspects of Ras function mediated by Rin1 are still not known, but it has been proposed that Rin1 coordinates signals from Ras and Abl (Afar *et al.*, 1997).

By using a related strategy, several groups screened for mutations that suppressed abnormal vulval and eye development caused by constitutively activated Ras in *C. elegans* and *Drosophila* respectively (Kornfeld *et al.*, 1995; Sundaram *et al.*, 1995; Therrien *et al.*, 1995). The screens revealed homologs of a novel gene named KSR-1 (kinase suppressor of ras-1). KSR-1 appears to be a novel member of the protein kinase superfamily with sequence motifs present in both serine/threonine and tyrosine protein kinases but it is most similar to the Raf family kinases with 30-40% sequence identity. Genetic studies indicate that KSR-1 lies downstream of *ras* but it is not known whether *KSR-1* functions upstream of *raf* or in a separate signaling pathway. In addition, two hybrid studies or bacterially expressed fusion proteins have not detected any interaction between Ras and KSR-1 (Therrien *et al.*, 1995).

AF-6 and Canoe are two structurally related proteins found recently to bind GTP-Ras (Kuriyama *et al.*, 1996). Genetic studies in *Drosophila* have linked *Canoe*, the Drosophila homolog of mammalian AF6, to eye development (Matsuo *et al.*, 1997). The function of these proteins is not known but they contain the GLGF motif shared among several proteins that associate with cellular junctions. This raises the possibility that AF6/Canoe coordinate signaling events at the plasma membrane to remodeling of the cytoskeleton. AF-6 is also found as the fusion partner in a number of translocations found commonly in acute lymphoblastic and myelocytic leukemias (Prasad *et al.*, 1993).

In addition to these well-defined pathways downstream of Ras, several other putative effectors of Ras have been identified. In some cases the evidence for Ras effector function of these proteins is limited to the observation of GTP-dependent interaction *in vitro*. For example, Ras has been found to interact with the Jun N-terminal kinase (JNK) and with Jun itself, both of which have been implicated in the regulation of apoptosis in some circumstances (Alder *et al.*, 1995). For some, the interaction with candidate effectors is observed *in vivo* upon co-immunoprecipitation, but these

experiments are often done under conditions in which the Ras target is overexpressed (Chen *et al.*, 1996). Additionally, Ras has been shown to interact with Bcl-2, a strong pro-survival factor, in cells over-expressing both proteins (Chen *et al.*, 1996). Ras is also known to interact with and activate the PKC ζ (Diaz-Meco *et al.*, 1994). PKCζ is an atypical PKC isoform that is insensitive to diacylglycerol and calcium. Several studies suggest that PKCζ can play a role in Ras-mediated signal transduction (Diaz-Meco *et al.*, 1994; Berra *et al.*, 1995). As with Raf-1, it has an amino-terminal regulatory domain which binds Ras-GTP and association with Ras can be stimulated *in vivo* by platelet derived growth factor (PDGF) (Diaz-Meco *et al.*, 1994). Whether or not PKCζ is activated directly by Ras is not known. However, there is evidence to suggest that PKCζ is involved in the protection of some cells from apoptosis (Diaz-Meco *et al.*, 1996). In addition, PKCζ has been reported to have mitogenic effects in Ras-dependent oocyte maturation (Dominguez *et al.*, 1992) and serum-stimulated mitogenic signaling in mammalian cells (Berra *et al.*, 1993). Regulatory domains of PKCζ associate with Ras-GTP, indicating that Ras-GTP localizes PKCζ to the plasma membrane, where it may be activated by PIP_3. Raf activation by PKC was not blocked by dominant inhibitory mutants of Ras, indicating that PKC activates Raf by a mechanism distinct from that initiated by the RTK pathway (Marais *et al.*, 1998). To date, Raf appears to be the only Ras target protein for which genetic studies confirm its fundamental role in Ras signaling in a normal context. Nevertheless, the interaction of Ras with these target proteins is likely to be important for mediating some function of Ras in cells.

1.9 THE BIOLOGY OF RAS PROTEINS

ras gene products are plasma membrane-associated guanyl nucleotide-binding proteins which play an important role in regulating intracellular signaling pathways that control normal and transformed cell growth. In various mammalian cells, Ras proteins regulate differentiation, cell cycle progression into S phase and immunological responses (Lowy and Willumsen, 1993). It has become apparent that small GTPases of this type control a wide variety of cellular processes, serving to regulate much of the diverse signaling linking extracellular signals to intracellular effector molecules (Lowy and Willumsen, 1993; Marshall *et al.*, 1995).

1.9.1 RAS AND DEVELOPMENT

Ras plays a crucial role in development by regulating diverse cellular processes in invertebrates and vertebrates. For example, Ras controls the specification of vulval or tail structures in the nematode *Caenorhabditis elegans*, the specification of neuronal and non-neuronal cell fates in *Drosophila* and the choice between proliferation and differentiation in PC12 cells (Marshall *et al.*, 1995; Wassarman *et al.*, 1995; Sundaram *et al.*, 1996).

Several previous studies have implicated Ras in development and suggest that different Ras homologues could preferentially mediate distinct cellular processes. K-Ras, but not H- or N-Ras plays an essential role in mouse development (Johnson *et al.*, 1997; Koera *et al.*, 1997; Umanoff *et al.*, 1995). K-Ras is induced during differentiation of pluripotent embryonal stem cells. The expression of K-Ras during early embryogenesis is limited temporally in a tissue-specific distribution (Pells *et al.*, 1997). Phenotypic analysis of K-Ras -/- mice shows defects in myocardial cell proliferation and neuronal-programmed cell death (Koera *et al.*, 1997; Johnson *et al.*, 1997). The erythroid cells that form these embryos are able to achieve end-stage differentiation within the hepatic microenvironment (Koera *et al.*, 1997; Johnson *et al.*, 1997). K-Ras has been shown to specifically interact with microtubules (Thissen *et al.*, 1997) and disrupt basolateral polarity in colon epithelial cells (Yan *et al.*, 1997).

In addition to its role as a negative regulator of Ras activity, NF1 regulates proliferation and survival of precursors and lineage-restricted myeloid progenitors in response to multiple cytokines by modulating Ras output (Zhang *et al.*, 1998). Loss of function of NF1 gene is found in some patients with Juvenile chronic myelogenous leukemia (JCML). Deficiency in NF1 also induces myeloproliferative disease through Ras-mediated hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Largaespada *et al.*, 1996). Similarly, NF -/- mouse embryos show aberrant growth of hematopoietic cells, suggesting that NF1 is required to downregulate Ras activation in myeloid cells exposed to GM-CSF, IL-3, or stem cell factor (Bollag *et al.*, 1996). Lastly, NF1 inactivation cooperates with N-Ras in lymphogenesis by a mechanism independent of its GTPase activity (Mangues *et al.*, 1998). Not only do these

observed cooperations highlight the importance of searching for additional functions of NF1, but they also implicate the importance of Ras regulators in development.

Another negative regulator of Ras, p120GAP, has also been knocked out in mice (Henkemeyer *et al.*, 1995). This mutation affects the ability of endothelial cells to organize into a highly vascularized network and results in extensive neuronal cell death. Interestingly, mutations in p120GAP and NF1 genes synergize such that embryos homozygous for mutations in both genes show an exacerbated GAP phenotype. Hence, it appears that both p120GAP and NF1 act to regulate Ras activity during development (Henkemeyer *et al.*, 1995).

One group has investigated the biological functions of the mammalian SH2/SH3 adaptor protein Grb2 by creating a Grb2 knock-out mouse (Cheng *et al.*, 1998). Analyses of mutant embryonic stem cells, embryos and chimeras revealed that Grb2 is required during embryogenesis for the differentiation of endodermal cells and formation of the epiblast. Further studies by this group provided genetic evidence for a mammalian Grb2-Sos-Ras signaling pathway that has multiple functions in embryogenesis. Altogether, these genetic studies provide compelling evidence for a role of Ras in development.

1.9.2 RAS IN CELL PROLIFERATION AND DIFFERENTIATION

Ras is involved in the regulation of cell proliferation and differentiation (Rapp *et al.*, 1994). The prevalence of Ras proteins in malignancies suggests a role in the growth of normal cells. While activated Ras leads to transformation of fibroblasts, dominant negative mutants inhibit their growth (Fieg and Cooper, 1988). Furthermore, the transformation of fibroblasts induced by microinjection of Ras is accompanied by membrane ruffling and fluid-phase pinocytosis (Bar-Sagi and Feramisco, 1986). These effects are reversed by the microinjection of specific antibodies to Ras. In addition to eliciting these mitogenic responses, Ras is required for the action of other mitogens. The mitogenic response of NIH3T3 cells to serum-derived growth factors was blocked by microinjection of the rat monoclonal antibody Y13-259 (Mulcahy *et al.*, 1985) or by the expression of dominant negative mutants (Cai *et al.*, 1990). Similarly, the Y13-259

antibody blocked the transforming potential of membrane-bound tyrosine kinases such as *src*, *fms* and *fes*, but not cytoplasmic kinases mos or Raf (Smith *et al.*, 1986).

In other cell types, microinjection of Ras induces differentiation. The best described case is the differentiation of PC 12 cells, in which Ras mimics the effects of NGF (Barbacid, 1987). Furthermore, these effects were blocked by dominant negative Ras mutants or anti-Ras monoclonal antibodies (Hagag *et al.*, 1986; Szeberenyi *et al.*, 1990). In neurons, neurite outgrowth is also induced by Ras (Borasio *et al.*, 1989). In a different cell system, the differentiation of F9 embryonal carcinoma cells into endoderm-like cells is induced by Ras, by a c-jun-dependent pathway (Yamaguchi-Iwai *et al.*, 1990). This response is typically elicited by retinoic acid and cyclic adenosine 3'-5'-monophosphate (cAMP).

The decisive event in cell fate and determination by Ras signaling is the fine-tuning of the strength and duration of signal. For example in PC 12 cells (pheochromocytoma), the strength and duration of Ras signaling determines whether these cells proliferated or differentiate. Treatment of PC12 cells with EGF causes a transient activation of Ras-Erk signaling and results in cell proliferation, while the addition of nerve growth factor (NGF) to these cells results in a sustained activation of the cascade and neuronal differentiation (Qui and Greene, 1992; Marshall, 1995). NGF treatment also results in the negative regulation of the cell cycle (Buchkovich and Ziff, 1994;Yan and Ziff, 1995). In addition, expression of constitutive active versions of Ras or Raf causes these cells to differentiate as observed in the case of NGF treatment (Troppmair *et al.*, 1992; Qui and Green, 1992; Wood *et al.*, 1993; Rapp *et al.*, 1994; Wixler *et al.*, 1996). Thus, in PC12 cells transient activation of Ras-Erk signaling induces cell proliferation, whereas sustained activation causes these cells to differentiate and gradually stop cycling (Qui and Greene, 1992; Buchkovich and Ziff, 1994).

1.9.3 RAS AND THE CELL CYCLE

By employing constitutively active and dominant negative mutants of Ras and Raf, it has been shown that the Ras-Raf-Erk cascade plays a central role in the regulation of cell proliferation by growth factors (Mulcahy *et al.*, 1985; Feig and Cooper, 1988; Kolch *et al.*, 1991; Bruder *et al.*, 1992; Dobrowolski *et al.*, 1994; Muszynski *et al.*, 1995;

Winston *et al.*, 1996; Aktas *et al.*, 1997; Kerkhoff and Rapp, 1997; Kerkhoff *et al.*, 1998). Growth factors stimulate the entry of arrested cells into the cell cycle. After passage through the G1 phase the cells replicate their DNA and subsequently divide. Growth factors are necessary for the progression of cells through G1 phase until they reach the restriction point where they become independent of growth factor signaling and start an autonomous program of DNA replication and cell division (Pardee *et al.*, 1989). The phosphorylation of the RB protein by cdk4/6 or cdk2 kinase activity is regarded as the central molecular event at the restriction point (Sherr *et al.*, 1996). Several events are required for the activation of Cdks: Cdks interact with a cyclin, Cdks are phosphorylated at a central threonine residue by CDK-activating kinase (CAK), and Cdks are dephosphorylated at inhibitory phosphorylation sites in their N-terminal region by cdc25 phosphatase (Sebastian *et al.*, 1993; Sherr and Roberts, 1995; Draetta *et al.*, 1997). Furthermore, the cdk inhibitor proteins, like p16 or p27 have to be released from the cyclin-Cdk complexes (Sherr and Roberts, 1995). Several cell cycle targets of Ras signaling include cyclin D1, c-Myc, p27Kip1 and cdc25a proteins, all of which have been described to mediate the proliferative response. In the case of the anti-proliferative response, p21cip1 Cdk inhibitor is the target of Ras signaling.

1.9.3.1 Cyclin D1 Over-Expression is Induced by Constitutive Ras-Erk Signaling

Growth factor stimulation of cells induces the expression of cyclin D1 as a delayed early gene (Matsushime *et al.*, 1991). Cyclin D1 is known to associate with the cdk4 and cdk6 kinases and is required for their activation (Sherr *et al.*, 1995). Cells expressing constitutively activated mutants of Ras or Raf display increased levels of cyclin D1 protein (Lavoie *et al.*, 1996; Kang *et al.*, 1996; Winston *et al.*, 1996; Filmus *et al.*, 1994; Albanese *et al.*, 1995; Arber *et al.*, 1996), a finding that led to the proposal that the Ras-Erk cascade regulates the expression of the cyclin D1 gene (Filums *et al.*, 1994; Liu *et al.*, 1995; Winston *et al.*, 1996; Kerkhoff and Rapp, 1997; Sewing *et al.*, 1997; Lloyd *et al.*, 1997). Cyclin D1 therefore provides an important link between Ras signaling and the cell cycle machinery.

In order for cells to divide, growth factor stimulation is required as well as attachment to extracellular matrix (Bottazzi *et al.*, 1997). Ras appears to provide either of these signals in that Ras-transformed cells can proliferate in the presence of growth

factors whilst unattached or in the absence or reduced levels of growth factors whilst attached. Recent findings reveal how Ras can overcome these cell-cycle controls, which is likely to be key to the role of Ras in tumorigenesis. For instance, adhesion signals and mitogens were found to synergize in their activation of the ERK pathway (Miyamoto *et al.*, 1996; Zhu *et al.*, 1995). Constitutive activation of the ERK pathway appears sufficient for anchorage and mitogen-independent growth of fibroblasts, as cells expressing activated MEK can form colonies in soft agar and proliferate in the absence of serum (Mansour *et al.*, 1994; Cowley *et al.*, 1994). Activated Ras appears to mimic this effect by constitutively activating the Erk pathway irrespective of whether the cells are adherent or not (Cowley *et al.*, 1994). Since constitutive activation of the Ras-ERK pathway also results in the induction of cyclin D1 expression, the connection between Ras and cyclin D appears to be important in mediating anchorage-independent growth of cells. Accordingly, cyclin D1 has been shown to be required for Ras-induced anchorage-independent growth (Liu *et al.*, 1995) and in some cell lines, but not all, expression of cyclin D1 is sufficient to confer anchorage independent growth (Zhu *et al.*, 1996; Resnitzky *et al.*, 1997). Additionally, Ras induction of cyclin D1 in attached cells also appears to be important for its mitogenic response. The expression of Ras results in a shortened G1, an effect that requires cyclin D1 and is mimicked by the exogenous expression of cyclin D1 (Liu *et al.*, 1995). This ability of activated Ras to induce cyclin D1 levels in the absence of anchorage or mitogens reflects what appears to be a physiological role of endogenous Ras in mediating growth factor and extracellular matrix signaling.

1.9.3.2 Repression of p27kip1 Expression by Ras-Erk Signaling

Ras also plays a crucial role in control of p27kip1 expression. p27kip1 belongs to a class of CDK inhibitors (Sherr and Roberts, 1995). p27kip1 interacts with cyclin/cdk complexes and thereby inhibits the catalytic activity of the complex. In fibroblast cells arrested by serum starvation, high levels of p27kip1 are detected (Nourse *et al.*, 1994; Hengst and Reed, 1996). In proliferating murine fibroblasts cells expressing an inducible constitutive active version of the c-Raf protein, p27Kip1 protein levels are reduced upon activation of the oncogenic Raf protein (Kerkhoff and Rapp, 1997). Furthermore, in rat embryo fibroblast cell line (REF52) infected with c-Myc- and Ras-expressing viruses

(Leone *et al.*, 1997), p27kip1 expression levels are completely down-regulated. Addition of serum to serum-starved cells drives these cells back into the cell cycle and results in the highly reduced expression of p27kip1 protein (Nourse *et al.*, 1994; Aktas *et al.*, 1997). The down-regulation of p27kip1 expression was shown to be blocked by the over-expression of RasN17, a dominant negative Ras mutant protein (Aktas *et al.*, 1997). One proposed explanation of how the Ras signaling pathway mediates p27kip1 protein is based on the observation that Erk is able to phosphorylate p27kip1 *in vitro* and that phosphorylated p27kip1 cannot bind to and inhibit the kinase activity of cdk2 immune complexes (Kawada *et al.*, 1997; Alessandrini *et al.*, 1997).

1.9.3.3 Robust Ras-Erk Signaling Induces p21cip1 Expression

Previous studies have reported that constitutive active versions of Ras or Raf are growth inhibitory for cells and that in order to proliferate in the presence of oncogenic Ras or Raf a second oncogene has to be activated or a tumor suppressor gene inactivated (Hirakawa and Ruley, 1988; Ridley *et al.*, 1988; Lloyd *et al.*, 1997). However, recent studies in NIH3T3 fibroblasts showed that the activation of an inducible oncogenic version of c-Raf1 is sufficient to drive cells arrested by serum starvation back into the cell cycle (Kerkhoff and Rapp, 1997). Interestingly, in the same cells, a similar Raf construct was shown to block cell proliferation when activated (Samuels and McMahon, 1994; Pumiglia and Decker, 1997; Woods *et al.*, 1997; Sewing *et al.*, 1997).

Transformation experiments in several epithelial cell lines as well as primary cells demonstrated that activation of the Ras-Erk signaling cascade is insufficient to transform cells (Oldham *et al.*, 1996). The molecular mechanisms involved in the resistance of primary cells to transformation by Ras have only recently been addressed. Two groups have shown that the activation of Ras signaling pathways in two different primary cells is actually growth inhibitory and that this is due to the induction of CDKIs (Lloyd *et al.*, 1997; Serrano *et al.*, 1997). In primary rat Schwann cells, it was previously reported that the introduction of activated Ras results in cell-cycle arrest (Ridley *et al.*, 1988). It has now been demonstrated that the activation of the Ras-Erk pathway in these cells leads to the induction, in a p53-dependent manner, of the universal CDKI p21Cip1 (Lloyd *et al.*, 1997). These cells are growth arrested in the G1 phase and display certain properties of transformation in that they develop refractile morphology and show increased motility.

In a separate study, Serrano *et al.* (1997) reported that primary fibroblasts of rodent and human origin also respond to activated Ras with a growth arrest. This inhibition of cell growth is associated with an induction of p21Cip1 and with an increase in the levels of the CDKI p16 INK4a, which specifically bind to and inhibit the kinases associated with cyclin D, the critical mediators of Rb inactivation.

As described above, the activation of an inducible oncogenic version of c-Raf-1 is sufficient for cell cycle progression in arrested fibroblasts, and activated Ras induces cell cycle arrest in primary cells. These differences in results were recently explained by the observation that the activation of constitutive low Raf signals leads to cell proliferation and that very high constitutive Raf signals lead to a cell cycle arrest (Sewing *et al.*, 1997; Woods *et al.*, 1997; Kerkhoff and Rapp, 1998). The molecular basis for this difference was found to be the strong induction of p21cip1 expression which was induced by robust signals. p21cip1 is a cdk inhibitor and is expressed at low levels in serum starved or density arrested fibroblasts (Sherr and Roberts, 1995; Macleod *et al.*, 1995; Kerkhoff and Rapp, 1997). The addition of serum results in increased levels of p21cip1 expression. When over-expressed, the p21cip1 protein inhibits the kinase activity of cyclin/cdk complexes and by this blocks the progression through the cell cycle (Xiong *et al.*, 1993; El-Deiry *et al.*, 1993; Di Leonardo *et al.*, 1994; Macleod *et al.*, 1995). Evidence indicating that the cell cycle arrest induced by high intensity Raf signals arises from the high p21cip1, is apparent in p21-deficient fibroblasts whereby these signals do not lead to a cell cycle arrest (Woods *et al.*, 1997). These experiments led to the conclusion that p21cip1 is a target of the Ras signaling pathway. Hence, Ras can also mediate anti-proliferative effects since Ras activation can induce p21cip expression and G1 arrest (Olson *et al.*, 1998). These findings demonstrate how quantitative differences in the activity of a signaling pathway can have opposing effects on the cell cycle and show a possible means whereby a cell can utilize the same pathway to elicit different biological outcomes.

1.9.3.4 Ras and Senescence

Senescence is a process cells enter towards the end of their proliferative lifespan. Interestingly, normal fibroblasts growth arrested by Ras exhibit a phenotype indistinguishable from senescence (Serrano *et al.*, 1997). The cells develop a distinctive

flat morphology, express specific markers and cease proliferation. This process is associated with the induction of the CDKI proteins p16 and p21 Cip1 and the loss of these proteins' activities results in either immortalized cells or an extended lifespan depending on the species and/or type of cell (Wynford-Thomas *et al.*, 1997). The limited lifespan of most cells appears to be a key restraint to tumor formation. Hence, if inappropriate Ras activity results in the premature activation of the senescent process or a cell-cycle arrest, this would provide a protective mechanism to counteract their danger, as growth-inhibited cells have less chance of accumulating additional deleterious mutations.

1.9.3.5 Ras-Erk Signaling Induces c-myc Expression

c-Myc expression is induced early after growth factor treatment of arrested cells (Muller *et al.*, 1984). The gene is expressed during the entire cell cycle (Waters *et al.*, 1991). c-Myc is a DNA-binding protein which is involved in transcriptional control of gene expression and has been shown to be essential for cell proliferation (Amati and Land, 1994). Over-expression of the c-Myc protein in arrested Rat1a cells results in the activation of cyclinE/cdk2 complexes and subsequent entry into DNA replication (Steiner *et al.*, 1995; Peres-Roger *et al.*, 1997). Also, co-expression of Ras and c-Myc induces cyclin-E-dependent kinase activity and transition to S phase (Leone *et al.*, 1997). In agreement, it has been shown that high Myc levels cooperate with Ras (Land *et al.*, 1983) and Raf (Rapp *et al.*, 1985, 1994; Cleveland *et al.*, 1994) in cell transformation. These observations led to the conclusion that the Ras signaling cascade may not regulate the *c-myc* gene. However, by using dominant negative and constitutive active mutants of the Ras-Erk pathway, it was recently shown that the *c-myc* gene is regulated by the Ras signaling cascade (Kerhoff and Rapp, 1998). In addition, expression of the N-terminal domain of Raf, which binds to Ras and inhibits its function, leads to a significant reduction of the induction of *c-myc* expression following serum stimulation of arrested NIH3T3 cells. Furthermore, the activation of a constitutive active mutant of Raf-1 was found to be sufficient to induce *c-myc* gene expression within 2-6 hours. This data provides evidence in support of an involvement of the Ras-Erk cascade in the process of c-myc activation. However, how the Ras-Erk signaling cascade leads to the induction of c-myc expression has not been fully described and is still subject to speculation.

1.9.3.6 Phosphorylation and Activation of cdc25A Phosphatase by c-Raf-1

The activity of cdks is not only regulated by the association with cyclins and cdk inhibitor proteins, but also by phosphorylation (Draetta *et al.*, 1997; Sebastian *et al.*, 1993). cdks must be dephosphorylated at key residues in order to become activated. In mammalian cells, the cdks are dephosphorylated by the cdc25 phosphatases, namely cdc25A, B, C (Jinno *et al.*, 1994). All three cdc25 phosphatases have been shown to exist in complexes with Raf-1 (Galaktionov *et al.*, 1995), an interaction that may be facilitated by the 14-3-3 proteins (Conklin *et al.*, 1995). For cdc25A, which plays a pivotal role in the early cell cycle (Jinno *et al.*, 1994), it has been shown that Raf-1 directly phosphorylates and activates the phosphatase (Galaktionov *et al.*, 1995).

1.9.4 RAS AND TRANSFORMATION

Ras genes were first identified by virtue of their ability to induce foci of transformed cells within a quiescent monolayer of immortalized fibroblasts. These cells were shown to exhibit many attributes of transformation in that they displayed a reduced growth factor requirement, a loss of contact inhibition, anchorage-independent growth and formed tumors when injected into nude mice/athymic mice (Barbacid, 1987). Further studies however, demonstrated that activated Ras was unable to form foci when introduced into non-immortalized primary fibroblasts but required the expression or loss of activity of a second cooperating gene (Weinberg, 1989). Such a requirement for more than one genetic defect to transform a primary cell was in accordance with the multistep nature of tumor development.

Ras mutations are found in many human tumors, and the frequency of these mutations is the highest among any genes in human cancers (Barbacid, 1987; Hunter 1997). Ras-transformed fibroblasts show typical anchorage-independent growth and morphological changes. These phenotypes are thought to be caused by Ras-induced gene expression and rearrangement of the cytoskeleton and cell adhesions. While Raf is the only effector protein for which genetic evidence is available regarding its activity as a candidate effector, Ras-induced transformation cannot be explained by the effect of Raf alone.

Several independent studies have provided convincing evidence to suggest the presence of several Ras signaling pathways which can account for the ability of Ras to transform cells. Direct evidence for the involvement of multiple signaling pathways downstream of Ras comes from the identification of effector domain mutants, which are defective in interaction, and activation of Raf (White *et al.*, 1995). These mutants, namely Ras (G12V, E37G) and Ras (G12V, Y40C), no longer activate Raf-1 or Erk, but retain their ability to bind to other putative Ras targets (Khosravi-Far *et al.*, 1996). Combining this initial finding with subsequent reports, the important results are as follows: Ras (G12,V, T35S) stimulates the Raf pathway but does not transform NIH3T3 cells; Ras (G12V, E37G) binds RalGDS but not Raf and cooperates with Ras (G12,V, T35S) in a focus forming assay; Ras (G12V, Y40C) binds PI3-K, stimulates acting rearrangement and cooperates with either of the above two alleles in a focus formation assay (White *et al.*, 1995; Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997). Additionally, in a DNA synthesis assay, cooperation between Ras (G12V, Y40C) and Ras (G12,V, T35S) has been observed (Joneson *et al.*, 1996). Although the non-Raf effectors that mediate the transforming actions of these two mutants have not been clearly described, an effector pathway leading to Rac1 may be important. This is supported by the following evidence: Ras (G12V, Y40C) caused a transformed morphology similar to Rho proteins, retained the ability to activate JNK (Khosravi-Far *et al.*, 1996), and induced membrane ruffling in REF52 cells which could be blocked by a dominant negative Rac1 (Joneson *et al.*, 1996).

Recent studies employing these Ras effector mutants have attempted to address the question of how Ras binding to Raf-1 mediates Ras transforming activity, and the role of other candidate Ras effectors in transformation (Marte and Downward, 1997; Rodriguez-Viciana *et al.*, 1997; Winkler *et al.*, 1997; Stang *et al.*, 1997). Although these effector mutants provided a useful means in identifying and characterizing Ras-mediated signal pathways, they remain incompletely characterized with respect to their ability to bind to and activate various Ras effectors relative to wild-type Ras or other activated forms of Ras. In addition, given differences in binding, cell type and cell-based assays employed in these studies, interpreting the resulting data should be exercised with caution.

1.9.4.1 Multiple Signaling Pathways Lead to Ras-Mediated Cell Transformation

The best-described Ras effector signaling pathway is the Ras-Raf-Mek-Erk protein kinase cascade. In this case activation of Ras leads to the recruitment of Raf to the plasma membrane where it is activated and subsequently triggers the Ras-Erk signaling cascade. Several observations support the idea that Raf plays an important role in Ras-induced transformation (Leevers and Marshall, 1992). However, although Raf plays a critical role in deregulating the mitogenic signaling in Ras-induced transformation, there is recent evidence which suggests that Raf alone is not sufficient to cause the cytoskeletal and morphological changes in Ras-induced transformation (White *et al.*, 1995; Joneson *et al.*, 1996; Rodriguez-Viciania *et al.*, 1997). An engineered form of Raf-1 which is targeted to the plasma membrane, Raf-CAAX, fails to induce cytoskeletal rearrangement in some fibroblasts and endothelial cells under conditions where activated Ras can induce membrane ruffling. Furthermore, the involvement of other effectors in Ras-induced transformation was indicated by the finding that Raf-binding defective mutants of Ras, which can interact with other effectors, possess some but not full transforming activities.

Another effector signaling pathway involved in Ras-induced transformation is mediated by PI3-K. PI3-K interacts with Ras-GTP and becomes activated (Kodaki *et al.*, 1994; Rodriguez-Viciania *et al.*, 1994, 1996). PI3-K plays a crucial role in the regulation of the actin cytoskeleton by growth factors such as PDGF and insulin (Kotani *et al.*, 1994; Wennstrom *et al.*, 1994; Nobes *et al.*, 1995). By employing various effector mutants of Ras, Rodriguez-Viciania *et al.* (1997) were able to demonstrate that the activation of PI3-K is necessary for actin cytoskeletal rearrangement in the Ras-induced transformation and that it is mediated by the activation of Rac. Since the activated form of PI3-K alone is insufficient to cause cellular transformation of fibroblasts, cooperative signaling pathways such as that mediated by Raf, appear to be necessary for the PI3-K-mediated pathway in Ras-induced transformation.

RalGDS has also been implicated as a key player in Ras-mediated transformation of cells. As described earlier, RalGDS as well as Rgl and Rlf, bind activated Ras in the yeast two-hybrid system. A role for Ral in regulation of phospholipase D and in actin cytoskeletal rearrangements via interactions with its putative effector RalBP1, has been suggested (Jiang *et al.*, 1995; Cantor *et al.*, 1995). Evidence that RalGDS serves as a

positive regulator of Ras-mediated transformation originates from several independent observations. First, co-expression of isolated Ras-binding domain of Rgl and Rlf inhibited Ras, but not Raf, transforming activity in NIH3T3 cells (Okazaki *et al.*, 1996; Peterson *et al.*, 1996). Second, while constitutively activated Ral alone does not cause transformed foci, one study shows that its co-expression enhanced Ras transformation, whereas dominant negative Ral impaired Ras focus-forming activity (Urano *et al.*, 1996). These effects were not dramatic however, and a separate study did not find a significant regulation of Ras transforming activity by RalA or RalB proteins (White *et al.*, 1996). Third, co-expression of RalGDS cooperated synergistically with activated Raf-1 to induce transformation of NIH3T3 cells (White *et al.*, 1996). It is still not clear whether the growth promoting activity of RalGDS was due to its activation of Ral proteins. However, it has been proposed that RalGDS activation of Ral may in turn lead to either activation of CDC42/Rac or phospholipase D (Jiang *et al.*, 1995). The discovery of a putative Ral effector RalBP1 as a novel GAP for CDC42 and Rac, suggests that RalBP1 might regulate these Ras-related GTPases (Cantor *et al.*, 1995). It is also possible, however, that RalGDS may have other functions distinct form its RalGEF activity.

1.9.5 RAS AND APOPTOSIS

In several cell types, especially T lymphocytes and fibroblasts, Ras signaling is known to induce apoptosis (Kauffman-Zeh *et al.*, 1997; Fukasawa *et al.*, 1997). Under conditions of stress, either loss of matrix adhesion (Nikiforov *et al.*, 1996; McGIll *et al.*, 1997), tamoxifen treatment (Vater *et al.*, 1996), tumor necrosis factor (TNF) treatment (Trent *et al.*, 1996), or over-expression of an activated Ras oncogene in fibroblasts, Ras promotes apoptosis. In some cases, v-ras promotes fibroblast apoptosis even in the absence of obvious stress (Fukasawa *et al.*, 1997).

Several groups have gathered evidence to suggest that loss of p53 relieves the induction of apoptosis in activated Ras-expressing fibroblasts (Fukasawa *et al.*, 1997; Vater *et al.*, 1996; Nikiforov *et al.*, 1996). Constitutively activated Ras and Raf are known to induce expression of the cyclin-dependent kinase inhibitors p16INK4A and p21 WAF1/Cip1 through mechanisms involving p53 (Lloyd *et al.*, 1997; Serrano *et al.*, 1997). The pro-apoptotic effect of Ras is believed to be associated with cell-cycle

regulation and is part of a protective response of cells against excessive activation of the Ras-ERK pathway. For example, strong activation of the Ras-ERK pathway in Swiss 3T3 cells results in the apoptosis of cells in S phase but the arrest of cells in other parts of the cell cycle (Fukasawa *et al.*, 1995). Although Ras mediated mitogen signaling is transient and weak, it is unlikely that these responses of the cell to strong constitutive action of the Ras-ERK pathway are relevant to normal Ras signaling. However, they may be important in protecting cells from the transforming effects of mutational activation of the *ras* proto-oncogenes, which could result in a much stronger and more prolonged activation of ERK.

The decision of cells to undergo apoptosis or growth arrest in response to apoptotic stimuli is believed to depend on the presence of p21WAF1/Cip1. For instance, whether cells arrest or apoptose in response to DNA damage has been shown to be dependent upon whether the p21 checkpoint is intact. This is supported by experiments which demonstrate that p21-/- cells dye rather than arrest in response to ultraviolet irradiation (Waldman *et al.*, 1997). Likewise, the decision to arrest or apoptose in response to oncogenic Ras may depend on which checkpoints are functional. Hence, the ability of Ras to control cell cycle check points suggests an indirect mechanism by which Ras may influence apoptosis.

In T cells, Ras is activated following the stimulation of the antigen receptor or IL-2 receptor. This can either lead to proliferation or to apoptosis depending on whether other stimuli are acting simultaneously (Latinis *et al.*, 1997; Gomez *et al.*, 1996, 1997). Over-expression of activated Ras in Jurkat cells leads to increased expression of the ligand for Fas (FasL), whereas dominant negative Ras inhibits FasL expression in response to antigen receptor stimulation (Latinis *et al.*, 1997). It has been suggested that Ras promotes apoptosis by inducing autocrine activation of the Fas pathway (Gulbins *et al.*, 1995). In PC12 cells, Ras activation is also linked to the induction of apoptosis whereby the cells are rescued from apoptosis after expression of a dominant negative Ras mutant (Ferrari *et al.*, 1994). Hence on one hand it is possible that two different Ras-mediated pathways are activated by an external stimulus, one leading to proliferation and the other to apoptosis. On the other hand, Ras may simultaneously induce both proliferation and apoptosis, the latter blocked by the action of survival factors, or Ras may induce either proliferation or apoptosis, depending on external signals.

