Analysis of activation of different Ras family members by hematopoietic growth factors and antigen receptors

by

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Abstract

Many members of the Ras superfamily of GTPases have been implicated in the regulation of hematopoietic cell growth, survival, differentiation, cytokine production, motility, vesicle-trafficking, and phagocytosis. The well-known p21 Ras proteins H-Ras, N-Ras, K-Ras 4A and K-Ras 4B are also frequently mutated in human cancer and leukemia. Besides the p21 Ras proteins, the "Ras" subfamily of the Ras superfamily includes R-Ras, TC21 (R-Ras2), M-Ras (R-Ras3), Rap1A/B, Rap2A/B, and RalA/B. They exhibit remarkable overall amino acid identities, especially in the regions interacting with the guanine nucleotide exchange factors that catalyze their activation. In addition, there is considerable sharing of various downstream effectors and of GTPase activating proteins that down-regulate their activity, resulting in overlap in their regulation and effector function. Relatively little is known about the physiological functions of individual Ras family members. The structural and functional similarities have meant that commonly used research tools fail to discriminate between the different family members, and that functions previously attributed to one family member may be shared with other members of the Ras family. However, evidence is emerging that Ras isoforms have specific functions, and it has been suggested that the differential localization of Ras proteins in different parts of the cell membrane may affect their downstream signaling.

In this study, we tested the hypothesis that the localization of some isoforms of Ras to lipid rafts would dictate their susceptibility to activation by four external stimuli, the ligation of the antigen receptors of B or T lymphocytes (BCR, TCR), and stimulation of the receptors for the hematopoietic growth factors IL-3 and CSF-1. While both types of stimuli activated different subsets of Ras proteins, we found that the localization of the Ras proteins to lipid rafts affected the activation of Ras isoforms by the growth factors, but had no influence on their activation by antigen receptors. We show that the BCR uses a PLC-γ dependent pathway to activate p21 Ras proteins, and that depletion of proteins of the diacylglycerol-responsive RasGRP family of exchange factors by prolonged exposure to phorbol ester correlated with reduced activation of p21 Ras. Thus, antigen receptors appear to signal through a preferred set of exchange factors which in turn exhibit differential activity towards Ras substrates.

This study provides the first evidence that Ras proteins, and in particular two members of the closely related p21 Ras proteins, can be activated differentially by physiological stimuli. The significance of the preferential activation of some Ras proteins over others remains to be

determined. Our data also imply that it cannot be generally predicted from the localization to membrane domains of receptors and Ras proteins which of the isoforms will be activated. This raises questions about the functions of membrane microdomains.

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Abbreviations

APC	antigen presenting cell
BCR	B cell receptor
βc	common beta chain of the IL-3, IL-5, and GM-CSF receptors
BODIPY® TR	N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indiazene-3-yl)phenoxy)acetyl)
cAMP	cyclic adenosine monophosphate
CSF	colony-stimulating factor
DAG	diacylglycerol
DH	dbl-homology
DNA	deoxyribonucleic acid
DRM	detergent-resistant membrane
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
Erk	extracellular signal regulated kinase
FACS	fluorescence activated cell sorter
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GDP	guanosine diphosphate
GDS	guanine nucleotide dissociation stimulator
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophge colony-stimulating factor
GRF	guanine nucleotide releasing factor
GRP	guanine nucleotide releasing protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP	horseradish peroxidase
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranoside
IRES	internal ribosomal entry site
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAP	mitogen activated protein

MAPK	mitogen activated protein kinase
MEK	MAP/Erk kinase
MEKK	MEK kinase
MES	2-[N-Morpholino]ethanesulfonic acid
MKK	MAP kinase kinase
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa B
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PdBu	phorbol-12,13-dibutyrate
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI	phosphatidylinositol
PI-3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
RA	Ras association
RBD	Ras binding domain
TBS-N	Tris-buffered sodium chloride solution with Nonidet P-40
TCR	T cell receptor

Preface

Thesis format

This thesis is divided into five chapters. The first chapter introduces the regulation and functions of Ras proteins. Chapter II describes the methods and materials used in chapters III and IV. Chapters III and IV describe and discuss data obtained during the course of this study. Chapter V contains a summary of general conclusions that can be drawn from this study as well as future directions.

Publications obtained during the course of this thesis

Ehrhardt, A., David, M., and Schrader, J.W. (in preparation). Growth factors preferentially activate Ras isoforms that localize outside lipid rafts.

Ehrhardt, A., Ehrhardt, G.R.A., and Schrader, J.W. (submitted). Localization to rafts does not determine the activation of Ras isoforms by ligation of the B or T cell antigen receptors.

Ehrhardt, A., Ehrhardt, G.R.A., Guo, X., and Schrader, J.W. (2002). Ras and relatives - job sharing and networking keep an old family together. *Exp. Hematol.* 30:1-18 (review)

Schrader, J.W., Schallhorn, A., Grill, B., and Guo, X. (2002). Activation of small GTPases of the Ras and Rho family by growth factors active on mast cells. *Mol. Immunol.* 38:1181-1186 (review)

Babic, I., Schallhorn, A., Lindberg F.P., and Jirik, F.R. (2000): SHPS-1 induces aggregation of Ba/F3 pro-B cells via an interaction with CD47. *J. Immunol.* 164:3652-3658

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THE TRUTH IS OUT THERE

CHAPTER 1 Introduction

1.1 Introduction to the Ras family of GTPases

1.1.1 Ras proteins as molecular switches

The history of the Ras family of proteins dates back almost four decades to observations that viruses could cause tumor formation in mice and rats (Harvey, 1964; Kirsten and Mayer, 1967). The viral genes responsible were called *"ras"*, for rat sarcoma, and turned out to be mutated versions of genes that encode enzymes with intrinsic GTPase activity. Thus, Ras proteins function as molecular switches with the "on" and "off" states determined by whether they are bound to GDP ("off" position), or GTP ("on" position). Inactive, GDP-bound Ras proteins are activated by interaction with members of a large structurally diverse class of proteins termed "guanine-nucleotide exchange factors" (GEFs) which catalyze the release of GDP. The lost GDP is then rapidly replaced by the more abundant GTP (Lenzen et al., 1998). This exchange of GDP for GTP results in an allosteric change in two key regions of the GTPase termed "Switch I" and "Switch II" (Boriack-Sjodin et al., 1998). Switch I is part of the so-called "effector loop" and enables the binding of a variety of different effector proteins when Ras is in its GTP-bound configuration. Various "GTPase activating proteins" (GAPs) also bind to Ras proteins in their GTP-bound state. They act as negative regulators by greatly enhancing the low intrinsic GTPase activity of the Ras proteins, resulting in hydrolysis of GTP to GDP and causing an allosteric change of the Ras to the inactive "off" state. Cell surface receptors can stimulate the activation of Ras proteins by at least two mechanisms. One involves the recruitment of GEFs to receptors with the help of adapter proteins such as Grb2 and Shc. The other mechanism involves receptor-induced production of second messengers such as calcium or diacylglycerol (DAG) which may activate GEFs (Fig. 1.1).

