University of Alberta

Molecular Mechanisms of RasGRP Regulation

by

Yong Zheng 

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

Spring, 2006

Your file Votre référence
ISBN: 0-494-14073-9
Our file Notre référence
ISBN: 0-494-14073-9

To my parents

In eternal gratitude

For the nest, the compass, and the wings

Abstract

Ras, a small guanosine triphosphatase, plays a central role in signaling pathways that control T and B cell development, antigen receptor activation of lymphocyte proliferation, cytokine gene induction and development of T cell anergy.

Ras oscillates between the GTP-bound active state and the GDP-bound inactive state. The balance between the two states is directly controlled by two groups of proteins: guanine nucleotide exchange factors (GEFs) activate Ras by promoting the GTP loading on Ras, whereas GTPase-activating proteins (GAPs) inactivates Ras by enhancing its intrinsic GTPase activity of Ras.

The Ras guanyl nucleotide-releasing proteins (RasGRPs) represent a novel family of GEFs that have restricted expression including lymphocytes. Besides the catalytic domain, RasGRPs also contain an EF-hand motif, a putative proline-rich region, and a diacylglycerol (DAG)-binding C1 domain, which mediates the membrane recruitment of RasGRPs during lymphocyte activation. The molecular mechanisms underlying RasGRP activation are still poorly understood, however, previous studies implied multiple levels of regulation. In my study, I investigated three potential modes of RasGRP regulation including protein kinase C (PKC)-mediated regulatory phosphorylation, protein-protein interaction mediated by the Grb2 family members, and calcium-dependent regulation.

Firstly, in the RasGRP phosphorylation study, I identified RasGRP1 and RasGRP3 as *in vitro* PKC substrates. I then identified RasGRP3 threonine 133 as

an *in vivo* PKC-regulated phosphorylation site in primary B cells using mass spectrometry and a phospho-peptide antibody. I also showed the homologous threonin in RasGRP1 was also an *in vivo* phosphorylation site in primary T cells while RasGRP4 might be regulated by other means.

Secondly, I showed for the first time that RasGRP1 preferentially bound to the N-terminal Src homology 3 (SH3) domain of the growth factor receptor-bound protein 2 (Grb2) *in vitro,* probably through its PXXP motif. Moreover, in resting Jurkat T cells, RasGRP1 interacted with GST-fused Grb2-related adaptor downstream of Shc (Gads), a hematopoietic-specific adaptor in Grb2 family involved in lymphocyte development and activation.

Finally, I showed the right EF-hand of RasGRP1 seemed less important for calcium binding *in vitro*. However, RasGRP1 EF-hand mutants did not attenuate PMA-induced Erk activation in Rat2 fibroblasts despite the disruption of calcium binding *in vitro*.

Acknowledgement

I wish to express sincere appreciation to my supervisor Dr. James C. Stone, whose enlightened mentoring were instrumental and inspiring. Thanks go to all the members in Dr. Stone's laboratory for their assistance throughout my study. Thanks also go to my collaborators in Dr. Liang Li's laboratory, whose expertise is essential for the success of this work. In addition, thanks are due to my committee members, Dr. Michael C. Schultz and Dr. David T. Stuart, for their suggestions and comments. I wish to acknowledge my gratitude to external faculty members Dr. Jane McGlade, Dr. Colin C. Anderson, and Dr. Hanna Ostergaard, whose critical eye was helpful in the preparation of this thesis.

I offer sincere thanks to my wife, Yue, for her unshakable faith in me and her willingness to endure with me the vicissitudes of my endeavors. I also wish to thank my daughter, Helen, who supported me spiritually with her purest smiles.

The study was supported by grants from the Canadian Institutes of Health Research to Dr. James C. Stone and from the Protein Engineering Network of Centers of Excellence and Alberta Cancer Board to Dr. Liang Li and Dr. James C. Stone.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Akt	AKT8 retrovirus proto-oncogene (also known as protein kinase B)
ATP	adenosine triphosphate
BCR	B cell receptor
Btk	Bruton's tyrosine kinase motif
cRas	cellular Ras
C3G	Crk SH3-binding guanyl nucleotide releasing protein
CalB	calmodulin binding domain
cAMP	cyclic adenosine monophosphate
CAPRI	Ca^{2+}-promoted Ras activator
cDNA	complementary DNA
CDK	cyclin-dependent protein kinase
C. elegans	*Caenorhabditis elegans*
CREB	cyclic AMP response element binding protein
Dbl	diffuse B-cell lymphoma
DAG	diacylglycerol
dCTP	deoxycytosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
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
Gads	Grb2-related adaptor downstream of Shc (also known as Mona)
GAP	GTPase activating proteins
GDP	guanosine diphosphate
GEF	guanyl nucleotide exchange factor
GPCR	G-protein coupled receptors
Grap	Grb2-related adaptor protein
Grb2	growth factor receptor bound protein 2
GRD	GAP-related domain
GRF	guanyl nucleotide releasing factor
GRP	guanyl nucleotide releasing protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
H-Ras	Harvey Ras
HA	hemaglutinin
HEK293	human embryonic kidney 293 cells
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IgG	immunoglobin G
IKK	IκB kinase
IMAC	immobilized metal ion affinity column
IP3	inositol triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
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)
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MBP	maltose-binding protein
mRNA	messenger RNA
MS	mass spectrometry
N-Ras	neuronal Ras
NGF	nerve growth factor
NP40	nonylphenoxy polyethoxy ethanol
OA	okadaic acid
PAGE	polyacrylamide gel electrophoresis
PAS	protein A sepharose
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGS	protein G sepharose
PH	pleckstrin homology
Pi	inorganic phosphate
PI3K	phosphoinostide 3 kinase
PIP_2	phosphosphatidyl 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
Raf	rapidly growing fibrosarcoma
Ras	rat sarcoma
RASAL	Ras GTPase activating-like protein
Rbc7	rat brain cDNA #7
RBD	Ras binding domain
REM	Ras exchange motif

Rin	Ras interacting
RNA	ribonucleic acid
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulphate
SH2	Src-homology2
SH3	Src-homology3
Shc	Src homology 2 domain containing transforming protein
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
Sos	son of sevenless
Tec	tyrosine kinse expressed in hepatocellular carcinoma
TCR	T cell receptor
vRas	viral Ras

CHAPTER 1

INTRODUCTION

Chapter 1

1.1 Overview

At the surface of cells, many different kinds of receptors are expressed which allow the cell to respond to signals provided by its environment. Activation of these receptors leads to a variety of biochemical events in which a large group of small GTPases play essential roles for the propagation and divergence of signaling pathways.

The ubiquitous classical Ras proteins (H-Ras, N-Ras, K-Ras) belong to the Ras superfamily of small GTPases, which also consist of many Ras-related proteins. Mutations in Ras proteins are frequently involved in human cancers, making it an important research area in which great efforts have been made to illustrate the mechanism of Ras regulation and signaling. It is now well appreciated that, by oscillating between inactive GDP-bound forms and active GTP-bound forms, Ras proteins function as regulated GTPase switches that are activated by a diverse spectrum of extracellular stimuli, and which themselves activate a number of effector pathways. Ras proteins have been implicated in many important cellular functions including cell differentiation, cell survival and senescence. However, despite the great improvement in our understanding of the mechanism of Ras signaling over 30 years, many critical questions remain to be answered.

1.2 Ras history

Ras oncogenes were originally identified as the transforming elements of Harvey and Kirsten murine sarcoma viruses (Ha-MSV and Ki-MSV). In 1964, Harvey noticed that the passage of Ha-MSV through laboratory strains of rats can cause the rapid production of tumors (Harvey 1964; Kindig and Kirsten 1967). A similar observation was later made in 1967 by Kirsten and his colleagues using a different strain of murine sarcoma virus (Ki-MSV) (Kindig and Kirsten 1967). However, it was not until early the 1980s that people realized that the highly oncogenic activities of these viruses depended

on newly acquired sequences, a result of viral transduction of cellular genes. In a foci formation assay, only the subgenomic DNA fragments of Ha-MSV containing cell-derived sequences efficiently transform NIH 3T3 cells (Lowy and Willumsen 1993).

These findings lead to the subsequent identification of the Ras proteins. Using antibodies isolated from Ha-MSV and Ki-MSV infected rats, Langbeheim and colleagues detected a 21,000-dalton phosphoprotein (p21) expressed by virus in infected cells. This protein is also a normal component of untransformed cells. In addition, the gene coding for Ha-MSV p21 locates at the region of viral DNA known to be of cellular origin. Therefore, the p21 oncogene of Ha-MSV, Ki-MSV, or other closely related viruses is derived from a proto-oncogenic form present in normal cells (Lowy and Willumsen 1993). The genes encoding p21 protein are now referred to as *ras* genes, for *rat sarcoma* genes.

1.3 Ras genes in mammalian cells

Following the identification of viral *ras* genes, three structurally and functionally related *ras* genes were cloned and characterized in mammalian cells, namely H-*ras*, K-*ras* and N-*ras*. They are located on different chromosomes and all consist of four exons and a 5' noncoding exon (exon φ) with wide differences in the intron sizes and sequences. H-*ras* is the proto-oncogene homolog of Ha-MSV oncogene, and Ki-*ras* is similar to K-MSV oncogene (Lowy and Willumsen 1993). Additionally, K-*ras* gene can undergo alternative splicing of its fourth exon to generate two forms of p21: K-Ras4A and K-Ras4B, whose only differences lie in their COOH-terminal 25 amino acids. The third *ras* gene, N-*ras*, was identified by transfecting DNA from neuroblastoma cells into NIH-3T3 cells, and thus has no retroviral origin. In addition to the three functional *ras* genes, two pseudogenes, Ha-*ras*-2 and Ki-*ras*-1 have been found in various mammalian cells (Barbacid 1987; Lowy and Willumsen 1993).

The three *ras* genes are not only highly conserved in all mammalian species studied, but also share a great amount of conservation between yeast and humans, providing evidence for the essential role of *ras* genes in critical cellular process. In support of this,

some yeast Ras alleles are able to transform mouse NIH3T3 fibroblasts with high efficiency. Similarly, human Ras proteins have been shown to be able to complement nonviable yeast mutants and induce phenotypic alterations (Lowe and Goeddel 1987; Lowy and Willumsen 1993).

Despite the sequence and functional similarities, increasing evidence suggest that the differences in expression pattern, plasma membrane interaction, and effector preference might be different for the four Ras proteins (see discussion below).

1.4 Abnormal Ras activation and malignancies

In the 1980s the causative role of *ras* oncogenes in human cancers became well appreciated. Ras proteins bind guanine nucleotides (GDP or GTP) with high affinity and possess intrinsic GTPase activity. By oscillating between the GDP-bound inactive form and GTP-bound active form, a mechanism that is under the tight control of a group of regulatory proteins, Ras proteins act as a molecular switch in signal transduction (Lowy and Willumsen 1993). Point mutations that impair GTPase activity or facilitate GTP/GDP exchange result in the transforming activity of Ras due to constitutive activation. The first evidence came from a transformation assay, where high molecular weight genomic DNA from chemically transformed cells induced morphological transformation of NIH-3T3 cells, as well as many other cell lines, suggesting the existence of dominant acting transforming genes in these cells. One of these genes was later identified as the mutationally activated and oncogenic version of the Ha-*ras* gene in a human tumor (Lowy et al. 1993). In the EJ/T24 bladder carcinoma cell line, a point mutation that altered the twelfth codon of the Ras from glycine to valine converts the normal proto-oncogene Ras into its active oncogenic form (Lowy and Willumsen 1993). Further mutagenesis studies show that mutations at position 12, 13, 59, 61 and 63 are associated with impaired GTPase activity, whereas mutations at codon 16, 17, 116, 117, 119, 144 and 146 cause increased guanine nucleotide exchange (Malumbres and Pellicer 1998). Upon these observations, the involvement of Ras in human malignancies soon became subject of intensive research scrutiny. Now *ras* genes are no doubt one of the

most frequently activated oncogenes detected in human malignancies, associated with up to 30% of all human tumors (Bos 1989; Mulder et al. 1989; Salhab et al. 1989; Mangues and Pellicer 1992; Rodenhuis 1992; Rodenhuis and Slebos 1992) (Table 1.1). Some of the highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%); in thyroid tumors (50%); and in myeloid leukemia (30%) (Bos 1989; Hirsch-Ginsberg et al. 1990; Imamura et al. 1993; Sweetenham 1994). Most mutations happen in codon 12, 13, or 61, or so called hot loci (Bos 1989). Ras mutations are also commonly observed in experimentally induced tumors in animals. However, present studies show that they are apparently rare and do not perform an important role in the pathogenesis of naturally occurring tumors in animals (Rotkiewicz et al. 2004). Whether other mutations in other functional areas of these genes, or if there are other mechanisms involved in their activation in non-human animals needs further study.

Different *ras* genes are activated in different human cancers. K-ras mutations have been found preferentially in adenocarcinomas and are often detected in pancreatic cancers, cholangiocarcinomas, colorectal cancers and in lung cancers. H-ras is often found mutated in bladder cancer, cutaneous squamous cell carcinomas, and squamous head and neck tumors. N-ras mutation are more frequent in acute leukemias (mainly of the myeloblastic cell type) and in the myelodysplastic syndromes (Bos 1989).

Other than point mutations, cell transformation can be also achieved by overexpression of normal Ras proteins. Increasing the absolute amount of non-mutated Ras, thus the overall Ras-GTP level, has been shown to transform NIH 3T3 fibroblasts (Scheele et al. 1995), and more importantly, to cause tumorgenesis in transgenic mice (Hua et al. 1997).

Now, a considerable research effort has been made to define the underlying mechanism by which Ras mediates its actions in normal and neoplastic cells. Illustration of this mechanism may give the hope to development of antagonists of Ras as novel approaches for cancer treatment.

1.5 Ras relatives

Ras serves as a prototype for a large group of small GTPases that share structural and functional homology, the Ras superfamily proteins. Members of this family, which are all between 20-35 KDa, are ubiquitously expressed in all eukaryotic species and regulate a wide range of cellular functions. The enzymatic properties of most of the different members of the Ras superfamily are regulated by the same fundamental mechanism: the GTP/GDP cycle.

The Ras superfamily of proteins is now comprised of over 150 small GTPases and, in terms of both primary sequence and biological activity, it can be divided into six subfamilies, the Ras, Rho, Ran, Rab, Arf, and Kir/Rem/Rad subfamilies (Bourne et al. 1990; Bos 1997). The relationship of Ras superfamily members has been illustrated in a phylogenetic tree (Figure 1-1).

This thesis will focus only on the Ras subfamily, in which 19 proteins have been identified so far as members of this subfamily. Comparative analysis of the primary sequences of these proteins further divided them, as defined by the presence of C. elegans orthologs, into five subgroups, the Ras (H-Ras, N-Ras, K-Ras4A, K-Ras4B), Rap (Rap1A, Rap1B, Rap2A and Rap2B), R-Ras (R-Ras, R-Ras2/TC21), Ral (RalA and RalB), and the newly identified M-Ras (M-Ras/R-Ras3 and Rheb). A characteristic feature of this family is that all the members use a similar effector domain, the so-called switch I region (see discussion on Ras structure below) to interact with their downstream molecules. Recently, several new Ras-like GTPases have been described and included in the Ras subfamily. They lack the characteristic features of Ras proteins, such as prenylation signals (Rin and Rit), or typical effector domains (Rheb, Rhes, Dexras1, NOEY2, and κB-Ras1/2) (Reuther and Der 2000). The functional importances of these proteins has not yet been well characterized.

1.5.1 Ras subgroup

The four proteins in the Ras subgroup (H-Ras, N-Ras, K-Ras4A, and K-Ras4B) are often considered the classical Ras proteins, since they are the first group of Ras proteins identified. These proteins are highly related, exhibiting about 85% sequence homology

between any protein pair (Lowy and Willumsen 1993). As determined by histochemical analysis using specific Ras antibodies, all four Ras proteins are concurrently expressed in most mouse and human tissues, but substantial variations in protein levels are found between tissues as well as during development (Muller et al. 1983; Furth et al. 1987; Leon et al. 1987). For example, H-ras has a particular pattern of expression, with more expression in brain, muscle and skin. N-ras is expressed more in thymus and testis, and K-ras is most prevalent in gut, lung and thymus (Leon *et al.* 1987; Shen *et al.* 1987; Lowy and Willumsen 1993). This observation leads to the question of whether each Ras protein functions differently or their roles overlap. To solve this question, mice that are homozygous null for H-ras alone, N-ras alone, or H-ras and N-ras were generated. Surprisingly, none of these knock-out mice showed detectable defects in development, growth, and fertility (Umanoff et al. 1995; Esteban et al. 2001), suggesting a redundant role between the different Ras proteins. Nevertheless, further analysis on N-ras deficient mice showed reduced number of CD8 single positive thymocytes and decreased thymocyte proliferation *in vivo*. Moreover, TCR signaling and IL-2 production in KO mice are also impaired, indicating a specific role of N-ras in T cell activation and development (de Castro et al. 2003). In contrast to mice lacking H-ras and N-ras, K-ras knock-out mice showed profound defects in hematopoiesis (Johnson, L. *et al.* 1997), myocardial cell proliferation, and neuronal cell survival (Koera et al. 1997), and died progressively between day 12 and 14 of gestation (Johnson, L. *et al.* 1997; Koera *et al.* 1997), demonstrating the selective requirement for this protein. Therefore, it is possible that different Ras isoforms function specifically in a tissue-specific and developmental stage-specific manner.

Throughout this dissertation, the four classical Ras proteins will be collectively referred to Ras unless specially noted.

1.5.2 Rap subgroup

The Rap subgroup consists of two pairs of related proteins: Rap1 (Rap1A and Rap1B) and Rap2 (Rap2A and Rap2B). While the homology between Rap1 and Rap2 proteins is only about 60%, the A and B isoforms for each Rap protein show at least 90% identity.

Overall, Rap1 is extremely similar to H-Ras at the amino acid level, especially at the effector domain (Takai et al. 2001), and both Rap1 and Ras can bind to the effectors Raf-1, B-Raf, RalGDS and AF-6 (Bos et al. 2001). Compared to Ras, this ubiquitously expressed small GTPase has a remarkably diverse subcellular localization including endocytic, lysosomal vesicles and perinuclear structures (Pizon et al. 1994). Some plasma membrane localization of Rap1 has also been reported (Gao et al. 2001; Ohba et al. 2003). A wide range of extracellular stimuli can activate Rap1 through integrins, receptor tyrosine kinases, G protein–coupled receptors, antigen receptors, and other transmembrane proteins (Boussiotis et al. 1997; McLeod et al. 1998; Quilliam et al. 2002; Ribeiro-Neto et al. 2002; Sebzda et al. 2002). Several GEFs have been shown to activate Rap. Two GEFs, MR-GEF (M-Ras-regulated GEF) and PDZ-GEF, function as Rap-specific GEFs in vivo, whereas the dual specificity GEF Ras guanyl nucleotide-releasing protein 3 (RasGRP3) acts on both Ras and Rap1 (Ebinu *et al.* 1998; Rebhun *et al.* 2000a; Rebhun *et al.* 2000b).

Originally, the mammalian Rap1 cDNA (once called K-rev for Ki-Ras revertant) was identified based on its ability to revert the transformation morphology in fibroblasts induced by an oncogenic mutant of K-Ras (Kitayama et al. 1989). Moreover, high levels of activated Rap1 have been detected in anergic T cells (Cook *et al.* 1993; Boussiotis *et al.* 1997; Hu, C. D. *et al.* 1997)), prompting the hypothesis that Rap1 antagonises TCR signaling by sequestering Ras effectors, such as Raf-1, in nonproductive complexes (Zwartkruis and Bos 1999; Stork 2003). However, the competitive binding of Rap1 to Raf-1 can only be seen in cells overexpressing certain mutants of Rap1 (Bos *et al.* 2001). Furthermore, whether Rap1 activates or inhibits the Erk/MAP kinase pathway and cell proliferation appears to be cell type-dependent (Bos *et al.* 2001; Schmitt and Stork 2001; Klinger *et al.* 2002; Laroche-Joubert *et al.* 2002; Sebzda *et al.* 2002).

Recent studies established additional roles of Rap1 that are essential for a particularly wide range of biological processes. Most of Rap1 functions are adhesion-related, including regulatory effects on morphogenesis, cell-cell adhesion, actin polymerization, and cell migration (Rebstein et al. 1993; Asha et al. 1999; Knox and Brown 2002; Tohyama et al. 2003; McLeod et al. 2004). Moreover, several reports showed that expressing constitutively active Rap1 promotes integrin-mediated cell

adhesion in T cells, B cells, and myeloid cells (Katagiri *et al.* 2000; Abraham 2002; Sebzda *et al.* 2002; Shimonaka *et al.* 2003; Katagiri *et al.* 2004; McLeod *et al.* 2004). In budding yeast, the Rap1 orthologue Bud1 (Rsr1) is necessary for bud site selection (Chant et al. 1991; Chant and Pringle 1991). Recently, the importance of the Rap1 signaling pathway has been shown for the first time in mammalian cells by the analysis of knockout mice for C3G, which is a Rap1 GEF and required for integrin-mediated cell adhesion and cellular spreading (Ohba et al. 2001). $C3G^{-/-}$ homozygous mice died before embryonic day 7.5. The $C3G^{-/-}$ fibroblast cell line prepared from these mouse embryo showed impaired cell adhesion, delayed cell spreading and accelerated cell migration, which can be suppressed by expression of active Rap (Ohba *et al.* 2001).

In contrast to Rap1, the signaling role of Rap2 is still elusive. It has been thought to be functionally analogous to Rap1 since some of the effectors of Ras and Rap1, such as Raf-1, RalGDS, and PI3K, also interact with Rap2 (Ohba *et al.* 2000a). However, the effector domain of Rap2, although similar, is different from those of Ras and Rap1, suggesting it may interact with effectors specific for Rap2 and thereby signaling to distinct pathways.

1.5.3 Ral subgroup

The members of Ral subgroup (RalA and RalB) recently have attracted much attention, mostly due to the finding that the Ral guanine nucleotide dissociation stimulator (RalGDS), a Ral-specific GEF, is regulated by direct binding to Ras (as well as several other Ras-like GTPases) (Hofer et al. 1994; Spaargaren and Bischoff 1994). Moreover, two structurally related RalGEFs, Rgl and Rlf, are also found to be activated by interacting with Ras, establishing the RalGEF-Ral pathway as an effector pathway of Ras (Kikuchi *et al.* 1994; Wolthuis *et al.* 1996; Wolthuis *et al.* 1998a; Wolthuis *et al.* 1998b). Unlike the interaction between Ras and Raf, the Ras binding of RalGDS involves both the switch I and II regions of Ras (Huang, L. et al. 1998).

However, activation of Ral seems controlled by both Ras-dependent and Ras-independent events. For instance inhibition of Ras activation by dominant-negative Ras (Ras17N) had little effect on Ral-GTP levels (Hofer et al. 1998). In stead, Ral activation in platelets is mediated by Ca^{2+} ionophore ionomycin-induced increase of

intracellular Ca^{2+} level (Hofer *et al.* 1998; Wolthuis *et al.* 1998a).

The function of Ral is largely unclear. There is circumstantial evidence that Ral may have a function in regulating the cytoskeleton through its interaction with RIP1 (also known as RLIP or RalBP1), a GTPase-activating protein specific for the small GTPases Cdc42 and Rac (Symons 1996). The involvement of Ral in endocytosis and/or receptor downregulation have also been postulated (Yamaguchi et al. 1997; Ikeda et al. 1998).

1.5.4 R-Ras subgroup

Originally identified based on its similarity with Ras, R-Ras defines a subgroup of protein in the Ras subfamily that consists of a pair of closely related proteins: R-Ras and R-Ras2/TC21 (Lowe and Goeddel 1987; Reuther and Der 2000).

The two GTPases have a 26-amino acid NH_2-terminal extension and differ from other members of the Ras subfamily at the highly divergent C-terminal sequences. The core effector domain of R-Ras comprising 9 amino acids (amino acids 30-40) is identical to that of Ras (Self et al. 1993). As a result, all of the effectors identified for R-Ras, including phosphatidylinositol 3-kinase (PI3K), Ral-GDS/Rlf/Rgl, the guanine nucleotide exchange factor (GEF) Ras-GRF, and Nore, are shared with Ras (Spaargaren and Bischoff 1994; Spaargaren et al. 1994; Herrmann et al. 1996; Gotoh et al. 1997; Marte et al. 1997; Self et al. 2001). Similar to oncogenic Ras, the constitutively active mutant of R-Ras is able to transform, although weakly, NIH3T3 cells, stimulate cell proliferation, and give rise to tumors in nude mice (Lowe and Goeddel 1987; Cox et al. 1994; Yu and Feig 2002). Moreover, cells expressing R-Ras have an elevated basal level of MAP kinase, seeming to suggest that R-Ras can activate transduction pathways of Ras (Cox *et al.* 1994). However, unlike classical Ras proteins, activation of MAP kinase pathway by R-Ras or TC21 seems to be Raf-1 independent, suggesting the involvement of R-Ras-specific effectors (Graham et al. 1996; Huff et al. 1997). Guanine nucleotide exchange of R-Ras and TC21 is promoted by RasGRF, C3G, CalDAG-GEFI, and RasGRP1 and RasGRP2 both in 293T cells and *in vitro* (Ohba *et al.* 2000b; Yamashita *et al.* 2000).

R-Ras has unique cellular functions. One of the major activities of R-Ras is to affect integrin-mediated adhesion. The expression of a constitutively active R-Ras has been

shown to convert cell lines that grow in suspension into highly adherent cells by increasing the affinity of the prototype platelet integrin, αIIbβ3, for soluble ligand (Zhang, Z. et al. 1996). This activity of R-Ras requires the effector loop and the prenylation site, as well as the proline-rich sequence in the hypervariable region of R-Ras (Oertli *et al.* 2000; Wang, B. *et al.* 2000a; Hughes *et al.* 2001). R-Ras has also been implicated in cell spreading, cell survival and haptotactic migration, a cellular migration process requires the interaction between integrin and its ligand (Suzuki, J. *et al.* 1997; Osada *et al.* 1999; Berrier *et al.* 2000; Suzuki, J. *et al.* 2000). Overexpression and mutation of TC21 have been reported in tumor tissues or cell lines (Saez et al. 1994; Huang, Y. et al. 1995; Clark et al. 1996).

1.5.5 M-Ras subgroup

M-Ras proteins, discovered by a search of the NCBI expressed sequence tag data base for Ras-related gene products, are the new additions to the Ras subfamily. They show wide expression in brain, heart, skeleton muscle, thymus, spleen, and cell lines of hematopoietic origin, as well as in epithelial cells. (Kimmelman *et al.* 1997; Matsumoto, K. *et al.* 1997; Ehrhardt *et al.* 1999). The constitutively active form of M-Ras induced the formation of microspikes, focus formation, anchorage-independent growth, and moderate activation of Erk in fibroblasts (Kimmelman *et al.* 1997; Matsumoto, K. *et al.* 1997).

Although misleadingly referred as R-Ras3 by one of the two independent groups that cloned it, (Kimmelman *et al.* 1997; Matsumoto, K. *et al.* 1997), M-Ras actually differs at the carboxyl terminus from both TC21/R-Ras2 and R-Ras (Ehrhardt *et al.* 1999). Moreover, M-Ras only weakly binds to some of the common effectors for classical Ras and R-Ras such as RalGDS and Raf-1 (Matsumoto, K. *et al.* 1997; Ehrhardt *et al.* 1999). The regulators of M-Ras and R-Ras are also different. For example, the Ras guanine nucleotide exchange factor (GEF) son of sevenless (Sos) activates M-Ras but not R-Ras (Gotoh *et al.* 1997; Quilliam *et al.* 1999; Ohba *et al.* 2000b). Ras GAPs such as p120 GAP, and NF-1 regulate both M-Ras and R-Ras, whereas R-Ras GAP stimulates R-Ras, but not M-Ras (Ohba *et al.* 2000b). These data may suggest that M-Ras mediates activation of distinct signaling pathways that function

in parallel to those downstream of Ras (Ehrhardt *et al.* 1999).

Another member identified in this group based on sequence similarity is Rheb (Ras homolog enriched in brain) (Yamagata et al. 1994). The exact cellular functions of M-Ras subgroup proteins are still largely unknown. Some studies suggest Rheb may function to antagonize v-Ras signaling, although it is remain to be determine whether this is the physiological role for this Ras-related protein (Del Villar et al. 1996; Clark et al. 1997).

1.6 Protein structures of Ras

1.6.1 Primary structure

The protein encoded by each ras gene is approximately 21KD in size, containing 188 (H-Ras, K-Ras4A and N-Ras) or 189 (K-Ras4B) amino acids (Lowy and Willumsen 1993). Amino acid sequence analysis of all four Ras species reveals the presence of four distinct domains required for Ras functions: residues 1-85, 85-165, 166-185, and at the very carboxyl terminal end, the CAAX motif (residue 186-189; C: cysteine, A: aliphatic amino acid, and X: serine or methionine) (Figure 1.2). The first 165 residues are highly homologous among all Ras proteins (with the first 85 residues being identical and 79% of the next 78 residues being identical) and account for the GTP hydrolysis activity of Ras (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984; Temeles et al. 1985; Lowy and Willumsen 1993). In the region encompassing the next 20 residues lies the so-called heterogeneous region because it is the most divergent region among all Ras proteins with only 4% sequence identity (Lowy and Willumsen 1993). However, in each Ras species, this region is highly conserved between human and mouse, making it very likely that this region is responsible for isoform-specific functions of Ras proteins (Lowy and Willumsen 1993). While some studies seem to support this idea, others showed codons 166-179 are dispensable for the transforming activity of activated Ras, suggesting that the isoform-specific function of each Ras protein is a more complex story. (Willumsen *et al.* 1984b). In addition, the amino terminal end, residues 64-76, 93-108, and 124-138 have been shown to be unimportant for structural integrity of Ras

or its effector function (Barbacid 1987). Finally, the last functional domain, the CAAX motif, is present in all Ras proteins to direct post-translational modification (see discussion below) required for Ras membrane association.

1.6.2 Three dimension structure

Crystallographic analyses of Ras have allowed us to gain considerable insight into the biochemical and cellular activities of Ras (Krengel et al. 1990; Pai et al. 1990; Lai et al. 1993). The crystal structures of both the wildtype and mutated Ras proteins complexed to a slowly hydrolyzing GTP analogue has been solved at high resolution (de Vos *et al.* 1988; Pai *et al.* 1990; Tong *et al.* 1991). Data analysis shows that Ras consists of six beta sheet strands connected by hydrophilic loops and alpha helices (Figure 1.3). The central beta sheets comprise the hydrophobic core of the protein. The structural core of Ras is conserved in all members of G-proteins, reflecting the significant sequence identity (Bourne *et al.* 1990; 1991). Surprisingly, the structure of mutated Ras appears to be almost identical as that of the wildtype protein, with the only significant differences found in one of the loops (L4) and in the vicinity of the gamma-phosphate of bound GTP (Krengel *et al.* 1990).

Three distinct structural motifs are responsible for the ability of Ras to bind to guanine nucleotides. These motifs are polypeptide loop regions termed L1 (also known as P-loop; residues 10-17), L2 (residues 27-37) and L4 (residues 59-66) (Pai *et al.* 1990; Bourne *et al.* 1991). Protein sequences of these loop regions are found conserved in other G-proteins such as EF-Tu and Gα subunits (Bourne *et al.* 1991).

L1 region is critically involved in the binding to the α- and β- phosphated GTP. The glycine residue located in position 12 (Gly12) of Ras is found conserved in all guanosine and some adenine nucleotide binding proteins (Moller and Amons 1985). It is located close to the 'arginine finger' of the GAP proteins, which complements the active site of Ras. Any amino acid substitutions at this position would sterically interfere with the geometry of the transition state in which GTP is hydrolyzed in the presence of the GAP fingers (Krengel *et al.* 1990; Tong *et al.* 1991). Interestingly, Gly12 is one of the loci frequently found mutated in oncogenic Ras proteins. Replacement of serine at position 17 by asparagine generates a Ras mutant protein (Ras17N) with largely reduced affinity

for GTP. Ras17N has been widely used as dominate negative mutant to suppress endogenous Ras activity in many cell lines (Feig and Cooper 1988a). L1 is relatively rigid and does not undergo conformational changes upon GTP binding and hydrolysis (Brunger et al. 1990; Milburn et al. 1990).

L2 and L4 are involved in binding to the Mg^{2+} and to the γ–phosphate in the GTP-bound form (Tong *et al.* 1991; Lowy and Willumsen 1993). In contrast to L1, these two loop regions are highly flexible and account for most of the conformational changes during GTP binding, which are essential for the switch function of Ras (Ma and Karplus 1997a). The L1 and L4 regions, including certain adjacent residues that also contribute to the switch function, are thus also known as switch I and switch II (switch I: residues 30-38; switch II: residues 60-76), respectively (Wittinghofer and Pai 1991; Ma and Karplus 1997b). Hydrophilic residues are found on the external surface of the protein. When complexed with GTP, direct hydrogen bonds are formed between the γ phosphate of GTP and the backbone atoms of threonine 35 (Thr35) in switch I and glycine60 (Gly60) and glutamine61 (Gln61) in switch II. Threonine 35 also coordinates Mg^{2+} ion. These interactions lead to the localized yet relatively large conformational changes in the two switches to facilitate effector binding (Ma and Karplus 1997a). The switch I region is the main effector binding site responsible for interaction with p120GAP and other downstream effectors. It is sensitive to mutations that impair transforming ability (Sigal *et al.* 1986a; Sigal *et al.* 1986b; DeClue *et al.* 1991). Switch II region is partially involved in binding to GEFs and are critical for the intrinsic GTPase activity of Ras. Especially, the Gln61, also a hot spot for mutations in cancers, has a vital role in catalysis. It directly interacts with a specific residue (Arg789) of GAP p120 to allow the nucleophilic attack of a water molecule that is crucial for GTP hydrolysis. Mutations of this residue therefore result in reduced stabilization of the transition state and impaired GTPase activity (Krengel *et al.* 1990).

1.7 Biochemical properties of Ras

1.7.1 Nucleotide binding

The Ras proteins bind magnesium complexes of guanine nucleotides with high affinity [K_d approximately 10^{11} M^{-1}] to form an extremely stable complex (Hoshino et al. 1987; John et al. 1990; Rensland et al. 1991). The GDP-bound Ras proteins, which exist predominant in resting cells, are at their inactive state. Releasing of GDP from Ras leads to the binding of Ras to the relatively prevalent free GTP in cytosol, which induces a rapid conformational change that turns Ras on (Bos 1997). The intrinsic GTP hydrolysis activity of Ras converts GTP to GDP and turns Ras off (Figure 1.4) (Scolnick et al. 1979; Shih et al. 1980; Bos 1997). In addition to GTP and GDP, Ras also forms complex with dGTP, ppGpp and several nonphysiological analogs of GTP and GDP *in vitro* (Scolnick *et al.* 1979; Tucker *et al.* 1986).

As determined by structural analysis, different residues are responsible for GDP and GTP binding. In the GDP-bound state, a serine at position 17 directly corrdinates with Mg^{2+} and the β-phosphate of GDP. Several other amino acids near the binding site also contribute to GDP binding indirectly through four water molecules. In the GTP-bound state, coordination between the γ-phosphate and a second conserved threonine at position 35 replaces the coordination with two of the four water molecules (Krengel *et al.* 1990; Tong *et al.* 1991). The negative effect of Mg^{2+} on GDP dissociation is essential for guanine nucleotide binding and GTPase activity (Hall and Self 1986; John et al. 1993). In line with this idea, the Mg^{2+} coordination site is highly conserved among GTP-binding proteins. Mutation of the first Mg^{2+} coordination site to asparagine in Ras (RasS17N) abolishes the cation's effect on GDP dissociation,

1.7.2 GTP hydrolysis

The intrinsic Ras GTPase activity results in the slow hydrolysis of bound GTP leaving the Ras proteins complexed with GDP (Feuerstein *et al.* 1987; John *et al.* 1990). Mutations on residue 12 or 61 affect this rate, resulting in a 3-9 fold increase in the half-life of Ras-GTP complex (1-5 hours) (Feuerstein *et al.* 1987; John *et al.* 1990). The GTPase activity seems to be limited in the first 164 amino acids since a truncated

version of Ras contains only the first 166 amino acid behaves the same as the full length protein in terms of biochemical properties (Gideon et al. 1992; Mistou et al. 1992).

High resolution X-ray crystallographic analysis of Ras crystallized with a nonhydrolyzable GTP analog, as well as caged GTP, which photolyzes to GTP after UV exposure and then undergoes hydrolysis in situ in the crystal, provides many hints for the otherwise debatable mechanism of GTP hydrolysis (Pai *et al.* 1990; Schlichting *et al.* 1990; Prive *et al.* 1992). The hydrolysis probably involves an inversion of the γ-phosphate and an in-line attack on the γ-phosphate by the hydroxyl group from a nearby catalytic water molecule (Pai *et al.* 1990). A hydrolyzing conformation of the GTP-binding pocket has been proposed (Kraut 1988). The conserved residue Gln61 is thought to locate in this pocket and activate the critical water molecule for hydrolysis in Ras. Mutations at this residue have been shown to reduce GTPase rate by 10-fold and prevent responsiveness to RasGAPs (Der et al. 1986; Vogel et al. 1988).

1.7.3 Spontaneous guanine nucleotide exchange

In normal Ras GTP/GDP cycles, dissociation of GDP leads to rapid binding of GTP and thus activation of Ras proteins. This is because the concentration of free GTP in cytosol is much higher than that of GDP. Besides, the intrinsic dissociation rate of GTP is very low (approximately 10^{-5} moles per second dissociating per mole of complex) (John *et al.* 1990). Mutations in residues 14, 16, 59, 61, 83, 116, 119, 144, 146 have all been shown to affect the rate of spontaneous GTP loading of Ras, resulting in high proportion of Ras-GTP (Patel et al. 1992; Zhang, K. et al. 1992).

1.7.4 Posttranslational modification and membrane localization of Ras

An important property of Ras proteins that is known to be a key requirement for their transforming properties is that Ras proteins are constitutively associated with the inner side of the plasma membrane (Willumsen *et al.* 1984a; Willumsen *et al.* 1984c). Membrane localization allows Ras to interact with upstream activators and downstream targets. This process is achieved by a series of posttranslational modifications at the C termini of newly synthesized Ras proteins. The initial step is to attach a C15 polyisoprenyl (farnesyl) moiety to a conserved cysteine at position 186 in the CAAX

motif (see below) (Casey et al. 1989). This is followed by the proteolytic removal of the AAX and the carboxymethylation of the newly generated farnesylated Ras C-terminal cysteine to give a more hydrophobic C-terminal end in Ras proteins (Gutierrez et al. 1989). Cooperating with a secondary membrane targeting signal provided by the adjacent hypervariable region (see below) of Ras, the protein stably associated with the plasma membrane. In H-Ras, N-Ras, and K-Ras4A, this signal contains one or two palmitoylated cysteine residues within the hypervariable region (Hancock et al. 1989), whereas in K-Ras4B, the signal consists of a polybasic domain containing multiple lysine residues (Figure 1.2) (Hancock et al. 1990). Different membrane anchors among various Ras proteins might provide a mechanistic explanation for their preferred intracellular localizations, thus functional specificity, in distinct membrane domains.

Besides plasma membrane, the presence of functional H- and N-Ras, but not K-Ras in endomembrane compartments such as the endoplasmic reticulum and the Golgi apparatus has recently been reported (Choy et al. 1999; Chiu et al. 2002), giving rise to the notion that Ras proteins localized in plasma membrane, reticulum, and Golgi can signal differently (Chiu *et al.* 2002).

1.8 Pathways that activate Ras

Ras serves as a point of convergence of multiple signaling pathways stimulated by a great variety of extracellular signals including growth factors, cytokines, hormones and neurotransmitters. These extracellular signals activate Ras through the stimulation of different type of receptors at the surface of the cells, such as tyrosine kinase receptors, cytokine receptors, immune cell receptors, and subunits of heterotrimeric G protein coupled receptors (GPCR) (Malumbres and Pellicer 1998; Vojtek and Der 1998).

1.8.1 Protein tyrosine kinase signaling pathway

The mitogen-activated protein kinase (MAPK) pathway induced by receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) is one of the best-characterized pathways activated by Ras. Extracellular signals such as EGF stimulate the dimerization and autophosphorylation of EGFR on several specific

tyrosine residues near the C terminus, providing docking sites for many Src homology 2 (SH2)-containing proteins such as Grb2, Shc, phosphoinostide 3 kinase (PI3K), phospholipase C-γ (PLC-γ), p120 RasGAP, and SH-PTP2 tyrosine phosphatase (Syp) (Ullrich and Schlessinger 1990; Pawson 1995; Bonfini *et al.* 1996). Grb2 forms stable complex with Sos. Direct recruitment of Grb2/Sos complex to RTK allows Sos to interact with Ras and activate it (see detailed discussion below).

1.8.2 G-protein coupled receptor signaling pathway

The superfamily of GPCRs comprises a large group of structurally diverse receptors for many hormones, paracrines, neurotransmitters, and neuromodulators with important physiological functions. It has been estimated that ~80% of known hormones and neurotransmitters activate cellular signal transduction mechanisms by activating GPCRs (Liao et al. 1990; Kristiansen 2004). Upon binding of extracellular ligands, these seven-transmembrane-spanning receptors couple to one or several subtypes of G protein which reside at the intracellular side of the plasma membrane to trigger intracellular signaling events (Marinissen and Gutkind 2001; Armbruster and Roth 2005). The Heterotrimeric guanine triphosphate-binding proteins (G proteins) consist of a Gα subunit and the Gβγ complex, both of which can activate effectors. The Gα subunits are 40kDa proteins that bind guanine nucleotides. In resting cells, the GDP-bound Gα subunits form a stable complex with the Gβγ subunits. Ligand binding to GPCR induces GTP-GDP exchange on Gα subunit, resulting in the dissociation of Gα subunit from Gβγ subunits. Gα subunits in their GTP-bound form activate target effectors such as adenylate cyclase or PLC-β, resulting in cAMP synthesis or PIP_2 hydrolysis, respectively (Clapham and Neer 1997; Marinissen and Gutkind 2001; Armbruster and Roth 2005).

The Gα subunit promoted metabolism of PIP_2 produces two secondary messengers: DAG and IP_3. The former mediates membrane translocation and activation of C1 domain containing proteins such as PKCs, which is thought to activate MAPK pathway by directly phosphorylating Raf-1 in many cell types (Sozeri *et al.* 1992; Kolch *et al.* 1993; Gudermann *et al.* 2000). IP_3 leads to the increase of intracellular free calcium,

which may stimulate Ras through activating RasGRF, a calcium-regulated RasGEF in brain (Berridge *et al.* 1993; Marinissen and Gutkind 2001).

The Gβγ subunits, once dissociated from Gα subunit, can activate Ras-MAPK pathway in a PI3K inhibitor sensitive, yet PKC-independent manner (Crespo *et al.* 1994a; Crespo *et al.* 1994b; Mattingly *et al.* 1994; Marinissen and Gutkind 2001). The effect was found to be dependent on PI3Kγ, a PI3K isotype activated by both the Gα and Gβγ subunits (Lopez-Ilasaca *et al.* 1997a; Lopez-Ilasaca *et al.* 1997b). Ras activation by Gβγ subunits also requires a tyrosine kinase, adaptors such as Shc and Grb2, and Sos (Lopez-Ilasaca *et al.* 1997a).

1.8.3 Immune receptor-mediated Ras activation

Upon antigen ligation, immune receptors expressed on the surface of T cells, B cells, natural killer cells, mast cells and platelets initiate the formation of multiprotein signaling complexes, which propagate the signal to activate many distal signaling transduction pathways including the Ras/MAP kinase cascade.

The T cell receptor is built of six different polypeptide chains. The specificity of ligand binding is dictated by the clonotypic TCRα and TCRβ chains which form a heterodimer to recognize peptide-MHC complex directly (Call and Wucherpfennig 2004). TCRαβ stably associates with CD3 chains, which are arranged into three dimers: γε, δε, and ζζ (Call and Wucherpfennig 2004). Each CD3 chain contains immunoreceptor tyrosine-based activation motifs (ITAMs) that posse a pair of conserved tyrosine residues. Stimulation of the TCR with antigen-MHC complex or antibodies directed against TCR subunits results in the rapid phosphorylation on these tyrosines by the Src-family kinase Lck. This event is prerequisite for initiation of TCR signaling (Call and Wucherpfennig 2004). Lck activation requires its exposure to CD45, a tyrosine phosphates involved in Lck activation through catalyzing the dephosphorylation of the negative regulatory site on Lck (Hermiston *et al.* 2002). Once fully phosphorylated by Lck, ITAMs serve as docking sites to recruit SH2 domain-containing protein tyrosine kinases (PTKs) such as ZAP-70. ZAP-70 recruitment leads to its activation by Lck phosphorylation and by autophosphorylation on specific tyrosines, which in turn allow

ZAP-70 to recruit and tyrosine-phosphorylate various positive and negative signaling effectors to the TCR complex. These include α-tubulin, Sam-68, Vav-1, VHR, Shc, Gab2, LAT, and SLP-76 (Elder 1998). LAT and SLP-76 are two adapter proteins essential for TCR signaling. They serve as the nucleation points for the formation of the multiprotein signaling complexes to allow intermolecular interactions to occur (Figure 1.5). One of the proteins activated by LAT and SLP-76-mediated recruitment is phospholipase C-γ1 (PLC-γ1). Upon activation, PLC-γ1 hydrolyses PIP_2 to produce two secondary messengers: DAG and IP_3. DAG activates PKC and is required for activation of Ras in T cells, while IP_3 stimulates Ca^{2+} mobilization. LAT and SLP-76 deficient mice share similar phenotypes, including defects in PLC-γ1 activation and Ca^{2+} mobilization and Ras/MAP kinase activation (Wange 2000; Bunnell *et al.* 2003; Huang, Y. and Wange 2004).

The mechanism for B cell receptor-mediated Ras activation is very similar to that found in T cells. However, a different set of PTKs and adaptors are involved in signal initiation and propagation. The BCR is also a multiprotein structure. It consists of an antigen binding-membrane immunoglobulin (mIg) and a disulfide-linked heterodimer of the Igα and Igβ proteins, each contain a ITAM motif within their cytoplasmic tail (Dal Porto *et al.* 2004). Upon BCR aggregation, ITAM is tyrosine-phosphorlylated by src-family tyrosine kinases such as Lyn, providing binding sites for other PTKs such as Syk. Syk in turn recruit and phosphorylates the adaptor protein B cell linker protein (BLNK). BLNK is structurally homologous to the SLP-76 in T cells. Like SLP-76, it provides a major platform for the assembly of multiple effector molecules. One of these is PLC-γ2, another PLC isoform that catalyzes PIP_2 hydrolysis to produce DAG and IP_3 (Kurosaki 2002; Dal Porto *et al.* 2004).

Despite the clear link between PTK activation and the phosphatidylinositol pathway, less is known about how PTK activation and DAG production in T cells are linked to GTP loading of Ras (Downward *et al.* 1990). It was originally thought occurs as a result of PKC-mediated RasGAP inhibition (Downward et al. 1990). However, this hypothesis can not explain why PMA treatment did not activate Ras in other cell types. Moreover, the T cell receptor induced Ras activation can not be completely blocked by PKC

inhibitors, suggesting a secondary PKC-independent pathway to Ras (Burgering et al. 1993; Izquierdo et al. 1994). By parallel to the model of RTK activation of Ras in growth factor receptor signaling, tyrosine kinases-dependent membrane recruitment of Grb2/Sos was suggested to be responsible for immune receptor-induced, PKC-independent activation of Ras. However, solid evidence is still missing in support of this model in lymphocytes (see discussion below). Also, this model can not be used to explain why TCR-induced Ras activation requires PLC-γ1 and can be efficiently stimulated by DAG analogs.

The discovery of RasGRP, a RasGEF with a DAG-binding domain, provided a sound link between DAG signaling and Ras activation in lymphocytes. DAG-mediated recruitment of RasGRP is thought to lead to its activation of Ras (Ebinu *et al.* 1998; Dower *et al.* 2000; Ebinu *et al.* 2000; Oh-hora *et al.* 2003). However, the exact molecular mechanism underlining RasGRP regulation is still not fully understood and appears to be more complex than originally thought.

As discussed above, the regulation of Ras involves a complex network of interacting pathways. The work describe here adds new levels of complexity to this signaling system.

1.9 Ras signaling from internal membrane systems

In addition to transducing extracellular signals across the plasma membrane through various cell surface receptors, Ras has been shown recently to also signal from internal membrane systems such as the endosomes and Golgi apparatus (Chiu *et al.* 2002).

As discussed before, Ras contains a CAAX motif at its C-terminus which undergoes a series of lipid modification processes which are required for membrane association (Hancock *et al.* 1990). At least some of the enzymes involved in CAAX processing are located in the ER and Golgi membrane (Hancock *et al.* 1990; Dai *et al.* 1998). Therefore, CAAX processing targets Ras to these internal membrane systems, from where Ras further traffics to the plasma membrane (Hancock *et al.* 1990; Michaelson *et al.* 2001). The hypervariable region of Ras is also involved in directing different Ras proteins to

distinct subdomains on the plasma membrane, as well as to various intracellular compartments, which is thought to be the basis for specificity in Ras signaling (Michaelson *et al.* 2001; Prior *et al.* 2001).

Following cellular activation by growth factor receptors, such as the EGFR, these receptors are internalized from the cell surface and sorted either to late endosomes and lysosomes for degradation or to recycling compartments via clathrin-dependent and -independent pathways (Di Fiore and Gill 1999; Mineo *et al.* 1999). This process has been implicated in the downregulation of EGFR signaling (Di Fiore and Gill 1999). However, using fluorescent probes and live cell imaging, several groups provided evidence showing that the internalized EFGRs are still active (Mineo *et al.* 1999; Wouters and Bastiaens 1999; Sorkin *et al.* 2000). In addition, Grb2, Shc, and specific isoforms of Ras have been found co-localized with EGFR on endosomes (Pol *et al.* 1998; Chiu *et al.* 2002; Jiang, X. and Sorkin 2002; Roy *et al.* 2002).

In addition to signaling from endosomes, Ras is also found to signal from the Golgi apparatus when stimulated with growth factors. Compared to the rapid and transient Ras activation on plasma membrane, signaling from Golgi is delayed but sustained (Chiu *et al.* 2002). More surprisingly, TCR engagement-induced Ras activation appeared to primarily happen on the Golgi apparatus (Bivona and Philips 2003). Further analysis suggested that Ras activation on the Golgi was Src- and PLC-γ1-dependent. Moreover, the diffusible secondary messenger calcium is thought to mediate the translocation of the signal from cell surface to Golgi. Therefore, RasGRP1, the RasGEF with both DAG-binding C1 domain and calcium-binding EF-hand motif, has been suggested to be responsible for Ras activation on the Golgi, whereas the Ca^{2+}-activated Ras-GAP CAPRI (calcium-promoted Ras inactivator) is thought to inactivate Ras on the plasma membrane (Bivona *et al.* 2003). In support of this idea, overexpressed RasGRP1 has been shown to accumulate primarily on Golgi upon TCR ligation in the Jurkat T cell line (Bivona *et al.* 2003).

The biological significance of Ras signaling from internal membrane systems is still under investigations. By pharmacological interference that distinguished EGFRs within endosomes from EGFRs within the plasma membrane, it has been shown that activation of EGFRs specifically on endosomes may promote cell survival via phosphatidylinositol

3-kinase (Wang, Y. *et al.* 2002). Activation of over-expressed Ras on Golgi also induced differentiation of PC12 cells, transformed fibroblasts and mediated radio-resistance (Bivona *et al.* 2003).

1.10 Regulators of Ras activity

As a molecular switch to connect extracellular stimuli to intracellular output, Ras needs to rapidly cycle between GDP- and GTP-bound states. However, the high affinity binding of Ras for GDP and GTP, and the low intrinsic GTP hydrolysis make the spontaneous conversion of GTP- to GDP-bound Ras an extremely slow process with an estimated k_{cat}=0.0028 min^{-1} (Temeles *et al.* 1985). In cells, GDP/GTP cycling is controlled by two main classes of regulatory proteins. Guanine-nucleotide-exchange factors (GEFs) accelerate guanyl nucleotide exchange activity of Ras by 100-fold, and thus function as Ras activators (Malumbres and Pellicer 1998). On the other hand, GTPase-activating proteins (GAPs), which enhance the intrinsic GTPase activity by more than 10^5-fold, promote formation of the inactive GDP-bound form (Jones, S. *et al.* 1991; Gideon *et al.* 1992).

Guanine nucleotide exchange is thought to be the limiting step in Ras activation since the nucleotide exchange rate is slower than the rate of GTP hydrolysis (Feig and Cooper 1988b). Moreover, the intracellular concentration of free GTP is much higher that that of GDP. Therefore, Ras that disassociates from the nucleotide will quickly form a complex with GTP, suggesting that GDP disassociation leads to Ras activation (John *et al.* 1990).

The crystal structure of Ras in complex with the catalytic domain of Sos1 provides insights on the molecular mechanism of Ras GEF action (Boriack-Sjodin et al. 1998). Sos uses two mechanisms to catalyze the GDP-release from Ras. First, an alpha-helix structure from Sos inserts into Ras to displace the Switch 1 region of Ras and open up the nucleotide-binding site. Second, side chains from leucine938 and glutamine942 in this helix of Sos interact with Ras, resulting a distorted conformation of the switch 2 region. Changes in the chemical environment of the binding site then cause the

disruption of the binding for the phosphate groups of the nucleotide and the associated magnesium ion. After promoting nucleotide dissociation, Sos forms a stable complex with nucleotide-free-Ras. The Sos-Ras complex adopts a conformation that does not block the binding of GTP or GDP. Notably, the N17 Ras has an abnormally low affinity for GTP, thus it can form stable complex with Sos (Feig and Cooper 1988a). Overexpression of N17 Ras leads to depletion of endogenous Ras GEF in cells and thus may used as a dominant-negative tool in dissecting Ras signaling pathways.