While the role of Ras in apoptosis in any cell type is not clearly understood, the connections between Ras and the JNK pathway appear to be important in this regard. At present, the connection between JNK and Ras with regards to apoptosis is still not clear. The cellular consequences of JNK activation may be cell type-specific or depend on the activation of complementary pathways. The question of whether JNK activation is a cause or consequence of apoptosis remains to be addressed (Xia *et al.*, 1995; Juo *et al.*, 1997; Lenczwski *et al.*, 1997). Il-2 deprivation correlates with an increase in JNK1 activity directly related to the induction of apoptosis (Cerezo *et al.*, 1999). By contrast, activation of the ERK pathway blocks JNK activation and promotes cell survival (Xia *et al.*, 1995). It has also been demonstrated that inhibition of JNK activation can impair Ras transformation, suggesting a growth-promoting role for this kinase (Amar *et al.*, 1997).

1.9.6 RAS AND CELL SURVIVAL

There is also a substantial amount of evidence which implicates Ras in the protection of cells from apoptosis. In epithelial cells, activated Ras expression has been shown to protect strongly from apoptosis induced by detachment from the extracellular matrix (Frisch *et al.*, 1994; Rak *et al.*, 1995) or by the expression of the adenovirus E1A protein (Lin *et al.*, 1995). In sensory neurons NGF, which promotes neuronal survival, acts via Ras and PI3-K (Klesse *et al.*, 1998). Activated Ras has also been observed in growth factor stimulated T and B cells (Gomez *et al.*, 1996; Graves *et al.*, 1992). The mechanism by which Ras protects epithelial cells from detachment-induced and other forms of apoptosis has been found to involve the Ras effector PI3-K. In this case, PI3-K activates PKB/Akt, and not the conventional Ras-Erk pathway (Khwaja *et al.*, 1997). Hence, the anti-apoptotic activity of Ras has been linked to its ability to activate PI3-K. The PI3-K mediated survival signal is achieved through the activation of Akt/PKB, a kinase activated by PIP_2 (Franke *et al.*, 1997; Marte *et al.*, 1997; Cerezo *et al.*, 1998). There is also evidence, however, that Akt/PKB can be activated in a PI3-K-independent fashion, suggesting that Akt/PKB protection from apoptosis can occur without PI3-K activation (Cerezo *et al.*, 1998). Akt/PKB activation is involved in prevention of apoptosis in IL-4-stimulated cells because over-expression of wild-type or constitutively active Akt mutants protects cells from IL-4 deprivation-induced apoptosis (Cerezo *et al.*,

1998). Furthermore, in IL-4 deprived cells, over-expression of a constitutively active Akt mutant correlates with inhibition of JNK2 activity (Cerezo *et al.*, 1998). Akt/PKB inhibits the activation of caspases, which are required for the apoptotic response to serum withdrawal (Kennedy *et al.*, 1997). One mechanism proposed for the protection of cells by Akt/PKB against apoptosis is the phosphorylation and inactivation of Bad, a pro-apoptotic Bcl-2 family member (Del Peso *et al.*, 1997; Datta *et al.*, 1997). PI3-K/Akt is also implicated as playing a crucial role in mediating the aberrant survival of Ras transformed cells in the absence of attachment and mediates matrix-induced survival of normal cells (Frisch *et al.*, 1994; Khwaja *et al.*, 1997). In addition, IL-2 and IL-3-dependent cells are protected from starvation-induced apoptosis by activated Ras through upregulation of Bcl-2 and Bcl-X expression (Kinoshita *et al.*, 1995; Gomez *et al.*, 1998). This protection is believed to be due to the association of Raf with Bcl-2 (Wang *et al.*, 1994, 1996).

1.10 RAS SIGNALING: FUTURE PROSPECTS

The Ras signaling pathway mediates several apparently conflicting cellular responses, such as proliferation, apoptosis, growth arrest, differentiation, and senescence, depending on the duration and strength of the external stimulus and on cell type. The ability of Ras to mediate various effects in cells could reflect the use of these different pathways under different circumstances and in different cell types. In addition, it is becoming apparent that signaling through the same pathway in the same cell type could result in different biological outcomes depending on the presence or absence of other regulatory molecules and also the amplitude and persistence of signal. Ras, in some cases, appears to control opposing pathways regulating cell growth and cell arrest and even cell death within the same cell (Mulcahy *et al.*, 1995; Kaufmann-Zeh *et al.*, 1997; Lloyd *et al.*, 1997). Biochemical, genetic, and structural studies have only begun to provide clues as to how Ras can control a plethora of developmental and physiological processes in different cells in vertebrates and invertebrates. There are currently three broad themes that have emerged. First, different effects of Ras activation can originate from the activation of parallel effector pathways. Second, quantitatively different levels and duration of Ras activity can also affect the outcomes of Ras activation. Third,

different cellular contexts and distinct sets of transcription factors or other Ras targets that interpret a common signal in different ways can determine how the Ras signal is interpreted.

Whereas Ras signaling was initially proposed as a linear Ras-Raf-Mek-Erk cascade, recent findings indicate that this is not the case. What is clear is that Ras does not simply activate Raf. To date, Raf remains the major effector of Ras function. While Ras and Raf would appear to be sufficient for signaling from the membrane to the nucleus, over these last few years new components have been identified continually, gradually increasing the complexity of the simple view of this pathway. Several recent papers have added new proteins that conspire with Ras, Raf and receptors to transduce signals (Therrien *et al.*, 1995, Sieburth *et al.*, 1998). For example, CNK and Sur-8, both share similar genetic properties and they both act in multiple receptor pathways utilizing Ras and Raf. Both act downstream of Ras and upstream of Raf based on double mutant studies. These two proteins join two other recently identified molecules, 14-3-3 proteins and KSR, found as modifiers of the Ras-Erk cascade, that also seem to act downstream of Ras and upstream or parallel to Raf.

The function of Ras and its downstream kinase cascade is central to numerous cellular processes. The diversity of Ras target proteins and the necessity for activation of multiple effector pathways for malignant transformation by Ras unveils new directions for the design of additional therapeutic interventions that may negate Ras transformation without abolishing all of Ras function. Future challenges will be to identify the importance of the various candidate Ras effectors as well as defining their contribution to Ras function. In this case, questions relating to the regulation of Ras function via effector utilization remain to be addressed. Also, the identification of additional proteins which link various extracellular stimuli to Ras function, will prove important in understanding more clearly the regulation and function of Ras in various biological processes.

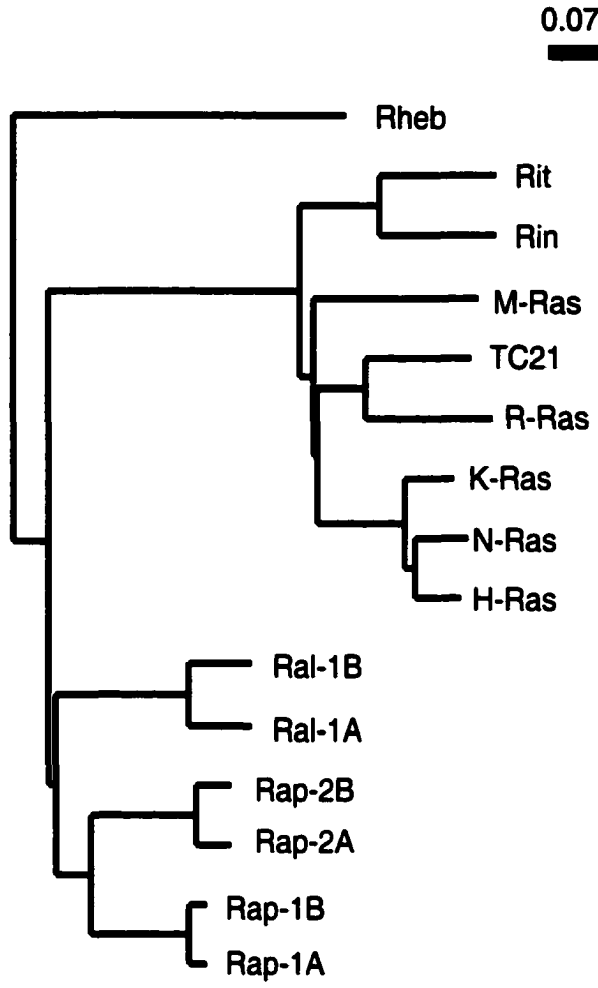

Figure 1-1. Sequence similarity between members of the Ras Subfamily. Alignment of the primary sequences (human) of members of the Ras subfamily was conducted using the ClustalW program. Using a distance matrix generated by the resulting multiple alignments, the above dendogram was constructed. The branch lengths are proportional to the estimated divergence along each branch (Adapted from Campbell *et al.*, 1998).

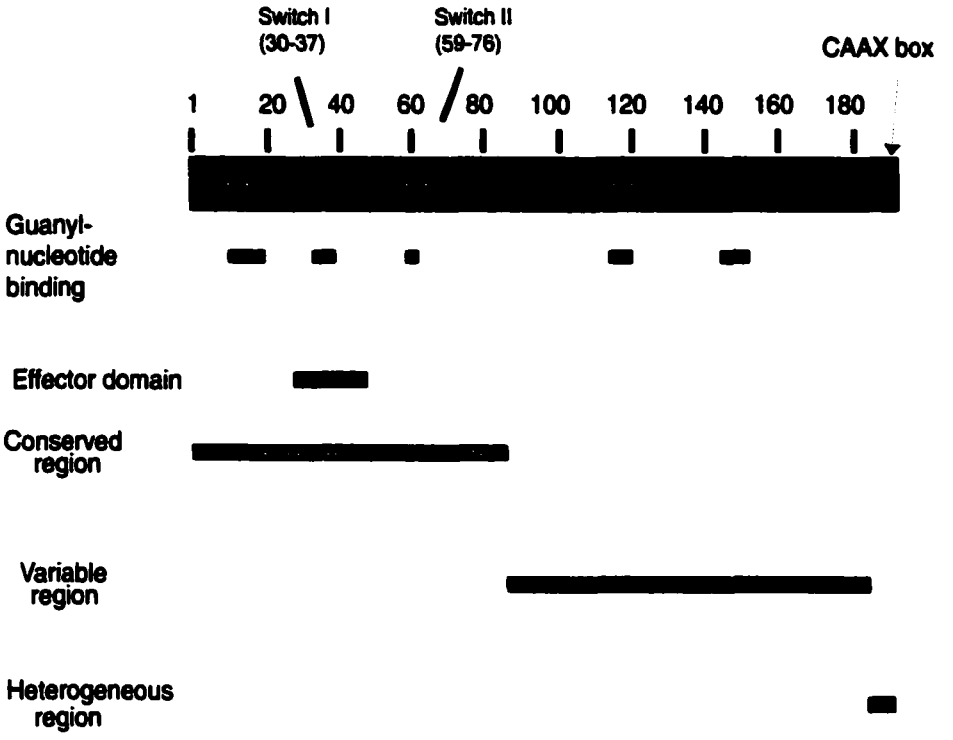

Figure 1-2. The Primary Structure and Functional Domains of Ras. Schematic representation of the structural and functional domains described in mammalian Ras proteins. The numbers correspond to the 189 amino acids found in p21Ras. Ras can be divided into four structural domains: an N-terminal conserved region (amino acids 1-86), a middle variable region (amino acids 86-166), a C-terminal hypervariable or heterogeneous domain (167-185), and a CAAX box (residues 186-189). In each case the bars represent the residues which comprise the respective functional regions described on the left. The stars (☆) show some of the residues in Ras that are mutated in cancer. Mutations in naturally occurring Ras oncogenes have been localized in residues 12 (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982), 13 (Bos, 1989), 59 (Dhar et al., 1982; Tsuchida et al., 1982), and 61 (Taparowsky et al., 1982; Brown et al., 1984). Mutations in codons 63 (Fasano et al., 1984), 116 (Walter et al., 1986), and 119 (Sigal et al., 1986) are also known to be transforming.

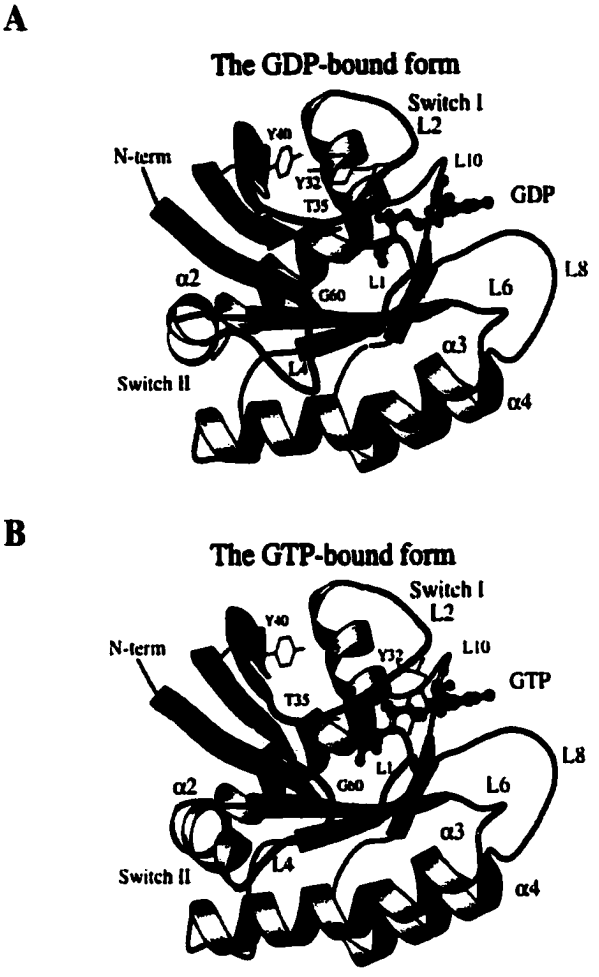

Figure 1-3. The Resolved 3-dimensional structure of Ras bound to GDP and GTP. A) A schematic diagram of Ras bound to GDP. The coordinates taken are from the Protein Data bank entry 1Q28 (Tong *et al.*, 1991). B) A schematic representation of GTP-bound Ras. The structure was taken from the protein Databank entry 5P21 (Pai *et al.*, 1990). The switch segments, loop1 (L1), loop2 (L2), loop4 (L4), loop8 (L8), GDP and GTP are labeled. The side chains of Tyr-32 and Tyr-40 are shown to demonstrate the conformational change that occurs in the switch I region. This figure was adapted from Ma and Karplus (1997).

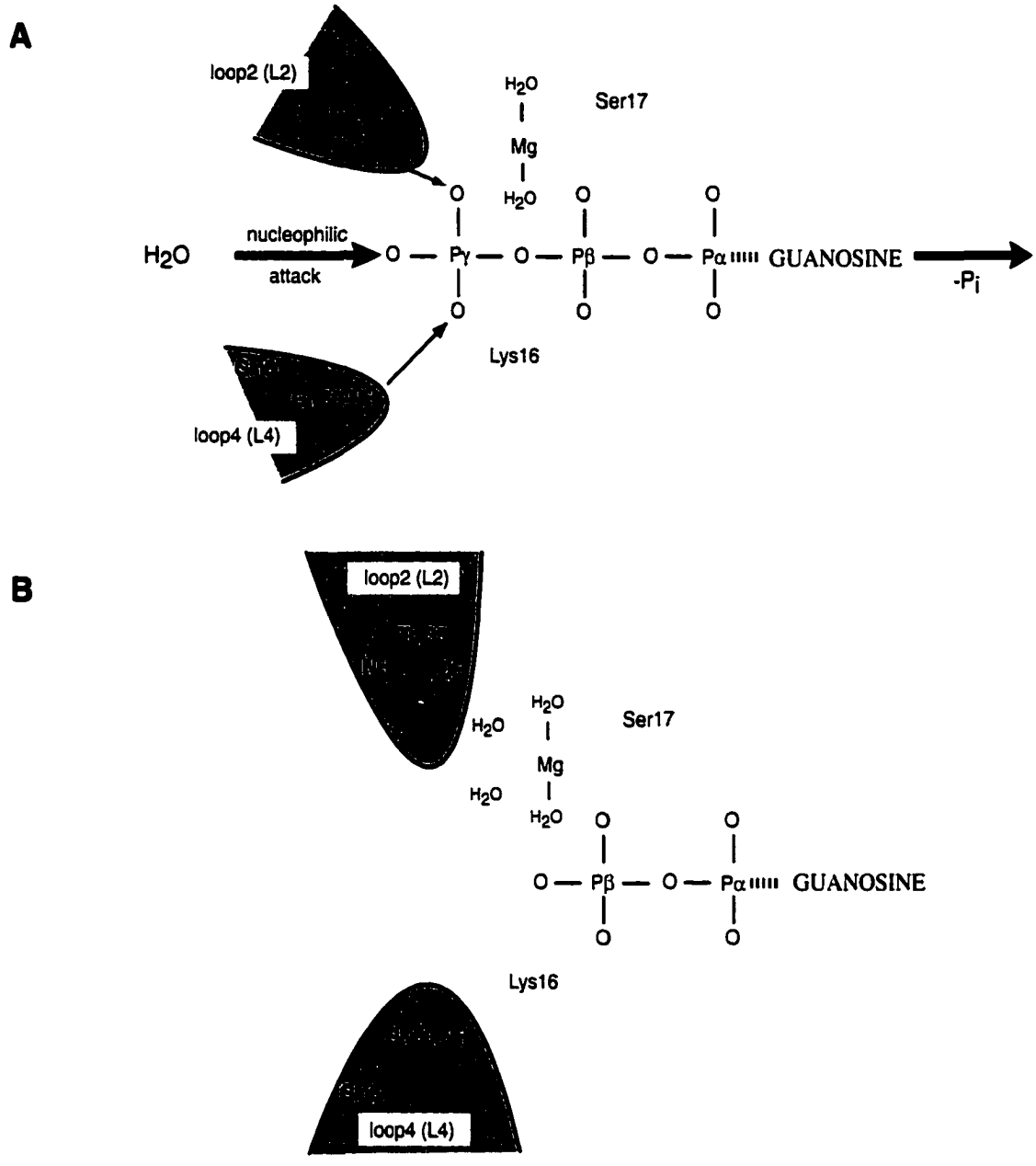

Figure 1-4. Biochemical activities of Ras. The above diagram depicts the proposed mechanism of GTP hydrolysis illustrating the changes in interactions between nucleotide and protein occuring upon GTP hydrolysis. A) GTP-bound Ras and the events that occur following in-line nucleophilic attack by water. B) Structural changes that occur in L2 and L4 of Ras after GTP hydrolysis (adapted from Boriack-Sjodin *et al.*, 1998).

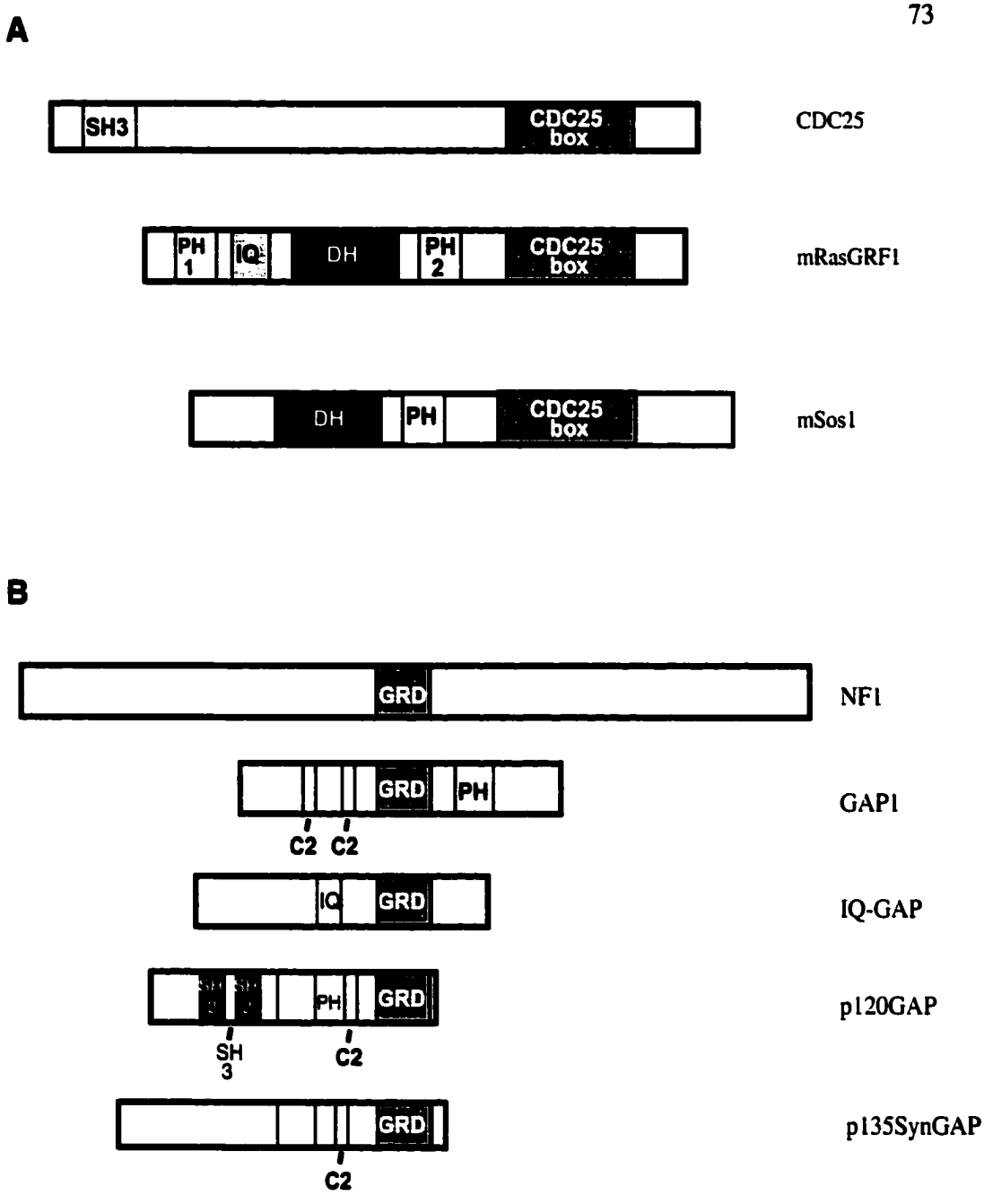

Figure 1-5. The mammalian families of RasGEFs and RasGAPs. A) Schematic representation of the members of the CDC25 family of RasGEFs. The structural domains of members of the different subfamilies of RasGEFs are illustrated. B) Diagram illustrating the structural regions of some of several RasGAPs. Each RasGAP illustrated represents a distinct subfamily of GAPs based on their structural similarity. DH, Dbl homology domain; PH, pleckstrin homology domain; IQ, ilimaquinone motif; C2, C2 domain; SH2, Src homology region 2 domain; SH3, Src homology region 3 domain; GRD, GAP-related domain.

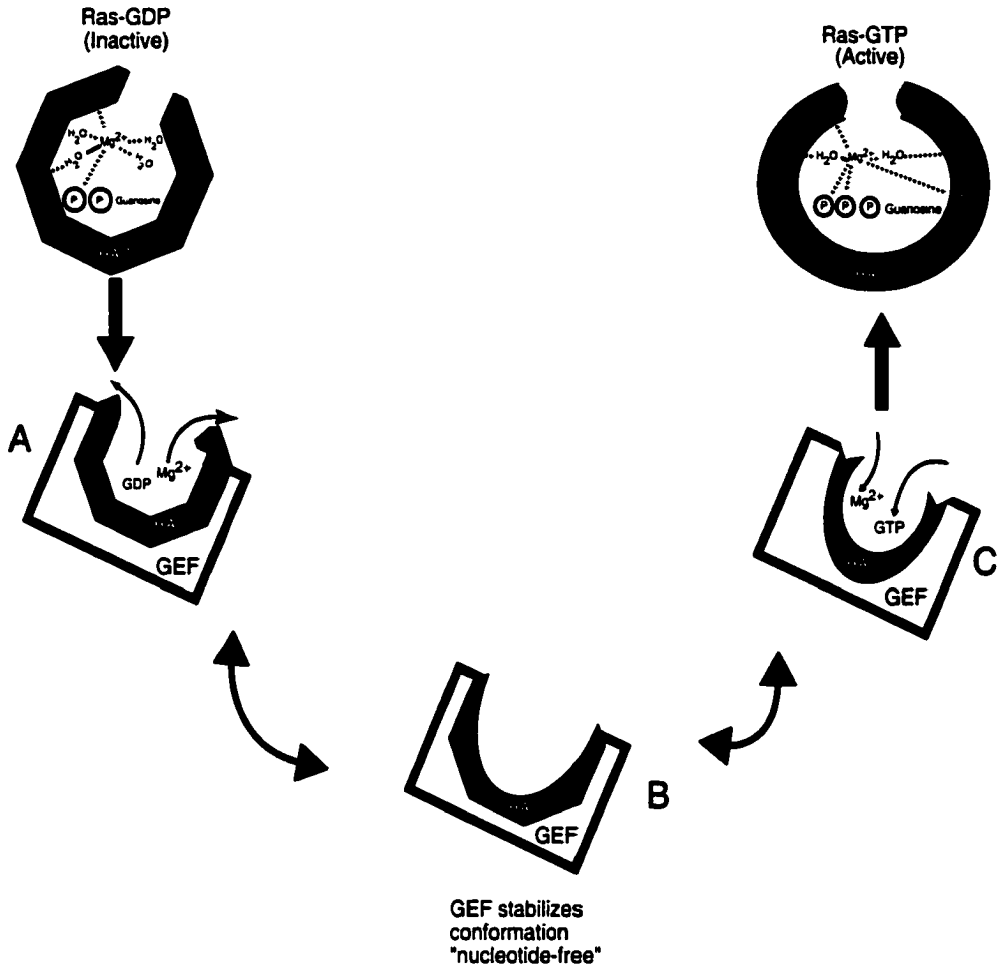

Figure 1-6. The Mechanism of GEF-mediated nucleotide exchange. Ras interconverts between an inactive (GDP-bound), transient (nucleotide-free) and active (GTP-bound) states. In the inactive state, Mg^{2+} coordinates directly with the β-phosphate of GDP and a conserved residue S17 and indirectly with other amino acids through four water molecules. The coordination of two of the water molecules is replaced by direct coordination with the γ-phosphate and a second conserved Thr (T35) residue in the active state.

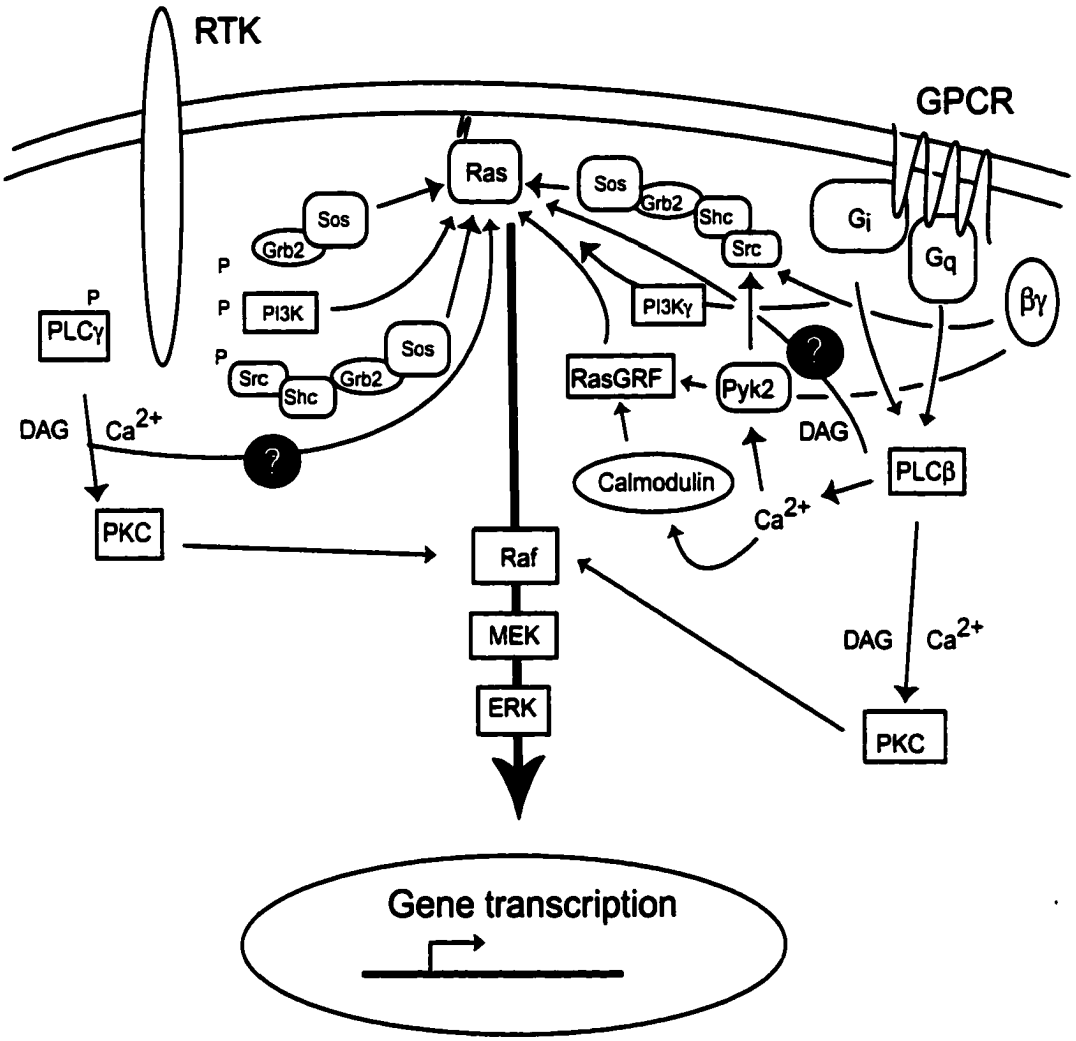

Figure 1-7. Receptor activation of the Ras signaling cascade. Schematic representation of the mechanisms by which receptor tyrosine kinases (RTK) or G-protein coupled receptors (GPCR) lead to the activation of Ras signaling. In both cases, Ras serves to relay extracellular ligand-stimulated signals to intracellular signaling pathways thereby providing a link between the cell surface and the nucleus. The question mark (?) refers to an unidentified link between the two molecules involved.

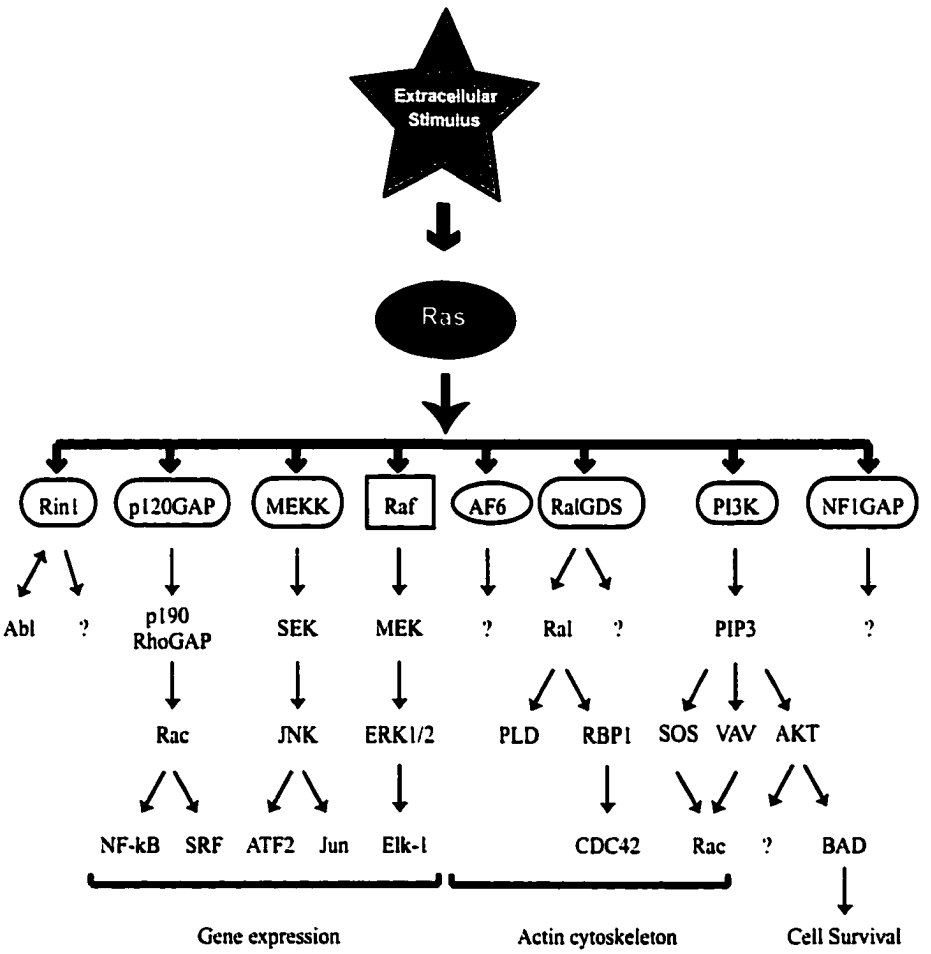

Figure 1-8. Ras effector signaling pathways. Ras can activate multiple effector pathways that contribute to Ras function. The above diagram depicts the current outlook of the downstream targets of each of the Ras effectors. PLD, phospholipase D; PIP3, phosphatidylinositol trisphosphate; SRF, serum response factor, RBP1, Ral binding protein 1.

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1.13 HYPOTHESIS

In this study I examine the biological, genetic and biochemical characteristics of a transforming cDNA isolated from a rat brain cDNA expression library. Based on my sequence analysis I proposed that the encoded protein, RasGRP, was related to the CDC25 class of Ras activators. RasGRP contains a pair of atypical EF hands and a putative diacylglycerol-binding C1 domain. These observations led to the hypothesis that RasGRP represents a novel family of Ras-specific exchange factors. Our findings have also led us to formulate the hypothesis that extracellular signals utilize calcium and diacylglycerol to recruit RasGRP to the membrane where it can interact with and activate Ras. Since stimuli expected to activate RasGRP are also expected to activate PKC, I have also explored the possibility that RasGRP is subject to regulation by reversible protein phosphorylation.

The exclusive expression of RasGRP in the brain and in lymphoid cells suggests a role for RasGRP in physiological functions attributed to these specific cell types. In either case, signals known to increase diacylglycerol and calcium are responsible for the activation of Ras, which eventually affects cell survival, synaptic plasticity in the case of neurons, or T cell activation in the case of T lymphocytes. In this dissertation, I characterize a novel family of Ras exchange factors. I test the hypothesis that RasGRP links extracellular signals which increase the intracellular concentrations of diacylglycerol and calcium to Ras activation in T cell and neurons. Specifically, I test the hypothesis that increases in diacylglycerol levels, recruits RasGRP to the membrane where it interacts with and activates Ras.