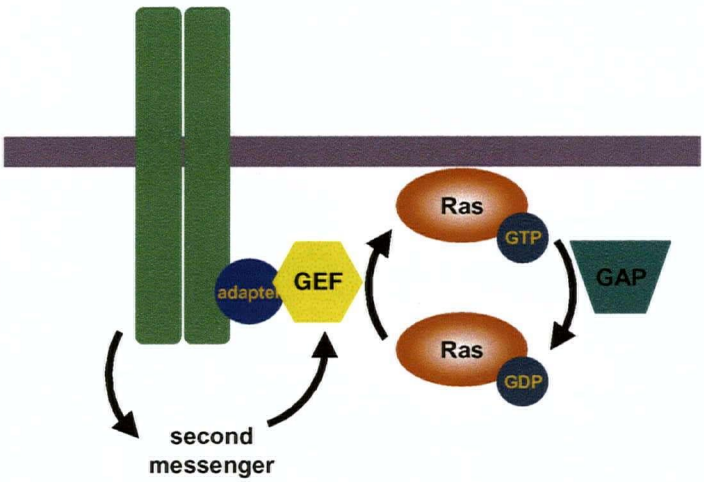

Figure 1.1. ***General model of receptor-mediated Ras activation.*** Note that although drawn differently, Ras is at the plasma membrane (in purple) regardless of its GDP- or GTP-bound state. The receptor is represented by green bars. The receptor may recruit the adapter/GEF complex after activation.

The Ras superfamily of proteins now includes over 150 small GTPases. It comprises six subfamilies, the Ras, Rho, Ran, Rab, Arf, and Rad/Gem/Kir (RGK) subfamilies. The Ras subfamily contains 13 members that fall into four subgroups (Fig. 1.2). The first group includes the first Ras proteins discovered, H-Ras and K-Ras. Together with N-Ras, these comprise the p21 Ras or classical Ras proteins. K-Ras occurs in two alternatively spliced forms, termed K-Ras 4A and K-Ras 4B. The p21 Ras proteins are closely related and exhibit about 85% sequence identity. Members of the other three subgroups R-Ras, Rap and Ral, all share 40-50% amino acid identity with p21 Ras. In addition, several Ras-like GTPases have been described and included in the Ras subfamily despite the lack of characteristic features of Ras proteins such as prenylation signals (Rin and Rit) or typical effector domains (Rheb, Rhes, Dexras1, NOEY2, Di-Ras1/2, and κB-Ras1/2) (Reuther and Der, 2000). These proteins have not yet been well characterized.

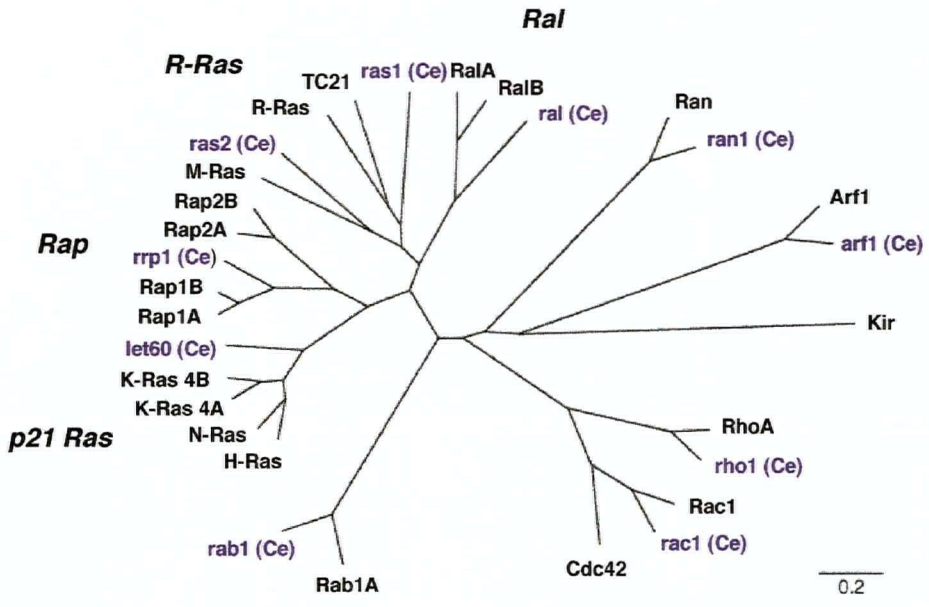

Figure 1.2. ***Phylogenetic tree of Ras family members.*** Human and *C. elegans* Ras sequences were obtained from the NCBI and Wormbase (www.wormbase.org) databases and aligned using the ClustalW program at www.ebi.ac.uk/clustalw. A phylogenetic tree was obtained by analysis of the alignment at www.genebee.msu.su/services/phtree_full.html.

Members of the Rho, Arf, Rab, RGK, and Ran sub-families have roles in cell motility, regulation of the actin cytoskeleton, vesicle transport and nuclear import. As these proteins were not part of the present study, they will not be discussed further.

1.1.2 Post-translational modifications regulate trafficking

Ras subfamily members exhibit a carboxy-terminal CaaX motif, where a cysteine is followed by two aliphatic residues and one random amino acid. This motif is a target for processing by enzymes which results in the addition of a carboxy-terminal prenyl group. This group, either a farnesyl or a geranylgeranyl moiety, is involved in anchoring Ras to membranes (Casey, 1995). However, prenylation alone is insufficient for functional anchorage of Ras proteins into the inner

leaflet of the plasma membrane (Hancock et al., 1990). Further lipid modification or the presence of multiple basic residues at the carboxy-terminus are required. The carboxy-termini of H-Ras, N-Ras, and K-Ras 4A include cysteine residues that undergo palmitoylation (Hancock et al., 1989; Hancock et al., 1990). K-Ras 4B lacks sites for palmitoylation but exhibits multiple positively charged lysines which are thought to stabilize membrane localization by interacting with negatively charged head groups of membrane phospholipids. The nature of the carboxy-termini also dictate the route by which Ras proteins reach the plasma membrane (Fig. 1.3). The palmitoylated carboxy-termini of H-Ras and N-Ras are required for their transport to the plasma membrane via the Golgi (Apolloni et al., 2000; Choy et al., 1999). The polybasic carboxy-terminus of K-Ras 4B dictates a route to the membrane which bypasses the Golgi but which may involve binding of K-Ras 4B to tubulin (Apolloni et al., 2000; Thissen et al., 1997).

1.1.3 Plasma membrane and lipid rafts

The plasma membrane was originally described using a model where membrane proteins can freely move in a two-dimensional lipid bilayer ("fluid mosaic"). However, it has been recognized that although most membrane lipids are randomly distributed, some lipids, including sphingolipids (sphingomyelin and glycosphingolipids) tend to cluster together, because their longer and more saturated, straighter hydrocharbon chains allow for stronger hydrophobic interactions. These can also more tightly intercalate cholesterol molecules. Thus, sphingolipids and cholesterol form tightly packed, "liquid-ordered" microdomains, or "rafts", that are surrounded by more fluid, "liquid-disordered" membrane. Because of the tight packing, lipid rafts are relatively resistant to solubilization by some detergents. They also have relatively low buoyant densities and can be separated from the disordered membranes by sucrose density gradient centrifugation. Rafts that are isolated based on these properties have been designated

"detergent-resistant" or "detergent-insoluble" membranes (DRM or DIM) (reviewed in Simons and Toomre, 2000).