By a three-dimensional structural study on a transition-state-mimicking complex formed between Ras-GDP and the catalytic fragment of p120GAP, the mechanism for Ras GAP promoting Ras GTP hydrolysis is also proposed (Mittal et al. 1996). According to the structure, RasGAPs align the catalytically important Gln61 in the switch II region of Ras. They also insert a so-called arginine finger residue (R789) into the active site of the GTPase and directly participate in Ras-GTP hydrolysis by stabilizing emerging negative charges during the transition state (Scheffzek et al. 1997).

1.10.1 Positive regulation of Ras by RasGEFs

RasGEFs were first identified in lower eukaryotes. In yeast *Saccharomyces cerevisiae* the CDC25 gene product was genetically mapped as being upstream of Ras and is an activator of the RAS/cyclic AMP pathway. It was subsequently confirmed to possess RasGEF activity (Jones, S. *et al.* 1991; Lai *et al.* 1993). Before long, mammalian homologs of CDC25 were cloned 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).

So far, 3 major families of GEFs responsible for Ras activation have been described in mammalian cells and will be discussed. They are the closely related Sos1 and Sos2 proteins that are similar to the *Drosophila* gene product *Son of Sevenless*, highly homologous GRF1 and GRF2 proteins, and the RasGRP family members (Ebinu *et al.* 1998; Vojtek and Der 1998; Quilliam *et al.* 2002; Downward 2003; Arozarena *et al.* 2004). They all contain a highly conserved domain with GEF activity related to that of

the *Saccharomyces cerevisiae* protein CDC25 (Camonis *et al.* 1986; Boy-Marcotte *et al.* 1989).

1.10.1.1 SOS

The ubiquitously expressed son of sevenless (Sos) was first identified in *Drosophila melonogaster* (dSos) and is the best-studied RasGEF that mediates growth factor receptor-induced activation of Ras (Simon et al. 1991; Li, N. et al. 1993; Myung et al. 2000). Mammalian cells express two closely related Sos proteins, Sos1 and Sos2 (Rogge et al. 1991; Bowtell et al. 1992; Chardin et al. 1993). Sequence analysis reveals distinct functional domains including a N-terminal Dbl homology (DH) domain, followed by a pleckstrin homology (PH) domain, a CDC25 box, and a C-terminal proline-rich region (Figure 1.6).

The Dbl homology (DH) or RhoGEF domain consists of an ~150 amino acid region that functions as GEF for Rho and Rac family GTPases (Quilliam et al. 1995; Hoffman and Cerione 2002). In COS-1 cells, DH domain is required for ectopically expressed Sos to activate Rac (Nimnual et al. 1998). The DH domain is invariably followed by a PH domain, which is a segment of approximately 100 amino acids involved in mediating protein-protein interaction and membrane translocation. While not absolutely required for catalysis of nucleotide exchange, the presence of PH domain appears to greatly increase catalytic efficiency of the DH domain in many cases (Luttrell et al. 1995; Hoffman and Cerione 2002). The CDC25 box is highly conserved among different RasGEFs and functions to mediate the catalytic activity (Boguski and McCormick 1993). The CDC25 box in Sos acts on all three forms of Ras proteins (Leonardsen *et al.* 1996).

A characteristic feature of Sos is the presence of multiple proline-rich regions that mediate binding to SH3 domain containing adaptors such as Grb2 and Nck (Buday 1999; Wunderlich et al. 1999). In resting cells, Grb2 binds to the C-terminal proline-rich region of Sos via its SH3 domain to form a relatively stable complex in cytosol. Upon RTK activation by growth factors such as EGF, the SH2 domains of Grb2 bind to phospho-tyrosines on intracellular domains of activated receptors directly, leading to membrane recruitment and activation of Sos (Simon et al. 1991; Egan et al. 1993; Li, W. et al. 1994). In some cases, the recruitment of Grb2/Sos complexes by

tyrosine-phosphorylated RTKs is indirect and requires additional adaptors. One example is the Shc protein. Shc binds to tyrosine-phosphorylated RTK through its SH2 domain, and to Grb2 SH2 domain via its phosphotyrosine-containing motif. Thus, Shc functions as a bridge to recruit Grb2/Sos complex and contribute to Ras activation (Bonfini *et al.* 1996; Luzi *et al.* 2000).

Notably, Sos itself is also a target for multiple site regulatory phosphorylation (Corbalan-Garcia *et al.* 1996). In human peripheral blood T lymphoblasts, phorbol ester treatment caused rapid Sos phosphorylation on serine/threonine residues in an Erk-dependent manner. Phosphorylation disassociated Grb2 from the Grb2/Sos complex, and thus suppressed Sos membrane localization (Buday *et al.* 1995). Similar observations were also made in insulin receptor or growth factor receptor-mediated Ras activation (Corbalan-Garcia *et al.* 1996; Waters *et al.* 1996). Thus, Erk-dependent Sos phosphorylation may represent a negative feedback mechanism responsible for downregulation of Ras signaling following cellular activation. However, in the Jurkat T cells, while the basal level of Sos phosphorylation seems to depend on Erk, TCR ligation-stimulated Sos phosphorylation was Erk-independent, and instead relied on the activity of CD45, Lck, and PKC (Zhao *et al.* 1997). Moreover, TCR engagement-induced phosphorylation of Sos failed to cause the dissociation of Grb2 from Sos, suggesting the phosphorylation may happen on different sites by a different group of kinases, and thus has different regulatory effects on Sos (Zhao et al. 1997). Similar to this idea, a recent study showed that growth factor stimulation resulted in Sos phosphorylation on tyrosine residues. Tyrosine-phosphorylation stimulated the Rac GEF activity of Sos and was catalyzed by Abl, a non-receptor tyrosine kinase implicated in actin remodeling (Sini *et al.* 2004). Therefore, different cell surface receptors may couple distinct kinases to Sos phosphorylation and the consequences of Sos phosphorylation depends, at least in part, on sites of its phosphorylation.

In contrast to the evident involvement of Sos in RTK signaling, the role of Sos in mediating immune receptor-mediated Ras activation is still controversial. The Grb2/Sos model was originally used to explain TCR-mediated Ras activation in T lymphocytes. Indeed, membrane-targeting of Sos by a chemical inducer that mimicks the action of Grb2 leads to rapid Ras activation in T lymphocytes (Holsinger et al. 1995). Moreover,

physical contacts between Grb2 and Sos have also been reported in activated T cells (Ravichandran et al. 1995). However, at least under certain circumstances, TCR induced Ras activation does not seem to require recruitment of Sos to receptor complexes (Osman *et al.* 1995). Also, the Grb2/Sos model cannot explain the observation that TCR-mediated Ras activation requires PLC-γ1 activity, which can be efficiently mimicked by PMA treatment (Wardenburg *et al.* 1996; Clements *et al.* 1998; Yablonski *et al.* 1998), suggesting the presence of a DAG regulated Ras activator, which was later identified as RasGRP1 (see discussion later). In Sos1/Sos2 double-deficient DT40 chicken B cells, EGER-mediated Ras and subsequent Erk activation is completely blocked. In contrast, BCR-mediated Ras-Erk activation is largely intact, indicating that there is unlikely a dominant role of Sos in BCR-mediated Ras signaling in this system (Oh-hora *et al.* 2003).

1.10.1.2 RASGRFs

The Ras guanyl nucleotide releasing factor (RasGRF) family of RasGEFs also consists of two closely related proteins of 140kDa: RasGRF1 (also known as CDC25Mm) and RasGRF2 (Shou *et al.* 1992; Fam *et al.* 1997). Unlike Sos, RasGRFs have restricted tissue expression. RasGRF1 is predominantly expressed in the brain with particular localization to the synaptic junction, whereas RasGRF2 is relatively more widely expressed (Shou *et al.* 1992; Fam *et al.* 1997; Zippel *et al.* 1997).

The primary structure of these proteins shows the presence of several regulatory motifs that are presumably involved in protein-protein interactions and other diverse signaling control mechanisms. RasGRF1 and RasGRF2 both contain two PH domains, an ilimaquinone (IQ) motif, a DH domain and a CDC25 box (Figure 1.6). The IQ motif is a small stretch of tandem isoleucine and glutamine residues first identified as a CaM binding motif in neuromodulin (Alexander et al. 1988), and later characterized in myosins (Cheney and Mooseker 1992). Proteins that contain IQ motifs typically bind calmodulin in a calcium-dependent manner, although there are some exceptions. The IQ motif mediates the binding of RasGRFs to Ca^{2+}/CaM in response to Ca^{2+} elevation. Calcium influx leads to membrane translocation and activation of RasGRF, probably through the cooperation of a N-terminal pleckstrin homology (PH) domain (Farnsworth

et al. 1995; Fam *et al.* 1997). RasGRFs also contain a putative coiled-coil structure which consists of two or three amphipathic helices in parallel that may mediate the formation of homo- or heterodimers (Lupas *et al.* 1991).

Interestingly, RasGRF1 activation is also regulated by multi-site phosphorylation. The full activation of the Ras exchange factor activity of mouse Ras-GRF1 by muscarinic receptors requires direct phosphorylation of two serine residues located just upstream of the CDC25 box, at position 898 and 916 (Mattingly and Macara 1996; Yang, H. *et al.* 2003). However, how RasGRF1 catalytic activity is regulated by phosphorylation is still unclear.

Since neither RasGRF proteins is expressed in lymphoid tissues, they are not likely to mediate Ras activation in lymphocytes (Fam *et al.* 1997; Sturani *et al.* 1997; Zippel *et al.* 1997).

1.10.1.3 RASGRPs

A genetic screen searching for cDNAs that can enhance Ras signaling led to the discovery of the first member of the third class of RasGEF, Ras guanyl nucleotide releasing protein 1 (RasGRP1) (Ebinu *et al.* 1998). RasGRP1 is distinguished from other classes of RasGEFs in that it contains a dual function regulatory region that consists of a calcium-binding EF-hand motif and a DAG-binding C1 domain, suggesting an integration of two secondary messenger pathways. Further genetic analysis established an essential role of RasGRP1 in coupling TCR signaling to Ras activation (Dower *et al.* 2000; Ebinu *et al.* 2000). With the discovery of additional family members, RasGRP1 now serves as the prototype for a family consisting of four structurally related yet functionally distinct members.

RasGRP1

RasGRP1, also known as CalDAG-GEFII, was initially isolated as rbc7 (rat brain cDNA #7) from a rat cDNA library in a genetic screen that searched for cDNA, when ectopically expressed, capable of enhancing the transforming activity of Ras in Rat2 fibroblasts (Ebinu *et al.* 1998). Subsequent sequence analysis of rbc7 indicated that it was a 5' and 3' truncated version of a larger normal gene product, RasGRP1 (Ebinu *et al.*

1998). The human gene encoding RasGRP1 localized to 15q15 as identified by in situ hybridization (Bottorff *et al.* 1999).

Full-length RasGRP1 protein consists of 795 amino acids. Sequence alignment predicted the presence of a Ras exchange motif (REM) and a catalytic CDC25 box similar to those found in other RasGEFs such as RasGRF1 and Sos1. Biochemical analysis confirmed RasGRP1 as an activator for Ras and R-Ras (Ebinu *et al.* 1998; Toki *et al.* 2001). However, RasGRP1 also contains several regulatory domains that distinguish it from other RasGEFs. N-terminal to the CDC25 in RasGRP1 lies an atypical pair of EF-hands that binds calcium *in vitro* and a C1 domain that is similar to the DAG-binding C1 domains found in PKCs. The C1 domain mediates membrane translocation of PKC via binding to DAG in the plasma membrane (Huang, Y. and Wange 2004). Moreover, there is a proline-rich region with the PXXP concensus sequence between CDC25 box and the EF-hands (Ebinu *et al.* 1998; Tognon *et al.* 1998), indicating RasGRP1 may be regulated by SH3-mediated mechanisms. A similar domain structure reoccurs in all RasGRP family members (Figure 1.7).

RasGRP1 has a restricted expression profile with relatively abundant expression found in thymus, T cells, B cells, brain, spleen, and skin (Ebinu *et al.* 1998; Rambaratsingh *et al.* 2003). Whereas the role of RasGRP1 in brain is still largely unknown, the analysis on *Rasgrp1* null mice has given insight on its functional significance in thymus. Thymocytes from *Rasgrp1*$^{-/-}$ mice failed to activate the Ras/MAP kinase cascade in response to TCR stimulation. In accordance with this observation, the mutant mice are defective in T cell positive selection, which requires active TCR signaling, resulting in a remarkable decrease in the number of $CD4^{+}/CD8^{-}$ and $CD4^{-}/CD8^{+}$ single positive thymocytes. However, B cell development is intact. These data established an essential role of RasGRP1 in linking TCR activation to Ras signaling (Dower *et al.* 2000).

The characterization of a mouse strain with a spontaneous mutant in the *Rasgrp1* gene resulting in loss of RasGRP1 expression demonstrated the essential role of RasGRP1 in maintaining self-tolerance (Layer *et al.* 2003). Aged mice develop an autoimmune syndrome very similar to the human disease systemic lupus erythematosus (SLE) typified by auto-antibody production, tissue lymphocyte infiltration, and enlarged

spleens (Bini *et al.* 1990). This observation provides a novel genetic model of autoimmune disease. Mutant mice also accumulate autoreactive T cells and activated B cells and have defects in TCR-mediated Ras activation and proliferation. Although the molecular mechanism whereby RasGRP1 normally maintains immune tolerance is still unclear, the phenotypes of these mutant mice share many similarities with those of mice that carry a point mutation in the LAT adaptor protein, which prevents this molecule from activating PLC-γ1 (Sommers *et al.* 2002; Layer *et al.* 2003). A potential role of RasGRP1 signaling is also suggested in T-cell lymphomagenesis. A genetic screen using a PCR-based method for host DNA sequence that occasionally targeted by retroviruses identified RasGRP1 as a candidate oncogene in mouse T-cell lymphomas (Kim, R. *et al.* 2003).

RasGRP1 is also expressed in B cells, but the biological significance is still under investigation. There is evidence, however, showing that overexpressing RasGRP1 in an immature B cell line promotes BCR-induced apoptosis, suggesting a potential role of RasGRP1 in deletion of self-reactive B cells to maintain immune tolerance (Guilbault and Kay 2004). Notably, a similar role of RasGRP1 has also been suggested in T cells (Layer *et al.* 2003).

Another question regarding RasGRP regulation is related to the attenuation of its activity following activation. As a DAG-regulated protein, the exchange factor activity of RasGRP1 is sensitive to the local availability of DAG. DAG kinases (DGKs) stop DAG signaling by converting it to phosphatidic acid. Nine mammalian DGK isoforms have been discovered and further classified into five subtypes based on the presence of different structural motifs. In addition to the common catalytic domain, they all contain a C1 domain and are recruited by DAG in response to TCR stimulation (Topham and Prescott 1999; 2001; Jones, D. R. *et al.* 2002; Cipres *et al.* 2003; Sanjuan *et al.* 2003). DGKα is one of the major DGK isoforms in T lymphocytes (Sanjuan et al. 2001). Inhibition of endogenous DGKα activity by overexpressing a kinase-dead mutant of this kinase isoform in a T cell line resulted in sustained RasGRP1 membrane association and Erk activation (Jones, D. R. et al. 2002). On the other hand, expressing a constitutively active mutant of DGKα suppressed RasGRP membrane translocation and Ras activation

in a Jurkat T cell line (Sanjuan et al. 2003). Therefore, it is proposed that DGKα may indirectly inhibit RasGRP1-mediated Ras signaling by DAG downregulation. However, reports from other groups suggested that DGKζ, another DGK isoform enriched in T cells, was responsible for the suppression of RasGRP1-dependent Ras activation. Overexpression of DGKζ, but not other DGKs, inhibited Ras-Erk activation and expression of activation marker CD69 upon TCR triggering (Zhong *et al.* 2002). DGKζ was also found co-immunoprecipitated and co-localized with RasGRP1 in HEK293 cells (Topham and Prescott 2001). More importantly, loss of DGKζ expression in mouse T cells resulted in more efficient signaling from the TCR to the Ras-ERK cascade both *ex vivo* and *in vivo*, without interfering with the TCR-induced calcium influx. A more vigorous and effective T cell response was also observed in DGKζ deficient mice challenged with pathogen (Zhong *et al.* 2003). Collectively, these observations demonstrate that DGKs plays an essential role in terminating TCR signaling, possibly through interfering with RasGRP1 activation.

RasGRP2

RasGRP2 was first identified as HCDC25L, by large-scale genomic sequencing, and as CalDAG-GEFI, by a differential display method (Kedra et al. 1997; Kawasaki et al. 1998). Another group that independently cloned CalDAG-GEFI also identified an additional 5'-coding exon. The longer form of CalDAG-GEFI, generated as a result of alternative splicing, was termed RasGRP2 (Figure 1.7) (Clyde-Smith *et al.* 2000). The *Rasgrp2* gene is mapped to chromosome 11q13 in human genome encodes a protein of 671 residues with the predicated MW of 72 kDa.

RasGRP2 is widely expressed in heart, brain, placenta, lung, spleen, liver, thymus and some T cells and B cells. It acts mostly on Rap1 and, to a lesser extent, N-Ras and K-Ras, but not H-Ras, R-Ras, or RalA (Kawasaki *et al.* 1998; Ohba *et al.* 2000a).

Unlike other RasGRP family members, the C1 domain of RasGRP2 has a low affinity to DAG and thus may not mediate its membrane association. Instead, it contains an N-terminal consensus sequences for co-translational myristoylation and posttranslational palmitoylation, and is constitutively localized to the plasma membrane

by N-terminal acylation. The membrane translocation also depends on actin dynamics and is mediated by direct binding to F-actin, suggesting a role in spatial activation of Rap1(Caloca *et al.* 2004).

RasGRP2 was first implicated in integrin signaling in megakaryocytes, the bone marrow cells from which platelets are derived (Eto et al. 2002). Subsequent genetic analysis of gene-targeted mice identified RasGRP2 as an important regulator of platelet functions (Crittenden *et al.* 2004). The integrin-dependent aggregation of platelets in RasGRP2-knockout mice was severely compromised despite the normal expression level of integrin receptor on cell surface. Further analysis showed that this defect was due to poor activation of Rap1, which is required for platelet activation. Consistent with this observation, RasGRP2 has been found associated with Rap-GTP in platelets (Crittenden *et al.* 2004). In addition to the essential role in platelet adhesion, RasGRP2 has also been suggested to be responsible for coupling the muscarinic acetylcholine receptor (mAChR) to Erk activation in neuronal PC12 cells (Guo *et al.* 2001). The signal transduction activated by M_1 mAChR couples $G_{q/11}$ to PLC-β activation and is involved in learning and memory (Marino *et al.* 1998).

RasGRP3

RasGRP3, also known as CalDAG-GEFIII or KIAA0846, was identified as a Ras and Rap dual specificity GEF during a data base search for new GEFs for the Ras family G proteins (Figure 1.7) (Yamashita *et al.* 2000). Sequence alignment shows high amino acid sequence homology among these three RasGRPs, with the highest homology found between RasGRP1 and RasGRP3 (Yamashita *et al.* 2000). The gene encoding RasGRP3 is localized on human chromosome 2p23 and is predicated to give rise to a product of 84 KDa in size consisting of 692 residues.

The C1 domain of RasGRP3 binds to phorbol ester with high affinity with the cooperation of anionic phospholipids (Lorenzo *et al.* 2001). DAG binding recruits RasGRP3 to plasma membrane and causes its activation.

RasGRP3 is found selectively expressed in B cells, but not T cells, suggesting that RasGRP3 might function downstream of the BCR (Teixeira *et al.* 2003). This idea is supported by the observation that BCR-induced Ras signaling is defective in B cell lines

deficient for RasGRP3 expression. Furthermore, in PLC-γ2-deficient B cells, BCR-induced RasGRP3 membrane translocation and Ras activation were greatly decreased. However, this phenotype was suppressed by expressing a membrane-attached form of RasGRP3 (Oh-hora *et al.* 2003).

In addition to RasGRP3, B cells also express RasGRP1, hinting that these two RasGRPs might function redundantly. Moreover, RasGRP1 and RasGRP3 may function in coordination to contribute to receptor signaling and regulation of cross-talking between T cells and B cells (Coughlin *et al.* unpublished data).

Other than immune cells, expression of RasGRP3 in endothelial cells has also been reported. It is thought to function as an angiogenesis-regulated protein downstream of the vascular endothelial growth factor (VGFR) in these cells (Roberts *et al.* 2004).

RasGRP4

RasGRP4 was simultaneously cloned as a mast cell-specific and myeloid leukemia-specific Ras activator by two independent groups (Figure 1.7) (Reuther *et al.* 2002; Yang, Y. *et al.* 2002). It resides on a region of human chromosome 19q13.1 that had not been sequenced by the Human Genome Project.

Expression of RasGRP4 has only been reported in mast cells. Abnormally spliced transcripts have been found in patients with asthma or mastocytosis, as well as the HMC-1 cell line derived from a MC leukemia patient, making it the first RasGRP family member correlated with human disease (Yang, Y. *et al.* 2002). In asthma and other inflammatory disorders, the release of prostaglandin D_2 (PGD_2), a neuromodulator in the brain, by activated mast cells is thought to be the cause of these diseases (Lewis *et al.* 1982). RasGRP4 has been shown to control the production of PGD_2 (Li, L. *et al.* 2003).

As a unique family of Ras activators that are implicated in various aspects of immune responses, the RasGRP family has drawn rapidly increasing research interests. Thus, illustration of the molecular mechanisms governing the regulation of these exchange factors would greatly improve our understanding on the function of Ras in immune system. In the original model, DAG-mediated membrane recruitment is thought to activate all RasGRPs except for RasGRP2. However, this model does not account for the other regulatory domains found in all RasGRPs, i.e. the EF-hand motif

(see Chapter 4 for detailed discussion) and a putative PXXP motif (see Chapter 4 for detailed discussion). Moreover, there is evidence suggesting regulation modes other than DAG-mediated recruitment. For example, RasGRP3 was found to undergo PKC-dependent phosphorylation upon BCR stimulation (see Chapter 3 for detailed discussion) (Teixeira *et al.* 2003). In addition, the Rap1 GEF activity of RasGRP2 seems to be regulated by calcium signal (Clyde-Smith *et al.* 2000). In this study, I explored three possible regulation modes of RasGRPs.

It is unlikely the three families of RasGEFs discussed above are the whole story for Ras activators in cells. A recent report described another novel candidate RasGEF, CNrasGEF. It is mainly expressed in brain and associates with plasma membrane through its PDZ domain, a protein- and lipid-binding motif. CNrasGEF also contains cNMP (cAMP/cGMP) binding (cNMP-BD) domains and promotes Ras-GTP loading in cells in response to cAMP and cGMP (Pham *et al.* 2000; Pak *et al.* 2002). CNrasGEF also stimulates Rap activity in a cAMP-independent manner (Kuiperij *et al.* 2003). However, the physiological role of CNrasGEF has not been fully explored.

1.10.2 Negative regulation of Ras by RasGAPs

The overall extent of downstream Ras signaling depends not only on the degree of activation by RasGEFs, but also on the deactivation regulated by RasGAPs. At least five RasGAPs have been discovered, these include p120GAP, Neurofibormin (NF1), the members of the GAP1 family, IQGAP1, and SynGAP (Vogel *et al.* 1988; Schaber *et al.* 1989; Baba *et al.* 1995; Cullen *et al.* 1995; Yamamoto *et al.* 1995; Malumbres and Pellicer 1998; Bar-Sagi and Hall 2000). All RasGAPs share a structurally conserved region called the GAP related domain (GRD). This region amplifies the intrinsic GTPase activity of Ras, thereby favoring the deactivation of Ras (Schaber *et al.* 1989).

1.10.2.1 P120GAP

p120GAP was the first GAP to be discovered and serves as the prototype for RasGAPs (Trahey and McCormick 1987; Vogel *et al.* 1988). It is ubiquitously expressed in human tissues. Several distinct domains have been identified in p120GAP, including a C-terminal GAP related domain (GRD) that selectively binds Ras in its GTP form, a

central PH domain and two N-terminal SH2 domains that flank a single SH3 domain (Henkemeyer *et al.* 1995). The SH2 domain of p120GAP provides it with the adaptor-like functions which not only allows association with various autophosphorylated RTKs, but also many other intracellular phospho-tyrosine containing proteins such p190 RhoGAP, a Rho-specific GAP (Ellis *et al.* 1990; Kaplan *et al.* 1990; Carpino *et al.* 1997). p120GAP, upon binding to RTKs, also undergoes tyrosine phosphorylation, allowing it to provide docking sites to recruit a variety of signaling proteins (Ellis *et al.* 1990; Medema *et al.* 1996). The SH3 domain of p120GAP is believed to play an effector role in Ras-dependent signal transduction (Ellis et al. 1990; Duchesne et al. 1993). Disruption of p120GAP in mice led to vascular system defects and neuronal apoptosis (Henkemeyer *et al.* 1995). PDGF stimulation of fibroblasts derived from mutant mouse embryos led to an abnormally large increase in the level of Ras-GTP and in the duration of MAP kinase activation compared with wild-type cells, demonstrating the negative role of p120GAP in regulating Ras activity (van der Geer *et al.* 1997). Interestingly, a Ras regulation-independent function of p120GAP has also been demonstrated in a p120GAP-deficient cell line. These cells showed defects in establishing complete cell polarity and migration into the wound. Association of p120GAP with p190RhoGAP is critical for this process (Kulkarni *et al.* 2000).

1.10.2.2 NF1

Neurofibromatosis type-1 (NF-1), also called von Recklinghausen disease, is caused by loss of the *Nf1* gene, a tumor suppressor gene identified by positional cloning (Ballester et al. 1990). NF-1 is one of the most common tumor predisposition syndromes affecting the nervous system (Ballester et al. 1990; Friedman 1999). Disruption of a *Nf1* allele is often followed by somatic mutation of the remaining allele (Wallace et al. 1990). The *Nf1* gene encodes a large 220–250 kDa cytoplasmic protein NF1 that contains a central GRD. The GRD of NF1 binds Ras with high affinity and induces a 100,000-fold increase in the rate of GTP hydrolysis (Bollag and McCormick 1992). Loss of NF1 in a variety of both human tumor and *Nf1*-deficient mouse cells is also associated with increased Ras activity, Ras effector activation, and proliferative phenotype, which can be reversed by direct inhibition of Ras and its effectors, or by replacement of the

NF1 GRD in these cells (Sherman *et al.* 2000; Bajenaru *et al.* 2001; Hiatt *et al.* 2001; Ingram *et al.* 2001). NF1 inactivation in myeloid precursors resulted in hypersensitivity to mitogenic cytokines, increased cell proliferation and myeloid leukemogenesis (Dasgupta and Gutmann 2003). Interestingly, expressing the GRD of p120GAP failed to restore normal growth and cytokine signaling in primary Nf1-deficient cells, although inactivation of both p120GAP and NF1 in mice appeared to have a synergistic effect (Henkemeyer et al. 1995; Hiatt et al. 2001). This suggests a non-redundant role of NF1 in regulation of Ras activity. Consistent with this hypothesis, p120GAP null mice and NF1 null mice have distinct phenotypes (Henkemeyer *et al.* 1995). NF1 has also been shown to be phosphorylated by a cAMP-dependent protein kinase and may be involved in cAMP- and protein kinase A (PKA)-mediated gene transcription (Izawa *et al.* 1996; The *et al.* 1997).

1.10.2.3 GAP1

GAP1 is the first member of the GAP1 family of RasGAPs that consists of a group of structurally and functionally related proteins: GAP1m and GAP1IP4BP (Malumbres and Pellicer 1998). Both proteins contain an N-terminal calcium-dependent phospholipid-binding C2 domain that mediates membrane translocation in a Ca^{2+}-dependent manner (Fukuda *et al.* 1997). In addition, GAP1 possesses a PH domain, which is thought to interact with PI3K product, phosphatidylinositol 3,4,5-triphosphate (PIP_3). This interaction leads to the sequestration of GAP1 activity, and thus may contribute to TCR-mediated Ras activation (Lockyer *et al.* 1999).

1.10.2.4 IQGAP1

IQGAP1 was identified a decade ago as cDNA clone predicting a 1657-amino acid protein that contains a region with extensive sequence similarity to the catalytic domain of RasGAPs (Weissbach et al. 1994). The subsequent discovery of IQGAP2 establishes the IQGAP family of RasGAPs comprising a small group of eukaryotic proteins, with representatives in species as divergent as yeast and mammals (Brill *et al.* 1996; Machesky 1998; Briggs and Sacks 2003). IQGAP1 gets its name for containing four N-terminal calmudulin-binding IQ motifs, and thus was thought to link Ras and

Ca^{2+}/calmudulin mediated signaling processes (Cheney and Mooseker 1992; Weissbach *et al.* 1994; Briggs and Sacks 2003). IQGAP1 also possesses multiple protein-interacting domains that mediate its binding to many protein targets, such as CDC42, Rac, actin, E-cadherin, β-catenin and CLIP-170 (Briggs and Sacks 2003). Therefore, IQGAP1 functions as a scaffolding protein to participate in many fundamental cellular processes including cell-cell adhesion, β-catenin-mediated transcription, cell migration, regulation of actin, microtubule function, the MAP kinase cascade, and Ca^{2+}/calmodulin signaling (Briggs and Sacks 2003). A recent report suggested that IQGAP1 regulated the neuronal cytoskeleton in a PKC phosphorylation-dependent manner (Li, Z. *et al.* 2005). The physiological role of IQGAP1 in immune cells is not clear, although expression of IQGAP1 was found up-regulated in peripheral blood T cells from patients with atopic dermatitis, a chronic pruritic skin condition usually beginning in infancy (Matsumoto, Y. *et al.* 2002).

1.10.2.5 SynGAP

SynGAP is a brain-specific RasGAP enriched postsynaptic densities (PSD) at glutamatergic synapses, where it is present as an abundant component of a large macromolecular complex that also includes NMDA receptor and PSD-95, a protein implicated in the organization of synaptic structure (Kim, J. H. *et al.* 1998). The GAP catalytic domain lies in the central region of the primary structure. A PH and a C2 domain are situated at the N-terminal region. In the C-terminal region, there is a proline-rich region and a QTRV sequence conforming the binding to PDZ domain, a 80 amino acid sequence involved in mediating protein-protein and sometimes protein-lipid interactions (Kim, J. H. *et al.* 1998; Dev 2004). SynGAP is thought to regulate Ras activity at synaptic junctions. Analysis of SynGAP deficient mice suggested an important role of this protein in regulation of spine and synapse formation in hippocampal neurons (Vazquez *et al.* 2004).

It should be noted that although overexpression of RasGAPs usually results in the reduction of endogenous Ras-GTP, and thus the prevention of transformation, certain forms of Ras mutants are not sensitive to the inhibitory effects of RasGAPs, due to the mutation in the effector domain of Ras (Scheffzek *et al.* 1997).

The contribution of RasGAPs to the activation of Ras upon antigen receptor stimulation is still poorly characterized. In T cells, the Ras-GTP accumulation following TCR ligation is correlated with the inhibition of p120GAP in a PKC-independent manner (Izquierdo *et al.* 1992). It was speculated that adapter proteins such as DOK may be involved in p120GAP activity suppression. The hemopoietically expressed adaptor DOK (downstream of tyrosine kinase) is one of the major tyrosine-phosphorylated molecules in v-src-transformed cells (Yamanashi and Baltimore 1997). It is found associated with p120GAP upon phosphorylation. Disruption of DOK expression in mice resulted in aberrant hemopoiesis and Ras/MAP kinase activation. Moreover, all DOK knockout mice spontaneously developed transplantable CML-like myeloproliferative disease due to increased cellular proliferation and reduced apoptosis, demonstrating a negative role of DOK in regulation of Ras activaiton (Niki et al. 2004). Therefore, it is thought p120GAP inhibition during lymphocyte activation may be mediated by mechanisms that inhibit or reverse DOK phosphorylation. Similar to that of p120GAP, T cells from mice lacking NF1 showed enhanced Ras activation, which is associated with thymic and splenic hyperplasia, and an increase in the absolute number of immature and mature T-cell subsets compared with T cells from control mice (Ingram *et al.* 2002). These results identify NF1 as another regulator of Ras activation in T cells.

1.11 Ras effectors

The GTP-bound form of Ras initiates downstream signaling events by interacting directly with a great number of distinct target proteins. These target proteins, or effectors, include the well-studied MAP kinase kinase kinase Raf-1, the Ral guanine nucleotide dissociation stimulator (RalGDS), phosphatidylinositol 3-kinase (PI3K), phospholipase C ε (PLC-ε), and the recently identified Ras inhibitor RIN1 and the polarity protein AF6/Canoe (Vojtek and Der 1998; Downward 2003).

The region of Ras involved in effector binding is the effector loop (residues 30-38), some flanking residues, and the switch II region (residues 60-76). The exchange of Ras-GDP by GTP induces a conformational change in Ras switch I and switch II region

that allows effector binding (Polakis and McCormick 1993; Fujita-Yoshigaki *et al.* 1995; Moodie *et al.* 1995). Moreover, the residues 26, 31, 41, 45, 46, 48, 49, and 53, flanking the effector loop, are proven to be essential for effector binding, although no conformational changes have been found in these residues upon GTP binding by Ras (Marshall 1993).

1.11.1 RAF

Although three members, A-Raf, B-Raf, and Raf-1, have been identified in the Raf serine/threonine protein kinase family, Raf-1 (herein referred to as Raf; Figure 1.8) has been the best studied member of the family (Wood *et al.* 1992; Lee *et al.* 1996; Wojnowski *et al.* 1997).

Dominant-negative mutants of Raf can inhibit transformation caused by activated Ras, identifying Raf as an essential signal transducer downstream of Ras (Kolch et al. 1991). Direct interaction between Ras and Raf in a GTP-dependent manner has been reported *in vitro* and in the yeast two-hybrid system (Van Aelst *et al.* 1993; Vojtek *et al.* 1993; Warne *et al.* 1993; Zhang, X. F. *et al.* 1993). However, the mechanism by which Raf becomes activated by Ras is still not fully understood. Raf associates with GTP-bound normal Ras and the oncogenic V12 and L61 forms of Ras with equal affinity through a N-terminal region called the Ras-binding-domain (RBD) which recognizes the switch I region of Ras (Chuang *et al.* 1994; Wittinghofer and Nassar 1996). However, simple binding of Ras to Raf is not sufficient for Raf activation (Zhang, X. F. *et al.* 1993). Further analysis suggests the requirement of a secondary interaction between a cystein-rich zinc-finger region in Raf and regions of Ras that include switch 2 (Brtva *et al.* 1995; Cutler *et al.* 1998; Zhang, Y. Y. *et al.* 1998). While it is clear that Ras functions to recruit Raf to plasma membrane, the activation process of Raf is highly complex and involves membrane recruitment, dimerization or oligomerization, binding to other proteins, conformational changes and phosphorylation/dephosphorylation events resulting in kinase activation/release from repression (Dhillon and Kolch 2002; Wellbrock et al. 2004).

The protein kinase Mek is the widely accepted substrate of Raf. Together with Erk, they constitute the conserved signaling module Raf/Mek/Erk kinase cascade functioning

as one of the major Ras effector pathways (Dent *et al.* 1992; Howe *et al.* 1992; Kyriakis *et al.* 1992; Huang, W. *et al.* 1993; Warne *et al.* 1993; Baccarini 2005). In response to binding of extracellular ligands such as growth factors, cytokines and hormones to their cell-surface receptors, the Ras-GTP complex recruits Raf through direct binding to the plasma membrane where subsequent events lead to its phosphorylation and activation (Warne *et al.* 1993; Wellbrock *et al.* 2004). Active Raf in turn phosphorylates the serine/threonine protein kinase Mek on two serines at positions 218 and 222, which lie in the activation loop of Mek (Howe *et al.* 1992; Kyriakis *et al.* 1992; Johnson, N. L. *et al.* 1996). The phosphorylation gives rise to increased kinase activity by inducing conformational changes in Mek (Johnson, N. L. *et al.* 1996). Activated Mek then phosphorylates and activates the MAP kinase Erk, which goes on to phosphorylate a wide range of cellular targets leading to a variety of biochemical changes. One of the functions of phospho-Erk1/2 is to translocate into the nucleus, where it regulates a number of transcription factors, such as Elk1, leading to changes in gene transcription and, thus, changes in cellular behavior. However, it is should be noted that the overall outcome of Ras signaling is the result of a complex array of signaling pathways, where cross-talk among different pathways is frequent (Campbell *et al.* 1998).

Depending on the cellular context, the Ras/Raf/Mek/Erk pathway is essential for diverse biological functions including cell growth, survival and differentiation predominantly through the regulation of transcription, metabolism and cytoskeletal rearrangements. Loss of Raf-1 expression in mice resulted in embryonic death around midgestation (Pritchard *et al.* 1996). Embryo tissues from *Raf-1* knockout mice showed increase apoptosis, suggesting one of its major functions is anti-apoptosis (Huser et al. 2001; Mikula et al. 2001). In immune cells, the Ras/Raf/Mek/Erk pathway is also essential for proliferation and differentiation programs. Transgenic mice expressing a dominate negative mutant of Ras, RasN17, which inhibits the Ras/Raf/Mek/Erk cascade, showed a complete block in TCR-induced proliferation of mature thymocytes. At the same time, the antigen-specific positive selection, but not the negative selection, is compromised in these mice (Swan et al. 1995). Importantly, this selective inhibition on positive selection can be replicated by expressing a catalytically inactive form of Mek in thymocytes (Alberola-Ila et al. 1995; Swan et al. 1995).

Lymphocyte development and immune response depends on the induced expression of a set of immediate early genes such as *c-fos* and *egr-1* upon the stimulation of lymphocyte antigen receptors (Kaptein et al. 1995; Niiro and Clark 2002). The transcription factors for the immediate early genes, such as Elk-1, are substrates for MAPKs in lymphocytes (Chai and Tarnawski 2002). Elk-1 is a ternary complex factor (Tcf) that regulates the transcription factor serum response factor (SRF). Activation of the Ras/Raf/Mek/ Erk cascade is necessary and sufficient for its induction (Turner and Cantrell 1997; Hao et al. 2003). The MAPK pathway also controls serine phosphorylation of STAT3 (signal transducer and activator of transcription 3), which is involved in cytokine-mediated gene transcription (Zhong *et al.* 1994b; 1994a; Ihle 1996). STAT3 activation is initiated by cytokine-activated Janus kinases (JAKs), which regulate tyrosine phosphorylation of the molecule to induce dimerization of STAT3. However, serine phosphorylation of STAT3 catalyzed by Erk is required for its transcriptional activity (Ihle 1996; Ng and Cantrell 1997). By linking multiple kinase pathways, STAT3 integrates signals from cytokine receptors and antigen receptors in T cells. This process is essential for lymphocytes to make the correct proliferation/differentiation decisions during an immune response. Other targets for MAPKs in lymphocytes include proteins with diverse functions such as the tyrosine kinase p56Lck and oncoprotein18 (Op18)/stathmin, a regulator of microtubule dynamics (Marklund et al. 1993).

Substrates other than Mek have also been reported for Raf. For example, Raf has been proposed to activate the transcription factor nuclear factor NF-κB indirectly in a Mek-independent manner (Li, S. and Sedivy 1993; Janosch et al. 1996; Ikenoue et al. 2003). In addition, two cell cycle regulators, phosphatase CDC25C and the tumor-suppressor protein retinoblastoma (RB), were shown to be phosphorylated by Raf, although physiological relevance of this is unclear (Galaktionov et al. 1995). Moreover, Raf has been reported to bind to BCL2-associated athanogene-1 (BAG1), an anti-apoptotic protein that binds to another survival factor called BCL2 (Wang, H. G. *et al.* 1996a; Wang, H. G. *et al.* 1996b). BAG1 is proposed to mediate the interaction between Raf and pro-apoptotic protein Bcl2-antagonist of cell death (BAD), leading to the phosphorylation and activation of the latter and thereby stimulates cell survival (Troppmair and Rapp 2003).

1.11.2 PI3K

The cellular function of Ras in immune cells can not be completely explained by Ras/Raf/Mek/Erk signaling pathway. For example, in T cells, expression of dominant negative N17Ras prevents TCR and Ras activation of the nuclear factor of activated T cells (NFAT), a transcription factor regulating a variety of T cell functions, but without interfering with the ERK pathway (Hao *et al.* 2003; Macian 2005). In mast cells, FcεR1 activation of NFAT requires the activation of Ras, but not Erk (Turner and Cantrell 1997). Moreover, Mek activity is not required for TCR induced cytokine production of mature T cells whereas Ras signals are indispensable (Alberola-Ila et al. 1995; Swan et al. 1995). These results suggest the involvement of multiple effector pathways in mediating Ras function in immune cells.

The Rho family GTPase Rac is found involved in mediating of Ras regulation of NFAT activity. It is thought that Rac functions downstream of Ras and growth factor receptors (Ridley *et al.* 1992). Although there is no consensus as to how Ras is able to control Rac proteins and actin polymerization, there is evidence in fibroblasts suggesting the involvement of the phosphoinositide 3-kinases (PI3Ks), a lipid kinase implicated in the regulation of the actin cytoskeleton by growth factors such as PDGF and insulin (Kotani *et al.* 1994).

PI3Ks constitute a family of evolutionarily conserved lipid kinases that catalyze the phosphorylation of the inositol 3' hydroxyl group of phosphatidyl inositol (4,5)-bisphosphate (PIP_2) to give phosphatidyl inositol (3,4,5)-triphosphate (PIP_3), which targets many signaling molecules including Akt/protein kinase B (PKB), RacGEF (e.g. Vav and Sos), Bruton's tyrosine kinase (Btk), phosphoinositide-dependent kinases (PDK), integrin-linked kinase (ILK), atypical PKCs, and phospholipase Cγ (Wymann and Pirola 1998). PI3K proteins are divided into three classes according to structural characteristics and substrate specificity (Domin *et al.* 1997; Fruman *et al.* 1998). Only class I PI3K proteins (herein referred to as PI3K; Figure 1.8) are Ras effectors and will be discussed. They interact with Ras in a GTP-dependent manner and are activated both *in vitro* and *in vivo* as a result of this interaction (Vandenabeele *et al.* 1994).

PI3K consists of a p110 catalytic subunit and a p85 regulatory subunit. The p85 subunit contains two SH2 domains that constitutively binds to the N-terminal domain of

the p110 subunit to suppress its enzymatic activity (Wymann and Pirola 1998). The activation of PI3K is regulated by several mechanisms. The p110 subunit has been shown to associate with Ras *in vivo*, which induces a conformational change that results in an increase of the enzyme activity (Fruman *et al.* 1998). The Ras-PI3K interaction also promotes PI3K membrane translocation, allowing interactions with its lipid substrates. In addition, binding of the SH2 domains of p85 subunit to phospho-tyrosines on many adaptor proteins releases the inhibitory effect of p85 subunit on p110 subunit and also contributes to PI3K activation (Fruman *et al.* 1998; Wymann and Pirola 1998). Once activated, PI3K binds to multiple signaling proteins such as Akt, Sos, and Vav to regulate diverse cellular functions (Franke *et al.* 1997; Han *et al.* 1998). Two PI phosphatases, namely "phosphatase and tensin homologue deleted on chromosome 10" (Pten) and SH2 domain-containing 5 inositol phosphatase (SHIP), provide a negative regulatory mechanism for PI3K activity in immune cells by limiting the amount of PIP_3 in cells. Pten catalyses the opposite reaction to PI3Ks, whereas SHIP removes the 5-phosphate from the inositol ring of PIP_3 to generate PIP_2 (Koyasu 2004). A deficiency of Pten or SHIP leads to hyperactivation of PI3K-mediated signaling pathways in many cell types and often results in cellular transformation (Di Cristofano *et al.* 1998; Helgason *et al.* 1998; Huber *et al.* 1998).

PI3K plays an important role in mitogenic signaling and cell survival, cytoskeletal remodeling, metabolic control and vesicular trafficking (Wymann and Pirola 1998). PI3K knockout mice die between embryonic days 9.5 and 10.5, providing evidence for the essential role of PI3K in regulating cell growth and survival (Bi et al. 1999; Bi et al. 2002). The function of PI3K in immune cells is still not fully characterized. In B cells, the p85 regulatory subunit has been found to associate with the B cell receptor, where it acts on its target PKB/Akt (Han et al. 1998; Donjerkovic and Scott 2000). Consistent with this observation, mice deficient for PI3K p85 subunit showed impaired activation of Akt (Suzuki, H. et al. 2003). This serine/threonine kinase phosphorylates multiple targets, which: 1) prevent Bad from binding and inhibiting the anti-apoptotic protein Bcl-xL; 2) inactivate caspase-9 (Casp9) to block caspase cascade activation; and 3) activate IκB kinase (IKK) to cause NF-κB release and its stimulation of expression of anti-apoptotic genes (Bellacosa et al. 2005). Moreover, loss of p110 subunit or p85

subunit in mice resulted in intrinsic defects in B lineage cells including impaired proliferation in response to BCR, lipopolysaccharide or CD40 stimulation, indicating the critical role of PI3K in B cells (Fruman *et al.* 1999a; Fruman *et al.* 1999b). Many of the phenotypes observed in mice lacking PI3K have been seen in many knockout mice, such as those lacking B cell linker protein (BLNK), PKCβ, phospholipase C-γ2, Vav-1/2, and CD19 (Engel *et al.* 1995; Leitges *et al.* 1996; Pappu *et al.* 1999; Wang, D. *et al.* 2000b; Tedford *et al.* 2001). It is thus suggested that PI3K functions in the same signaling pathway as these molecules in B cells. In T cells, the PI3K/PKB pathway is thought to be responsible for IL-2 induced T cell proliferation (Brennan *et al.* 1997). However, there are reports showing that PI3K is neither required nor sufficient for Ras-induced/Rac-regulated transcription factor responses in T cells (Parry *et al.* 1997; Reif *et al.* 1997). In contrast to obvious B cell phenotypes, only subtle phenotypic changes have been observed in T cells from PI3K deficient mice (Clayton *et al.* 2002; Jou *et al.* 2002; Okkenhaug *et al.* 2002). There is evidence suggesting PI3K may be invlovled in the determination of TCR signaling threshold (Okkenhaug *et al.* 2002). However, the precise role of Ras-PI3K pathway in immune cells requires further investigation.

1.11.3 RAL GEFs

The discovery of RalGDS, along with its two close relatives, Rgl, and Rgl2/Rlf, greatly improved the knowledge of the function and regulation of Ral (Ral A and B). These three ubiquitously expressed RalGEFs exhibit Ras-binding properties and have exchange activity towards Ral *in vivo* (Wolthuis *et al.* 1996; Bos 1997; Wolthuis *et al.* 1997; Wolthuis and Bos 1999). Importantly, growth factor receptor-induced activation of Ral is inhibited by dominant-negative Ras, placing the RalGEF-Ral pathway downstream of Ras (Wolthuis *et al.* 1998a).

Genetics studies in fibroblasts using a Ras mutant that selectively binds to RalGDS, but not Raf or PI3K, demonstrated that the Ras/RalGDS/Ral pathway contributes to transformation (Feig *et al.* 1996). However, very little is know about downstream effectors of Ral. In a yeast two-hybrid screen, Ral has been found associated with the Ral-binding protein (RalBP), a GAP for the Rho-like GTPases Cdc42 and Rac. RalBP

negatively regulates Cdc42 and Rac GTPases, is involved in the regulation of the cytoskeleton, and is essential for Ras-induced oncogenic transformation (Symons 1996). Another Ral-associated protein, phospholipase D1 (PLD1), associates *in vitro* with the N-terminus of Ral independently of the nucleotide bound to Ral. PLD1 produces phosphatidic acid (PA) by hydrolysis of phosphatidylcholine (PC) and is also implicated in vesicular trafficking (Jiang, H. *et al.* 1995). The role of Ral family GTPases as effectors of Ras signaling in lymphocytes has not yet been investigated.

1.12 Future prospects of Ras study

Despite the great progress we have made in our understanding on the complex physiological function of Ras, as well as the complex network that regulates it, much remains to be revealed. For example, further detailed analysis is required for the implications of both upstream regulators and downstream effectors of Ras in human cancers, which may provide new targets and protocols for therapy. As discussed above, three groups of proteins, Raf, PI3K, and RalGDS, have been identified as major Ras effectors. Although the Ras/Raf/Mek/Erk pathway has been shown to be important for Ras oncogenic signaling, it appears to be more potent at transforming murine cells than human cells. In contrast, the less understood RalGDS protein might be responsible for transformation of human cells, suggesting that distinct mechanisms have been used by Ras to transform murine and human (Hamad *et al.* 2002). Moreover, as more and more Ras effectors have been described over the past few years, more insight into biological functions of Ras can be gained (Malumbres and Barbacid 2003). For instance, AF6/Rsb1/canoe binds Ras in a GTP-dependent manner and is associated with cellular junctions and Notch signaling (Kuriyama *et al.* 1996). Rin1, a Ras effector that forms a stable complex with Ras and Abl, appears to function through competitive inhibition of Ras-Raf binding and links Ras with Abl tyrosine kinase signaling (Hu, H. *et al.* 2005). No doubt with the development of chip array gene-expression analyses, this area of Ras study is certain to add more complexity to the elaborate network of Ras signaling.

Most mouse and human tissues concurrently express all of the three major classes of Ras proteins, H-Ras, N-Ras, and K-Ras. Whether they play redundant roles or have

distinct functions is poorly understood. Observations made on gene targeted mice provide great insights into the functional assignments of each Ras form. Surprisingly, mice without H-Ras or N-Ras alone, or without both proteins, survive with only minor phenotypes, suggesting that both loci are dispensable for normal mouse development, growth, and fertility (Umanoff *et al.* 1995; Johnson, L. *et al.* 1997; Koera *et al.* 1997; Esteban *et al.* 2001). By contrast, K-Ras null mice die progressively during mid-gestation due to foetal liver defects and anemia (Johnson, L. *et al.* 1997; Koera *et al.* 1997). These observations argue for both distinct functions and redundant roles of each Ras protein. However, given the fact that the expression level of individual Ras protein appears to be tissue- and developmental stage-specific, the functional divergence of each protein, should be further studied in the context of particular tissues and developmental stages.

There is accumulating evidence suggesting that Ras is under spatial regulation in cells. As previously discussed, each Ras protein undergoes different N-terminal post-translational modifications which might anchor them to different membrane subdomains. For example, interfering with the function of caveolin, an integral membrane protein implicated in cholesterol homeostasis, impairs the signaling activity of H-Ras, but not K-Ras4B at the inner leaflet of plasma membrane. This raises the question as to how the different mechanisms of membrane anchoring of Ras proteins could confer functional specificity. One possibility is that different subdomains of membrane may serve as platforms to recruit a distinct group of regulators and effectors for the assembly of signaling complexes. For instance, RTKs and members of the MAP kinase cascade have been shown to be enriched in specialized cholesterol-rich membrane domains known as caveolae (Okamoto *et al.* 1998).

Ras was thought to signal solely from the plasma membrane. More recently, the application of new cell imaging techniques such as fluorescence resonance energy transfer (FRET) using spectral variants of GFP in living cells provided emerging evidence indicating that Ras may also signal from internal membrane systems such as the Golgi, ER, and vesicular compartments following RTK activation (Bivona and Philips 2003). In T cells, H-Ras activation upon TCR stimulation appears to happen predominantly on Golgi, and depends on the activity of PLC and RasGRP1 (Bivona *et al.*

2003). Although the physiological significance remains to be determined, these observations add an unexpected complexity to Ras signaling. Thus, the knowledge on the precise subcellular location of Ras and its different effectors during cellular activations will be valuable in understanding compartmentalized Ras signaling.

1.13 Thesis overview

As previously discussed, Ras activation is essential for TCR and BCR signaling in immune cells. The discovery of RasGRP family members and subsequent genetic analysis on RasGRP deficient mice establishes the critical role of RasGRPs in coupling TCR and BCR signaling to Ras activation. In order to regulate Ras, the activities of RasGRPs are also need to be closely regulated. The C1 domains of RasGRPs bind to the secondary messenger DAG and provide a mechanism for RasGRP membrane recruitment upon immune receptor ligation. Although there are many evidences supporting this idea, whether DAG-mediated membrane recruitment is sufficient to activate RasGRPs is unknown. Moreover, other mechanisms may be required to fine tune RasGRP activities. Previous studies from our lab showed RasGRP3 underwent serine/threonine phosphorylation upon activation in BCR-stimulated B cells. In addition, sequence analysis indicates the presence of a potential PXXP motif in RasGRP1, and an atypical pair of EF hands in all RasGRP members. In the well-known Ras GEF Sos, the PXXP motif provides a binding site for the SH3 domains of adaptor protein Grb2, which indirectly membrane recruits Sos to activated growth factor receptors via the interaction between the SH2 domain of Grb2 and phospho-tyrosines in the cytoplasmic tails of receptors. The presence of EF hands provide a possible integration of calcium signaling and DAG signaling. It is possible that RasGRPs are under multiple modes of regulations. In this thesis, I sought to study the molecular mechanisms of RasGRP regulations, and specifically asked three questions:

1) What's the role of serine/threonine phosphorylation in RasGRP activation?
2) Does RasGRP1 interact with Grb2 family members via its PXXP motif?
3) Is the EF hand motif in RasGRP1 functional?

TABLE 1.1 Activation of Ras signaling pathways in different tumors

Defect or mutation	Tumor Type	Frequency (%)
Ras mutation	Pancreas	90 (K)
	Lung adenocarcinoma (non-small-cell)	35 (K)
	Coloectal	45 (K)
	Thyroid (Follicular)	55 (H, K, N)
	Thyroid (Undifferentiated papillary)	60 (H, K, N)
	Seminoma	45 (K, N)
	Melanoma	15 (N)
	Bladder	10 (H)
	Liver	30 (N)
	Kidney	10 (H)
	Myelodysplastic syndrome	40 (K, N)
	Acute myelogenous leukaemia	30 (N)
BRAF mutation	Melanoma	66
	Colorectal	12
EGFR overexpression	Most carcinomas	>50
ERBB2 amplification	Breast	30
PTEN loss	Glioblastoma multiforme	20-30
	Prostate	20
	Pancrease	40
AKT2 amplification	Ovarian	12
	Pancreas	10
PI3K amplification	Ovarian	40

H, K and N refer to H-Ras, K-Ras and N-Ras, respectively.