CHAPTER 2
MATERIALS AND METHODS

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2.1 ANTIBODIES

H176 is derived from rabbits immunized with the amino-terminal rat RasGRP peptide (residues 1-15). J32 was raised in rabbits by injecting the catalytic domain of rat RasGRP (residues 49 to 473). Antibodies were purified by affinity selection using immobilized recombinant rat RasGRP. These antibodies were made and purified by others in the lab (Bottorff, D. and Stang, S.). m133 and m199 monoclonal antibodies were raised and purified by others in Dr. J. Stone's lab.

2.2 PLASMID CONSTRUCTS AND RECOMBINANT PROTEINS

rbc7HA, rbc7ΔDAG. The pBabePuro vector (Morgenstern *et al.*, 1990; Land, 1990) encodes for puromycin resistance and was used to transmit rbc7 and v-H-Ras cDNA sequences. An epitope-tagged version of rbc7, rbc7HA, was constructed by inserting a hemaglutinin (HA) epitope (5'-TATGATG TTCCTGATTATGCTAGCCTC-3') immediately upstream of the stop codon. rbc7ΔDAG was constructed by inverted

PCR mutagenesis with primer DG-1 (5'-TGGAAAGCCTAG GCCCAGC-3') and primer DG-2 (5' AAGAAACGATCCAAGAGCCCC-3') and Xho1 linearized rbc7HA as a PCR template. Primer DG-1 represents the antisense sequence of rbc7 corresponding to the region immediately upstream of the DAG-binding domain. Primer DG-2 corresponds to the sense strand of the region immediately downstream of the DAG-binding domain.

GST-7HA. For the construction of GST-7HA, the *Bam*H1/*Sal* 1 rbc7HA fragment was isolated from pBabePuro-rbc7HA and subcloned into the *Bam*H1/*Sal* 1 sites of the pGEX 4T3 (Pharmacia) bacterial expression vector. To adjust the reading frame and shift it 2+ nucleotides, GST-7HA was linearized with *Bam*H 1 restriction enzyme and then incubated with Klenow and dNTPs (250 μM) to form blunt ends. To form polymers of linkers at each end, the blunt-ended DNA molecule was ligated in the presence of non-phosphorylated Xho1 linker monomers (5'-dCCCTCGAGGG-3', New England Biolabs). The DNA was resolved by agarose gel electrophoresis and the linear fragment was gel purified by electro-elution into dialysis tubes. To recircularize the DNA, the linear fragment was heated to 65 °C and then allowed to cool slowly. Subsequently, the DNA was reintroduced into *E. Coli* and ampicillin-resistant transformants were isolated from plates containing LB and ampicillin. Rapid preparations of plasmid DNA were therafter performed.

GST-DAG. To construct GST-DAG, GST-7HA was digested with *Bam* H1 and *Avr* II, which removed the DNA sequence encoding residues 1 to 537 of RasGRP. To form blunt-ends, dNTPs (250 μM) and 0.5 μl Klenow (10U/ul, GIBCO-BRL) were added after the digestion. To inactivate the Klenow enzyme, the DNA was treated with phenol-chloroform-isopropanol (PCI), and precipitated by the addition of 1/10 volume 3M sodium acetate and 2X volume 95 % ethanol. Thereafter, the DNA was resolved by agarose (1%) gel electrophoresis. The linear DNA fragment containing the GST vector and the DAG domain (residues 538-598 of RasGRP) was thereafter gel purified. To recircularize the GST-DAG sequence, the DNA was heated to 65 °C and then slow cooled.

ΔEF-RasGRP, ΔN-RasGRP, ΔC-RasGRP, ΔEFΔDAG-RasGRP. ΔEF-RasGRP was constructed by deleting the fragment of rbc7HA cDNA lying between the *Pfml*1 and *Apa* I sites. Other investigators in the lab made ΔEF-RasGRP. ΔN-RasGRP and ΔC-RasGRP were constructed by making rbc7-RasGRP recombinants. For ΔN-RasGRP, the N-terminal fragment of rbc7 (*Bam*HI to *Apa* I), was ligated to the C-terminal fragment of full-length RasGRP (*Apa*I to *Sal* I). For ΔC-RasGRP, a fragment containing the C-terminus of rbc7 (*Apa* I to *Sal*I) was ligated to a DNA fragment containing the N terminus of RasGRP (*Bam*HI to *Apa* I). ΔEFΔDAG-RasGRP was constructed in the context of rbc7. A *Pfml*1-*Nhe*I fragment containing the EF and DAG domains was removed and the HA-tagged C-terminal end was reconstructed with the use of PCR primers. Other investigators in the lab made this construct.

RasGRP catalytic domain (pET7). The catalytic domain of RasGRP (residues 49-471) was fused to a His6-tag in the pET21a vector and expressed in *E. coli.* Soluble proteins were subsequently purified by nickel chromatography and stored in buffer A + glycerol at –80 °C until used. pET7 was constructed and purified by others in the lab.

RasGRF1 (p30). The plasmid encoding the catalytic domain of RasGRF1 (p30) was a generous gift from Dr. D Lowy. The 6x His-tagged sequence of p30 was expressed in Sf9 cells and purified by nickel chromatography (Leonardsen *et al.*, 1996). Recombinant p30 was expressed and purified by D. Bottorff in our lab. The protein was then dialyzed into buffer A (20 mM Tris 7.5, 100 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM DTT, 10% glycerol) and stored at –80 °C until use.

Mouse Brain cytosol. Whole brains from 6-8 week old mice (Jackson labs) were homogenized in homogenization buffer [50 mM Tris-chloride pH 9, 1mM $MnCl_2$, 1mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was centrifuged at 100 000g for 60 minutes, and the supernatant obtained was recentrifuged at 120 000g for 45 minutes. This supernatant, referred to as mouse brain cytosol, was stored at –80 °C until needed.

GST-EF1⁻, GST-EF2⁻, GST-EF1⁻EF2⁻. BamH1/Sal 1 fragments of rbc7HA (referred to as EF1⁻, EF2⁻ and EF1⁻EF2⁻) containing mutations in the EF hand alleles were kindly constructed and provided by Dr. J. Stone. The EF1⁻ allele has quadruple alanine substitutions of the calcium-binding residues at positions 483, 485, 487 and 494. EF2⁻ contains similar alanine substitutions in the second EF hand at positions 510, 512, 514, and 521. EF1⁻EF2⁻ has all eight substitutions. To construct GST-EF1⁻, GST-EF2⁻ and GST-EF1⁻EF2, the BamH1/Sal 1 fragments were subcloned into the BamH1/Sal 1 sites of pGEX 4T3 (Pharmacia) bacterial expression vector.

2.3 CELL CULTURE AND VIROLOGY

Rat2 fibroblasts were maintained at 37 °C in a 5 % CO_2 incubator in non-selective medium, Dulbecco's Eagle medium (DMEM-Gibco BRL) supplemented with 10 % fetal bovine serum, L-glutamine, penicillin and streptomycin. To select for cells that expressed the retroviral vector pBabepuro, cells were subcultured in non-selective medium supplemented with puromycin (2.5 ug/ml).

Purified plasmids were transfected into a retroviral packaging cell line, Bosc 23 cells (Pear *et al.*, 1993), by the calcium phosphate precipitation method to generate helper-free retrovirus stocks. The virus was collected 48 hours post-transfection and filtered through 0.45 um pore-size filters (Millipore) to remove cells. Polybrene was added to a concentration of 8 ug/ml to facilitate virus attachment. Rat cells were plated at a density of 2×10^5 in 25 cm^2 culture flasks the day before infection. To infect rat2 cells, the virus stocks were added to the cultured cells and incubated for 1 hour. Thereafter, the flasks were incubated in non-selective medium at 37 °C for another 48 hours. After this, the cells were sub-cultured in selective medium which was replaced every three days until colonies could be counted and morphology examined for transformation. Colonies were treated with trypsin, pooled and maintained in selective medium.

The rv68BUR mutant cell line which is heterozygous for an activating mutation in Mek1 (Bottorff *et al.*, 1995) and rat2 cells overexpressing *c-H-ras*, were infected with virus stocks as described above. rv68BUR-infected cells were maintained in DMEM supplemented with puromycin (2.5 ug/ml). Infected-rat2 cells overexpressing *c-H-ras*

were maintained in DMEM supplemented with G418 (0.65 mg/ml geniticin [Gibco-BRL]) and puromycin (2.5 ug/ml).

Jurkat T cells were maintained in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA), L-glutamine, penicillin, streptomycin and β-mercaptoethanol. Cells were cultured in a 37 °C, 10% CO_2 incubator.

2.3.1 In vivo metabolic labeling of Rat2 cells with ^{32}P orthophosphate

Cells were plated at a density of 2 x 10^6/ 25 cm^2 culture flask in non-selective medium supplemented with puromycin (2.5 ug/ml). After overnight incubation at 37 °C, the cells were rinsed twice with DMEM containing 1/20th the normal amount of phosphate and 0.5 % serum. Thereafter, each 25 cm^2 culture flask was labeled with 2 ml of 1/20th phosphate DMEM supplemented with 0.5 mCi of ^{32}P (Du Pont-NEN) for 4 hours. After labeling, cultures were either treated with 100 nM PMA or DMSO for 10 minutes. At the end of the treatment periods, the cells were washed once with ice-cold PBS and lysed in 0.5 ml of cold Raf lysis buffer [150 mM NaCl, 20 mM Tris-Cl pH 8.0, 10 % glycerol, 1 % NP-40, 10 mM NaF, 40 mM β-glycerophosphate, 1 % (v/v) Aprotinin, 1 % (v/v) leupeptin, 1 % (v/v) pefabloc, 1 % (v/v) sodium vanadate]. The lysates were cleared by centrifugation at room temperature for 5 minutes at 15, 800 x g. Immunoprecipitations were performed by incubating the supernatant with 50 ul of protein A Sepharose and 1 ug of anti-HA polyclonal antibody (Bellco) or 0.5 ug ERK-1 (Santa Cruz) at 4 °C for 60 minutes. The immune complexes were collected by centrifugation for 1 minute at 4 °C at 15, 800 x g, washed twice with Raf lysis buffer, twice with Tris/LiCl (0.5 M LiCl, 20 mM Tris-Cl pH 7.5) and once again with Raf lysis buffer and eluted with 2X SDS sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore). Results were quantified by phosphor-imager analysis and then exposed to X-ray film at -80 °C with an intensifier screen. Filter-bound proteins corresponding to rbc7HA or ERK-1 bands were excised and digested with Trypsin as described in the tryptic phosphopeptide mapping methods.

2.3.2 IN VIVO METABOLIC LABELING OF RAT2 CELLS WITH ^{35}S-METHIONINE

Rat2 cells expressing ΔN-RasGRP, ΔC-RasGRP, rbc7HA, RasGRP were seeded at 1.8 x 10^6 / 25 cm^2 flask. Cells were then pulse-labeled for 2 hours in 2ml of methionine-free medium containing 200 uCi of [^{35}S] methionine (DuPont-NEN). The cells were subsequently washed once with ice cold PBS and lysed in 0.5 ml of lysis buffer supplemented with protease inhibitors [20mM Tris-Cl (pH 8.0), 150mM NaCl, 1% NP-40 (v/v), 10% glycerol, 10mM NaF, 40mM β-glycerophosphate, 1% aprotinin, 1% leupeptin, 1% pefabloc and 1% sodium vanadate]. Cell debris and nuclei were removed by centrifugation at 14,000 rpm (15,800g) for 10 minutes at 4 °C in a microcentrifuge. To reduce nonspecific binding of the labeled proteins to the protein A-Sepharose beads, the supernatant was preincubated with 50ul of 10% *Staphylococcus aureus* for 30 minutes at 4 °C. The lysates were then cleared by centrifugation at 14,000 rpm for 5 minutes at 4 °C. To determine the amount of label incorporation, trichloroacetic acid (TCA) precipitation was performed. Briefly, 2ul of labeled lysate was added to 2 ml of 10% TCA and left on ice for 10 minutes. Thereafter, the samples were vortexed and the precipitated proteins were recovered onto glass microfiber disks (Whatmann G/F) by flitration under a vacuum. After washing the filters once with 10% TCA and 95% ethanol, the radioactivity associated with TCA precipitated proteins was determined by scintillation counting.

Immunoprecipitation of radiolabeled protein

Immunoprecipitations were performed by incubating supernatants from above (matched for TCA precipitable counts) with 50 ul of protein A-Sepharose beads and 30ul of 133 monoclonal antibody or no primary antibody. Incubation was carried out by gently shaking the samples overnight at 4 °C. The beads were then washed 3 times by centrifugation and resuspension in RIPA buffer [20mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA], and then boiled for 5 minutes in reducing SDS sample buffer. The immunoprecipitated proteins were then resolved by SDS-PAGE on a 10% SDS polyacrylamide gel. Gels were subsequently fixed and lightly stained for 1 hour in a solution containing 10% (v/v) acetic acid, 45% (v/v) methanol and a pinch of Coomassie Brilliant Blue R-250 (Sigma). Radiofluorography was performed by treating the gel with EN^{3}HANCE (NEN-Du Pont),

for 1 hour. The gel was then incubated in Milli Q water for 30 minutes before drying. The dried gel was then exposed to X-ray film at –70 °C.

2.4 BIOLOGICAL ASSAYS

2.4.1 FOCUS TO DRUG-RESISTANT COLONY RATIO

Cells were infected and after 48 hours they were trypsinized and then half the culture was either sub-cultured in puromycin-supplemented medium to generate drug-resistant colonies or in non-selective medium to allow for the emergence of transformed foci. The drug-resistant colonies were scored after 7 days by staining the cells with a 0.7% crystal violet stain in 70% ethanol. The non-selective medium was changed every 3 days and after 21 days foci were counted by staining with crystal violet.

2.4.2 SOFT AGAR ASSAY

1×10^5 cells were plated in non-selective medium containing 0.3% agar supported by a 0.6% agar layer. Cells were then incubated at 37 °C, 5% CO_2 for 14 days and colonies were photographed.

2.4.3 SATURATION DENSITY EXPERIMENT

Pools of cells expressing the parental retrovirus vector pBabePuro (no insert) or rbc7HA were plated at a density of 5×10^4 in 25 cm^2 culture flasks. Triplicate flasks were harvested and counted with a Coulter counter on the days 3, 5, 7, 11 and 15.

2.4.4 MORPHOLOGICAL TRANSFORMATION BY RBC7 AND C-H-RAS

Drug resistant colonies of rat2 cells expressing either rbc7, over-expressing *c-H-ras*, or both were selected after infection with retrovirus vectors and typical colonies were examined.

2.4.5 TUMORIGENICITY ASSAY

2×10^5 rat 2 cells expressing *v-H-ras*, rbc7, and an empty puro vector pBabepuro, were injected subcutaneously into *scid beige* mice (3 mice per cell type). The mice were monitored for the progression of tumors until a diameter of 2 cm and thereafter terminated.

2.4.6 NORTHERN BLOT ANALYSIS OF RASGRP EXPRESSION IN RATS

Total RNA was isolated from various rat tissues using the TRIzol extraction method (Bethesda Research Laboratories). 10 ug of total RNA was mixed with 25 ul of sample buffer [0.75 ml deionized formamide, 0.15 ml 100 mM [3-(N-morpholino) propanesulfonic acid] MOPS, 0.24 ml formaldehyde, 0.1 ml deionized RNAse-free H2O, 0.1 ml glycerol, 0.08 ml 10 % (w/v) bromophenol blue] and heated for 15 minutes at 65°C. After adding 1 ul of ethidium bromide solution to each sample, they were loaded on a 1% agarose-formaldehyde gel and then resolved by electrophoresis at 100 V for 2-3 hours in electrophoresis buffer (20 mM MOPS, 50 mM sodium acetate, 10 mM EDTA adjusted to pH 7.0 and autoclaved). After electrophoresis, the gel was photographed and rinsed in DEPC and transferred to a nitrocellulose membrane as described (Fourney *et al.*, 1988). After the transfer, the RNA was UV crosslinked to the membrane in a Stratalinker using the "autolink" setting. Prehybridization was carried out for 4 hours at 65 °C in "7% solution" (0.5 M $NaPO_4$ pH7.1, 7 % SDS, 2 mM EDTA, 0.1 % $NaPP_i$ pH 8.0). The solution was thereafter discarded and fresh buffer containing 4.3 x 10^7 cpm of rbc7 probe (specific activity 8.4 x 10^{14}cpm/ug) was added. Hybridization was for 48 hours by incubation at 65 °C with gentle shaking. Thereafter, the membrane was washed twice for 20 minutes in 1X SSC, 0.1% (w/v) SDS at room temperature followed by two 20 minute washes in 0.1X SSC, 0.1% SDS at 50-55 °C. For autoradiographic analysis, filters were exposed to Kodak X-Ray film at –80 °C overnight with an intensifying screen.

2.5 IN VITRO GUANYL NUCLEOTIDE EXCHANGE ASSAYS

2.5.1 RAS-GDP DISSOCIATION ASSAY

To load Ras with labelled GDP, 1.0 ug of recombinant c-H-Ras was incubated with [^{3}H] GDP (33.5 Ci/mMole) in the presence of bovine serum albumin (0.2 mg/ml), 1 mM dithiothreitol (DTT), 1 mM EDTA and 20 mM HEPES pH 7.2 for 10 minutes at 30 °C. To stabilize the complex and prevent the dissociation of [^{3}H] GDP, 11 ul of 100 mM $MgCl_2$ was added after 10 minutes to give a final concentration of 10 mM and the mixture was immediately cooled on ice. To measure GDP dissociation catalyzed by RasGRP, the Ras-[^{3}H] GDP complex was incubated with 100 uM unlabeled GTP and

either buffer A (20 mM Tris 7.5, 100 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM DTT, 10% glycerol), 3.0 ug of RasGRP or 0.5 ug of p30GRF1 in a final reaction volume of 50 ul. The reaction was performed at 30 °C for 30 minutes and was terminated by placing the samples on ice. The reaction mixture was immediately added to protein A-Sepharose beads coated with Y13-259 anti-Ras antibody (Santa Cruz). Immunoprecipitation of c-H-Ras was then carried out at 4 °C for 1 hour. Thereafter, the beads were washed with ice cold TMN buffer (20 mM Tris pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl) and the amount of label associated with Ras was quantified by scintillation counting.

2.5.2 RAS-GTP ASSOCIATION ASSAY

To measure the association of GTP with Ras, 1.0 ug of recombinant c-H-Ras complexed with unlabelled GDP was incubated with 1.25 nmole [α^{32}P] GTP (8 Ci/mMole) and either buffer A, 2.7 ug RasGRP (residues 49-471) or 0.44 ug p30GRF1 in a final reaction volume of 50 ul for 10 minutes at 30 °C. Immunoprecipitations using the Y13-259 anti-Ras antibody were performed as above. To effect the maximal exchange, buffer A containing 2.0 mM EDTA (50 ul final volume) was incubated with c-H-Ras for 10 minutes at 30 °C followed by the addition of $MgCl_2$ to a final concentration of 20 mM $MgCl_2$. Results are expressed as a percentage of maximum.

2.5.3 MEMBRANE RAS-GUANYL NUCLEOTIDE ASSOCIATION ASSAY

To assay guanyl nucleotide association with membrane-bound Ras, membrane fractions were suspended in 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$ (2.5 X 10^{+5} cell equivalents/ul). Membranes (25 ul) were then incubated with 1 ul [α^{32}P] GTP (3000 Ci /mMole final concentration 66 nM) in a final volume of 50 ul. After incubation at 30 °C for 1 minute to allow exchange of resident guanyl nucleotide with the radiolabel led GTP, the reaction was diluted in ice-cold buffer (50 mM Tris pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% v/v NP40) containing 1 ug of anti-Ras antibody Y13-259. After incubation at 0 °C for 1 hour, Ras-guanyl nucleotide complexes were recovered using protein A-Sepharose beads coated with rabbit anti-rat IgG. Guanyl nucleotide was released by heating at 80 °C in 1.0 M potassium phosphate (pH 3.4). Following chromatography of polyethylenimine plates, total guanyl nucleotide (GTP plus GDP) was quantified by phosphor-imager analysis. Background values obtained when no Y13-259 antibody was present were used to correct the experimental values. To determine the

maximal degree of Ras activation in these preparations, membranes were exposed to 5.0 mM EDTA for 5 minutes at 30 °C followed by addition of excess $MgCl_2$, before lysis and precipitation. To demonstrate the specificity of the above assay, membrane preparations were pre-incubated with antibodies (J32) raised against the catalytic domain of RasGRP (residues 49 to 473) or with pre-immune antibodies from the same rabbit. To demonstrate that J32 antibodies specifically inhibited RasGRP, exchange assays were performed with purified proteins in buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 1.0 mM DTT, 1.0 mM $MgCl_2$, 10% v/v glycerol and either 1.6 pmoles recombinant full-length Sos or 1.96 pmoles RasGRP catalytic domain. These low amounts of Ras GEF were used to increase the likelihood that the antibody was in molar excess. Total IgG was 62 pmoles/reaction but the amount of neutralizing antibody is an unknown fraction of the total.

2.6 IN VIVO RAS-GTP LEVELS

2.6.1 MEASUREMENT OF IN VIVO RAS-GTP IN RESPONSE TO PMA

Cells were seeded at 2 x 10^6/ 25 cm^2 culture flask in non-selective medium. Following overnight incubation at 37 °C, the cells were rinsed twice with DMEM containing 1/20th the normal amount of phosphate and 0.5% serum. To facilitate the incorporation of label, cellular guanyl nucleotide pools were labeled for 4 hours in phosphate-reduced medium (1/20th the normal amount) containing 0.5 mCi $^{32}P_i$. Cultures were either treated for 2 minutes with 100 nM PMA or DMSO as a control. Thereafter, the cells were rinsed with ice-cold phosphate buffered saline supplemented with 20 mM $MgCl_2$ (PBS/20 mM $MgCl_2$) and lysed on ice in p21 lysis buffer [50 mM Tris 7.5, 20 mM $MgCl_2$, 150 mM NaCl and 0.5 % NP40, 1% v/v Aprotinin (Sigma), 1% v/v leupeptin, 1% v/v Pefabloc (Boehringer Mannheim)] containing the anti-Ras monoclonal antibody Y12-259. Lysates were cleared by centrifugation at 15, 800 x g for 3 minutes, and supernatants were incubated with 100 ul of charcoal suspension (10% w/v charcoal, 10 mg/ml BSA, in PBS/20 mM $MgCl_2$) to reduce free nucleotides. After mixing at 0 °C for 45 minutes, the charcoal was removed by centrifugation, and the lysate was added to 60 ul of protein A Sepahrose beads complexed with rabbit-anti-rat IgG

(RARIG-PAS). The mixture was then incubated for 1 hour at 0 °C. The immunoprecipitates were washed five times with p21 lysis buffer, three times with PBS/20 mM $MgCl_2$ and then suspended in 20 ul of 1 M KH_2PO_4 (pH 3.4). To dissociate Ras-bound guanyl nucleotide, the suspension was heated at 85-90 °C for 3 minutes, vortexed and centrifuged. 10ul of the supernatant was spotted onto a polyethyleneimine-cellulose (PEI) thin-layer chromatography plate (Brinkman Canada, Inc.). As controls, 0.5 ul of cold 10 mM GDP and 10 mM GTP standards were also spotted onto PEI plates. The plates were developed with 1 M KH_2PO_4 (pH 3.4) and GDP and GTP were quantified by phosphor-imager analysis (Fuji Bas). Unlabelled guanyl nucleotide standards were visualized with UV. The percent of Ras-GTP was calculated as GTP/(1.5 GDP + GTP) x 100% (Gibbs, 1995).

2.7 LIGAND BINDING

2.7.1 [^{3}H] PDBU FILTER BINDING ASSAY

To measure the ability of [^{3}H] PDBu to bind to DAG domain of RasGRP, a filtration-binding assay (Tanaka *et al.*, 1986) was performed with some modifications. GST-DAG fusion protein (containing residues 538-598 of RasGRP) was expressed in *E.coli* and purified to near homogeneity. The standard reaction was conducted in a plastic tube. The reaction mixture (0.2 ml) contained 20 mM Tris-Cl (pH 7.5), 0.15 mM $CaCl_2$, 0.5 % dimethyl sulfoxide (DMSO) 100 ug/ml phosphatidylserine, 30 nM [^{3}H] PDBu (13.4 Ci/mmol) and either 2 ul of buffer (50 mM Tris-Cl pH 9.0, 1 mM PMSF) or 50 ug of GST (negative control), 50 ug GST-DAG, 50 ug mouse brain cytosol (positive control) (Dunphy *et al.*, 1981). Phosphatidylserine (Sigma) in chloroform was dried under a stream of nitrogen and suspended in 40 mM Tris-Cl (pH 7.5) by vortexing, and sonicated with a microtip sonicator using three-30 second bursts before adding to the protein mixture. To start the reaction, 100 ul of the dispersed lipids was added to 100 ul of the protein solution and incubated at 30 °C for 20 minutes. The reaction was stopped by adding 4 ml of ice-cold 0.5 % DMSO. The mixture was then poured onto Whatman GF/F glass-fiber filters which had been presoaked in 0.3 % polyethylenimine solution for one hour to efficiently trap the radioactive material and reduce non-specific binding.

Filters were placed under a high vacuum suction apparatus and washed three times with 3 ml of ice-cold 0.5 % DMSO. The filters were dried and the amount of [^{3}H] PDBu bound was determined by scintillation counting.

2.7.2 ^{45}CA BINDING EXPERIMENT

^{45}Ca binding was determined as described (Maruyama *et al.*, 1984) with some minor modifications. Overnight cultures of *E.coli* (BL21DE3) transformed GST, GST-7HA, GST-EF1$^-$, GST-EF2$^-$ or GST-EF1$^-$EF2$^-$ were diluted 1:10 in fresh Lauria-Bertani medium [1 % (w/v) NaCl, 1 % (w/v) Bactotryptone, 5 % (w/v) Yeast extract] and grown for 1 hour at 37 °C before adding isopropyl-1-thio-β-D-galactoside (IPTG) to 1 mM and the cells were grown for an additional 4 hours. After 4 hours of growth, cells were pelleted and washed once with PBS and resuspended in 1 ml PBS containing 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF). Thereafter, 100 ul was treated with 100 ul 2X SDS sample buffer followed by boiling at 100 °C for 5 minutes. Total lysates were resolved in a 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Biorad) in Towbin transfer buffer (39 mM glycine pH 8.3, 48 mM Tris base, 20% methanol). To wash away transfer buffer, the membrane was washed three times (20 minutes/wash) in a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH6.8). Thereafter, the membrane was probed with the same buffer containing 1 mCi/L ^{45}Ca for 10 minutes. The membrane was then rinsed with distilled water for 2 minutes and dried between two sheets of Whatmann No. 1 paper to remove excess water. After drying the membrane at room temperature for 2 hours, audioradiographs were obtained by exposure to Kodak Scientific X-ray film at -80 °C for 12-48 hours with an intensifying screen. To show that equal amounts of protein were being expressed, a parallel SDS-polyacrylamide gel was run and stained with Coomassie Brilliant Blue R-250 (Sigma).

2.8 SUB-CELLULAR FRACTIONATION

Jurkat T cells were suspended in hypotonic buffer (20 mM Tris-Cl pH 7.5, 5mM EGTA, 2mM EDTA, 0.3% β-mercaptoethanol, 10 mM NaF) and disrupted in a glass

homogenizer. After removing nuclei and unbroken cells by centrifugation at 2,000g, cell homogenates were separated into P100 and S100 by ultra-centrifugation at 100,000g for 60 minutes. For detection of RasGRP, samples were resolved by SDS-PAGE and immunoblotted with H176 IgG.

2.9 PHOSPHORYLATION STUDIES

2.9.1 TRYPTIC PHOSPHOPEPTIDE MAPPING

Phosphopeptides were generated by tryptic digestion of protein immobilized to Immobilon-P membrane. To increase the efficiency of elution of the peptides from the membrane and block nonspecific absorption of trypsin, the radiolabelled bands were excised and soaked in 300 ul of 0.5 % polyvinylpyrolidone [PVP-360 (Sigma)] in 100mM acetic acid for 30 minutes at 37 °C. The membrane was washed five times with 1 ml of H_2O and once with 1 ml of freshly made 50 mM NH_4HCO_3. Digestion was achieved by incubating the membranes at 37 °C for 2 hours in 200 ul of 50 mM NH_4HCO_3 with 10 ug of trypsin. Following the incubation period, a further 10 ug of trypsin was added and the samples were incubated overnight at 37 °C. Thereafter, 300 ul of H_2O was added, the samples were vortexed and lyophilized in a Speed-Vac to remove the ammonium bicarbonate. During this time fresh performic acid solution (100 ul of fresh 30% H_2O_2 and 900 ul of 98% formic acid) was mixed at room temperature for 1 hour and placed on ice until use. Once the membranes were dry, oxidation of the phosphopeptides was performed using performic acid to fully oxidize all methionine and cysteine residues to their single oxidation state. This is known to prevent formation of isomers that can separate during chromatography (Boyle *et al.*, 1991). 500 ul of fresh cold performic acid solution was added and each sample was vortexed and incubated on ice for 1 hour. Thereafter, 1 ml of water was added to dilute the samples since performic acid can cleave certain peptide bonds during extended incubation at higher temperatures. The samples were vortexed and lyophilized to completion for 3-4 hours in a Speed-Vac (Savant). The samples were resuspended in 10 ul of H_2O and 1-3 ul (100-200 counts) was spotted onto cellulose thin-layer chromatography plates. Peptide maps were generated by electrophoresis at pH 8.9 (1% NH_4HCO_3) for 27 minutes at 1 kV followed

by ascending chromatography (15 parts n-Butanol, 10 parts pyridine, 3 parts acetic acid, 12 parts H_2O). Peptides were visualized by autoradiography.

2.9.2 PHOSPHOAMINO ACID ANALYSIS

To determine the phosphoamino acid content of radiolabeled rbc7HA and ERK-1, filter-bound protein corresponding to rbc7HA and ERK-1 were excised from the Immobilon-P membrane and subjected to partial acid hydrolysis for 1 hour at 110 °C in 200 ul 5.7 N HCl. Thereafter, the membranes were removed, placed in fresh tubes and hydrolyzed at 110 °C for another 3 hours in 200 ul 5.7 N HCl. After hydrolysis, the samples were dried by lyophilization in a Speed-Vac and the remaining radiolabel was determined by Cerenkov counting. Hydrolysates were then resuspended in 5-10 ul of pH 1.9 buffer (25 parts 88% formic acid, 78 parts acetic acid and 897 parts H_2O) containing cold phosphoamino acid standards (0.66 mg/ml of each phosphoserine, phosphothreonine and phosphotyrosine diluted 1:3 (v/v) in deionized water) and 3 ul were spotted onto cellulose thin-layer chromatography plates. Phosphoamino acids were separated by two-dimensional electrophoresis. Electrophoresis in the first dimension is carried out in pH 1.9 buffer for 20 minutes at 1.5 kV followed by pH 3.5 (0.5 % pyridine, 5% acetic acid, 94.5% H_2O) for 13 minutes at 1.6 kV. To visualize the cold phosphoamino acid standards the plates were sprayed with 0.25% (w/v) Ninhydrin in acetone and left to dry at room temperature. The plates were then exposed to Kodak X-ray film with an intensifier screen at -80 °C.

2.10 PKC ASSAY

Protein kinase C assay was performed using reagents from a PKC assay kit (Amersham). A component mixture was prepared and consisted of equal volumes of calcium buffer (12 mM calcium acetate in 50 mM Tris/HCl [pH 7.5]), lipid (detergent dispersed solution of 8 mole% phosphatidylserine and 24 ug/ml PMA in 50 mM Tris/HCl (pH 7.5), DTT (30 mM DTT in Tris/HCl [pH 7.5]). The reaction mixture contained 25 ul of component mixture, 25 ul of MgATP buffer (150 uM ATP, 45 mM Mg acetate, [γ-^{32}P] ATP (0.04 uCi/ul), 1 ug of recombinant RasGRP (catalytic domain) and various amounts of PKC rat brain (Calbiochem). To start the reaction, MgATP buffer was added

last to the reaction mixture and the samples were incubated at 25 °C for 15 minutes upon which 100 ul of stop reagent was added (dilute acidic reaction-quenching reagent). After adding 2X SDS sample buffer, the proteins were resolved by SDS/PAGE and transferred to Immobilon-P. ^{32}P incorporation was detected by autoradiographic analysis.

Phosphorylation of synthetic peptides

Assays were performed as described for phosphorylation of recombinant protein except that synthetic peptides replaced the recombinant protein. Phosphorylated peptides were captured onto P81 phosphocellulose peptide binding paper (Amersham). Filters were washed two times with 10ml of 0.75M phosphoric acid for 10 minutes each. The level of phosphorylation was quantified by liquid scintillation counting. The CS2894 and CS2895 synthetic peptides were ordered from the Alberta Peptide Institute (University of Alberta).

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CHAPTER 3
RASGRP, A NOVEL RAS ACTIVATOR WITH CALCIUM- AND DIACYLGLYCEROL-BINDING MOTIFS

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Chapter 3

INTRODUCTION

3.1 INTRODUCTION

3.1.1 ISOLATION OF A cDNA INVOLVED IN THE RAS SIGNALING PATHWAY

A crucial step in elucidating the molecular mechanisms which regulate Ras is the identification and cloning of genes that direct Ras signaling in mammalian cells. We screened a rat brain cDNA expression library for cDNAs that could enhance the transforming potential of a weak *v-H-ras* effector mutant. We hoped to identify molecules that could accentuate Ras signaling. Using this approach, we were able to isolate a weakly transforming cDNA- rat brain cDNA #7 (rbc7). The sequence of the normal version of rbc7 was determined from rat brain cDNA PCR products and overlapping phage clones. The deduced normal product, which we call RasGRP, is predicted to consist of 795 residues with a size of 90.3 kDa. rbc7 is both a 5' and 3' truncated version of RasGRP. Subsequent analysis of rbc7 revealed that it is a novel element involved in the Ras signaling pathway. In particular, RasGRP represents a novel family of Ras guanyl-nucleotide exchange factor.

RESULTS

3.2 RESULTS

3.2.1 DISCOVERY AND SEQUENCE ANALYSIS OF RBC7/RASGRP

As described in the introduction, our initial discovery of rbc7 was based on the modest transforming activity it displayed in both parental rat2 cells and rat2 cells expressing Tyr32Arg *v-H-ras*. Using PCR methods, we obtained the DNA sequence of rbc7. To determine the presence of any similarity between rbc7 and other sequences, I searched various DNA and protein sequence databases. I found that the sequence with the highest computer-assigned score was the *C. elegans* o.r.f of unknown function F25B3.3. A computer-assisted sequence-homology search (BLAST search, PubMED) with the deduced amino acid sequence of rbc7 further revealed significant homology between rbc7 and the CDC25-family of exchange factors. These observations suggested that rbc7 is a novel member of the CDC25 family of exchange factors and that it transforms rat2 cells by the activation of endogenous Ras.