Another characteristic of lipid rafts is that they provide an environment for certain membrane proteins that have affinity for them, which isolates these proteins to some extent from proteins in the disordered membrane. The discovery that cell surface receptors such as the T and B cell receptors for antigen, and the IgE receptor, can localize to rafts (Cheng et al., 1999; Xavier et al., 1998; Field et al., 1995), and that these receptors also recruit important signaling modules to rafts, led to the theory that lipid rafts constitute signaling platforms for receptors that preferentially reside in these microdomains, or are translocated into them after ligand binding (Delon and Germain, 2000). Many proteins, and in particular lipid-modified proteins, have been shown to localize to lipid rafts. These include those with GPI-anchors, such as Thy-1, or palmitoylated proteins that can be either transmembrane proteins (such as LAT) or cytoplasmic, membrane attached proteins. The latter include Src family kinases, heterotrimeric G proteins, and those Ras proteins that are palmitoylated (Horejsi, 2002).

1.1.4 Association of Ras proteins with palmitoylated carboxy-termini with rafts

The differences in the carboxy-termini of Ras proteins dictate their insertion into different parts of the plasma membrane. Data from experiments where cholesterol-rich membrane domains were disrupted, as well as sucrose density gradient and electron microscopy data, indicate that H-Ras resides in lipid rafts whereas K-Ras 4B is excluded from rafts and instead localizes to the disordered plasma membrane (Fig. 1.3) (Prior et al., 2001; Roy et al., 1999). The localization of H-Ras to lipid rafts was shown to be essential for its activation of downstream effectors such as Raf-1 and PI-3 kinase (Jaumot et al., 2001; Roy et al., 1999). Thus, differences in localization of Ras proteins may regulate proximity to different types of receptors and GEFs which may result

in differences in susceptibility to activation by particular stimuli. For example, it might be predicted that palmitoylated Ras proteins such as H-Ras or N-Ras that are located in rafts, would likely be activated by stimulation of the B cell or T cell antigen receptors (BCR, TCR), or the receptor for IgE (FcεR), which are known to translocate to rafts upon activation (Cheng et al., 2001; Sheets et al., 1999; Xavier et al., 1998). Likewise, Ras proteins with stretches of basic amino acids near their carboxy-termini would be predicted to preferentially become activated by non-raft-associated receptors.

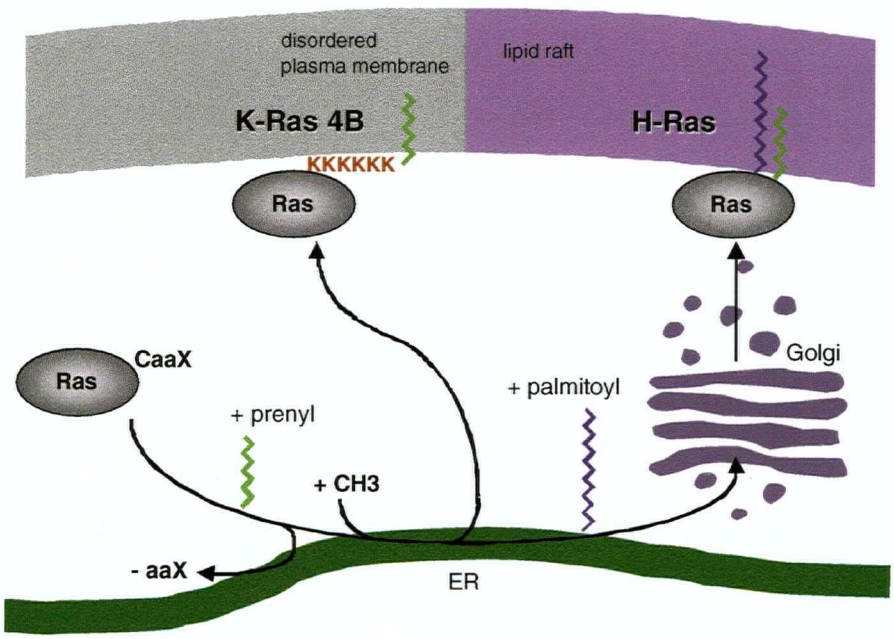

Figure 1.3. ***Differential trafficking and membrane localization of Ras proteins with palmitoylated or polybasic carboxy-termini.*** Prenylation occurs in the cytoplasm followed by proteolysis of the last three amino acids on Ras proteins (indicated by -aaX) on the endoplasmic reticulum (ER) membrane. After methylesterfication (indicated by +CH3), isoforms with stretches of basic residues near their C-termini take a relatively undefined route to the disordered plasma membrane that may involve microtubules. Other isoforms that are palmitoylated on cysteine residues near their C-termini traffic through the Golgi to lipid raft areas by the conventional exocytic pathway. Adapted from Apolloni et al., 2000.

1.2 Regulation of Ras subfamily GTPases

1.2.1 GEFs and their specificity

One simple answer to the question of why there are so many similar members of the Ras subfamily would be that they would be activated by different GEFs that were themselves activated by different stimuli. Indeed there are many GEFs, exhibiting a variety of domains which enable response to and integration of a large variety of signals. However, with few exceptions, most GEFs act on multiple members of the Ras subfamily, in some instances having GEF activity on members of the Ras superfamily outside the Ras subgroup, such as Rho proteins. Nevertheless, there is emerging evidence for considerable fine-specificity, with some GEFs even able to discriminate between members of the closely related p21 Ras group. Moreover, it is conceivable that differences within the Ras family in susceptibility to a particular GEF may be obscured by the experimental techniques based on over-expression, which for example may result in non-physiological localization of Ras proteins or GEFs. The activation of different Ras subfamily proteins by the various GEFs is summarized in table 1.1.

With one single exception discussed below, all GEFs activate Ras proteins through one or more catalytic domains homologous with the minimal Ras activation domain of CDC25, the yeast GEF that activates *Saccharomyces cerevisiae* RAS. A crystallographic structure of the interaction of the CDC25 domain of Sos1 and H-Ras has revealed the details of this interaction (Boriack-Sjodin et al., 1998). The various GEFs exhibit a large range of additional structural features which enable them to respond to many different signals. These signals include the phosphorylation of proteins and lipids, calcium fluxes, and the generation of cyclic nucleotides or diacylglycerol (DAG). Some GEFs, like Sos1/2 or RasGRF1/2, have domains with homology to the catalytic domain of Dbl, which exhibit GEF activity towards Rho subfamily proteins, in

addition to CDC25-like domains and thus act as GEFs for members of both the Ras and Rho subfamilies, potentially integrating their activation.

	H-Ras	N-Ras	K-Ras 4A	K-Ras 4B	M-Ras	R-Ras	TC21	Rap	Ral
Sos1	+	+	+	+	+	-	-	-	-
Sos2	+								
RasGRF1	+	-		-	+	(+)	+		-
RasGRF2	+					-		-	-
RasGRP1	+	+		+	(+)	+	+		
RasGRP2	-	+		+	-	(+)	(+)	(+)	
CalDAG-GEFI	-	-		-	-	(+)	(+)	+	-
RasGRP3	+				(+)	+	+	+	-
RasGRP4	+							-	
Epac1/2	-				-	-	-	+	
MR-GEF	-					-	-	+	-
RA-GEF I								+	-
RA-GEF II	-	-			-	-		+	-
C3G					-	(+)	(+)	+	
PLC epsilon	-				-	-		+	-
RalGDS, Rgl1/2/3	-								+
AND-34	-					+		+	+
smgGDS	-	-	-	+	+			+	+

Table 1.1 ***Summary of Ras family substrates for GEFs***. Good activation: +. Weak activation: (+). No activation: -. All references are in the text.