(Source: Downward J. (2003) Nature Reviews Cancer 3: 11-22)

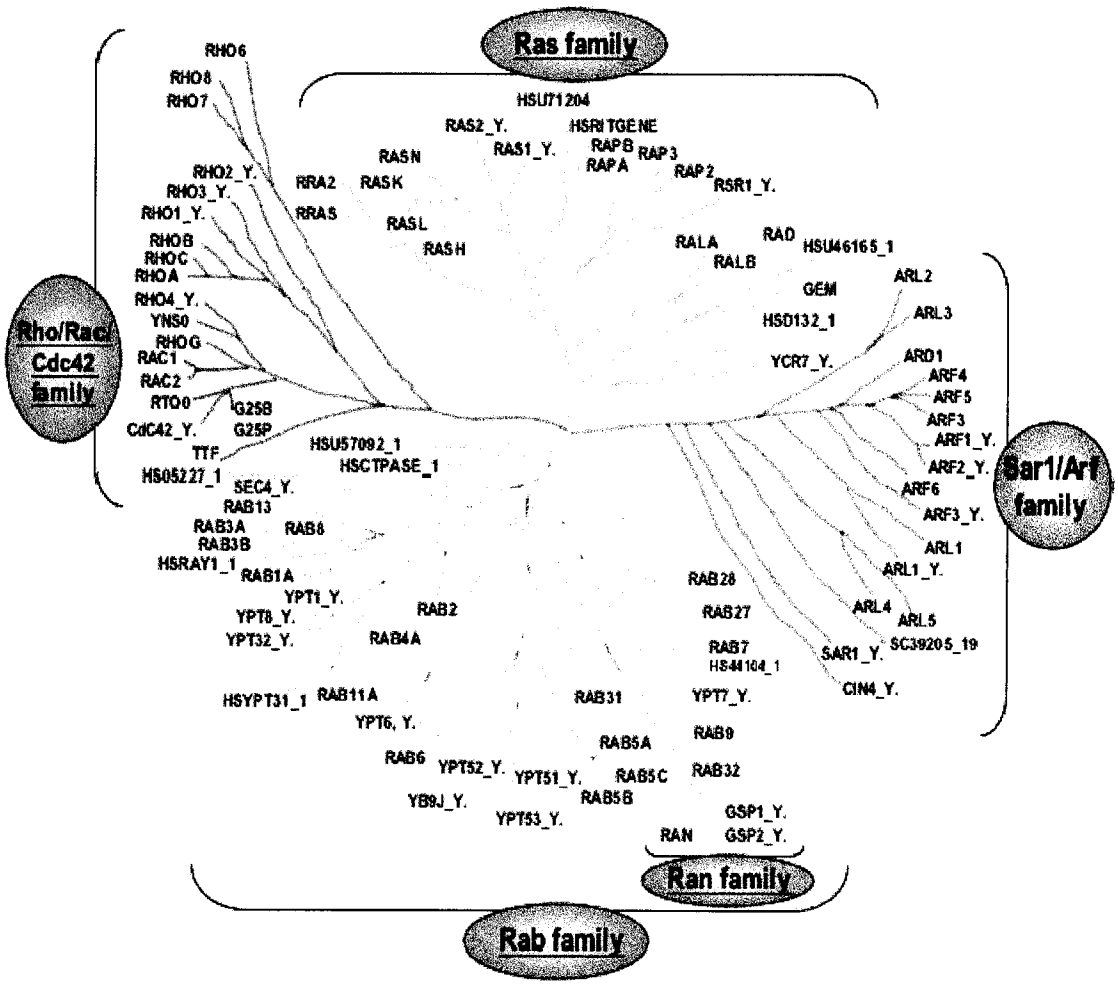


FIGURE 1. 1 Phylogenetic tree of Ras superfamily members.

(From: Takai et al. (2001) Physiol. Rev. 81 (1):153.)

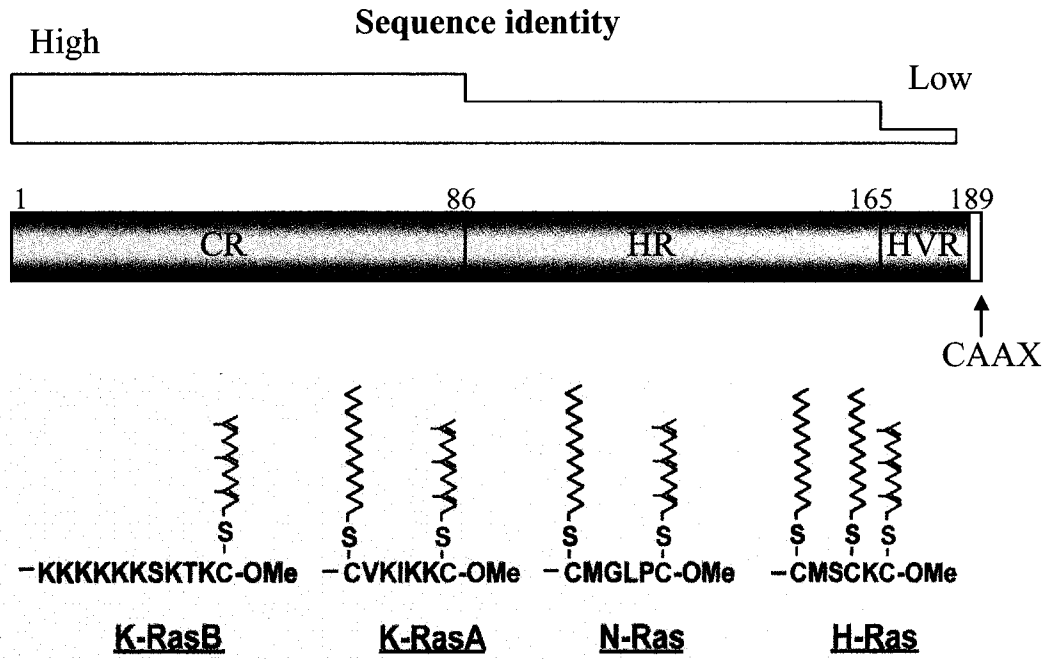


FIGURE 1.2 Sequence conservation between Ras proteins. Sequence identity from 0 to 100% is presented as a gradient from low to high. The sequences corresponding to the membrane anchor region of each human Ras protein are shown with their respective posttranslational modifications. Note: the farnesyl chain is longer than the palmitoyl chain. The CAAX box is indicated by arrow. Four structural regions are shown: 1-86: CR (conserved region); 87-165: HR (heterogeneous region); 166-185: HVR (hypervariable region); 186-189: CAAX

(Modified from: Bar-Sagi, D. (2001) Mol. Cell Biol. 21(5):1441-1443)

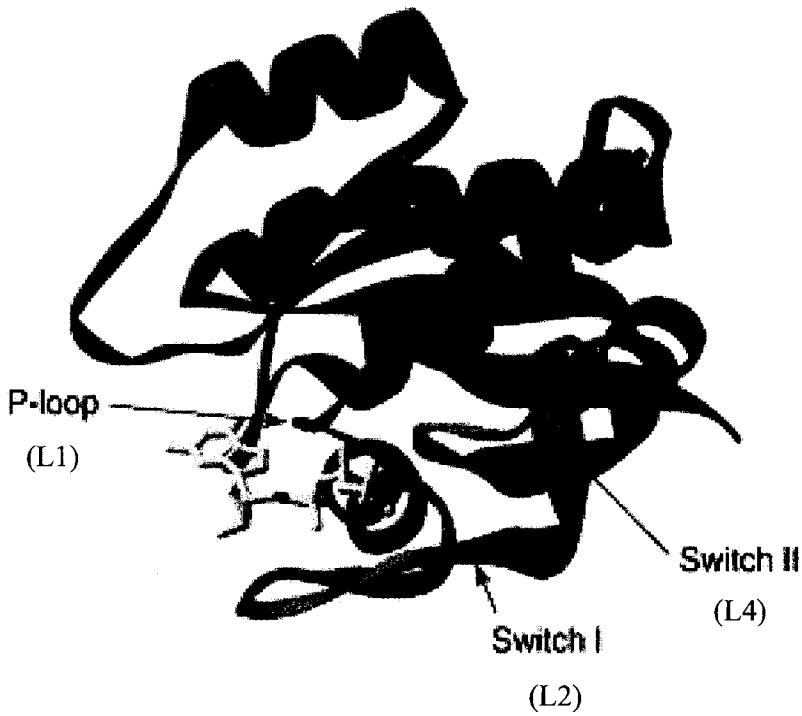


FIGURE 1.3 The crystal structure of Ras complex with GTP. Ribbon diagram of Ras bound to a GTP analog, GMPPNP. The P-loop/L1 (residues 10–17), Switch I/L2 (residues 30–38) and Switch II/L4 (residues 60–67) are shown in red, and the bound GTP and coordinated Mg^{2+} ion (stick representation) are shown in yellow.

(Adopted from Pai E. F. et al. (1990). Embo J. 9(8): 2351-9)

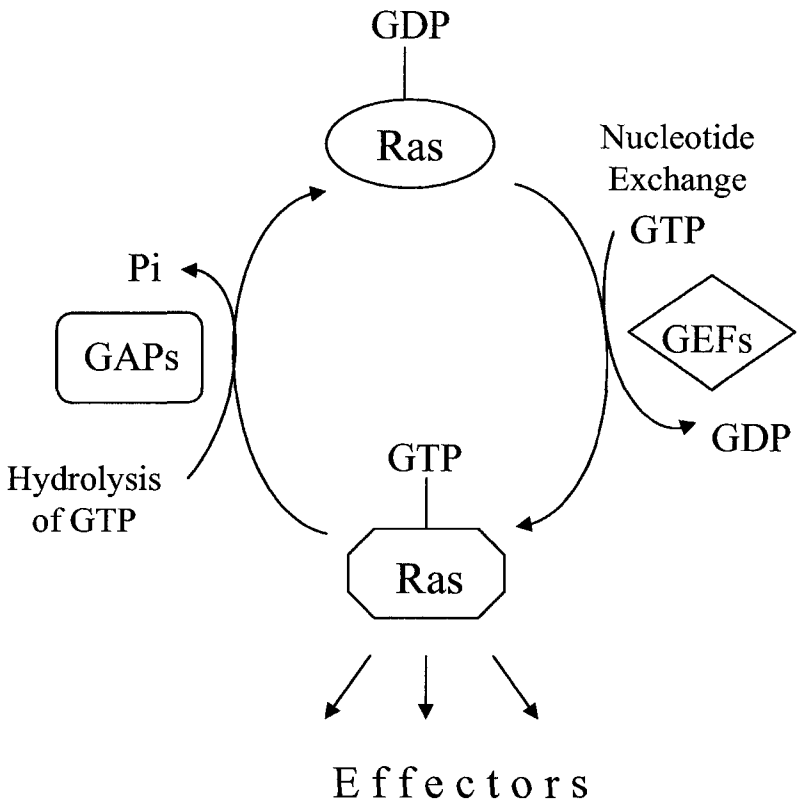


FIGURE 1.4 Mechanism of Ras regulation. The activity of Ras proteins is regulated by a cycle of guanine nucleotide binding and hydrolysis. Ras is in its active state when bound to GTP, in the inactive to GDP. GEFs promote dissociation of GDP and function as positive regulators; GAPs promotes intrinsic hydrolysis of GTP and act as negative regulators. Pi, inorganic phosphate.

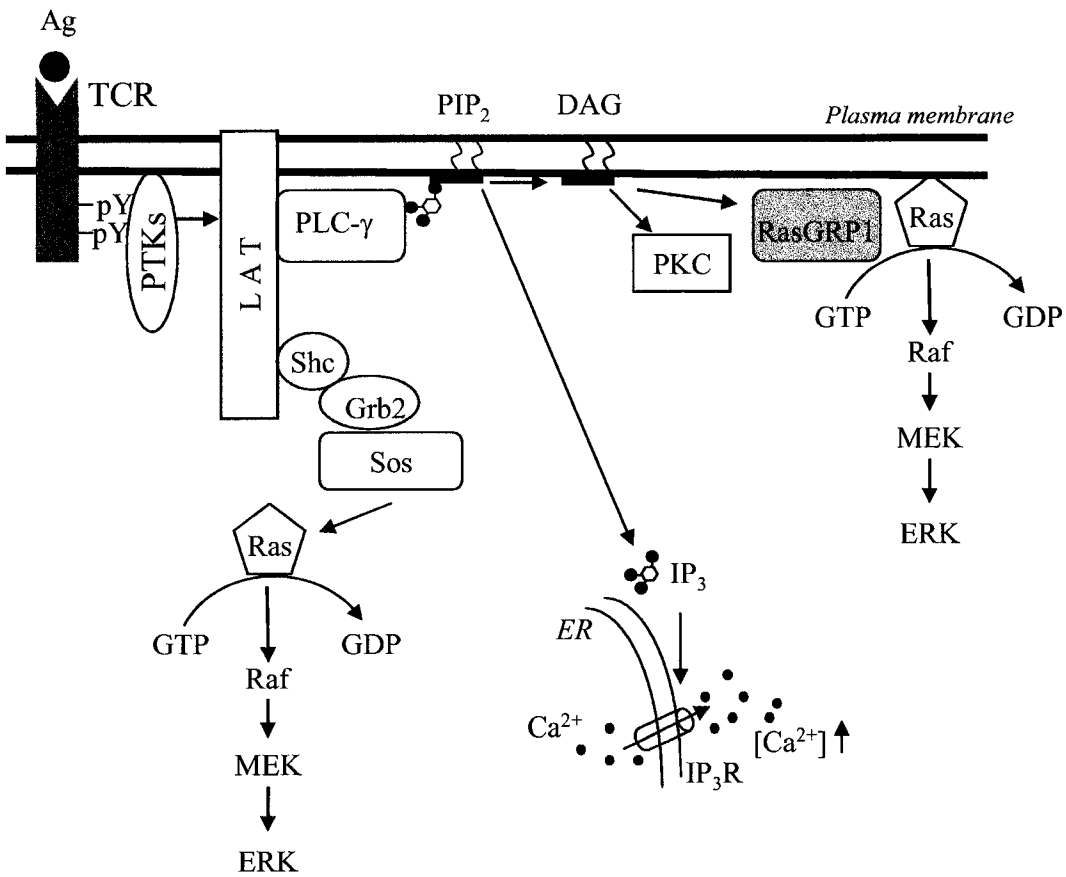


FIGURE 1.5 Mechanisms for Ras activation in T cells. Upon ligation, TCR activates a protein tyrosine kinase cascade (PTKs) resulting in the phosphorylation of adaptor proteins, notably LAT, which then recruit PLCs; this results in increased hydrolysis of PIP_2 to produce DAG which recruits RasGRP1 to plasma membrane. Tyrosine-phosphorylated LAT also recruits Shc and the Grb2/Sos complex to the plasma membrane. IP_3, inositol 1,4,5-triphosphate; PIP_2 phosphatidylinositol 4,5-diphosphate.

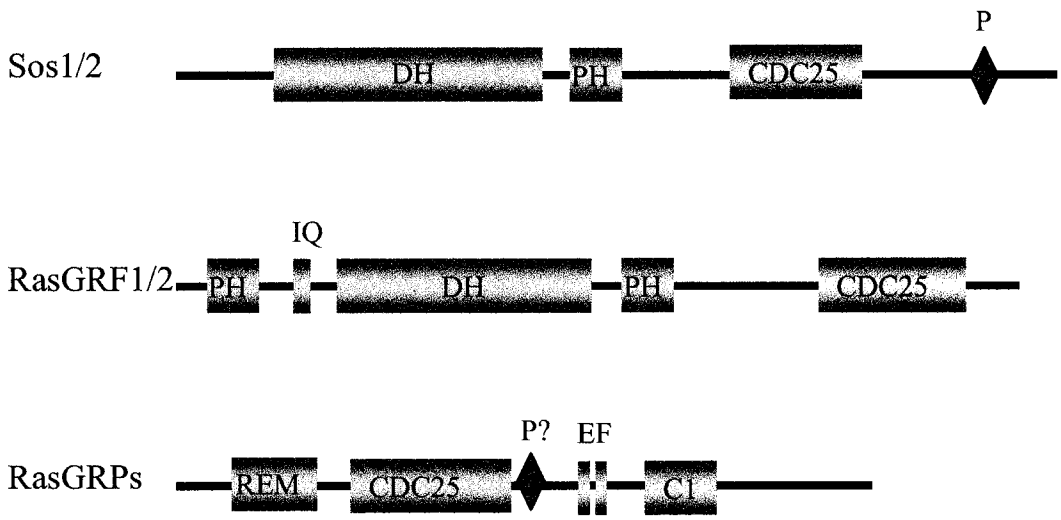


FIGURE 1.6 Schematic representation of Ras family guanine nucleotide exchange factors with their domain organization. DH, Dbl homology domain exhibiting GEF activity towards Rho and Rac subfamily proteins. CDC25, RasGEF catalytic domain. PH, pleckstrin homology. P, proline–rich sequence, representing SH3 domain binding sites. IQ, calmodulin binding. C1, DAG binding domain. REM, Ras exchange motif. EF, EF-hand.

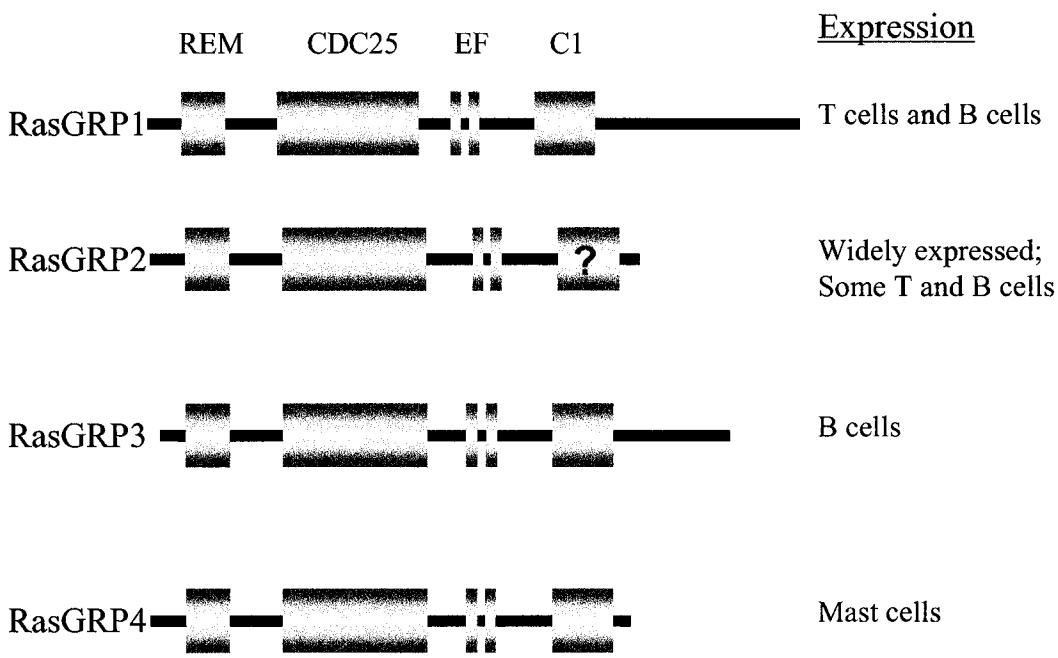


FIGURE 1.7 Schematic representation of RasGRP family members with their domain organization. The expression pattern of each RasGRP is also listed. The C1 domain of RasGRP2 has low affinity to DAG and phorbol ester, thus is marked with a question mark (?).

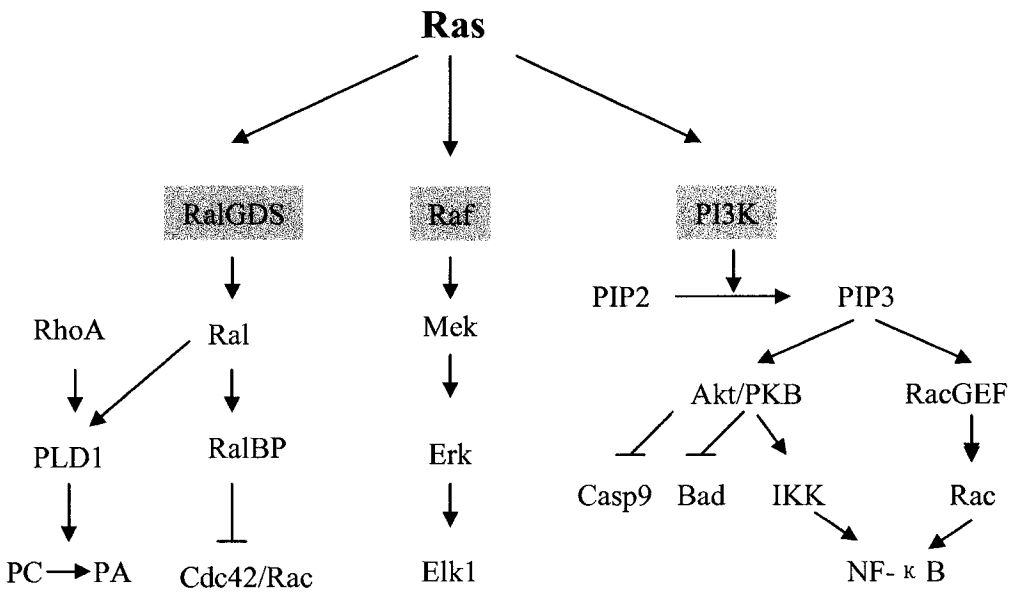


FIGURE 1.8 Ras activation of multiple effector-mediated signalling pathways. See text for details.

References

Abraham, R. T. (2002). "Antigen receptors rap to integrin receptors." Nat Immunol **3**(3): 212-3.

Alberola-Ila, J., K. A. Forbush, et al. (1995). "Selective requirement for MAP kinase activation in thymocyte differentiation." Nature **373**(6515): 620-3.

Alexander, K. A., B. T. Wakim, et al. (1988). "Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein." J Biol Chem **263**(16): 7544-9.

Armbruster, B. N. and B. L. Roth (2005). "Mining the receptorome." J Biol Chem **280**(7): 5129-32.

Arozarena, I., D. Matallanas, et al. (2004). "Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors." Mol Cell Biol **24**(4): 1516-30.

Asha, H., N. D. de Ruiter, et al. (1999). "The Rap1 GTPase functions as a regulator of morphogenesis in vivo." Embo J **18**(3): 605-15.

Baba, H., B. Fuss, et al. (1995). "GapIII, a new brain-enriched member of the GTPase-activating protein family." J Neurosci Res **41**(6): 846-58.

Baccarini, M. (2005). "Second nature: biological functions of the Raf-1 "kinase"." FEBS Lett **579**(15): 3271-7.

Bajenaru, M. L., J. Donahoe, et al. (2001). "Neurofibromatosis 1 (NF1) heterozygosity results in a cell-autonomous growth advantage for astrocytes." Glia **33**(4): 314-23.

Ballester, R., D. Marchuk, et al. (1990). "The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins." Cell **63**(4): 851-9.

Bar-Sagi, D. and A. Hall (2000). "Ras and Rho GTPases: a family reunion." Cell **103**(2): 227-38.

Barbacid, M. (1987). "ras genes." Annu Rev Biochem **56**: 779-827.

Bellacosa, A., C. C. Kumar, et al. (2005). "Activation of AKT kinases in cancer: implications for therapeutic targeting." Adv Cancer Res **94**: 29-86.

Berridge, C. W., A. F. Arnsten, et al. (1993). "Noradrenergic modulation of cognitive function: clinical implications of anatomical, electrophysiological and behavioural studies in animal models." Psychol Med **23**(3): 557-64.

Berrier, A. L., A. M. Mastrangelo, et al. (2000). "Activated R-ras, Rac1, PI 3-kinase and PKCepsilon can each restore cell spreading inhibited by isolated integrin beta1 cytoplasmic domains." J Cell Biol **151**(7): 1549-60.

Bi, L., I. Okabe, et al. (2002). "Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase." Mamm Genome **13**(3): 169-72.

Bi, L., I. Okabe, et al. (1999). "Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase." J Biol Chem **274**(16): 10963-8.

Bini, P., J. L. Chu, et al. (1990). "Analysis of autoantibodies to recombinant La (SS-B) peptides in systemic lupus erythematosus and primary Sjogren's syndrome." J Clin Invest **85**(2): 325-33.

Bivona, T. G., I. Perez De Castro, et al. (2003). "Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1." Nature **424**(6949): 694-8.

Bivona, T. G. and M. R. Philips (2003). "Ras pathway signaling on endomembranes." Curr Opin Cell Biol **15**(2): 136-42.

Boguski, M. S. and F. McCormick (1993). "Proteins regulating Ras and its relatives." Nature **366**(6456): 643-54.

Bollag, G. and F. McCormick (1992). "Ras regulation. NF is enough of GAP." Nature **356**(6371): 663-4.

Bonfini, L., E. Migliaccio, et al. (1996). "Not all Shc's roads lead to Ras." Trends Biochem Sci **21**(7): 257-61.

Boriack-Sjodin, P. A., S. M. Margarit, et al. (1998). "The structural basis of the activation of Ras by Sos." Nature **394**(6691): 337-43.

Bos, J. L. (1989). "ras oncogenes in human cancer: a review." Cancer Res **49**(17): 4682-9.

Bos, J. L. (1997). "Ras-like GTPases." Biochim Biophys Acta **1333**(2): M19-31.

Bos, J. L., J. de Rooij, et al. (2001). "Rap1 signalling: adhering to new models." Nat Rev Mol Cell Biol **2**(5): 369-77.

Bottorff, D., J. Ebinu, et al. (1999). "RasGRP, a Ras activator: mouse and human cDNA sequences and chromosomal positions." Mamm Genome **10**(4): 358-61.

Bourne, H. R., D. A. Sanders, et al. (1990). "The GTPase superfamily: a conserved switch for diverse cell functions." Nature **348**(6297): 125-32.

Bourne, H. R., D. A. Sanders, et al. (1991). "The GTPase superfamily: conserved structure and molecular mechanism." Nature **349**(6305): 117-27.

Boussiotis, V. A., G. J. Freeman, et al. (1997). "Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1." Science **278**(5335): 124-8.

Bowtell, D., P. Fu, et al. (1992). "Identification of murine homologues of the Drosophila son of sevenless gene: potential activators of ras." Proc Natl Acad Sci U S A **89**(14): 6511-5.

Boy-Marcotte, E., F. Damak, et al. (1989). "The C-terminal part of a gene partially homologous to CDC 25 gene suppresses the cdc25-5 mutation in Saccharomyces cerevisiae." Gene **77**(1): 21-30.

Brennan, P., J. W. Babbage, et al. (1997). "Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F." Immunity **7**(5): 679-89.

Briggs, M. W. and D. B. Sacks (2003). "IQGAP1 as signal integrator: Ca2+, calmodulin, Cdc42 and the cytoskeleton." FEBS Lett **542**(1-3): 7-11.

Brill, S., S. Li, et al. (1996). "The Ras GTPase-activating-protein-related human protein IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases." Mol Cell Biol **16**(9): 4869-78.

Brtva, T. R., J. K. Drugan, et al. (1995). "Two distinct Raf domains mediate interaction with Ras." J Biol Chem **270**(17): 9809-12.

Brunger, A. T., M. V. Milburn, et al. (1990). "Crystal structure of an active form of RAS protein, a complex of a GTP analog and the HRAS p21 catalytic domain." Proc Natl Acad Sci U S A **87**(12): 4849-53.

Buday, L. (1999). "Membrane-targeting of signalling molecules by SH2/SH3 domain-containing adaptor proteins." Biochim Biophys Acta **1422**(2): 187-204.

Buday, L., P. H. Warne, et al. (1995). "Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos." Oncogene **11**(7): 1327-31.

Bunnell, S. C., V. A. Barr, et al. (2003). "High-resolution multicolor imaging of dynamic signaling complexes in T cells stimulated by planar substrates." Sci STKE **2003**(177): PL8.

Burgering, B. M., G. J. Pronk, et al. (1993). "Role of p21ras in growth factor signal transduction." Biochem Soc Trans **21**(4): 888-94.

Call, M. E. and K. W. Wucherpfennig (2004). "Molecular mechanisms for the assembly of the T cell receptor-CD3 complex." Mol Immunol **40**(18): 1295-305.

Caloca, M. J., J. L. Zugaza, et al. (2004). "F-actin-dependent translocation of the Rap1 GDP/GTP exchange factor RasGRP2." J Biol Chem **279**(19): 20435-46.

Camonis, J. H., M. Kalekine, et al. (1986). "Characterization, cloning and sequence analysis of the CDC25 gene which controls the cyclic AMP level of Saccharomyces cerevisiae." Embo J **5**(2): 375-80.

Campbell, S. L., R. Khosravi-Far, et al. (1998). "Increasing complexity of Ras signaling." Oncogene **17**(11 Reviews): 1395-413.

Carpino, N., D. Wisniewski, et al. (1997). "p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells." Cell **88**(2): 197-204.

Casey, P. J., P. A. Solski, et al. (1989). "p21ras is modified by a farnesyl isoprenoid." Proc Natl Acad Sci U S A **86**(21): 8323-7.

Chai, J. and A. S. Tarnawski (2002). "Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing." J Physiol Pharmacol **53**(2): 147-57.

Chant, J., K. Corrado, et al. (1991). "Yeast BUD5, encoding a putative GDP-GTP exchange factor, is necessary for bud site selection and interacts with bud formation gene BEM1." Cell **65**(7): 1213-24.

Chant, J. and J. R. Pringle (1991). "Budding and cell polarity in Saccharomyces cerevisiae." Curr Opin Genet Dev **1**(3): 342-50.

Chardin, P., J. H. Camonis, et al. (1993). "Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2." Science **260**(5112): 1338-43.

Cheney, R. E. and M. S. Mooseker (1992). "Unconventional myosins." Curr Opin Cell Biol **4**(1): 27-35.

Chiu, V. K., T. Bivona, et al. (2002). "Ras signalling on the endoplasmic reticulum and the Golgi." Nat Cell Biol **4**(5): 343-50.

Choy, E., V. K. Chiu, et al. (1999). "Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi." Cell **98**(1): 69-80.

Chuang, E., D. Barnard, et al. (1994). "Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues." Mol Cell Biol **14**(8): 5318-25.

Cipres, A., S. Carrasco, et al. (2003). "Regulation of diacylglycerol kinase alpha by phosphoinositide 3-kinase lipid products." J Biol Chem **278**(37): 35629-35.

Clapham, D. E. and E. J. Neer (1997). "G protein beta gamma subunits." Annu Rev Pharmacol Toxicol **37**: 167-203.

Clark, G. J., M. S. Kinch, et al. (1996). "Overexpression of the Ras-related TC21/R-Ras2 protein may contribute to the development of human breast cancers." Oncogene **12**(1): 169-76.

Clark, G. J., M. S. Kinch, et al. (1997). "The Ras-related protein Rheb is farnesylated

and antagonizes Ras signaling and transformation." J Biol Chem **272**(16): 10608-15.

Clayton, E., G. Bardi, et al. (2002). "A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation." J Exp Med **196**(6): 753-63.

Clements, J. L., B. Yang, et al. (1998). "Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development." Science **281**(5375): 416-9.

Clyde-Smith, J., G. Silins, et al. (2000). "Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor." J Biol Chem **275**(41): 32260-7.

Cook, S. J., B. Rubinfeld, et al. (1993). "RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts." Embo J **12**(9): 3475-85.

Corbalan-Garcia, S., S. S. Yang, et al. (1996). "Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2." Mol Cell Biol **16**(10): 5674-82.

Cox, A. D., T. R. Brtva, et al. (1994). "R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells." Oncogene **9**(11): 3281-8.

Crespo, P., N. Xu, et al. (1994a). "Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation. Evidence for a protein kinase C-independent pathway." J Biol Chem **269**(33): 21103-9.

Crespo, P., N. Xu, et al. (1994b). "Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits." Nature **369**(6479): 418-20.

Crittenden, J. R., W. Bergmeier, et al. (2004). "CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation." Nat Med **10**(9): 982-6.

Cullen, P. J., J. J. Hsuan, et al. (1995). "Identification of a specific Ins(1,3,4,5)P4-binding protein as a member of the GAP1 family." Nature **376**(6540): 527-30.

Cutler, R. E., Jr., R. M. Stephens, et al. (1998). "Autoregulation of the Raf-1 serine/threonine kinase." Proc Natl Acad Sci U S A **95**(16): 9214-9.

Dai, Q., E. Choy, et al. (1998). "Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum." J Biol Chem **273**(24): 15030-4.

Dal Porto, J. M., S. B. Gauld, et al. (2004). "B cell antigen receptor signaling 101." Mol Immunol **41**(6-7): 599-613.

Dasgupta, B. and D. H. Gutmann (2003). "Neurofibromatosis 1: closing the GAP between mice and men." Curr Opin Genet Dev **13**(1): 20-7.

de Castro, I. P., R. Diaz, et al. (2003). "Mice deficient for N-ras: impaired antiviral immune response and T-cell function." Cancer Res **63**(7): 1615-22.

de Vos, A. M., L. Tong, et al. (1988). "Three-dimensional structure of an oncogene protein: catalytic domain of human c-H-ras p21." Science **239**(4842): 888-93.

DeClue, J. E., J. C. Stone, et al. (1991). "A ras effector domain mutant which is temperature sensitive for cellular transformation: interactions with GTPase-activating protein and NF-1." Mol Cell Biol **11**(6): 3132-8.

Del Villar, K., D. Dorin, et al. (1996). "C-terminal motifs found in Ras-superfamily G-proteins: CAAX and C-seven motifs." Biochem Soc Trans **24**(3): 709-13.

Dent, P., W. Haser, et al. (1992). "Activation of mitogen-activated protein kinase kinase

by v-Raf in NIH 3T3 cells and in vitro." Science **257**(5075): 1404-7.
Der, C. J., B. T. Pan, et al. (1986). "rasH mutants deficient in GTP binding." Mol Cell Biol **6**(9): 3291-4.
Dev, K. K. (2004). "Making protein interactions druggable: targeting PDZ domains." Nat Rev Drug Discov **3**(12): 1047-56.
Dhillon, A. S. and W. Kolch (2002). "Untying the regulation of the Raf-1 kinase." Arch Biochem Biophys **404**(1): 3-9.
Di Cristofano, A., B. Pesce, et al. (1998). "Pten is essential for embryonic development and tumour suppression." Nat Genet **19**(4): 348-55.
Di Fiore, P. P. and G. N. Gill (1999). "Endocytosis and mitogenic signaling." Curr Opin Cell Biol **11**(4): 483-8.
Domin, J., F. Pages, et al. (1997). "Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin." Biochem J **326 (Pt 1)**: 139-47.
Donjerkovic, D. and D. W. Scott (2000). "Activation-induced cell death in B lymphocytes." Cell Res **10**(3): 179-92.
Dower, N. A., S. L. Stang, et al. (2000). "RasGRP is essential for mouse thymocyte differentiation and TCR signaling." Nat Immunol **1**(4): 317-21.
Downward, J. (2003). "Targeting RAS signalling pathways in cancer therapy." Nat Rev Cancer **3**(1): 11-22.
Downward, J., J. D. Graves, et al. (1990). "Stimulation of p21ras upon T-cell activation." Nature **346**(6286): 719-23.
Duchesne, M., F. Schweighoffer, et al. (1993). "Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling." Science **259**(5094): 525-8.
Ebinu, J. O., D. A. Bottorff, et al. (1998). "RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs." Science **280**(5366): 1082-6.
Ebinu, J. O., S. L. Stang, et al. (2000). "RasGRP links T-cell receptor signaling to Ras." Blood **95**(10): 3199-203.
Egan, S. E., B. W. Giddings, et al. (1993). "Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation." Nature **363**(6424): 45-51.
Ehrhardt, G. R., K. B. Leslie, et al. (1999). "M-Ras, a widely expressed 29-kD homologue of p21 Ras: expression of a constitutively active mutant results in factor-independent growth of an interleukin-3-dependent cell line." Blood **94**(7): 2433-44.
Elder, M. E. (1998). "ZAP-70 and defects of T-cell receptor signaling." Semin Hematol **35**(4): 310-20.
Ellis, C., M. Moran, et al. (1990). "Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases." Nature **343**(6256): 377-81.
Engel, P., L. J. Zhou, et al. (1995). "Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule." Immunity **3**(1): 39-50.
Esteban, L. M., C. Vicario-Abejon, et al. (2001). "Targeted genomic disruption of H-ras

and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development." Mol Cell Biol **21**(5): 1444-52.

Eto, K., R. Murphy, et al. (2002). "Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling." Proc Natl Acad Sci U S A **99**(20): 12819-24.

Fam, N. P., W. T. Fan, et al. (1997). "Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras." Mol Cell Biol **17**(3): 1396-406.

Farnsworth, C. L., N. W. Freshney, et al. (1995). "Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF." Nature **376**(6540): 524-7.

Feig, L. A. and G. M. Cooper (1988a). "Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP." Mol Cell Biol **8**(8): 3235-43.

Feig, L. A. and G. M. Cooper (1988b). "Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins." Mol Cell Biol **8**(6): 2472-8.

Feig, L. A., T. Urano, et al. (1996). "Evidence for a Ras/Ral signaling cascade." Trends Biochem Sci **21**(11): 438-41.

Feuerstein, J., H. R. Kalbitzer, et al. (1987). "Characterisation of the metal-ion-GDP complex at the active sites of transforming and nontransforming p21 proteins by observation of the 17O-Mn superhyperfine coupling and by kinetic methods." Eur J Biochem **162**(1): 49-55.

Franke, T. F., D. R. Kaplan, et al. (1997). "PI3K: downstream AKTion blocks apoptosis." Cell **88**(4): 435-7.

Friedman, J. M. (1999). "Epidemiology of neurofibromatosis type 1." Am J Med Genet **89**(1): 1-6.

Fruman, D. A., R. E. Meyers, et al. (1998). "Phosphoinositide kinases." Annu Rev Biochem **67**: 481-507.

Fruman, D. A., S. B. Snapper, et al. (1999a). "Phosphoinositide 3-kinase knockout mice: role of p85alpha in B cell development and proliferation." Biochem Soc Trans **27**(4): 624-9.

Fruman, D. A., S. B. Snapper, et al. (1999b). "Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha." Science **283**(5400): 393-7.

Fujita-Yoshigaki, J., M. Shirouzu, et al. (1995). "A constitutive effector region on the C-terminal side of switch I of the Ras protein." J Biol Chem **270**(9): 4661-7.

Fukuda, M., T. Kojima, et al. (1997). "Regulation by bivalent cations of phospholipid binding to the C2A domain of synaptotagmin III." Biochem J **323 (Pt 2)**: 421-5.

Furth, M. E., T. H. Aldrich, et al. (1987). "Expression of ras proto-oncogene proteins in normal human tissues." Oncogene **1**(1): 47-58.

Galaktionov, K., C. Jessus, et al. (1995). "Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation." Genes Dev **9**(9): 1046-58.

Gao, X., T. Satoh, et al. (2001). "Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras." J Biol Chem **276**(45): 42219-25.

Gibbs, J. B., I. S. Sigal, et al. (1984). "Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules." Proc Natl Acad Sci U S A **81**(18): 5704-8.

Gideon, P., J. John, et al. (1992). "Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: the C-terminal domain of GAP is not sufficient for full activity." Mol Cell Biol **12**(5): 2050-6.

Gotoh, T., Y. Niino, et al. (1997). "Activation of R-Ras by Ras-guanine nucleotide-releasing factor." J Biol Chem **272**(30): 18602-7.

Graham, S. M., A. B. Vojtek, et al. (1996). "TC21 causes transformation by Raf-independent signaling pathways." Mol Cell Biol **16**(11): 6132-40.

Gudermann, T., R. Grosse, et al. (2000). "Contribution of receptor/G protein signaling to cell growth and transformation." Naunyn Schmiedebergs Arch Pharmacol **361**(4): 345-62.

Guilbault, B. and R. J. Kay (2004). "RasGRP1 sensitizes an immature B cell line to antigen receptor-induced apoptosis." J Biol Chem **279**(19): 19523-30.

Guo, F. F., E. Kumahara, et al. (2001). "A CalDAG-GEFI/Rap1/B-Raf cassette couples M(1) muscarinic acetylcholine receptors to the activation of ERK1/2." J Biol Chem **276**(27): 25568-81.

Gutierrez, L., A. I. Magee, et al. (1989). "Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis." Embo J **8**(4): 1093-8.

Hall, A. and A. J. Self (1986). "The effect of Mg2+ on the guanine nucleotide exchange rate of p21N-ras." J Biol Chem **261**(24): 10963-5.

Hamad, N. M., J. H. Elconin, et al. (2002). "Distinct requirements for Ras oncogenesis in human versus mouse cells." Genes Dev **16**(16): 2045-57.

Han, J., K. Luby-Phelps, et al. (1998). "Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav." Science **279**(5350): 558-60.

Hancock, J. F., A. I. Magee, et al. (1989). "All ras proteins are polyisoprenylated but only some are palmitoylated." Cell **57**(7): 1167-77.

Hancock, J. F., H. Paterson, et al. (1990). "A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane." Cell **63**(1): 133-9.

Hao, S., T. Kurosaki, et al. (2003). "Differential regulation of NFAT and SRF by the B cell receptor via a PLCgamma-Ca(2+)-dependent pathway." Embo J **22**(16): 4166-77.

Harvey, J. J. (1964). "An Unidentified Virus Which Causes the Rapid Production of Tumours in Mice." Nature **204**: 1104-5.

Helgason, C. D., J. E. Damen, et al. (1998). "Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span." Genes Dev **12**(11): 1610-20.

Henkemeyer, M., D. J. Rossi, et al. (1995). "Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein." Nature **377**(6551): 695-701.

Hermiston, M. L., Z. Xu, et al. (2002). "Reciprocal regulation of lymphocyte activation by tyrosine kinases and phosphatases." J Clin Invest **109**(1): 9-14.

Herrmann, C., G. Horn, et al. (1996). "Differential interaction of the ras family GTP-binding proteins H-Ras, Rap1A, and R-Ras with the putative effector molecules Raf kinase and Ral-guanine nucleotide exchange factor." J Biol Chem

271(12): 6794-800.

Hiatt, K. K., D. A. Ingram, et al. (2001). "Neurofibromin GTPase-activating protein-related domains restore normal growth in Nf1-/- cells." J Biol Chem **276**(10): 7240-5.

Hirsch-Ginsberg, C., A. C. LeMaistre, et al. (1990). "RAS mutations are rare events in Philadelphia chromosome-negative/bcr gene rearrangement-negative chronic myelogenous leukemia, but are prevalent in chronic myelomonocytic leukemia." Blood **76**(6): 1214-9.

Hofer, F., R. Berdeaux, et al. (1998). "Ras-independent activation of Ral by a Ca(2+)-dependent pathway." Curr Biol **8**(14): 839-42.

Hofer, F., S. Fields, et al. (1994). "Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator." Proc Natl Acad Sci U S A **91**(23): 11089-93.

Hoffman, G. R. and R. A. Cerione (2002). "Signaling to the Rho GTPases: networking with the DH domain." FEBS Lett **513**(1): 85-91.

Holsinger, L. J., D. M. Spencer, et al. (1995). "Signal transduction in T lymphocytes using a conditional allele of Sos." Proc Natl Acad Sci U S A **92**(21): 9810-4.

Hoshino, M., D. J. Clanton, et al. (1987). "Interaction of ras oncogene product p21 with guanine nucleotides." J Biochem (Tokyo) **102**(3): 503-11.

Howe, L. R., S. J. Leevers, et al. (1992). "Activation of the MAP kinase pathway by the protein kinase raf." Cell **71**(2): 335-42.

Hu, C. D., K. Kariya, et al. (1997). "Coassociation of Rap1A and Ha-Ras with Raf-1 N-terminal region interferes with ras-dependent activation of Raf-1." J Biol Chem **272**(18): 11702-5.

Hu, H., J. M. Bliss, et al. (2005). "RIN1 is an ABL tyrosine kinase activator and a regulator of epithelial-cell adhesion and migration." Curr Biol **15**(9): 815-23.

Hua, V. Y., W. K. Wang, et al. (1997). "Dominant transformation by mutated human ras genes in vitro requires more than 100 times higher expression than is observed in cancers." Proc Natl Acad Sci U S A **94**(18): 9614-9.

Huang, L., F. Hofer, et al. (1998). "Structural basis for the interaction of Ras with RalGDS." Nat Struct Biol **5**(6): 422-6.

Huang, W., A. Alessandrini, et al. (1993). "Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation." Proc Natl Acad Sci U S A **90**(23): 10947-51.

Huang, Y., R. Saez, et al. (1995). "A novel insertional mutation in the TC21 gene activates its transforming activity in a human leiomyosarcoma cell line." Oncogene **11**(7): 1255-60.

Huang, Y. and R. L. Wange (2004). "T cell receptor signaling: beyond complex complexes." J Biol Chem **279**(28): 28827-30.

Huber, M., C. D. Helgason, et al. (1998). "Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells." Embo J **17**(24): 7311-9.

Huff, S. Y., L. A. Quilliam, et al. (1997). "R-Ras is regulated by activators and effectors distinct from those that control Ras function." Oncogene **14**(2): 133-43.

Hughes, P. E., B. Oertli, et al. (2001). "R-Ras regulation of integrin function." Methods Enzymol **333**: 163-71.

Huser, M., J. Luckett, et al. (2001). "MEK kinase activity is not necessary for Raf-1 function." Embo J **20**(8): 1940-51.

Ihle, J. N. (1996). "STATs and MAPKs: obligate or opportunistic partners in signaling." Bioessays **18**(2): 95-8.

Ikeda, M., O. Ishida, et al. (1998). "Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral." J Biol Chem **273**(2): 814-21.

Ikenoue, T., Y. Hikiba, et al. (2003). "Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors." Cancer Res **63**(23): 8132-7.

Imamura, N., A. Kuramoto, et al. (1993). "Detection of high incidence of H-RAS oncogene point mutations in acute myelogenous leukemia." Am J Hematol **43**(2): 151-3.

Ingram, D. A., K. Hiatt, et al. (2001). "Hyperactivation of p21(ras) and the hematopoietic-specific Rho GTPase, Rac2, cooperate to alter the proliferation of neurofibromin-deficient mast cells in vivo and in vitro." J Exp Med **194**(1): 57-69.

Ingram, D. A., L. Zhang, et al. (2002). "Lymphoproliferative defects in mice lacking the expression of neurofibromin: functional and biochemical consequences of Nf1 deficiency in T-cell development and function." Blood **100**(10): 3656-62.

Izawa, I., N. Tamaki, et al. (1996). "Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) by cAMP-dependent protein kinase." FEBS Lett **382**(1-2): 53-9.

Izquierdo, M., J. Downward, et al. (1992). "Role of protein kinase C in T-cell antigen receptor regulation of p21ras: evidence that two p21ras regulatory pathways coexist in T cells." Mol Cell Biol **12**(7): 3305-12.

Izquierdo, M., S. J. Leevers, et al. (1994). "The role of protein kinase C in the regulation of extracellular signal-regulated kinase by the T cell antigen receptor." Eur J Immunol **24**(10): 2462-8.

Janosch, P., M. Schellerer, et al. (1996). "Characterization of IkappaB kinases. IkappaB-alpha is not phosphorylated by Raf-1 or protein kinase C isozymes, but is a casein kinase II substrate." J Biol Chem **271**(23): 13868-74.

Jiang, H., J. Q. Luo, et al. (1995). "Involvement of Ral GTPase in v-Src-induced phospholipase D activation." Nature **378**(6555): 409-12.

Jiang, X. and A. Sorkin (2002). "Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells." Mol Biol Cell **13**(5): 1522-35.

John, J., H. Rensland, et al. (1993). "Kinetic and structural analysis of the Mg(2+)-binding site of the guanine nucleotide-binding protein p21H-ras." J Biol Chem **268**(2): 923-9.

John, J., R. Sohmen, et al. (1990). "Kinetics of interaction of nucleotides with nucleotide-free H-ras p21." Biochemistry **29**(25): 6058-65.

Johnson, L., D. Greenbaum, et al. (1997). "K-ras is an essential gene in the mouse with partial functional overlap with N-ras." Genes Dev **11**(19): 2468-81.

Johnson, N. L., A. M. Gardner, et al. (1996). "Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death." J Biol Chem **271**(6): 3229-37.

Jones, D. R., M. A. Sanjuan, et al. (2002). "Expression of a catalytically inactive form of

diacylglycerol kinase alpha induces sustained signaling through RasGRP." Faseb J **16**(6): 595-7.

Jones, S., M. L. Vignais, et al. (1991). "The CDC25 protein of Saccharomyces cerevisiae promotes exchange of guanine nucleotides bound to ras." Mol Cell Biol **11**(5): 2641-6.

Jou, S. T., N. Carpino, et al. (2002). "Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex." Mol Cell Biol **22**(24): 8580-91.

Kaplan, D. R., D. K. Morrison, et al. (1990). "PDGF beta-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex." Cell **61**(1): 125-33.

Kaptein, J. S., C. L. Yang, et al. (1995). "Synergy between signal transduction pathways is obligatory for expression of c-fos in B and T cell lines: implication for c-fos control via surface immunoglobulin and T cell antigen receptors." Immunobiology **193**(5): 465-85.

Katagiri, K., M. Hattori, et al. (2000). "Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase." Mol Cell Biol **20**(6): 1956-69.

Katagiri, K., M. Shimonaka, et al. (2004). "Rap1-mediated lymphocyte function-associated antigen-1 activation by the T cell antigen receptor is dependent on phospholipase C-gamma1." J Biol Chem **279**(12): 11875-81.

Kawasaki, H., G. M. Springett, et al. (1998). "A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia." Proc Natl Acad Sci U S A **95**(22): 13278-83.

Kedra, D., E. Seroussi, et al. (1997). "The germinal center kinase gene and a novel CDC25-like gene are located in the vicinity of the PYGM gene on 11q13." Hum Genet **100**(5-6): 611-9.

Kikuchi, A., S. D. Demo, et al. (1994). "ralGDS family members interact with the effector loop of ras p21." Mol Cell Biol **14**(11): 7483-91.

Kim, J. H., D. Liao, et al. (1998). "SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family." Neuron **20**(4): 683-91.

Kim, R., A. Trubetskoy, et al. (2003). "Genome-based identification of cancer genes by proviral tagging in mouse retrovirus-induced T-cell lymphomas." J Virol **77**(3): 2056-62.

Kimmelman, A., T. Tolkacheva, et al. (1997). "Identification and characterization of R-ras3: a novel member of the RAS gene family with a non-ubiquitous pattern of tissue distribution." Oncogene **15**(22): 2675-85.

Kindig, D. A. and W. H. Kirsten (1967). "Virus-like particles in established murine cell lines: electron-microscopic observations." Science **155**(769): 1543-5.

Kitayama, H., Y. Sugimoto, et al. (1989). "A ras-related gene with transformation suppressor activity." Cell **56**(1): 77-84.

Klinger, M., O. Kudlacek, et al. (2002). "MAP kinase stimulation by cAMP does not require RAP1 but SRC family kinases." J Biol Chem **277**(36): 32490-7.

Knox, A. L. and N. H. Brown (2002). "Rap1 GTPase regulation of adherens junction positioning and cell adhesion." Science **295**(5558): 1285-8.

Koera, K., K. Nakamura, et al. (1997). "K-ras is essential for the development of the

mouse embryo." Oncogene **15**(10): 1151-9.
Kolch, W., G. Heidecker, et al. (1993). "Protein kinase C alpha activates RAF-1 by direct phosphorylation." Nature **364**(6434): 249-52.
Kolch, W., G. Heidecker, et al. (1991). "Raf-1 protein kinase is required for growth of induced NIH/3T3 cells." Nature **349**(6308): 426-8.
Kotani, K., K. Yonezawa, et al. (1994). "Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling." Embo J **13**(10): 2313-21.
Koyasu, S. (2004). "Role of class IA phosphoinositide 3-kinase in B lymphocyte development and functions." Biochem Soc Trans **32**(Pt 2): 320-5.
Kraut, J. (1988). "How do enzymes work?" Science **242**(4878): 533-40.
Krengel, U., L. Schlichting, et al. (1990). "Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules." Cell **62**(3): 539-48.
Kristiansen, K. (2004). "Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function." Pharmacol Ther **103**(1): 21-80.
Kuiperij, H. B., J. de Rooij, et al. (2003). "Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2." Biochim Biophys Acta **1593**(2-3): 141-9.
Kulkarni, S. V., G. Gish, et al. (2000). "Role of p120 Ras-GAP in directed cell movement." J Cell Biol **149**(2): 457-70.
Kuriyama, M., N. Harada, et al. (1996). "Identification of AF-6 and canoe as putative targets for Ras." J Biol Chem **271**(2): 607-10.
Kurosaki, T. (2002). "Regulation of B cell fates by BCR signaling components." Curr Opin Immunol **14**(3): 341-7.
Kyriakis, J. M., H. App, et al. (1992). "Raf-1 activates MAP kinase-kinase." Nature **358**(6385): 417-21.
Lai, C. C., M. Boguski, et al. (1993). "Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins." Mol Cell Biol **13**(3): 1345-52.
Laroche-Joubert, N., S. Marsy, et al. (2002). "Protein kinase A-independent activation of ERK and H,K-ATPase by cAMP in native kidney cells: role of Epac I." J Biol Chem **277**(21): 18598-604.
Layer, K., G. Lin, et al. (2003). "Autoimmunity as the consequence of a spontaneous mutation in Rasgrp1." Immunity **19**(2): 243-55.
Lee, J. E., T. W. Beck, et al. (1996). "Regulation of A-raf expression." Oncogene **12**(8): 1669-77.
Leitges, M., C. Schmedt, et al. (1996). "Immunodeficiency in protein kinase cbeta-deficient mice." Science **273**(5276): 788-91.
Leon, J., I. Guerrero, et al. (1987). "Differential expression of the ras gene family in mice." Mol Cell Biol **7**(4): 1535-40.
Leonardsen, L., J. E. DeClue, et al. (1996). "Rasp21 sequences opposite the nucleotide binding pocket are required for GRF-mediated nucleotide release." Oncogene **13**(10): 2177-87.
Lewis, R. A., N. A. Soter, et al. (1982). "Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE." J Immunol **129**(4): 1627-31.