The sequence of rbc7 and RasGRP is shown in Figure 3-1A. Further cloning and DNA sequence analysis done in Dr. J. Stone's lab established that rbc7 is a 5' and 3' truncated version of a larger sequence we named Ras guanyl nucleotide releasing protein (RasGRP). rbc7 and RasGRP contain 550 and 795 amino acids respectively.

The RasGRP cDNA sequence is predicted to encode several recognizable polypeptide motifs. These include a sequence conserved among exchange factors that can act on Ras or Ras-related proteins, Ras-exchange motif (REM) (Camus *et al.*, 1995), a CDC25-related catalytic domain (Boguski and McCormick, 1993), two EF hand-like motifs (Strynadka and James, 1989) and a putative diacylglycerol-binding C1 domain (Ono *et al.*, 1989). The amino acid sequence of the catalytic domain of RasGRP is

similar to both RasGRF1 and Sos1 (Figure 3-1B). However, a notable difference between RasGRP and other exchangers is the presence of the atypical pair of EF hands and the C1 domain.

Alignment of the CDC25 box of RasGRP, RasGRF1 and Sos1 revealed a high degree of similarity (Figure 3-2A). The CDC25 box of RasGRP is 50% similar to the same region in RasGRF1 and Sos. RasGRP contains the critical residues required for the interaction with and activation of Ras (Park *et al.*, 1994; Camus *et al.*, 1995). The REM sequence is 54% similar to the conserved motif found in RasGRF1 and Sos1, however, RasGRP has an insertion of 7 amino acids (Figure 3-2A). The two loops of the EF hands of RasGRP (Figure 3-2B), which contain the key residues for binding calcium, share 79 % homology with the EF hand domains of calcineurin B (Aitken *et al.*, 1984). Both EF hands in RasGRP possess the necessary charged residues required for the coordination of calcium-binding (Strynadka and James, 1989). The putative DAG-binding C1 domain shares 68% similarity with the DAG-binding C1 domain of PKCδ (Aris *et al.*, 1993; Figure 3-2C). In addition, the C1 domain of RasGRP has the necessary residues for the binding of diacylglycerol (Kazanietz *et al.*, 1995; Hurley *et al.*, 1997). These observations led to the hypothesis that RasGRP belongs to a novel family of guanyl nucleotide exchange factor. In particular, the presence of the atypical EF hands and the putative DAG-binding C1 domain suggested that calcium and diacylglycerol normally regulate RasGRP.

3.2.2 BIOLOGICAL PROPERTIES OF RBC7

To characterize the transforming properties of rbc7, rat2 cells expressing rbc7 or an epitope-tagged version, rbc7HA, were compared to parental rat2 cells or rat2 cells expressing the empty vector pBabePuro using a number of assays.

One property of transformed fibroblasts is the capacity for anchorage-independent growth. As shown in Figure 3-3A, the colonies of rbc7 and *v-H-Ras* expressing cells grew to a similar size. However, the colony-forming efficiency of rbc7 was not strictly comparable to that of rat2 cells transformed by *v-H-Ras* , in that they took longer to grow. rat2/puro did not grow in soft agar.

As a measure of the transforming potential of rbc7, I assessed its focus-forming activity in rat fibroblasts. rbc7-expressing rat2 cells produced foci and drug-resistant colonies at a ratio of about 0.17. The morphology of foci observed were Ras-like although they developed after 3 weeks compared to 1 week for *v-H-ras* (Figure 3-3B).

Malignant transformation is characterized by a loss of density-dependent growth inhibition. To determine the effect of rbc7 expression on the growth rate and density-dependent growth of rat cells, I monitored their proliferative capacity over several days. rbc7 and puro-expressing cells were plated in multiple flasks at a low cell density. On various days, the number of cells was determined in triplicate cultures by Coulter counting. Control cells grew to confluence reaching a saturation cell density within 10 days (Figure 3-3C). rbc7-expressing cells, however, grew to a much higher cell density over the same time period and displayed a marked increase in growth rate.

As an independent test of transformation, cells were assessed for their ability to form tumors in immune-deficient mice. rbc7-expressing cells and control cells were inoculated subcutaneously and monitored for the formation of tumors. While mice inoculated with rat cells expressing the retroviral vector pBabepuro were negative for tumors after 90 days (duration of experiment), tumors were observed in all mice injected with rbc7-expressing cells after 39 days compared to 12 days for *v-H-ras* transformed cells.

To investigate whether rbc7 interacts genetically with elements of the Ras pathway, we expressed rbc7 in two different cell lines. We observed a fully transformed morphological phenotype when rbc7 was expressed in rat cells that over-express *c-H-ras*. Similar results were observed when rbc7 was expressed in the rv68BUR cell line which is hypersensitive to transformation by *v-H-ras*.

My results show that rbc7 is qualitatively similar to but weaker than an activated version of Ras, *v-H-ras*. Furthermore, our genetic analysis suggests that rbc7 can interact with elements of the Ras signaling pathway, in support of my hypothesis that rbc7 encodes a Ras activator.

3.2.3 BIOCHEMICAL ANALYSIS OF RASGRP FUNCTIONAL DOMAINS

3.2.3.1 RasGRP is a Ras-specific guanyl nucleotide releasing protein

To determine whether RasGRP could activate Ras *in vitro*, I investigated the ability of recombinant RasGRP (catalytic domain) to display Ras-guanyl nucleotide exchange activity in an *in vitro* biochemical assay. Ras was first loaded with [^{3}H] GDP. Subsequently, RasGDP complexes were incubated with excess unlabeled GTP and recombinant RasGRP. As controls I used a known Ras activator, RasGRF1 (catalytic domain), or buffer alone. After the reaction had been incubated for 30 minutes at 30 °C, the dissociation of [^{3}H] GDP was monitored by immunoprecipitating Ras onto protein A Sepharose beads that had been coated with rat anti-rabbit IgG and Y13-259 antibody. As shown in Figure 3-4A, purified RasGRP stimulated 98% GDP-dissociation compared to 18% by buffer. Similarly, RasGRF1 (p30= catalytic domain) stimulated a high level of GDP-dissociation as expected. I assessed next the ability of RasGRP to stimulate the binding of [α-^{32}P] GTP to c-H-Ras. Both RasGRP and RasGRF1, but not buffer, stimulated GTP-association with Ras by 40% and 51% respectively (Figure 3-4B). Using this [α-^{32}P] GTP association assay, the specificity of RasGRP activity was also examined by testing its ability to stimulate GTP-association with Ras-related proteins, Rho and R-Ras. RasGRP was not active on bacterially expressed Rho or on a GST fusion protein containing R-Ras (data not shown).

To address the possibility that RasGRP functions as a Ras-specific guanyl nucleotide releasing protein *in vivo*, I measured the proportion of GTP-bound Ras in rat2 cells. Cells were metabolically labeled for four hours with [^{32}P] orthophosphate and Ras was immunoprecipitated with the Y13-259 monoclonal antibody. Ras-bound nucleotides were eluted, resolved by thin-layer chromatography and the radioactive guanyl nucleotides were quantitated by phosphor-imager analysis (Figure 3-5A, B). Compared to control cells which showed 12% GTP, rbc7-expressing cells displayed 31% GTP. To explore the functional significance of the putative DAG-binding C1 domain, I examined the response of these cells to a DAG analogue, phorbol myristate acetate (PMA). In cells expressing the empty vector, PMA treatment for 2 minutes had no significant effect on Ras-GTP levels. However, in rbc7-expressing cells, PMA treatment for 2 minutes increased Ras-GTP levels to 41% (Figure 3-5B).

3.2.3.2 The EF hands of RasGRP bind ^{45}Ca in vitro

RasGRP contains two EF hand motifs. This structure is atypical in that the linker region between the EF hands is 15 residues in length rather than the 20 to 30 residues typically found. To determine whether these helix-loop-helix motifs could bind calcium, we constructed several GST-fusion proteins that contained either wild-type rbc7 or mutants that either had alanine substitutions which destroyed the first EF hand (GST-EF1$^-$), the second EF hand (GST-EF2$^-$) or both EF hands (GST-EF1$^-$EF2$^-$). Proteins were expressed in *E. coli* and after lysis in SDS, total cell lysates were resolved by SDS/PAGE, transferred to nitrocellulose and probed with ^{45}Ca. My results indicated that both GST-rbc7HA and GST-EF1$^-$ bound ^{45}Ca (Figure 3-6A). When the second EF hand was mutated, ligand-binding was not observed. These results suggest that the EF hands of RasGRP bind calcium, and further demonstrate that the second EF hand binds calcium with a higher affinity than the first EF hand.

3.2.3.3 The C1 domain of RasGRP binds phorbol dibutyrate in vitro

To determine whether the C1 domain of RasGRP binds diacylglycerol, I examined the capability of recombinant protein containing the C1 domain of RasGRP to bind a radioactive phorbol ester [^{3}H] PDBu *in vitro*. GST fusion proteins containing the putative DAG-binding C1 domain of RasGRP (GST-DAG) were expressed in *E.coli* and purified. GST or GST-DAG was incubated with [^{3}H] PDBu and phosphatidylserine multilamellar vesicles and ligand-binding was determined by using a filtration assay. GST did not bind [^{3}H] PDBu, however, GST-DAG displayed ligand-binding activity (Figure 3-6B). As controls, I performed the same experiment looking at the phorbol-ester binding capability of mouse brain cytosol, known to contain diacylglycerol-binding PKC. As expected, the mouse brain cytosol displayed [^{3}H] PDBu-binding activity and the buffer did not.

3.2.4 PROPERTIES OF RBC7ΔDAG

To explore the role of the C1 domain, we studied the properties of rbc7ΔDAG, a mutant containing a deletion of the DAG-binding C1 domain. To measure the transforming potential of rbc7ΔDAG, I assessed its focus-forming activity in rat

fibroblasts. rbc7ΔDAG -expressing rat2 cells produced foci and drug-resistant colonies at a ratio of 0.0 (0/155). Compared to rbc7-expressing rat2 cells, these results indicate that the DAG-binding domain is necessary for the transforming capability of rbc7.

To explore further the functional role of the C1 domain, Drell Bottorff in our lab examined the effects of rbc7 versus rbc7ΔDAG expression on Ras signaling to ERK as well as the subcellular localization of rbc7HA in response to phorbol ester. rbc7HA facilitated sustained activation of ERK in response to PMA treatment and rbc7ΔDAG did not (Ebinu *et al.*, 1998). In addition, rbc7HA translocated to the membrane in response to PMA. No changes in the subcellular localization of rbc7ΔDAG were observed.

3.2.5 EXPRESSION OF RASGRP MRNA IN THE ADULT RAT

In an effort to gain insight into the normal function of RasGRP, I surveyed a wide variety of normal tissues for evidence of expression by using Northern blot analysis (Figure 3-7). Analysis of total RNA prepared from brain, liver, lung, intestine, heart, muscle, spleen and uninfected rat2 fibroblasts indicated that a putative mRNA of approximately 5.6 kb was expressed only in the brain using a ^{32}P-labeled rbc7 probe. This result suggests that the RasGRP transcript is expressed in a tissue specific-manner and normally functions in the brain.

DISCUSSION

3.3 DISCUSSION

In this study, I report the identification of a novel signal transduction element that activates Ras. Amino acid sequence similarity to the CDC25 family of exchangers suggested that RasGRP acts as a positive regulator of Ras proteins. Although RasGRP is similar to the two other classes of exchange factors, based on the sequences of their CDC25-related catalytic domains, RasGRP is distinct in that it possesses two polypeptide motifs, an atypical pair of EF hands and a DAG-binding C1 domain. The deduced structure of RasGRP led us to propose that it functions in a signal transduction pathway that links upstream calcium and lipid signals to downstream Ras.

3.3.1 EXPRESSION OF RBC7 ALTERS THE BIOLOGICAL PROPERTIES OF RAT CELLS

Ectopic expression of rbc7, a truncated version of RasGRP, altered the growth properties of rat fibroblasts. rbc7-expressing rat2 cells exhibit a higher saturation density, modest anchorage-independent growth and a tumorigenic phenotype. Cells that over-express *c-H-ras*, or that were hypersensitive to transformation (rv68BUR), displayed a fully transformed morphology when engineered to express rbc7. These findings suggest that RasGRP is involved in the Ras signaling pathway and supported the hypothesis that it is a positive regulator of Ras.

3.3.2 RasGRP activates Ras *in vitro* and *in vivo*

The catalytic domain of RasGRP functions as a Ras-specific guanyl nucleotide releasing protein *in vitro*. In rat2 cells, ectopic expression of rbc7 increases Ras-GTP levels. Furthermore, PMA treatment induces a higher level of RasGTP in these cells. This rbc7-mediated increase in Ras-GTP correlated with the sustained activation of ERK. These results suggest that RasGRP functions as a DAG-responsive Ras-specific guanyl nucleotide releasing protein and that activation of c-H-Ras by RasGRP triggers the Ras signaling pathway.

3.3.3 RasGRP binds ^{45}Ca *in vitro*

EF hands are helix-loop-helix motifs that are found in a variety of proteins and are known to coordinate the binding of calcium. These proteins can either be calcium storage depots or, like calmodulin, calcium-dependent allosteric regulators (Clapham, 1995). In RasGRP, the EF hands have an atypical structure in that they possess a short spacer region between the calcium binding loops. I have shown that RasGRP binds calcium. In addition, my results suggest that the second EF hand has a higher affinity for calcium.

3.3.4 The C1 domain binds phorbol ester *in vitro*

Diacylglycerol-binding C1 domains are structures that facilitate the binding of proteins to membranes (Zhang *et al.*, 1995). An example of such a domain is found in PKC. C1 domains have conserved cysteine and histidine residues that coordinate with zinc and a defined pocket between two loops that bind lipid (Ono *et al.*, 1989). In some C1 domain-containing proteins, the C1 domain does not bind phorbol esters. The evidence presented here suggests that the C1 domain of RasGRP binds a diacylglycerol analog, and is important for the transforming activity of RasGRP.

3.3.5 The C1 Domain Contributes to Transforming Activity, Subcellular Localization and Ras-ERK Signaling

A central question, crucial for understanding biological signal transduction, is the manner by which ligand-activated receptors stimulate downstream signaling molecules. Membrane recruitment is one general model that has been shown to play a major role in the activation of several signaling molecules such as Sos1. For the Sos family of

exchangers, targeting to the membrane is necessary and sufficient for the activation of the Ras signaling pathway in mammalian cells as well as in yeast (Aronheim *et al.*, 1994). Sos1 is targeted to the plasma membrane whereby it is recruited into the vicinity of membrane-bound Ras. This facilitates its ability to regulate Ras activity. RasGRF1 and RasGRF2 have been shown to be activated by calcium and there is evidence which demonstrates that RasGRF2 translocates to the periphery upon activation (Fam *et al.*, 1997). By analogy, the predicted structure of RasGRP and the results of our experiments with rbc7 suggest that RasGRP translocates to the plasma membrane and that this membrane recruitment is dependent upon the DAG-binding C1 domain. In support of this model, rbc7ΔDAG did not translocate to the membrane fraction nor did it promote sustained activation of ERK (Ebinu *et al.*, 2000). In addition, the DAG-binding C1 domain was shown to be required for RasGRP-mediated sustained activation of ERK.

Activation of RasGRP leads to sustained activation of ERK in rat fibroblasts. There are several reports which state that sustained activation of ERK is associated with its translocation to the nucleus, whereas transient activation is not (Chen *et al.*, 1992; Traverse *et al.*, 1992; Dikic *et al.*, 1994). Thus, transient activation will have very different consequences for gene expression compared with sustained activation because nuclear accumulation of active ERK will result in phosphorylation of transcription factors (Hill *et al.*, 1993; Marais *et al.*, 1993; Xing *et al.*, 1996). Interestingly, in the Aplysia model system for short- and long-term memory, referred to as short- and long-term facililation respectively, it has been observed that ERK activation and nuclear translocation is associated with and necessary for the establishment of long-term but not short-term facilitation in *Aplysia* (Martin *et al.*, 1997; English *et al.*, 1997). Perhaps, one role for RasGRP in the brain is to regulate ERK activity in response to signals that generate Ca^{2+} and diacylglycerol. More specifically, RasGRP may be responsible, at least in part, for the sustained activation of ERK which is necessary and required for synaptic plasticity and long-term memory (Martin *et al.*, 1997; English *et al.*, 1997). Such a role could involve either RasGRP binding to diacylglycerol at the membrane, and/or the activation of PKC resulting in the phosphorylation and modulation of RasGRP-mediated ERK activation. It is also worthy to note that PKC activation can become sustained depending on the combination and levels of lipid messengers and mediators in the membrane (Nishizuka, 1995). This sustained activation of PKC allows

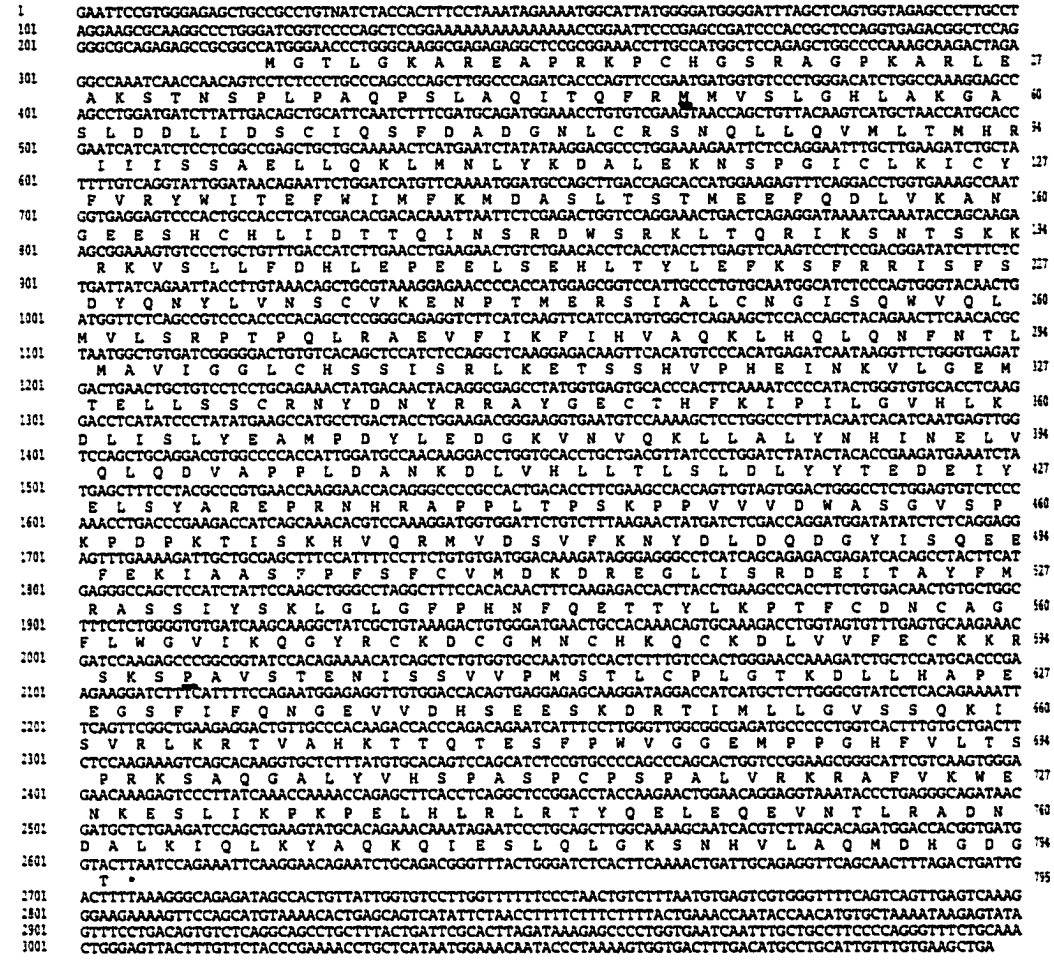
it to play a role in normal processes that occur during long term cellular responses even after both Ca^{2+} and DAG have returned to basal levels. Thus, during such periods of transient increases in Ca^{2+} and DAG, RasGRP could potentially serve as a substrate of constitutively activated PKC, and as a result lead to the sustained activation of ERK.

3.3.6 RasGRP is expressed in the brain

The restricted expression of RasGRP within the nervous system and especially in neurons of the rat hippocampus is intriguing (Ebinu *et al.*, 1998). In neurons, Ras signaling promotes differentiation, axonal growth and cell survival (Finkbeiner and Greenberg, 1996). Neurotrophins also stimulate other signaling proteins such as PLCγ and they effect synaptic behaviors which may or may not involve Ras (Borasio *et al.*, 1993). Expression of RasGRP may allow neurons to integrate receptor-mediated signals from neighboring neurons or supporting glial cells and to impart these messages into changes in biochemical activities that ultimately lead to a programmed biological response.

The interaction of extracellular factors with cell surface receptors triggers a cascade of intracellular biochemical events that modulate both function and expression of target proteins. Growth factors are linked by protein tyrosine kinase receptors to the activation of PLCγ. Hormones are linked by serpentine receptors and heterotrimeric G proteins to the activation of PLCβ. In both cases, activation of PLCβ/PLCγ leads to PIP_2 hydrolysis and results in the production of diacylglycerol and IP_3 which serves to increase intracellular calcium levels. In diverse signaling systems, increases in intracellular calcium and diacylglycerol levels are linked to various cellular responses through their coactivation of protein kinase C at the plasma membrane (Divecha and Irvine, 1995). Based on our findings, signals that generate calcium and diacylglycerol might also be linked to the Ras signaling pathway by RasGRP (Figure 3-8).

A



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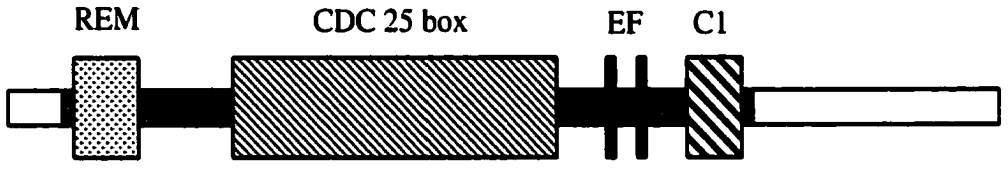


Figure 3-1. Nucleotide and predicted amino-acid sequence of rbc7/RasGRP. A) The sequence of the normal RasGRP coding sequence was deduced from the sequence of the original rbc7 isolate and from overlapping cDNA clones. RasGRP and rbc7 differ at two internal sites: Asp404 is Asn in rbc7 and Gly 576 is Asp in rbc7. The Met and Pro residues are underlined and represent the first and last residues of the deduced rbc7product. rbc7 encodes a 550 residue, 63.4kd product. RasGRP encodes 795 residue 90.3kd product. **B)** Domain map of rbc7/RasGRP. The REM box (▨), CDC25 box (▧), EF hands (■) and C1 domain (▧) are shown . The regions found in RasGRP but absent in rbc7 are shown as white rectangles at the 5' and 3' ends of rbc7 which itself is shaded (■).

A

REM

```
F25B3.3        .elltedqlv arcvecfdvd eedevedief vdalflshqw lsdslslith fvnfy

                       *                                *  *        *  *
RasGRP     58  .KGASLDDLI DSCIQSFDAD .GNLCRSNQL LQVMLTMHRI IISSAELLQK LMNLY
RasGRF1   632  IRYASVERLL ER.......L TDLRFLSIDF LNTFLHSYRV FTDAVVVLDK LISIY
mSOS-1          6KEGTVLKLI ER.......L TYHMYADPNF VRTFLTTYRS FCRPQELLSL LIERF
```

CDC25 SCR #1

```
F25B3.3        fvqa spsdistsls hidyrvlsri

                      *    **  *
RasGRP     201  FDHL EPEELSEHLT YLEFKSFRRI
RasGRF1   1005  FENH SAMEIAEQLT LLDHLVFKSI
mSOS-1     793  LLTL HPIEIARQLT LLESDLYRAV
```

CDC25 SCR #2

```
F25B3.3        ra eilvkfvhva khlrkinnfn tlmsvvggit hssvarlakt y

               *                  * * *             *   **  *
RasGRP     271  RA EVFIKFIHVA QKLHQLQNFN TLMAVIGGLC HSSISRLKET S
RasGRF1   1077  RA STIEKWVAVA DICRCLHNYN AVLEITSSIN RSAIFRLKKT W
mSOS-1     865  RV AVVSRIIEIL QVFQELNNFN GVLEVVSAMN SSPVYRLDHT F
```

CDC25 SCR #3

```
F25B3.3        kfr ipiigvhlkd lvaincsgan

                    * * *       * *
RasGRP     349  HFK IPILGVHLKD LISLYEAMPD
RasGRF1   1156  PC. VPYLGMYLTD LAFLEEGTPN
mSOS-1     942  PC. VPFFGIYLTN ILKTEEGNPE
```

B

EF-Hands

```
F25B3.3        vdavfkhy dhdrdgfisqee fqliagn.........fpfidafvni dvdmdgqiskde lktyfmaa

                   * * * * *****  *                            ***  * **  *   *
RasGRP     475  VDSVFKNY DLDQDGYISQEE FEKIAAS.........FPF..SFCVM DKDREGLISRDE ITAYFMRA
Cal.B       91  LRFAFRIY DMDKDGYISNGE LFQVLKM -[14]- VDK..TIINA DKDGDGRISFEE FCAVVGGL
```

C

C1 Domain

```
F25B3.3        hnfhettflt pttcnhcnkl lwgilrqgfk ckdcglavhs ccksnavaec

               * *    *   ***** *    ***  ***   * ***** *   *   *   *
RasGRP     542  HNFQETTYLK PTFCDNCAGF LWGVIKQGYR CKDCGMNCHK QCKDLVVFEC
PKCδ       231  HRFKVHNYMS PTFCDHCGSL LWGLVKQGLK CEDCGMNVHK KCREKVANLC
```

Figure 3-2. Structural comparison of RasGRP with other proteins. A) Alignment of the REM box and structural conserved regions (SCR) within the catalytic domain of RasGRP with RasGRF1 and mSos-1. B) The EF hands are aligned with those of calcineurin B subunit (Aitken *et al.*, 1984). C) The C1 domain of RasGRP is aligned with that of PKCδ (Aris *et al.*, 1993). In all alignments, residues that are identical or chemically similar are bold; those that are identical are marked with an asterisk. In the alignments of the REM and CDC25 boxes, residues that are underlined in RasGRP are identical or similar to either Sos1 or RasGRF. In the alignment of the EF hands, the underligned residues are those that interact with calcium. In the alignment of the C1 domain, the underligned residues are those that coordinate with zinc atoms and are conserved in all diacylglycerol-binding C1 domains. The nematode sequence F25B3.3 is also aligned with RasGRP (Accession # 1262950). Similarities between RasGRP and the nematode sequence are found throughout the entire sequence and have not been highlighted

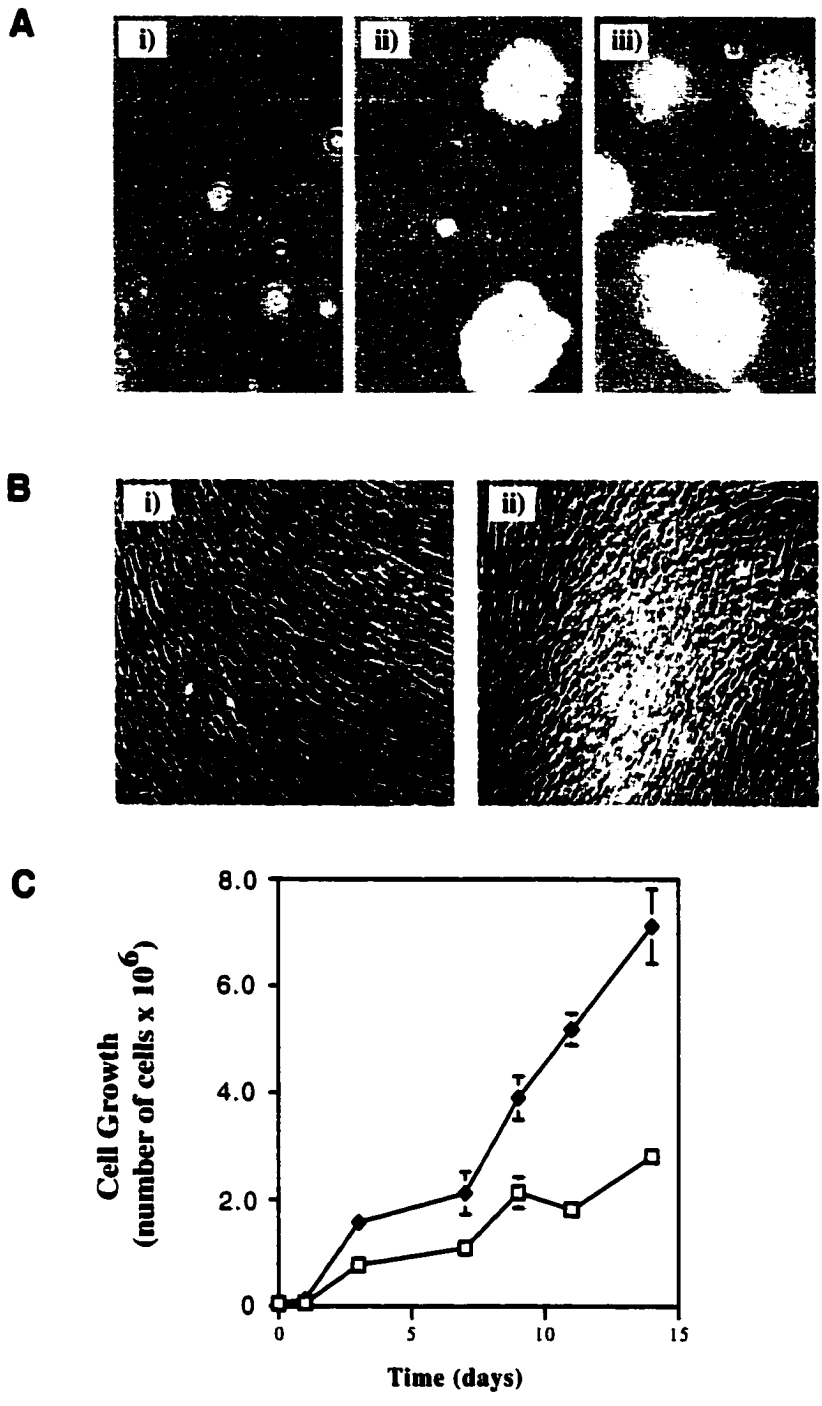


Figure 3-3. rbc7-expressing rat2 cells display transforming phenotypes. A) Anchorage-dependent growth of rat2 cells expressing i) pBabepuro empty vector ii) rbc7 and iii) v-H-Ras. Cells were plated in soft agar and photographed after 14 days of incubation. B) Appearance of transformed foci in rbc7-expressing rat2 cells. Morpholgy of rat fibroblasts infected with either i) empty retrovirus vector pBabepuro or ii) rbc7 21 days after incubation. C) Density-dependent growth curve of rat2 cells expressing either pBabepuro (—□—) or rbc7 (—•—). Pools of cells were plated at a density of 1 x 10^5. Triplicate flasks were harvested and counted with a coulter counter on the days indicated days.

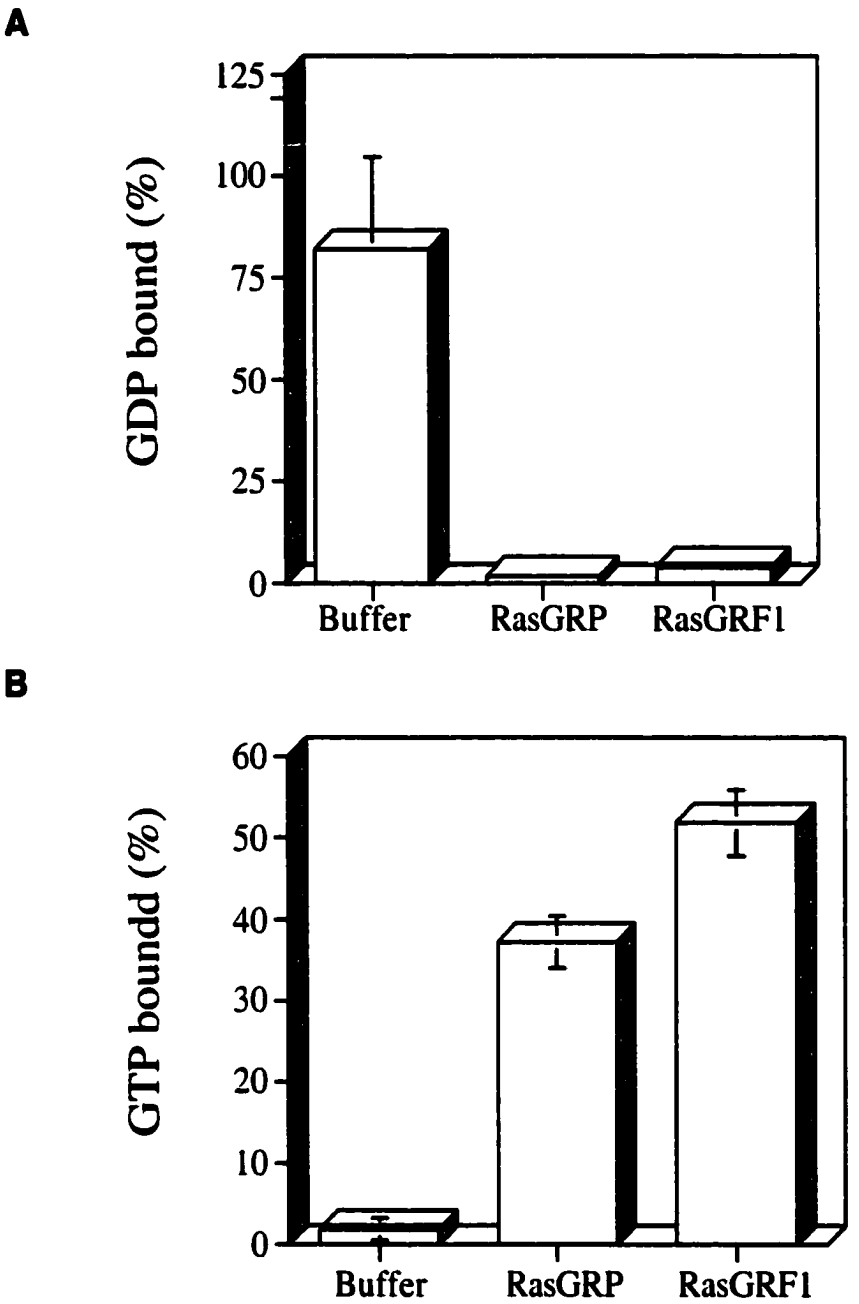
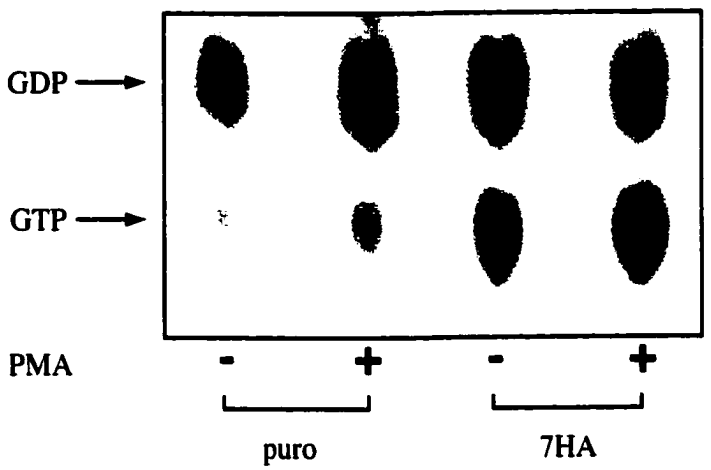


Figure 3-4. Evidence that RasGRP functions as a nucleotide exchange factor for c-Ha-Ras in vitro. A) RasGRP promotes dissociation of Ras-GDP. Purified RasGRP was incubated with [^{3}H] GDP-bound c-Ha-Ras for 30 minutes. Nucleotide release reactions contained 1 ug RasGRP, 1 ug labelled Ras-GDP 100 uM unlabeled GTP and was measured using an immunoprecipitation procedure. B) RasGRP promotes association of Ras and GTP. Ras was incubated with either buffer, RasGRP (catalytic domain) or p30 RasGRF1 (catalytic domain) in the presence of [α^{32}P] GTP. The association of Ras and GTP was monitored after 10 minutes using an immunoprecipitation procedure as described in Chapter 2 (materials and methods). Values are the average of three determinations with the standard error of the mean indicated.