1.2.1.1 RasGRF and mSos

RasGRF1 and RasGRF2 (Cen et al., 1992; Fam et al., 1997; Martegani et al., 1992; Wei et al., 1992) differ in specificity as RasGRF1 appears to selectively activate H-Ras but not N-Ras or K-Ras 4B (Jones and Jackson, 1998) and also acts on M-Ras and R-Ras (Ohba et al., 2000b). RasGRF2 activates H-Ras but not R-Ras (Gotoh et al., 2001), although additional Ras proteins precipitated by Y13-259 (see section 1.4.2) may also be activated (Fam et al., 1997). RasGRF1/2 contain an IQ domain which binds calmodulin. However, while calmodulin-binding

appears to be required for activation of Erk, it was shown not to modulate Ras activation (de Hoog et al., 2000). Mammalian Sos1 and Sos2 are orthologs of the *D. melanogaster* gene product *son-of-sevenless* which functions upstream of Ras in R7 photoreceptor cell differentiation (Bowtell et al., 1992; Chardin et al., 1993). mSos1/2 contain pleckstrin homology (PH) domains which interact with membrane lipids, and catalytic domains for activation of both Ras proteins and members of the Rho family. Within the Ras subfamily, mSos1 is active on H-Ras, N-Ras, K-Ras 4A and 4B, and M-Ras, but not on R-Ras or TC21 (Nielsen et al., 2001; Ohba et al., 2000b). mSos2 activates H-Ras (Liu et al., 1993).

1.2.1.2 RasGRP

The RasGRP family of GEFs is defined by the presence of calcium- and DAG-binding domains and has four members. RasGRP1 (also referred to as CalDAG-GEF II) activates H-Ras, N-Ras, K-Ras 4B, R-Ras, TC21, and weakly M-Ras (Ohba et al., 2000b; Tognon et al., 1998). In contrast, RasGRP2 activates N-Ras and K-Ras 4B, but not H-Ras. It also efficiently activates Rap after stimulation with TPA and calcium, whereas the GEF activity towards N-Ras is partially inhibited by the presence of calcium (Clyde-Smith et al., 2000). CalDAG-GEF I is an alternatively spliced form of RasGRP2 which lacks amino-terminal myristoylation and palmitoylation and may thus localize differently in membranes. CalDAG-GEF I has been shown to activate Rap1 and very weakly R-Ras *in vivo* and *in vitro* (Clyde-Smith et al., 2000; Ohba et al., 2000b; Yamashita et al., 2000). CalDAG-GEF I promotes nucleotide exchange on N-Ras *in vitro*, but neither H-Ras, N-Ras or K-Ras 4B are activated by CalDAG-GEF I *in vivo* (Clyde-Smith et al., 2000; Kawasaki et al., 1998b). RasGRP3 (also known as CalDAG-GEF III) activates H-Ras, R-Ras, Rap1A, and Rap2A, but not RalA (Lorenzo et al., 2001; Yamashita et al., 2000). A fourth member of the RasGRP family, RasGRP4, can activate H-Ras *in vitro*, an action which is inhibited by the presence of calcium, but does not activate Rap1 (Reuther et al.,

2002; Yang et al., 2002). While the expression of the various GEFs in different types of hematopoietic cells has not been studied exhaustively, the expression pattern of members of the RasGRP family in these cells seems to be more restrictive than that of mSos1. RasGRP1 is only found in T and B cells, and there is a possibility that RasGRP3 may be a B cell-specific GEF (James Stone, personal communication). RasGRP4 is expressed in mast cells but not in lymphocytes (Yang et al., 2002), but was also found in leukemic blasts in a patient with acute myeloid leukemia (AML) and myeloid cell lines (Reuther et al., 2002).

1.2.1.3 GEFs for Rap and Ral

Some GEFs appear to activate only Rap. Two exchange factors which are regulated by cAMP, Epac1 and Epac2 (cAMP-GEFII/I) activate Rap1A but not H-Ras or R-Ras (Kawasaki et al., 1998a). MR-GEF, RA-GEF I (PDZ-GEF), and RA-GEF II also seem to selectively act on Rap, although there are conflicting data on the ability of RA-GEF I to activate H-Ras (Gao et al., 2001; Rebhun et al., 2000a). RA-GEF I has also been reported to be involved in induction of Ras activation by the β1 adrenergic receptor, a G protein-coupled receptor (Pak et al., 2002). MR-GEF and RA-GEF II appear to bind specifically to GTP-bound M-Ras and are thus potentially specific effectors of M-Ras (see below). Recently, a novel isoform of phospholipase C, PLCε, has been shown to have GEF activity on Rap1A but not Rap2A, H-Ras, R-Ras, M-Ras, RalA, Rit, Rin, or Rheb (Jin et al., 2001). By virtue of one of its two Ras association (RA) domains, PLCε also functions as a downstream effector of activated Ras (see below).

Another family of GEFs appear to be exclusively active on Ral proteins. Interestingly, all of these RalGEFs are effectors of other members of the Ras subfamily and are discussed below. The potential links between p21 Ras and its activation of GEFs for other members of the Ras subfamily are shown in figure 1.4.

1.2.1.4 GEFs with broad specificity and putative GEFs

Some exchange factors exhibit broader specificity within the Ras superfamily. C3G was found in a search for Crk-interacting proteins (Tanaka et al., 1994). It activates Rap1/2, R-Ras, TC21 and the Cdc42-related protein, TC10 (Chiang et al., 2001; Ohba et al., 2000b). Several proteins were identified that exhibit relatively low homology of their GEF domains with other CDC25-like domains. These are the related BCAR1, BCAR3/NSP2, SHEP1/Chat/NSP3, and NSP1 proteins (Quilliam et al., 2002). The murine ortholog of BCAR3, AND-34, was shown to activate RalA, Rap1A, and R-Ras but not H-Ras (Gotoh et al., 2000). However, other studies failed to detect GEF activity of this protein (Quilliam et al., 2002).

A protein termed smgGDS is the GEF with the broadest specificity. It was the first mammalian exchange factor to be identified (Kaibuchi et al., 1991). However, in that it consists almost entirely of a series of Armadillo repeats, and lacks a CDC25-like domain altogether, smgGDS is structurally unrelated to any other GEF. It acts not only on many members of the Ras subfamily like K-Ras 4B, Rap1A/B and Ral, but also on members of the Rho family like RhoA, Cdc42, and Rac (Takai et al., 1993). Intriguingly, smgGDS fails to activate H-Ras, N-Ras or K-Ras 4A, leading to the suggestion that it requires a polybasic carboxy-terminus on its substrate. However, there is evidence that basic residues on substrates are necessary but not sufficient for smgGDS exchange activity (Vikis et al., 2002). Another unusual feature of smgGDS is its ability to bind to dominant active Ras proteins in yeast-two-hybrid assays (Vikis et al., 2002), which is not characteristic of GEFs (Lenzen et al., 1998) and may indicate that smgGDS possibly also functions as a scaffold.