Li, L., Y. Yang, et al. (2003). "RasGRP4 regulates the expression of prostaglandin D2 in human and rat mast cell lines." J Biol Chem **278**(7): 4725-9.

Li, N., A. Batzer, et al. (1993). "Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling." Nature **363**(6424): 85-8.

Li, S. and J. M. Sedivy (1993). "Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex." Proc Natl Acad Sci U S A **90**(20): 9247-51.

Li, W., R. Nishimura, et al. (1994). "A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase." Mol Cell Biol **14**(1): 509-17.

Li, Z., D. E. McNulty, et al. (2005). "IQGAP1 promotes neurite outgrowth in a phosphorylation-dependent manner." J Biol Chem **280**(14): 13871-8.

Liao, C. F., W. P. Schilling, et al. (1990). "Cellular responses to stimulation of the M5 muscarinic acetylcholine receptor as seen in murine L cells." J Biol Chem **265**(19): 11273-84.

Lockyer, P. J., S. Vanlingen, et al. (1999). "Tissue-specific expression and endogenous subcellular distribution of the inositol 1,3,4,5-tetrakisphosphate-binding proteins GAP1(IP4BP) and GAP1(m)." Biochem Biophys Res Commun **255**(2): 421-6.

Lopez-Ilasaca, M., P. Crespo, et al. (1997a). "Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma." Science **275**(5298): 394-7.

Lopez-Ilasaca, M., W. Li, et al. (1997b). "Requirement of phosphatidylinositol-3 kinase for activation of JNK/SAPKs by PDGF." Biochem Biophys Res Commun **232**(2): 273-7.

Lorenzo, P. S., J. W. Kung, et al. (2001). "Phorbol esters modulate the Ras exchange factor RasGRP3." Cancer Res **61**(3): 943-9.

Lowe, D. G. and D. V. Goeddel (1987). "Heterologous expression and characterization of the human R-ras gene product." Mol Cell Biol **7**(8): 2845-56.

Lowy, D. R., M. R. Johnson, et al. (1993). "Cell transformation by ras and regulation of its protein product." Ciba Found Symp **176**: 67-80; discussion 80-4.

Lowy, D. R. and B. M. Willumsen (1993). "Function and regulation of ras." Annu Rev Biochem **62**: 851-91.

Lupas, A., M. Van Dyke, et al. (1991). "Predicting coiled coils from protein sequences." Science **252**(5010): 1162-4.

Luttrell, L. M., T. van Biesen, et al. (1995). "G beta gamma subunits mediate mitogen-activated protein kinase activation by the tyrosine kinase insulin-like growth factor 1 receptor." J Biol Chem **270**(28): 16495-8.

Luzi, L., S. Confalonieri, et al. (2000). "Evolution of Shc functions from nematode to human." Curr Opin Genet Dev **10**(6): 668-74.

Ma, J. and M. Karplus (1997a). "Ligand-induced conformational changes in ras p21: a normal mode and energy minimization analysis." J Mol Biol **274**(1): 114-31.

Ma, J. and M. Karplus (1997b). "Molecular switch in signal transduction: reaction paths of the conformational changes in ras p21." Proc Natl Acad Sci U S A **94**(22): 11905-10.

Machesky, L. M. (1998). "Cytokinesis: IQGAPs find a function." Curr Biol **8**(6): R202-5.

Macian, F. (2005). "NFAT proteins: key regulators of T-cell development and function." Nat Rev Immunol **5**(6): 472-84.

Malumbres, M. and M. Barbacid (2003). "RAS oncogenes: the first 30 years." Nat Rev Cancer **3**(6): 459-65.

Malumbres, M. and A. Pellicer (1998). "RAS pathways to cell cycle control and cell transformation." Front Biosci **3**: d887-912.

Mangues, R. and A. Pellicer (1992). "ras activation in experimental carcinogenesis." Semin Cancer Biol **3**(4): 229-39.

Marinissen, M. J. and J. S. Gutkind (2001). "G-protein-coupled receptors and signaling networks: emerging paradigms." Trends Pharmacol Sci **22**(7): 368-76.

Marino, M. J., S. T. Rouse, et al. (1998). "Activation of the genetically defined m1 muscarinic receptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells." Proc Natl Acad Sci U S A **95**(19): 11465-70.

Marklund, U., G. Brattsand, et al. (1993). "Serine 25 of oncoprotein 18 is a major cytosolic target for the mitogen-activated protein kinase." J Biol Chem **268**(20): 15039-47.

Marshall, M. S. (1993). "The effector interactions of p21ras." Trends Biochem Sci **18**(7): 250-4.

Marte, B. M., P. Rodriguez-Viciana, et al. (1997). "R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways." Curr Biol **7**(1): 63-70.

Matsumoto, K., T. Asano, et al. (1997). "Novel small GTPase M-Ras participates in reorganization of actin cytoskeleton." Oncogene **15**(20): 2409-17.

Matsumoto, Y., T. Oshida, et al. (2002). "Identification of highly expressed genes in peripheral blood T cells from patients with atopic dermatitis." Int Arch Allergy Immunol **129**(4): 327-40.

Mattingly, R. R. and I. G. Macara (1996). "Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta gamma subunits." Nature **382**(6588): 268-72.

Mattingly, R. R., A. Sorisky, et al. (1994). "Muscarinic receptors transform NIH 3T3 cells through a Ras-dependent signalling pathway inhibited by the Ras-GTPase-activating protein SH3 domain." Mol Cell Biol **14**(12): 7943-52.

McGrath, J. P., D. J. Capon, et al. (1984). "Comparative biochemical properties of normal and activated human ras p21 protein." Nature **310**(5979): 644-9.

McLeod, S. J., R. J. Ingham, et al. (1998). "Activation of the Rap1 GTPase by the B cell antigen receptor." J Biol Chem **273**(44): 29218-23.

McLeod, S. J., A. J. Shum, et al. (2004). "The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes." J Biol Chem **279**(13): 12009-19.

Medema, J. P., G. J. Pronk, et al. (1996). "Insulin-induced tyrosine phosphorylation of a M(r) 70,000 protein revealed by association with the Src homology 2 (SH2) and SH3 domains of p120GAP and Grb2." Cell Growth Differ **7**(4): 543-50.

Michaelson, D., J. Silletti, et al. (2001). "Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding." J Cell Biol **152**(1): 111-26.

Mikula, M., M. Schreiber, et al. (2001). "Embryonic lethality and fetal liver apoptosis in

mice lacking the c-raf-1 gene." Embo J **20**(8): 1952-62.
Milburn, M. V., L. Tong, et al. (1990). "Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins." Science **247**(4945): 939-45.
Mineo, C., G. N. Gill, et al. (1999). "Regulated migration of epidermal growth factor receptor from caveolae." J Biol Chem **274**(43): 30636-43.
Mistou, M. Y., E. Jacquet, et al. (1992). "Mutations of Ha-ras p21 that define important regions for the molecular mechanism of the SDC25 C-domain, a guanine nucleotide dissociation stimulator." Embo J **11**(7): 2391-7.
Mittal, R., M. R. Ahmadian, et al. (1996). "Formation of a transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins." Science **273**(5271): 115-7.
Moller, W. and R. Amons (1985). "Phosphate-binding sequences in nucleotide-binding proteins." FEBS Lett **186**(1): 1-7.
Moodie, S. A., M. Paris, et al. (1995). "Different structural requirements within the switch II region of the Ras protein for interactions with specific downstream targets." Oncogene **11**(3): 447-54.
Mulder, M. P., W. Keijzer, et al. (1989). "Activated ras genes in human seminoma: evidence for tumor heterogeneity." Oncogene **4**(11): 1345-51.
Muller, R., D. J. Slamon, et al. (1983). "Transcription of c-onc genes c-rasKi and c-fms during mouse development." Mol Cell Biol **3**(6): 1062-9.
Myung, P. S., N. J. Boerthe, et al. (2000). "Adapter proteins in lymphocyte antigen-receptor signaling." Curr Opin Immunol **12**(3): 256-66.
Ng, J. and D. Cantrell (1997). "STAT3 is a serine kinase target in T lymphocytes. Interleukin 2 and T cell antigen receptor signals converge upon serine 727." J Biol Chem **272**(39): 24542-9.
Niiro, H. and E. A. Clark (2002). "Regulation of B-cell fate by antigen-receptor signals." Nat Rev Immunol **2**(12): 945-56.
Niki, M., A. Di Cristofano, et al. (2004). "Role of Dok-1 and Dok-2 in leukemia suppression." J Exp Med **200**(12): 1689-95.
Nimnual, A. S., B. A. Yatsula, et al. (1998). "Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos." Science **279**(5350): 560-3.
Oertli, B., J. Han, et al. (2000). "The effector loop and prenylation site of R-Ras are involved in the regulation of integrin function." Oncogene **19**(43): 4961-9.
Oh-hora, M., S. Johmura, et al. (2003). "Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor signaling." J Exp Med **198**(12): 1841-51.
Ohba, Y., K. Ikuta, et al. (2001). "Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis." Embo J **20**(13): 3333-41.
Ohba, Y., K. Kurokawa, et al. (2003). "Mechanism of the spatio-temporal regulation of Ras and Rap1." Embo J **22**(4): 859-69.
Ohba, Y., N. Mochizuki, et al. (2000a). "Rap2 as a slowly responding molecular switch in the Rap1 signaling cascade." Mol Cell Biol **20**(16): 6074-83.
Ohba, Y., N. Mochizuki, et al. (2000b). "Regulatory proteins of R-Ras, TC21/R-Ras2, and M-Ras/R-Ras3." J Biol Chem **275**(26): 20020-6.
Okamoto, T., A. Schlegel, et al. (1998). "Caveolins, a family of scaffolding proteins for

organizing "preassembled signaling complexes" at the plasma membrane." J Biol Chem **273**(10): 5419-22.

Okkenhaug, K., A. Bilancio, et al. (2002). "Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice." Science **297**(5583): 1031-4.

Osada, M., T. Tolkacheva, et al. (1999). "Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival." Mol Cell Biol **19**(9): 6333-44.

Osman, N., S. C. Lucas, et al. (1995). "A comparison of the interaction of Shc and the tyrosine kinase ZAP-70 with the T cell antigen receptor zeta chain tyrosine-based activation motif." J Biol Chem **270**(23): 13981-6.

Pai, E. F., U. Krengel, et al. (1990). "Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis." Embo J **9**(8): 2351-9.

Pak, Y., N. Pham, et al. (2002). "Direct binding of the beta1 adrenergic receptor to the cyclic AMP-dependent guanine nucleotide exchange factor CNrasGEF leads to Ras activation." Mol Cell Biol **22**(22): 7942-52.

Pappu, R., A. M. Cheng, et al. (1999). "Requirement for B cell linker protein (BLNK) in B cell development." Science **286**(5446): 1949-54.

Parry, R., G. Smith, et al. (1997). "Activation of the PI3K effector protein kinase B following ligation of CD28 or Fas." Biochem Soc Trans **25**(4): S589.

Patel, G., M. J. MacDonald, et al. (1992). "Alternate mechanisms of ras activation are complementary and favor and formation of ras-GTP." Oncogene **7**(2): 283-8.

Pawson, T. (1995). "Protein-tyrosine kinases. Getting down to specifics." Nature **373**(6514): 477-8.

Pham, N., I. Cheglakov, et al. (2000). "The guanine nucleotide exchange factor CNrasGEF activates ras in response to cAMP and cGMP." Curr Biol **10**(9): 555-8.

Pizon, V., M. Desjardins, et al. (1994). "Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex." J Cell Sci **107 (Pt 6)**: 1661-70.

Pol, A., M. Calvo, et al. (1998). "Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment." FEBS Lett **441**(1): 34-8.

Polakis, P. and F. McCormick (1993). "Structural requirements for the interaction of p21ras with GAP, exchange factors, and its biological effector target." J Biol Chem **268**(13): 9157-60.

Prior, I. A., A. Harding, et al. (2001). "GTP-dependent segregation of H-ras from lipid rafts is required for biological activity." Nat Cell Biol **3**(4): 368-75.

Pritchard, C. A., L. Bolin, et al. (1996). "Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene." Curr Biol **6**(5): 614-7.

Prive, G. G., M. V. Milburn, et al. (1992). "X-ray crystal structures of transforming p21 ras mutants suggest a transition-state stabilization mechanism for GTP hydrolysis." Proc Natl Acad Sci U S A **89**(8): 3649-53.

Quilliam, L. A., A. F. Castro, et al. (1999). "M-Ras/R-Ras3, a transforming ras protein regulated by Sos1, GRF1, and p120 Ras GTPase-activating protein, interacts

with the putative Ras effector AF6." J Biol Chem **274**(34): 23850-7.
Quilliam, L. A., R. Khosravi-Far, et al. (1995). "Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins." Bioessays **17**(5): 395-404.
Quilliam, L. A., J. F. Rebhun, et al. (2002). "A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases." Prog Nucleic Acid Res Mol Biol **71**: 391-444.
Rambaratsingh, R. A., J. C. Stone, et al. (2003). "RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes." J Biol Chem **278**(52): 52792-801.
Ravichandran, K. S., U. Lorenz, et al. (1995). "Interaction of Shc with Grb2 regulates the Grb2 association with mSOS." Ann N Y Acad Sci **766**: 202-3.
Rebhun, J. F., A. F. Castro, et al. (2000a). "Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction." J Biol Chem **275**(45): 34901-8.
Rebhun, J. F., H. Chen, et al. (2000b). "Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral." J Biol Chem **275**(18): 13406-10.
Rebstein, P. J., G. Weeks, et al. (1993). "Altered morphology of vegetative amoebae induced by increased expression of the Dictyostelium discoideum ras-related gene rap1." Dev Genet **14**(5): 347-55.
Reif, K., S. Lucas, et al. (1997). "A negative role for phosphoinositide 3-kinase in T-cell antigen receptor function." Curr Biol **7**(5): 285-93.
Rensland, H., A. Lautwein, et al. (1991). "Is there a rate-limiting step before GTP cleavage by H-ras p21?" Biochemistry **30**(46): 11181-5.
Reuther, G. W. and C. J. Der (2000). "The Ras branch of small GTPases: Ras family members don't fall far from the tree." Curr Opin Cell Biol **12**(2): 157-65.
Reuther, G. W., Q. T. Lambert, et al. (2002). "RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia." J Biol Chem **277**(34): 30508-14.
Ribeiro-Neto, F., J. Urbani, et al. (2002). "On the mitogenic properties of Rap1b: cAMP-induced G(1)/S entry requires activated and phosphorylated Rap1b." Proc Natl Acad Sci U S A **99**(8): 5418-23.
Ridley, A. J., H. F. Paterson, et al. (1992). "The small GTP-binding protein rac regulates growth factor-induced membrane ruffling." Cell **70**(3): 401-10.
Roberts, D. M., A. L. Anderson, et al. (2004). "A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters." Mol Cell Biol **24**(24): 10515-28.
Rodenhuis, S. (1992). "ras and human tumors." Semin Cancer Biol **3**(4): 241-7.
Rodenhuis, S. and R. J. Slebos (1992). "Clinical significance of ras oncogene activation in human lung cancer." Cancer Res **52**(9 Suppl): 2665s-2669s.
Rogge, R. D., C. A. Karlovich, et al. (1991). "Genetic dissection of a neurodevelopmental pathway: Son of sevenless functions downstream of the sevenless and EGF receptor tyrosine kinases." Cell **64**(1): 39-48.
Rotkiewicz, T., S. Slodki, et al. (2004). "The role of ras genes in the neoplasmic transformation in animals." Pol J Vet Sci **7**(1): 47-52.
Roy, S., B. Wyse, et al. (2002). "H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis." Mol Cell Biol **22**(14): 5128-40.

Saez, R., A. M. Chan, et al. (1994). "Oncogenic activation of human R-ras by point mutations analogous to those of prototype H-ras oncogenes." Oncogene **9**(10): 2977-82.

Salhab, N., D. J. Jones, et al. (1989). "Detection of ras gene alterations and ras proteins in colorectal cancer." Dis Colon Rectum **32**(8): 659-64.

Sanjuan, M. A., D. R. Jones, et al. (2001). "Role of diacylglycerol kinase alpha in the attenuation of receptor signaling." J Cell Biol **153**(1): 207-20.

Sanjuan, M. A., B. Pradet-Balade, et al. (2003). "T cell activation in vivo targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation." J Immunol **170**(6): 2877-83.

Schaber, M. D., V. M. Garsky, et al. (1989). "Ras interaction with the GTPase-activating protein (GAP)." Proteins **6**(3): 306-15.

Scheele, J. S., J. M. Rhee, et al. (1995). "Determination of absolute amounts of GDP and GTP bound to Ras in mammalian cells: comparison of parental and Ras-overproducing NIH 3T3 fibroblasts." Proc Natl Acad Sci U S A **92**(4): 1097-100.

Scheffzek, K., M. R. Ahmadian, et al. (1997). "The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants." Science **277**(5324): 333-8.

Schlichting, I., S. C. Almo, et al. (1990). "Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis." Nature **345**(6273): 309-15.

Schmitt, J. M. and P. J. Stork (2001). "Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1." Mol Cell Biol **21**(11): 3671-83.

Scolnick, E. M., A. G. Papageorge, et al. (1979). "Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses." Proc Natl Acad Sci U S A **76**(10): 5355-9.

Sebzda, E., M. Bracke, et al. (2002). "Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling." Nat Immunol **3**(3): 251-8.

Self, A. J., E. Caron, et al. (2001). "Analysis of R-Ras signalling pathways." J Cell Sci **114**(Pt 7): 1357-66.

Self, A. J., H. F. Paterson, et al. (1993). "Different structural organization of Ras and Rho effector domains." Oncogene **8**(3): 655-61.

Shen, W. P., T. H. Aldrich, et al. (1987). "Expression of normal and mutant ras proteins in human acute leukemia." Oncogene **1**(2): 157-65.

Sherman, L. S., R. Atit, et al. (2000). "Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma Schwann cells but not fibroblasts." J Biol Chem **275**(39): 30740-5.

Shih, T. Y., A. G. Papageorge, et al. (1980). "Guanine nucleotide-binding and autophosphorylating activities associated with the p21src protein of Harvey murine sarcoma virus." Nature **287**(5784): 686-91.

Shimonaka, M., K. Katagiri, et al. (2003). "Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow." J Cell Biol **161**(2): 417-27.

Shou, C., C. L. Farnsworth, et al. (1992). "Molecular cloning of cDNAs encoding a

guanine-nucleotide-releasing factor for Ras p21." Nature **358**(6384): 351-4.
Sigal, I. S., J. B. Gibbs, et al. (1986a). "Identification of effector residues and a neutralizing epitope of Ha-ras-encoded p21." Proc Natl Acad Sci U S A **83**(13): 4725-9.
Sigal, I. S., J. B. Gibbs, et al. (1986b). "Mutant ras-encoded proteins with altered nucleotide binding exert dominant biological effects." Proc Natl Acad Sci U S A **83**(4): 952-6.
Simon, M. A., D. D. Bowtell, et al. (1991). "Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase." Cell **67**(4): 701-16.
Sini, P., A. Cannas, et al. (2004). "Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation." Nat Cell Biol **6**(3): 268-74.
Sommers, C. L., C. S. Park, et al. (2002). "A LAT mutation that inhibits T cell development yet induces lymphoproliferation." Science **296**(5575): 2040-3.
Sorkin, A., M. McClure, et al. (2000). "Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy." Curr Biol **10**(21): 1395-8.
Sozeri, O., K. Vollmer, et al. (1992). "Activation of the c-Raf protein kinase by protein kinase C phosphorylation." Oncogene **7**(11): 2259-62.
Spaargaren, M. and J. R. Bischoff (1994). "Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap." Proc Natl Acad Sci U S A **91**(26): 12609-13.
Spaargaren, M., G. A. Martin, et al. (1994). "The Ras-related protein R-ras interacts directly with Raf-1 in a GTP-dependent manner." Biochem J **300 (Pt 2)**: 303-7.
Stork, P. J. (2003). "Does Rap1 deserve a bad Rap?" Trends Biochem Sci **28**(5): 267-75.
Sturani, E., A. Abbondio, et al. (1997). "The Ras Guanine nucleotide Exchange Factor CDC25Mm is present at the synaptic junction." Exp Cell Res **235**(1): 117-23.
Suzuki, H., S. Matsuda, et al. (2003). "PI3K and Btk differentially regulate B cell antigen receptor-mediated signal transduction." Nat Immunol **4**(3): 280-6.
Suzuki, J., Y. Kaziro, et al. (1997). "An activated mutant of R-Ras inhibits cell death caused by cytokine deprivation in BaF3 cells in the presence of IGF-I." Oncogene **15**(14): 1689-97.
Suzuki, J., Y. Kaziro, et al. (2000). "Positive regulation of skeletal myogenesis by R-Ras." Oncogene **19**(9): 1138-46.
Swan, K. A., J. Alberola-Ila, et al. (1995). "Involvement of p21ras distinguishes positive and negative selection in thymocytes." Embo J **14**(2): 276-85.
Sweet, R. W., S. Yokoyama, et al. (1984). "The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity." Nature **311**(5983): 273-5.
Sweetenham, J. W. (1994). "Mutated genes in myeloid leukemias." Exp Hematol **22**(1): 5-6.
Symons, M. (1996). "Rho family GTPases: the cytoskeleton and beyond." Trends Biochem Sci **21**(5): 178-81.
Takai, Y., T. Sasaki, et al. (2001). "Small GTP-binding proteins." Physiol Rev **81**(1): 153-208.
Tedford, K., L. Nitschke, et al. (2001). "Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling." Nat Immunol **2**(6): 548-55.

Teixeira, C., S. L. Stang, et al. (2003). "Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3." Blood **102**(4): 1414-20.

Temeles, G. L., J. B. Gibbs, et al. (1985). "Yeast and mammalian ras proteins have conserved biochemical properties." Nature **313**(6004): 700-3.

The, I., G. E. Hannigan, et al. (1997). "Rescue of a Drosophila NF1 mutant phenotype by protein kinase A." Science **276**(5313): 791-4.

Tognon, C. E., H. E. Kirk, et al. (1998). "Regulation of RasGRP via a phorbol ester-responsive C1 domain." Mol Cell Biol **18**(12): 6995-7008.

Tohyama, Y., K. Katagiri, et al. (2003). "The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration." Mol Biol Cell **14**(6): 2570-82.

Toki, S., H. Kawasaki, et al. (2001). "Guanine nucleotide exchange factors CalDAG-GEFI and CalDAG-GEFII are colocalized in striatal projection neurons." J Comp Neurol **437**(4): 398-407.

Tong, L. A., A. M. de Vos, et al. (1991). "Crystal structures at 2.2 A resolution of the catalytic domains of normal ras protein and an oncogenic mutant complexed with GDP." J Mol Biol **217**(3): 503-16.

Topham, M. K. and S. M. Prescott (1999). "Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions." J Biol Chem **274**(17): 11447-50.

Topham, M. K. and S. M. Prescott (2001). "Diacylglycerol kinase zeta regulates Ras activation by a novel mechanism." J Cell Biol **152**(6): 1135-43.

Trahey, M. and F. McCormick (1987). "A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants." Science **238**(4826): 542-5.

Troppmair, J. and U. R. Rapp (2003). "Raf and the road to cell survival: a tale of bad spells, ring bearers and detours." Biochem Pharmacol **66**(8): 1341-5.

Tucker, J., G. Sczakiel, et al. (1986). "Expression of p21 proteins in Escherichia coli and stereochemistry of the nucleotide-binding site." Embo J **5**(6): 1351-8.

Turner, H. and D. A. Cantrell (1997). "Distinct Ras effector pathways are involved in Fc epsilon R1 regulation of the transcriptional activity of Elk-1 and NFAT in mast cells." J Exp Med **185**(1): 43-53.

Ullrich, A. and J. Schlessinger (1990). "Signal transduction by receptors with tyrosine kinase activity." Cell **61**(2): 203-12.

Umanoff, H., W. Edelmann, et al. (1995). "The murine N-ras gene is not essential for growth and development." Proc Natl Acad Sci U S A **92**(5): 1709-13.

Van Aelst, L., M. Barr, et al. (1993). "Complex formation between RAS and RAF and other protein kinases." Proc Natl Acad Sci U S A **90**(13): 6213-7.

van der Geer, P., M. Henkemeyer, et al. (1997). "Aberrant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap." Mol Cell Biol **17**(4): 1840-7.

Vandenabeele, P., V. Goossens, et al. (1994). "Functional requirement of the two TNF receptors for induction of apoptosis in PC60 cells and the role of mitochondria in TNF-induced cytotoxicity." Circ Shock **44**(4): 196-200.

Vazquez, L. E., H. J. Chen, et al. (2004). "SynGAP regulates spine formation." J Neurosci **24**(40): 8862-72.

Vogel, U. S., R. A. Dixon, et al. (1988). "Cloning of bovine GAP and its interaction with oncogenic ras p21." Nature **335**(6185): 90-3.

Vojtek, A. B. and C. J. Der (1998). "Increasing complexity of the Ras signaling pathway." J Biol Chem **273**(32): 19925-8.

Vojtek, A. B., S. M. Hollenberg, et al. (1993). "Mammalian Ras interacts directly with the serine/threonine kinase Raf." Cell **74**(1): 205-14.

Wallace, M. R., D. A. Marchuk, et al. (1990). "Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients." Science **249**(4965): 181-6.

Wang, B., J. X. Zou, et al. (2000a). "R-Ras contains a proline-rich site that binds to SH3 domains and is required for integrin activation by R-Ras." J Biol Chem **275**(7): 5222-7.

Wang, D., J. Feng, et al. (2000b). "Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors." Immunity **13**(1): 25-35.

Wang, H. G., U. R. Rapp, et al. (1996a). "Bcl-2 targets the protein kinase Raf-1 to mitochondria." Cell **87**(4): 629-38.

Wang, H. G., S. Takayama, et al. (1996b). "Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1." Proc Natl Acad Sci U S A **93**(14): 7063-8.

Wang, Y., S. Pennock, et al. (2002). "Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival." Mol Cell Biol **22**(20): 7279-90.

Wange, R. L. (2000). "LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways." Sci STKE **2000**(63): RE1.

Wardenburg, J. B., C. Fu, et al. (1996). "Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function." J Biol Chem **271**(33): 19641-4.

Warne, P. H., P. R. Viciana, et al. (1993). "Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro." Nature **364**(6435): 352-5.

Waters, S. B., D. Chen, et al. (1996). "Insulin and epidermal growth factor receptors regulate distinct pools of Grb2-SOS in the control of Ras activation." J Biol Chem **271**(30): 18224-30.

Weissbach, L., J. Settleman, et al. (1994). "Identification of a human rasGAP-related protein containing calmodulin-binding motifs." J Biol Chem **269**(32): 20517-21.

Wellbrock, C., M. Karasarides, et al. (2004). "The RAF proteins take centre stage." Nat Rev Mol Cell Biol **5**(11): 875-85.

Willumsen, B. M., A. Christensen, et al. (1984a). "The p21 ras C-terminus is required for transformation and membrane association." Nature **310**(5978): 583-6.

Willumsen, B. M., R. W. Ellis, et al. (1984b). "Further genetic localization of the transforming sequences of the p21 v-ras gene of Harvey murine sarcoma virus." J Virol **49**(2): 601-3.

Willumsen, B. M., K. Norris, et al. (1984c). "Harvey murine sarcoma virus p21 ras protein: biological and biochemical significance of the cysteine nearest the carboxy terminus." Embo J **3**(11): 2581-5.

Wittinghofer, A. and N. Nassar (1996). "How Ras-related proteins talk to their effectors." Trends Biochem Sci **21**(12): 488-91.

Wittinghofer, A. and E. F. Pai (1991). "The structure of Ras protein: a model for a universal molecular switch." Trends Biochem Sci **16**(10): 382-7.

Wojnowski, L., A. M. Zimmer, et al. (1997). "Endothelial apoptosis in Braf-deficient

mice." Nat Genet **16**(3): 293-7.
Wolthuis, R. M., B. Bauer, et al. (1996). "RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein." Oncogene **13**(2): 353-62.
Wolthuis, R. M. and J. L. Bos (1999). "Ras caught in another affair: the exchange factors for Ral." Curr Opin Genet Dev **9**(1): 112-7.
Wolthuis, R. M., N. D. de Ruiter, et al. (1997). "Stimulation of gene induction and cell growth by the Ras effector Rlf." Embo J **16**(22): 6748-61.
Wolthuis, R. M., B. Franke, et al. (1998a). "Activation of the small GTPase Ral in platelets." Mol Cell Biol **18**(5): 2486-91.
Wolthuis, R. M., F. Zwartkruis, et al. (1998b). "Ras-dependent activation of the small GTPase Ral." Curr Biol **8**(8): 471-4.
Wood, K. W., C. Sarnecki, et al. (1992). "ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK." Cell **68**(6): 1041-50.
Wouters, F. S. and P. I. Bastiaens (1999). "Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells." Curr Biol **9**(19): 1127-30.
Wunderlich, L., A. Goher, et al. (1999). "Requirement of multiple SH3 domains of Nck for ligand binding." Cell Signal **11**(4): 253-62.
Wymann, M. P. and L. Pirola (1998). "Structure and function of phosphoinositide 3-kinases." Biochim Biophys Acta **1436**(1-2): 127-50.
Yablonski, D., M. R. Kuhne, et al. (1998). "Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell." Science **281**(5375): 413-6.
Yamagata, K., L. K. Sanders, et al. (1994). "rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein." J Biol Chem **269**(23): 16333-9.
Yamaguchi, A., T. Urano, et al. (1997). "An Eps homology (EH) domain protein that binds to the Ral-GTPase target, RalBP1." J Biol Chem **272**(50): 31230-4.
Yamamoto, T., T. Matsui, et al. (1995). "A novel GTPase-activating protein for R-Ras." J Biol Chem **270**(51): 30557-61.
Yamanashi, Y. and D. Baltimore (1997). "Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok." Cell **88**(2): 205-11.
Yamashita, S., N. Mochizuki, et al. (2000). "CalDAG-GEFIII activation of Ras, R-ras, and Rap1." J Biol Chem **275**(33): 25488-93.
Yang, H., D. Cooley, et al. (2003). "Phosphorylation of the Ras-GRF1 exchange factor at Ser916/898 reveals activation of Ras signaling in the cerebral cortex." J Biol Chem **278**(15): 13278-85.
Yang, Y., L. Li, et al. (2002). "RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function." J Biol Chem **277**(28): 25756-74.
Yu, Y. and L. A. Feig (2002). "Involvement of R-Ras and Ral GTPases in estrogen-independent proliferation of breast cancer cells." Oncogene **21**(49): 7557-68.

Zhang, K., A. G. Papageorge, et al. (1992). "Mechanistic aspects of signaling through Ras in NIH 3T3 cells." Science **257**(5070): 671-4.

Zhang, X. F., J. Settleman, et al. (1993). "Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1." Nature **364**(6435): 308-13.

Zhang, Y. Y., T. A. Vik, et al. (1998). "Nf1 regulates hematopoietic progenitor cell growth and ras signaling in response to multiple cytokines." J Exp Med **187**(11): 1893-902.

Zhang, Z., K. Vuori, et al. (1996). "Integrin activation by R-ras." Cell **85**(1): 61-9.

Zhao, H., Y. Y. Li, et al. (1997). "T cell receptor-induced phosphorylation of Sos requires activity of CD45, Lck, and protein kinase C, but not ERK." J Biol Chem **272**(34): 21625-34.

Zhong, X. P., E. A. Hainey, et al. (2003). "Enhanced T cell responses due to diacylglycerol kinase zeta deficiency." Nat Immunol **4**(9): 882-90.

Zhong, X. P., E. A. Hainey, et al. (2002). "Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta." J Biol Chem **277**(34): 31089-98.

Zhong, Z., Z. Wen, et al. (1994a). "Stat3 and Stat4: members of the family of signal transducers and activators of transcription." Proc Natl Acad Sci U S A **91**(11): 4806-10.

Zhong, Z., Z. Wen, et al. (1994b). "Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6." Science **264**(5155): 95-8.

Zippel, R., N. Gnesutta, et al. (1997). "Ras-GRF, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system." Brain Res Mol Brain Res **48**(1): 140-4.

Zwartkruis, F. J. and J. L. Bos (1999). "Ras and Rap1: two highly related small GTPases with distinct function." Exp Cell Res **253**(1): 157-65.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

2.1 Reagents

The PKC inhibitors Go6976 and Ro318220 were purchased from Calbiochem (San Diego, CA). Okadaic acid (OA) was either purchased from Calbiochem or was a gift from Dr. Charles Holmes (University of Alberta). Phorbol 12-myristate 13-acetate (PMA) was purchased from SigmaAldrich (St Louis, MO).

Concanavalin A was purchased from Pharmacia (Kirkland, QC) and lipopolysaccaride (LPS) from Sigma-Aldrich, (St. Louis, MO).

2.2 Antibodies

For immunoblotting, we used the pan-Ras antibody (clone RAS10) from Upstate Biotechnology (Lake Placid, NY). Anti-PKC-θ (#P15120), anti-PKC-β (#P17720), and anti-PKC-δ (#NP-997704) were purchased from Transduction Laboratories (Mississauga, ON, Canada). The phospho p44/p42 ERK antibody (#9101) was from Cell Signaling Technology (Beverly, MA). The Rap1 (121) and ERK1 antibodies (#K-23) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Stimulatory goat anti-human immunoglobulin M (IgM) $F(ab')_2$ fragment was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-mouse IgM (μ-chain specific) was purchased from Southern Biotechnology Associates (Birmingham, AL). Mouse- and human-reactive anti-CD3 antibodies 145-2C11 and OKT3 were purified from hybridomas.

RASGRP-specific Antibodies Polyclonal anti-RasGRP1 (H176) and anti-RasGRP3 (L247-9) antibodies have been described (Ebinu *et al.* 2000; Teixeira

et al. 2003), as has m199, a rodent-specific anti-RasGRP1 monoclonal antibody (Puente *et al.* 2000). The m133 monoclonal antibody was derived from mice that had been immunized with rat RasGRP1 and it reacts with human as well as rodent protein. However, the epitope maps to the highly conserved EF-hand region and the antibody also immune-precipitates RasGRP3. Despite this cross-reaction, note that RasGRP1 (90kDa) and RasGRP3 (78 kDa) can be readily distinguished by their electrophoretic mobility. The m404 anti-RasGRP3 monoclonal antibody was derived from mice immunized with bacterially expressed full-length human RasGRP3. RasGRP1 and RasGRP were immune-precipitated with one or more monoclonal antibodies before immunoblotting with polyclonal antibodies, except in the experiment described in Fig 2B, where RasGRP3 was immune-precipitated with polyclonal phospho-specific antibodies.

To generate antiserum that selectively recognizes Thr133 phosphorylated RasGRP3, rabbits were immunized with an 11-residue phosphopeptide (WMRRVpTQRKKV) coupled to KLH (Alberta Peptide Institute, Edmonton, AB). The resulting serum, CV4, was diluted and used directly without purification. Similarly, an 11-residue phosphopeptide (WSRKLpTQRIKS) was used to generate 3B7, an antiserum that recognizes RasGRP1 phosphorylated on Thr184. In this case, it was necessary to affinity purify specific antibodies using the phosphopeptide coupled to BSA. For clarity in the text and figures, we have termed these antibodies "anti-3/pThr133" (reacts with RasGRP3 phosphorylated on Thr133) and "anti-1/pThr184" (reacts with RasGRP1 phosphorylated on Thr184).

2.3 Plasmid constructs and recombinant proteins

2.3.1 RasGRPs and mutants

RasGRP1, T184A. Full length mouse wildtype RasGRP1 cDNA and its mutant containing a threonine 184 to alanine (Thr184Ala) were cloned as BamHI/SalI fragments into pBabePuro expression vector encoding for puromycin

resistance (Morgenstern and Land 1990). Subsequently, the DNA was introduced into *E. Coli.* and ampicillin-resistant transformants were isolated from plates containing LB and ampicillin. Plasmid DNA used for virus packaging and fibroblast cell infection was thereafter prepared using QIAGEN Plasmid Maxi Kit (QIAGEN, Mississauga, ON).

RasGRP1-mEF1 and RasGRP1-mEF2. Full length mouse RasGRP1 cDNA containing a glutamic acid 494 to alanine (Glu494Ala, EF1) or a glutamic acid 521 to alanine (Glu521Ala, EF2) were generated using PCR-mediated point mutagenesis and cloned as BamHI/SalI fragments into pBabePuro expression vector encoding for puromycin resistance.

GST-EF1$^-$, GST-EF2$^-$, GST-EF1$^-$EF2$^-$. BamHI/SalI fragments of rbc7HA (referred to as EF1$^-$, EF2$^-$ and EF1$^-$EF2$^-$) containing mutations in the EF-hand alleles were contrasted by another investigator in the lab. 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. BamHI/SalI fragments were prepared from cDNA containing these mutations and inserted into pGEX 4T3 (Pharmacia) bacterial expression vector.

MBP-RasGRP1-3PA. Full length mouse wildtype RasGRP1 cDNA containing three point mutations (prolines at position 442, 445, and 448 were replaced by alanines) were cloned as BamHI/SalI fragments into pMAL-c2 expression vector for expression of maltose binding protein (MBP) fusion protein, MBP-RasGRP1-3PA. The construct was transformed into *E. Coli.* strain BL21DE3 (Stratagene) and expressed by induction of T7 polymerase at mid-logarithmic growth by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). MBP fusion proteins were purified from cell lysate by chromatography using amylose resin (New

England Biolabs). The protein was then dialyzed into buffer A (20mM Tris 7.5, 100mM NaCl, 1.0mM $MgCl_2$, 1.0mM DTT, 10% glycerol) and stored at -80°C until use.

RasGRP3, T133A, T133E. Full-length human RasGRP3 cDNA (GenBank accession no. KIAA0846) was also cloned as BamHI/SalI fragment into the pBabePuro vector. RasGRP3 cDNAs containing a threonine133 to alanine (T133A) point mutation or a threonine133 to glutamic acid (T133E) were generated using a PCR method, sequence verified and incorporated into pBabePuro.

RasGRP4. Full-length RasGRP4 cDNA (GenBank accession no. AY048119) was cloned as a EcoRI/SalI fragment into the retrovirus vector pBabeHygro (Morgenstern and Land 1990).

MBP-RasGRP3, MBP-T133A, MBP -T133S; MBP-RasGRP1, MBP -T184A. The BamHI/SalI fragments of full length human RasGRP3 cDNA and its two point mutants, T133A (threonine133 to alanine) and T133S (threonine133 to serine) were cloned into the pMAL-c2 vector (Teixeira *et al.* 2003) for expression of MBP fusion proteins by IPTG induction in BL21DE3. The full length mouse RasGRP1 cDNA and the T184A (threonine184 to alanine) point mutation were similarly cloned into pMAL-c2 vector.

GFP-RasGRP3, GFP-T133A. The N-terminal GFP-tagged version of RasGRP3 and the T133A mutant were constructed by inserting the BamHI/SalI digested inserts into pEGFP-bos vector. The pEGFP-bos vector introduces the Bos promoter from pEF-BOS into pEGFP-C1 (Clonetech) to enhance GFP expression.

2.3.2 Baculovirus constructs

Baculoviruses expressing RasGRP3, PKC-α, PKC-θ, PKC-β_2, or PKC-δ were constructed by other investigators in the lab using the Bac-to-Bac system, and proteins were expressed in *Sf9* cells, according to the manufacturer's instructions (Gibco BRL, Grand Island, NY).

2.3.3 GST-Grb2 and its mutants; a GST-Grap, and GST-Gads

The constructs encoding for GST-Grb2 full-length (Grb2), GST-Grb2 full-length with point mutations (E39K, P49L, and S90N), isolated domains of Grb2 (GST-Grb2 N-SH3, GST-Grb2-C-SH3, and GST-Grb2-SH2) were generously provided by Dr. Zhixiang Wang (Department of Cell Biology, University of Alberta). These plasmids were constructed by inserting corresponding cDNA fragments into pGEX-4T3 vector (Amersham Biosciences) and sequenced.

DNA corresponding to the full-length Grap (Genebank accession number: AAH35856) and Gads (Genebank accession number: O75791) were amplified by polymerase chain reaction (PCR) using Jurkat genomic DNA as the templates. The PCR products were subsequently BamHI/Sal I cloned into pGEX4T3 vectors and were transformed into E. Coli. for GST fusion protein expression and purification.

2.4 Cell culture and virology

2.4.1 Cell culture

Jurkat human T cell and Ramos human B cell lines were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, $5x10^{-5}$ M β-mercaptoethanol and antibiotics at 37°C in a 10% CO_2 atmosphere, as described previously (Ebinu *et al.* 2000; Teixeira *et al.* 2003). Rat2 fibroblast were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose with 10% fetal bovine serum, and antibiotics at 37°C in a 10% CO_2 atmosphere. To generate primary B cell culture, spleens from B6 x 129/J mixed background mice were

homogenized in RPMI medium using sterilized frosted slides. After two washes with fresh media, splenocytes were counted and allowed to proliferate in media containing 20 μg/ml LPS for 72 hours to generate B cell cultures. Likewise, to generate primary T cell cultures, splenocytes were incubated in media containing 5 μg/ml conconavalin A plus 20 ng/ml IL-2 for 48 hours, then maintained in media containing 20 ng/ml IL-2 for 4 days.

2.4.2 Gene transfer

To establish stable Rat2 lines expressing RasGRPs and mutants, purified pBabePuro constructs were converted to retrovirus forms with a high-efficiency packaging system as previously described (Pear *et al.* 1993). Briefly, the retroviral packaging cell line, Bosc23 cells were seeded at $1x10^6$ cells per 60-mm cell culture dish the day before transfection. The plasmid constructs were transfected into the cells using a calcium-phosphate precipitation method to generate helper-free retrovirus stocks. The resulting retroviruses were collected 48 hours post-transfection and filtered through 0.45 um pore-size filter (Millipore) to remove cells. Polybrene was added to a concentration of 8 μg/ml to facilitate virus attachment. Rat2 fibroblasts were seeded the day before infection at 2×10^5 cells into 25 cm^2 culture flask. To infect Rat2 cells, 1ml of virus stock was added into the medium of cultured cells and incubated for 48 hours at 37°C. Cells were then subcultured in selective medium until colonies could be counted. Colonies were pooled and maintained in selective medium.

For transient transfection of HEK293 cells with GFP constructs, 1×10^5 cells were plated per 60-mm dish into dishes containing glass cover slips for over night to allow attachment. Cells were then transfected with plasmid DNA by calcium-phosphate–mediated precipitation as previously described (Chen and Okayama 1987). Briefly, 2-5 μg GFP plasmid constructs or empty vector was mixed with 250 μl of 0.25 M $CaCl_2$ before the dropwise addition of 250 μl of 2 x BES buffer (50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, pH 6.96). After 10

to 20 minutes, the DNA-calcium phosphate complex was added to 60-mm dishes. Cultures were incubated at 37°C overnight in 5% CO_2. After 14 hours, cells were rinsed twice with medium before the addition of fresh medium.

Sf9 cells were maintained in SF900II medium (Gibco-BRL) at 27°C. To infect cells with baculovirus, *sf9* cells were seeded at approximately $1x10^7$ cells per T75 cell culture flask the day before infection. The next day, 2 ml of virus stock were added into culture medium. Cells were treated 48 hours post-infection with PMA, to stimulate PKCs, or okadaic acid, to induce RasGRP3 phosphorylation, then harvested.

2.5 Sequence alignments

Sequence alignments were performed with Macvector 7.1 (Accelrys Inc., San Diego, CA).

2.6 Mass spectrometry analysis

Two methods were used to prepare phosphorylated RasGRP3 for mass spectrometry analysis:

1) RasGRP3 purified from *E.coli.* was phosphorylated with PKC using an immune-complex kinase assay (see "Immune-complex kinase assay" for details) and resolved by 7.5% SDS-PAGE.

2) *In vivo* phosphorylated RasGRP3 was purified by immune-precipitation from OA-treated *sf9* cells infected with a RasGRP3-expressing baculovirus. To obtain enough material for mass spectrometry analysis, 30 x T75 cell culture flasks were infected with baculovirus for two days at 27°C. Cells were resuspended in 10 ml original medium and stimulated with 1 μM OA for 45 minutes, then OA + 100 nM PMA for 15 minutes, to cause the hyper-phosphorylation of intracellular proteins

including RasGRP3. Untreated cells were used for the negative control. Mock-infected *sf9* cells were used for a control for immune-precipitation. Post-nuclear supernatant were then prepared using lysis buffer B (20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 40 mM beta-glycerolphosphate, 1 mM sodium orthovanadate, plus protein inhibitors). To immune-precipitate RasGRP3, 100 μg RasGRP3 polyclonal antibodies (L247-9) were incubated with cell lysates overnight on ice. Protein was then collected with protein A sepharose (PAS), washed 4 times lysis buffer B, and subject to 7.5% SDS-PAGE.

The mass spectrometry analysis was described elsewhere (Liu *et al.* 2004). Briefly, the SDS-PAGE gel was rinsed with MilliQ water once and stained with Biosafe (Biorad, Hecules, CA) for about 1 hour, followed by destaining with MilliQ water. The protein band was then excised using a clean razor blade, reduced and digested with trypsin. After in-gel digestion, eluted phosphorylated peptides were enriched by open tubular immobilized metal ion affinity chromatography (IMAC). Peptides eluted from IMAC were subjected to MALDI MS and MS/MS analysis on an Applied Biosystems MDS-Sciex QSTAR Pulsar QqTOF instrument equipped with an orthogonal MALDI source employing a 337 nm nitrogen laser (Mississauga, ON Canada). ProteinProspector Tools MS-Digest and MS-Product (http://prospector.ucsf.edu/) were used for peptide mapping and calculation of the theoretical protonated mass values of peptides (phosphorylated and non-phosphorylated).

2.7 Immune-complex kinase assays

As a source of active PKC, *Sf9* cells were infected with PKCθ-, PKCβ_2-, or PKCδ-expressing baculovirus or they were mock infected for 48 hours. Before harvesting, cells were treated with 100 nM PMA for 15 minutes. Cells were lysed in lysis buffer B, and nuclear-free supernatants were incubated over night on ice with or without 4 μg anti-PKCθ, anti-PKCβ, or anti-PKCδ antibody. After 1 hour

collection on PAS beads (25 µl packed-bead volume), immune complexes were washed 6 times with lysis buffer B and once with T/M buffer (20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$). To each reaction (25 µl packed-bead volume) was added 25 µl reaction mixture containing equal volumes (6.0 µl) of lipid (0.2 mg/ml diolein, 1 mg/ml phosphatidylserine; Avanti Polar Lipids, Alabaster, AL), 30 mM dithiothreitol (DTT), 12 mM $Ca(CH_3COO)_2$, and 6 µg/µl protein substrate. Lipids were prepared by mixing equal volumes of diolein and phosphatidylserine (dissolved in chloroform), dried under N_2, and resuspended in 50 mM Tris-HCl, pH 7.5. To each reaction, 1 µl [γ^{32}P]ATP (3000 Ci/mmol [11 100 x 1010 Bq/mmol]; Amersham Biosciences, Baie d'Urfa, QC, Canada) was added before incubation for 15 minutes at 30°C. Reactions were stopped by the addition of an equal volume of 2 x SDS sample buffer. Proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

Note: some PKC isoforms, such as PKCβ, seem "sticky" and cause non-specific binding to PAS beads during immune-precipitation. To avoid this, PAS beads were incubated with anti-PKCβ antibody for 2 hours followed by 1-hour incubation with 100 µg BSA to minimize non-specific binding. The beads were washed once and mixed with nuclear-free supernatant over-night. A stringent washing condition was also used to prevent non-specific binding. Specifically, the beads were sequentially washed 3 times with lysis buffer B, once with lysis buffer B + 500 mM NaCl, once with lysis buffer B + 0.1% SDS, twice with lysis buffer B, and once with T/M buffer. The immune-complex kinase assay was then carried out as described above.

To prepare MBP-RasGRP3 for mass spectrometry, the assay used unlabeled ATP and reactions were scaled up. Usually, for each reaction, 30 µg PKC antibody were used to immune-precipitate PKC from lysates prepared from 5 x T75 flasks of PKC-expressing baculovirus infected *sf9* cells. PKC was then collected with 150 µl bead volume of PAS bead and washed 6 times with lysis buffer B and once with T/M buffer. The beads were evenly divided into 6 eppendorf tubes and aspirated to remove excess buffer. In each tube, the beads were mixed with a reaction mixture containing 6.0 µl lipid, 6.0 µl 30 mM DTT, 6.0 µl 12 mM $Ca(CH3COO)_2$, 5.0 µl cold

ATP (ATP.Magnesium, Amersham Biosciences), and 6-10 μg/μl protein substrate. The reactions were incubated for 30 minutes at 30°C, stopped by adding equal amount of 2 x SDS sample buffer. After boiling for 5 minutes, the samples were subjected to 7.5% SDS-PAGE.

2.8 Detection of phosphorylated RasGRP1 and RasGRP3 by immune-precipitation and immunoblotting

After stimulation, T and B cells were lysed in buffer B and nuclei-free protein lysates were prepared. To prevent dephosphorylation of RasGRP1 and RasGRP3 during immune-precipitation, we added 1μM okadaic acid to the lysis buffer B. To precipitate RasGRP1 or RasGRP3, 90% protein lysates were incubated overnight on ice with 50 μg corresponding monoclonal antibodies (m133 for RasGRP1 and m404 for RasGRP3; sometimes m133 is also used for RasGRP3) followed by crosslinking to protein G-sepharose (PGS, Amersham Biosciences) on ice for 2 hours. After three washes with lysis buffer B, 80% of bead-associated RasGRP1 or RasGRP3 was separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed over night at 4°C with anti-1/pThr184 (3B7-78 at 1:100 dilution) or anti-3/pThr133 (CV4 serum at 1:200 dilution), for detection of phospho-RasGRP1 and phospho-RasGRP3, respectively. The remaining 20% of each bead-associated RasGRP1 or RasGRP3 were resolved by a parallel SDS-PAGE and detected with corresponding non-phospho-antibodies (A176 for RasGRP1 and L247-9 for RasGRP3). The remaining protein lysates were used for signaling assays and Ras activation assay.

In some cases, the immune-precipitations were performed reciprocally, i. g., phosphorylated RasGRP3 was immune-precipitated by anti-3/Thr133 (CV4) antibody and probed with L247-9 antibody.

To detect phosphorylated RasGRP1, $2x10^7$ Jurkat or primary mouse T cells were used per assay. For phosphorylated RasGRP3, $5x10^7$ Ramos or primary mouse B cells were used per assay.

2.9 Ras and Rap activation assay, signaling assays, and inhibitor studies

Ras activation was assayed by comparing the amount of Ras-GTP and the amount of total Ras in cell lysates, as described previously (Ebinu *et al.* 2000). Briefly, 90% of the nuclei-free protein lysates prepared from stimulated cells was incubated with GST-RBD (Ras-binding domain) fusion protein bound to glutathione beads to collect GTP-bound Ras. Bead-associated Ras was detected in an immunoblotting protocol following SDS-PAGE separation. The remaining 10% of each lysate was probed with the anti-Ras antibody to verify that similar amounts of total Ras were present in each lysate and with phospho-Erk antibody to assess the level of Erk activation. Rap1 activation was similarly determined by RalGDS pull down assay as previously described (Franke *et al.* 1997). Some immunoblot bands were quantified using NIH ImageJ 1.32j program (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2004).

For cell stimulation, PMA was used at 100 nM. Soluble anti-human IgM and anti-mouse IgM were used to stimulate Ramos and mouse B cells, respectively. Likewise, anti-human CD3 (OKT3), and anti-mouse CD3 (clone 145-2C11) were used to stimulate Jurkat and mouse T cells, respectively. CD3 is a component of TCR. Stimulatory antibodies were used at 10 μg/ml. Pan-PKC inhibitor Ro318220 was used at 10 μM and conventional PKC inhibitor Go6976 was used at 20 μM. Cells were pre-treated with inhibitors for 15 minutes before stimulation.

2.10 Proliferation assays

Rat2 cells expressing vector, wild type RasGRP3, or mutant RasGRP3 (Thr133Ala) were seeded at 2 x 10^4 cells per well in 24-well tissue culture plates in medium containing 10 % FBS followed by incubation at 37°C. Cells were then harvested later the same day and counted with a Coulter counter or grown for three days in media containing 0.5% FBS plus 100 nM PMA or solvent, then counted.

2.11 Intracellular imaging of RasGRP3

24 hours after transfection, HEK-293 cells transiently transfected with empty vector, GFP-RasGRP3, or GFP-T133A were treated at 37°C for 10 minutes with 100 nM PMA or solvent. After fixation in 4% paraformaldehyde, cells were mounted in Cytoseal-60 from Richard-Allen Scientific (Kalamazoo, MI). Fluorescent images were taken with a Zeiss 510 inverted laser scanning confocal microscope (LSM; Carl Zeiss, Oberkochen, Germany). A minimum of five 0.45 μM slices were acquired for each cell. Tiff images were processed with Adobe Photoshop 7.0.

2.12 *In vitro* Ca^{45} binding assay

Ca^{45} binding of RasGRP1 EF mutants was determined as previously described (Maruyama *et al.* 1984) with some minor modifications. Purified MBP-RasGRP1 and its EF-hand mutants were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Biorad) in Towbin transfer buffer (39 mM glycine pH8.3, 48 mM Tris base, 20% methanol). To wash away transfer buffer, the membrane was washed 3 times (20 minutes/wash) in a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl pH 6.8. Thereafter, the membrane was probed with the same buffer containing 1 mCi/L Ca^{45} for 10 minutes. The membrane was then rinsed with distilled water for 2 minutes and dried between two sheets of

Whatman No. 1 paper to remove excess water. After drying the membrane at room temperature for 2 hours, radioautographs were obtained by exposure to Kodak Scientific X-ray film at -80°C for 12-48 hours with an intensifying screen. To show the equal loading of proteins, the membrane was stained with Ponceau Red S and scanned.