A



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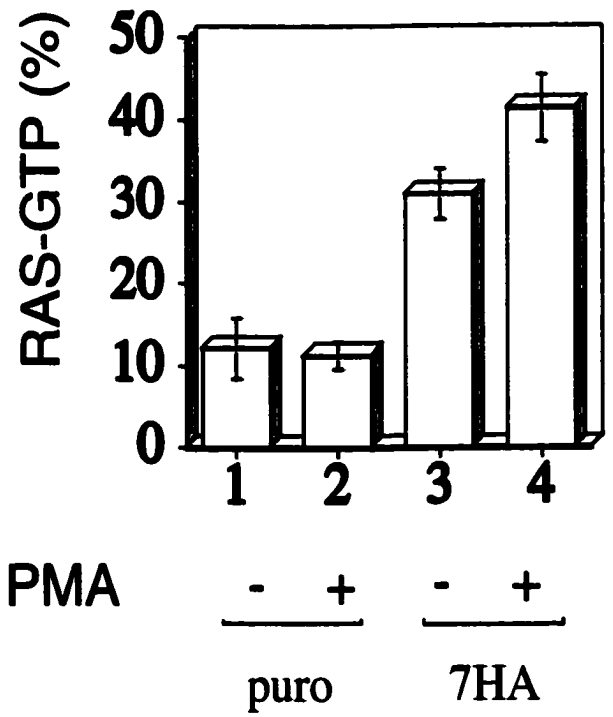

Figure 3-5. RasGRP activates c-H-Ras in vivo. A) Autoradiogram of guanyl nucleotides associated with ras. Rat cells expressing empty puro vector (puro) or rbc7HA (7HA) were labeled with ^{32}Pi and Ras-GTP levels were measured by chromatography on polyethyleneimine plates. B) Results were quantitated by phosphorimager analysis and %Ras-GTP was calculated as described in Chapter 2 (Materials and Methods).

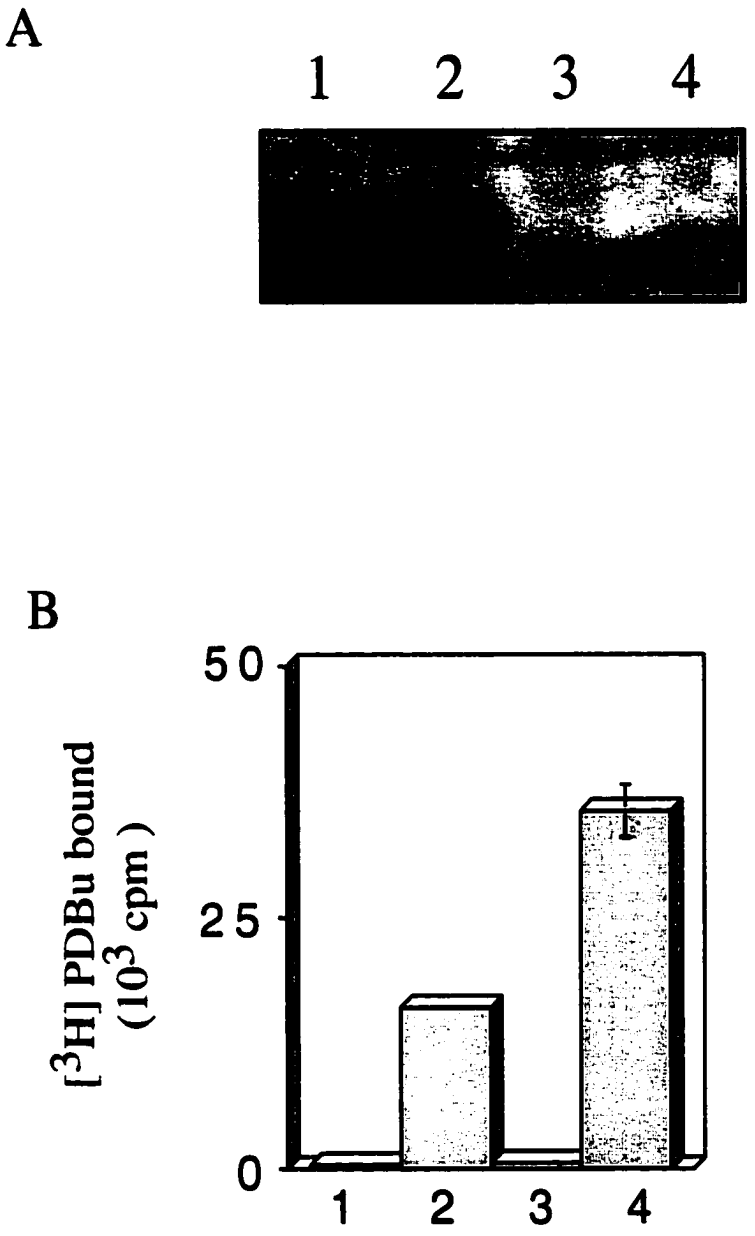

Figure 3-6. Biochemical analysis of rbc7. A) The EF hands of RasGRP bind ^{45}Ca in vitro. GST-rbc7 proteins were resolved by SDS/PAGE, blotted to nitrocellulose and then probed with ^{45}Ca. 1) wildtype; 2) GST-EF1$^-$, (the first EF hand mutated); 3) GST-EF2$^-$, (the second EF hand mutated); 4) GST-EF1$^-$EF2$^-$ (both EF hands mutated). B) The C1 domain of RasGRP interacts with phorbol dibutyrate (PDBu). Various proteins were incubated with labeled PDBu and ligand complexed with protein was assessed by filter binding. 1) buffer control; 2) 50 ug soluble mouse brain protein; 3) 50ug GST protein; 4) 50ug GST-DG protein. Values are the average of three determinations with the standard error of the mean indicated.

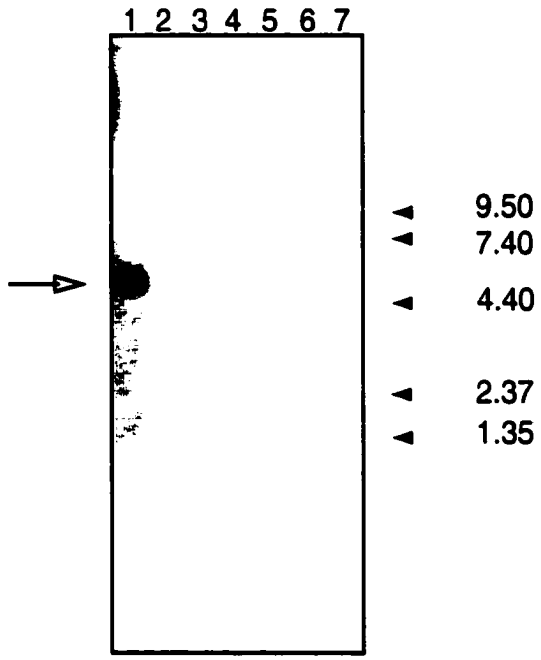

Figure 3-7. Expression of RasGRP mRNA in adult rat tissues. Total RNA was separated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. Filter was hybridized with rbc7 cDNA labeled with [^{32}P] dCTP. Tissues examined included 1) brain, 2) spleen, 3) Intestine, 4) skeletal muscle, 5) heart, 6) lung and 7) rat2 cells. Markers indicated by the arrows are in kilobases. The open arrow represents a 5.6 kb transcript.

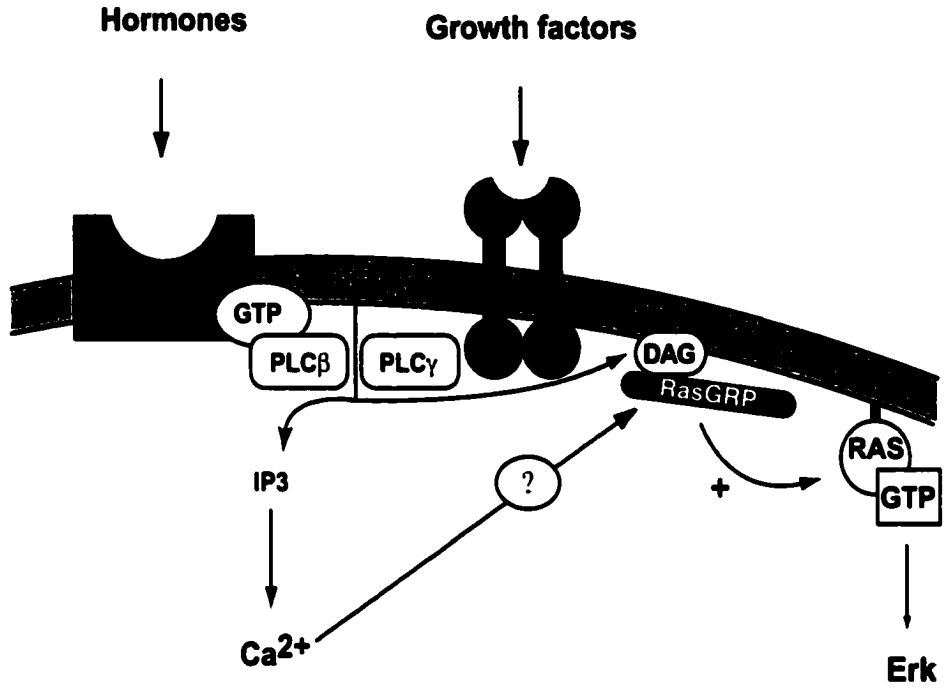

Figure 3-8. Regulation of Signaling by RasGRP. Extracellular signals utilize calcium and diacylglycerol (DAG) to recruit RasGRP to the plasma membrane where it can interact with Ras and catalyze Ras activation. Thus, in cells that express RasGRP, upstream signaling events that generate calcium and diacylglycerol messengers can be linked to Ras output. The role of calcium in regulating RasGRP remains to be determined and this is indicated by the question mark "?".

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CHAPTER 4
RASGRP LINKS T CELL RECEPTOR SIGNALING TO RAS

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Chapter 4

INTRODUCTION

4.1 INTRODUCTION

A crucial event in the mounting of immune responses to most antigens is the clonal activation of antigen-specific T lymphocytes. T cells are able to recognize and respond to antigenic stimuli through the clonotypically expressed T cell receptor (TCR). Engagement of the TCR by antigens or other stimulatory ligands, such as monoclonal antibodies, triggers a cascade of biochemical events which culminate in T cell activation, growth and differentiation. Several groups have outlined a unifying model for the process of signal transduction through the TCR. While our current understanding of the relevant events following TCR stimulation is far from complete, a substantial amount of evidence indicates that an increase in protein tyrosine kinase (PTK) activity represents the initial transduction event induced by TCR crosslinkage. Early events such as activation of tyrosine phosphorylation, elevation of intracellular calcium, activation of lipid-dependent kinases, and activation of Ras and its downstream kinase cascade are known to occur (Weiss and Littman, 1994; Cantrell, 1996). Significant gaps remain in our understanding of TCR signaling, particularly in how the early tyrosine phosphorylation events couple receptor activation to subsequent cellular events. This dissertation provides insight into the as yet incomplete picture with respect to the

biochemistry of TCR-mediated signaling, with particular emphasis on the molecular mechanisms coupling TCR signaling to Ras activation in antigen stimulated T cells.

4.1.1 T CELLS AND THE T CELL ANTIGEN RECEPTOR

The TCR regulates a diverse array of biological responses. During T cell development, signals transduced through the TCR play determinant roles in the positive selection of cells capable of interacting with foreign antigens and also eliminating those cells which recognize self antigens. Mature T cells are activated by interactions with antigen presenting cells (APC) displaying antigenic peptides bound to self-major histocompatibility (MHC) complex–encoded molecules (Weiss and Litman, 1994). Hence activation refers to a highly pleiotropic set of cellular responses that include G0 to G1 phase transition of the cell cycle, and the expression of high affinity receptors for the T cell growth factor interleukin 2 (IL-2).

The primary function of T cells is to recognize and respond to foreign antigenic peptides bound to major histocompatibility (MHC) molecules on antigen presenting cells. Depending on the predisposition of the responding T cells, the triggering of the TCR leads to either cytotoxicity against antigen-expressing cells, as in the case of a cytotoxic T cell, or to the recruitment and activation of other cells of the immune system through the production and secretion of bioactive lymphokines, as in the case of helper T cells. The helper T cells can be subdivided into two distinct subclasses, Th1 and Th2, which secrete distinct sets of lymphokines having quite different effects on the immune response. If the appropriate secondary signals are present, the stimulation of the TCR also results in the progression through the cell cycle and clonal expansion.

Aside from the antigenic signal, T cells require a variety of accessory signals for optimal activation, including molecular interactions with cell-surface molecules of the APCs via CD2, CD5 and CD4/CD8 and CD28 coreceptors. As an effect of TCR signaling that occurs in response to antigen recognition, both APCs and T cells secrete a variety of mediators, including cytokines that also exert their biological effects via specific cell-surface markers. The coordinated production of all these factors on the

differentiation, function and proliferation of lymphoid is essential for the regulation of the immune response.

4.1.1.1 The Structure of the T Cell Receptor

The TCR is a multisubunit complex composed of two clonotipic glycosylated polypeptide chains ($\alpha\beta$ or $\gamma\delta$ heterodimers) which confer antigen specificity to the receptor (Weissman, 1994; Figure 4-1). This heterodimer is expressed at the cell surface in non-covalent association with at least six invariant chains in three pairs: CD3 ($\varepsilon\gamma$), CD3 ($\varepsilon\delta$) and a disulfide-linked homo- or heterodimer of the ζ, η, or γ chains (Weiss, 1993). All three CD3 subunits are non-polymorphic proteins that belong to the immunoglobulin supergene family. The ζ, η, and γ chains are unrelated to the CD3 chains.

The TCR is an oligomeric unit that can be functionally separated into two subunits, the ligand-binding domain and the signal transducing subunits. The ligand-binding domain consists of the $\alpha\beta$ heterodimer, which recognizes and binds to foreign antigenic peptides bound to major histocompatibility (MHC) molecules on antigenic presenting cells. The signal transducing subunit comprises the CD3 complex, and the ζ chains. Each of these signal transducing TCR subunits contains conserved immunotyrosine-based activation motifs (ITAMs) within their cytoplasmic domains (Reth, 1989; Cambier, 1995). The consensus sequence for an ITAM is $YXXL(X)_{6-8}YXXL$. The primary function of the ITAM is to mediate interactions with intracellular signaling molecules.

4.1.1.2 T Cell Receptor-coupled Signaling Pathways

The TCR $\alpha\beta$ heterodimer, together with the T cell-specific CD4 or CD8 coreceptors, are essential for recognition of the MHC antigen complex, while the CD3 components, in concert with CD4 or CD8, direct the intracellular events (Weiss, 1993). At the molecular level, engagement of the TCR is known to elicit a variety of intracellular changes, including early events at the plasma membrane and through cytosplasmic and eventually nuclear events, culminating in transcription of a number of genes (Weiss, 1993). One of the earliest signaling events coupled to the TCR is the activation of several protein tyrosine kinases and the subsequent tyrosine phosphorylation

of multiple intracellular proteins (Weiss, 1993; Wange and Samelson, 1996). Activation of Lck and Fyn, two members of the Src family of protein tyrosine kinases expressed in T cells, appears to be the proximal event in this pathway. It has been proposed that either one or both of these enzymes phosphorylate tyrosine residues found within the ITAMs located in the cytoplasmic tails of the TCRζ and CD3 chains (Iwashima *et al.*, 1994; van Oers *et al.*, 1996). Each ITAM contains two tyrosine residues, which, upon phosphorylation create high affinity binding sites for the tandem SH2 domains of ZAP-70, a Syk family PTK. Lck or Fyn then phosphorylates the bound ZAP-70, resulting in its activation. Hence one function of the src family of tyrosine kinases in TCR signaling is the phosphorylation of membrane-associated ITAMs, a consequence of which is the recruitment and activation of ZAP-70 (Iwashima *et al.*, 1994; Wange *et al.*, 1995; van Oers *et al.*, 1996; Kong *et al.*, 1996).

Two direct consequences of protein tyrosine phosphorylation have been described. First, tyrosine phosphorylation is known to activate enzymes. For example, ZAP-70 must be phosphorylated for full enzymatic activity (Chan *et al.*, 1995; Wange *et al.*, 1995). Also, PLCγ1, which upon activation hydrolyzes phosphoinositides to generate products that result in an increase in intracellular calcium stores, is responsible for subsequent activation of PKC (Rhee and Bae, 1997). Second, tyrosine phosphorylation provides binding sites for SH2 containing proteins (Pawson, 1995). Of particular importance are the adaptor molecules Shc, Grb2, LAT and SLP-76, all of which contain protein-protein interacting domains

4.1.2 CONSEQUENCES OF TCR-INDUCED TYROSINE PHOSPHORYLATION

Several downstream signaling pathways, which are indirectly controlled by tyrosine phosphorylation, have been documented. The signaling events downstream of protein tyrosine phosphorylation following TCR engagement include activation of the phosphatidyl inositol pathway, activation of Ras and that of several serine/threonine protein kinases and phosphatases. The coordination of these proceedings is crucial for T cell activation.

4.1.2.1 Activation of phosphoinositide turnover pathways

Stimulation of TCR proliferation requires the activation of multiple signaling pathways which are coordinated to drive specific events during the course of the cell cycle. One of the main transducers activated by the TCR is PLCγ1, which is responsible for hydrolyzing PIP_2 into the second messengers inositol 1,4,5-trisphosphate (IP_3), which increases intracellular calcium, and diacylglycerol (DAG). The importance of these two second messengers is evident from the fact that T cell activation can be achieved by combining a Ca^{2+} ionophore to provide a Ca^{2+} signal with a phorbol ester which is known to activate PKC in T cells (Downward *et al.*, 1990). The hydrolysis of PIP_2, therefore, initiates two separate signaling pathways, both of which are essential for T cell activation (Figure 4-1).

As described earlier, one of the major signaling pathways through which the tyrosine kinases act is mediated by phospholipase C (PLC) (Weiss *et al.*, 1991). Within 1min of TCR stimulation, PLCγ is recruited to the plasma membrane, via protein-protein interactions with LAT, and becomes tyrosine phosphorylated (Weiss *et al.*, 1991). This event is important for PLCγ activation and is associated with an increase in PLCγ catalytic activity (Weiss and Littman, 1994). While it is not known which specific tyrosine kinase activates PLC, there is some evidence that PLCγ is phosphorylated by Lck or Fyn (Liao *et al.*, 1993). Alternatively, PLC may be phosphorylated by ZAP-70 or the Tec family of tyrosine kinases (Isakov, 1993). As previously noted, PLC is responsible for the hydrolysis of PIP_2, resulting in generation of IP_3, a common second messenger in signal transduction. IP_3 is involved in a well-described effect of TCR activation, the mobilization of Ca^{2+} from intracellular stores (Berridge, 1993). Increase in intracellular Ca^{2+} stores leads to the activation of the calcium/calmodulin-dependent serine phosphatase calcineurin (Crabtree and Clipstone, 1994). The importance of calcineurin was revealed when it was identified as the target of the immunosuppressants cyclosporine A and FK506 (Schreiber and Crabtree, 1992). Indeed, expression of a catalytically active form of calcineurin mimics the effects of calcium ionophore in T cell activation and, in conjunction with activated Ras, provides sufficient stimulus to induce IL-2 transcription in Jurkat T cells (Woodrow *et al.*, 1993). The target of calcineurin is the family of NFAT transcription factors (Clipstone and Crabtree, 1992). All NFAT isoforms translocate to the nucleus in response to calcium-dependent signals, and all also

share the ability to interact with AP-1 and to bind cooperatively to the composite NFAT/AP-1 site in the IL2- gene. Of particular importance is the finding that mice bearing a targeted disruption of the NFAT1 gene develop splenomegaly with hyperproliferation of both B and T cells (Hodge *et al.*, 1996; Xanthoudakis *et al.*, 1996). These results indicated that NFAT1 might participate in a previously unrecognized negative regulatory pathway during T cell activation. Another calcium/calmodulin-dependent enzyme activated after TCR stimulation, CAM-kinase II, has been reported to play an inhibitory role in IL-2 induction (Nghiem *et al.*, 1994; Hama *et al.*, 1995). The relationship between these two calcium/calmodulin-dependent pathways is not well understood.

The second product of PIP_2 hydrolysis is DAG. Previous studies reported that phorbol esters were mitogenic for lymphocytes (Touraine *et al.*, 1977; Abb *et al.*, 1979). Having identified PKC as a major phorbol ester receptor (Nel *et al.*, 1983), the role for this enzyme in T cells became apparent. In agreement, high levels of PKC activity were found in human peripheral blood lymphocytes (PBL) and PKC was subsequently shown to be activated in response to various T cell agonists including PMA and IL-2 (Farrar and Anderson, 1985; Kaibuchi *et al.*, 1985).

The use of monoclonal antibodies to the TCR-CD3 complex lead to the identification of PKC as a downstream signaling molecule following TCR stimulation. Further studies illustrated that PKC translocation and substrate phosphorylation were an important consequence of T cell activation via TCR-CD3 complex (Nel, 1990). Hence, following an increase in DAG and Ca^{2+} levels in response to TCR stimulation, PKC becomes activated. Upon activation, PKC translocates from the cytosol to the plasma membrane and is rapidly cleaved, leading to proteolytic degradation (Clemens *et al.*, 1992). Several isoforms of PKC are thought to be involved in T cell proliferation. PLCγ can activate PKCα within 10 minutes of stimulation, whereas PKCβ activation is sustained and probably occurs via DAG activated through phospholipase A2 or calcium-mediated mechanisms (Szamel *et al.*, 1995). In T lymphocytes, the PKC isoform primarily responsible for AP-1 activation is PKCθ (Baier-Bitterlich *et al.*, 1996).

4.1.2.2 Activation of Ras

PKC phosphorylates and activates a wide range of proteins. It can activate Raf-1 (Kolch *et al.*, 1993) and possibly MEKK (Blumer *et al.*, 1994) directly. However, stimuli that activate Erk do not necessarily go through PKC. An alternative or interdependent pathway by which external stimuli activate the MAP kinases is through members of the Ras superfamily.

TCR stimulation leads to the rapid accumulation of active Ras-GTP complexes (Downward *et al.*, 1990). The activity of Ras proteins appears to be essential for TCR signaling and for cellular response, such as TCR and PKC regulation of NFAT, and IL-2 synthesis in activated T cells (Baldari *et al.*, 1993; Rayter *et al.*, 1992; Woodrow *et al.*, 1993a, 1993b). The importance of the Ras-Erk signaling pathway in T cells is underscored by studies of this pathway in antigen-unresponsive T cells, also referred to as anergic T cells (Fields *et al.*, 1996; Li *et al.*, 1996). These cells have a block in the activation of the Ras-Erk cascade and fail to produce IL-2. The importance of Ras was demonstrated further by co-transfection experiments which examined the consequences of expressing constitutively active or dominant inhibitory Ras mutants on the activity of co-expressed reporter genes for different transcription factors. Expression of constitutively active mutants of Ras replaced the requirement for PKC stimulation in the activation of NFAT and IL-2 synthesis during T cell activation. Furthermore, when expressed in T cells, oncogenic Ras (v-Ras) can activate transcriptional factors such as AP-1 and also synergize with a calcium signaling pathway to activate NFAT and the IL-2 gene (Woodrow *et al.*, 1993; Rayter *et al.*, 1992). The contribution of Ras in NFAT inductions is thought to reflect the involvement of Ras in the regulation of AP-1, although it is also possible that Ras signals can induce posttranslational modifications of NFAT proteins and thus directly modify their transcriptional activity. Dominant inhibitory mutants of Ras were shown to specifically block TCR signaling (Baldari *et al.*, 1992, 1993; Rayter *et al.*, 1992; Woodrow *et al.*, 1993). Moreover, expression of a dominant negative mutant of Ras inhibited several activation pathways, suggesting that Ras activity might be necessary at more than one point in the transduction of signals in T lymphocytes (Wotton *et al.*, 1993). The critical role of Ras in T cell development has also been described (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995; Swat *et al.*, 1996; Crompton *et al.*, 1996).

In T cells, phorbol esters which activate PKC can mimic aspects of TCR signaling and induce the accumulation of active Ras-GTP complexes (Downward *et al.*, 1990). There are two proposed mechanisms for Ras regulation: one PKC-dependent and the other PKC-independent. The molecular mechanism of the PKC-independent pathway is not clearly defined but it is known to be mediated by tyrosine phosphorylation. This is supported by the observation that when PKC signaling is blocked, TCR signaling to Ras still occurs (Izquierdo *et al.*, 1992). Furthermore, a potent tyrosine kinase inhibitor, herbimycin A, blocks the PKC-independent activation of Ras (Ohtsuka *et al.*, 1996). The PKC-dependent mechanism is compatible with previous studies in which PKC mediates TCR regulation of Raf-1 and Erk (Nel *et al.*, 1990; Siegel *et al.*, 1990).

Several models have been described in an attempt to explain how the TCR regulates the activation of Ras. One proposed pathway for Ras activation involves the tyrosine phosphorylation, by ZAP-70, of a membrane-associated adaptor, LAT (Izquierdo *et al.*, 1995). The tyrosine phosphorylation of LAT allows it to interact with another adaptor protein, Grb2, through Grb2's SH2 domain. As mentioned earlier, Grb2 has two SH3 domains and both of these can bind to Sos. Sos–Grb2 complexes exist in unstimulated cells and it is thought, based on studies in other cell types, to be the relocation of Sos, to the membrane, as LAT is membrane-bound, that results in increased Ras activation (Figure 4-1; Izquierdo *et al.*, 1995). However, this has not been clearly demonstrated in T cells.

Another pathway, by which TCR signaling may affect Ras, is through the activation of PKC. In T cells, direct activation of PKC through treatment with phorbol esters results in Ras activation (Downward *et al.*, 1990). Since stimulation via TCR activates PKC, it has been proposed that PKC indirectly regulates Ras activity. A considerable increase in Ras-GTP has been observed in addition to a diminished activity of GAPs, due to direct (or indirect) phosphorylation of GAPs by PKC (Downward *et al.*, 1990; 1992). In T cells, the observed stimulation of Ras via TCR or by phorbol ester correlated with a rapid decrease in the level of GAP activity.

It is still not clear how GAPs are regulated. The most probable mechanism for regulation of GAPs would be direct phosphorylation by PKC or other kinases involved in the PKC-independent stimulation of Ras. Inhibition of GAPs by receptor tyrosine kinases has been reported (Schlessinger, 1993; Polakis and McCormick, 1993).

Alternatively, the regulation of GAPs might involve phosphorylation of further proteins regulating GAPs. Plasma membrane associated p62dok, phosphorylated on tyrosine in response to receptor stimulation, and cytosolic p190, phosphorylated predominantly on S/T, were identified as putative regulators of p120GAP (Carpino *et al.*, 1997; Molloy *et al.*, 1989; Anderson *et al.*, 1990; Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauzkas *et al.*, 1990; Moran *et al.*, 1991). Association of p120GAP with p190 inhibits its GTPase activating activity suggesting that, upon T cell activation, inhibition of GAP activity could occur (Downward *et al.*, 1992).

The function of calcium, PKC and Ras signals in TCR signaling has been studied in the context of IL-2 production in response to TCR stimulation. The role of calcium and PKC in T cells has been explored by examining the consequences of stimulating T cells with calcium ionophores that elevate intracellular calcium and phorbol esters that activate PKC. The results of such experiments demonstrated that the combination of these two pharmacological activators could substitute for TCR triggering in the induction of the IL-2 gene, a fact suggesting that TCR control of calcium and PKC was sufficient to explain TCR control of T cell activation. It is now recognized, however, that in T cells phorbol esters are very effective at activating Ras. In addition, it is becoming apparent that many of the effects of phorbol ester previously attributed to PKC are in fact Ras mediated (Downward *et al.*, 1990).

In Chapter 3, my results showed that RasGRP is expressed in a tissue-specific manner. In particular, RasGRP mRNA was detected in the brain. While RasGRP is expressed in the brain, a RasGRP cDNA was isolated by Dr. R. Kay's group from a T cell library (Tognon *et al.*, 1998). Subsequent studies showed that RasGRP mRNA (Tognon *et al.*, 1998; Kawasaki *et al.*, 1998) and protein (Ebinu *et al.*, 2000) is expressed in lymphoid cells and a variety of T cell lines. The failure to detect RasGRP in the thymus and spleen by Northern blot analysis in my earlier experiments (Figure 3-7) is attributed to technical difficulties. Given that RasGRP is expressed in lymphoid cells, I decided to test the hypothesis that RasGRP links TCR signaling and phorbol esters to Ras.

RESULTS

4.2 RESULTS

4.2.1 RasGRP Activates Ras in Jurkat T cells

The activities of RasGRP *in vivo* were initially characterized by studies involving ectopic expression of RasGRP in rat2 fibroblasts. Since RasGRP is normally expressed in T cells (Tognon *et al*, 1998; Kawasaki *et al.*, 1998), I wanted to establish a role for RasGRP in T cells. Given that TCR receptors stimulate the production of DAG and Ca^{2+}, the two proposed regulators of RasGRP activity, I asked whether RasGRP is involved in mediating TCR signaling to Ras. To address this question, I developed an *in vitro* assay to measure the total activities associated with Ras guanyl nucleotide exchange factors (GEFs) in Jurkat T cell membrane fractions.

Jurkat T cells can be stimulated with antibodies such as OKT3, which recognize the extracellular region of the ε chain of CD3, a component of TCR. Treatment with soluble OKT3 activates Ras within 5 minutes in Jurkat T cells. To measure total RasGEF activities associated with this Ras activation, equal numbers of cells were stimulated with OKT3. Control cells were left untreated. Following a 5-minute incubation at 37 °C, cells were resuspended in a hypotonic buffer and membranes were prepared by dounce homogenization followed by ultracentrifugation at 100,000g. To measure total RasGEF activity, membranes from treated and control cells were incubated at 30 °C for 1 minute

in the presence of [α^{32}P]GTP. After incubation, the amount of [α^{32}P]GTP associated with endogenous Ras was measured by immunoprecipitating Ras with the Y13-259 monoclonal antibody. Thereafter, guanyl nucleotides were eluted from Ras, resolved by thin layer chromatography and quantified by phosphor-imager analysis. As a positive control, and to determine the maximal level of exchange, membranes prepared from unstimulated cells were incubated at 30 °C for 5 minutes in the presence of EDTA, to chelate Mg^{2+} and stimulate GDP dissociation, thus allowing for [α^{32}P]GTP to associate with all functional Ras. Ras-GTP complexes were then locked-on by the addition of excess Mg^{2+}.

Stimulation of Jurkat T cells with OKT3 for 5 minutes resulted in a prompt 5.9-fold increase in the ability of subsequently isolated membranes to support the transfer of [α^{32}P]GTP to endogenous, membrane-bound Ras (Figure 4-2A). In both the stimulated and untreated membranes, much of this newly associated guanyl nucleotide was converted to GDP during the course of the reaction, a phenomenon that I attribute to the presence of Ras GAPs in these membrane preparations. Ras is constitutively associated with the plasma membrane and we found TCR stimulation does not affect the amount of Ras in T cell membrane preparations (data not shown).

4.2.2 Effect of purified anti-RasGRP antibodies (J32T) on Ras activation in Jurkat membrane fractions

To determine what fraction of total RasGEF activity is attributed to the activity of RasGRP, I assessed the ability of OKT3 treated Jurkat T cells to stimulate guanyl nucleotide exchange in the presence of antigen affinity purified antibodies (J32) directed against the catalytic domain of RasGRP (residues 49-473). Membranes from stimulated and unstimulated cells were preincubated with immune or preimmune serum from the same rabbit for 5 minutes on ice. These membrane fractions were then assayed for their ability to catalyze the association of [α^{32}P]GTP with endogenous Ras. I found that stimulation of Jurkat T cells with OKT3 for 5 minutes resulted in a 5.9-fold increase in [α^{32}P]GTP -association with endogenous Ras (Figure 4-2A). Importantly, I found that stimulated labeling of Ras could be blocked by adding purified antibodies (J32) directed against RasGRP catalytic domain (Figure 4-2A). There was no significant effect of the purified antibodies on basal guanyl nucleotide exchange activity.

4.2.3 Effect of J32 on RasGRP and Sos activities *in vitro*

As previously noted, the catalytic domain of RasGRP is highly conserved among the RasGEFs. Since the antibodies used in the above experiment were directed against this highly conserved catalytic domain, I questioned the specificity of these antibodies. The only other known RasGEFs expressed in T cells are the Sos proteins. To demonstrate the specificity of the affinity purified antibodies directed against the catalytic domain of RasGRP, I conducted an *in vitro* exchange assay using recombinant RasGRP and Sos. Purified RasGRP and Sos proteins were preincubated with pre-immune and immune serum for 5 minutes on ice. Thereafter, the RasGEFs were tested for their ability to catalyze the association of [α^{32}P]GTP with recombinant Ras. In this *in vitro* experiment with purified proteins, J32 antibodies specifically inhibit the Ras GEF activity of recombinant RasGRP but not that of Sos (Figure 4-2B).