1.2.1.5 Features of Ras that can influence GEF specificity

There are recent insights into the molecular features that determine the specificity of GEFs for particular Ras family members. Differences in the helix 3 region of H-Ras and R-Ras (residues 91-103 in H-Ras) appear to account for the ability of RasGRF1 but not Sos1 to act on R-Ras (Tian and Feig, 2001). The ability of RasGRF1 to activate H-Ras but not K-Ras 4B appears to depend on residues in the carboxy-terminus (Jones and Jackson, 1998). Another factor affecting GEF specificity may be the nature of the prenyl groups. R-Ras is usually not activated by RasGRF2, but when the most carboxy-terminal residue is changed from leucine to serine (as in H-Ras), R-Ras is then farnesylated instead of geranylgeranylated and becomes more responsive to RasGRF2 (Gotoh et al., 2001).

1.2.2 GTPase activating proteins

GAPs negatively regulate GTPases by catalyzing the hydrolysis of bound GTP through a mechanism involving insertion of an arginine side-chain into the active site of Ras (Ahmadian et al., 1997). This results in an allosteric change of Ras into its inactive configuration. p120 RasGAP was the first protein shown to bind to Ras in a GTP-dependent fashion. p120 RasGAP is known to associate with p190 RhoGAP, thereby regulating activity of Rho proteins (Ellis et al., 1990; McGlade et al., 1993; Settleman et al., 1992). p120 RasGAP was also shown to associate with BCR/Abl in chronic myelogeneous leukemia (CML) cell lines (Druker et al., 1992). Four other types of GAPs have been identified, the product of the neurofibromatosis gene Nf1, SynGAP, the recently cloned DAP1/2 (Kim et al., 1998; Wang et al., 2002; Xu et al., 1990), and the GAP1 family of proteins. This family of GAPs is comprised of GAP1m, GAP1(IP4BP) (also known as R-Ras GAP or GAPIII), RASAL, and CAPRI (Allen et al., 1998; Baba et al., 1995;

Cullen et al., 1995; Lockyer et al., 2001; Maekawa et al., 1994; Yamamoto et al., 1995). Both GAP1m and GAP1(IP4BP) can be regulated via binding to inositol phosphates. GAP1m may interconnect the heterotrimeric and monomeric G proteins by directly binding to $G\alpha_{12}$ (Jiang et al., 1998; Wittinghofer, 1998). CAPRI is the only GAP which is regulated by calcium by virtue of a functional C2 domain that distinguishes it from other members of the GAP1 family (Lockyer et al., 2001). p120 RasGAP, GAP1m, and Nf1 exhibit activity towards p21 Ras, M-Ras, R-Ras, and TC21 but not Rap1A (Ohba et al., 2000b). DIP1/2 is active on K-Ras, R-Ras, and TC21 but not on Rap1A (Wang et al., 2002), GAP1(IP4BP) is only active on R-Ras and Rap1A (Wittinghofer, 1998), and CAPRI has only been tested for activity on p21 Ras (Lockyer et al., 2001).

1.3 Ras effectors

Activation of members of the Ras subfamily leads to their interaction with a variety of downstream effector proteins. These effectors may be broadly grouped into GEFs for other Ras superfamily members, protein or lipid kinases, GAPs (described above), and a still expanding group of other effectors with poorly characterized functions. The effectors of p21 Ras and some of their downstream functions are summarized in figure 1.4.

1.3.1 GEFs as effectors of the Ras subfamily

Interest in the Ral GTPases RalA and RalB increased when GEFs for these proteins were found to be effectors of Ras. These RalGEFs include RalGDS, Rgl, Rlf/Rgl2, RPM/Rgl3, the PH-domain containing RalGPS/RalGEF2, and Rgr, the GEF portion of the Rsc fusion protein (Albright et al., 1993; D'Adamo et al., 1997; de Bruyn et al., 2000; Ehrhardt et al., 2001; Kikuchi

et al., 1994; Rebhun et al., 2000b; Shao and Andres, 2000; Spaargaren and Bischoff, 1994; Wolthuis et al., 1996). Through their RA domains, these RalGEFs interact with various activated members of the Ras subfamily including Ras, Rap, and Rit. RalGPS proteins have not yet been shown to actually bind GTP-Ras. Activated TC21 was shown to interact with RalGDS and Rgl, but there are conflicting data on whether this association can mediate activation of RalA (Murphy et al., 2002; Rosario et al., 2001). The RA domains of RalGEFs are required for Ras-mediated activation of Ral, placing Ral downstream of Ras. However, activation of Ral that is independent of Ras but dependent on calcium has also been demonstrated (Hofer et al., 1994).

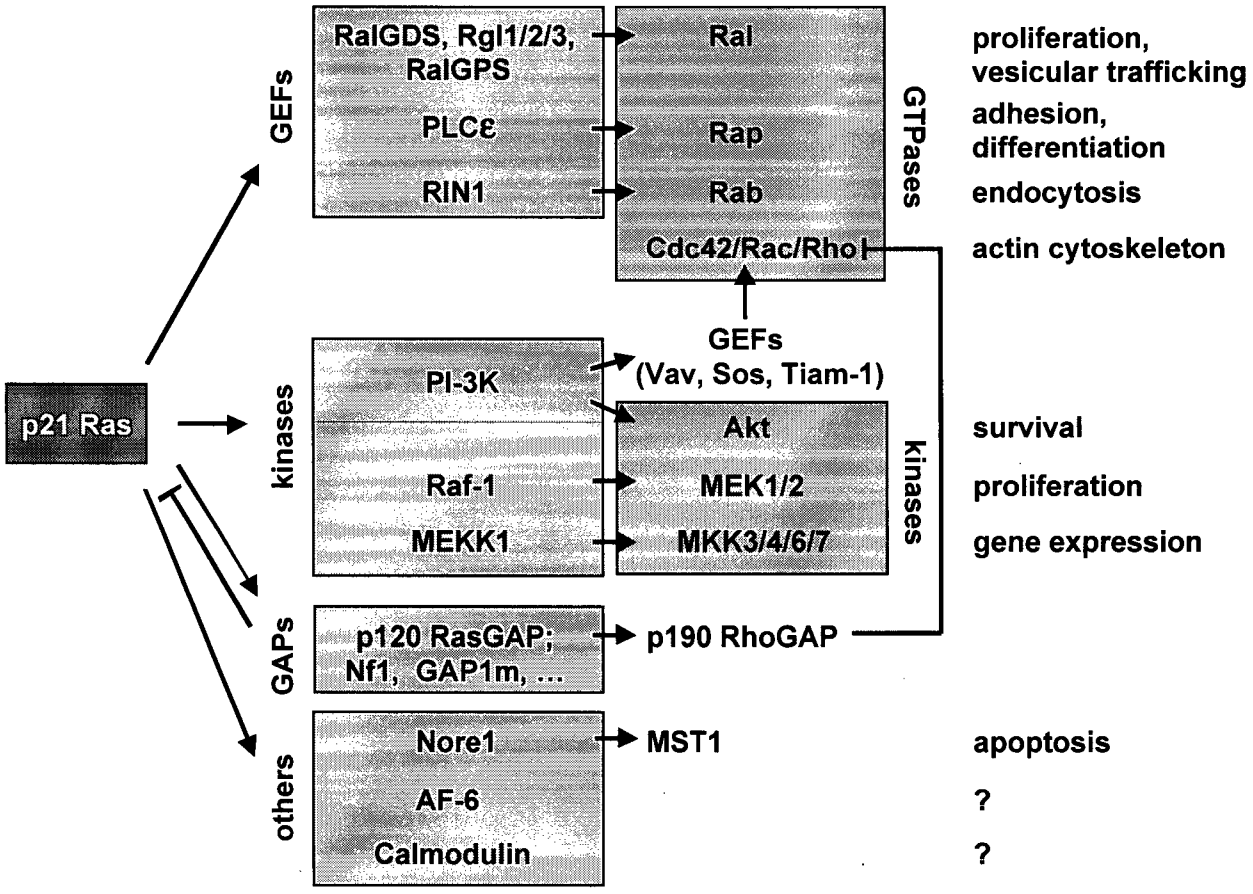

Figure 1.4. ***General downstream effectors of Ras proteins and their functions.***