2.13 *In vitro* GST pull-down assay

2.13.1 Purification of GST-fusion proteins from E. coli

To purify GST fusion proteins, the following protocol was followed: GST constructs and empty vector were transformed into competent *E. coli.* (BL21DE3) and one transformant from each transformation reaction were picked to inoculate 2 ml over night LB cultures. On day two, 25 ml of LB were inoculated with 250 µl over night culture and grown at 37°C until the OD600 reached 0.4-0.6. Following addition of IPTG (final concentration: 0.5 µM), the cultures were further grown at 37°C for 2 hours. The cells were harvested by centrifugation and the supernatants were removed. The cell pellets were resuspended in 5 ml GST-lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100, 10% glycerol, 100 mM sodium fluoride, 1 mM Sodium pyrophosphate, 1 mM sodium vanadate) to which was added 2 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM Pefabloc (Zhou *et al.* 2004). Cells were lysed by incubating on ice with 10 µg/ml lysozyme followed by sonication 6 x 20 seconds. After centrifugation at 10,000g x 30 minutes, the supernatants were transferred into new eppendorf tubes and stored as 1 ml aliquots at -80°C. To prepare beads bound with GST fusion protein for each pull down reaction, 50 µl glutathione-Sepharose bead slurry (Amersham Biosciences) was washed three times with GST lysis buffer, mixed with 500 µl of cell lysate on a rotating wheel at 4°C for 30 minutes, and washed again with GST lysis buffer for three times. To determine the yield of purification, 10% of the beads bound with

GST fusion proteins were resolved on a 15% SDS-PAGE gel using the BioRad mini-gel system and stained with Coomassie blue.

Note: since the yield of soluble GST-Gads is very low (less than 10%), large-scale LB culture is required to give enough material to work with. For each pull-down reaction, I induced 1 liter of LB culture with 0.1 μM IPTG at room temperature overnight.

2.13.2 GST pull-down assay

For GST pull-down assay, beads bound with an equal amount of GST fusion proteins or GST alone were mixed either with 1ug purified MBP, as negative control, or MBP fusion proteins or with cell lysates prepared as indicated (1mg total cellular protein) on a rotating wheel in cold room for 2 hours. Samples were then washed 6 times with GST binding buffer and boiled in SDS sample buffer for 5 minutes. The eluted proteins were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with corresponding antibodies.

References

Chen, C. and H. Okayama (1987). "High-efficiency transformation of mammalian cells by plasmid DNA." Mol Cell Biol **7**(8): 2745-52.

Ebinu, J. O., S. L. Stang, et al. (2000). "RasGRP links T-cell receptor signaling to Ras." Blood **95**(10): 3199-203.

Franke, B., J. W. Akkerman, et al. (1997). "Rapid Ca2+-mediated activation of Rap1 in human platelets." Embo J **16**(2): 252-9.

Liu, H., J. Stupak, et al. (2004). "Open tubular immobilized metal ion affinity chromatography combined with MALDI MS and MS/MS for identification of protein phosphorylation sites." Anal Chem **76**(14): 4223-32.

Maruyama, K., T. Mikawa, et al. (1984). "Detection of calcium binding proteins by 45Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis." J Biochem (Tokyo) **95**(2): 511-9.

Morgenstern, J. P. and H. Land (1990). "Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line." Nucleic Acids Res **18**(12): 3587-96.

Pear, W. S., G. P. Nolan, et al. (1993). "Production of high-titer helper-free retroviruses by transient transfection." Proc Natl Acad Sci U S A **90**(18): 8392-6.

Puente, L. G., J. C. Stone, et al. (2000). "Evidence for protein kinase C-dependent and -independent activation of mitogen-activated protein kinase in T cells: potential role of additional diacylglycerol binding proteins." J Immunol **165**(12): 6865-71.

Teixeira, C., S. L. Stang, et al. (2003). "Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3." Blood **102**(4): 1414-20.

Zhou, D., C. Noviello, et al. (2004). "Growth factor receptor-bound protein 2 interaction with the tyrosine-phosphorylated tail of amyloid beta precursor protein is mediated by its Src homology 2 domain." J Biol Chem **279**(24): 25374-80.

CHAPTER 3

PHOSPHORYLATION OF RASGRP3 ON THREONINE 133 PROVIDES A MECHANISTIC LINK BETWEEN PKC AND RAS SIGNALING SYSTEMS IN B CELLS

A version of this chapter has been published: Zheng Y, Liu H, Coughlin J, Zheng J, Li L, Stone JC. **(2005)** Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. *Blood*, **105**(9):p.3648-3654.

Chapter 3

3.1 Introduction

Diacylglycerol (DAG) acts as a secondary messenger molecule located in the inner leaflet of the plasma membrane, linking ligand receptor events to internal biochemical signaling events and biologic processes (Kazanietz *et al.* 2000). The cellular provider of DAG is PLC, which cleaves phosphatidylinositol bis-phosphate (PIP_2) to produce DAG and IP_3 upon receptor-mediated activation. Conventional (α, β_1, β_2, γ) and novel (δ, ε, θ, η) forms of PKCs have been thought to be the major receptor of DAG (Liu, W. S. and Heckman 1998). Binding of PKCs C1 domains to DAG leads to membrane recruitment and activations of PKCs. Conventional forms of PKC also require calcium for activation, whereas the activation of novel forms is calcium-independent. C1 domains are protein modules containing specifically arranged cysteine and histidine residues that coordinate zinc. They bind to DAG with low nanomolar affinity and thus serve as membrane recruitment modules. The presence of acidic membrane lipids such as phosphatidylserine is often required during DAG-binding by the C1 domain. The C1 domains of conventional and novel PKC forms also bind the phorbol ester class of DAG analog, which has been extensively studied *in vivo* as a tumor promoter and used *in vitro* as an artificial DAG stimulus in various experimental protocols. It should be noted that not all C1 domains bind DAG and its analog. Atypical forms of PKC, such as ι and ζ, are not regulated by DAG or calcium (Kazanietz 2002).

Theoretically, Ras activity can be determined by the balance between the rate of GTP hydrolysis, stimulated by RasGAPs, and the rate of nucleotide exchange, stimulated by RasGEFs. In human peripheral blood lymphocytes and the Jurkat T cell line, stimulation with anti-TCR antibodies, which lead to phosphatidylinositol hydrolysis and DAG production, caused rapid GTP loading on to Ras (Downward *et al.* 1990). Furthermore, strong and prompt Ras activation was observed after

treatment with the DAG analogue PMA. These observations link TCR stimulation to DAG generation and Ras activation in lymphocytes. Subsequent studies suggested that DAG-dependent Ras activation upon TCR ligation may due to decreased RasGAP activity, rather than increased RasGEF activity, as guanyl nucleotide exchange was constitutively high in the permeable cells prepared from PMA-treated cells. Since PKCs are thought to be the major transducer of the DAG signal, a PKC-dependent downregulation of RasGAP activity mechanism had been proposed, although the mechanistic details were never uncovered. However, a sizable fraction of the Ras activation in lymphocytes was indeed eliminated by PKC-inhibitors, while a PKC-independent component was also apparent (Balboa *et al.* 1992; Cantrell *et al.* 1992; Izquierdo *et al.* 1992).

Subsequent work in many labs, including important work by some of the authors cited above (Downward *et al.* 1990), suggest the DAG or PKC-dependent Ras activation does not appear to be a common mechanism in most non-lymphoid cells. In most cells, Ras is regulated by the recruitment of the RasGEF Sos to the plasma membrane by means of adaptor proteins and tyrosine phosphorylated docking sites (Downward 1996).

The discovery of C1 domain-containing RasGRP1, and the demonstration that this RasGEF protein is selectively expressed in certain immune cells, provided a convenient explanation for the original and unique observations on DAG activation of Ras in lymphocytes (Ebinu *et al.* 1998; Tognon *et al.* 1998; Dower *et al.* 2000; Ebinu *et al.* 2000). The catalytic domain of RasGRP1 is similar to that of Sos. In addition, RasGRP1 possesses several regulatory domains including a C1 domain that binds DAG and DAG analogues with high affinity, a pair of atypical EF-hands that bind calcium *in vitro*, and a putative proline-rich region that may bind to SH3 domain-containing proteins (Ebinu *et al.* 1998; Tognon *et al.* 1998; Lorenzo *et al.* 2000). Although the functional significance of the EF-hands and the proline-rich region are still unclear, it has been confirmed that the C1 domain facilitates

RasGRP1 membrane recruitment by DAG and interaction with substrate Ras (Ebinu *et al.* 1998; Tognon *et al.* 1998; Dower *et al.* 2000; Ebinu *et al.* 2000).

The study of RasGRP1 in Jurkat T cells and the analysis of *Rasgrp1* null mutant mice has provided substantial evidence that this RasGEF links TCR-PLC-γ1-DAG pathway to Ras signaling (Ebinu *et al.* 1998; Dower *et al.* 2000; Ebinu *et al.* 2000; Priatel *et al.* 2002). Nonetheless, this new model for Ras activation in T cells did not account for the original evidence from inhibitor studies that PKC was somehow involved.

With the discovery of RasGRPs, as well as several other C1 domain-containing protein families including Unc-13 proteins (Lackner *et al.* 1999; Nurrish *et al.* 1999), protein kinase D (Rozengurt *et al.* 1995), chimerins (Caloca *et al.* 2001; Wang and Kazanietz 2002), and at least one form of DAG kinases (Lackner *et al.* 1999; Nurrish *et al.* 1999; Shindo *et al.* 2001; Kazanietz 2002), PKCs are no longer thought of as the exclusive receptor of DAG signaling. However, these discoveries also raise the thought that DAG may recruit different classes of protein to the same membrane compartment to allow physical and functional interactions. A harmonious hypothesis emerged from our analysis of the Ras signaling in the Ramos B cell line (Teixeira *et al.* 2003). RasGRP3, which has a similar domain structure to RasGRP1, is expressed in B cells and functions to activate Ras downstream of BCR-PLC-γ2-DAG pathway (Oh-hora *et al.* 2003; Coughlin *et al.* 2005). However, an unexpected observation provided a possible resolution to the long-standing puzzling role of PKC in Ras signaling in lymphocytes; stimulation of BCR in Ramos B cells resulted in high stoichiometry, multi-site phosphorylation of RasGRP3 contemporaneously with Ras activation. Furthermore, with PMA or BCR stimulation, in the presence or absence of various PKC inhibitors, we found a striking concordance between RasGRP3 phosphorylation and Ras activation. Extrapolation from these B cell studies to T cells provides a plausible explanation for the original TCR signaling studies: membrane accumulation of DAG leads to

recruitment of both RasGRP1 and one or more forms of PKC, with regulatory phosphorylation of the former by the latter (Teixeira *et al.* 2003).

Objectives

Here we sought to map the BCR-regulated, PKC-catalyzed phosphorylation sites in RasGRP3, and investigate the functional significance of this modification. The implication of RasGRP3 phosphorylation was also studied in RasGRP1 and RasGRP4.

3.2 Results

3.2.1 RasGRP3 is an in vitro substrate of PKCθ and PKCδ

The first question we asked was which PKC isoform(s) could phosphorylate RasGRP3. To address this question, we tested whether candidate PKC isoforms can directly phosphorylate RasGRP3 and RasGRP1 in an immune-complex kinase assay. The nPKC family member PKCθ is expressed in Ramos B cells and most T cell lines. When transiently co-expressed with RasGRP3 in HEK293 cells, PKCθ, but not PKCα, causes the activation of RasGRP3, as well as the elevation of basal Ras-Erk signaling level (Teixeira *et al.* 2003). PKCδ, another novel class PKC isoform, is found physically associated with RasGRP3 in a PMA-dependent manner and caused its phosphorylation when ectopically expressed in HEK293 cells (Brodie *et al.* 2004). Moreover, the $PKC\beta^{-/-}$ mice exhibited a defect in B cell development, suggesting that PKCβ is a critical component of the BCR signaling machinery (Leitges *et al.* 1996; Kang *et al.* 2001; Antony *et al.* 2003; Saijo *et al.* 2003; Thuille *et al.* 2004). We thus focused on these three PKC candidate isoforms first. Parental MBP and MBP-RasGRP3 were each individually incorporated into immune complex kinase reactions with bacculovirus-expressed PKCs serving as the modifying enzymes. [^{32}Pγ]-ATP was used as cofactor to radiolabel product and the reaction was monitored by autoradiography after resolution of products by SDS/PAGE. Sf9 cells that were mock-infected and immuneprecipitations that lacked the anti-PKC antibodies served as negative controls. Immunoprecipitated $PKC\beta_2$, PKCθ, and PKCδ phosphorylated MBP-RasGRP3 but not parental MBP (Figure 3.1; data not shown). In contrast, PKCα purified from infected cells did not phosphorylate RasGRP3 (Data not shown). These data confirmed that RasGRP3 was the direct *in vitro* substrate of certain PKC isoforms. Moreover, MBP-RasGRP1 is also phosphorylated *in vitro* by $PKC\beta_2$, PKCθ, and PKCδ, but not PKCα (Figure 3.8; data not shown).

3.2.2 RasGRP3 is phosphorylated by PKCθ on threonine 133 in vitro.

Analysis of the primary sequence showed a total of 35 threonine residues and 57 serine residues in RasGRP3. Among them, five threonines and 23 serines are predicted to be potential threonine/serine-specific protein kinase phosphorylation sites, as judged by the significant score when we searched the human RasGRP3 sequence with the protein database motif engine Netphos 2.0 (table 3.1-1 and -2). A traditional approach used for the analysis of phosphoproteins is to label the protein with ^{32}P to monitor phosphorylation followed by proteolytic cleavage and the subsequent analysis of the resulting peptides by two-dimensional phosphopeptide maps of phosphorylated proteins. However, this technology is evidently hampered by the inability to actually identify the phosphopeptide and pinpoint the phosphorylation site. Recent advances in mass spectrometry based methods have started a new era for the analysis of post-translational modifications including phosphorylation due to higher sensitivity, selectively, and speed than most biochemical techniques (Liu, H. *et al.* 2004; Zeller and Konig 2004; Loyet *et al.* 2005; Vierra-Green *et al.* 2005). The concept was quite simple: phosphorylation of a polypeptide results in an easily detectable 80 Dalton increase of the molecular weight of the target peptide. However, phosphorylation level for a given protein on a specific site may often be low, leading to low relative quantities of phosphopeptides in a tryptic digestion. In addition, non-phosphorylated peptides usually produce higher signals in the mass spectrometer than its phosphorylated version, thus making phosphopeptide detection rather difficult. To circumvent these setbacks in our analysis of the PKC phosphorylation sites in RasGRP3, we combined mass spectrometry with the open tubular immobilized metal affinity chromatography (IMAC) technique. This technique greatly improves the detection and identification of phosphopeptides by separating them from non-phosphorylated species. Two strategies were used to produce phosphorylated RasGRP3 protein for mass spectrometry analysis.

Strategy 1:

We first generated a truncated version of RasGRP3 protein containing only the C-terminal region of RasGRP3 (residue 543 to 689) fused to MBP protein. This is because the relative small size (16KDa) of the RasGRP3 fragment makes it an ideal target for mass spectrometry parameterization. In addition, high expression level of this fragment in *E. Coli.*, provides sufficient amount of material for subsequent mass spectrometry analysis. The RasGRP3 C-terminal fragment was able to be *in vitro* phosphorylated by PKCθ, as determined by ^{32}P incorporation in an immune-complex kinase assay (data not shown).

Purified RasGRP3 C-terminal fragment fused to MBP was then *in vitro* phosphorylated by PKCθ in an large scale immune complex kinase assay in the presence of unlabelled Mg^{2+}-ATP instead of [$^{32}P\gamma$]-ATP. Phosphoprotein was resolved by SDS/PAGE and subjected to in-gel digestion with trypsin. Peptides were eluted and phosphorylated peptides were enriched by open tubular IMAC before analysis by MALDI-TOF mass spectrometry (All mass spectrometry analyses were performed by investigators in Dr. Liang Li's group, Department of Chemistry, University of Alberta). We observed one phosphorylated peptide represented by doubly charged ion at m/z 1914.66 (669-GpTEFELDQDEGEEpTR-683) in phosphorylated RasGRP3 C-terminal fragment. A peptide at m/z 1754.73, which represents the de-phosphorylation of the phosphopeptide as the result of losing two phosphate groups (2x 80 Dalton), was also observed (Figure 3.2). This phosphopeptide was selected for tandem mass spectroscopy (MS/MS) analysis for detailed structural elucidation and sequence confirmation. In this method, particular tryptic peptides selected during mass spectropic analysis are broken down and these peptide fragment ions are further analyzed by a second round of mass spectrometry. The MS/MS analysis confirmed the two threonines at positions 670 and 682 were the *in vitro* substrate of PKCθ (data not shown). Nevertheless, it should be noted that truncation might cause conformational changes in this portion of

the protein leading to changes in the accessibility of certain Thr/Ser residues to cause artificial phosphorylation sites.

Interestingly, in addition to phosphorylation, a methylation on the last glutamic acid (Glu687) was also observed. In eukaryotic cells, protein methylation usually happens on carboxyl groups or on the side-chain nitrogens of certain amino acids. This modification is often involved in cellular stress responses and the aging/repair of proteins (Yang, X. J. 2005). However, the methylation of glutamic acid has not been reported in proteins expressed from *E. Coli.* (Aletta *et al.* 1998). Alternatively, it could be an unexpected gel electrophoresis-related protein modification that preferentially methylates glutamic acid (Haebel *et al.* 1998). Thus, precautions should be taken during sample preparation to minimize artificial protein modifications.

Upon the optimizing of the protocol, full length RasGRP3 was expressed as MBP fusion protein and used as the *in vitro* substrate of PKCθ. Phosphoprotein was then subjected to in-gel tryptic digestion and mass spectrometry analysis. This time, we repeatedly observed two related tryptic peptides represented by singly charged ions at m/z 739.36 (131-RVpTQR-135) and 867.45 (131-RVpTQRK-136) in phosphorylated RasGRP3. These two phosphopeptides were then selected for MS/MS analysis. The MS/MS spectrum along with peak assignments for m/z 739.36 is shown in Figure 3.3A. A fragment ion at m/z 641.42 resulting from loss of the phosphomoiety (H_3PO_4, M.W. = 98) from the parental ion (phosphopeptide at m/z739.36) was observed, verifying single phosphorylation of this peptide. The observed m/z values of other reduced fragment ions were also listed in Figure 3.3C, and were compared with their predicted theoretical values. The results further confirmed that RasGRP3 is phosphorylated on Thr133 *in vitro*. Similar data were collected for the other related ion (data not shown).

Strategy 2:

In the second strategy, to mimic the *in vivo* phosphorylation of RasGRP3, the protein was expressed as untagged native protein in sf9, an insect cell line using a baculovirus expression system. In this method, sf9 cells were infected with baculovirus carrying a RasGRP3 open reading frame. Before cell lysis, the cells were pretreated for 1 hour with 1uM okadaic acid (OA), a potent and specific cell-permeating inhibitor of protein phosphatases that completely inhibits phosphatase 2A at nanomolar concentrations and type-1 phosphatases at 1 mM (Dounay and Forsyth 2002). Treatment with OA results in a significant non-specific increase in total cellular protein phosphorylation, and is often utilized in the identification of target molecules of intracellular signal transduction pathways that become phosphorylated in response to environmental stimuli (Dounay and Forsyth 2002). RasGRP3 was then immune-precipitated from cell lysates, separated by SDS/PAGE, in-gel digested by trypsin, and subjected to mass spectrometry. Seven phosphopeptides were observed (Table 3.2). Further MS/MS analysis confirmed nine phosphorylated threonines or serines from these phosphopeptides. The relative positions of these peptides in RasGRP3 are shown in Figure 3.4A. The MS/MS spectrum of phosphopeptide at m/z 867.45 (131-RVpTQRK-136) is also shown (Figure 3.4B).

Among all the observed phosphorylation sites, the threonine at position 133 (Thr133) is of particular interest for several reasons.

1. Thr133 is the only residue that was phosphorylated by two different strategies;
2. It corresponds to a consensus PKC phosphorylation sequence RXXS/T, where R represents arginine, X is any amino acid except for proline; S is serine; T is threonine;
3. It is one of the residues that has the most significant score in the Netphos 2.0 search;

4. Mammalian forms of RasGRP1 and RasGRP2 both have threonine at the corresponding site while the *C. elegans* orthologue has serine (Figure 3.5B);
5. The adjacent basic residues of Thr133 in RasGRP3 is also conserved in RasGRP1, 2, 3, and worm RasGRP. RasGRP4, which is expressed in mast cells and cell lines derived from the myeloid lineage (Reuther *et al.* 2002; Yang, Y. *et al.* 2002; Li *et al.* 2003), lacks this sequence and has several prolines in the region (Figure 3.5B).

Thr133 is located near the amino terminal region of the CDC25 box (Figure 3.5A, B). According to the three-dimensional model structure of the Ras-GEF domain of RasGRP3 built by homology modeling based on a crystal structure of SOS, Thr133 is located in a flexible loop opposite to the Ras binding site in the CDC25 box (data not shown). Introducing a negatively charged phosphate group on Thr133 may induce a conformational change in the Ras binding pocket of RasGRP3, thus alters its ability to bind Ras.

Taken together, these data indicated that RasGRP3 Thr133 is directly phosphorylated *in vitro* by PKCθ and it is also one of the OA-induced phosphorylation sites *in vivo*.

3.2.3 Alanine substitution at position 133 of RasGRP3 blocked in vitro phosphorylation by PKCθ and PKCδ

To confirm that Thr133 was a major site of phosphorylation by PKCθ *in vitro*, we generated a mutant cDNA containing a substitution encoding alanine at position 133. Parental MBP, MBP-RasGRP3 and MBP-RasGRP3-Thr133Ala (T133A) were each individually incorporated into immune-complex kinase reactions with bacculovirus-expressed PKCθ serving as the modifying enzyme, as in 3.2.1. [^{32}Pγ]-ATP was again used as cofactor to radiolabel product and the reaction was monitored by autoradiography after resolution of products by SDS/7.5%PAGE. Sf9

cells that were mock-infected and immune- precipitations that lacked the anti-PKCθ antibody served as negative controls. Consistent with results in Figure 3.1, PKCθ-dependent phosphorylation of MBP-RasGRP3 was detected with this assay (Figure 3.6A). In contrast, labeling of parental MBP and mutant RasGRP3 was undetectable. Interestingly, when Thr133 is replaced by a serine residue, the phosphate incorporation catalyzed by PKCθ is similar to that of wildtype (Figure 3.6B). Recent work with RasGRP3 using an ectopic expression system in HEK293 cells has provided evidence that the novel form PKCδ physically interacts with RasGRP3 and regulates it (Brodie *et al.* 2004). Thus, we performed similar experiments with PKCδ. The result showed that PKCδ also directly phosphorylated RasGRP3, but not T133A mutant (Figure 3.7).

RasGRP1 Thr184 is partially responsible for in vitro phosphorylation by PKCβ₂ and PKCδ, but not PKCθ.

RasGRP1 has a threonine at the site corresponding to the threonine 133 in RasGRP3, which is position 184 (Figure 3.4B). To test whether Thr184 is also a major phosphorylation site responsible for RasGRP1 phosphorylation, we generated a RasGRP1 mutant containing a substitution encoding alanine at position 184. MBP-RasGRP1 and MBP-RasGRP1-Thr184Ala (T184A) were *in vitro* phosphorylated by indicated PKC isoforms. Surprisingly, despite the poor total ^{32}P-labeling on the wildtype protein, the phosphorylation of T184A by PKCθ was not affected (Figure 3.8A). In comparison, alanine substitution caused a 50% decrease in PKCδ-catalyzed phosphate incorporation in the T184A mutant, implying a second phosphorylation site in this protein (Figure 3.8 C). Notably, when PKCβ$_2$ was tested, a PKCβ$_2$-dependent increase of basal phosphorylation of RasGRP1 and T184A was observed. The elevated basal level of phosphorylation might be caused by the non-specific binding of PKCβ$_2$ to protein G sepherose beads during immune-precipitation, and could be decreased by blocking the beads with 5% BSA solution and extra washing steps. Nevertheless, PKCβ$_2$-catalyzed ^{32}P-incorporation

of T184A was, again, decreased by 50% when compared to that of the wild type RasGRP1 (Figure 3.8B). These results suggested that Thr184 of RasGRP1 is not likely a PKCθ phosphorylation site, but may serve as an important phosphorylation site for PKCβ_2 and PKCδ. In addition, the weak all over phosphate labeling on RasGRP1 catalyzed by PKCθ might indicate that this kinase was not a major form in T cells able to regulate RasGRP1 activity via phosphorylation.

Collectively, these results confirm that Thr133 in RasGRP3 is a major phosphorylation site for nPKC forms *in vitro*. The homologous site Thr184 in RasGRP1 is also partially responsible for RasGRP1 phosphorylation by PKCβ_2 and PKCδ.

3.2.4 RasGRP3 is phosphorylated on threonine 133 in vivo.

RasGRP3 phosphorylation on Thr133 occurs in B cells after stimulation and is correlated with Ras-Erk signaling.

In order to confirm that RasGRP3 Thr133 is an *in vivo* phosphorylation site, we generated polyclonal anti-phospho-peptide antibodies, anti-3/pThr133. Ramos B cells were left unstimulated, treated with anti-IgM antibodies to stimulate the BCR, or treated with PMA to activate DAG signaling proteins directly. To ensure the specificity of our assay, RasGRP3 was immune-precipitated from lysates with monoclonal antibodies, resolved by SDS/10%PAGE, and blotted with anti-3/pThr133 antibodies.

The weak reactivity observed with RasGRP3 from untreated Ramos B cells could reflect either a low level of basal phosphorylation or weak reactivity of the antibodies with the non-phosphorylated RasGRP3 (Figure 3.9A). Much stronger reactivity was observed 5 minutes after treatment with anti-IgM antibodies or 10 minutes after treatment with PMA. Both of these treatments resulted in strong Ras and Erk activation, as expected. To confirm the results, we also showed that anti-3/pThr133 antibodies selectively immune-precipitated RasGRP3 only after cell stimulation (Figure 3.9B).

The Ramos B cells used in these experiments are human Burkitt lymphoma cells carrying various mutations and expressing high level of RasGRP3 (Teixeira *et al.* 2003). To confirm that BCR crosslinking-induced RasGRP3 Thr133 phosphorylation also happens under physiological conditions in normal B cells, primary mouse B cells were stimulated with anti-IgM antibody or PMA, and the phosphorylation of RasGRP3 was analyzed. The results confirmed RasGRP3 phosphorylation was coincident with Ras-Erk signaling in BCR- and PMA-stimulated primary B cells (Figure 3.9C).

PKC-dependent RasGRP3 phosphorylation on Thr133 in B cells.

Since RasGRP3 Thr133 is an *in vitro* substrate for PKCs, we tested if its *in vivo* phosphorylation is sensitive to PKC inhibition. As shown in Figure 3.10B, both the pan-PKC inhibitor Ro318220 and the cPKC-selective inhibitor Go6976 largely blocked both anti-3/pThr133 reactivity and Erk activation in BCR-stimulated cells. For comparative purposes, we also looked at the effect of inhibitors on PMA-induced RasGRP3 phosphorylation in Ramos B cells (Figure 3.10A). In this case, we observed substantial inhibition of anti-3/p-Thr133-reactivity with Ro318220 but negligible inhibition with Go6976. Despite this difference, the inhibition of PMA-induced Thr133 phosphorylation roughly matched the effect on PMA-induced Erk activation.

Together, these data demonstrate that Thr133 of RasGRP3 is an *in vivo* BCR-regulated, PKC-dependent phosphorylation site in normal B cells.

3.2.5 RasGRP3 Thr133Ala is functionally impaired in an ectopic expression system.

RasGRP3 Thr133Ala blocks PMA-induced Ras-Erk activation in Rat2 cells.

To determine the functional significance of Thr133 phosphorylation for RasGRP3 activity *in vivo*, we generated a mutant RasGRP3 that contains an alanine substitution at position 133 (T133A). Wildtype and mutant RasGRP3 were

expressed in Rat2 cells using a retrovirus vector system. Rat2 cells do not express endogenous RasGRP3 that might complicate the analysis. With the expression of wildtype RasGRP3, Rat2 cells can respond to DAG analogue treatment to activate Ras and Erk (Figure 3.11A) as has been reported previously (Lorenzo *et al.* 2001). When we tested Rat2 cells that express RasGRP3 T133A, we found that PMA-induced Ras and Erk activation were impaired relative to wildtype, although signaling was higher than seen in cells expressing empty vector. As expected, the mutant protein exhibited no reactivity with the anti-3/pThr133 antibody.

Phosphorylation events often cause a conformational change in the protein by introducing a negative charge. Thus, to mimic the effect of phosphorylation, we generated a phospho-mimic mutant of RasGRP3 by replacing Thr133 with the negatively charged glutamic acid (T133E). When ectopically expressed in Rat2 cells, RasGRP3 T133E failed to restore the PMA-induced Ras-Erk activation. However, it did increase the basal level of Erk activation in unstimulated cells (Figure 3.12A). Moreover, PMA-induced Erk activation in cells expressing T133E was not sensitive to PKC inhibitor treatment (Figure 3.12B).

RasGRP3 Thr133Ala blocks PMA-induced Rap activation in Rat2 fibroblasts.

RasGRP3 has been reported to catalyze activation of the Ras-related GTPase Rap1A (Yamashita *et al.* 2000). Although the significance of this function of endogenous RasGRP3 is uncertain, we have found that the T133A mutant was defective relative to wildtype in PMA-induced Rap1 activation, at least when ectopically expressed in Rat2 cells (Figure 3.13).

RasGRP3 Thr133Ala causes a defect in PMA-induced growth of Rat2 fibroblasts.

Unlike RasGRP1 and RasGRP4, RasGRP3 does not cause apparent morphological changes associated with activated Ras in Rat2 cells. Nonetheless, RasGRP3 expression does influence cellular proliferation. Growth of parental Rat2 cells is strictly dependent on serum growth factors. In medium containing 10%

serum Rat2 cells undergo mitosis daily, but in medium containing 0.5% serum, very little net increase in cell number is observed over three days. PMA does not substitute for serum in Rat2 cells and in fact may be slightly detrimental to cell recovery. However, when Rat2 cells are expressing wildtype RasGRP3, PMA can induce substantial growth over three days (Figure 3.14A). This effect was largely abrogated by expressing T133A mutation in these cells.

RasGRP3 Thr133Ala does not block PMA-induced membrane recruitment.

DAG-mediated membrane recruitment of RasGRP3 is critical for its activation. Thus, it is possible that the Thr133 phosphorylation facilitates membrane binding. To test this hypothesis, we transiently expressed the GFP-tagged wildtype and T133A mutant in HEK293 cells, and studied their subcellular localizations using confocal microscopy. As shown in the representative images of the cells, both wildtype and T133A mutant rapidly translocated to plasma membrane and perinuclear region upon PMA treatment (Figure 3.14B).

Taken together, these data demonstrated that the Thr133 phosphorylation is necessary for the *in vivo* function of RasGRP3, although it is not required for the membrane localization of the protein.

3.2.6 Implications of RasGRP3 Thr133 phosphorylation for regulation of RasGRP1 and RasGRP4

RasGRP1 in T cells is subject to PKC-dependent regulatory phosphorylation on Thr184 upon PMA and TCR stimulation

RasGRP3 amino acid Thr133 corresponds to RasGRP1 Thr184 (Figure 3.4B), so we generated a second polyclonal antibody, anti-1/pThr184, that recognizes phosphorylated RasGRP1. Extracts were prepared from unstimulated Jurkat T cells, from cells treated with PMA and from cells stimulated with a stimulatory antibody that recognizes CD3, a component of TCR. As we predicted, anti-1/pThr184 antibodies recognized RasGRP1 only in stimulated cells (Fig 3.15A). Ro318220

and Go6976 individually inhibited phosphorylation of RasGRP1 in Jurkat T cells after immune receptor stimulation (Figure 3.15B). This result was confirmed in primary mouse T cells (Fig 3.15C). Again, there was a good correspondence between inhibition of phosphorylation and inhibition of Erk activation. However, functional analysis of the RasGRP1 Thr184Ala mutation has so far not revealed a signaling defect and ongoing work indicates that regulation of RasGRP1 by phosphorylation may be complex.

RasGRP4 is regulated by other means

RasGRP4 is not highly conserved in the region corresponding to the sites phosphorylated in RasGRP1 and RasGRP3. Rather than a consensus PKC phosphorylation sequence, RasGRP4 has several prolines in the corresponding region (Figure 3.4B), arguing that this protein cannot be subject to the same sort of regulation. We noted a slight activation of Erk in empty vector cells exposed to PMA (Figure 3.16). This response likely reflects a direct activation of Raf by PKC that occurs without Ras activation, although it may depend on basal Ras-GTP levels. Erk activation is much more robust in RasGRP4-expressing Rat2 cells as a result of Ras activation. Strikingly, PMA-induced Erk activation in Rat2 cells expressing RasGRP4 was completely resistant to PKC inhibitors (Figure 3.16).

Together, these data indicate that Thr184 in RasGRP1 is also a TCR-regulated PKC-dependent phosphorylation site *in vivo*, while RasGRP4 may be regulated by other means.

3.3 Discussion

The engagement of BCR by antigen activates PLCγ-2, leading to DAG production. Accumulation of membrane DAG is expected to recruit and activate various PKCs, RasGRPs and other C1 domain proteins (Kazanietz 2002). The PKC and RasGRP3 pathways were thought to operate separately. However, our previous

studies raised the possibility that DAG might recruit both proteins to the same membrane compartment to facilitate regulatory phosphorylation of RasGRP3 by PKCs (Teixeira *et al.* 2003). B cells express both RasGRP1 and RasGRP3, although the analysis of mutant chicken DT40 pre-B cells indicates that the latter protein is the major Ras activator responding to BCR signals (Oh-hora *et al.* 2003). B cells also express a variety of PKC isoforms. Both conventional and novel classes of PKCs are regulated by DAG. However, studies on knock-out mice suggested an isoform-specific functional role of different PKCs. For example, PKCβ null mutant mice have impaired humoral response and their B cells show defective *in vitro* proliferation (Leitges *et al.* 1996; Saijo *et al.* 2002). PKCδ mutant B cells fail to establish an antigen-tolerant state, a process requiring active BCR signaling and Erk activation, and thus predispose mice to an auto-immune disease (Mecklenbrauker *et al.* 2002; Miyamoto *et al.* 2002).

Using an *in vitro* immune-complex kinase assay, we demonstrated that RasGRP3 was directly phosphorylated by PKCθ and PKCβ in a previous study, and by PKCδ, in this study. We have also shown that co-transfection of activation mutant PKCθ and RasGRP3 cDNAs into HEK293 cells resulted in phosphorylation of the latter protein and cooperative Ras signaling (Teixeira *et al.* 2003). Furthermore, although PKCθ expression is typically associated with T cells, we showed that Ramos B cells express this isozyme. For these reasons, in the present study we first mapped the position of phosphate incorporated into RasGRP3 by PKCθ *in vitro* using mass spectrometry. These studies provided physical evidence for phosphorylation on Thr133. The result was confirmed by the observation that recombinant RasGRP3 bearing the Thr133Ala substitution was virtually inert as a substrate for PKCθ. This RasGRP3 substitution also blocks phosphorylation by PKCδ. The latter observation is intriguing in light of the evidence that this PKC isozyme is an effective physical partner of RasGRP3 in HEK293 cells (Brodie *et al.* 2004) and the proposal that BCR signaling through PKCδ is required for induction of B cell antigen tolerance (Mathis and King 2002). Recently it was shown that PKCδ

plays a role in BCR-induced $pp90^{rsk}$ activation and subsequent phosphorylation of the CREB transcription factor (Blois *et al.* 2004). The involvement of RasGRP3-Erk intermediates between PKCδ and $pp90^{rsk}$ could neatly explain these observations.

To verify that RasGRP3 Thr133 phosphorylation is a true *in vivo* event upon BCR stimulation in B cells, we developed phosphopeptide antibodies specific for phospho-Thr133 in RasGRP3. In both Ramos B cell line and mouse primary B cell culture, phosphorylation was more intense after PMA treatment. Importantly, PKC inhibitors that interfered with RasGRP3 phosphorylation on Thr133 also diminished Ras-Erk signaling. In our PMA stimulation protocol, only the pan-PKC inhibitor Ro318220 was effective at blocking RasGRP3 phosphorylation, providing evidence for the role of an nPKC. The results observed after BCR stimulation are more puzzling. Although the pan-PKC inhibitor was the most effective at blocking RasGRP3 phosphorylation, we have consistently found that this drug was less effective than the cPKC-selective drug Go6976 at blocking BCR-stimulated signaling. We previously demonstrated that this anomaly can be seen at the level of Ras-GTP and can be duplicated with a second pan-PKC inhibitor, Bisindolylmaleimide (Teixeira *et al.* 2003). Possibly, pan-PKC inhibitors have effects on other Ras regulators in Ramos B cells such as Ras GAPs, as originally proposed for T cells (Downward *et al.* 1990).

Consistent with the idea that Thr133 phosphorylation represents a positive regulatory modification in RasGRP3, we found that the Thr133Ala mutant protein has reduced Ras-Erk signaling in Rat2 cells after brief PMA treatment. RasGRP3 Thr133Ala-expressing cells also exhibited defective PMA-induced growth, which provides a longer-term functional assay. However, we cannot exclude the possibility that Thr133Ala substitution has some effect on RasGRP3 other than preventing phosphorylation. We did find that GFP-tagged wildtype and Thr133Ala mutant forms of RasGRP3 had similar sub-cellular distribution when expressed in HEK293 cells and showed similar recruitment to cellular membranes in response to

PMA. In some respects, then, the mutant form behaves normally and may serve as a specific model of non-phosphorylated RasGRP3. Recently, BCR-induced phosphorylation of RasGRP3 on Thr133 was demonstrated in the DT40 chicken pre-B cell line (Aiba *et al.* 2004). This modification was dependent upon PLC-γ2 and PKC activity. Furthermore, the Thr133Ala mutation impaired Ras activation as shown in the present study.

Sequence analysis showed that the Thr133 is also conserved in RasGRP1, corresponding to Thr184. This argues, by analogy to our study on RasGRP3 phosphorylation, that Thr184 regulatory phosphorylation is required for RasGRP1 activation downstream of TCR. Indeed, we showed that PKCβ, PKCθ, and PKCδ, but not PKCα can *in vitro* phosphorylate Thr184. Analysis using phosphopeptide antibodies specific for phospho-Thr184 of RasGRP1 in T cells shows *in vivo* phosphorylation of this site after both TCR stimulation and treatment with DAG agonist. Again, PKC inhibitors block both phosphorylation and Ras-Erk signaling, consistent with the idea that phosphorylation of RasGRP1 requires both membrane recruitment and regulatory phosphorylation by a membrane-recruited PKC for activation. PKCθ. plays an important role in TCR signaling and is an obvious candidate for a RasGRP1 regulator. However, the effectiveness of Go6976 implies a role for cPKC and our mutagenesis studies indicate that the Thr184Ala mutation does not significantly impair RasGRP1 phosphorylation by PKCθ. *in vitro* (Figure 3.8). Alternatively, RasGRP1 might be phosphorylated on multiple sites upon activation. We showed that phosphorylation by PKCδ and PKCβ on RasGRP1 Thr184Ala mutant was decreased by 50% when comparing to that of wildtype, arguing the presence of another PKC-regulated phosphorylation sites.

The position of Thr133 suggests that phosphorylation might affect guanyl nucleotide exchange activity. Our analysis of RasGRP4 offers an interesting comparison. This species has an unusual density of prolines in this region and lacks a homologous PKC phosphorylation site, implying this protein is not subject to PKC-regulated phosphorylation. In line with this idea, RasGRP4 signaling is

insensitive to PKC inhibitor treatment. We speculate the RasGRP4 is constitutively in the activated state, as a result of the structural effect of these prolines. Consistent with this idea, when we individually express all four family members in the same retrovirus vector in Rat2 cells, RasGRP4 is readily the most transforming species.

Phosphorylation may be a common mechanism for RasGEF regulation. Erk phosphorylates Sos, and this may provide a negative feedback mechanism to uncouple Ras signaling in later stages of growth factor responses (Porfiri and McCormick 1996). RasGRF1, a neuronal RasGEF is phosphorylated and positively regulated by cAMP-dependent kinase, at a site close to that corresponding to Thr133 in RasGRP3 (Mattingly 1999; Yang, H. *et al.* 2003). It has been reported that RasGRP3 is phosphorylated by a src-type protein tyrosine kinase in HEK 293 cells as part of a EGF receptor- pp60src-PLC-γ1- RasGRP3-Rap2B-PLC-ε pathway (Stope *et al.* 2004). Using our anti-RasGRP3 antibodies, however, we found no expression of RasGRP3 in these cells. Furthermore, we were unable to demonstrate BCR-induced tyrosine phosphorylation of immune-precipitated RasGRP3 in Ramos cells, using an anti-phosphotyrosine antibody in an immunoblotting assay (Stone lab, unpublished).

Since the present work completed, Roose and colleagues showed that kinase-dead PKCθ could bind to RasGRP1, RasGRP2, and RasGRP3 when expressed in 293T cells. In addition, introduction of kinase-active PKCθ, but not PKCα, in a Jurkat T cell line resulted in efficient upregulation of the activation marker CD69. The presence of sufficient wild-type RasGRP1 is required for this effect, suggesting a PKCθ-RasGRP1-Ras/Erk-CD69 pathway (Roose *et al.* 2005). They also confirmed the TCR- and PMA-induced RasGRP Thr184 phosphorylation in these cells using the anti-phosphoThr184 antibody described in this dissertation (Roose *et al.* 2005).

The functional significance of PKC-mediated regulatory phosphorylation of RasGRPs is still not clear. Theoretically, this RasGEF could be regulated only by DAG-mediated membrane recruitment, as may be the case in RasGRP4. One

possibility is that while the level of membrane recruitment of RasGRP3 (or PKC) may be a linear function of DAG membrane concentration, the membrane concentration of active, phosphorylated RasGRP may be non-linear, as it would depend on the surface concentrations of PKC and substrate RasGRP3, as well as the activities of phosphatases. The ability to respond to DAG in a non-linear fashion might effectively turn an otherwise analogue system into a digital switch, a useful feature for a biochemical pathway that is used to control procession through various stages of lymphocyte differentiation, activation and death. The present identification of RasGRP phosphorylation sites provides a specific link in the PKC-RasGRP axis, from which further mechanistic and physiological insights may be gained.

Table 3.1-1 Predication of PKC phosphorylation sites (Serine) in RasGRP3

Residue	Position	Sequence	Netphos 2.0 Score
Serine	116	EKHVSLIDI	0.967
	125	SSIPSYDWM	0.903
	139	RKKVSKKGK	0.981
	167	LEHKSFRRI	0.781
	172	FRRISFTDY	0.996
	252	LSHSSISRL	0.888
	254	HSSISRLKE	0.511
	261	KETHSHLSS	0.815
	264	HSHLSSEVT	0.992
	280	ELVSSNGNY	0.645
	334	MHQLSVTLS	0.752
	385	EPRNSKSQP	0.962
	391	SQPTSPTTP	0.931
	427	KLVESVFRN	0.917
	441	DGYISQEDF	0.994
	483	LRAKSQLHC	0.727
	552	ARAPSLSSG	0.974
	554	APSLSSGHG	0.771
	555	PSLSSGHGS	0.575
	563	SLPGSPSLP	0.949
	597	LVTGSSRKI	0.670
	598	VTGSSRKIS	0.712
	602	SRKISVRLQ	0.996

Table 3.1-2 Predication of PKC phosphorylation sites (Threonine) in RasGRP3

Residue	Position	Sequence	Netphos 2.0 Score
Threonine	11	GKAATLDEL	0.792
	133	MRRVTQRKK	0.992
	394	TSPTTPNKP	0.733
	610	QRATTSQAT	0.905
	671	VDRGTEFEL	0.933

Table 3.2 Phosphopeptides identified in RasGRP3 immunoprecipitated from okadaic acid-treated sf9 cells

Peptide Sequences	Charges	Confirmed Sites
1-MG**pS**p**S**GLG-8	2	Ser3, Ser4
131-RV**pT**Q-134	1	Thr133
219-AEIV**pT**-223	1	Thr223
256-LKETH SHLSSEVT-268	1	-
550-APSLSSGHG**pS**L PG**pS**PSLPPAQDE-572	3	Ser558, Ser562
590-AITLV**pT**GS**pS**RKI**pS**VR-604	2	Thr594, Ser597, Ser601
669-GTEFELDQDEG EE**pT**RQDGEDG-689	1	Thr682

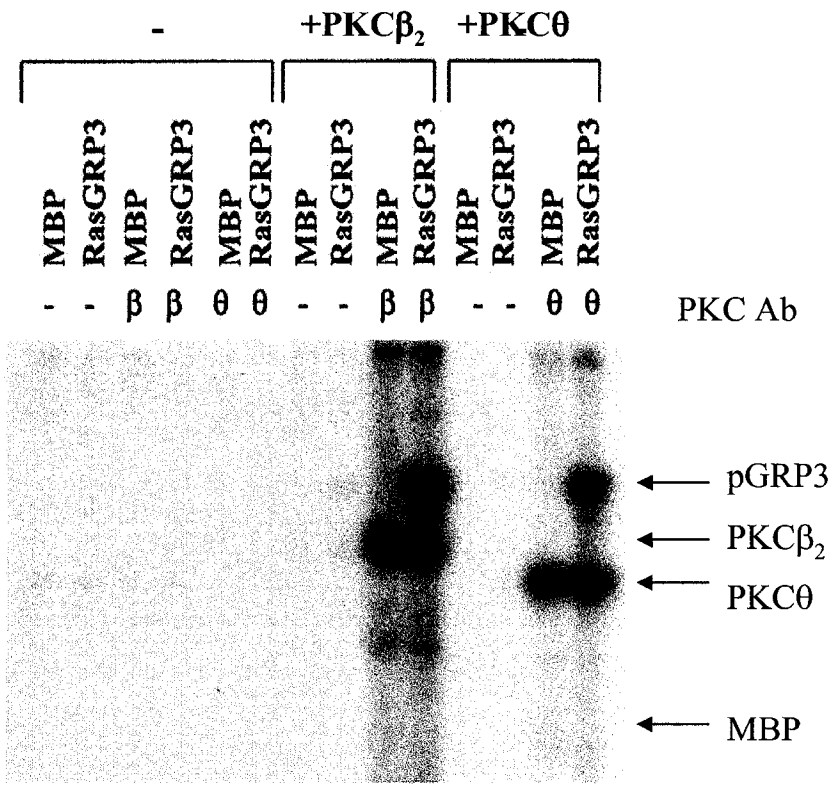


FIGURE 3.1 RasGRP3 is directly phosphorylated by PKC-θ and PKC-β_2. *Sf9* cells were mock infected or infected with PKC-θ or PKC-β_2 expressing baculovirus. Lysates were either immunoprecipitated with anti–PKC-θ, anti–PKC-β, or primary antibody was omitted, as a control. An immunocomplex kinase assay was conducted using either a 43-kDa MBP or a 127-kDa MBP-RasGRP3 fusion protein followed by resolution by SDS-PAGE and autoradiography. The positions of MBP-RasGRP3, autophosphorylated PKC-θ or PKC-β_2, and MBP are shown. Autoradiographic exposure was for 20 minutes. Results are representatives of three independent experiments.

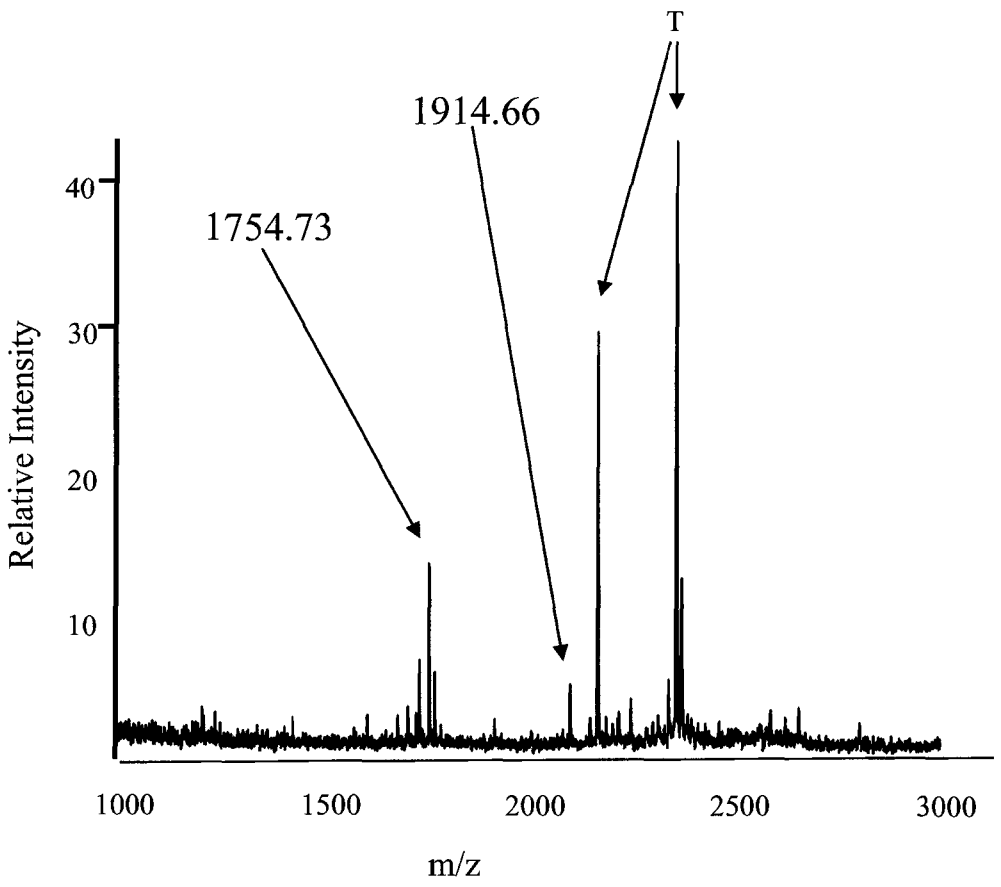
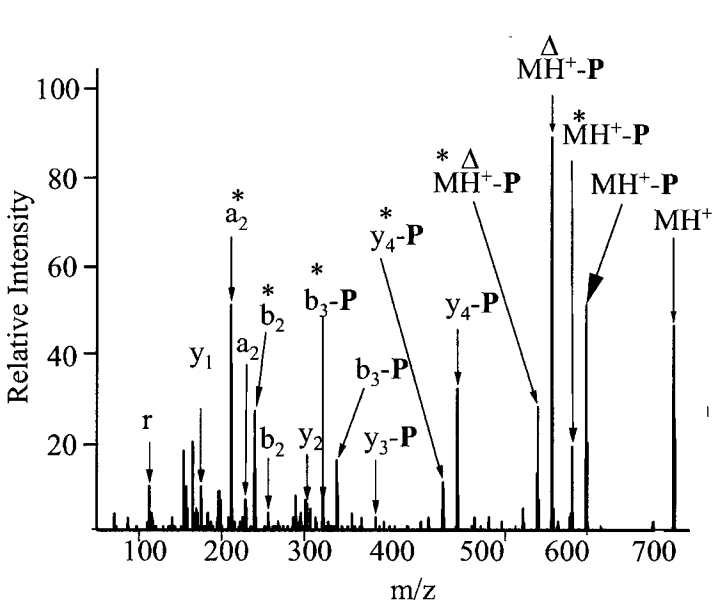
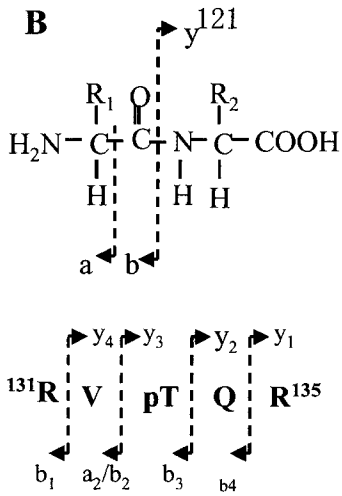


FIGURE 3.2 PKCθ phosphorylates RasGRP3 C-terminal fragment at Thr670 and Thr682. RasGRP3 C-terminal fragment fused to MBP protein was *in vitro* phosphorylated by PKCθ in an immune-complex kinase assay followed by in-gel tryptic digestion and MALDI-TOF mass spectrometry analysis. The mass spectrum of phosphopeptide m/z 1914.66 (589-GpTEFELDQDEGEEpTR-603) is shown. The peaks corresponding to the detected phosphopeptide and its non-phosphorylated version are labeled with their m/z value. T, peptides resulted from tryptic autocleavages. Results are representative of two independent experiments.

A



B



C

Ions	Sequences	T. m/z	O. m/z	Ions	Sequences	T.m/z	O.m/z
MH^+	RVpTQR	739.36	739.44	b_3-P	RVT	339.21	339.25
MH^+-P	RVTQR	641.38	641.42	b_3-P*	RVT*	322.19	322.23
MH^+-P*	RVTQR*	624.36	624.41	y_2	QR	303.18	303.19
MH^+-PΔ	RVTQRΔ	599.36	599.42	b_2	RV	256.18	256.22
MH^+-P*Δ	RVTQR*Δ	582.34	582.38	b_2*	RV*	239.15	239.20
y_4-P	VTQR	485.28	485.34	a_2	RV	228.18	228.21
y_4-P*	VTQR*	468.26	468.28	a_2*	RV*	211.18	211.18
y_3-P	TQR	386.22	386.25	y_1	R	175.12	175.14

FIGURE 3.3 Threonine 133 in RasGRP3 is phosphorylated *in vitro* by PKCθ. **(A)** MS/MS spectrum of phosphopeptide m/z 739.36 (131-RVpTQR-135) derived from analysis of RasGRP3 that was subjected to phosphorylation by recombinant PKCθ. The fragment ions are labeled with peak assignments (a, b or y ions) conforming to the common notation used for peptide fragment ions. The peak labeled MH+ corresponds to the protonated ion of the phosphorylated parental tryptic peptide. Peaks resulting from neutral loss of a phosphate group from an ion are marked by "**-P**". *, denotes the loss of a "-NH3". Δ, denotes the loss of a "-C(NH)2". r, stands for immonium ion of arginine. **(B)** Generic (top) and specific (bottom) structural representations of a, b and y fragment ions are shown. **(C)** The corresponding peptide sequences (Sequences), the theoretical m/z value (T. m/z) and the observed m/z value (O. m/z) of each fragment ion in Figure1A are shown. The information derived from these fragment ions confirms that Thr133 in this peptide is phosphorylated. Results are representatives of two independent experiments.