4.2.4 RasGRP activates Ras and Ras-Erk signaling in response to TCR signaling

The presence of RasGRP in T cells suggests a role for RasGRP in regulating the activity of Ras during TCR signaling. By immunoblot analysis, RasGRP has been detected in several T cell lines as well as in primary mouse thymocytes (Ebinu *et al.*, 2000). To establish a role for RasGRP in T cells, other investigators in the lab determined the effects of excess RasGRP on TCR signaling to Ras. Using a bovine papiloma-based vector with an inducible promoter, human RasGRP cDNAs were over-expressed in Jurkat T cells (Ebinu *et al.*, 2000). Over-expression of RasGRP was shown to enhance OKT3-induced levels of Ras-GTP. This increase in the accumulation of Ras-GTP complexes was sustained when compared to Jurkat cells expressing physiological levels of RasGRP. We also found that over-expression of RasGRP enhanced acute activation of Ras by PMA. In this experiment, the level of phospho-ERK paralleled the level of Ras-GTP. In an independent study, we determined the effect of RasGRP over-expression on the level of Erk activation at various concentrations of OKT3. Using the phosphorylation state of Erk as a marker for its activation, we found that RasGRP increased the absolute amount activated Erk observed at a saturating amount of antibody.

Additionally, over-expression of RasGRP decreased the concentration of OKT3 required to elicit a given level of Erk phosphorylation (Ebinu *et al.*, 2000).

TCR stimulation results in the activation of PLCγ which ultimately leads to an increase in intracellular calcium and DAG. Agonists that increase calcium and DAG levels in rat fibroblasts, such as endothelin-1, are known to activate RasGRP (Ebinu *et al.*, 1998). To determine whether PLCγ activation in response to TCR ligation is required for RasGRP activation, we examined the effects of a specific PLCγ inhibitor (U73122) on TCR-induced Ras activation in Jurkat T cells. When OKT3-treated Jurkat T cells were preincubated with the U73122 PLCγ inhibitor, we found that Ras activation was dramatically reduced (Ebinu *et al.*, 2000). Specifically, inhibition was 2.8-fold when saturating amounts of OKT3 was used and 5.3-fold in the presence of sub-optimal amounts of OKT3. The specificity of this inhibition was demonstrated by showing that the inert analogue U73343 had no effect.

4.2.5 Subcellular redistribution of RasGRP in response to TCR signaling

Based on studies in rat fibroblasts which ectopically expressed RasGRP, we proposed a model whereby RasGRP relocates to the plasma membrane in response to extracellular stimuli that increase DAG and calcium signals. To test this model in cells that normally express RasGRP, I asked whether the subcellular localization of RasGRP was affected in response to TCR activation. Membrane (P100) and cytosolic (S100) fractions were prepared from OKT3-stimulated and control cells using sub-cellular fractionation techniques. RasGRP from untreated cells was found in both the soluble and particulate fractions (Figure 4-3A). After 5 minutes of OKT3 treatment, a reproducible (n= 7) decrease in the relative amount of soluble RasGRP was detected over a time period of 2 hours. In a similar time-course experiment performed by other investigators in the lab, this change in RasGRP fractionation behavior closely paralleled the activation of Ras (Ebinu *et al.*, 2000).

4.2.6 RasGRP is required for CREB activation in primary thymocytes

To ascertain the function of RasGRP, our lab generated RasGRP knock-out mice. These mice were phenotypically normal, but displayed certain defects with respect to T

cell development and TCR signaling (Dower *et al.*, 2000). In particular, thymocytes of RasGRP -/- mice failed to develop into mature CD4+ or CD8+ cells. Of particular interest was the finding that thymocytes of RasGRP -/- mice failed to activate Erk in response to DAG analogues PMA and bryostatin-1. These findings are consistent with the idea that RasGRP mediates the activation of the Ras-Erk cascade in response to phorbol esters.

Previous studies demonstrated that transcriptionally active cAMP-responsive element-binding protein (CREB) is requried for T cell activation of normal murine T cells following stimulation of the TCR (Barton *et al.*, 1996). Quiescent T cells contain inactive, unphosphorylated CREB. In response to TCR engagement, CREB becomes activated by the phosphorylation of Ser 133. Importantly, transgenic mice expressing a dominant negative unphosphorylated form of CREB exhibit a profound T cell proliferative defect characterized by G1 cell cycle arrest, significant decrease in IL-2 synthesis, and defective transcriptional induction of multiple Fos and Jun proteins (Barton *et al.*, 1996). These findings were consistent with earlier reports which demonstrated that T cell activation results in CREB phosphorylation and increased CREB DNA-binding activity (Wolberg *et al.*, 1994; Xie *et al.*, 1993, 1995).

Until recently, the signaling pathways that regulate CREB phosphorylation following TCR ligation were not known. In a recent report by Muthsuamy and Leiden (1998), CREB was shown to be activated by a PKC-Ras-Raf-MEK-RSK2 pathway. To determine whether RasGRP is responsible for activating Ras and ultimately CREB in response to TCR signals, in collaboration with other investigators in the lab, I asked whether CREB is activated in RasGRP +/+ versus -/- primary thymocytes in response to phorbol esters. Dissociated thymocytes from +/+, +/- and -/- mice were stimulated for 10 minutes with PMA and bryostatin-1, a heterocyclic lactone that also acts a DAG analogue. Thereafter, cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody specific for the active phosphorylated form of CREB. In both PMA and bryostatin-1-treated thymocytes, there was a significant induction of CREB phosphorylation in +/+ and +/- RasGRP thymocytes when compared to untreated cells (Figure 4-3B). However, in the -/- mutant thymocytes the activation of CREB was significantly reduced. As a loading control, total cell lysates were probed with an anti-Erk antibody to detect total ERK levels in an immnuoblot (Dower *et al.*, 2000).

4.2.7 Over-expression of RasGRP potentiates IL-2 production in Jurkat T cells

While TCR signaling can ultimately lead to IL-2 synthesis and secretion in a normal T cell response, treatment of Jurkat T cells with soluble OKT3 does not. Instead, a calcium ionophore and a DAG analogue such as PMA, which are thought to activate the calcineurin and PKC pathways respectively, can be used to stimulate IL-2 in Jurkat T cells. To determine the effects of RasGRP over-expression on IL-2 production in Jurkat T cells, we measured IL-2 levels in RasGRP-overepxressing Jurkat T cells. We found that by over-expressing RasGRP, significantly more IL-2 was synthesized when compared to control cells upon dual stimulation (Ebinu *et al.*, 2000). Interestingly, this effect was also observed when a combination of calcium ionophore and bryostatin-1 was used. Insignificant amounts of IL-2 were produced when no agonist or only one agonist was used.

DISCUSSION

4.3 DISCUSSION

The signaling events downstream of protein tyrosine phosphorylation following TCR stimulation include activation of the phosphatidyl inositol pathway, activation of Ras and that of several serine/threonine protein kinases and phosphatases. These events have been casually linked to a variety of cellular responses, of which the best characterized one is the transcriptional induction of the interleukin-2 (IL-2) gene in T cells. The calcium signaling system that cooperates with Ras is apparently controlled by Src family kinases and is mediated by the calcium phosphatase, calcineurin. The signal transmitted to the nucleus is proposed to involve the regulation of a Raf-MEK-Erk kinase cascade. At least two intracellular pathways for regulation of this cytoplasmic protein kinase cascade coexist in T cells. One is mediated by Ras, the other by protein kinase C. A cross-talk between the T cell receptor-stimulated mechanisms appears to modulate cellular responses.

The presence of RasGRP in T cells suggests a role for RasGRP in regulating the activity of Ras during TCR signaling. In support of this, we found that over-expression of RasGRP in T cells leads to more pronounced and sustained levels of Ras activation in response to TCR signaling. In addition, independent experiments conducted by others in the lab showed that Jurkat T cells that over-express RasGRP are hyper-sensitive to TCR-

Ras-Erk signaling and hyper-sensitive to agonist-induced IL-2 secretion (Ebinu *et al.*, 2000). Using a novel *in vitro* assay to measure RasGEF activity, I demonstrated that total RasGEF activity is increased in response to OKT3 treatment. Additionally, this TCR-induced increase in RasGEF activity is completely blocked by purified antibodies which were raised against the catalytic domain of RasGRP. These antibodies are specific for RasGRP and have no effect on the exchange activity of Sos, a ubiquitously expressed Ras activator. This data suggests that RasGRP activates Ras in response to TCR signaling. Additionally, it provides a role for RasGRP in the biological responses attributed to T cell activation, since over-expression of RasGRP potentiated IL-2 synthesis.

The generation of RasGRP knock-out mice has provided insight into the function of RasGRP in thymocytes. Dower *et al.* (2000) have shown that thymocytes of RasGRP null mice fail to develop into single positive mature T cells. These findings suggest that RasGRP is involved in thymocyte development. In addition, thymoctes from RasGRP -/- mice fail to activate Ras and ERK in response to DAG and bryostatin-1. These findings indicate that RasGRP is required for the activation of the Ras-Erk pathway in response to phorbol esters. They also confirm the results described in this dissertation and support a role for RasGRP in TCR signaling. In agreement, my analysis of the transcription factor CREB denotes a role for RasGRP in the activation of the Ras-ERK-CREB signaling pathway in T cells. Given the importance of CREB in T cell activation these observations confirm the role of RasGRP in TCR signaling.

Our model based on studies in rat fibroblasts suggests that RasGRP relocates to the plasma membrane in response to signals that increase intracellular diacylglcyerol levels. In accordance with this model, I demonstrated that the subcellular localization of RasGRP is affected by TCR signaling. Significantly, this redistribution of RasGRP to the membrane fraction closely paralleled the activation of Ras in a time course experiment. Moreover, other investigators in the lab showed that a PLCγ inhibitor diminished TCR-induced activation of Ras. This data supports our original model whereby the activity of RasGRP is regulated by its subcellular localization in the cell. These studies also provide a novel mechanism for the activation of Ras in T cells whereby receptor stimulation leads to activation of PLC-γ1 followed by the generation of membrane DAG, RasGRP membrane recruitment and Ras activation. One could propose that PLC-γ1-generated free cytoplasmic calcium regulates RasGRP through its EF hands. However, we

observed that RasGRP can activate Ras *in vivo* even in the presence of EGTA plus BAPTA/AM, compounds that sequester extracellular and intracellular calcium, respectively (Stang and Stone, unpublished data). In addition, our model may help explain several facts about TCR signaling including the observation that stimulation of PLC-γ1 by ectopically expressed G-protein-coupled receptors leads to T cell activation (Desai *et al.*, 1990).

Engagement of the TCR results in the activation of PLCγ and the consequent generation of the second messengers DAG and IP_3. While the latter stimulates calcium mobilization, DAG directly activates the PKC family of related S/T kinases, which have been widely studied in various signaling systems (Szamel and Resch, 1995). Despite extensive studies using phorbol esters, which bind to and activate novel and conventional PKCs, the importance of PKC activation in TCR signaling remains obscure. This is primarily due to the fact that although PKC stimulates Ras activation, TCR-induced Ras activation is most entirely PKC-independent (Izquierdo *et al.*, 1992). Thus, much of the literature on PKC effects is confounded by superimposed Ras-mediated phenomena.

Conversion of Ras-GTP back to inactive state is brought about by GTP hydrolysis, an event mediated by GAPs. In T cells, stimulation by anti-CD3 antibodies or by PKC activators rapidly increases Ras-GTP from 5-80% (Downward *et al.*, 1990). Such increases in RasGTP are also apparent in fibroblasts stimulated with insulin (Burgering *et al.*, 1991). In these cells the exchange rate has been reported to be extremely high, hence inhibition of GAP activity may explain the accumulation of RasGTP. One implication is that Ras is dynamically cycling between GDP and GTP forms. In unstimulated cells, the GTPase reaction is faster than the GTP/GDP exchange rate, so that the GDP form of Ras is more abundant. To increase the fraction of Ras in the GTP state from 10-90 %, would require a 100-fold decrease in the ratio of exchange rate to GTPase rate (exchange rate/GTPase rate =100) (McCormick, 1988). To date, the changes in GAP activity observed following T cell activation are much less than this (6-fold). Therefore, there is no extant data that provides a full quantitative model for this mode of Ras activation in T cells.

In this dissertation, I describe a novel phorbol ester receptor present in T cells. The finding that RasGRP activates Ras in response to TCR signaling suggests that the effects previously attributed to PKC may in fact be RasGRP-mediated. Although our

findings do not address the role of Sos in TCR signaling, nor do they preclude a role for PKC down-regulation of Ras GAPs, they do suggest that RasGRP plays a significant role in coupling TCR signals to Ras. In either case, several forms of Ras regulation might allow a common Ras switch to differentially respond to diverse external stimuli and developmental cues, and thereby orchestrate distinct biochemical changes and biological responses. This is in agreement with studies in other cell types whereby the amplitude or duration of Ras signaling is varied according to the combination of regulatory mechanisms deployed.

Ras-Erk signaling is known to play important roles at a number of stages in the life of a T cell. Early growth and maturation in the thymus are dependent on a pre-TCR complex (Malissen and Malissen, 1996) and on Ras-Erk signaling (Crompton *et al.*, 1996; Swan *et al.*, 1995). After T cell maturation, autocrine and paracrine action of IL-2 is thought to contribute to Ras activation (Graves *et al.*, 1992; Gomez *et al.*, 1997). RasGRP could facilitate Ras activation during these stages, as well.

Of particular interest is the finding that the DAG analogue bryostatin-1 binds to (Lorenzo *et al.*, 2000) and activates RasGRP (Ebinu *et al.*, 2000). Bryostatin-1 is currently being tested in clinical trials as a cancer chemotherapeutic agent for certain types of leukemia, lymphoma and melanoma (Pluda *et al.*, 1996). The cytostatic effects of bryostatin-1 are attributed to the activation and degradation of PKC. However, given that RasGRP is expressed in certain lymphoid cells, our results suggest that RasGRP mediates some of the effects of bryostatin-1. In addition to the observations by Dower *et al.* (2000), our results will definitely necessitate the re-evaluation of the current clinical trials that aim to exploit the cytostatic effects of byrostatin-1. Importantly, the ability of RasGRP to activate the Ras-ERK pathway in T cells could be used to regulate hematopoesis and the immune response.

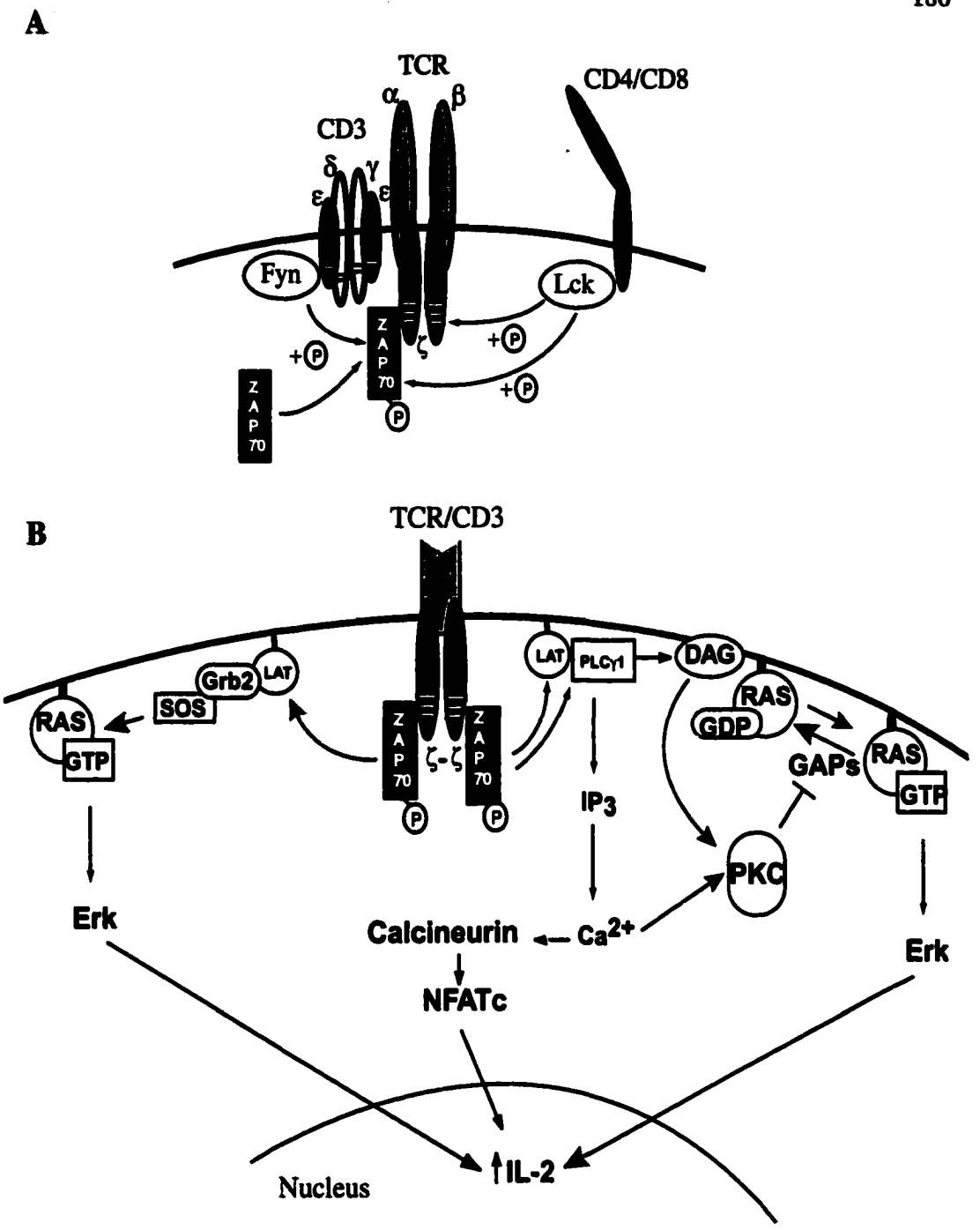

Figure 4-1. The T cell receptor and TCR-coupled signaling pathways. A) The T cell receptor complex (TCR) and early signaling events that occur following TCR-engagement. Cross-linking of the TCR together with the accessory molecules CD4 or CD8, results in the formation of a TCR complex which results in the recruitment of the kinases Fyn, Lck, Zap70, which are activated by phosphorylation as indicated. B) TCR-coupled signaling pathways and proposed models for the activation of Ras upon TCR stimulation. Engagement of the TCR leads to the activation of several pathways that lead to transcriptional activation. Two mechanisms have been proposed to explain how Ras is activated following TCR engagement. One states that Grb2/Sos complexes are recruited to the mebrane-bound tyrosine phosphorylated adaptor molecule LAT. Another model states that activation of Ras is mediated by the inhibition of GAPs by PKC.

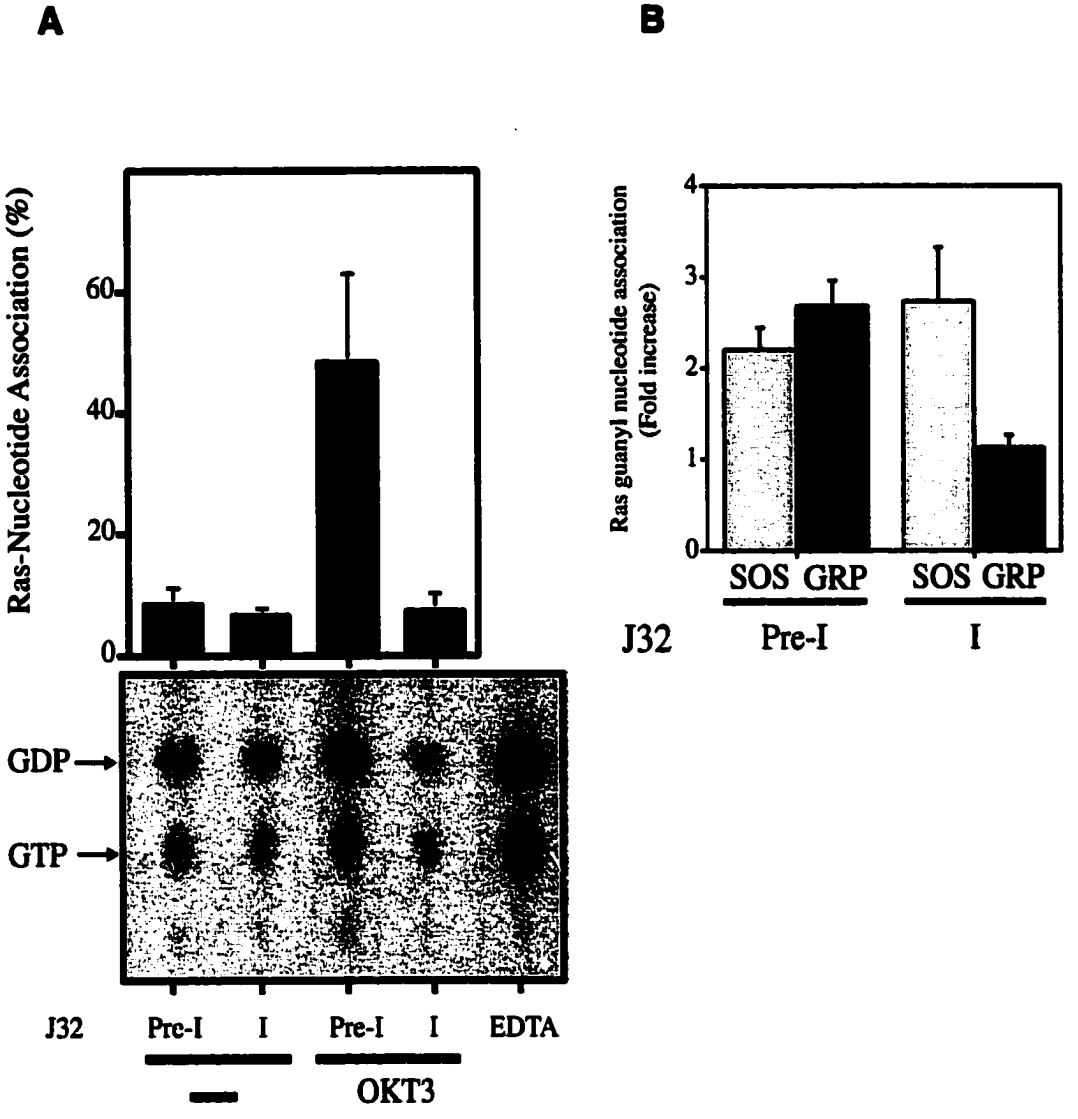

Figure 4-2. RasGRP activates Ras in TCR-stimulated T cell membranes. A) The effect of anti-RasGRP antibodies on total RasGEF activity in resting and activated T cell membranes was examined. Membranes from untreated and OKT3 treated (5 minutes) T cells were assessed for their ability to stimulate the transfer of exogenous radiolabeled guanyl nucleotide onto endogenous Ras. Membranes were preincubated for 12 minutes at 0 °C with IgG prepared from pre-immune (Pre-I) and immune serum (I) containing anti-RasGRP antibodies raised against the catalytic domain of RasGRP. Membranes were then examined for their ability to catalyze guanyl nucleotide exchange by incubation at 30 °C for 1 minute. Ras-associated guanyl nucleotide was detected by thin layer chromatography and the results were quantified by phosphorimager analysis. B) The effect of anti-RasGRP antibodies on the catalytic activity of recombinant Sos and RasGRP (catalytic domain). In vitro Ras guanyl nucleotide exchange assays were performed as described in Chapter 2 (Materials and Methods), and the effect of anti-RasGRP antibodies on the activity of recombinant Sos and RasGRP (catalytic domain) was measured.

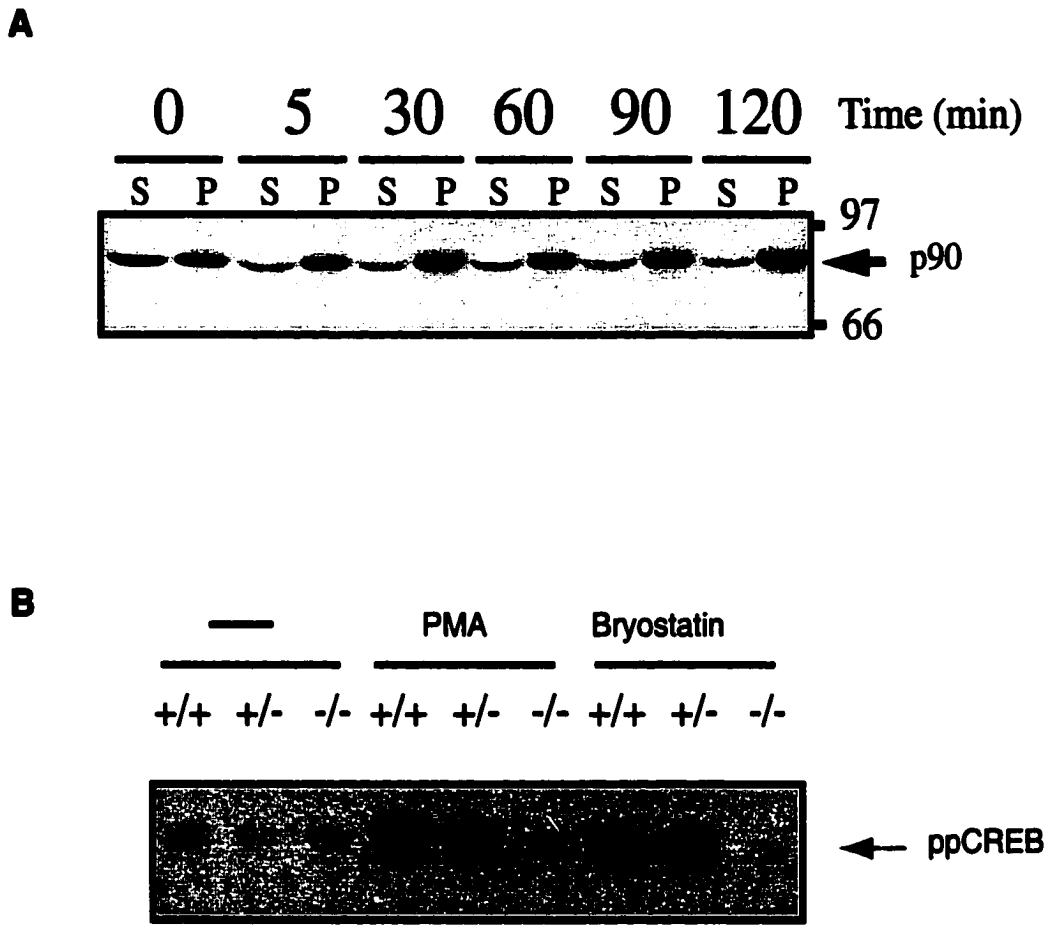

Figure 4-3. Properties RasGRP in TCR-stimulated T cells and primary murine thymocytes. A) Subcellular localization of RasGRP in T cells. In a time course experiment, OKT3-treated and control T cells were lysed by Dounce homogenization in hypotonic buffer and separated into particulate (P) and soluble (S) fractions by ultracentrifugation at 100 000g. RasGRP was detected by immunoblotting with the H176 anti-RasGRP peptide antibody. B) The activation state of CREB in primary thymocytes from RasGRP wild-type and null mice. Dissociated primary thymocytes from wild-type (+/+), heterozygous (+/-) and null (-/-) RasGRP mice were stimulated with PMA and bryostatin-1 for 15 minutes at 37 °C. Cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-CREB (ppCREB) antibody (New England Biolabs).

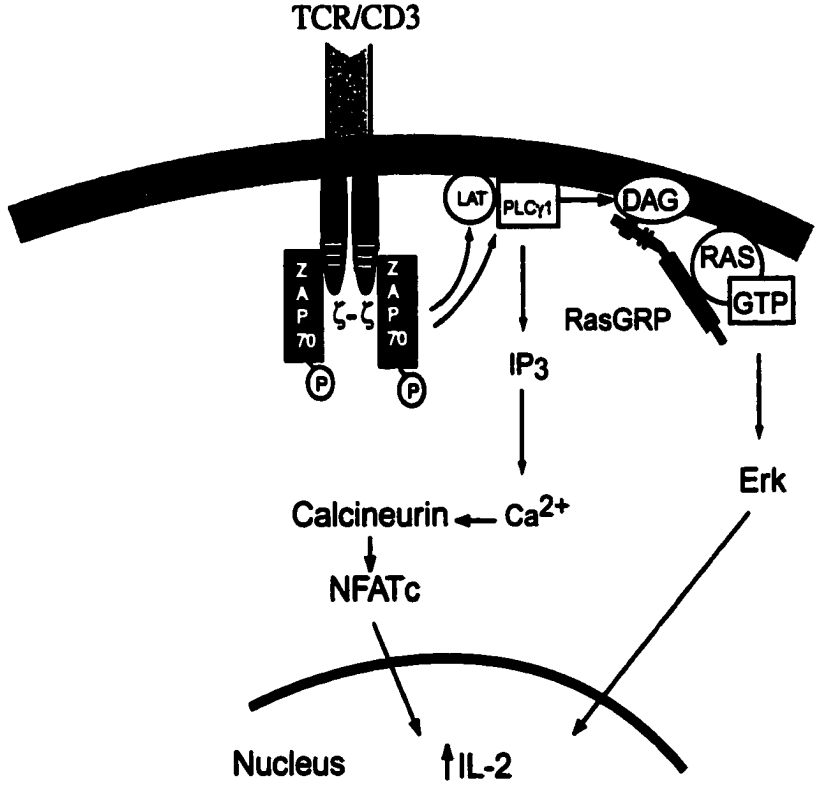

Figure 4-4. RasGRP links T cell receptor signaling to Ras. In response to TCR stimulation several tyrosine phosphorylation-dependent events are triggered. Consequently, tyrosine kinases such as ZAP-70 are recruited to the TCR complex and activated. Activation of ZAP-70 allows for the phosphorylation of several of its substrates including the adaptor protein LAT and PLC-γ1. Activation of PLC-γ1 leads to the generation of membrane DAG, RasGRP membrane recruitment and Ras activation.

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CHAPTER 5
STRUCTURE-FUNCTION RELATIONSHIPS OF RASGRP

Chapter 5

5.1 INTRODUCTION

In the present study I sought to identify mechanisms that control the catalytic activity of RasGRP. I demonstrate that various truncation mutants lacking either the amino- or carboxyl-terminal domain, or both, display differential abilities to activate c-H-Ras when compared with that of the full-length protein. These results suggest that certain structural features impose constraints on the catalytic activity of RasGRP. Because of the likely importance of RasGRP in physiological processes in the brain and in lymphoid cells, I decided to conduct a study of the structure-function relationships of RasGRP.

5.2 RESULTS AND DISCUSSION

5.2.1 EFFECT OF RASGRP TRUNCATION MUTANTS ON RAS SIGNALING

To characterize the roles of the different domains of RasGRP in regulating its activity, we generated a series of expression plasmids encoding HA-tagged RasGRP truncations mutants (Table 5-1). When stably expressed in rat2 cells, each of the expression plasmids gave rise to a polypeptide of the expected molecular weight, as determined by immunoblotting and immunoprecipitating with anti-RasGRP antibodies, J32 and m133 respectively (Figure 5-1).

To investigate the ability of the RasGRP mutants to activate Ras, I compared their transforming activity in a focus to drug resistant colony assay. As a positive control, the oncogenic form of Ras, v-H-Ras, was used. In the presence of full-length RasGRP, rat2 cells expressing this protein produced foci and drug-resistant colonies at a ratio of about 0.002 (n=3) (Table 5-1). The morphology of the cells was flat and non-transformed.

To examine the role of the N- and C- termini in the regulation of the activity of RasGRP, I constructed N- and C-terminal truncation mutants of the full-length protein (Table 5-1). ΔN-RasGRP contains a truncated N-terminus resembling that of rbc7, and a normal C-terminal end. ΔC-RasGRP contains a C-terminal truncation, such that the C terminus resembles that of rbc7, and its N terminus is normal. When ectopically expressed in rat2 cells both these proteins were stable (Figure 5-1). To assess the effects of these truncations, I compared their abilities to form transformed foci. ΔN-RasGRP-expressing rat2 cells displayed a focus to drug-resistant colony ratio of 0 (n=2). These cells did not produce any foci and the morphology of the cells was flat and non-transformed. Interestingly, ΔC-RasGRP-expressing cells displayed a focus to drug-resistant colony ratio of 0.012. As described earlier, when rat2 cells were engineered to express an N- and C-terminal truncated mutant, rbc7, the focus to drug-resistant colony ratio was 0.17. rbc7-expressing cells were highly efficient in inducing the formation of foci. Altogether, these observations suggest that the C terminus of RasGRP imposes autoinhibitory regulations of the transforming potential of RasGRP. It is worth noting that depending upon the antibodies used to detect RasGRP in an immunoblot or immunoprecipitation protocol, small differences in the levels of steady state proteins were detected as observed in Figure 4-1. The level of protein expression, however, did not correlate with the transforming potential of the various RasGRP cDNAs studied.

5.2.2 Effect of an EF hand deletion mutant on the transforming activity of RBC7

The two EF hands of RasGRP were shown to bind calcium with different relative affinities (Figure 3-7). To examine further the role of the EF hands in regulating the activity of RasGRP, Dr. Stone constructed a deletion mutant of rbc7, which lacked both EF hands (ΔEF-7HA). When ectopically expressed in rat2 cells, this protein was stable (Figure 5-1). To assess the effects of this EF hand deletion on the activity of rbc7, I examined the ability of these cDNAs to induce the formation of transformed foci.

Interestingly, ΔEF-7HA-expressing cells displayed an approximate 3-fold increase in the ability to form foci when compared to wild-type rbc7, which displayed a transforming potential of 0.10 in this experiment (n=3). ΔEF-7HA-expressing cells had a focus to drug-resistant colony ratio of 0.30 (n=3) and their morphology appeared even more transformed than *v-H-Ras* transformed rat2 cells.

EF hands can serve as sensors of changes in the concentration of intracellular calcium (Ikura, 1996). These motifs are generally thought to function in pairs, which allows for cooperative changes in conformation, following the binding of calcium, and the subsequent exposure of hydrophobic surfaces that can participate in intra- and/or intermolecular interactions. The EF hands of RasGRP bind calcium, and could potentially serve as a calcium responsive element that could alter intramolecular interactions within RasGRP, or alter interactions of RasGRP with other proteins. The evidence presented here suggests that the EF hands negatively regulate the transforming activity of RasGRP. The precise role of the EF hands and the mechanism by which they function remains elusive. Further studies will need to be done to unequivocally establish and provide a mechanism for how these atypical EF hands function.