Unlike RalGDS and Rlf, RPM/Rgl3 appears to function as a negative regulator of the p21 Ras path (see below). In *Drosophila*, Ral was recently suggested to function downstream of Rap rather than of Ras, despite the fact that the *Drosophila* ortholog of the Rgl proteins, RGL, can interact with both activated Ras and Rap (Mirey et al., 2003).

Two proteins MR-GEF and RA-GEF II that bind selectively to GTP-Bound M-Ras and are thus potential effectors of M-Ras are known to function as RapGEFs. The effect on Rap1A of the recruitment of MR-GEF or RA-GEF II by activated M-Ras appears to be complex and potentially involves increases in Rap1A activity at the plasma membrane but decreases in the cytoplasm (Gao et al., 2001; Rebhun et al., 2000a). Another newly identified Rap1A-specific GEF is PLCε which acts as an effector of H-Ras and TC21 (Murphy et al., 2002) (see above). Over-expression of H-Ras Q61L stimulated PLCε-mediated hydrolysis of $PI(4,5)P_2$ in a calcium-dependent manner (Kelley et al., 2001).

The SH2 domain-containing protein RIN1 interacts with activated H-Ras and was shown to block Ras-induced transformation, possibly by competing with Ras for Raf-1 (Han and Colicelli, 1995). By virtue of its Vps9p-like RabGEF domain, RIN1 functions as a GEF for Rab5A and potentiates Ras-mediated endocytosis (Tall et al., 2001).

1.3.2 Protein and lipid kinases

The best characterized signal transduction pathway downstream of p21 Ras is the one that leads to activation of members of the mitogen-activated protein (MAP) kinase family and in particular of Erk1/2. This cascade is initiated by binding of p21 Ras to members of the Raf family of serine/threonine kinases, Raf-1, A-Raf, and B-Raf (Vojtek et al., 1993; Zhang et al., 1993). Raf-1 is most efficiently activated by activated K-Ras 4B, followed by activated K-Ras

4A, N-Ras, and H-Ras (Voice et al., 1999). R-Ras and M-Ras are very poor activators of the Raf-Erk pathway (Kimmelman et al., 1997; Marte et al., 1997; Quilliam et al., 1999). There is conflicting evidence on the ability of TC21 to activate Raf and Erk (Graham et al., 1996; Movilla et al., 1999; Rosario et al., 1999). Activation of B-Raf, but not of Raf-1, by Rap1 was reported to lead to sustained activation of Erk after stimulation of PC12 cells with NGF (York et al., 1998).

The serine/threonine kinase MEKK-1 was originally identified as an upstream regulator of MEK1/2 and Erk1/2, but was later shown to more potently activate the stress-activated MAP kinases JNK and p38 (Lange-Carter et al., 1993; Xu et al., 1996). Experiments with dominant active and dominant-negative mutants of p21 Ras demonstrated that MEKK-1 probably functions downstream of p21 Ras (Lange-Carter and Johnson, 1994). Consistent with this, MEKK-1 binds directly to the effector domain of activated p21 Ras (Russell et al., 1995). MEKK-1 is also part of the 700 kDa IKK complex that is involved in the activation of NFκB (Lee et al., 1998).

Phosphatidylinositol (PI)-3 kinase is another effector of Ras subfamily members and has kinase activity against both lipids and proteins. Its catalytic p110 subunit interacts directly with GTP-bound Ras (Rodriguez-Viciana et al., 1994). By catalyzing the production of 3' phosphorylated phosphatidylinositols, PI-3 kinase targets proteins containing PH domains to the plasma membrane where they can be activated (e.g. by phosphorylation) and mediate their respective effects. These proteins include the kinases Akt and Btk (reviewed in Katso et al., 2001). Activated R-Ras and M-Ras may increase the activity of PI-3 kinase more efficiently than H-Ras, which in turn is more efficient than K-Ras 4B (Kimmelman et al., 2000; Marte et al., 1997; Yan et al., 1998). PI-3 kinase is likely one of the major effectors for M-Ras or R-Ras, as neither are efficient at activating the MAP kinases Erk, JNK and p38 (Kimmelman et al., 2000; Marte et al., 1997; Quilliam et al., 1999). The increases in activity of PI-3 kinase induced by growth

factors and cytokines are not entirely dependent on activation of Ras family members. IL-4 stimulation does not result in activation of Ras but does result in increased activity of PI-3 kinase via IRS-2 (Satoh et al., 1991; Welham et al., 1997).

Ras subfamily proteins are also able to mediate activation of Rho subfamily proteins via PI-3 kinase. $PI(3,4,5)P_3$ produced by PI-3 kinase can bind to the PH domains of mSos1/2, Vav, and Tiam-1, all of which stimulate nucleotide exchange on Rac (Han et al., 1998; Michiels et al., 1997; Nimnual et al., 1998). Rac1 is more efficiently activated by K-Ras 4B compared to H-Ras, leading to increased cell motility of cells expressing activated K-Ras 4B (Voice et al., 1999; Walsh and Bar-Sagi, 2001).

1.3.3 Effectors with negative regulatory functions

We and others have recently identified an effector of M-Ras, H-Ras, and Rit, that shares homology with RalGDS, Rgl1, and Rgl2 (Rlf) and was termed RPM or Rgl3 (Ehrhardt et al., 2001; Shao and Andres, 2000). This protein functions as an exchange factor for RalA and RalB, but unlike Rgl1 and Rgl2, it does not synergize with Ras to activate Elk-1 and inhibits growth of transformed fibroblasts, indicating a potential role for RPM/Rgl3 as a tumor suppressor.

RIN1, at least when overexpressed, exhibits a negative regulatory effect on p21 Ras action that may be due to its competition for p21 Ras with Raf (Han and Colicelli, 1995). RIN1 exhibits a Vps9p RabGEF domain and activates Rab5 to stimulate receptor-mediated endocytosis (Tall et al., 2001).

Nore1 was identified in a yeast 2-hybrid screen as a protein that interacts with GTP-bound H-Ras. The presence of a DAG-binding site and of several potential SH3-domain binding sites

suggests that this protein is an adapter protein (Vavvas et al., 1998). Nore1 also efficiently interacts with activated M-Ras and K-Ras 4B (Ehrhardt et al., 2001; Ortiz-Vega et al., 2002). Recently, Nore1 and three other highly homologous proteins, RasSF1A, RasSF1C, and *C. elegans* T24F1.3, were shown to interact with the pro-apoptotic kinase, MST1. The recruitment of Nore1/MST1 complexes to activated Ras proteins provided a possible explanation for the apoptotic effects observed after over-expression of these activated Ras proteins in Jurkat, NIH3T3, or 293 cells (Khokhlatchev et al., 2002; Vos et al., 2000).