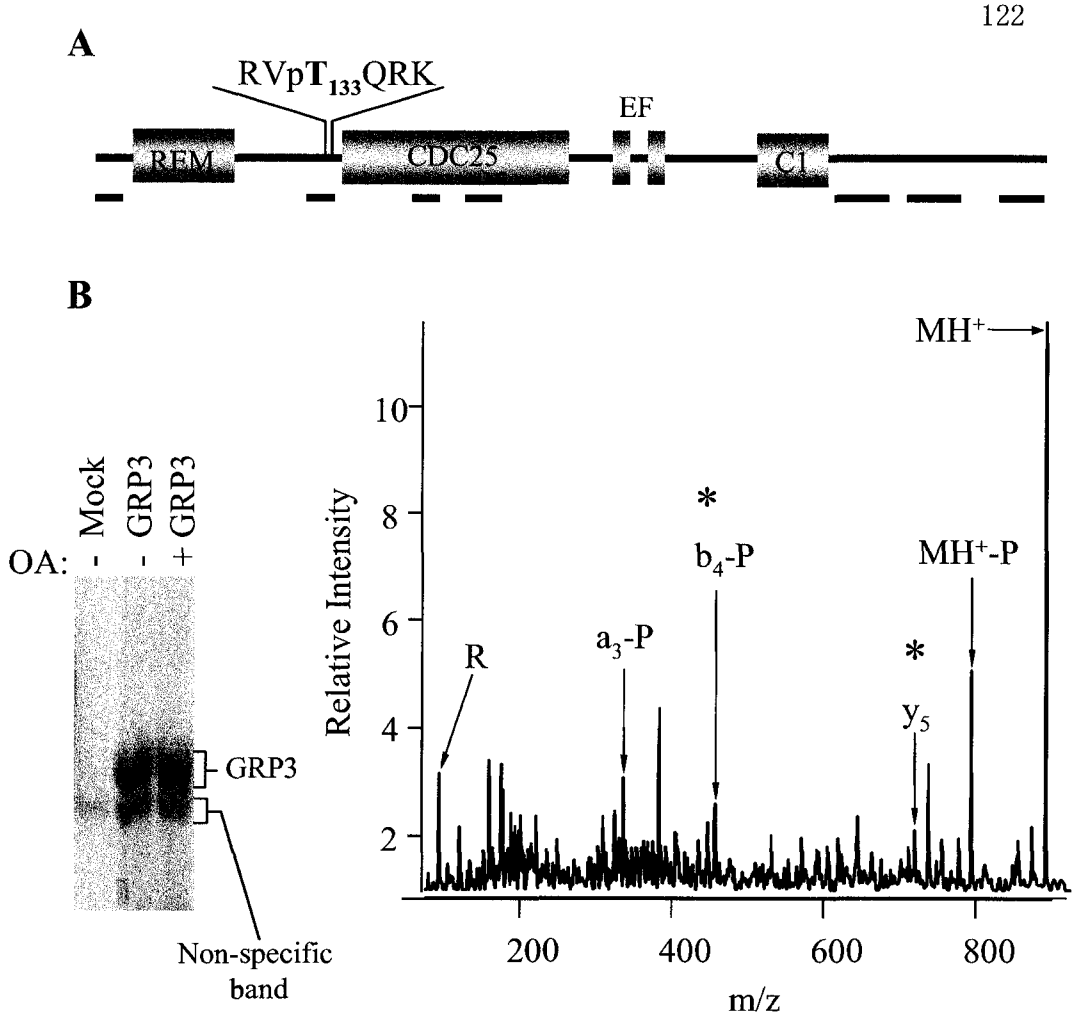


FIGURE 3.4 Mass spectrometry analysis of RasGRP3 expressed in sf9 cells. (A) A schematic diagram of RasGRP3 with positions of identified phosphopeptides shown. Sf9 cells were infected with recombinant baculovirus expressing RasGRP3 for two days then treated with 1uM okadaic acid for 1 hour before lysis. RasGRP3 were immunprecipitates from cell lysates and analyzed with mass spectrometry. Relative positions of phosphopeptides (bars) are shown. **(B)** MS/MS of phosphopeptide 131-RVpTQRK-135 (m/z at 867.45) in RasGRP3. Coomassie blue staining of RasGRP3 immunprecipitates were shown (Left). The peak labeled MH+ corresponds to the protonated ion of the phosphorylated parental tryptic peptide. Peaks resulting from neutral loss of a phosphate group from an ion are marked by "-P". *, denotes the loss of a "-NH3". Results are representative of two independent experiments. R, stands for immonium ion of arginine.

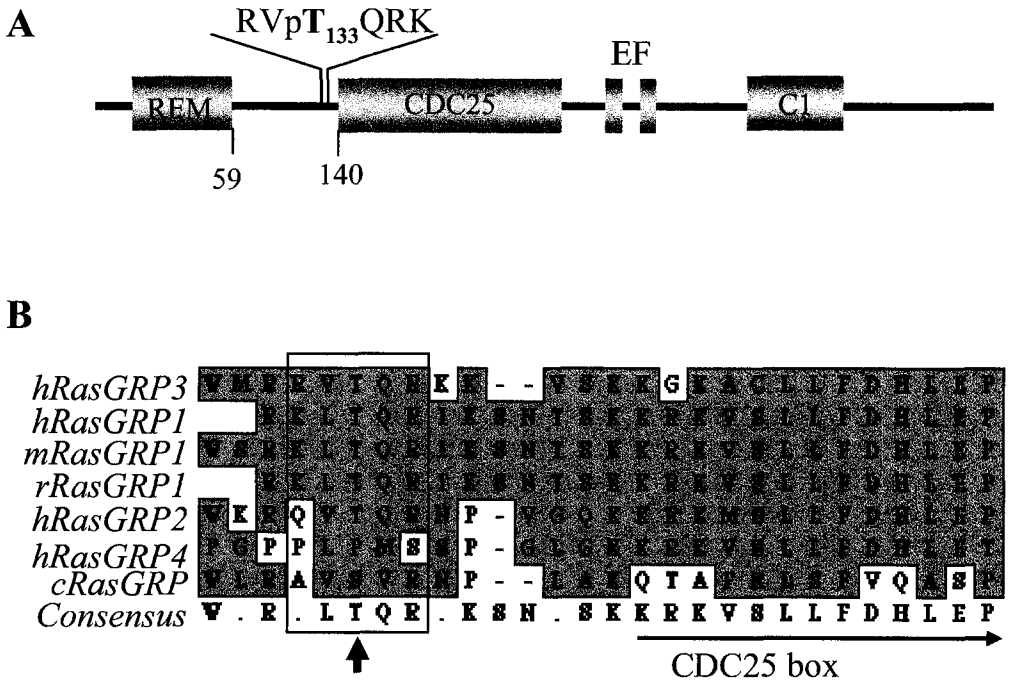


FIGURE 3.5 Thr133 is conserved in RasGRP family members. (A) A schematic diagram of RasGRP3 with the position of the deduced phosphorylation and the sequence of the diagnostic peptide shown. REM: Ras exchange motif; CDC25: Ras activator domain; EF: calcium-binding domain; C1: DAG-binding domain. **(B)** The region surrounding the PKC phosphorylation site in human RasGRP3 is shown aligned with other RasGRP family members including mouse and rat RasGRP1. cRasGRP, *C.elegans* RasGRP. Consensus sequence is summarized at the bottom (consensus). The position of the partially conserved Thr is shown with a vertical arrow while the box highlights flanking basic residues. The amino terminal region of the CDC25 box is also indicated.

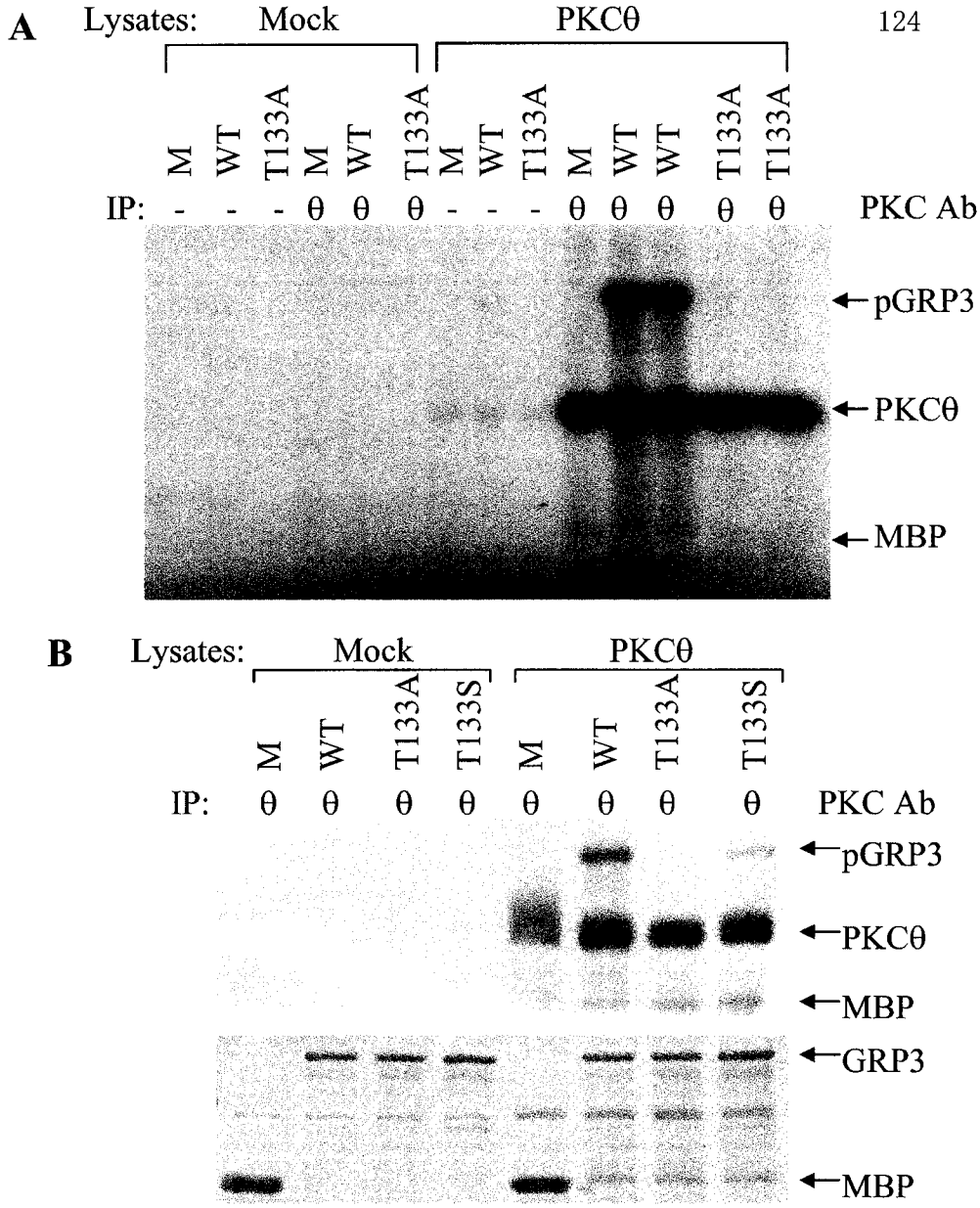


FIGURE 3.6 RasGRP3 Thr133Ala substitution blocks *in vitro* phosphorylation by PKCθ while serine replacement does not. (A) The effect of a RasGRP3 Thr133Ala substitution on phosphorylation by PKCθ is shown. Parental MBP (M), MBP-RasGRP3 (WT) or MBP-RasGRP3 Thr133Ala (T133A) were used as substrates in an immune complex kinase assay, as described in Materials and Methods. Arrows show, from top to bottom, phosphorylation of MBP-RasGRP3 (pGRP3), auto-phosphorylation of PKCθ and the position of parental MBP protein. Some lanes are in duplicate. **(B)** Similar experiments as in **(A)** were performed with Thr133Ser (T133S). Bottom: Coomassie staining of recombinant MBP fusion proteins. Results are representative of three independent experiments.

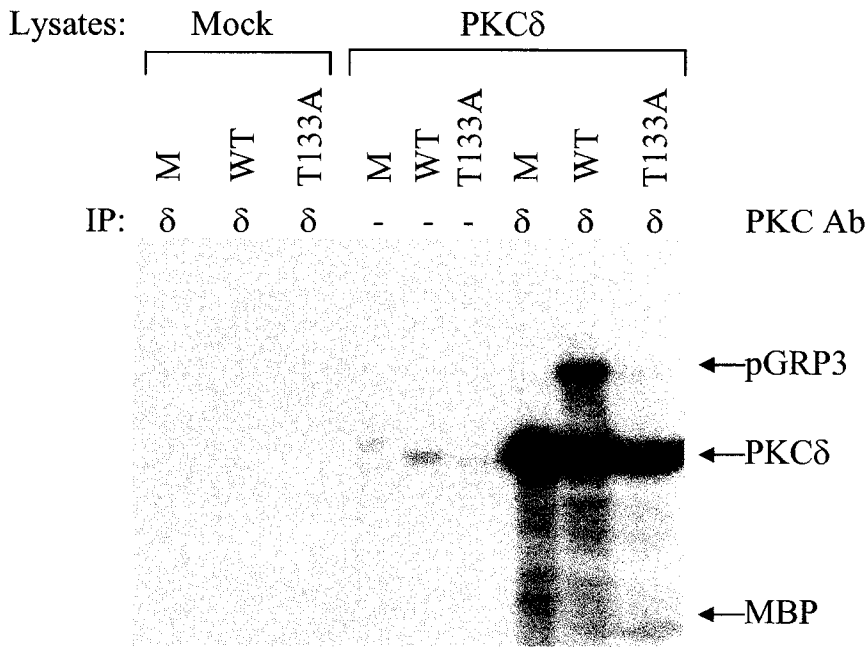


FIGURE 3.7 RasGRP3 Thr133Ala substitution also blocks *in vitro* phosphorylation by PKCδ. Similar experiments as in Figure 3.6A were performed with PKCδ. Results are representative of three independent experiments.

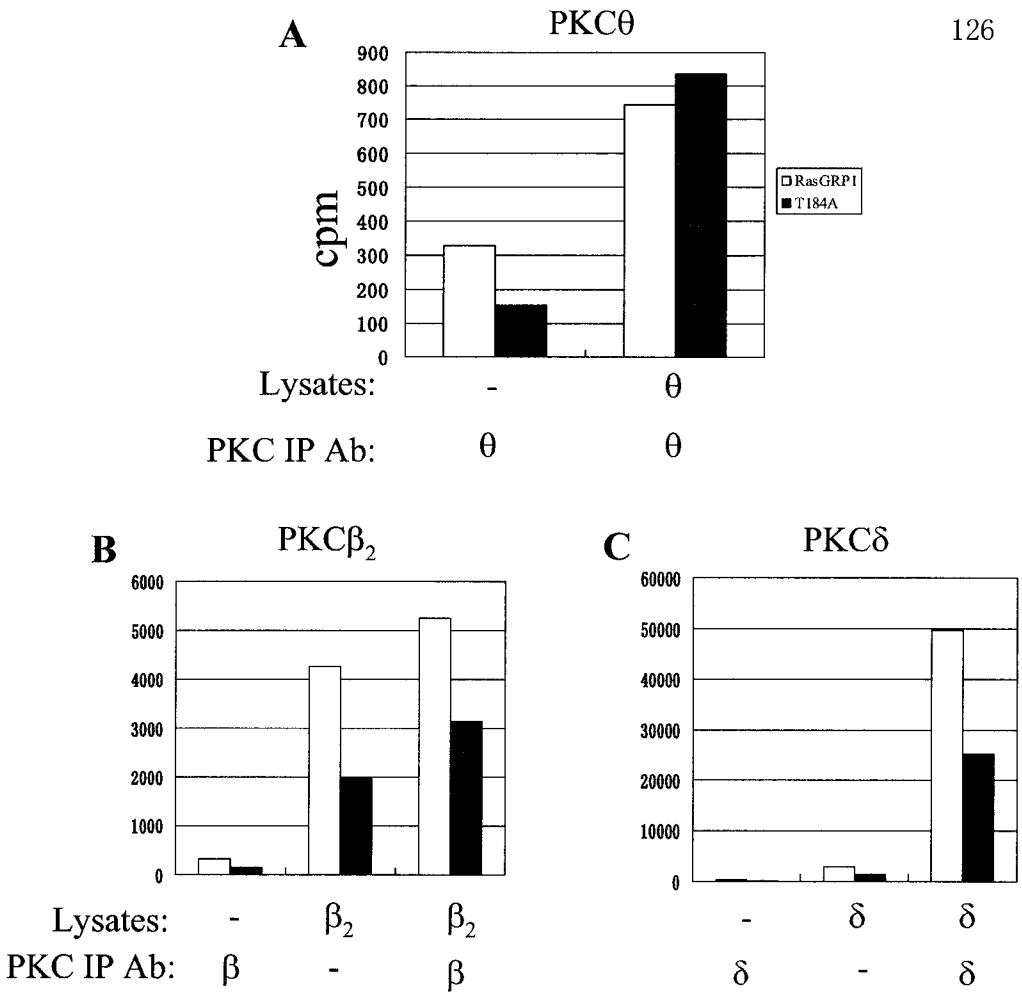


FIGURE 3.8 RasGRP1 Thr184Ala substitution decreased *in vitro* phosphorylation by PKCδ and PKCβ$_2$ by 50%. The effects of a RasGRP1 Thr184Ala substitution on *in vitro* phosphorylation by PKCθ **(A)**, β_2 **(B)** and δ **(C)** are shown. MBP-RasGRP1 (RasGRP1) or MBP-RasGRP1 Thr184Ala (T184A) were used as substrates in an immune complex kinase assay. PKC isoforms θ, β_2, and δ were immuno-precipitated with isoform-specific monoclonal antibody (PKC IP Ab) as indicated from Sf9 cell lysates prepared from cells infected with empty baculovirus or baculovirus expressing corresponding PKC isoforms (Lysates). Precipitated PKCs were used to phosphorylate MBP-RasGRP1 (RasGRP1) or MBP-RasGRP1 Thr184Ala (T184A) in an immune complex kinase assay. Phosphorylated proteins were separated on SDS/PAGE and coomassie-stained. Corresponding bands were excised. The [^{32}pγ]-ATP incorporations were measured by cherenkov counting on a scintillation counter. Cpm, counts per minute. Results are representatives of two independent experiments.

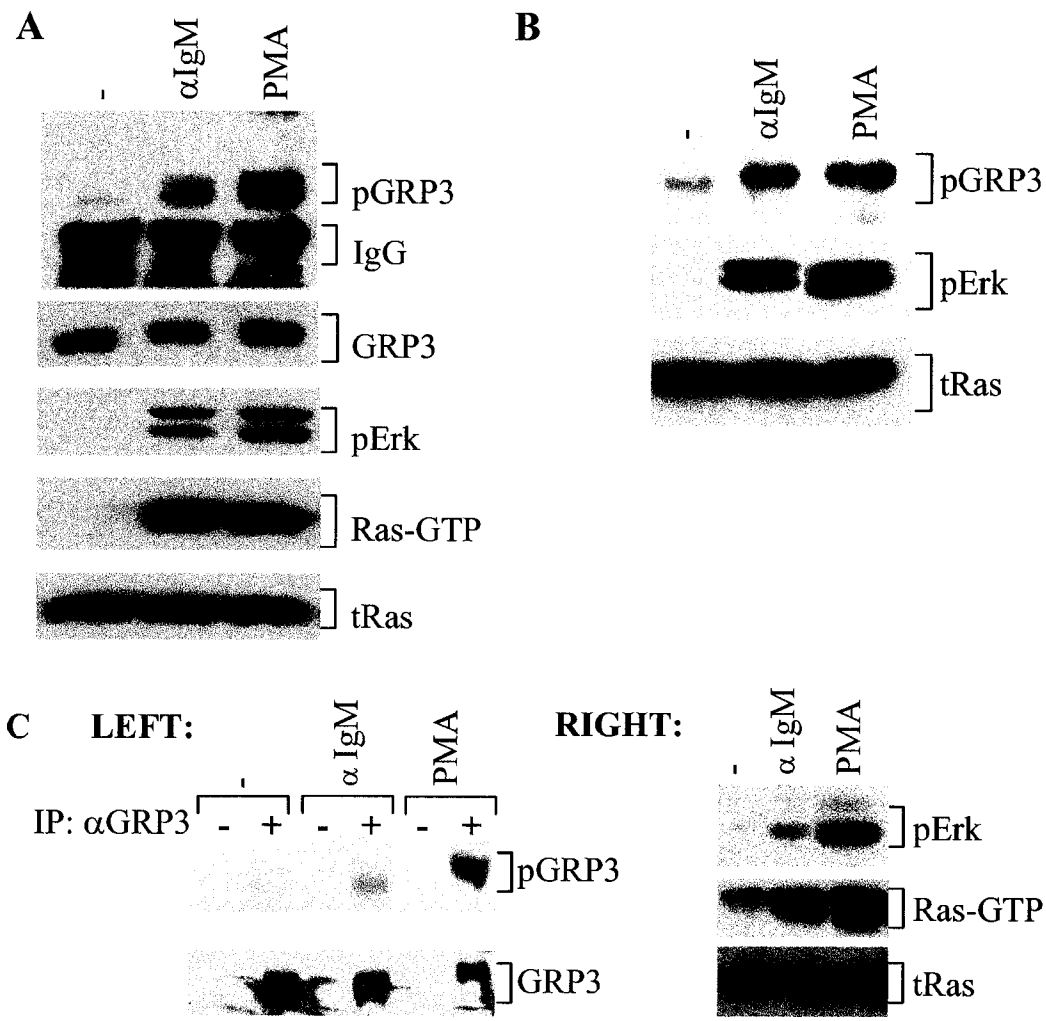


FIGURE 3.9 RasGRP3 phosphorylation on Thr133 occurs in B cells after stimulation and is correlated with Ras-Erk signaling. **(A)** Ramos B cells were left untreated or stimulated with PMA for 10 minutes or anti-IgM for 5 minutes and the levels of Thr133-phosphorylated RasGRP3 (pGRP3), RasGRP3 (GRP3), phosphorylated Erk , Ras-GTP and total Ras (tRas) were determined. **(B)** Ramos B cells were treated as above. Phosphorylated RasGRP3 was immune-precipitated with anti-3/pThr133 antibodies and precipitated RasGRP3 was then detected by immunoblotting using anti-GRP3 antibody. Levels of phospho-Erk and total Ras in lysates were also determined. **(C)** Mouse B cells were stimulated with anti-IgM antibodies for 5 minutes or PMA for 10 minutes. RasGRP3 phosphorylation and RasGRP3 recovery were determined as above (left panel) as were the levels of phospho-Erk, Ras-GTP and total Ras in lysates (right panel). Results are representatives of three independent experiments.

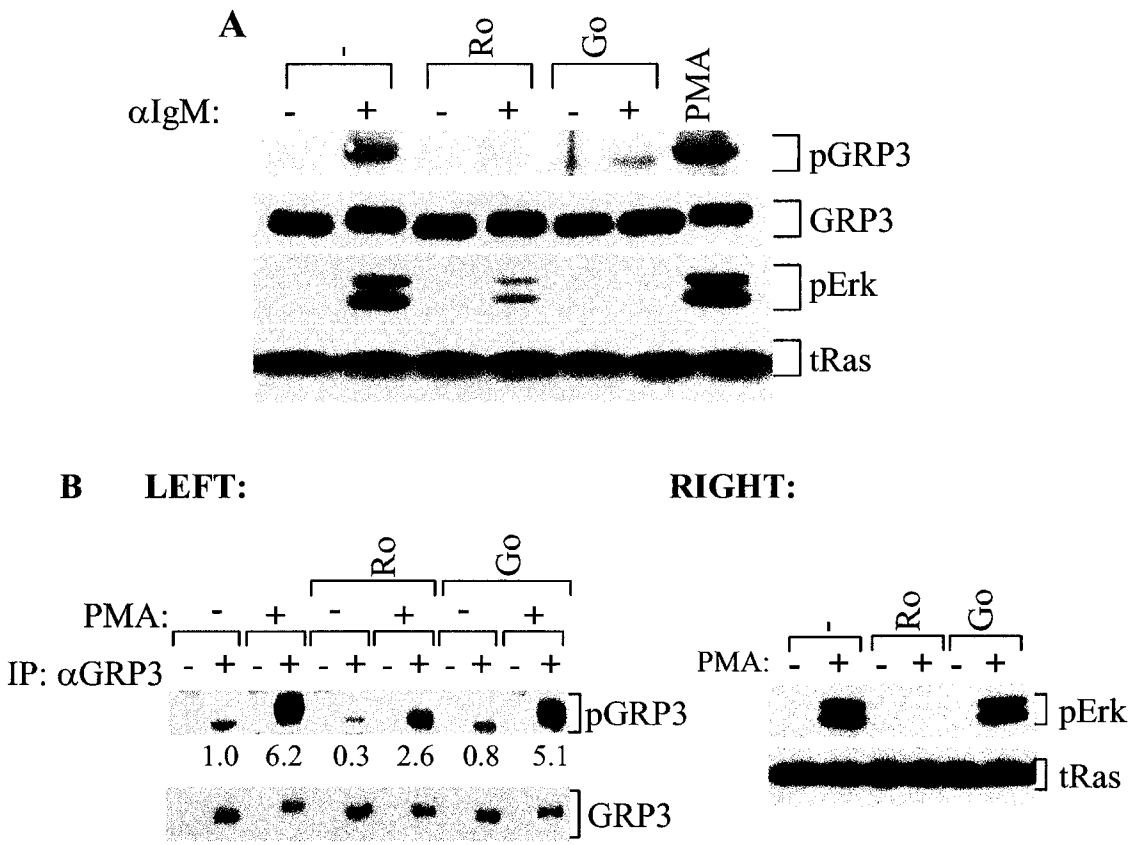


FIGURE 3.10 PKC-dependent RasGRP3 phosphorylation on Thr133 in B cells. (A) Ramos B cells were pretreated with inhibitors followed by stimulation with anti-IgM antibodies, as indicated, and lysates were probed for Thr133-phosphorylated RasGRP3 (pGRP3), total RasGRP3 (GRP3) and phosphorylated Erk. PMA treated cells served as a positive control. **(B)** Ramos B cells were pretreated as in (A), then stimulated with PMA for 10 minutes followed by analysis of RasGRP3 (left panel) and other signaling molecules (right panel). Numbers are quantification of band intensity. Ro, Ro318220; Go, Go6976. Results are representative of at least two independent experiments.

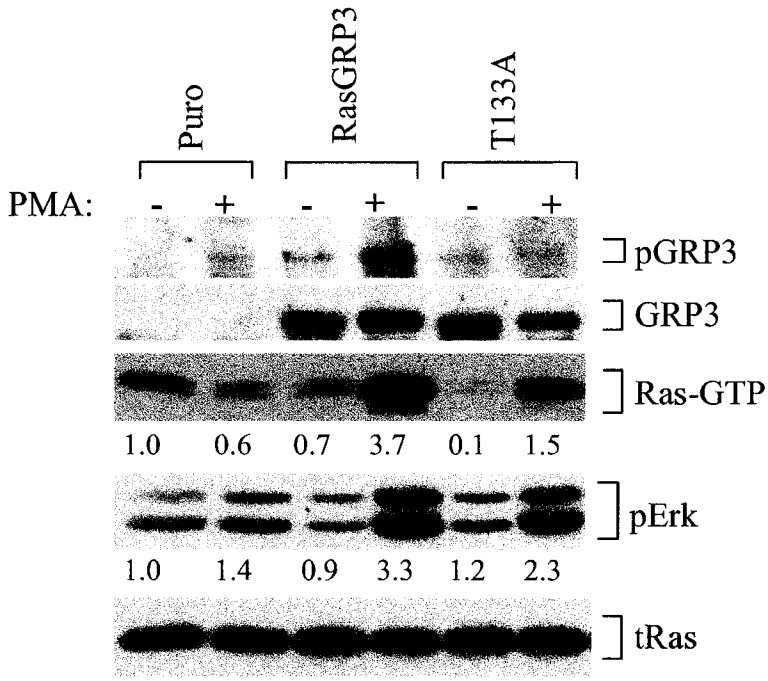


FIGURE 3.11 RasGRP3 Thr133Ala expressed in Rat2 cells is defective at PMA-induced Ras-Erk signaling. Rat2 cells expressing empty vector (Puro), RasGRP3 or RasGRP3Thr133Ala (T133A) were either left unstimulated or stimulated with PMA for 10 minutes. Lysates were assayed for Thr133-phosphorylated RasGRP3 (pGRP3), RasGRP3 (GRP3); phospho-Erk (pErk), Ras-GTP and total Ras (tRas) by immunoblotting. Numbers are quantification of band intensity. Results are representative of three independent experiments.

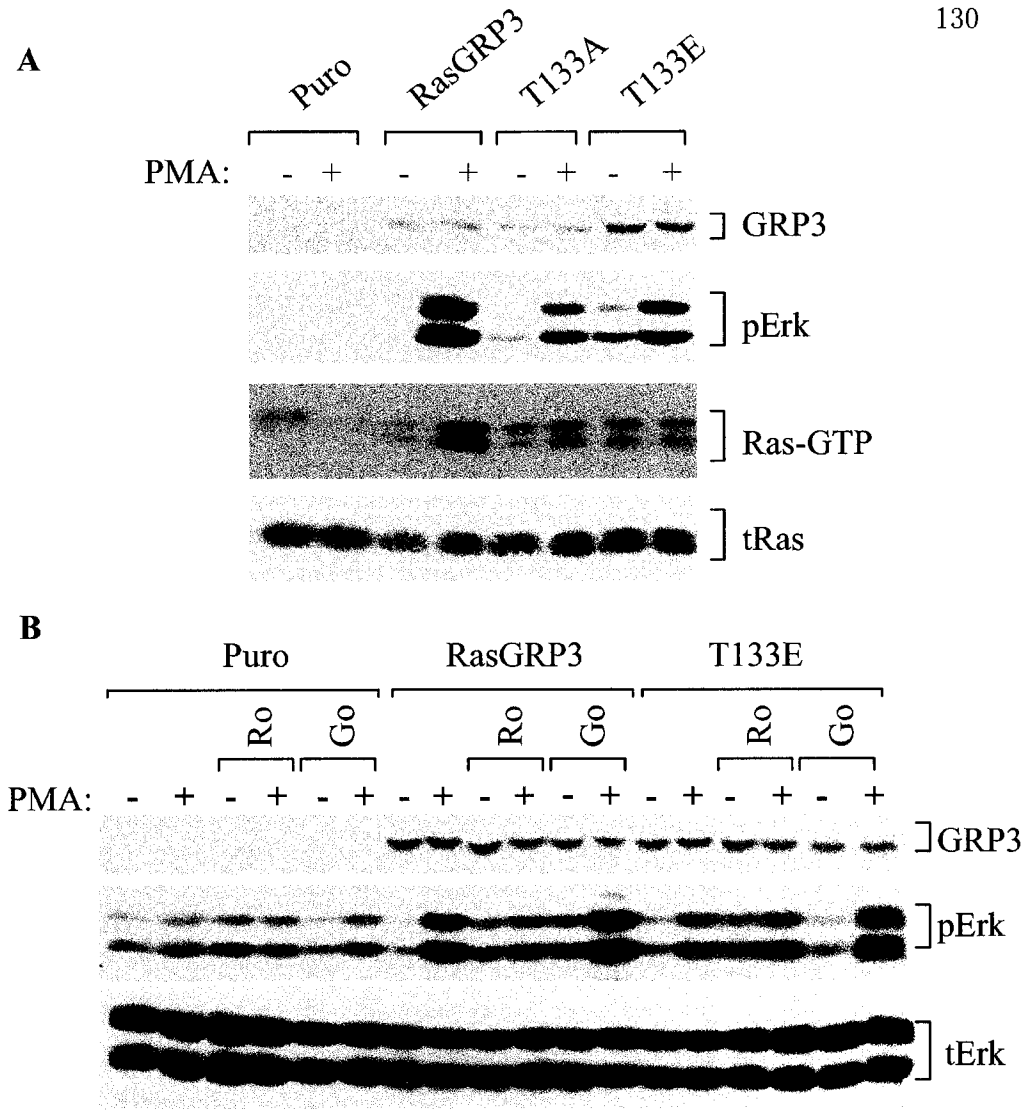


FIGURE 3.12 RasGRP3 phospho-mimic mutant Thr133Glu partially rescued signaling defects of Thr133Ala mutant in Rat2, and is not sensitive to PKC inhibition. (**A**) Rat2 cells expressing empty vector (Puro), RasGRP3, RasGRP3Thr133Ala (T133A), and RasGRP3Thr133Glu (T133E) were either left unstimulated or stimulated with PMA for 10 minutes. Lysates were assayed for RasGRP3 (GRP3), phospho-Erk (pErk), Ras-GTP and total Ras (tRas) by immunoblotting. (**B**) Rat2 expressing Puro, RasGRP3, and T133E mutant were pretreated with PKC inhibitors Ro318220 (Ro) or Go6976 (Go) for 15 minutes, followed by PMA treatment for 10 minutes. Cell lysates were prepared and analyzed for RasGRP3, phospho-Erk, and total Erk (tErk). Results are representative of two independent experiments.

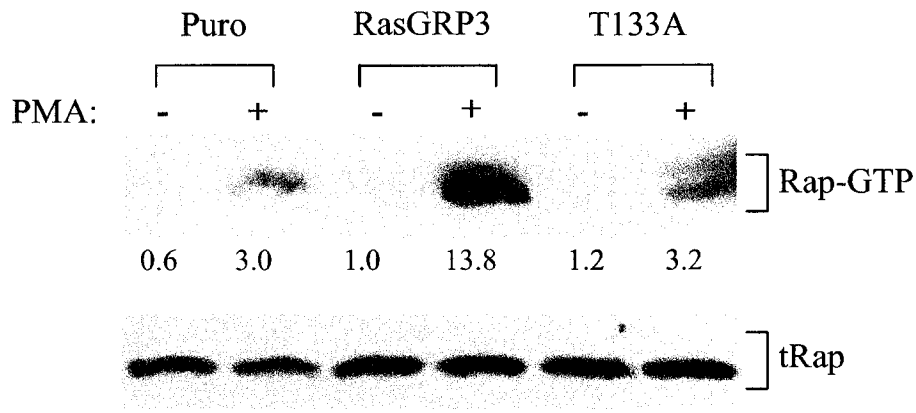


FIGURE 3.13 RasGRP3 Thr133Ala expressed in Rat2 cells is defective at PMA-induced Rap activation. Rat 2 cells similar to those in Figure 3.11 were assayed for Rap-GTP and total Rap (tRap). Numbers are quantification of band intensity. Results are representative of two experiments.

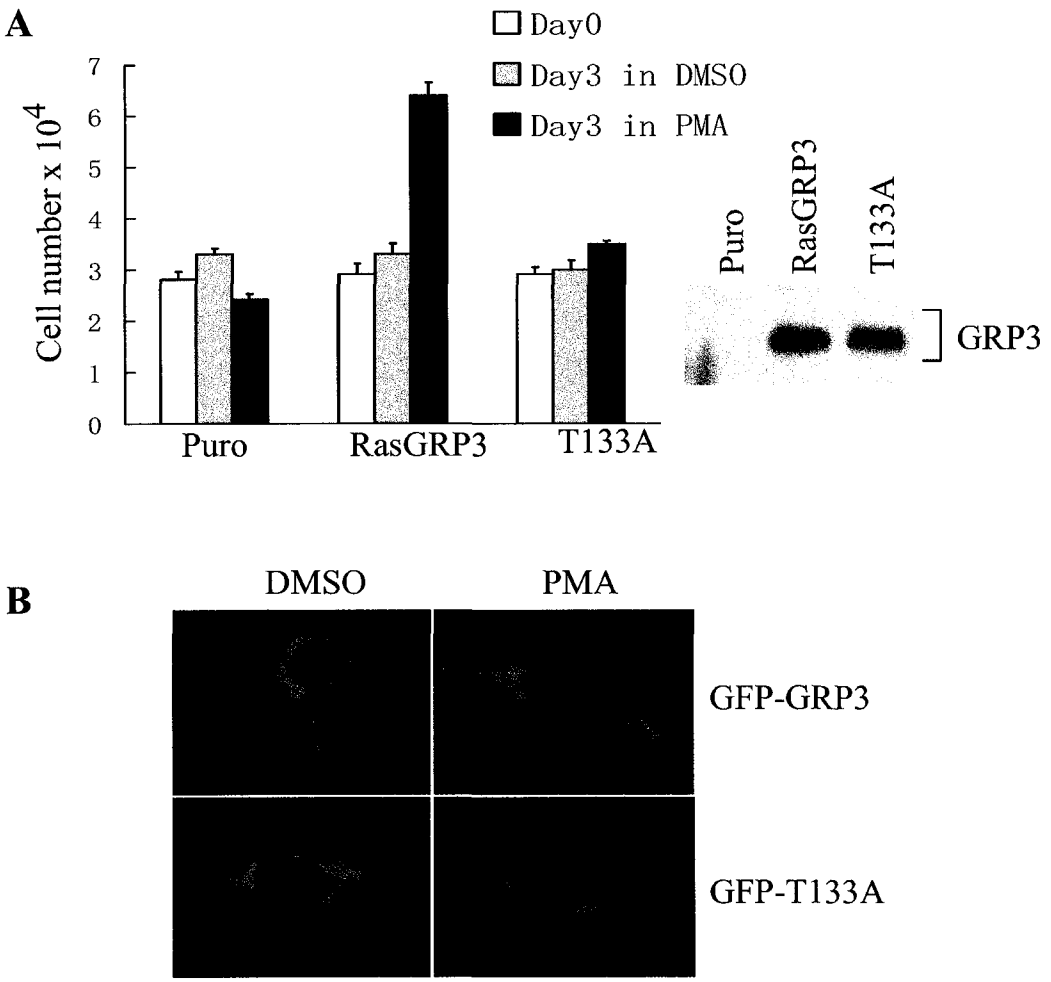


FIGURE 3.14 RasGRP3 Thr133Ala mutant blocks PMA-dependent growth in Rat2 cells while not inhibiting RasGRP3 membrane recruitment. (A) Rat2 cells similar to those in Figure 3.11 were plated in complete medium. One set of plates was harvested later the same day, while two sets were incubated for three days in medium containing 0.5% FBS alone or supplemented with 100nM PMA before performing cell counts. Values represent the means ± standard deviations of triplicates within a single experiment ($P<0.01$). RasGRP3 expression levels in these cells were estimated using anti-RasGRP3 antibodies (right panel). Results are representative of three independent experiments. **(B)** HEK293 cells were transiently transfected with either GFP-RasGRP3 or the alanine mutant (GFP-T133A) cDNA for 48 hours. cells were then treated with DMSO or PMA (100nM) for 10 minutes and fixed. Confocal images were taken. Cells shown are representatives from three independent experiments.

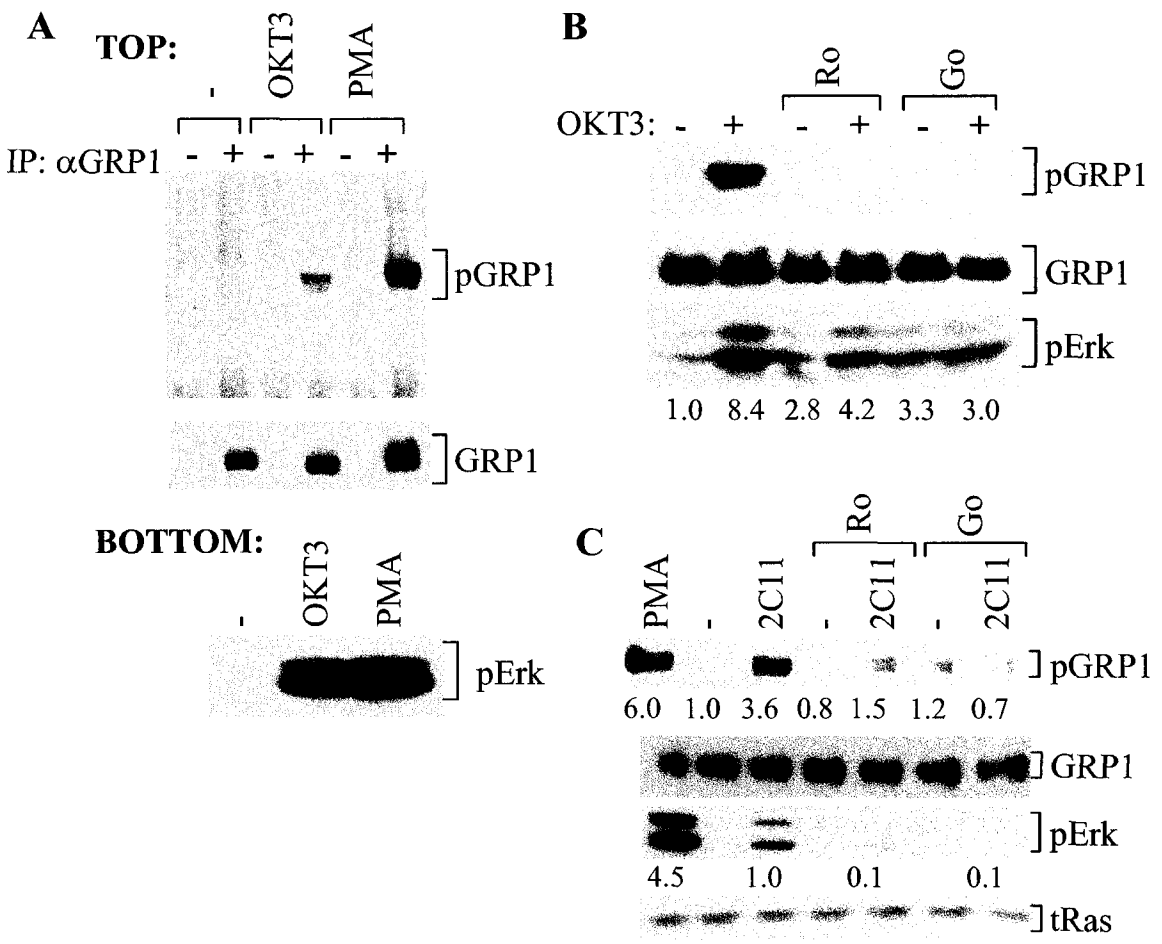


FIGURE 3.15 RasGRP1 in T cells is subject to PKC-dependent regulatory phosphorylation on Thr184. (**A**) Jurkat T cells were left untreated or were stimulated with anti-TCR antibodies (OKT3) for 5 minutes or PMA for 10 minutes and lysates were probed for Thr184-phosphorylated RasGRP1 (pGRP1) and total RasGRP1 (GRP1; top panel). Signaling to Erk was also monitored (bottom panel). (**B**) Jurkat T cells were pre-treated with PKC inhibitors and stimulated with the anti-TCR antibody OKT3. Lysates were assayed for phospho-RasGRP1 and total RasGRP1, as above. Lysates were also probed for phospho-Erk to monitor signaling. (**C**) Mouse T cells were pretreated with inhibitors and stimulated with anti-TCR antibodies (2C11) or PMA, and lysates were probed for signaling molecules as above. Numbers are quantification of band intensity. Numbers are quantification of band intensity. Results are representative of at least two independent experiments.

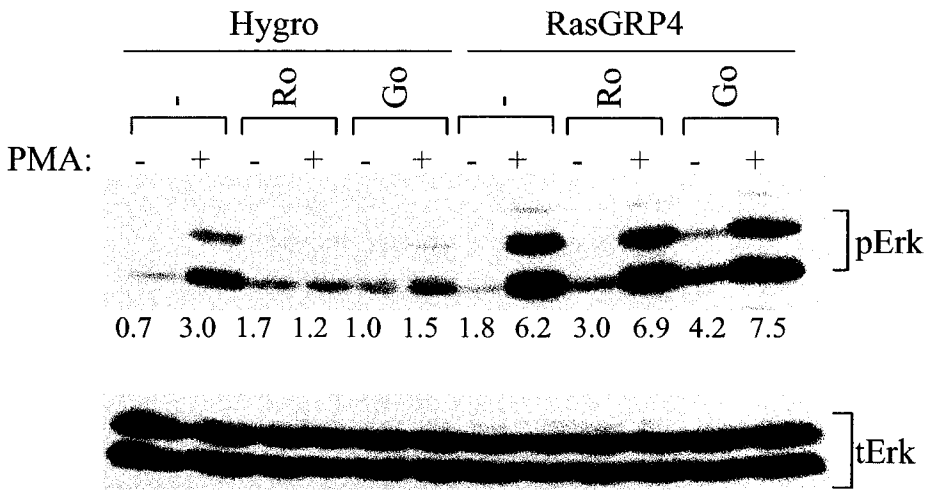


FIGURE 3.16 RasGRP4 is regulated by other means. Rat2 cells expressing empty vector pBabeHygro (Hygro) or RasGRP4 in the same vector were pre-treated with inhibitors, followed by PMA treatment, as indicated. Total cell lysates were blotted for phospho-Erk to monitor Ras-Erk signaling and total Erk (tErk) as a loading control. Numbers are quantification of band intensity. Results are representative of at least two independent experiments.

References:

Aiba, Y., M. Oh-hora, et al. (2004). "Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation." Proc Natl Acad Sci U S A **101**(47): 16612-7.

Aletta, J. M., T. R. Cimato, et al. (1998). "Protein methylation: a signal event in post-translational modification." Trends Biochem Sci **23**(3): 89-91.

Antony, P., J. B. Petro, et al. (2003). "B cell receptor directs the activation of NFAT and NF-kappaB via distinct molecular mechanisms." Exp Cell Res **291**(1): 11-24.

Balboa, M. A., M. Izquierdo, et al. (1992). "Analysis of different protein kinase C-dependent events in T cells from allogeneic bone marrow transplantation recipients." Clin Exp Immunol **87**(3): 478-84.

Blois, J. T., J. M. Mataraza, et al. (2004). "B cell receptor-induced cAMP-response element-binding protein activation in B lymphocytes requires novel protein kinase Cdelta." J Biol Chem **279**(29): 30123-32.

Brodie, C., R. Steinhart, et al. (2004). "PKCdelta associates with and is involved in the phosphorylation of RasGRP3 in response to phorbol esters." Mol Pharmacol **66**(1): 76-84.

Caloca, M. J., H. Wang, et al. (2001). "Phorbol esters and related analogs regulate the subcellular localization of beta 2-chimaerin, a non-protein kinase C phorbol ester receptor." J Biol Chem **276**(21): 18303-12.

Cantrell, D. A., J. D. Graves, et al. (1992). "T lymphocyte activation signals." Ciba Found Symp **164**: 208-18; discussion 218-22.

Coughlin, J. J., S. L. Stang, et al. (2005). "RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling." J Immunol **175**(11): 7179-84.

Dounay, A. B. and C. J. Forsyth (2002). "Okadaic acid: the archetypal serine/threonine protein phosphatase inhibitor." Curr Med Chem **9**(22): 1939-80.

Dower, N. A., S. L. Stang, et al. (2000). "RasGRP is essential for mouse thymocyte differentiation and TCR signaling." Nat Immunol **1**(4): 317-21.

Downward, J. (1996). "Control of ras activation." Cancer Surv **27**: 87-100.

Downward, J., J. D. Graves, et al. (1990). "Stimulation of p21ras upon T-cell activation." Nature **346**(6286): 719-23.

Ebinu, J. O., D. A. Bottorff, et al. (1998). "RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs." Science **280**(5366): 1082-6.

Ebinu, J. O., S. L. Stang, et al. (2000). "RasGRP links T-cell receptor signaling to Ras." Blood **95**(10): 3199-203.

Haebel, S., T. Albrecht, et al. (1998). "Electrophoresis-related protein modification: alkylation of carboxy residues revealed by mass spectrometry." Electrophoresis **19**(5): 679-86.

Izquierdo, M., J. Downward, et al. (1992). "Role of protein kinase C in T-cell antigen receptor regulation of p21ras: evidence that two p21ras regulatory pathways coexist in T cells." Mol Cell Biol **12**(7): 3305-12.

Kang, S. W., M. I. Wahl, et al. (2001). "PKCbeta modulates antigen receptor signaling via regulation of Btk membrane localization." Embo J **20**(20): 5692-702.

Kazanietz, M. G. (2002). "Novel "nonkinase" phorbol ester receptors: the C1 domain connection." Mol Pharmacol **61**(4): 759-67.

Kazanietz, M. G., M. J. Caloca, et al. (2000). "Pharmacology of the receptors for the phorbol ester tumor promoters: multiple receptors with different biochemical properties." Biochem Pharmacol **60**(10): 1417-24.

Lackner, M. R., S. J. Nurrish, et al. (1999). "Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release." Neuron **24**(2): 335-46.

Leitges, M., C. Schmedt, et al. (1996). "Immunodeficiency in protein kinase cbeta-deficient mice." Science **273**(5276): 788-91.

Li, L., Y. Yang, et al. (2003). "RasGRP4 regulates the expression of prostaglandin D2 in human and rat mast cell lines." J Biol Chem **278**(7): 4725-9.

Liu, H., J. Stupak, et al. (2004). "Open tubular immobilized metal ion affinity chromatography combined with MALDI MS and MS/MS for identification of protein phosphorylation sites." Anal Chem **76**(14): 4223-32.

Liu, W. S. and C. A. Heckman (1998). "The sevenfold way of PKC regulation." Cell Signal **10**(8): 529-42.

Lorenzo, P. S., M. Beheshti, et al. (2000). "The guanine nucleotide exchange factor RasGRP is a high -affinity target for diacylglycerol and phorbol esters." Mol Pharmacol **57**(5): 840-6.

Lorenzo, P. S., J. W. Kung, et al. (2001). "Phorbol esters modulate the Ras exchange factor RasGRP3." Cancer Res **61**(3): 943-9.

Loyet, K. M., J. T. Stults, et al. (2005). "Mass spectrometric contributions to the practice of phosphorylation site mapping through 2003: a literature review." Mol Cell Proteomics **4**(3): 235-45.

Mathis, D. and G. L. King (2002). "B-cell signaling: protein kinase Cdelta puts the brakes on." Curr Biol **12**(16): R554-6.

Mattingly, R. R. (1999). "Phosphorylation of serine 916 of Ras-GRF1 contributes to the activation of exchange factor activity by muscarinic receptors." J Biol Chem **274**(52): 37379-84.

Mecklenbrauker, I., K. Saijo, et al. (2002). "Protein kinase Cdelta controls self-antigen-induced B-cell tolerance." Nature **416**(6883): 860-5.

Miyamoto, A., K. Nakayama, et al. (2002). "Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta." Nature **416**(6883): 865-9.

Nurrish, S., L. Segalat, et al. (1999). "Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites." Neuron **24**(1): 231-42.

Oh-hora, M., S. Johmura, et al. (2003). "Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor signaling." J Exp Med **198**(12): 1841-51.

Porfiri, E. and F. McCormick (1996). "Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1." J Biol Chem **271**(10): 5871-7.

Priatel, J. J., S. J. Teh, et al. (2002). "RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation." Immunity **17**(5): 617-27.

Reuther, G. W., Q. T. Lambert, et al. (2002). "RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia." J Biol Chem **277**(34): 30508-14.

Roose, J. P., M. Mollenauer, et al. (2005). "A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells." Mol Cell Biol **25**(11): 4426-41.

Rozengurt, E., J. Sinnett-Smith, et al. (1995). "Protein kinase D (PKD): a novel target for diacylglycerol and phorbol esters." Mutat Res **333**(1-2): 153-60.

Saijo, K., I. Mecklenbrauker, et al. (2002). "Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha." J Exp Med **195**(12): 1647-52.

Saijo, K., I. Mecklenbrauker, et al. (2003). "B cell immunity regulated by the protein kinase C family." Ann N Y Acad Sci **987**: 125-34.

Shindo, M., K. Irie, et al. (2001). "Diacylglycerol kinase gamma is one of the specific receptors of tumor-promoting phorbol esters." Biochem Biophys Res Commun **289**(2): 451-6.

Stope, M. B., F. Vom Dorp, et al. (2004). "Rap2B-dependent stimulation of phospholipase C-epsilon by epidermal growth factor receptor mediated by c-Src phosphorylation of RasGRP3." Mol Cell Biol **24**(11): 4664-76.

Teixeira, C., S. L. Stang, et al. (2003). "Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3." Blood **102**(4): 1414-20.

Thuille, N., T. Gruber, et al. (2004). "Protein kinase C beta is dispensable for TCR-signaling." Mol Immunol **41**(4): 385-90.

Tognon, C. E., H. E. Kirk, et al. (1998). "Regulation of RasGRP via a phorbol ester-responsive C1 domain." Mol Cell Biol **18**(12): 6995-7008.

Vierra-Green, C. A., H. T. Orr, et al. (2005). "Identification of a novel phosphorylation site in ataxin-1." Biochim Biophys Acta **1744**(1): 11-8.

Wang, H. and M. G. Kazanietz (2002). "Chimaerins, novel non-protein kinase C phorbol ester receptors, associate with Tmp21-I (p23): evidence for a novel anchoring mechanism involving the chimaerin C1 domain." J Biol Chem **277**(6): 4541-50.

Yamashita, S., N. Mochizuki, et al. (2000). "CalDAG-GEFIII activation of Ras, R-ras, and Rap1." J Biol Chem **275**(33): 25488-93.

Yang, H., D. Cooley, et al. (2003). "Phosphorylation of the Ras-GRF1 exchange factor at Ser916/898 reveals activation of Ras signaling in the cerebral cortex." J Biol Chem **278**(15): 13278-85.

Yang, X. J. (2005). "Multisite protein modification and intramolecular signaling." Oncogene **24**(10): 1653-62.

Yang, Y., L. Li, et al. (2002). "RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function." J Biol Chem **277**(28): 25756-74.