5.2.3 The transforming ability of the catalytic domain of RasGRP

Given the importance of the EF hands and DAG domain in mediating the activity of RasGRP, we decided to study the transforming potential of an rbc7 mutant (ΔEFΔDAG-7HA) which lacked both regulatory domains. This mutant was constructed by Dr. J. Stone. When examined in a focus-forming assay, rat2 cells expressing ΔEFΔDAG-7HA displayed a significantly higher (4.5-fold) transforming potential when compared to wild-type rbc7-expressing cells (Table 5-1). The focus to drug-resistant colony ratio was 0.77 (n=3). These observations indicate that both of these domains negatively regulate the activity of RasGRP.

To examine further the role of the structural domains of RasGRP, I carried out a mutational analysis of the EF hand, DAG, N- and C-termini of RasGRP (Table 5-1). The analysis of the EF and DAG domains was carried out in the context of the N- and C-terminal truncated version of RasGRP, rbc7. The N- and C-terminal analysis was conducted in the context of the full-length protein. Using a genetic approach, we

established that the C-terminus plays a significant role in negatively regulating the transforming activity of RasGRP. Similarly, studies of the EF and DAG domains demonstrate that deletion of these regulatory sequences significantly increases the transforming activity of RasGRP. These observations suggest that the regulatory sequences of RasGRP, as well as the C-terminal end, impose constraints on the catalytic activity of RasGRP.

Two mechanisms could explain the results described above. One mechanism would involve intramolecular interactions and the other mechanism would involve intermolecular interactions. The deployment of intramolecular interactions as a mechanism for regulating the activity of proteins is a common biological phenomenon. One classic example for such a system, is that of the src family of tyrosine kinases. In this case, intramolecular interactions between the SH2 and SH3 domains regulate the enzymatic activity of this family of kinases (Moarefi *et al.*, 1997; Sicheri *et al.*, 1997; Xu *et al.*, 1997). In this dissertation, the results observed suggest such a mechanism for the negative regulation of the activity of RasGRP towards Ras. While the molecular determinants involved in the autoinhibitory control of RasGRP activity remain to be determined, the observations reported here suggest that the C-terminus and the EF hands are involved in the downmodulation of RasGRP activity. This idea is consistent with other findings which indicate that the RasGEF Sos is also subject to negative regulation by intramolecular interactions (Corbalan-Garcia *et al.*, 1998).

The role of the C-terminus in regulating the activity of RasGRP is intriguing due to the presence of a putative coiled-coil motif in this region (Tognon *et al.*, 1998). Coiled-coil domains are known to mediate intra- and/or intermolecular interactions (Lupas, 1996). The finding that a C-terminal deletion of RasGRP increases its transforming activity suggests that RasGRP might associate with another protein which negatively regulates its activity (Figure 5-2). Future experiments will need to address the question of whether the C-terminus of RasGRP interacts with other proteins.

In chapter 3, I described evidence which argued for the importance of membrane recruitment of RasGRP for its exchange activity towards Ras. In support of this notion, a DAG deletion mutant failed to activate Ras in response to PMA and did not induce the formation of foci in a transformation assay (Ebinu *et al.*, 1998). The observation that the catalytic domain of RasGRP (ΔEFΔDAG-7HA) is extremely efficient in activating Ras as

observed in the transformation assay, indicates that the stable association of RasGRP with the plasma membrane is not critical for its activity and that transient interaction between RasGRP and Ras may be sufficient for its catalytic activity *in vivo*. Alternatively, the catalytic domain of RasGRP may in fact have a higher affinity for Ras due to less conformational constraints than its full-length counterpart. These observations do not refute the initial model whereby the C1 domain of RasGRP is necessary for its signaling properties in response to diacylglycerol analogues. Instead, from a regulatory view, they suggest that the downmodulation of full-length RasGRP can be explained in two ways. First, the enzymatic activity of RasGRP can represent a basal state which is upregulated by signals that increase Ca^{2+} and DAG. Second, the enzymatic activity of RasGRP may represent a constitutive state which serves the physiological needs of the cell. The evidence presented in this dissertation supports the latter case in that only a fraction of the full catalytic activity potential of RasGRP appears to contribute to diacyglycerol-mediated Ras activation.

5.2.4 M199 AND M133 EPITOPE MAPPING

In vitro, antibodies of defined specificities have commonly been used to identify functionally important regions of signaling molecules. For example, the residues of the Ras amino acid sequence, which constitute the epitope for monoclonal antibody Y-13-259, have been of particular interest since microinjection of this antibody into NIH 3T3 cells and certain other fibroblastic cell lines blocks serum-stimulated DNA synthesis (Mulcahy *et al.*, 1985). Y13-259 also causes reversion of the transformed phenotype induced by a number of oncogenes and inhibits Ras stimulated adenylate cyclase activity in yeast cells (Smith *et al.*, 1984; Shimizu *et al.*, 1983). In this case, deletion mutants of Ras were used to localize the epitope recognized by Y13-259. Specifically, a stretch of amino acid residues from positions 63-73 in Ras were defined more precisely as the epitope for the Y13-259 antibody (Shimizu *et al.*, 1983).

Others in the lab have generated antibodies which recognize full-length RasGRP in both an immunoblot and immunoprecipitation protocol. Because of the likely importance of RasGRP in physiological processes in the brain and in lymphoid cells, and to facilitate future biochemical studies, I decided to map the epitopes of two monoclonal

antibodies, m199 and m133. m199 was raised against a synthetic peptide corresponding to residues in the C-terminus of RasGRP. m133 was directed against the EF hands of RasGRP. Other investigators in the lab were responsible for generating and purifying these monoclonal antibodies.

To define more clearly the regions recognized by these monoclonal antibodies, m133 and anti-HA polyclonal antiserum were used to immunoprecipitate RasGRP from rat2 cells expressing an HA-tagged EF hand or a C-terminal RasGRP deletion mutant. As a control, immunoprecipitations were also carried out from rbc7HA-expressing cells. The immunoprecipitated proteins were resolved by SDS PAGE and immunoblotted with the J32 antibody. When m133 was used for immunoprecipitation, the protein encoded by the ΔEF-7HA deletion mutant was undetectable, whereas the rbc7HA protein was readily detected in rbc7HA-expressing rat2 cells (Figure 5-3A). These results suggest that the m133 epitope lay between the EF hands of RasGRP (residues 475 to 529, Figure 5-3C).

To map the m199 epitope, lysates prepared from rat2 cells expressing full-length, ΔN- RasGRP, ΔC-RasGRP, ΔDAG-7HA, ΔEF-7HA mutants were resolved by SDS PAGE and immunoblotted with m199 antisera. As shown in Figure 5-3B, m199 only detected full-length RasGRP and ΔN-RasGRP. Note that ΔN- and ΔC-RasGRP were created in the context of the full-length protein and ΔDAG- and ΔEF-7HA were constructed in the context of rbc7. These results demonstrate that m199 recognizes the C-terminus of RasGRP (residues 550 to 795, Figure 5-3C).

The specificity of binding of monoclonal antibodies to unique antigenic determinants makes them ideal tools for the detailed analysis of protein structure and function. Since these antibodies have been mapped to specific regions of RasGRP, they will provide a powerful probe for further studies on the regulatory mechanisms for RasGRP activity. For example, questions pertaining to the influence of these antibodies on the intrinsic biochemical properties of RasGRP can be addressed. In addition, given the specificity towards RasGRP, we should be able to define the biological effects of these antibodies on processes such as TCR signaling as well as signaling in neurons. These results will provide clues as to the importance of these domains in regulating the activity and function of RasGRP.

Monoclonal antibodies also facilitate the resolution of related but distinct antigens. Given the presence of several RasGRP species (Nagase *et al.*, 1998, Kawasaki *et al.*, 1998; Kedra *et al.*, 1997), if the encoded RasGRP proteins contain unique antigenic

determinants, monoclonal antibodies might also permit discrimination among these gene products. We do not yet know the chemical nature of the antigenic determinants that are recognized by these antibodies or whether each antibody recognizes primary sequence or a conformational determinant. Nonetheless, they will prove valuable reagents for elucidating properties of RasGRP that are important for its function.

5.2.5 RBC7 IS PHOSPHORYLATED IN RAT FIBROBLASTS

Based on our results, PMA potentiates the activity of rbc7 *in vivo* by recruitment to the plasma membrane where it interacts with Ras. However, it is well established that PMA can also activate protein kinase C *in vitro* and *in vivo*. These observations raise the question as to whether protein kinase C can phosphorylate and modulate RasGRP activity.

The predicted sequence of RasGRP contains several consensus protein phosphorylation sites. These include putative sites for serine and threonine phosphorylation by PKC, as well as several putative sites for phosphorylation by ERK (Figure 3-1A). To determine whether rbc7 is phosphorylated *in vivo*, rat2 cells expressing rbc7HA were labeled with ^{32}Pi. Cells were treated for various times with 100 nM PMA or DMSO as a control. rbc7HA was isolated by immunoprecipitation methods, resolved by SDS/PAGE, transferred to Immobilon-P and exposed on film. As a control, I performed immunoprecipitation with anti-ERK antibody since this protein is phosphorylated in response to PMA treatment. As shown in Figure 5-4A, rbc7HA has a basal level of phosphorylation. Upon PMA treatment for 2 minutes I observed a 4-fold induction of phosphorylation (data not shown). PMA treatment for 10 minutes resulted in an even higher level of induced phosphorylation, approximately 10-fold over DMSO treated cells (Figure 5-4 A,B). To determine whether rbc7HA is phosphorylated in response to a more physiological stimulus, I treated rbc7-expressing rat cells with 100 nM endothelin-1 for various time periods. In rat cells endothelin-1 treatment results in weak activation of ERK and also stimulates phospholipid breakdown and increased levels of diacylglycerol and calcium in rat fibroblasts (Daub *et al.*, 1996). Endothelin induced a 4-fold induction of phosphorylation after 2 minutes and 7-fold induction after 10 minutes (data not shown). As a control, I examined ERK phosphorylation in response to PMA treatment. I found a correlation between PMA-induced rbc7HA phosphorylation and

ERK activation (Figure 5-4 A,B). Although phosphorylation correlated with rbc7 signaling, it is not clear from these studies whether phosphorylation of rbc7 is functionally important.

As a first step towards identifying the kinase(s) responsible for the phosphorylation *in vivo*, I determined the nature of the residues that were phosphorylated. rbc7HA-expressing rat2 cells were labeled with $^{32}P_i$ for four hours and then treated with 100 nM PMA for 10 minutes. The expressed rbc7HA protein was immunoprecipitated, resolved by SDS/PAGE and transferred to Immobilon-P. Phosphamino acid analysis of the filter-bound proteins indicated that the phosphorylated protein contained both serine and threonine phosphate (Figure 5-5A). As a control, I determined the phosphoamino acid content of phosphorylated ERK. As expected, our analysis indicated that ERK contained tyrosine and threonine phosphate (Figure 5-5A).

In an effort to identify the kinase responsible for rbc7HA phosphorylation, I examined the phosphorylation state of rbc7HA and ERK in the presence of several protein kinase inhibitors including staurosporine (2 uM), a non-specific protein kinase inhibitor. Interestingly, staurosporine pretreatment for 30 minutes followed by 10 minutes PMA treatment, caused a reduction in the level of PMA-induced rbc7HA phosphorylation as well as a decrease in the level of ERK activation as measured by its phosphorylation state (Figure 5-4 C,D). The PKC inhibitors chelerythrin chloride (CEC, 5 uM) and Go6976 (20 uM, specifically inhibits PKC isoforms α, β1 and μ) did not reduce PMA-induced phosphorylation of rbc7HA. I also found that in the absence of PMA treatment, inclusion of 500 nM okadaic acid [a potent protein phosphatase 2A (PP2A) phosphatase inhibitor] for 30 minutes stimulated a low level of rbc7HA phosphorylation. Okadaic acid treatment for 60 minutes stimulated a 1.5-fold increase in rbc7HA phosphorylation when compared to 30 minutes of okadaic acid treatment (Figure 5-4D).

One family of S/T protein kinases activated by tyrosine kinase signaling pathways through Ras is the ERK protein kinase family. As described earlier in Chapter 1, Sos can be regulated by feedback phosphorylation. In this case, Erk is known to phosphorylate Sos thus causing the dissociation of Sos/Grb2 complexes. The consensus sequence for ERK phosphorylation exists ten times in rbc7 protein (Figure 3-1A). I therefore considered the possibility that rbc7 is a substrate of ERK. To determine whether ERK is

responsible for rbc7HA phosphorylation, I repeated the experiment in the presence of a MEK specific inhibitor, PD98059 (50 uM). Our findings revealed that blocking the activation of ERK with PD98059 had no significant effect on the level of induced rbc7HA phosphorylation (Figure 5-4D). This suggests that ERK is not responsible for the induced phosphorylation.

In an effort to map the *in vivo* phosphorylation site(s) of rbc7HA, I carried out tryptic phosphopeptide analysis of the phosphorylated protein. Tryptic phosphopeptide maps were generated by digestion of filter-bound proteins with trypsin, followed by two-dimensional electrophoresis chromatography. As illustrated in Figure 5-5B, the tryptic map obtained from PMA treated rbc7HA-expressing cells exhibits four major phosphopeptides and two minor spots.

Having identified several putative PKC phosphorylation sites [XRXXS/TXRX] in rbc7 (Figure 5-6A), I wanted to ask whether rbc7HA is a substrate of PKC activity *in vitro*. To test this possibility, I examined the ability of PKC to phosphorylate recombinant RasGRP (catalytic domain) protein *in vitro*. Purified recombinant RasGRP (catalytic domain) was incubated with PKC from rat brain, [γ-^{32}P] ATP, calcium, diacylglycerol and dispersed phosphatidylserine vesicles. The reaction was incubated at 25 °C for 15 minutes, and the proteins were resolved and identified by SDS/PAGE and transferring to Immobilon-P, followed by autoradiographic analysis. As shown in Figure 5-6B, in the presence of increasing amounts of PKC, RasGRP becomes phosphorylated. To unequivocally determine whether the activity was due to Ca^{2+}/DAG activated PKC, the experiment was conducted in the absence of the PKC enzyme. As shown, no phosphorylation of RasGRP occurred.

To map the phosphorylation sites of RasGRP, I utilized synthetic peptides which contained the PKC consensus phosphorylation sites present in RasGRP. CS2894 was a synthetic peptide that contained residues ^{133}RKLTQRIK^{140}. CS2895 was a peptide that contained residues ^{137} QRIKSNTSKKRKV ^{149}. The underlined amino acids in each peptide correspond to residues which could potentially be phosphorylated by PKC. To determine whether CS2894 and CS2895 could serve as substrates for the activity of PKC *in vitro*, I conducted an *in vitro* kinase assay. Both CS2894 and CS2895 were phosphorylated by PKC (Figure 5-6C). Given that CS2894 contains one putative phosphorylation site, this result suggests that serine 136 of RasGRP is phosphorylated by

PKC. The results from the CS2895 peptide suggest that serine 141, threonine 143 and serine 144 are potential phosphorylation sites. Further studies will need to be conducted in order to establish the specificity and assess the importance of these results.

One of the major signal transduction pathways in the brain involves activation of protein kinase C. Stimulation of this family of S/T kinases has been implicated in the responses to various hormones, neurotransmitters and growth factors. The identity and molecular characterization of the physiological substrates of PKC, however, remains elusive. Importantly, PKC is activated by calcium and diacylglycerol second messengers both of which are proposed to regulate RasGRP. I have shown that the catalytic domain of RasGRP is a substrate of PKC activity *in vitro*. My studies suggest the intriguing possibility that RasGRP is a substrate of Ca^{2+}/ DAG activated PKC in the brain or in lymphoid cells. However, further studies are necessary to determine and assign a functional role for this phosphorylation.

Future experiments will need to determine whether the tryptic phosphopeptide map of PKC-phosphorylated RasGRP *in vitro* exhibits a pattern similar to that seen with rbc7HA phosphorylated *in vivo*. If the phosphorylation of rbc7HA *in vivo* is mediated by PKC or a kinase with identical site specificity, the tryptic phosphopeptides should comigrate. In addition, phosphoamino acid analysis of the four prominent phosphopeptides from rat2 cells expressing rbc7HA remains to be investigated.

Although little is known about RasGRP phosphorylation in the brain and the associated physiological consequences, there is evidence that provides a role for RasGRF1 in neurotransmission and plasticity *in vivo* (Finkbeiner and Greenberg, 1996). It has also been shown that following activation of muscarinic receptors *in vitro*, RasGRF1 becomes phosphorylated (Mattingly and Macara, 1996). In this case, RasGRF1 phosphorylation increases its exchange activity. Recent evidence indicates that mice lacking RasGRF1 are defective in some aspects of memory (Brambilla *et al.*, 1997). It was discovered that long-term potentiation was defective in certain brain regions. Interestingly, RasGRF1 was dispensable for various aspects of learning and memory formation in the hippocampus. This raises the intriguing possibility that the dispensability of RasGRF1 in hippocampal function may reflect the presence of multiple, functionally redundant Ras activators such as RasGRP.

I have shown that RasGRP is phosphorylated in rat fibroblasts and that this phosphorylation correlates with signaling to ERK. On the basis of my findings, I postulate that phosphorylation of RasGRP might constitute another mechanism for RasGRP regulation. Activation of PKC via increased levels of Ca^{2+} and DAG following PIP2 hydrolysis, may lead to RasGRP recruitment as well as the activation of kinases known to be regulated by Ca^{2+} and diacylglycerol, such as PKC and CaM kinase. Subsequent phosphorylation of RasGRP by PKC or some other unidentified kinase could potentially modulate RasGRP function and ultimately affect signaling from Ras to ERK.

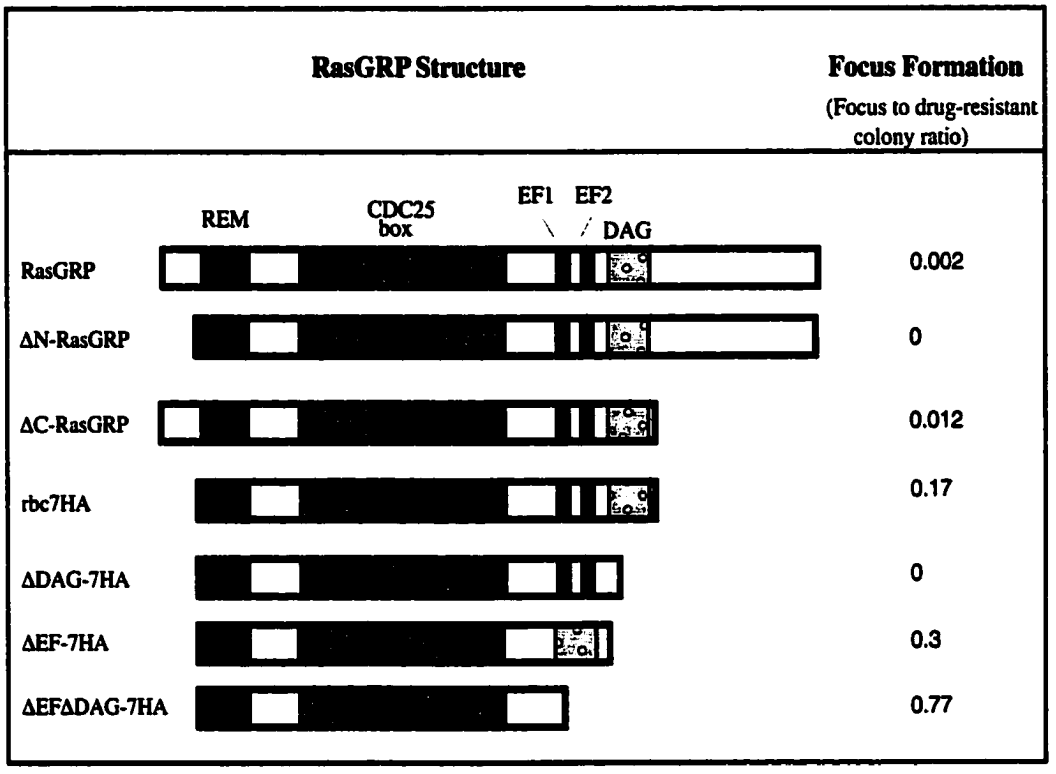
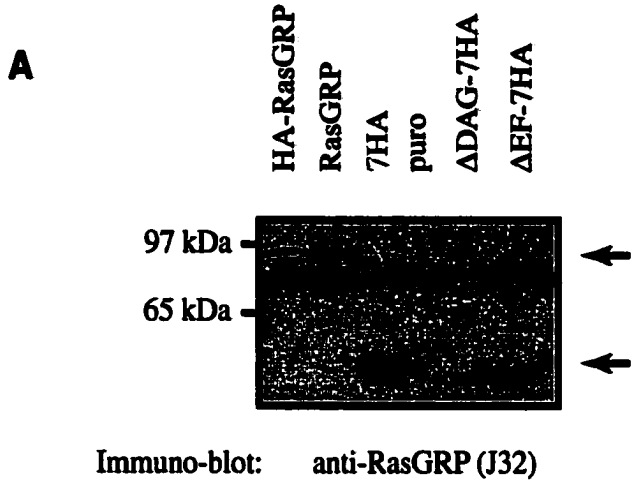


Table 5-1. Structural features and biological activities of various RasGRP mutants. Schematic representation of RasGRP mutant constructs is depicted. Regions corresponding to EF hands, DAG, CDC25 box and REM domains are indicated. RasGRP mutants were assayed for their ability to induce the formation of foci in rat2 fibroblasts. The focus to drug-resistant colony ratio was determined as described in Materials and Methods (Chapter 2). Values are the average of triplicate experiments.



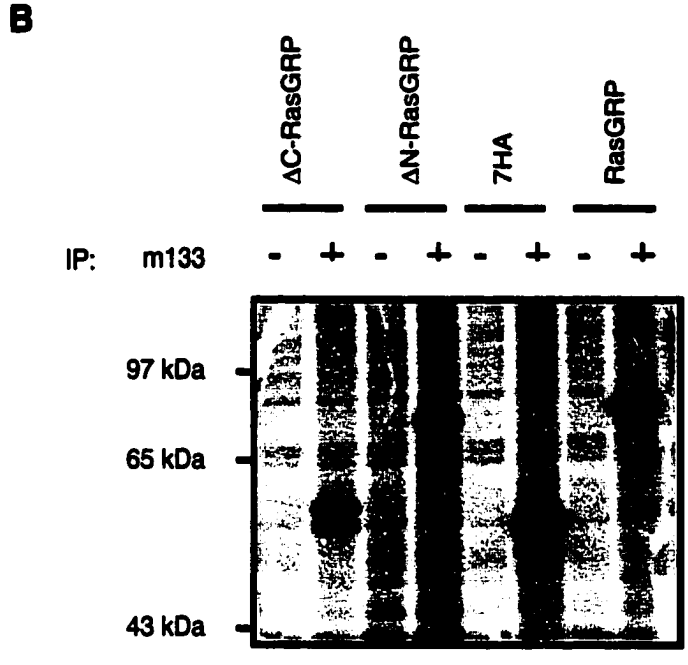


Figure 5-1. Expression of RasGRP mutants in rat fibroblasts. Rat2 fibroblasts were infected with retroviruses containing empty vector (puro), RasGRP, HA-RasGRP, rbc7HA (7HA), ΔN-RasGRP, ΔC-RasGRP, ΔEF-7HA and ΔDAG-7HA. Stable colonies were selected in puromycin-containing media. Cellular lysates were then prepared and resolved by SDS-PAGE. A) The expression of the indicated RasGRP mutants was detected by immunoblotting with J32 anti-RasGRP polyclonal antibody. B) Other RasGRP mutants were detected by immunoprecipitating the respective proteins from ^{35}S-met labeled cells with anti-RasGRP (m133) monoclonal antibody.

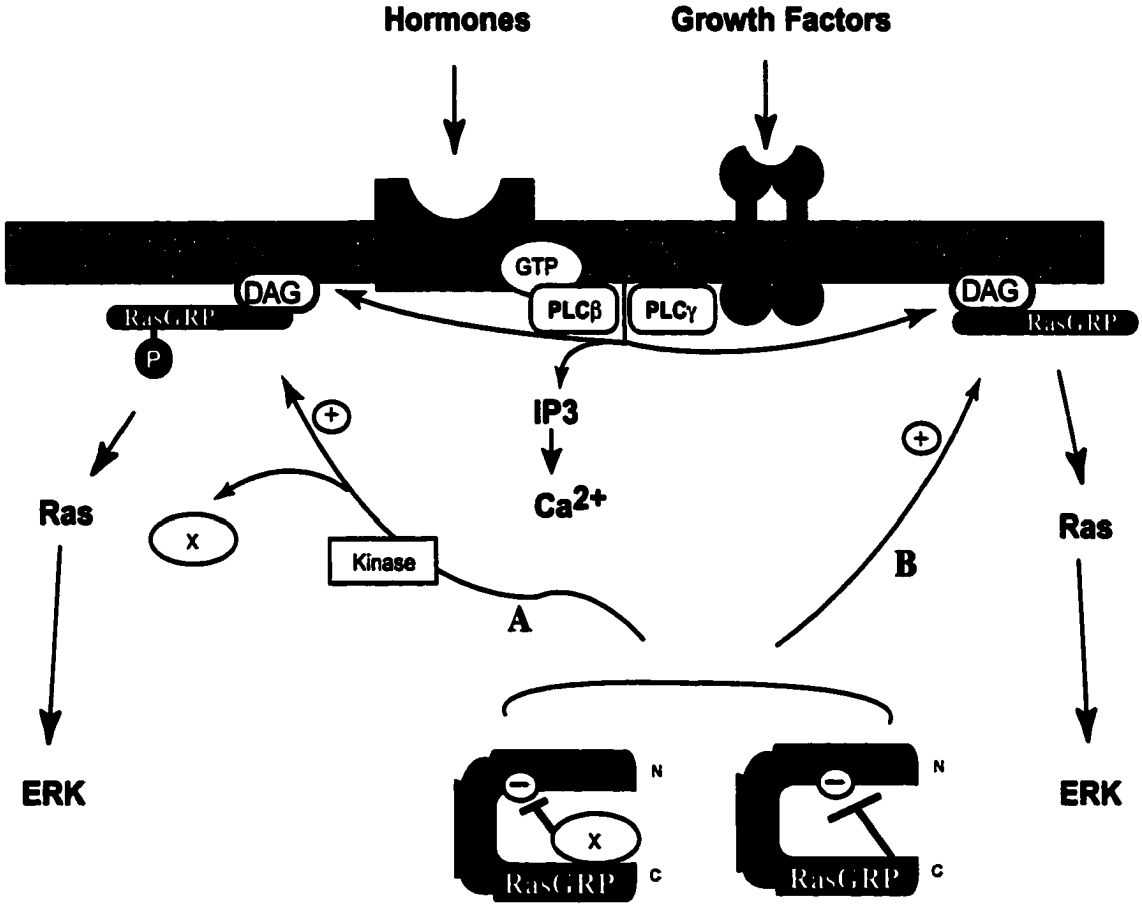


Figure 5-2. Proposed models for the regulation of RasGRP function. The above diagram illustrates a proposed model demonstrating how RasGRP function may be regulated by phosphorylation, inter- and/or intra-molecular interactions. A) In response to hormones or growth factors, intracellular increases in Ca^{2+} and DAG cause the activation of some unknown kinase which phosphorylates RasGRP. The phosphorylation of RasGRP causes a conformational change that relieves the inhibitory constraints (either inter- and/or intramolecular interactions) on RasGRP, and facilitates the membrane recruitment of RasGRP. B) Intracellular increases in DAG cause RasGRP to unfold and preferentially associate with membrane-bound DAG. In both cases, phosphorylation or increases in DAG could cause the displacement of an interacting protein "X" which inhibits RasGRP function.

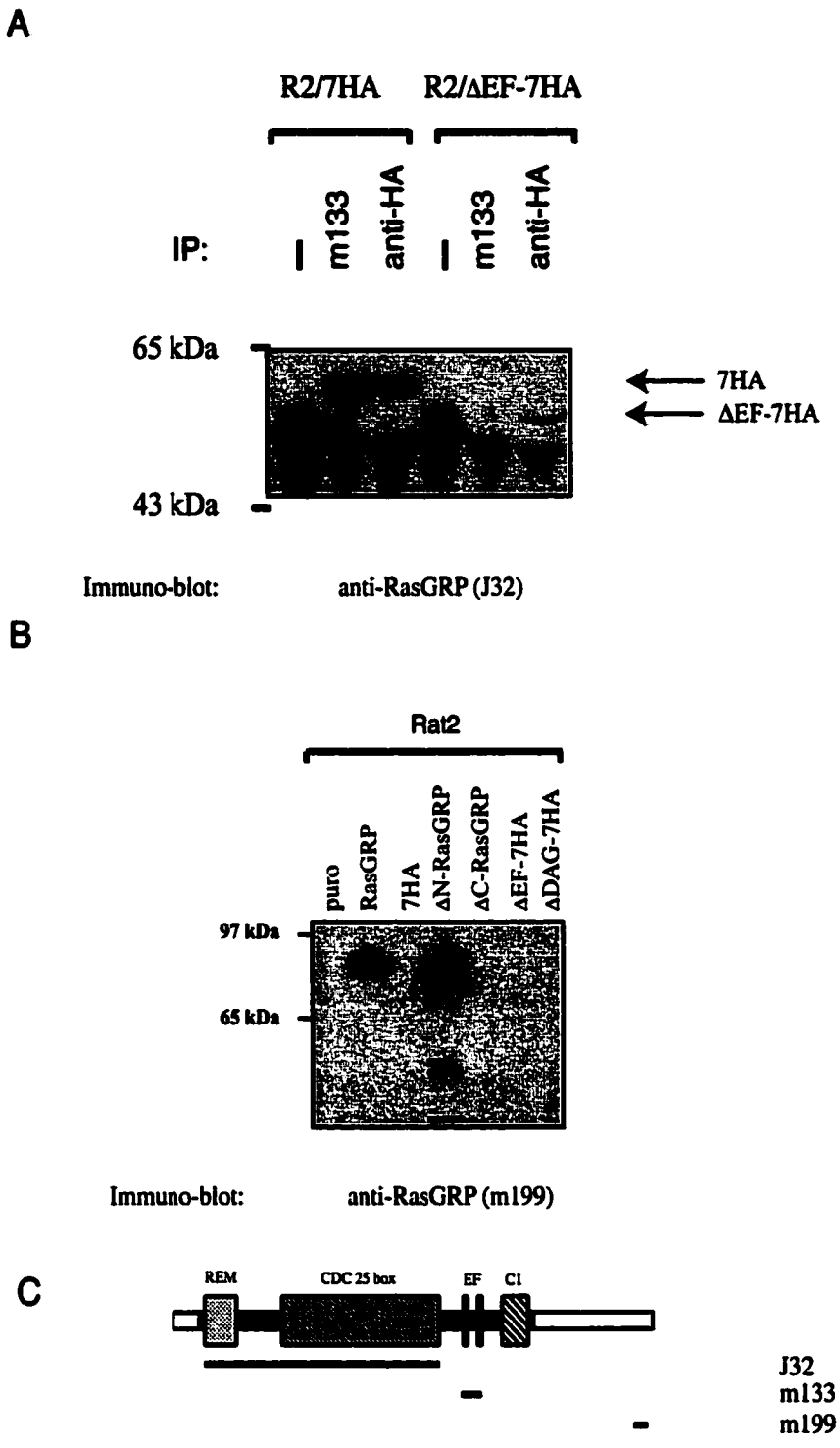


Figure 5-3. Epitope mapping of m199 and m133 anti-RasGRP monoclonal antibodies. A) anti-RasGRP (m133 monoclonal), anti-HA (polyclonal) or no antibody were used to immunoprecipitate HA-tagged RasGRP from rat2 cells expressing rbc7HA or ΔEF-7HA. Immunoprecipitated proteins were resolved by SDS PAGE and immunoblotted with J32 anti-RasGRP antibody. B) Cellular lysates were prepared from rat2 fibroblasts expressing the indicated RasGRP constructs. These lysates were then resolved by SDS-PAGE and immunoblotted with m199 anti-RasGRP monoclonal antibody. C) Schematic diagram of RasGRP illustrating the mapped epitopes of J32, m133 and m199 anti-RasGRP antibodies.

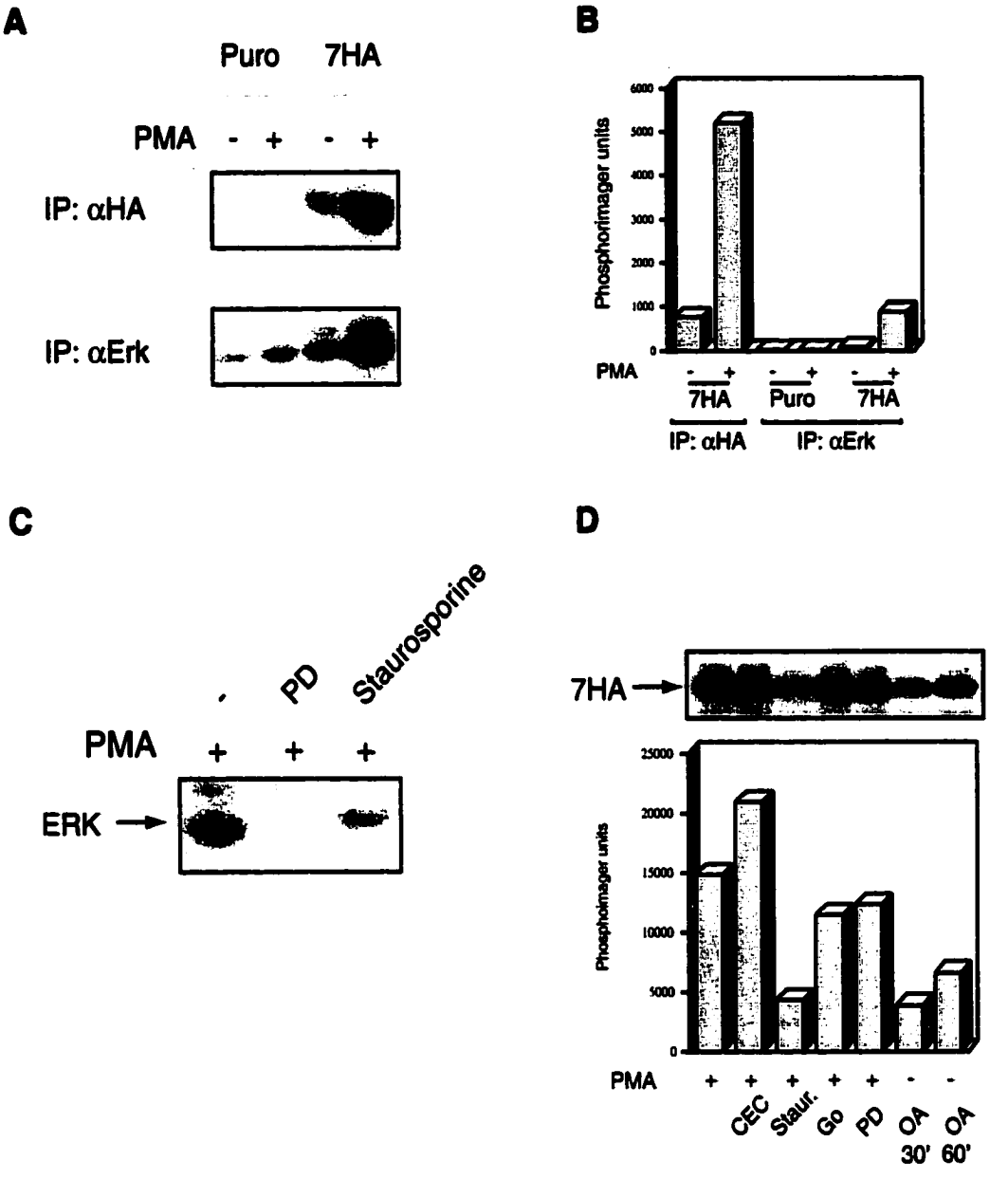


Figure 5-4. PMA-induced phosphorylation of rbc7 in rat2 cells. Control and rbc7HA-expressing cells were labeled with ^{32}Pi and treated with 100 nM PMA for 10 minutes. After the cells were lysed, rbc7HA (7HA) and ERK proteins were immunoprecipitated using anti-HA polyclonal and anti-ERK antibodies respectively. A) Top, autoradiogram of 7HA phosphorylation. Bottom, autoradiogram of ERK activity. B) Phosphorimager analysis of results shown in panel A. C) ERK is not responsible for PMA-induced phosphorylation of 7HA. Effect of PD 98059 (50 uM) and staurosporine (2 uM) on ERK activity. D) Effect of various protein kinase inhibitors on PMA-induced rbc7HA phosphorylation. rbc7HA-expressing cells were labeled with ^{32}Pi, pretreated for 30 minutes with chelerythrine chloride (CEC, 5 uM), staurosporine (2 uM), Go6976 (20 uM) and or for 90 minutes with PD 98059 (50 uM). The cells were then treated for 10 minutes with PMA (100 nM). 7HA protein was isolated by immunoprecipitation with anti-HA polyclonal antibody. Cells expressing rbc7HA were also treated with Okadiac acid (500 nM) for 30 and 60 minutes.