1.3.4 Effectors with uncertain functions

AF-6 is the mammalian homologue of the *Drosophila* protein Canoe, which is functionally linked with Ras in eye development. AF-6 preferentially binds to activated Rap1 and less efficiently to H-Ras, N-Ras, K-Ras, and M-Ras (Ehrhardt et al., 2001; Matsuo et al., 1999). AF-6 may physically link adhesion molecules at the plasma membrane and the cortical actin cytoskeleton (Boettner et al., 2000). Calmodulin was recently shown to specifically interact with activated K-Ras 4B but not with any other p21 Ras isoform (Villalonga et al., 2001). The significance of this interaction is unknown, but it is possible that it may inhibit the interaction of other effector proteins such as Raf-1 with K-Ras 4B.

1.4 Functions of Ras subfamily members

1.4.1 Redundancy?

There is a substantial degree of amino acid sequence identity between members of the Ras subfamily (40-95 % identity), with members of the p21 Ras sub-group sharing 85 % amino acid

identity. As discussed, there is also considerable overlap in the sets of GEFs that activate Ras proteins and downstream effectors that transmit their signals. Moreover, when overexpressed in a constitutively activated form, they produce similar phenotypes such as transformation. This observation led to speculations about functional redundancy within this family. However, it is becoming increasingly evident that different members of the Ras subfamily may have different biological functions which depend not only on differences in their affinities for regulators or effectors, but also in their precise subcellular localization.

1.4.2 Problems with interpretation of the literature

Most of the published information focuses on the function of p21 Ras. In most cases, the experiments used the monoclonal antibody Y13-259 or overexpression of dominant active or negative mutants of p21 Ras. However, it is now clear that the close structural similarities between members of the Ras family, and the widespread sharing of positive and negative regulators and effectors, mean that these experimental tools are inadequate to specifically assign functions to the various Ras family members. For example, while the monoclonal antibody Y13-259 is generally assumed to be specific for the four p21 Ras proteins, the epitope to which Y13-259 binds is composed of residues in Switch II which are very similar in other members of the subfamily such as M-Ras and TC21, resulting in crossreactivity (Ehrhardt et al., 1999; Feig, 1999). Microinjection of Y13-259 into cells blocks cell cycle progression (Mulcahy et al., 1985), but given its crossreactivity within the family, it can no longer be concluded that the observed effects were due specifically to inhibition of p21 Ras activity. Likewise, many experiments designed to assay activation of p21 Ras in response to extracellular stimuli measured the ratio of GTP to GDP in material that was immunoprecipitated from cells using Y13-259. Thus, it is unclear whether the published reports that growth factors like interleukin-3 (IL-3), steel locus factor (SLF), epidermal growth factor (EGF), or platelet-derived growth factor

(PDGF) activate p21 Ras (Duronio et al., 1992; Satoh et al., 1990a; Satoh et al., 1990b) are correct. The use of isolated Ras binding domains of effector proteins as affinity purification tools in "pull-down" assays (Taylor and Shalloway, 1996), together with the development of sensitive antibodies specific for each family member, will help to provide answers to these questions.

There are similar problems with conclusions about the functions of p21 Ras that are based on the use of over-expressed dominant negative mutants of p21 Ras. The affinity for GTP of the most commonly used dominant negative version of p21 Ras, the S17N mutant, is strongly reduced, resulting in a failure to release the GEF after binding. Thus, these mutants remain bound to GEFs, thereby sequestering them and preventing them from activating other Ras molecules in the cell. G15A mutants of p21 Ras function in a similar manner, but have an even higher affinity for GEFs (Feig, 1999). Most published reports on the effects of such dominant negative mutants of p21 Ras have concluded that the observed effects reflect interference with activation of p21 Ras. However, this is not necessarily the case because many GEFs which act on p21 Ras, and thus will be sequestered by dominant negative S17N or G15A p21 Ras mutants, also activate other members of the Ras subfamily or even Ras superfamily. For example, the ability of H-Ras S17N to sequester mSos1 could also directly block activation of Rac1, as mSos1 acts as a GEF for both Ras subfamily and Rho subfamily members. Thus, the demonstration that a function, for example the development of B or T lymphocytes is blocked by transgenic expression of H-Ras S17N (Iritani et al., 1997; Swan et al., 1995), alone does not establish that H-Ras or even p21 Ras as a group is necessary for normal T or B cell development. Additional evidence in support of a role for p21 Ras can be obtained by demonstrating that the effect of dominant negative Ras can be reversed by over-expression of an activated Ras effector, such as Raf, as reported by Iritani et al. (Iritani et al., 1997).

Another class of dominant inhibitory mutants act downstream of Ras by sequestering effector proteins. These have two types of mutations, one that maintains them in a constitutively active state with high affinity for effectors, and a second that prevents their association with the cell membrane. This results in sequestering of effectors in the cytoplasm where they cannot activate further downstream regulators. A third set of mutations in the Switch I region ("effector loop mutants") can restrict the range of effectors bound and thus sequester only a subset of all possible effectors (Feig, 1999). However, as there is overlap between effector-usage by different family members, this strategy will not provide information on the role of a given Ras isoform.

A second major source of information on the potential functions of Ras family members has come from experimental over-expression of constitutively active mutants of p21 Ras such as the G12V or Q61L mutants. However, this approach also has significant limitations. The effects of expression of chronically active Ras at supra-physiological levels may be quite different from those of physiological levels of activated Ras. Moreover, in physiological situations, activation of Ras may be very tightly regulated, for example decreasing at later stages of the cell cycle. Over-expression of a particular Ras family member may cause it to act on an effector which at physiological concentrations it activates only inefficiently or not at all. Over-expression may also result in mislocalization in the cell and lead to interaction with non-physiological effectors.

1.4.3 Role in development

Targeted disruption of individual *ras* genes in mice has been achieved for all three p21 Ras genes. However, with one exception, the phenotypes have not been obvious. It is not clear whether this reflects true overlaps in function, perhaps involving compensatory overexpression of other family members. Neither the *N-ras* or *H-Ras* genes, individually or in combination, are

essential for normal development, fertility, or hematopoiesis in mice (Esteban et al., 2001; Umanoff et al., 1995). However, N-Ras null fibroblasts exhibit an increased susceptibility to Fas and TNF-induced apoptosis. This phenotype was rescued by ectopic expression of N-Ras but not K-Ras, indicating a specific role for N-Ras in fibroblast survival (Wolfman and Wolfman, 2000). Mice lacking functional M-Ras are also viable and fertile and show no gross abnormalities (Wang and Schrader, unpublished observations). While the development of hematopoietic cells in H-Ras-, N-Ras-, or M-Ras-deficient mice appears grossly normal, a more detailed analysis is needed to see whether there are defects in the development of subsets of cells or in their function. Mice with non-functional *K-Ras* genes die *in utero* of anemia due to apoptosis in the fetal liver microenvironment (Johnson et al., 1997) or due to increased cell death in neuronal and heart tissues (Koera et al., 1997). It remains unclear if the observed embryonic lethality reflects the predominant expression of K-Ras rather than other forms of p21 Ras during development (Pells et al., 1997), or whether it reflects specific functions of K-Ras.