Zeller, M. and S. Konig (2004). "The impact of chromatography and mass spectrometry on the analysis of protein phosphorylation sites." Anal Bioanal Chem **378**(4): 898-909.

CHAPTER 4

IN VITRO INTERACTION OF RASGRP1 WITH GRB2 FAMILY MEMBERS

Chapter 4

4.1 Introduction

4.1.1 Presence of a putative proline-rich region in RasGRPs

Sequence analysis on RasGRP1 reveals the presence of a putative proline-rich region with a PXXP (X represents any amino acid) motif immediately downstream of the catalytic CDC25 box (Figure 4.1 A). Similar primary structures are found in all RasGRP family members (Figure 4.1 B). The PXXP motif is present in a plethora of intracellular proteins and serves as a binding site for SH3 domains to mediate intramolecular interactions between the PXXP- and SH3 domain-containing proteins, such as Grb2 family members (Ren *et al.* 1993). Binding of PXXP to the SH3 domains is a weak process with moderate specificity (Mayer 2001). Thus a PXXP motif can recognize several SH3 domain-containing proteins, favoring the formation of a nonlinear, complicated network consisting of multiple signaling proteins (Mayer 2001; Tong *et al.* 2002).

4.1.2 Grb2

Grb2 (growth factor receptor bound protein 2) is the most extensively studied adaptor protein involved in mediating Ras activation in growth factor-stimulated signaling pathways (Lowenstein et al. 1992; Chardin et al. 1995). It is made up of one SH2 domain flanked by two SH3 domains (Figure 4.2) (Lowenstein *et al.* 1992). As discussed in previous chapters, Grb2 mediates membrane translocation and activation of Sos by binding to the PXXP motif found in Sos via its two SH3 domains, while recognizing tyrosine-phosphorylated intracellular tails of RTKs via its SH2 domain (Lowenstein et al. 1992). Despite the structural similarity between the two SH3 domains, there is evidence showing they behave differently. For instance, although both SH3 domains are required for Sos binding, it was shown that the N-terminal SH3 domain contributes more than its C-terminal counterpart (Li *et al.*

1993; Rozakis-Adcock *et al.* 1993; Vidal *et al.* 1999).

In addition to Sos, Grb2 interacts with several proteins including c-Cbl, Gab1, Gab2, SLP-76, and Vav *in vivo* (Lim and Richards 1994; Lewitzky et al. 2001; Nishida et al. 2001; Berry et al. 2002; Yamasaki et al. 2003).

In T cells, Grb2 has been shown to be recruited to the membrane upon TCR ligation through binding to tyrosine-phosphorylated LAT via its SH2 domain (Weber *et al.* 1998; Zhang *et al.* 1998a; Zhang *et al.* 1998b; Zhang *et al.* 2000). Thus, Grb2 has the potential to recruit Sos and couple TCR signaling to Ras activation. Deletion of the Grb2-binding site of SLP-76 eliminates the ability of SLP-76 to increase IL-2 promoter activity upon TCR crosslinking (Zhang et al. 1998b). However, a direct role of Sos in mediating TCR-induced Ras activation *in vivo* still remains to be established (Buday *et al.* 1994; Reif *et al.* 1994; Jackman *et al.* 1995; Zhang *et al.* 2000).

4.1.3 The Grb2 family of adaptor proteins

The Grb2 family of adaptor proteins consists of two other members: Grap (Grb2-related adaptor protein) and Gads (Grb2-related adaptor downstream of Shc) (Liu, S. K. *et al.* 2001).

Grap

In contrast to Grb2, which is expressed ubiquitously, Grap is expressed mainly in hematopoietic cells. Grap shares ~60% amino acid sequence identity with Grb2 and possesses the same structural arrangement of SH3-SH2-SH3 domains (Figure 4.2) (Feng et al. 1996). The SH2 and SH3 domains of Grap and Grb2 appear to have overlapping ligand specificity. Similar to Grb2, Grap binds Sos1, primarily through its N-terminal SH3 domain (Feng *et al.* 1996). In contrast to the early embryonic death phenotype of Grb2 null mice, no apparent phenotypes were observed in Grap knockout mice (Cheng *et al.* 1998; Liu, S. K. *et al.* 2000). Therefore, Grap is proposed to function redundantly to amplify TCR activation of Ras.

Gads

Gads was cloned simultaneously by five independent groups using different screening methods (Liu, S. K. and McGlade 1998). Thus in addition to the more commonly used name, Gads is also known as Mona (Monocytic adaptor) (Bourette *et al.* 1998), Grap2 (Grb2-related adaptor protein 2) (Qiu *et al.* 1998; Asada *et al.* 1999)), GrpL (Grb2-related protein of the lymphoid system) (Law *et al.* 1999), or Grf40 (Grb2 family member of 40 kD) (Asada et al. 1999). Except for one SH2 domain and two flanking SH3 domains similar to those found in Grb2 and Grap, Gads also posses an additional 120-amino acid linker region located between SH2 domain and the C-terminal SH3 domain. This sequence contains proline-rich regions and has no significant homology to any known protein in the Genbank database (Figure 4.2). The functional significance of the linker region remains unclear. However, several studies showed the presence of a caspase cleavage site in the linker region and suggested a caspase cleavage-dependent role of Gads in modulating TCR signaling (Berry et al. 2001; Yankee et al. 2001).

Gads is mostly expressed in T lymphocytes (Liu, S. K. and McGlade 1998; Asada *et al.* 1999; Law *et al.* 1999). Coimmunoprecipitation experiments and mutagenesis analysis demonstrated that Gads used its SH3 domain to bind SLP-76, while its SH2 domain bound to LAT (Liu, S. K. and McGlade 1998; Asada *et al.* 1999; Law *et al.* 1999). Gads binds to SLP-76 with much higher affinity, compared to the weak interactions between Grb2 and SLP-76. Moreover, Jurkat T cells overexpressing a C-terminal SH3 domain-deletion mutant of Gads failed to produce interlukin-2 (IL-2) upon TCR activation (Liu, S. K. and McGlade 1998; Asada *et al.* 1999; Law *et al.* 1999). These observations establish Gads as a critical component of TCR signaling machinery, functioning to mediate the interaction between LAT and SLP-76 upon TCR stimulation (Liu, S. K. *et al.* 2000; Yoder *et al.* 2001). In support of this idea, genetic analysis on Gads-deficient mice showed profound developmental defects only in T lymphocytes, but not other hematopoietic lineages, demonstrating a pivotal role of Gads in T cell development (Zuniga-Pflucker and

Lenardo 1996; Yoder et al. 2001). In addition, in mice transgenically expressing a SH2 domain-deleted dominant-negative form of Gads, developmental defects in thymus were correlated with severely suppressed PLC-γ1 phosphorylation and Erk activation (Kikuchi et al. 2001). Notably, many of the phenotypes observed in Gads-deficient mice are also found in RasGRP1 null mice (Dower *et al.* 2000).

4.1.4 Hypothesis

Despite some evidence for membrane recruitment following TCR ligation, Sos cannot be activated by tyrosine phosphorylation in T cells (Reif *et al.* 1994; Jackman *et al.* 1995). Instead, RasGRP1 has been shown to be the major Ras activator in T cells linking TCR activation to Ras signaling (Dower *et al.* 2000). Based on the above observations, the identification of the putative PXXP motif in RasGRP1 raises an attractive hypothesis: in addition to forming a complex with Sos, Grb2 or its family members may also stably associate with RasGRP1, through interactions between the SH3 domain and the PXXP motif, to mediate its membrane translocation in response to TCR stimulation.

4.2 Results

4.2.1 Grb2 directly interacts with RasGRP1 in vitro via its N-terminal SH3 domain.

To examine the physical interaction between Grb2 and RasGRP1 *in vitro*, a GST pull-down assay was performed. The purified GST or GST-Grb2 fusion proteins were immobilized on glutathione-Sepharose beads and incubated with equal amounts of recombinant MBP or MBP-RasGRP1 fusion protein to allow potential *in vitro* complex formation. Following three washes of the beads, proteins precipitated with GST fusions bound to the beads were analyzed by SDS-PAGE and immunoblotting with an anti-MBP antibody. The nitrocellulose membrane was ponceau stained to monitor GST fusion loading. A clear binding was detected between MBP-RasGRP1 and GST-Grb2, while no binding of MBP-RasGRP1 to GST alone

was detected (Figure 4.3).

There is evidence showing that the two SH3 domains in Grb2 are different in terms of the relative affinity to their ligands. For example, the N-terminal SH3 domain contributes more to Sos binding (Li et al. 1993). To determine the relative binding affinity of each Grb2 domain for RasGRP1, GST pull-down assays were also carried out using domain fragments of Grb2 (i.e., GST-Grb2-N-SH3, GST-Grb2-SH2, and GST-Grb2-C-SH3). Results showed that the N-terminal SH3 domain of Grb2 bound to RasGRP1 with efficiency comparable to that of the full length Grb2, while only very limited interaction was detected between C-terminal SH3 and RasGRP1 (Figure 4.4 A). As expected, no binding beyond background with the SH2 domain was detected (Figure 4.4 A). Subsequently, a mutagenesis study was carried out to characterize the interactions between RasGRP1 and full-length Grb2 mutants containing loss-of-function amino acid substitutions in each SH2 and SH3 domains. P49L (proline 49 to leucine) is a N-terminal SH3 domain mutant (Vidal *et al.* 1999). S90N (serine 90 to asparagine) is a SH2 domain mutant, and G203K (glycine 203 to lysine) contains a mutation in the C-terminal SH3 domain (Northrop et al. 1996). Interestingly, all three SH3 domain mutants of Grb2 failed to interact with RasGRP1 while disruption of SH2 domain of Grb2 did not affect its binding to RasGRP1, suggesting both SH3 domains are required for binding (Figure 4.4 B).

Taken together, these data demonstrated that Grb2 directly interacts with RasGRP1 *in vitro* through its SH3 domains. While both N- and C-terminal SH3 domains are required for RasGRP1 binding, the N-terminal SH3 domain has a much higher affinity toward RasGRP1.

4.2.2 The PXXP motif in RasGRP1 is partially involved in Grb2 binding.

The *in vivo* function of Grb2 is mediated by the selective binding of the SH3 domains to the PXXP motif in its ligands. To determine whether Grb2 binds to RasGRP1 through the putative PXXP motif, a RasGRP1 mutant (3PA) was generated, by PCR-mediated mutagenesis, to contain three proline substitutions at position 442,

445, and 448 by alanine. The binding of wildtype Grb2 to 3PA was analyzed by GST pull-down assay. The disruption of the PXXP motif resulted in about 50% reduction in precipitated MBP-RasGRP (Figure 4.5), suggesting the involvement of other interactions in Grb2 binding of RasGRP1.

4.2.3 RasGRP1 preferentially interacts with recombinant Gads in Jurkat T cells.

Despite the apparent *in vitro* binding between Grb2 and RasGRP1, I failed to repeat the interaction *in vivo* (data not shown). Given the fact that the proteins of the Grb2 adapter family may have partially overlapping, yet distinct protein binding properties (Lewitzky *et al.* 2001), one possibility is that RasGRP1 may specifically associate with other Grb2 members *in vivo*. As previously discussed, there are two closely related members in the Grb2 family: Grap and Gads. The T-lymphocyte specific adaptor Gads is of special interest since it has been shown to be essential for TCR-mediated Ras activation (Liu, S. K. and McGlade 1998). Its function is required for tyrosine phosphorylation-dependent PLC-γ1 activation and DAG production. More importantly, Gads-deficient mice share similar phenotypes with RasGRP1 null mice (Dower *et al.* 2000; Yoder *et al.* 2001). Therefore, Gads may associate with RasGRP1 to mediate its membrane recruitment.

To test this hypothesis, I expressed GST fused to Grap and to Gads. *In vitro* binding of GST-Grap and GST-Gads to RasGRP1 MBP fusion were observed in a GST pull-down assay, demonstrating the overlapping ligand specificity among Grb2 family members (Figure 4.6 A). Next, I tested if endogenous RasGRP1 preferentially associated with one of the Grb2 family members. Jurkat cells (1 x 10^6/sample) were either left untreated or stimulated with anti-TCR antibody (OKT3). Cell lysates were prepared and incubated with bead associated GST, GST-Grb2, GST-Grap, or GST-Gads. As shown in Figure 4.6 B, both Grb2 and Grap, but not Gads, bound to endogenous Sos1, as previously reported, indicating the GST fusions are functionally active. When analyzed for RasGRP1, binding to Gads, but not Grap, was observed. RasGRP1 also precipitated with GST-Grb2, yet to a much less

extent comparing to that with GST-Gads. Moreover, the binding appears to be TCR-stimulation independent since TCR stimulation did not increase the amount of precipitated RasGRP1 (Figure 4.6 B). In addition, the binding of RasGRP1 to Gads seems to be calcium-independent since depletion of Ca^{2+} from the cell lysate with EGTA has no effects on binding (data not shown). This is consistent with the observation that mutations in the EF-hand motif of RasGRP1 that block *in vitro* calcium binding (see Chapter 5) did not suppress binding to Grb2 (data not shown).

Together, these data suggest that RasGRP1 preferentially associated with Gads but not Grap in both TCR stimulated and unstimulated Jurkat T cells.

4.3 Discussion

The presence of a putative PXXP motif in RasGRP1 suggests a possible involvement of SH3 domain-containing proteins in regulating RasGRP1 activity. In this study, I showed RasGRP1 directly interacts with Grb2 in vitro. The interaction requires both SH3 domains, as demonstrated by a point mutagenesis study where loss-of-function mutations in either of the two SH3 domains lowered the affinity of Grb2 for RasGRP1. Interestingly, when the two SH3 domains were tested separately, only the N-terminal SH3 domain showed high affinity for RasGRP1. One possible explanation is that the C-terminal SH3 domain does not interact with RasGRP1, but instead, functions to facilitate the binding by the N-terminal SH3 domain indirectly. The differential affinity toward ligand between the two SH3 domains of Grb2 has been reported in other proteins. For instance, only the N-SH3 domain of Grb2 has been shown to be essential for mediating the high affinity of binding to dynamin, a protein crucial for regulation of the endocytosis pathway (Liu, J. P. and Robinson 1995; Vidal et al. 1999).

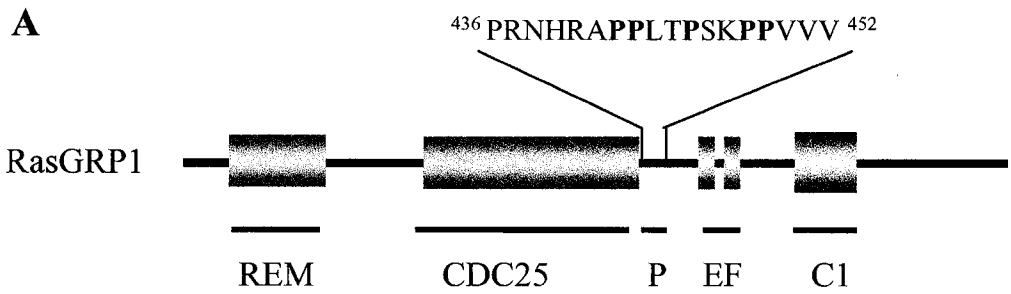
Binding to the SH3 domain is usually through a PXXP motif. Mutation of the putative PXXP motif in RasGRP1 decreased its affinity for Grb2, however, the effect is moderate (Figure 4.5). One possibility is that the presence of additional PXXP

motif(s) in other regions of RasGRP1 may also contribute to Grb2 binding. For example, Sos uses four PXXP motifs to bind Grb2. Each one of the motifs shows a low affinity to the SH3 domain of Grb2 (Garbay *et al.* 2000). Upon sequence analysis, several regions containing the PXXP consensus sequence have been found (Figure 4.7). Thus, whether they may contribute to SH3 domain binding needs to be tested by additional mutagenesis studies. Alternatively, it is apparent that not all SH3 domain-binding is mediated through PXXP motifs (Lewitzky *et al.* 2001; Kaneko *et al.* 2003). P*X*(V/I)(D/N)R*XX*KP is a classic example of such a motif, first identified in a deubiquitinating enzyme UBPY, a binding partner of the SH3 domain of mouse STAM2 (Kato *et al.* 2000; Kaneko *et al.* 2003). Therefore, the mapping of Grb2 binding site(s) on RasGRP1 might be extended to non-PXXP sequences.

I also showed recombinant that RasGRP1 indeed binds to GST-Grap and GST-Gads *in vitro*, demonstrating the overlap in ligand specificity among SH3 domain-containing proteins. However, using a pull-down assay in Jurkat T cell lysate, which is more physiologically relevant, I showed endogenous RasGRP1 is preferentially associated with recombinant Gads, and, to a much less extent, Grb2, but not Grap. This observation agrees with an essential role proposed for Gads in TCR signaling. Similar to the interaction between Grb2 and Sos, which form stable complex in resting cells, the interaction between Gads and RasGRP1 did not seem to require TCR signaling. Moreover, calcium depletion by EGTA in cell lysates could not disrupt the Gads/RasGRP1, excluding a potential structural role of the RasGRP1 EF-hand motif in regulating Gads binding.

Based on these observations, an exciting pathway can be proposed in analogy to the role of Grb2/Sos complex in coupling RTK signaling to Ras activation. Gads forms a stable complex with RasGRP1 in resting T cells. Upon TCR ligation, PTKs catalyzed tyrosine phosphorylation on transmembrane adaptors, such as LAT, will recruit the Gads/RasGRP1 complex to receptor proximal plasma membrane where RasGRP1 becomes activated and in turn, activates Ras. The secondary messenger DAG may facilitate this process by interacting with the C1 domain of RasGRP1.

To validate this model, an *in vivo* interaction has to be demonstrated between Gads and RasGRP1. Unfortunately, I failed to co-immuneprecipitate Gads with RasGRP1 in Jurkat T cells. A possible explanation for this lack of binding is that the stable complex between the two proteins in cells blocks the access of antibody to the epitope. Thus, an efficient antibody recognizing a different epitope would be helpful. Another possibility is that the role of Gads/RasGRP1-mediated Ras activation may be cell type-specific and function to couple receptors to distinct downstream pathway (Liu, S. K. et al. 2001). Thus, future directions might focus on characterization of *in vivo* interaction between Gads and RasGRP1 in other hematopoietic cell lineages.

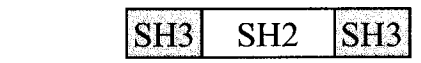


B

RasGRP1	...REPRNHRA**PPLTPSKPP**V
RasGRP2	...REPRNHRA**PPLTPSKPP**V
RasGRP3	...LEPRNSKS**PTSPTTPNKP**V
RasGRP4	...REPRC**PKSLPPSPFRAP**V

CDC25

FIGURE 4.1 Sequence comparison of the putative proline-rich region in RasGRPs. (**A**) Schematic representation of the putative proline-rich region and its sequence in mouse RasGRP1. (**B**) Comparison of the putative proline-rich region sequences of four RasGRPs (human sequence). The C-terminal end of CDC25 box is underlined. The proline residues are in bold and the putative proline-rich regions are also underlined.



	Expression	Phenotypes
Grb2	ubiquitous	Early embryonic lethality
Grap	Hematopoietic cells including T and B lymphocytes and myeloid linages	None reported
Gads	T lymphocytes, B cell progenitors, and myeloid lineages	Impairment of T cell development, and thymic selection

FIGURE 4.2 Schematic representation of the Grb2 family members. The expression patterns and phenotypes of null mice are also listed for each family member. SH3, src-homology 3; SH2, src-homology 2; linker: linker region (a 120 amino acid unique region found in Gads).

(Adopted from Liu SK *et al.* Oncogene. 2001; 20(44):6284-90)

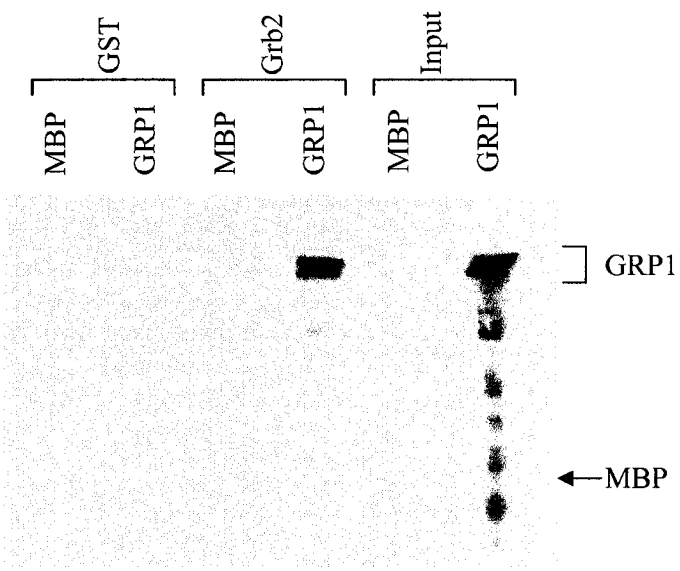


FIGURE 4.3 Grb2 binds to RasGRP1 *in vitro*. GST pull-down assay. GST alone or GST-Grb2 fusion protein (Grb2) were immobilized onto glutathione-Sepharose beads and incubated with MBP or MBP-RasGRP1 fusion protein (GRP1) for 1 hour on ice. The beads were then washed three times and the proteins co-precipitated with GST proteins were analyzed by SDS-PAGE and immunoblotting using antibody specific for RasGRP1. Position of MBP protein is indicated by arrow. Input, 1ug of recombinant MBP or MBP-RasGRP1 were directly loaded onto the SDS gel and analyzed by immunoblotting. Results are representative of three independent experiments.

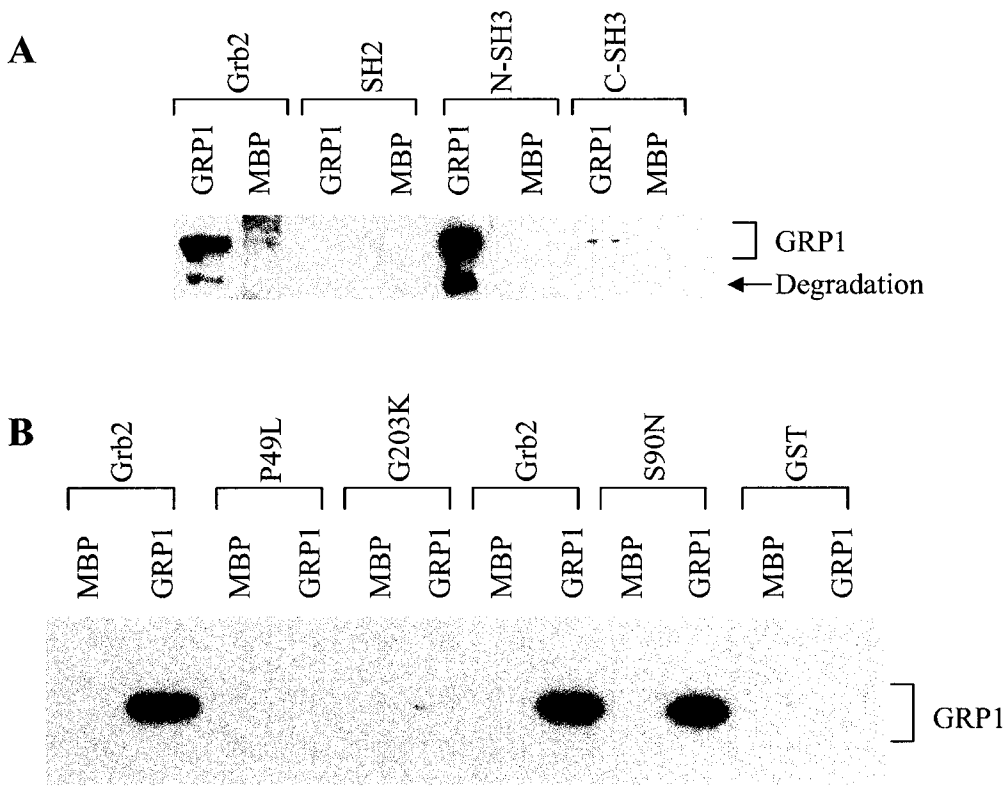


FIGURE 4.4 Grb2 binds RasGRP1 *in vitro* through its N-terminal SH3 domain. (**A**) GST fused wildtype Grb2 or isolated domains of Grb2 were subject to GST pull-down assay with MBP-RasGRP1 (GRP1), or with MBP as negative controls. (**B**) GST-Grb2 or its single point mutants (P49L, S90N, and G203K) were analyzed by GST pull-down assay. Results are representatives of three independent experiments.

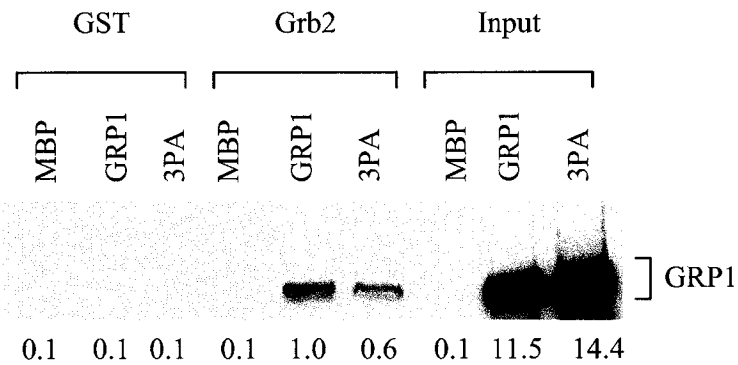


FIGURE 4.5 Mutants in the RasGRP1 proline-rich region partially inhibit *in vitro* interaction with Grb2. *In vitro* GST pull-down assay of GST alone or GST-Grb2 with MBP, MBP-RasGRP1 (GRP1), or MBP-RasGRP1 containing proline 442, 445, and 448 to alanine mutations (3PA). Numbers are quantification of band intensity. Results are representatives of three independent experiments.

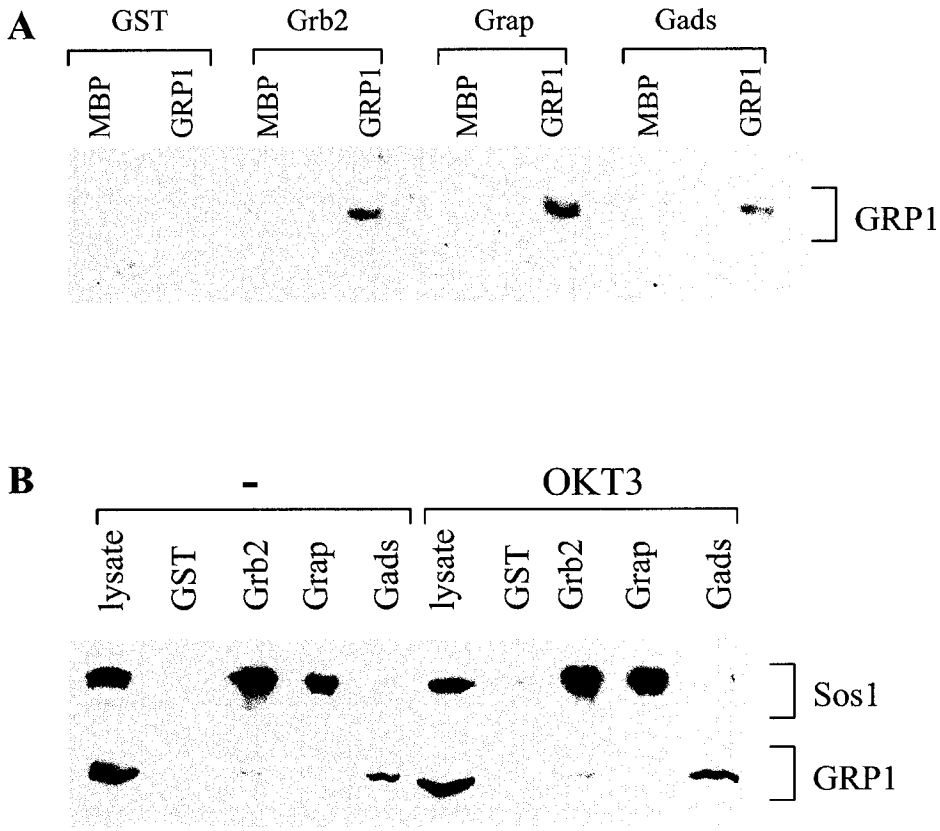
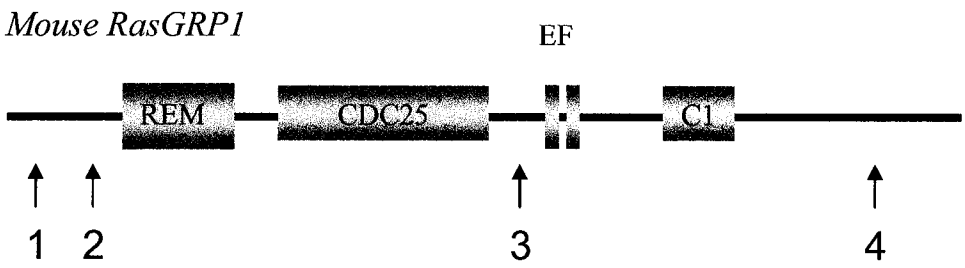


FIGURE 4.6 RasGRP1 binds to all Grb2 family members *in vitro*, but preferentially interacts with recombinant Gads in Jurkat. (A) GST pull-down analysis of binding between MBP-RasGRP1 and three Grb2 family members fused to GST. **(B)** Jurkat cells were left unstimulated or stimulated with anti-TCR antibody (OKT3) for 10 minutes. Nuclear-free cell lysates were prepared and incubated with GST alone, GST-Grb2, GST-Grap, or GST-Gads on ice for 1 hour. The proteins co-precipitated were analyzed by SDS-PAGE and immunoblotting using indicated antibodies. Results are representatives of three independent experiments.



No.	Start	Sequence
1	11	PRKP
2	34	PLPAQP
3	441	PPLTPSKPPV
4	708	PASPCPSP

FIGURE 4.7 PXXP consensus sequences in mouse RasGRP1. Relative positions of PXXP consensus sequences found in mouse RasGRP1 primary structure are indicated by arrows and numbered. The actual sequences are shown in the table. Start: position of the first residue of each PXXP sequence.

References

Asada, H., N. Ishii, et al. (1999). "Grf40, A novel Grb2 family member, is involved in T cell signaling through interaction with SLP-76 and LAT." J Exp Med **189**(9): 1383-90.

Berry, D. M., S. J. Benn, et al. (2001). "Caspase-dependent cleavage of the hematopoietic specific adaptor protein Gads alters signalling from the T cell receptor." Oncogene **20**(10): 1203-11.

Berry, D. M., P. Nash, et al. (2002). "A high-affinity Arg-X-X-Lys SH3 binding motif confers specificity for the interaction between Gads and SLP-76 in T cell signaling." Curr Biol **12**(15): 1336-41.

Bourette, R. P., S. Arnaud, et al. (1998). "Mona, a novel hematopoietic-specific adaptor interacting with the macrophage colony-stimulating factor receptor, is implicated in monocyte/macrophage development." Embo J **17**(24): 7273-81.

Buday, L., S. E. Egan, et al. (1994). "A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells." J Biol Chem **269**(12): 9019-23.

Chardin, P., D. Cussac, et al. (1995). "The Grb2 adaptor." FEBS Lett **369**(1): 47-51.

Cheng, A. M., T. M. Saxton, et al. (1998). "Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation." Cell **95**(6): 793-803.

Dower, N. A., S. L. Stang, et al. (2000). "RasGRP is essential for mouse thymocyte differentiation and TCR signaling." Nat Immunol **1**(4): 317-21.

Feng, G. S., Y. B. Ouyang, et al. (1996). "Grap is a novel SH3-SH2-SH3 adaptor protein that couples tyrosine kinases to the Ras pathway." J Biol Chem **271**(21): 12129-32.

Garbay, C., W. Q. Liu, et al. (2000). "Inhibitors of Ras signal transduction as antitumor agents." Biochem Pharmacol **60**(8): 1165-9.

Jackman, J. K., D. G. Motto, et al. (1995). "Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells." J Biol Chem **270**(13): 7029-32.

Kaneko, T., T. Kumasaka, et al. (2003). "Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain." J Biol Chem **278**(48): 48162-8.

Kato, M., K. Miyazawa, et al. (2000). "A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP." J Biol Chem **275**(48): 37481-7.

Kikuchi, K., Y. Kawasaki, et al. (2001). "Suppression of thymic development by the dominant-negative form of Gads." Int Immunol **13**(6): 777-83.

Law, C. L., M. K. Ewings, et al. (1999). "GrpL, a Grb2-related adaptor protein, interacts with SLP-76 to regulate nuclear factor of activated T cell activation." J Exp Med **189**(8): 1243-53.

Lewitzky, M., C. Kardinal, et al. (2001). "The C-terminal SH3 domain of the adapter protein Grb2 binds with high affinity to sequences in Gab1 and SLP-76

which lack the SH3-typical P-x-x-P core motif." Oncogene **20**(9): 1052-62.

Li, N., A. Batzer, et al. (1993). "Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling." Nature **363**(6424): 85-8.

Lim, W. A. and F. M. Richards (1994). "Critical residues in an SH3 domain from Sem-5 suggest a mechanism for proline-rich peptide recognition." Nat Struct Biol **1**(4): 221-5.

Liu, J. P. and P. J. Robinson (1995). "Dynamin and endocytosis." Endocr Rev **16**(5): 590-607.

Liu, S. K., D. M. Berry, et al. (2001). "The role of Gads in hematopoietic cell signalling." Oncogene **20**(44): 6284-90.

Liu, S. K. and C. J. McGlade (1998). "Gads is a novel SH2 and SH3 domain-containing adaptor protein that binds to tyrosine-phosphorylated Shc." Oncogene **17**(24): 3073-82.

Liu, S. K., C. A. Smith, et al. (2000). "The adaptor protein Gads (Grb2-related adaptor downstream of Shc) is implicated in coupling hemopoietic progenitor kinase-1 to the activated TCR." J Immunol **165**(3): 1417-26.

Lowenstein, E. J., R. J. Daly, et al. (1992). "The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling." Cell **70**(3): 431-42.

Mayer, B. J. (2001). "SH3 domains: complexity in moderation." J Cell Sci **114**(Pt 7): 1253-63.

Nishida, M., K. Nagata, et al. (2001). "Novel recognition mode between Vav and Grb2 SH3 domains." Embo J **20**(12): 2995-3007.

Northrop, J. P., M. J. Pustelnik, et al. (1996). "Characterization of the roles of SH2 domain-containing proteins in T-lymphocyte activation by using dominant negative SH2 domains." Mol Cell Biol **16**(5): 2255-63.

Qiu, M., S. Hua, et al. (1998). "Molecular cloning and expression of human grap-2, a novel leukocyte-specific SH2- and SH3-containing adaptor-like protein that binds to gab-1." Biochem Biophys Res Commun **253**(2): 443-7.

Reif, K., L. Buday, et al. (1994). "SH3 domains of the adapter molecule Grb2 complex with two proteins in T cells: the guanine nucleotide exchange protein Sos and a 75-kDa protein that is a substrate for T cell antigen receptor-activated tyrosine kinases." J Biol Chem **269**(19): 14081-7.

Ren, R., B. J. Mayer, et al. (1993). "Identification of a ten-amino acid proline-rich SH3 binding site." Science **259**(5098): 1157-61.

Rozakis-Adcock, M., R. Fernley, et al. (1993). "The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1." Nature **363**(6424): 83-5.

Strynadka, N. C. and M. N. James (1989). "Crystal structures of the helix-loop-helix calcium-binding proteins." Annu Rev Biochem **58**: 951-98.

Tong, A. H., B. Drees, et al. (2002). "A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules." Science **295**(5553): 321-4.

Vidal, M., N. Goudreau, et al. (1999). "Molecular and cellular analysis of Grb2 SH3 domain mutants: interaction with Sos and dynamin." J Mol Biol **290**(3): 717-30.

Weber, J. R., S. Orstavik, et al. (1998). "Molecular cloning of the cDNA encoding pp36, a tyrosine-phosphorylated adaptor protein selectively expressed by T cells and natural killer cells." J Exp Med **187**(7): 1157-61.

Yamasaki, S., K. Nishida, et al. (2003). "Gab1 is required for EGF receptor signaling and the transformation by activated ErbB2." Oncogene **22**(10): 1546-56.

Yankee, T. M., K. E. Draves, et al. (2001). "CD95/Fas induces cleavage of the GrpL/Gads adaptor and desensitization of antigen receptor signaling." Proc Natl Acad Sci U S A **98**(12): 6789-93.

Yoder, J., C. Pham, et al. (2001). "Requirement for the SLP-76 adaptor GADS in T cell development." Science **291**(5510): 1987-91.

Zhang, W., J. Sloan-Lancaster, et al. (1998a). "LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation." Cell **92**(1): 83-92.

Zhang, W., R. P. Trible, et al. (1998b). "LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation." Immunity **9**(2): 239-46.

Zhang, W., R. P. Trible, et al. (2000). "Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling." J Biol Chem **275**(30): 23355-61.

Zuniga-Pflucker, J. C. and M. J. Lenardo (1996). "Regulation of thymocyte development from immature progenitors." Curr Opin Immunol 8(2): 215-24.

CHAPTER 5

A PRELIMINARY STUDY ON THE ROLE OF THE EF-HANDS IN RASGRP1 ACTIVATION

Chapter 5

5.1 Introduction

5.1.1 Calcium as a universal secondary messenger

Calcium (Ca^{2+}) is ubiquitous intracellular signal known to play key roles in controlling numerous cellular processes including fertilization, secretion, contraction, cell-cycle progression, cell proliferation, apoptosis, learning and memory (Berridge *et al.* 2000; Bootman *et al.* 2001). Internal Ca^{2+} stores are held within the extensive membrane systems of the endoplasmic reticulum (ER), or the sarcoplasmic reticulum in muscle. Cells at the resting state have a Ca^{2+}concentration of 100 nM. In response to Ca^{2+}-mobilizing signals, the Ca^{2+} level can rise to about 1uM by calcium influx from external sources and release from internal stores, which is controlled by various channels such as the well-studied inositol 1,4,5-trisphosphate (IP_3) receptor and ryanodine receptor families (Berridge *et al.* 2000; Bootman *et al.* 2001). The Ca^{2+} signal generated by the release of Ca^{2+} from internal stores is then coupled to many Ca^{2+} sensing effector pathways including the Ras-MAP kinase pathway (Berridge *et al.* 2000; Cullen and Lockyer 2002).

5.1.2 Calcium regulation of Ras

The first hint of a signaling relationship between Ras GTPase and Ca^{2+} is dated back to the 80's where the Ca^{2+}-triggered terminal differentiation of mouse epidermal keratinocytes was found to be interfered by introduction of oncogenic Ras (Weissman and Aaronson 1985). In the phaeochromocytoma PC12 neuronal cells, calcium influx through voltage-sensitive calcium channels leads to activation of MAP kinase pathway, which is inhibited by a block of Ras activity, establishing a direct link between signaling by Ras and calcium (Rosen *et al.* 1994). Now it is well appreciated that the effects of calcium signaling on Ras activity is coupled by a group of Ca^{2+}-regulated RasGEFs, RasGAPs, and Ras effector proteins.

For example, an indirect mode for Ca^{2+}-dependent Ras activation would be mediated by the regulation of Sos activity by proline-rich tyrosine kinase 2 (Pyk2), a non-receptor tyrosine kinase that is highly expressed in the central nervous system (Lev *et al.* 1995; Dikic *et al.* 1996). Pyk2 is found to be tyrosine phosphorylated and activated by various signals that elevate intracellular Ca^{2+} concentration in neuronal and haematopoietic cells (Lev *et al.* 1995; Dikic *et al.* 1996). Once activated, Pyk2 forms a complex with Src and interacts with the Grb2–Sos complex to activate Ras. The identification of the IQ motif-containing RasGRFs as Ca^{2+}-dependent Ras GEFs in brain provides a direct link between calcium signaling and Ras activation (Martegani *et al.* 1992; Shou *et al.* 1992; Wei *et al.* 1992; Farnsworth *et al.* 1995; Fam *et al.* 1997). Proteins that contain IQ motifs bind calmodulin (CaM) in a Ca^{2+}-dependent manner (Munshi *et al.* 1996).

The effect of Ca^{2+} on Ras signaling can also be inhibitory, possibly through stimulating RasGAP activity (Chao *et al.* 1992; Medema *et al.* 1994; Ji and Carpenter 2000). For instance, in primary keratinoctyes, the basal level of Ras-GTP and EGF-stimulated ERK/MAPK activity were transiently inhibited, possibly by p120RasGAP, when switched to high Ca^{2+} media (Gawler *et al.* 1995a; Gawler *et al.* 1995b; Davis *et al.* 1996; Hernandez-Pineda *et al.* 1999). Another example for Ca^{2+}-regulated Ras GAP is the recently cloned Ca^{2+}-promoted Ras activator (CAPRI), which is a member of the GAP1 family of Ras-specific Ras GAPs with high expression in spleen, lymph node and muscle (Lockyer *et al.* 2001). It contains tandem C2 domains and rapidly translocates to the plasma membrane in response to Ca^{2+} signal to inactivate Ras; it thus may function to activate inputs from multiple players such as Pyk2 and Ca^{2+}-dependent GEFs (Lev *et al.* 1995; Berridge *et al.* 2000; Lockyer *et al.* 2001; Walker *et al.* 2004).

5.1.3 RasGRPs: The convergence of calcium and DAG signaling in Ras regulation?

In lymphocytes, the mobilization of intracellular free calcium is one major target

of immune receptor signaling and it is critical for the activation of lymphocytes (Healy *et al.* 1997; Dolmetsch *et al.* 1998). This process is promoted by the binding of IP3 receptor to its ligand, IP3, which is generated by PLC cleavage of PIP_2 upon immune receptor ligation (Nel 2002; Nel and Slaughter 2002). Calcium activates the protein phosphatase calcineurin, leading to dephosphorylation of the transcription factor NF-AT, which then cooperates with "basic/leucine zipper" transcription factors such as CREB and AP-1 to activate T cells (Alberola-Ila *et al.* 1997). As previously discussed, PIP_2 metabolism also produces another secondary messenger, DAG. The distinct features of the RasGRP family members make them a unique group of Ras activators that may mediate the convergence of Ca^{2+} and DAG signaling during lymphocyte activation. What distinguishes RasGRPs from other Ras GEFs is the presence of an atypical pair of calcium-binding EF-hands located downstream of the conserved catalytic CDC25 domain and a DAG-binding C1 domain.

EF-hands are highly conserved helix-loop-helix motifs consisting of two alpha helices, "E" and "F", joined by a canonical 12-residue interhelical sequence (loop) which coordinates the binding of a calcium molecule (Kretsinger and Nockolds 1973) (Figure 5.1). Functional EF-hands are usually found in pairs, or multiples of pairs, in a great variety of proteins. These proteins function not only in modulation of Ca^{2+} signals and calcium homeostasis, but also in intracellular Ca^{2+} signal transduction (Strynadka and James 1989; Skelton *et al.* 1994; Bhattacharya *et al.* 2004; Bunick *et al.* 2004). Proteins containing EF-hands can be grouped into two functional categories: regulatory or structural. Binding of calcium to regulatory EF-hand domain-containing proteins induces rearrangement of helices, which is transmitted to their target proteins, often catalyzing enzymatic reactions. The hydrophobic patch formed as a result of conformational change upon calcium binding in EF-hand containing proteins also functions to facilitate membrane association, as well as inter- and intra-protein interactions (Ikura 1996; Bhattacharya *et al.* 2004). One example for the regulatory EF-hand domain containing protein is the well-studied calcium sensor calmodulin which acts as a generic

calcium-dependent allosteric regulators to couple the calcium signal to the activities of many other signaling molecules such as kinases and phosphatases (Chattopadhyaya *et al.* 1992; Crivici and Ikura 1995; Nelson and Chazin 1998; Chin and Means 2000). In contrast, binding of calcium to structural EF-hand domain-containing proteins does not induce a significant conformational change. Structural EF-hand domains seem to play a role in buffering intracellular calcium levels, as exemplified by calbindin.

Interestingly, the EF-hands found in RasGRPs have an atypically short spacer between the pair of calcium-binding loops. The functional significance of this feature is not clear yet.

5.1.4 Hypothesis

Based on how other EF-hands function as calcium sensors (Ikura 1996), it is possible that binding of calcium induces a conformational change in RasGRPs that exposes a hydrophobic patch that facilitate membrane recruitment of RasGRP1 directly via interacting with membrane lipids, or indirectly through interacting with other signaling molecules. Alternatively, a somewhat speculative hypothesis involves calcium/EF-hand regulated protein stability. To test these hypotheses, the calcium-binding ability of the EF-hands in RasGRPs has to be characterized. The objective of my study is then to determine the calcium-binding ability of the EF-hands in RasGRP1, and to evaluate the functional importance of the EF-hands for RasGRP1 activity.

5.2 Results

5.2.1 In vitro calcium binding by RasGRP1 is abolished by single mutation in the left EF-hand.

Previous data from our lab suggested that RasGRP1 binds calcium *in vitro* (Ebinu *et al.* 1998). High affinity for Ca^{2+} in the EF-hands is frequently a result of cooperative binding to both sites with one of the two EF-hands having a higher affinity (Ikura 1996). Studies on RasGRP1 EF-hand mutants, in which four conserved glutamic acids critical for calcium coordination in each EF-hand were substituted by non-charged alanine, indicated that the right EF-hand has higher affinity compared to the other site (Linse *et al.* 1994; Ebinu *et al.* 1998; Fast *et al.* 2001). However, the low protein stability of these RasGRP1 EF-hand mutants implies a role of the EF-hands in regulating protein stability on one hand, and on the other hand, prevented further studies on the function of EF-hands. To solve this problem, I constructed two RasGRP1 EF-hand mutants containing a single point mutations in each of the EF-hand pairs. RasGRP1E494A contains an alanine substitution at position 494, whereas in RasGRP1E521A, alanine replaced the conserved glutamic acid at position 521 (Figure 5.2 A). The two mutants along with the wildtype protein were purified as MBP fusion proteins from *E. Coli.*, blotted onto nitrocellular membrane, and tested for calcium binding using the *in vitro* $^{45}Ca^{2+}$ binding assay. The affinity to $^{45}Ca^{2+}$ of each protein band on membrane was determined by autoradiography. As shown in Figure 5.2 B, the calcium binding by the wildtype protein is confirmed. However, the mutation in the left EF-hand completely blocked the calcium binding by RasGRP1 while alanine substitution in the right EF-hand has no effect. Therefore, the right EF-hand of RasGRP1 is less important for calcium binding, at least *in vitro*.

5.2.2 PMA-induced Ras-Erk activation is intact in fibroblasts expressing RasGRP1 EF-hand mutants

To study the functional consequence of the alanine substitution in the left

EF-hand in RasGRP1, we expressed wildtype RasGRP1 and the two EF-hand mutants (E494A and E521A) using a retrovirus vector system in Rat2 fibroblasts. Rat2 cells do not express endogenous RasGRP1 and expression of wildtype RasGRP1 endows Rat2 cells with the ability to couple DAG analogue treatment to Erk activation, as has been reported previously (Ebinu *et al.* 1998). We found that both mutants are stably expressed at comparable level to that of the wildtype protein in Rat2 cells (Figure 5.3 A), suggesting the mutants in EF-hands do not affect protein stability. The cells were stimulated with PMA for different durations of time and the PMA-dependent Erk activation was tested by immunoblotting with an anti-phosphoErk antibody. However, no defect in PMA-induced Erk activation was observed in cells expressing RasGRP1 mutants (Figure 5.3 B). Therefore, the data suggest that the activity of RasGRP1 does not depend on the presence of an intact EF-hand domain in the cellular system we tested.

5.3 Discussion

The presence of the calcium-binding EF-hand motifs and the DAG-binding C1 domain distinguishes RasGRPs from other Ras GEFs, but the functional significance of the EF-hands is still poorly understood. Using *in vitro* $^{45}Ca^{2+}$ binding assay, I confirmed the previous observation that RasGRP1 can bind calcium *in vitro*. In EF-hand containing proteins, high affinity for Ca^{2+} is frequently a result of positive cooperative binding to both sites, which requires a concerted movement of the helices in these EF-hand pairs (Linse and Chazin 1995; Zhang *et al.* 1995). In support of this idea, I showed that the left EF-hand seems to have relatively higher affinity for calcium since alanine substitution totally blocked calcium binding in the left, but not the right EF-hand (Figure 5.2 B). However, it is still not clear whether the EF-hands of RasGRP1 are functional *in vivo* with the physiological concentration of calcium, which requires a careful characterization of the calcium binding affinity.

As a preliminary attempt to test the function of the EF-hands in RasGRP1, I compared the signaling activities of the EF-hand mutants with the wildtype protein. If the mutation in the first EF-hand is able to attenuate or totally block the calcium response of RasGRP1, it should also attenuate Ras-Erk signaling. However, PMA-induced Erk activation was intact in Rat2 cells expressing either EF-hand mutants (Figure 5.3 B), suggesting the EF-hands are dispensable for RasGRP1 function under these circumstance. In agreement with this idea, deletion of the EF-hand pair in RasGRP1 seemed have no effect on transformation activity in Rat2 fibroblast (Tognon *et al.* 1998). However, transfection of CalDAG-GEFI, another RasGRP family member, produced an increase in GTP-bound Rap1A in 293T cells, which is further augmented by artificial calcium influx promoted by the Ca^{2+} ionophore A23187 (Kawasaki *et al.* 1998). Since immune receptor ligation-induced increase of intracellular free calcium concentration is much lower than that induced by ionophore, whether this observation is physiologically relevant needs to be further studied.

Taken together, the functional importance of the EF-hands to RasGRP regulation is still largely a mystery. Based on the studies on other EF-hand containing proteins, there are several possible mechanisms for the EF-hands to influence RasGRP activity. Firstly, the possible conformational changes in the EF-hands upon Ca^{2+} binding may facilitate membrane affiliation directly through hydrophobic patch formation, or allosterically, as in the case of recoverin, a calcium-sensing EF-hand containing protein in retinal rod cells (Flaherty *et al.* 1993). However, the calcium signal alone seems not sufficient in promoting RasGRP1 membrane translocation, since calcium ionophore treatment could not stimulate the membrane recruitment of RasGRP1 in HEK293 cells (data not shown). Cooperation between DAG and Ca^{2+} may be required. Secondly, the hydrophobic region found in the EF-hands may also provide binding site for other signaling molecules. For example, nuclear magnetic resonance (NMR) and X-ray studies of three Ca^{2+}-CaM and peptide complexes have revealed the significance of hydrophobic interactions of the two lobes of CaM with

the alpha-helical rod of the peptide (Chattopadhyaya *et al.* 1992; Ikura *et al.* 1992; Meador *et al.* 1993). Other examples include the interactions between calcineurin A and B (Griffith *et al.* 1995). In all cases, interaction is predominantly hydrophobic, but is complemented by acidic side chains from the EF-hands interacting with basic side chains of the target protein. Finally, while most EF-hand containing proteins undergo large conformational changes, some do not. One example of this latter kind is calbindin D_{9k}, a steroid-regulated calcium binding protein that functions to buffer intracellular Ca^{2+} (Kumar *et al.* 1989). Calcium binding induces only small conformational changes and may be responsible for the structural stability (Ikura 1996). Interestingly, two RasGRP EF-hand mutants with four conserved glutamic acids in each EF-hand replaced by alanine, respectively, show low protein stability in Rat2 cells. Unfortunately, it is difficult to predict from amino acid sequence data alone whether the EF-hand will undergo structural changes upon calcium binding.

My study only provides preliminary data on the biochemical property of the EF-hand in RasGRP1. A complete understanding of the role of the EF-hands in RasGRPs will depend on the identification of RasGRP binding partners and the elucidation of the structure of RasGRPs,

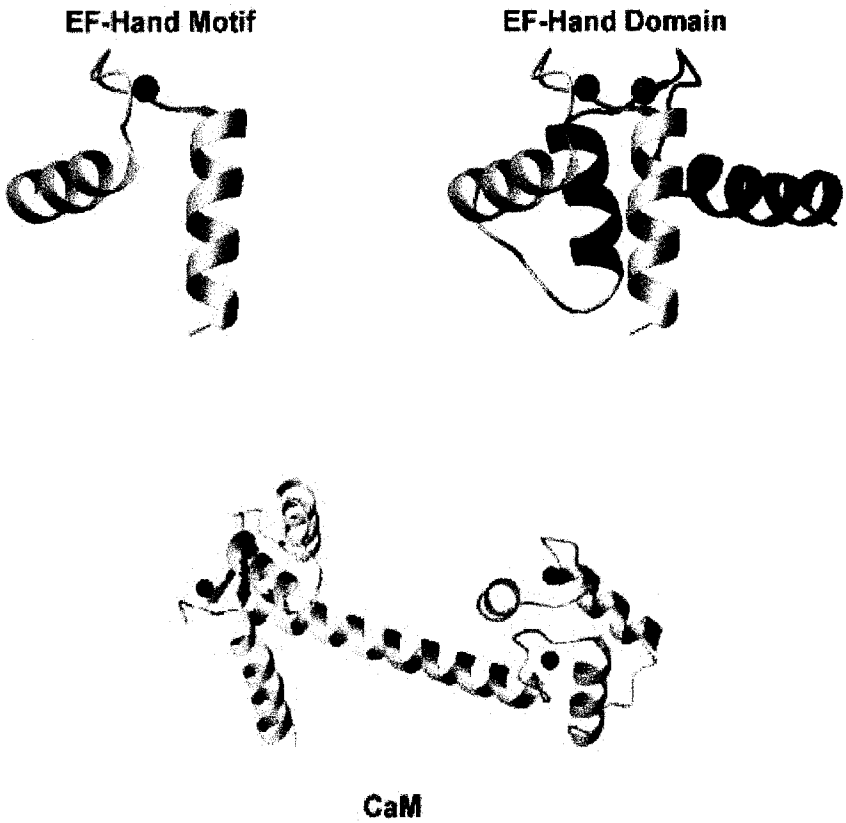


FIGURE 5.1 Basic structural features of EF-hand domain and Ca^{2+} binding proteins. The panels display structures of the isolated EF-hand motif, EF-hand domain from CaM, and intact CaM protein. Different colors are used to distinguish between the pairs of EF-hands and the two EF-hand domains of CaM. Calcium ion is shown in red. The figure was generated in MOLMOL.