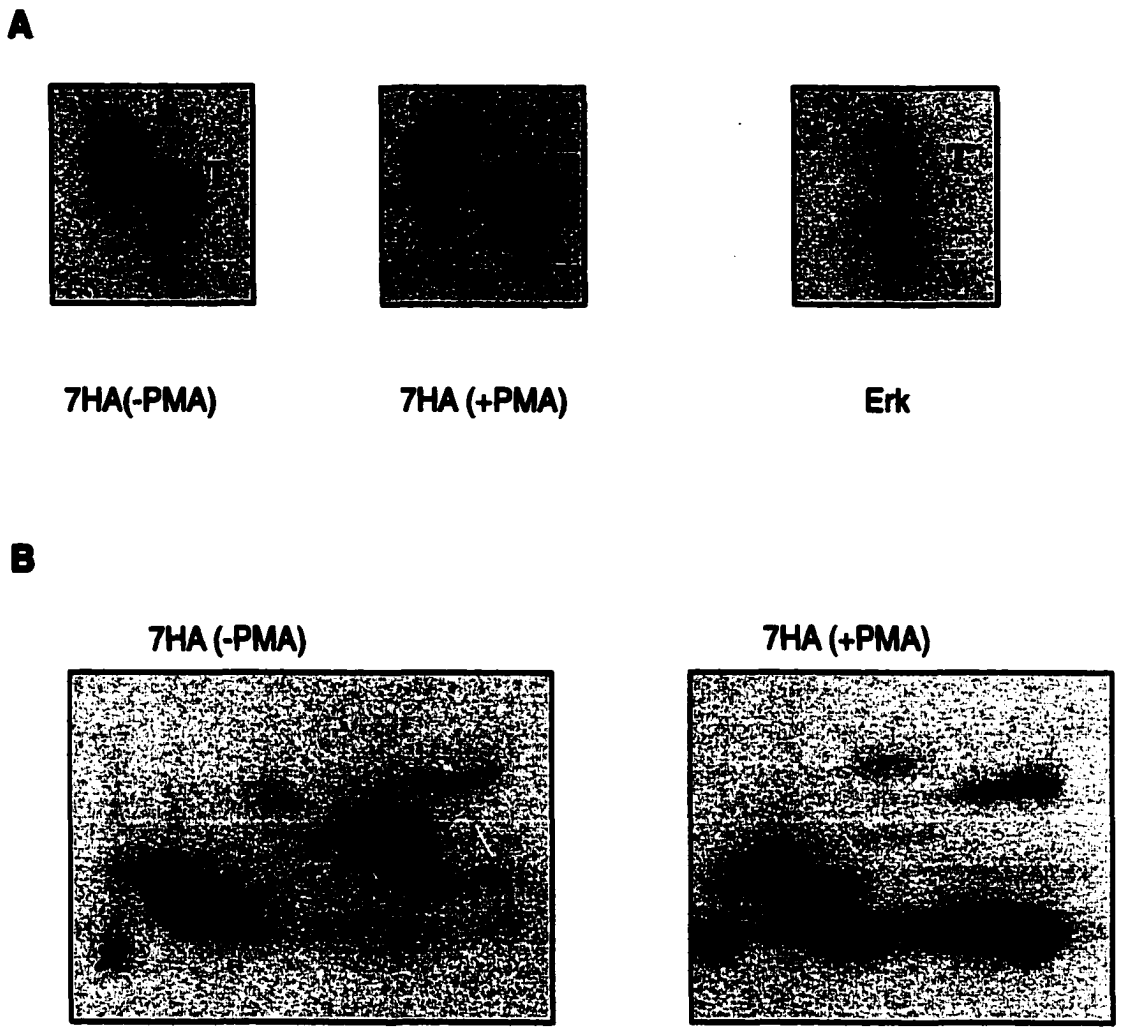


Figure 5-5. Characterization of PMA-induced phosphorylation of rbc7HA. A) Phosphoamino acid content of phosphorylated rbc7HA (7HA). Cells expressing 7HA were labeled with ^{32}Pi for four hours and then treated with 100 nM PMA for 10 minutes. Anti-HA immunoprecipitated proteins were resolved by SDS/PAGE, transferred to Immobilon-P and bands corresponding to 7HA were subjected to phosphoamino acid analysis as described in materials and methods. B) Tryptic phosphopeptide maps of rbc7HA phosphorylated in rat2 cells. Bands corresponding to 7HA on the Immobilon-P were excised and digested with trypsin. Peptide maps were obtained as described in material and methods.

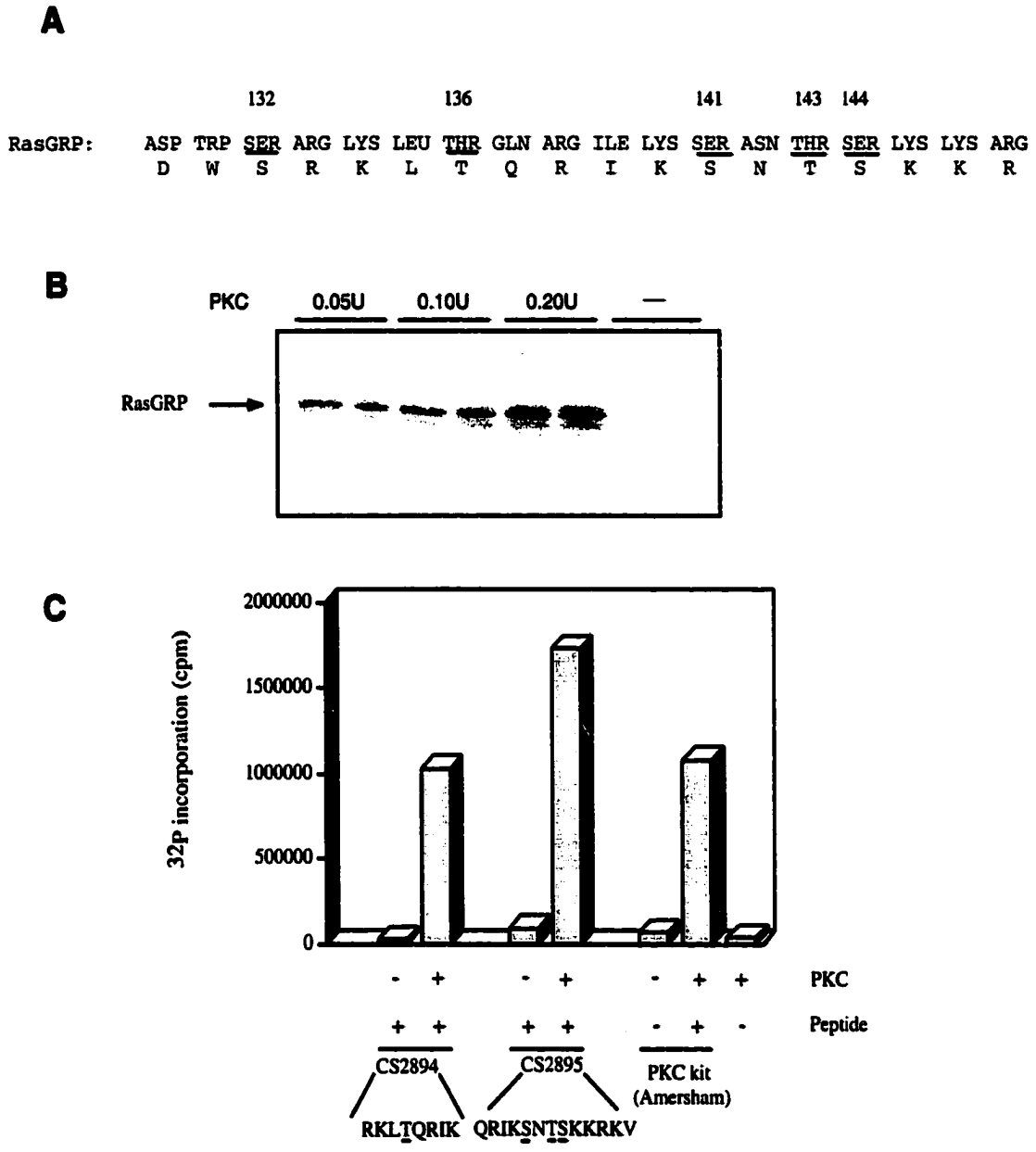


Figure 5-6. RasGRP is a substrate of PKC in vitro. A) A region containing putative PKC phosphorylation motifs is shown. Underlined residues represent potential phosphorylation sites. B) PKC (rat brain) phosphorylates RasGRP in vitro. [γ-32P] ATP incorporation into recombinant RasGRP protein was measured as described in materials and methods. 1 unit is described as the amount of enzyme required to transfer 1nmol of phosphate to histone III-S per minute. C). Synthetic peptides corresponding to RasGRP residues 133-140 (CS2894) and 137-149 (CS2895) were tested for their ability to serve as substrates of PKC in vitro. The reaction measured the incorporation of [γ-32P] ATP into the peptides in the presence or absence of PKC. Peptides were recovered by filter binding and after several washes the amount of radiolabel incorporated was measured in a liquid scintillation counter.

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CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Chapter 6

6.1 GENERAL DISCUSSION AND SUMMARY

6.1.1 STRUCTURE AND FUNCTION OF RASGRP

Our lab recently discovered a novel transforming cDNA, rbc7 (rat brain cDNA #7) from a rat brain cDNA library. The encoded protein functions in the Ras signaling pathway. Our analysis of rbc7 reveals that it is a 5' and 3' truncated version of a larger normal product, RasGRP. RasGRP is expressed normally in the brain and in lymphoid cells and is virtually nontransforming when expressed in rat fibroblasts. By sequence analysis, I identified several key functional domains of RasGRP- a Ras exchange motif (REM), a CDC25 catalytic box, a pair of atypical EF hands and a putative diacylglycerol-binding C1 domain. Using a variety of *in vitro* biochemical assays, I demonstrated that these domains display their predicted biochemical activities. I also studied the function of these domains *in vivo* and in collaboration with other investigators in the lab showed that they are important for the signaling properties of RasGRP in rat fibroblasts. Our results led to the hypothesis that RasGRP links extracellular signals that generate calcium and diacylglycerol to Ras signaling pathway in neurons and T cells.

6.1.2 REGULATION OF RASGRP ACTIVITY

6.1.2.1 Lipids

RasGRP possesses a DAG-binding C1 domain. The ability of RasGRP to bind DAG suggests that this is one mode of regulating the activity of RasGRP. In agreement, a deletion of the C1 domain abolished the ability of RasGRP to activate Ras in response to PMA treatment (Ebinu *et al.*, 1998). This deletion also abrogated the PMA-dependent membrane translocation of RasGRP. Hence, transient as well as sustained increases in diacylglycerol concentrations at the plasma membrane may be sufficient to induce membrane translocation of RasGRP and subsequent activation of Ras signaling.

6.1.2.2 Calcium

The presence of a pair of EF hands suggests that RasGRP may be subject to regulation by calcium. My analysis of the EF hands revealed that the wild-type and the EF1$^-$ mutant bound calcium, while EF2$^-$ and a double mutant (EF1$^-$EF2$^-$) did not. These results suggested that the second EF hand (EF2) is the higher affinity calcium-binding site. A deletion of both EF hands increases the transforming potential of rbc7, indicating that this domain may directly negatively regulate the activity RasGRP. In T cells, however, RasGRP can activate Ras in the presence of intra- and extra-cellular calcium chelators BAPTA/AM plus EGTA (Stang and Stone, unpublished data). Although these findings point towards an alternative role for this domain, they do not rule out the possibility that the EF hands indirectly inhibit RasGRP function. Further studies will be required to establish the importance of the EF hands and assess their role in regulating the catalytic activity, conformation (which could implicate protein-protein interactions), and subcellular distribution of RasGRP.

6.1.2.3 Phosphorylation

Although the C1 domain of RasGRP and membrane translocation plays a prominent role in regulating RasGRP, there may be additional mechanisms of regulating RasGRP function. Such modes would only be operative in cells where RasGRP is normally expressed i.e. T lymphocytes and neurons. One possible example of such regulation is by phosphorylation. In T cells, several kinases are activated in response to TCR stimulation. For example in Jurkat T cells, both Ca^{2+}-independent and total

activities of CaM-K IV are maximally increased within 1 minute of stimulation of the CD3 receptor (Hanissian *et al.*, 1993). In addition, CaM K II has been shown to downregulate both calcineurin and PMA-induced IL-2 expression (Hama *et al.*, 1995). Furthermore, agonists that activate PKA, such as cholera toxin, have been shown to block TCR-induced Ras activation (Tamir *et al.*, 1996). Other kinases that affect TCR signaling include PKC and casein kinase (Downward *et al.*, 1990, 1992; Izquidero *et al.*, 1992; Seldin *et al.*, 1995; Raman and Kimberly, 1998; Raman *et al.*, 1998). In neurons, several kinases including PKA, CaM kinase II and IV, and PKC are known to mediate processes such as synaptic plasticity, cell survival and long-term potentiation (Gosh and Greenberg, 1995; Chen and Tonegawa, 1997; Silva *et al.*, 1997). All of these biological processes require the activation of Ras-Erk signaling. Given that RasGRP contains several putative phosphorylation sites for these enzymes, it is possible that RasGRP is subject to regulation by phosphorylation.

In this dissertation I present evidence that the truncated form of RasGRP, rbc7, is phosphorylated on serine and threonine residues in response to phorbol ester treatment when ectopically expressed in rat fibroblasts. My studies show that phorbol ester-induced phosphorylation of rbc7 correlates with its ability to activate the Ras signaling cascade. In particular, I have investigated the possibility that RasGRP is subject to regulation by a phorbol ester-activated kinase (protein kinase C). I identified rbc7 as a substrate of PKC *in vitro*. Further studies must determine whether RasGRP is phosphorylated in neurons and T cells, as well as assign a functional role for this phosphorylation.

6.1.2.4 Intra- and/or Intermolecular interactions

Upon stimulation of cells with agonists that increase DAG, RasGRP primarily associates with membranes. I proposed that the physiological significance of this membrane translocation is to position RasGRP proximal to its membrane-bound substrate Ras. The presence of protein-protein interacting motifs, such as the coiled-coil motif, suggests that associated proteins may also regulate the activity of RasGRP. In this case, RasGRP may associate with proteins which sequester and inhibit its activity. In response to increases in DAG concentrations, RasGRP preferentially associates with membrane DAG allowing it to adopt an active conformation that facilitates its interaction with and activation of Ras. Note that intramolecular interactions between the domains of RasGRP

may also provide inhibitory constraints which are relieved upon DAG binding, or phosphorylation. Altogether, regulation by several independent mechanisms may provide exquisite fine-tuning for this family of exchange factor, ensuring low basal activity in the midst of complex intracellular signaling pathways.

Cellular responses often persist for a long period of time, and in some cases the sustained activation of Ras is essential for these processes. Depending on the genetic and developmental status of the cell, sustained Ras signaling can lead to proliferation, growth arrest, differentiation or sensitivity to the induction of apoptosis. Physiologically, the regulation of Ras activation may be brought about by several mechanisms discussed earlier. In either case, sustained increases in DAG levels in response to extracellular stimuli could lead to RasGRP-mediated sustained activation of Ras which is important for a variety of biological processes. Unusually persistent activation of Ras, such as that caused by active Ras mutants, or alternation of lipid metabolism may result in pathological responses such as tumorigenesis and abnormal cell cycle (Bos, 1989; Hanahan and Weinberg, 2000). Hence, spatiotemporal aspects and switch-off mechanisms of RasGRP activation must be explored further for full understanding of its role in various cellular responses.

6.1.3 PERSPECTIVES ON THE GRP FAMILY: A LINK BETWEEN PHORBOL ESTERS AND THE RAS SUPERFAMILY

6.1.3.1 The GRP family of guanyl nucleotide exchange factors

At present, 3 GRPs have been identified and classified into 2 groups based on substrate specificity. The best-characterized member of this family is RasGRP. As discussed earlier, RasGRP is expressed in the brain and in lymphoid cells. RasGRP is not expressed early in development, as determined by Western blot analysis of fetal versus adult mouse brain (Stang and Stone, unpublished data). The regulation of RasGRP is believed to occur through DAG and possibly calcium. RasGRP activates H-Ras, K-Ras, N-Ras, R-Ras and Ras2/TC21 but not Rho proteins (Ebinu *et al.*, 1998; Tognon et al., 1998; Ohba *et al.*, 2000). Recently, another GRP, which we call KGRP, was described (Nagase *et al.*, 1998). KGRP contains 689 amino acids and is expressed in skeletal muscle, testis, spleen, liver, pancreas, brain, heart, kidney and ovary by RT-PCR (Nagase *et al.*, 1998; Campbell *et al.*, 1998). We have evidence that KGRP is expressed in the brain early in development (Stang and Stone, unpublished data). KGRP displays

~54 % identity to RasGRP and activates Ras *in vivo* in response to DAG signals (Bottorff and Stone, unpublished data). Although KGRP is also expressed in T cells, the role of KGRP in T cell signaling remains to be established. A recent report indicated that KGRP, also referred to as CalDAG-GEFIII, can activate Ras, R-Ras, and Rap1 in 293T cells as well as *in vitro* (Yamashita *et al.*, 2000). Another member of the GRP family is RapGRP, also called HCDC25L (Kedra *et al.*, 1997). RapGRP encodes a 69kDa protein that displays ~40% identity to RasGRP. Although RapGRP displays a high degree of homology with RasGRP, its activity is specific towards the Rap proteins and R-Ras (Kawasaki *et al.*, 1998; Ohba *et al.*, 2000). RapGRP activates Rap in 293T cells in response to increases in DAG and Ca^{2+}, possibly by the recruitment of RapGRP to the plasma membrane (Kawasaki *et al.*, 1998). RapGRP is highly expressed in the brain, spleen, and thymus (Kawasaki *et al.*, 1998). Figure 6-1 depicts the structural and evolutionary relationships between the GRP family members.

In summary, members of the GRP family of GEFs are a single polypeptide, comprised of a conserved N-terminal catalytic domain, and a C-terminal regulatory region. Cloning of the first GRP, RasGRP, revealed several structural domains: a REM box, a catalytic domain, a pair of atypical EF hands, and a C1 domain, coiled-coil, proline-rich region. Each is a functional module, and many unrelated proteins are known to contain one or the other. The function of some of these domains has been established by biochemical and mutational experiments (Tognon *et al.*, 1998; Ebinu *et al.*, 1998). The REM motif confers specificity of GRPs towards members of the Ras superfamily; the catalytic region allows for the characteristic GEF activity of GRPs; the EF hands are known to bind calcium with different affinities although the functional significance remains to be established; the cysteine-rich C1 domain binds phorbol esters; the proline-rich and coiled-coil motifs are known to be involved in protein-protein interactions. It is worth noting that despite the high degree of sequence similarity among the GRP family members, there is no extant *in vivo* or *in vitro* data demonstrating the regulation of the all the GRP family members by calcium and diacylglycerol.

6.1.3.2 Model for the Regulation of signaling by RasGRP

Activation of GEFs can occur by several mechanisms. The best-characterized mechanism is the RTK-mediated pathway. Upon ligand-binding, RTKs undergo

autophosphorylation and some of the resulting phosphotyrosine residues provide docking sites for the adaptor proteins Grb2, Shc, FRS2, IRS1 and IRS2, and possibly Gab1, which are themselves tyrosine phosphorylated after receptor activation and are then able to bind Grb2 (Schlessinger, 1994; Pawson, 1995; Kouhara *et al.*, 1997; Yenush and White, 1997; Takahashi-Tezuka *et al.*, 1998; Holdago-Madruga *et al.*, 1996; Raabe *et al.*, 1996; Herbst *et al.*, 1996). Binding of Grb2 leads to recruitment of Grb2-Sos complexes to the plasma membrane where Sos can activate Ras. Additionally, Ras activation by tyrosine phosphorylation can occur following activation of integrins and cytokine receptors, implicating intracellular kinases, such as focal adhesion kinases (FAKs) and Janus Kinases (JAKs), which are activated by these receptors (Schlaepfer and Hunter, 1998; Weber-Nordt *et al.*, 1998; Hirano *et al.*, 1997; Leaman *et al.*, 1996).

Recently, G-protein-coupled receptors and some voltage-dependent Ca2+ channels have been shown to activate Ras signaling. Their action on exchange factors is complex and involves at least three mechanisms (Finkebeiner and Greenberg, 1996) which could act in concert. First, Ca2+ influx through L type voltage-sensitive Ca^{2+} channels and Gi- or Gq-coupled receptors results in activation of the non-RTK PYK2 and Src (Lev *et al.*, 1995; Dikic *et al.*, 1996). This would result in the recruitment of Grb2-Sos complexes to the membrane. This mechanism might also involve RTKs, since activated Src kinase can phosphorylate and activate EGF receptors, which allows them to bind Shc and, through Shc, Grb2-Sos complexes (Daub *et al.*, 1996, 1997; Rosen and Greenberg, 1996). Second, RasGRF can be activated by Ca^{2+} influx by means of its IQ motif, which in principle replaces the Grb2 binding domain found in Sos. The binding of Ca^{2+}-calmodulin complexes by the IQ domain is an essential step in the Ca^{2+}-dependent activation of the exchange factor (Farnsworth *et al.*, 1995). The third mechanism involves the release of G-protein βγ subunits upon GPCR activation which induces phosphorylation of RasGRF by an unknown S/T kinase, an essential step in its activation (Mattingly and Macara, 1996).

In this dissertation, I describe a novel mechanism for the activation of Ras signaling. This involves the newly identified RasGEF, RasGRP, which binds Ca^{2+} and DAG, better known as agonists of PKC. I propose a model whereby RasGRP links extracellular signals that generate Ca^{2+} and DAG to Ras activation. Specifically, increased levels of membrane DAG serve to recruit RasGRP to the plasma membrane

whereby RasGRP can interact with and activate Ras. I demonstrate that in T cells, RasGRP links TCR signaling to Ras activation. This finding may provide answers to numerous questions pertaining to the activation of Ras in response to phorbol esters and TCR stimulation. A model consistent with the biochemical and molecular genetic data is presented in Figure 6-2.

6.2 CONCLUSION

The discovery of this novel family of exchange factor is exciting. For a long time DAG signals linked to Ras activation were thought to be mediated solely by PKC. However, with the emergence of the GRPs, some biological effects attributed to PKC may in fact be GRP-mediated. The current dogma of TCR signaling involves diverging signaling systems with Sos and PKC-mediated pathways representing the major alternative branches. This dissertation provides evidence for a third, and perhaps essential pathway involving PLC-DAG-RasGRP-Ras. This pathway explains many perplexing aspects of Ras signaling in T cells, including the activation of Ras in response to phorbol esters.

From a clinical perspective, the finding that DAG analogues such as bryostatin-1 can activate RasGRP is appealing. Bryostatin-1 has shown useful clinical properties such as the growth arrest of certain tumor cells and the sensitization of some tumors to the induction of apoptosis (Pettit *et al.*, 1991; Vrana *et al.*, 1999). Additionally, bryostatin-1 can promote the growth of certain hematopoietic stem cells as well as activate lymphocytes against tumor cell targets (Scheid *et al.*, 1994; Berkow *et al.*, 1993). Bryostatin-1 is currently being tested in phase I and II human clinical trials for the treatment of certain types of cancer. Given that thymocytes of RasGRP -/- mice fail to activate Ras-Erk signaling in response to bryostatin-1 treatment, RasGRP could potentially serve as the major cellular target of bryostatin-1 (Dower *et al.*, 2000). Altogether, the results described here could have important practical implications since, unlike other Ras signaling pathways, the RasGRP-Ras-Erk pathway is subject to pharmacological manipulation as observed with bryostatin-1. Future efforts will need to focus on delineating the pathways and mechanisms involved in the regulation of RasGRP function in T cells and the brain.

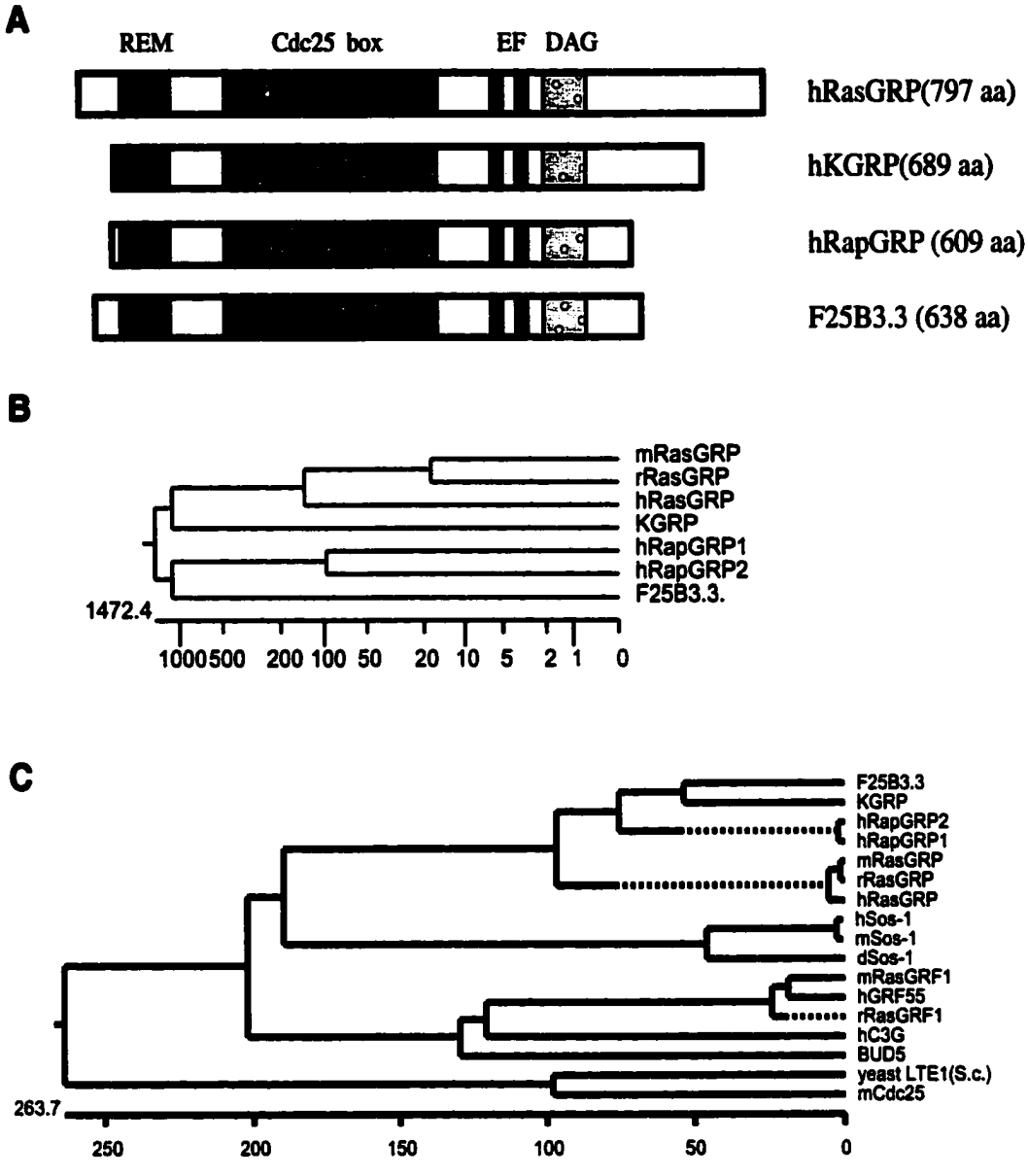

Figure 6-1. The GRP family of Ras-superfamily GEFs. (A) Structural features of members of the GRP family. (B) Evolutionary relationship among the GRP family. A phylogenetic tree analysis was carried out with the LASERGENE Software (DNAstar, Madison, WI). In the above phenograms, the numbers represent approximate distances between sequences. The numbers indicate the number of amino acids that differ between the sequences such that the branch lengths in the phenograms are proportional to the estimated divergence from an ancestral node. (C) A computer generated phylogenetic tree analysis was also performed for to compare the GRP family of exchangers and other known Ras activators. Genbank accession numbers of protein sequences are: hRasGRP, 5032025; mRasGRP, 7242201; F25B3.3, 1262950; rRasGRP, 3237381, hKGRP/KIAA0846, BAA74869, hRapGRP1, AAFo7219, hRapGRP2, AAF07220; mRasGRF1, 6755288; hGRF55, 4506433; rRasGRF1, 121515; hSos-1, 476780; mSos-1, 284779; dSos-1, 103188; yeast LTE1, 2144608; mCDC25, 882120; BUD5, 171141; hC3G, 474982.

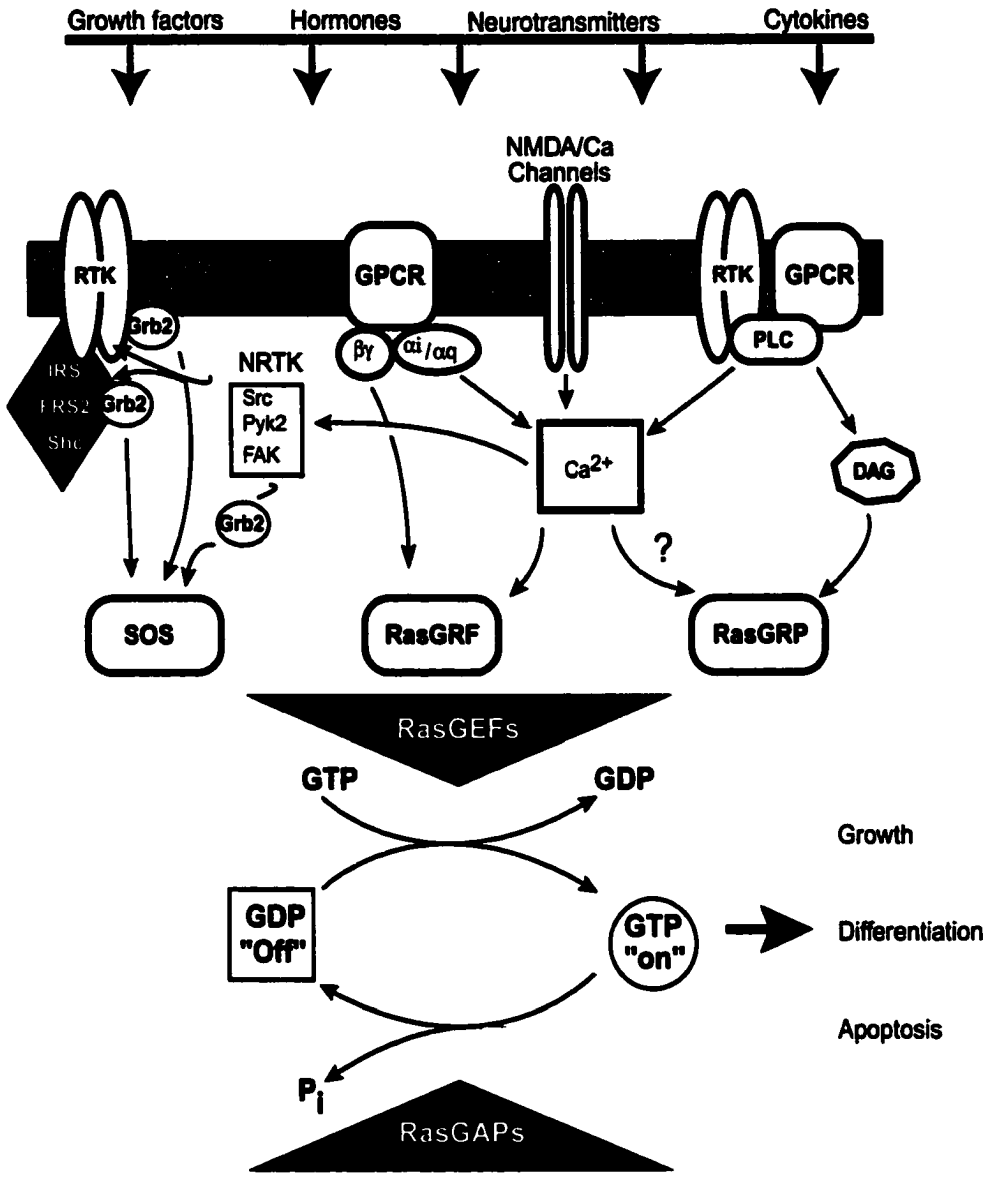

Figure 6-2. A novel signaling pathway that leads to the activation of Ras in neurons and T cells. The above diagram illustrates the signaling pathways that lead to the activation of Ras in response to a variety of extracellular stimuli. In each case, RasGEFs provide a link between the various extracellular stimuli and the activation of Ras. In this dissertation, I describe a novel signaling pathway whereby RasGRP links signals that increase intracellular DAG and possibly Ca^{2+} to the activation of Ras.

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