(Adopted from Bhattacharya S, et.al. Biochim Biophys Acta. 2004; 1742(1-3):69-79)

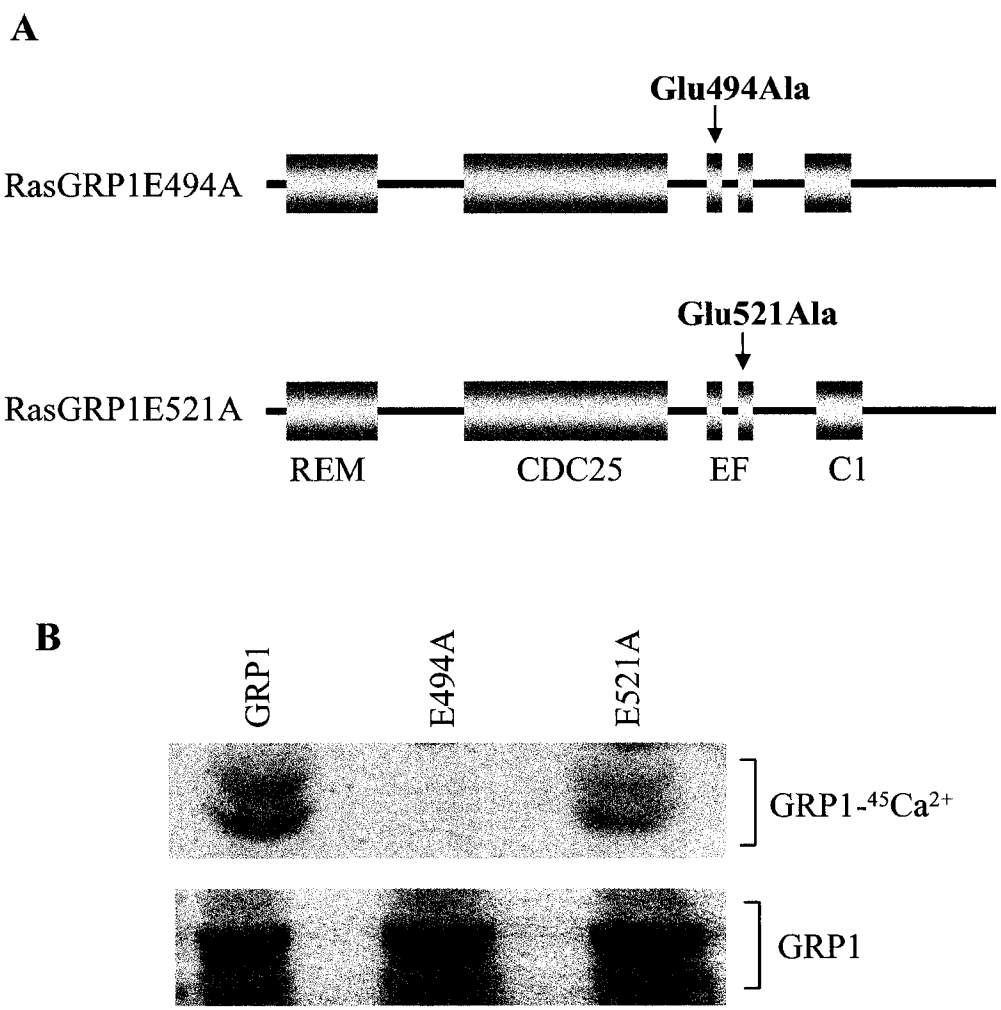


FIGURE 5.2 Mutation in the left EF hand blocks in vitro calcium binding in RasGRP1. **(A)** A schematic diagram of the two RasGRP1 mutants constructed with the position of the alanine substitution shown by arrows. **(B)** MBP-RasGRP1 (GRP1), MBP-RasGRP1E494A (E494A), and MBP-RasGRP1E521A (E521A) were resolved on SDS-PAGE, blotted onto nitrocellulose membrane and incubated with $^{45}Ca^{2+}$ in an *in vitro* binding assay. After washes, the ability of each protein to bind $^{45}Ca^{2+}$ was determined by autoradiography (top panel). The loading of the proteins were monitored by ponceau staining (bottom panel). The result shown is the representative of three independent experiments.

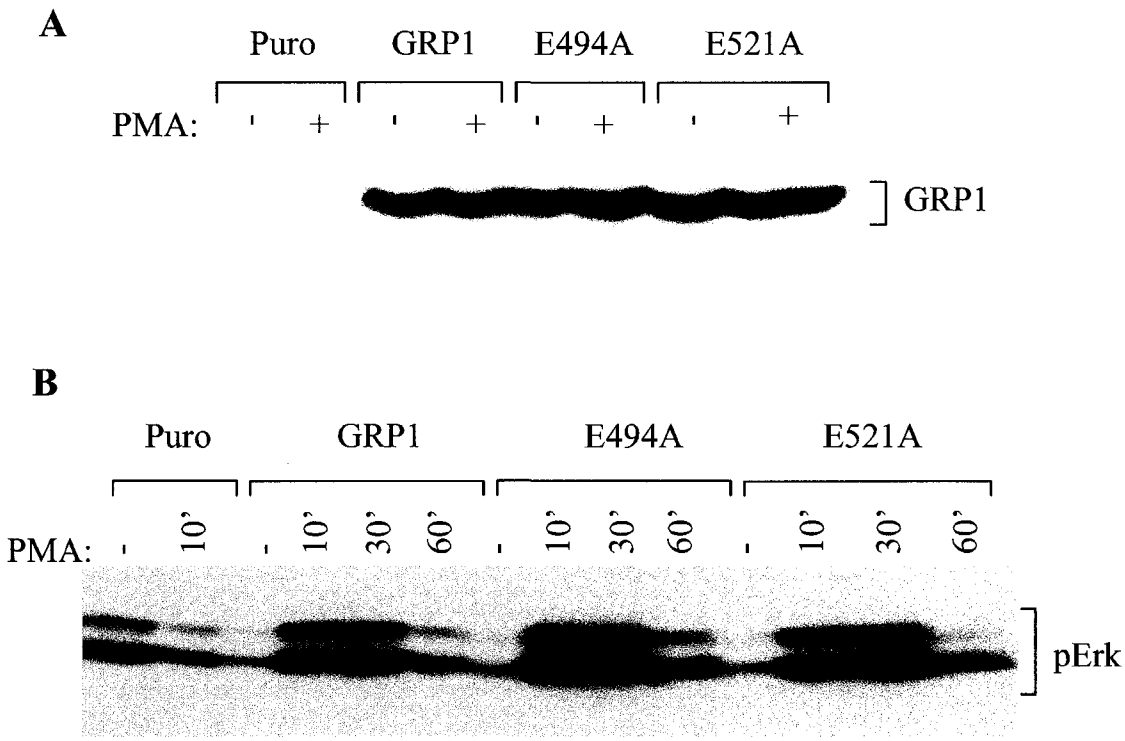


FIGURE 5.3 RasGRP1 EF hand mutants are functionally intact in an ectopic expression system. Rat2 fibroblasts expressing empty vector (Puro), RasGRP1, RasGRP1 EF mutants (E494A and E521A) were either left unstimulated or stimulated with 100nM PMA for indicated time. Lysates were assayed for RasGRP1 protein expression (**A**) and phospho-Erk (**B**) by immunoblotting. Results are representative of three independent experiments.

References:

Alberola-Ila, J., S. Takaki, et al. (1997). "Differential signaling by lymphocyte antigen receptors." Annu Rev Immunol **15**: 125-54.

Berridge, M. J., P. Lipp, et al. (2000). "The versatility and universality of calcium signalling." Nat Rev Mol Cell Biol **1**(1): 11-21.

Bhattacharya, S., C. G. Bunick, et al. (2004). "Target selectivity in EF-hand calcium binding proteins." Biochim Biophys Acta **1742**(1-3): 69-79.

Bootman, M. D., T. J. Collins, et al. (2001). "Calcium signalling--an overview." Semin Cell Dev Biol **12**(1): 3-10.

Bunick, C. G., M. R. Nelson, et al. (2004). "Designing sequence to control protein function in an EF-hand protein." J Am Chem Soc **126**(19): 5990-8.

Chao, T. S., K. L. Byron, et al. (1992). "Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor." J Biol Chem **267**(28): 19876-83.

Chattopadhyaya, R., W. E. Meador, et al. (1992). "Calmodulin structure refined at 1.7 A resolution." J Mol Biol **228**(4): 1177-92.

Chin, D. and A. R. Means (2000). "Calmodulin: a prototypical calcium sensor." Trends Cell Biol **10**(8): 322-8.

Crivici, A. and M. Ikura (1995). "Molecular and structural basis of target recognition by calmodulin." Annu Rev Biophys Biomol Struct **24**: 85-116.

Cullen, P. J. and P. J. Lockyer (2002). "Integration of calcium and Ras signalling." Nat Rev Mol Cell Biol **3**(5): 339-48.

Davis, A. J., J. T. Butt, et al. (1996). "The Ca2+-dependent lipid binding domain of P120GAP mediates protein-protein interactions with Ca2+-dependent membrane-binding proteins. Evidence for a direct interaction between annexin VI and P120GAP." J Biol Chem **271**(40): 24333-6.

Dikic, I., G. Tokiwa, et al. (1996). "A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation." Nature **383**(6600): 547-50.

Dolmetsch, R. E., K. Xu, et al. (1998). "Calcium oscillations increase the efficiency and specificity of gene expression." Nature **392**(6679): 933-6.

Ebinu, J. O., D. A. Bottorff, et al. (1998). "RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs." Science **280**(5366): 1082-6.

Fam, N. P., W. T. Fan, et al. (1997). "Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras." Mol Cell Biol **17**(3): 1396-406.

Farnsworth, C. L., N. W. Freshney, et al. (1995). "Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF." Nature **376**(6540): 524-7.

Fast, J., M. Hakansson, et al. (2001). "Symmetrical stabilization of bound Ca2+ ions

in a cooperative pair of EF-hands through hydrogen bonding of coordinating water molecules in calbindin D(9k)." Biochemistry **40**(33): 9887-95.

Flaherty, K. M., S. Zozulya, et al. (1993). "Three-dimensional structure of recoverin, a calcium sensor in vision." Cell **75**(4): 709-16.

Gawler, D. J., L. J. Zhang, et al. (1995a). "Mutation-deletion analysis of a Ca(2+)-dependent phospholipid binding (CaLB) domain within p120 GAP, a GTPase-activating protein for p21 ras." Biochem J **307 (Pt 2)**: 487-91.

Gawler, D. J., L. J. Zhang, et al. (1995b). "CaLB: a 43 amino acid calcium-dependent membrane/phospholipid binding domain in p120 Ras GTPase-activating protein." Oncogene **10**(5): 817-25.

Griffith, J. P., J. L. Kim, et al. (1995). "X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex." Cell **82**(3): 507-22.

Healy, J. I., R. E. Dolmetsch, et al. (1997). "Different nuclear signals are activated by the B cell receptor during positive versus negative signaling." Immunity **6**(4): 419-28.

Hernandez-Pineda, R., A. Chow, et al. (1999). "Kv3.1-Kv3.2 channels underlie a high-voltage-activating component of the delayed rectifier K+ current in projecting neurons from the globus pallidus." J Neurophysiol **82**(3): 1512-28.

Ikura, M. (1996). "Calcium binding and conformational response in EF-hand proteins." Trends Biochem Sci **21**(1): 14-7.

Ikura, M., G. M. Clore, et al. (1992). "Solution structure of a calmodulin-target peptide complex by multidimensional NMR." Science **256**(5057): 632-8.

Ji, Q. S. and G. Carpenter (2000). "Role of basal calcium in the EGF activation of MAP kinases." Oncogene **19**(14): 1853-6.

Kawasaki, H., G. M. Springett, et al. (1998). "A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia." Proc Natl Acad Sci U S A **95**(22): 13278-83.

Kretsinger, R. H. and C. E. Nockolds (1973). "Carp muscle calcium-binding protein. II. Structure determination and general description." J Biol Chem **248**(9): 3313-26.

Kumar, R., E. Wieben, et al. (1989). "The molecular cloning of the complementary deoxyribonucleic acid for bovine vitamin D-dependent calcium-binding protein: structure of the full-length protein and evidence for homologies with other calcium-binding proteins of the troponin-C superfamily of proteins." Mol Endocrinol **3**(2): 427-32.

Lev, S., H. Moreno, et al. (1995). "Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions." Nature **376**(6543): 737-45.

Linse, S., N. R. Bylsma, et al. (1994). "A calbindin D9k mutant with reduced calcium affinity and enhanced cooperativity. Metal ion binding, stability, and structural studies." Biochemistry **33**(41): 12478-86.

Linse, S. and W. J. Chazin (1995). "Quantitative measurements of the cooperativity in an EF-hand protein with sequential calcium binding." Protein Sci **4**(6):

1038-44.

Lockyer, P. J., S. Kupzig, et al. (2001). "CAPRI regulates Ca(2+)-dependent inactivation of the Ras-MAPK pathway." Curr Biol **11**(12): 981-6.

Martegani, E., M. Vanoni, et al. (1992). "Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a Saccharomyces cerevisiae RAS activator." Embo J **11**(6): 2151-7.

Meador, W. E., A. R. Means, et al. (1993). "Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures." Science **262**(5140): 1718-21.

Medema, J. P., M. W. Sark, et al. (1994). "Calcium inhibits epidermal growth factor-induced activation of p21ras in human primary keratinocytes." Mol Cell Biol **14**(11): 7078-85.

Munshi, H. G., D. J. Burks, et al. (1996). "Ca2+ regulates calmodulin binding to IQ motifs in IRS-1." Biochemistry **35**(49): 15883-9.

Nel, A. E. (2002). "T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse." J Allergy Clin Immunol **109**(5): 758-70.

Nel, A. E. and N. Slaughter (2002). "T-cell activation through the antigen receptor. Part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy." J Allergy Clin Immunol **109**(6): 901-15.

Nelson, M. R. and W. J. Chazin (1998). "Structures of EF-hand Ca(2+)-binding proteins: diversity in the organization, packing and response to Ca2+ binding." Biometals **11**(4): 297-318.

Rosen, L. B., D. D. Ginty, et al. (1994). "Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras." Neuron **12**(6): 1207-21.

Shou, C., C. L. Farnsworth, et al. (1992). "Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21." Nature **358**(6384): 351-4.

Skelton, N. J., J. Kordel, et al. (1994). "Signal transduction versus buffering activity in Ca(2+)-binding proteins." Nat Struct Biol **1**(4): 239-45.

Strynadka, N. C. and M. N. James (1989). "Crystal structures of the helix-loop-helix calcium-binding proteins." Annu Rev Biochem **58**: 951-98.

Tognon, C. E., H. E. Kirk, et al. (1998). "Regulation of RasGRP via a phorbol ester-responsive C1 domain." Mol Cell Biol **18**(12): 6995-7008.

Walker, S. A., S. Kupzig, et al. (2004). "Identification of a Ras GTPase-activating protein regulated by receptor-mediated Ca2+ oscillations." Embo J **23**(8): 1749-60.

Wei, W., R. D. Mosteller, et al. (1992). "Identification of a mammalian gene structurally and functionally related to the CDC25 gene of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **89**(15): 7100-4.

Weissman, B. and S. A. Aaronson (1985). "Members of the src and ras oncogene families supplant the epidermal growth factor requirement of BALB/MK-2 keratinocytes and induce distinct alterations in their terminal differentiation

program." Mol Cell Biol **5**(12): 3386-96.

Zhang, M., T. Tanaka, et al. (1995). "Calcium-induced conformational transition revealed by the solution structure of apo calmodulin." Nat Struct Biol **2**(9): 758-67.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6

6.1 Summary of PKC-mediated RasGRP phosphorylation

RasGRP3 was previously found to undergo PKC-inhibitor sensitive Serine/Threonine phosphorylation following BCR ligation. In this study, I further characterized the PKC-regulated phosphorylation of RasGRP3 and studied its implications in other RasGRP members.

Using mass spectrometry analysis, I provided physical evidence showing that Thr133 on RasGRP3 is a direct substrate of PKCθ, both *in vitro* and in phosphatase-inhibited insect cells. Sequence adjacent to Thr133 resembles the consensus sequence recognized by PKC. Further *in vitro* kinase labeling experiments and mutagenesis studies showed that alanine substitution of Thr133 completely blocked RasGRP3 *in vitro* phosphorylation by PKCθ, which could be rescued by serine substitution at this site. I also showed *in vitro* phosphorylation of RasGRP3 on Thr133 by PKCδ. The phospho-mutant of RasGRP3 (RasGRP3-T133A) was defective in PMA-induced Ras-Erk activation, Rap activation, and PMA-stimulated cell growth when ectopically expressed in Rat2 fibroblasts, demonstrating the functional significance of Thr133 phosphorylation in RasGRP3 activation. Subsequently, I generated a phosphopeptide antiserum specific for Thr133-phosphorylated RasGRP3 and used this reagent to show that Thr133 of RasGRP3 is phosphorylated *in vivo* in a PKC-dependent manner in BCR-stimulated mouse primary B cells. This result has been independently demonstrated in DT40 chicken B cells (Aiba et al. 2004).

RasGRP1 contains a homologous threonine at position 184 (Thr184). I generated another phosphopeptide antibody that specifically recognizes Thr184-phosphorylated RasGRP1 and showed this site is also subject to PKC-regulated phosphorylation in stimulated mouse primary T cells, consistent with the observation that recombinant RasGRP1 Thr184 was able to be *in vitro*

phosphorylated by PKCθ, PKCδ, and PKCβ_2. Moreover, the RasGRP1 mutant containing an alanine substitution at position 184 (RasGRP1-T184A) partially impaired *in vitro* phosphorylation by PKCβ_2 and PKCδ, although phosphorylation by PKCθ on RasGRP1 was still intact. Subsequent analysis on RasGRP1 phospho-mutant, however, failed to show any signaling defects in terms of PMA-stimulated Ras-Erk activation in Rat2 fibroblasts.

Sequence analysis shows that RasGRP4 is the only RasGRP family member to exhibit gross sequence variation around the homologous threonine site. It does not have the conserved threonine, but instead, has multiple proline residues in this region, suggesting RasGRP4 is regulated by other means. In agreement with this idea, I showed PMA stimulation of RasGRP4-dependent Ras-Erk activation was not sensitive to PKC inhibition in Rat2 cells.

6.2 Model for integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRPs

Based on the above observation, we proposed a model to show integration of DAG and PKC signaling pathways in B cells (Figure 6.1). In this model, BCR ligation activates protein tyrosine kinases such as Btk, Lyn, and Syk, eventually leading to the membrane recruitment and activation of a PLC-γ isoform, which in turn hydrolyzed PIP_2 to generate two secondary messengers: IP_3 and DAG. DAG recruits both RasGRP3 and some forms of PKC to the same membrane, which is followed by regulatory phosphorylation of the former on threonine 133 by the latter. This process may also require the formation of a multi-protein complex mediated by adaptors such as Grb2 to modulate RasGRP3 activity in greater efficiency. Moreover, the IP_3 stimulates intracellular calcium elevation through binding to IP_3R, which facilitates the membrane targeting of cPKCs, and perhaps RasGRP3. The potential roles of adaptor proteins and calcium in regulating RasGRP activity have

also been studied (see chapter 4 and 5 for details).

In T cells, where RasGRP3 expression is undetectable, PKC-dependent RasGRP1 phosphorylation may be responsible for regulation of Ras activation following TCR ligation. However, since mutagenesis study on RasGRP1 suggested the presence of additional phosphorylation sites, the regulation of Ras activation by PKC-mediated RasGRP1 phosphorylation may involve a more complex mechanism.

6.3 Future directions for studies of RasGRP regulatory phosphorylation

Regulatory phosphorylation as a means to fine-tune RasGEF activity is not unique to the RasGRP family members. In EGF-stimulated cells, Sos lies in a negative feedback pathway in which the serine/threonine phosphorylation of Sos by activated Erk results in disassociation of the Sos/Grb2 complex thereby limiting the extent of Ras activation (Buday et al. 1995; Cherniack et al. 1995; Waters et al. 1995a; Waters et al. 1995b). Moreover, the Rac GEF activity of Sos was recently found to be positively regulated by Abl–mediated tyrosine phosphorylation (Sini et al. 2004). Other examples include phosphorylation on both serine/threonine, as in the case of Tiam1 and RasGRF1 (Mattingly and Macara 1996; Fleming et al. 1999), and on tyrosine, as in the cases of Vav, Ras-GRF1 (Mattingly and Macara 1996; Crespo et al. 1997; Aghazadeh et al. 2000). Thus, examination of mechanisms that might underly the regulatory phosphorylation on RasGRPs could greatly improve our understanding on the complex signaling network governing Ras activation.

6.3.1 Identification of the kinases responsible for RasGRP phosphorylation

A critical step in understanding the biological significance of PKC-dependent RasGRP phosphorylation is to identify the responsible kinases. However, identification of the physiological function of individual PKC isoforms has been

restricted by the availability of only a few agents that inhibit or activate the isoforms with specificity, largely due to the structural similarity among PKC isoforms. Moreover, hematopoietic cells often express multiple PKC isoforms at the same time, which may play redundant roles. For example, I showed several PKC isoforms could efficiently phosphorylate RasGRP1 and RasGRP3 *in vitro*. However, the insight into the functional significance of individual PKC isoform can be gained from the genetic analysis of mice deficient for specific PKC isoforms. For example, PKCθ is expressed in a relatively selective manner in T lymphocytes, similar to the expression profile of RasGRP1. Characterization of PKCθ null mice indicates that PKCθ does not have an obvious role in T cell development, but it is essential for the activation, proliferation, and cytokine production of mature T cells. Following TCR ligation, PKCθ selectively translocates to the central region of the immunological synapse (IS) where it activates c-Jun N-terminal kinase and initiates the nuclear factor-κB cascade (Monks *et al.* 1998; Isakov and Altman 2002). PKCθ translocation to IS seems to depend on PI3K, Rac and actin cytoskeleton reorganization (Villalba et al. 2001). Thus, it will be interesting to know if RasGRP1 co-localizes with PKCθ in the IS during T cell activation. A recent study showed that the internal membranes, instead of the plasma membrane, are the primary sites for TCR induced RasGRP1 membrane localization (Bivona *et al.* 2003). Moreover, the C1 domain isolated from RasGRP1 was found concentrated in the ER and the Golgi body in a DAG-dependent manner in living Jurkat T cells, whereas the polypeptide consisting of the two C1 domains from PKCθ predominantly localized to plasma membrane, implying distinct functional roles of the two proteins (Carrasco and Merida 2004). Other PKC isoforms such as PKCδ may thus be responsible for RasGRP1 phosphorylation. However, it is important to note that in these studies, T cells were stimulated by activating antibodies, which cannot trigger some of the physiological T-cell responses that have only been seen following antigen presenting cell (APC) encounter (Monks *et al.* 1997). Therefore, further study should address the subcellular localization of endogenous RasGRP1 under more physiological

relevant conditions in T cells.

The phenotypes observed in mice deficient for PKCβ are characterized by impaired B cell activation and ineffective T-independent immune response, both requiring BCR signaling (Leitges *et al.* 1996). PKCδ-deficient mice show significant splenomegaly and lymphadenopathy and develop severe autoimmune disease (Mecklenbrauker *et al.* 2002). Interestingly, similar phenotypes are also seen in RasGRP1 null mice. Since some of the PKC isoforms may compensate for the loses of one specific isoform, understanding the distinct roles of each PKC isoform will require analyzing mice with multiple PKC-deficiencies.

PKCs may also regulate RasGRPs indirectly through activating intermediate kinases. One of the candidates is the protein kinase D (PKD) family of enzymes. DAG stimulated serine/threonine kinases expressed in many cell types including lymphocytes. PKD contains a catalytic domain different from that of all PKC family members, and a large inhibitory, regulatory domain, comprised of two Zinc fingers, and a PH domain (Figure 6.2) (Lint *et al.* 2002). Upon immune receptor conjugation, PKD is activated by phosphorylation on multiple sites by PKCs. Only a few potential substrates of PKD have been suggested so far, including RIN and PLC-γ1. Go6979, an inhibitor that blocks conventional PKC activity, also blocks PKD activity. More importantly, PKD activation is correlated with Ras/MAP kinase cascade activation. Thus, the PKC-dependent RasGRP activation may be mediated by phosphorylation of RasGRPs by PKD (Figure 6.2).

6.3.2 Mechanisms of regulatory phosphorylation

Although both RasGRP1 and RasGRP3 contain homologous threonine residues that can be phosphorylated upon stimulation, RasGRP1 seems also contain additional sites for serine/threonine kinases. No serine/threonine phosphorylation has been detected for RasGRP4 so far. These observations give rise to the hypothesis that different modes of serine/threonine phosphorylation may account for a specific role for each isoform. As the kinases responsible for this phosphorylation vary

depending on the stimulus, the biological role of this phosphorylation may also vary.

Phosphorylation may affect the GEF activity of RasGRPs by several mechanisms. According to the three dimensional structure model built by homology modeling based on a crystal structure of Sos, the conserved threonine phosphorylated in RasGRP1 or RasGRP3 is located in a flexible loop opposite to the Ras binding site in the catalytic cdc25 box of both proteins (Aiba *et al.* 2004). Introduction of a negative charge through threonine phosphorylation may thus cause conformational changes in critical residues that allosterically increase the Ras binding affinity or stabilize the catalytic site. An example for this type of regulation can be seen in the phosphorylation-dependent Vav activation, where phosphorylation on specific tyrosines is required for the binding of Vav to its substrate (Schuebel *et al.* 1996; Movilla and Bustelo 1999). Serine RasGEF activity of RasGRF1 is also positively regulated by phosphorylation (Mattingly and Macara 1996; Yang *et al.* 2003). Another mechanism of serine/threonine phosphorylation is to mediate inter- and intra-molecular interactions. In Sos, phosphorylation of serine within its carboxyl terminus domain induced by the growth factors has been thought to be involved in mediating intra-molecular interactions that lead to negative regulation of Sos (Corbalan-Garcia *et al.* 1998). Furthermore, phosphothreonine in RasGRPs may serve as the docking site for the assembly of signaling complex through specific adaptor proteins such as the 14-3-3 protein, a ubiquitously expressed phosphoserine/phosphothreoine-binding module participating in many protein kinase signaling pathways (Wilker and Yaffe 2004). Therefore, identification of binding partners specific to phosphorylated RasGRPs in activated T cells and B cells will provide essential information for the understanding of the mechanisms of RasGRP regulation.

6.3.3 Identification of additional phosphorylation sites

Like Sos and RasGRF1, regulation of RasGRPs by phosphorylation is very likely to involve more than one phosphorylation site. In my study, the mass

spectrometry analysis on RasGRP3 expressed in okadiac acid stimulated insect cells resulted in the identification of nine potential serine/threonine phosphorylation sites in addition to Thr133. Serine 3 is substituted by a conserved threonine in RasGRP1 and RasGRP2, while the serine 4 is not conserved. Threonine 223 is located in the CDC25 box, but not found in other RasGRPs, implying an isoform-specific function in RasGRP3. The rest of the potential phosphorylation sites are all identified in the long C-terminal tail of RasGRP3. Ser 558 is conserved in RasGRP1 and RasGRP2, but not in RasGRP4, whereas Ser 562 is only found in the RasGRP3 sequence. Site-directed mutagenesis study on these two sites suggested that neither is required for RasGRP3-dependent Ras-Erk activation in fibroblasts (data not shown). Whether they are required for RasGRP1 and RasGRP2 activity is not clear. Serine 597 and serine 601 are located close to a basic residue, making them candidate sites for PKCs. A RasGRP3 mutant that contains alanine substitution at Serine 597, 598, and 601 can only be poorly expressed ectopically in Rat2 cells, suggesting a role of these residues in maintaining protein stability (data not shown). Finally, threonine 682 is unique to RasGRP3. Alanine substitution on this site did not block *in vitro* ^{32}P-labeling by PKCs and had no affect on RasGRP3 activity (data not shown). Interestingly, none of the above phosphorylation sites was observed in bacterially expressed recombinant RasGRP3 *in vitro* phosphorylated by PKC. Therefore, it is possible that *in vivo* phosphorylation on these sites, if any, requires the involvement of additional regulatory molecules, which may increase the accessibility of these sites to PKC phosphorylation. Alternatively, protein kinases other than PKC isoforms may be responsible for phosphorylation on these sites.

A hint for multiple site phosphorylation on RasGRPs came from *in vitro* phosphorylation experiments on RasGRP1. Alanine replacement of threonine at position 184 in RasGRP1 resulted in an exact 50% loss of ^{32}P labeling on RasGRP1 mutant catalyzed by PKCδ and PKCβ, whereas PKCθ-dependent ^{32}P incorporation was not affected, suggesting the presence of another PKC site. This observation also implies that different PKC isoforms may regulate RasGRP through distinct

mechanisms by catalyzing phosphorylations on different sites. In support of this idea, phosphorylation of RasGRP3 stimulated by okadaic acid treatment, which activates many classes of kinases, seemed to negatively regulate RasGRP3 activity suggesting the presence of a negative phosphorylation site (Teixeira *et al.* 2003). It is possible that different cell surface receptors activate different kinases to phosphorylate RasGRP and that the consequences of RasGRP phosphorylation depends on sites of its phosphorylation. Thus, a comprehensive understanding of the role of phosphorylation in regulating RasGRP activity depends on the identification of more phosphorylation sites.

6.4 Summary of the study on Grb2 family member-mediated RasGRP1 regulation

By analogy to the Grb2/Sos model downstream of RTK signaling, it is proposed that during TCR signaling, Grb2, or its family members, binds to the putative PXXP motif of RasGRP1 via its SH3 domains to further regulate its activation. In this study, I showed a direct interaction between Grb2 and RasGRP1 in an *in vitro* GST pull-down assay. Similar to the Grb2 binding of Sos, both SH3 domains seem required for the binding of full-length Grb2 to RasGRP1, since mutations that disrupt either of the two SH3 domains blocked *in vitro* binding. However, binding assays using the isolated SH3 domains of Grb2 showed that the N-terminal SH3 domain, when tested alone, may have a much higher efficiency of binding to RasGRP1 than its counterpart at the C-terminus does. To determine the involvement of the PXXP motif in Grb2 binding, I disrupted the PXXP motif by replacing the conserved prolines with alanine. This RasGRP1 mutant still bound to Grb2, but showed a moderate decrease in binding affinity, comparing with that of the wildtype protein, suggesting the rest of the RasGRP1 sequence may have regions contribute to Grb2 binding.

Except for Grb2, lymphocytes also express the other two Grb2 family members, Grap and Gads. Gads has been shown to plays an essential role in TCR signaling. With the failure in demonstrating the *in vivo* interaction between Grb2 and RasGRP1, I tested the possibility that RasGRP1 associated with Grap and Gads. I showed that all three Grb2 family members bind to RasGRP1 *in vitro* with slightly different efficiency. However, the RasGRP1 in lysates of Jurkat T cells primarily bound to recombinant Gads, and, to a much less extent, Grb2. In contrast, no detectable binding of endogenous RasGRP1 to recombinant Grap was observed, although it bound to Sos1 with great efficiency in this experiment. Similar to the interaction between Grb2 and Sos, the binding between Gads and RasGRP1 did not require TCR signaling.

6.5 Future directions on identification of RasGRP binding partners

6.5.1 Identify potential RasGRP PXXP motif-binding partners

Despite the *in vitro* binding, co-immuneprecipitation between Grb2 or Gads and RasGRP1 was not successful, possibly due to the limitation of available immune reagents. Thus, a definitive conclusion concerning the *in vivo* interaction of RasGRP1 and Grb2 family members may depend on the use of alternative methods, such as the combination of co-immuneprecipitation and mass spectrometry. In addition, sequence analysis shows that other RasGRPs also contain multiple PXXP consensus sequences that potentially confer binding to the SH3 domain of Grb2 family members. Further experiments are needed to determine if these sites are functional.

It is possible that SH2/SH3 domain-containing adaptor proteins other than Grb2 family members function to bind and recruit RasGRPs in cells. Several adaptor proteins of this kind have been implicated in the regulation of Ras signaling. For example, Nck protein is a wildly expressed 47-kDa cytosolic molecules exclusively

composed of one SH2 and three SH3 domains. Overexpression of Nck caused transformation of mammalian fibroblasts (Chou and Hanafusa 1995). Nck is involved in regulation of actin cytoskeleton. However, several reports suggest that Nck associates with Sos *in vivo* through its SH3 domains (Hu *et al.* 1995; Okada and Pessin 1996; Wunderlich *et al.* 1999a). In addition, Nck-dependent activation of the *fos* promoter can be blocked by dominant negative Ras, leading to the speculation that, by analogy with Grb2, Nck is involved in Ras activation (Hu et al. 1995). This hypothesis is further supported by the observation that targeting the first two SH3 domains of Nck to the plasma membrane activated Erk1 in the absence of any growth factors, suggesting that recruitment of Sos to the membrane through Nck is sufficient for Ras and Erk1 activation (Gupta and Mayer 1998). Upon TCR stimulation, the tyrosine-phosphorylated SLP-76 scaffolding molecule recruits Nck adapter through its SH2 domains (Bubeck Wardenburg et al. 1998; Yablonski et al. 1998; Wunderlich et al. 1999b). Another candidate molecule for RasGRP proline-rich region binding is the Crk protein, which also consists of SH2 and SH3 domains. Crk is involved in a variety of signaling cascades such as those of growth factor receptor, integrin, TCR, BCR, and cytokines (Kiyokawa et al. 1997). During the early stage of lymphocyte activation, the SH2 domain of Crk transiently interacts with signal-transducing molecules, such as Cbl, ZAP-70, CasL, and STAT5. The output from Crk thus depends on the effector molecules recruited by its SH3 domains. The binding partners of Crk includes the RapGEF C3G, Abl tyrosine kinase, and the RasGEF Sos (Kiyokawa et al. 1997; Gelkop et al. 2003).

In addition to the SH3 domain, several other protein modules have also been shown to be able to recognize proline-rich sequences. The WW domain is a small 38 amino acid residue module containing two signature tryptophan (W) residues. Structural studies showed that the general rules of proline-rich motif recognition by WW domains were very similar to those used by SH3 domains. Therefore, these two domains can potentially bind overlapping sites (Koch et al, 1991; Harlan et al, 1994; van der Geer & Pawson, 1995; Einbond & Sudol, 1996; Lu et al, 1999). One

example of the WW domain-containing proteins is the ubiquitin protein ligase Nedd4. It binds to CNrasGEF, the cAMP activated Ras activator, and directs its ubiquitination (Kanelis *et al.* 2001; Pham and Rotin 2001). A similar mechanism may also account for the regulation of RasGRP stability. Interestingly, the binding of proline-rich sequences by some WW domains depends on the phosphorylation of adjacent serine/threonin residues, perhaps by proline-directed protein kinases such as MAP kinases (Yaffe *et al.* 1997). Other proline-rich motif-binding domains include the *Drosophila* enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domains, the GYF domains, and profilin domains (Bjorkegren *et al.* 1993; Mahoney *et al.* 1997; Nishizawa *et al.* 1998; Ball *et al.* 2002). Proteins containing these domains participate in distinct cellular processes such as transcription, RNA processing, protein trafficking, protein turnover, receptor signaling, and control of the cytoskeleton. Therefore, the proline-rich region of RasGRPs may mediate the binding to some of these proteins. To identify all the possible protein-protein interactions that involve RasGRPs, a systematic analysis of potential RasGRP binding partners using proteomic approaches such as mass spectrometry-based techniques on a proteome-wide scale is necessary. For example, the GST protein-fused PXXP motif of RasGRP1 can be used as the bait to pull down potential binding proteins in the lysates of stimulated Jurkat T cell line, which will be followed by protein profiling by mass spectrometry. Such information will be useful in understanding the role of the PXXP motif in regulation of RasGRP activity. Moreover, a novel small molecule named UCS15A has been recently reported to specifically block the SH3 domain-mediated protein-protein interaction, providing a potentially useful tool to dissect the functional role of the RasGRP PXXP motif in Ras signaling through pharmacological intervention (Sharma et al. 2001; Oneyama et al. 2002; Oneyama et al. 2003).

6.5.2 Other motifs in RasGRP1 that may be involved in protein-protein interaction

Another potentially interesting structural characteristic of RasGRP1 is the

presence of a putative coiled-coil domain. Using computational programs such as *COILS2* (Lupas *et al.* 1991; Lupas 1997) (http://www.ch.embnet.org/software/COILS_form.html), the coiled-coil domain is predicated near the end of the RasGRP1 C-terminal tail (residue 747-786). The coiled-coil domain consists of intimately-associated bundles of long alpha-helices. It is found in a diverse array of proteins, such as the transcription factor Jun and Fos, to mediate intramolecular and intermolecular interactions (Glover and Harrison 1995; Delahay and Frankel 2002). It is estimated that coiled-coil domain-containing proteins comprise 3-5% of the genomic complement and are implicated in a wide range of biological activities (Newman et al. 2000). In RasGRF1, a coiled-coil domain co-operates with IQ and PH domains for maximal stimulation of exchange activity by calmodulin-complexed calcium (Buchsbaum *et al.* 1996). Whether the coiled-coil domain of RasGRP1 play a functional role needs to be determined. Since the coiled-coil domain is lacking in other RasGRPs, it may be involved in the regulation of RasGRP1-specific functions.

6.6 Summary of the study on RasGRP1 EF-hands

The presence of the calcium-binding EF-hand motif makes RasGRPs a unique group of RasGEF. However, the functional significance of the EF-hand motif is far from clear. Using RasGRP1 as the model protein, I performed *in vitro* $^{45}Ca^{2+}$ binding assay in order to characterize the calcium-binding ability of each EF-hand. I showed that wildtype RasGRP1 bound Ca^{2+} *in vitro* and the left EF-hand contribute to most of the calcium binding as single point mutation completely blocked calcium binding. However, mutations in the EF-hands had no affect on RasGRP1 activation of Ras-Erk signaling in Rat2 fibroblasts, suggesting it is dispensable for RasGRP1 activity.

6.7 Future directions in understanding the role of EF-hands in RasGRPs

The functional role of EF-hands in RasGRPs is still a mystery. Although the first EF-hand bound calcium *in vitro*, it needs to be confirmed *in vivo* under physiological conditions. Determining the function of the RasGRP EF-hands depends on solving their structures, since the extent of conformational change that calcium binding induces in EF-hand proteins is a key biochemical property specifying Ca^{2+} sensor versus signal modulator function.

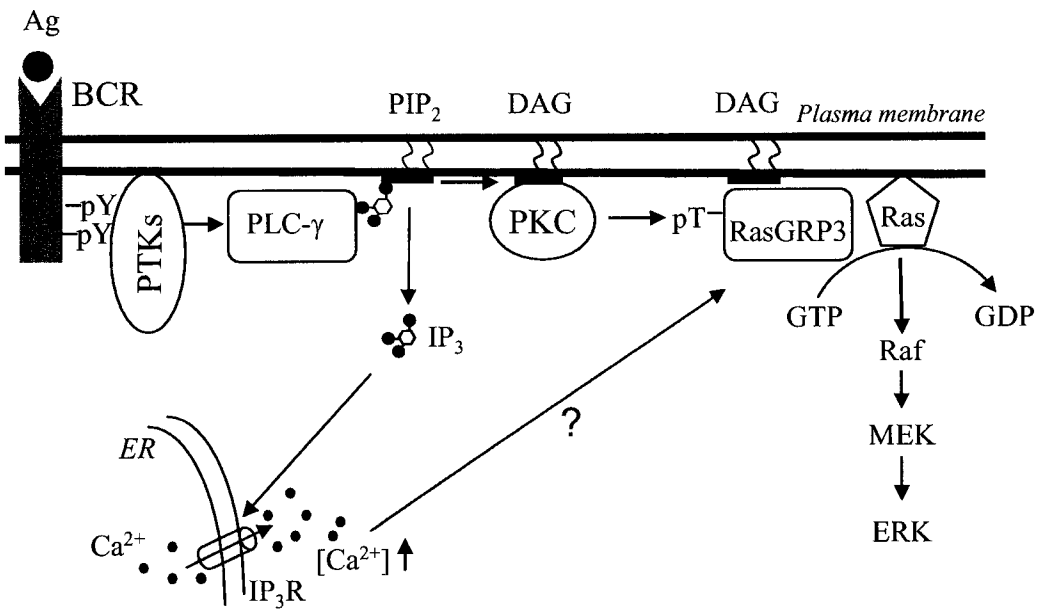
The atypically short distance between the two EF-hands of RasGRPs suggests that the EF-hands are involved in regulation of RasGRPs through a mechanism that does not require calcium binding. One of the examples is dystrophin, a cytoskeleton protein with scaffolding and calcium sensor functions. Dystrophin contains a WW domain and two adjacent EF-hand-like domains which form a composite surface that is required for dystrophin to bind the C-terminal Pro-Pro-X-Tyr motif in β-dystroglycan. The EF-hand region of dystrophin does not bind calcium, but functions to stabilize the fold of the WW domain and provides additional specificity in recognition of β-dystroglycan (Huang *et al.* 2000).

6.8 Concluding remarks

RasGEFs are often subjected to multiple modes of regulation. The activation of both Sos and RasGRF has been shown to involve complex interactions and cooperativity between multiple domains, with membrane localization being required but not always fully sufficient for activation (Schmidt and Hall 2002). This study presents evidence to show that the regulation of RasGRPs also requires multiple mechanisms. While the C1 domain-mediated membrane localization appears to have a dominant role in activating RasGRPs, regulatory phosphorylation is necessary for the full activity of certain RasGRPs. In addition, other modes of regulation, including protein-protein interaction and calcium-mediated regulation may also

operate to fine-tune the activity of RasGRPs. Therefore, RasGRPs function as the convergent point of distinct signaling pathways.

Importantly, some modes of RasGRP regulation may function cooperatively. For example, it is not clear how a particular RasGRP family member selectively activates certain Ras isoforms. It is possible that the phosphorylation of a RasGRP at a specific site alters its affinity towards specific adaptor proteins and thus changes its effector affinity, allowing the use of a single GEF at distinct times and locations. This sort of regulation mechanism has been observed in the coordination of Ras and Rac activity by Sos. The proline-rich region of Sos is shown to interact either with Grb2 or with E3b1, both adaptor proteins. When formed a complex with Grb2, Sos mediates Ras activation downstream of a receptor tyrosine kinase. Phosphorylation events result in shifting Sos to E3b1, which then couples Sos to Rac activation (Innocenti *et al.* 2002). Uncovering the mechanisms of these RasGRP regulation modes will thus allow better understanding of how different signaling events are integrated to achieve the temporal and quantitative aspects of Ras activation that are required to yield specific cellular outcomes.



FIGRUE 6.1 Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3. See details in text. pY, phospho-tyrosine; pT, phospho-threonine; IP_3R, IP_3 receptor; ER, endoplasmic reticulum. Question mark (?) indicates a putative pathway.

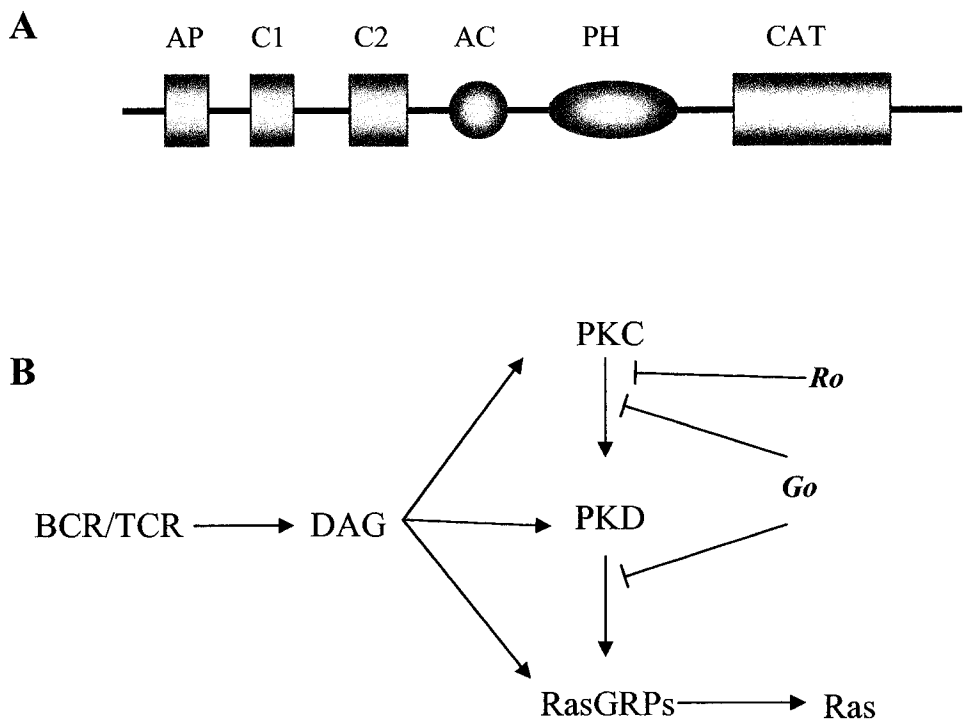


FIGURE 6.2 Proposed model for PKD mediated RasGRP phosphorylation. (**A**) Domain structure of PKD. AP: alanine and proline rich domain; CYS: cysteine-rich Zn finger domain; AC: acidic domain; PH: pleckstrin homology domain; CAT: catalytic domain. (**B**) Proposed model for indirect regulation of RasGRPs by PKC via PKD. Go: conventional PKC inhibitor; Ro: noval PKC inhibitor.

References

Aghazadeh, B., W. E. Lowry, et al. (2000). "Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation." Cell **102**(5): 625-33.

Aiba, Y., M. Oh-hora, et al. (2004). "Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation." Proc Natl Acad Sci U S A **101**(47): 16612-7.

Ball, L. J., T. Jarchau, et al. (2002). "EVH1 domains: structure, function and interactions." FEBS Lett **513**(1): 45-52.

Bivona, T. G., I. Perez De Castro, et al. (2003). "Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1." Nature **424**(6949): 694-8.

Bjorkegren, C., M. Rozycki, et al. (1993). "Mutagenesis of human profilin locates its poly(L-proline)-binding site to a hydrophobic patch of aromatic amino acids." FEBS Lett **333**(1-2): 123-6.

Bubeck Wardenburg, J., R. Pappu, et al. (1998). "Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76." Immunity **9**(5): 607-16.

Buchsbaum, R., J. B. Telliez, et al. (1996). "The N-terminal pleckstrin, coiled-coil, and IQ domains of the exchange factor Ras-GRF act cooperatively to facilitate activation by calcium." Mol Cell Biol **16**(9): 4888-96.

Buday, L., P. H. Warne, et al. (1995). "Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos." Oncogene **11**(7): 1327-31.

Carrasco, S. and I. Merida (2004). "Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes." Mol Biol Cell **15**(6): 2932-42.

Cherniack, A. D., J. K. Klarlund, et al. (1995). "Disassembly of Son-of-sevenless proteins from Grb2 during p21ras desensitization by insulin." J Biol Chem **270**(4): 1485-8.

Chou, M. M. and H. Hanafusa (1995). "A novel ligand for SH3 domains. The Nck adaptor protein binds to a serine/threonine kinase via an SH3 domain." J Biol Chem **270**(13): 7359-64.

Corbalan-Garcia, S., S. M. Margarit, et al. (1998). "Regulation of Sos activity by intramolecular interactions." Mol Cell Biol **18**(2): 880-6.

Crespo, P., K. E. Schuebel, et al. (1997). "Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product." Nature **385**(6612): 169-72.

Delahay, R. M. and G. Frankel (2002). "Coiled-coil proteins associated with type III secretion systems: a versatile domain revisited." Mol Microbiol **45**(4): 905-16.

Fleming, I. N., C. M. Elliott, et al. (1999). "Ca2+/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation." J Biol Chem **274**(18): 12753-8.

Gelkop, S., Y. Babichev, et al. (2003). "Involvement of crk adapter proteins in regulation of lymphoid cell functions." Immunol Res **28**(2): 79-91.

Glover, J. N. and S. C. Harrison (1995). "Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA." Nature **373**(6511): 257-61.

Gupta, R. W. and B. J. Mayer (1998). "Dominant-negative mutants of the SH2/SH3 adapters Nck and Grb2 inhibit MAP kinase activation and mesoderm-specific gene induction by eFGF in Xenopus." Oncogene **17**(17): 2155-65.

Hu, Q., D. Milfay, et al. (1995). "Binding of NCK to SOS and activation of ras-dependent gene expression." Mol Cell Biol **15**(3): 1169-74.

Huang, X., F. Poy, et al. (2000). "Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan." Nat Struct Biol **7**(8): 634-8.

Innocenti, M., P. Tenca, et al. (2002). "Mechanisms through which Sos-1 coordinates the activation of Ras and Rac." J Cell Biol **156**(1): 125-36.

Isakov, N. and A. Altman (2002). "Protein kinase C(theta) in T cell activation." Annu Rev Immunol **20**: 761-94.

Kanelis, V., D. Rotin, et al. (2001). "Solution structure of a Nedd4 WW domain-ENaC peptide complex." Nat Struct Biol **8**(5): 407-12.

Kiyokawa, E., N. Mochizuki, et al. (1997). "Role of Crk oncogene product in physiologic signaling." Crit Rev Oncog **8**(4): 329-42.

Leitges, M., C. Schmedt, et al. (1996). "Immunodeficiency in protein kinase cbeta-deficient mice." Science **273**(5276): 788-91.

Lint, J. V., A. Rykx, et al. (2002). "Getting to know protein kinase D." Int J Biochem Cell Biol **34**(6): 577-81.

Lupas, A. (1997). "Predicting coiled-coil regions in proteins." Curr Opin Struct Biol **7**(3): 388-93.

Lupas, A., M. Van Dyke, et al. (1991). "Predicting coiled coils from protein sequences." Science **252**(5010): 1162-4.

Mahoney, N. M., P. A. Janmey, et al. (1997). "Structure of the profilin-poly-L-proline complex involved in morphogenesis and cytoskeletal regulation." Nat Struct Biol **4**(11): 953-60.

Mattingly, R. R. and I. G. Macara (1996). "Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta gamma subunits." Nature **382**(6588): 268-72.

Mecklenbrauker, I., K. Saijo, et al. (2002). "Protein kinase Cdelta controls self-antigen-induced B-cell tolerance." Nature **416**(6883): 860-5.

Monks, C. R., B. A. Freiberg, et al. (1998). "Three-dimensional segregation of supramolecular activation clusters in T cells." Nature **395**(6697): 82-6.

Monks, C. R., H. Kupfer, et al. (1997). "Selective modulation of protein kinase C-theta during T-cell activation." Nature **385**(6611): 83-6.

Movilla, N. and X. R. Bustelo (1999). "Biological and regulatory properties of Vav-3, a new member of the Vav family of oncoproteins." Mol Cell Biol **19**(11): 7870-85.

Newman, J. R., E. Wolf, et al. (2000). "A computationally directed screen identifying interacting coiled coils from Saccharomyces cerevisiae." Proc Natl Acad Sci

U S A **97**(24): 13203-8.

Nishizawa, K., C. Freund, et al. (1998). "Identification of a proline-binding motif regulating CD2-triggered T lymphocyte activation." Proc Natl Acad Sci U S A **95**(25): 14897-902.

Okada, S. and J. E. Pessin (1996). "Interactions between Src homology (SH) 2/SH3 adapter proteins and the guanylnucleotide exchange factor SOS are differentially regulated by insulin and epidermal growth factor." J Biol Chem **271**(41): 25533-8.

Oneyama, C., T. Agatsuma, et al. (2003). "Synthetic inhibitors of proline-rich ligand-mediated protein-protein interaction: potent analogs of UCS15A." Chem Biol **10**(5): 443-51.

Oneyama, C., H. Nakano, et al. (2002). "UCS15A, a novel small molecule, SH3 domain-mediated protein-protein interaction blocking drug." Oncogene **21**(13): 2037-50.

Pham, N. and D. Rotin (2001). "Nedd4 regulates ubiquitination and stability of the guanine-nucleotide exchange factor CNrasGEF." J Biol Chem **276**(50): 46995-7003.

Schmidt, A. and A. Hall (2002). "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes Dev **16**(13): 1587-609.

Schuebel, K. E., X. R. Bustelo, et al. (1996). "Isolation and characterization of murine vav2, a member of the vav family of proto-oncogenes." Oncogene **13**(2): 363-71.

Sharma, S. V., C. Oneyama, et al. (2001). "UCS15A, a non-kinase inhibitor of Src signal transduction." Oncogene **20**(17): 2068-79.

Sini, P., A. Cannas, et al. (2004). "Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation." Nat Cell Biol **6**(3): 268-74.

Teixeira, C., S. L. Stang, et al. (2003). "Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3." Blood **102**(4): 1414-20.

Villalba, M., K. Bi, et al. (2001). "Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells." J Cell Biol **155**(3): 331-8.

Waters, S. B., K. H. Holt, et al. (1995a). "Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex." J Biol Chem **270**(36): 20883-6.

Waters, S. B., K. Yamauchi, et al. (1995b). "Insulin-stimulated disassociation of the SOS-Grb2 complex." Mol Cell Biol **15**(5): 2791-9.

Wilker, E. and M. B. Yaffe (2004). "14-3-3 Proteins--a focus on cancer and human disease." J Mol Cell Cardiol **37**(3): 633-42.

Wunderlich, L., A. Farago, et al. (1999a). "Characterization of interactions of Nck with Sos and dynamin." Cell Signal **11**(1): 25-9.

Wunderlich, L., A. Farago, et al. (1999b). "Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes." Eur J Immunol **29**(4): 1068-75.

Yablonski, D., L. P. Kane, et al. (1998). "A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK." Embo J **17**(19): 5647-57.

Yaffe, M. B., M. Schutkowski, et al. (1997). "Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism." Science **278**(5345): 1957-60.

Yang, H., D. Cooley, et al. (2003). "Phosphorylation of the Ras-GRF1 exchange factor at Ser916/898 reveals activation of Ras signaling in the cerebral cortex." J Biol Chem **278**(15): 13278-85.