1.4.4 Stimulation and inhibition of growth

The role of the Ras subfamily in general in transduction of mitogenic signals and proliferation is well established, although as noted, the specific roles of different members remain unclear due to problems with crossreactivity of experimental tools. Entry into the G_1 phase of the cell cycle and progression through G_1 is blocked by microinjection of dominant negative H-Ras S17N or monoclonal anti-Ras antibodies (Dobrowolski et al., 1994; Feig and Cooper, 1988; Mulcahy et al., 1985). H-Ras S17N blocks upregulation of cyclin D1, a positive regulator of cell cycle progression, after serum-starvation (Aktas et al., 1997). Upregulation of cyclin D1 may contribute to the transforming activities of activated Ras.

Over the last several years it has become increasingly evident that in addition to promoting cell growth and proliferation, p21 Ras may also induce growth arrest, apoptosis and senescence by induction of $p19^{ARF}$ or of cell cycle inhibitors such as $p16^{INK4a}$, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ (reviewed in Crespo and Leon, 2000; Pruitt and Der, 2001; Sherr, 2001), or by decreasing the expression of CDK4 (Lazarov et al., 2002). One mechanism of Ras-mediated growth arrest is through its induction of $p19^{ARF}$ which, via inactivation of Mdm2, causes accumulation of p53. Inhibition of cyclin-dependent kinases by $p16^{INK4a}$, $p21^{Cip1/WAF1}$, and $p27^{Kip1}$ and subsequent decreases in phosphorylation of Rb proteins is another way to achieve cell cycle arrest. In normal human fibroblasts, both p53 and $p16^{INK4a}$ are required for Ras-induced senescence. In the absence of $p19^{ARF}$, p53, or $p16^{INK4a}$, over-expression of activated p21 Ras results in transformation rather than inhibition of growth (Kamijo et al., 1997; Serrano et al., 1997). The apparent paradoxical ability of Ras to promote both cell cycle progression and arrest is in part explained by the ability of high levels of Raf activity to induce senescence, whereas lower levels promote cell cycle progression (Sewing et al., 1997; Woods et al., 1997). Thus, the levels of activated p21 Ras may determine whether its effects are anti- or pro-apoptotic. A third way that may be utilized by Ras proteins to achieve apoptosis is via the effector Nore1 and its interaction with the pro-apoptotic kinase MST1 (Khokhlatchev et al., 2002).

1.4.5 Differentiation

Ras proteins are critically involved in the development and function of hematopoietic cells. This is discussed in the context of specific cell types below (1.5.1).

1.4.6 Cell adhesion

Cells adhere to the extracellular matrix via integrins. During events such as cell migration or division, cells convert from being non-adherent to being adherent and vice versa through integrin activation or de-activation. Both H-Ras and Raf-1 were found in a screen for suppressors of integrin activation which provided an explanation for the less adhesive phenotype of Ras-transformed cells (Hughes et al., 1997). However, over-expression of H-Ras has also been reported to increase the activation of integrins and to induce adhesion, and expression of an S17N p21 Ras mutant blocked IL-3-induced adhesion and activation of β1 integrin (Shibayama et al., 1999). R-Ras also stimulates integrin activation (Zhang et al., 1996), an effect that was recently shown to be negatively regulated by Src (Zou et al., 2002). Rap1 also contributes to integrin-mediated cell adhesion (see 1.4.8) although the underlying mechanism is yet to be analyzed. Again a dominant negative Rap mutant blocked the IL-3-induced β1 integrin-dependent adhesion (Shibayama et al., 1999). However, since Rap can be activated by GEFs that are effectors of other Ras family members, it remains challenging to determine whether Rap and these other Ras proteins are acting in parallel or sequentially.

1.4.7 Functions of Ral

Relatively little is known about the functions of the Ral GTPases RalA and RalB of the Ras subfamily. Constitutively active RalA is not transforming in fibroblasts but it strongly enhances the transforming activities of activated Ras and Raf-1. Moreover, dominant negative mutants of Ral strongly inhibit the transforming activities of activated mutants of Ras and Ral (Urano et al., 1996). The mechanisms through which Ral mediates its effects are poorly understood. Active, GTP-bound Ral interacts with RalBP1 which acts as a GAP for the Rho-family GTPases Rac and Cdc42 and inhibits their activation (Cantor et al., 1995). RalBP1 is also known as

cytocentrin, a protein which shuttles between the cytosol and the nucleus, where it regulates the assembly of the mitotic spindle (Quaroni and Paul, 1999). A role for Ral in regulation of the cell cycle has been further substantiated by demonstration of its ability to upregulate expression of cyclin D1 (Henry et al., 2000). Ral GTPases are also involved in vesicular trafficking. Independent of their nucleotide binding, they interact with phospholipase D1 which is implicated in this process (Jiang et al., 1995; Luo et al., 1998). In its GTP-bound form, Ral is part of the exocyst, a protein complex which is required for tethering secretory vesicles to the plasma membrane in preparation for exocytic fusion (Moskalenko et al., 2002). Recent observations suggest the involvement of Ral in other cellular functions such as chemotaxis and receptor-mediated endocytosis (Nakashima et al., 1999; Suzuki et al., 2000). Ral can be activated by growth factors such as EGF and insulin, and there is also evidence that place Ral upstream of the Src tyrosine kinase in the response to stimulation with EGF or insulin (de Ruiter et al., 2000; Goi et al., 2000). In platelets, Ral activation by α-thrombin or other platelet agonists is thought to be dependent on calcium mobilization and correlates with calcium-dependent activation of Rap1, but not Ras (Wolthuis et al., 1998). The *Drosophila* Ral protein, DRal, appears to regulate developmental cell shape changes through inhibition of the JNK pathway (Sawamoto et al., 1999).

1.4.8 Functions of Rap

Rap1 was originally identified in a screen for genes that reverse the phenotype of fibroblasts transformed by K-Ras (Kitayama et al., 1991). This observation and other data on the antagonism between Rap1 and Ras in insulin signal transduction (Okada et al., 1998) and in T cell anergy (see below), led to the notion that Rap antagonised Ras by competing for Raf and thus inhibiting Ras-dependent activation of Erk (Cook et al., 1993). However, growth factor-induced activation of Rap1 does not correlate with the repression of Ras-dependent Erk

activation (Zwartkruis et al., 1998). Moreover, Rap1 was found to be important for NGF-induced differentiation of PC12 cells by the sustained activation of B-Raf and Erk by Rap1. In contrast, in the same cells Ras was involved in proliferation through a transient Erk activation (York et al., 1998). It is unlikely that the physiological activation of Rap1 by growth factors is solely concerned with the modulation of Ras signaling and the interference with Ras activation by Rap may be an artifact of over-expression.

Most functions of Rap1 relate to cell adhesion. For example, Rap1 has been reported to induce cell spreading (Tsukamoto et al., 1999), to regulate inside-out signaling to integrins (Caron et al., 2000; Reedquist et al., 2000), to control the correct positioning of adherens junction markers in *Drosophila* wing epithelium (Knox and Brown, 2002), and to control the formation of the immunological synapse at the interface between a T cell and an APC (Katagiri et al., 2002).

Loss-of-function mutations of Rap1 in *Drosophila* result in lethality (Hariharan et al., 1991). Rap1 function was shown to be required during fly embryogenesis, imaginal development, and oogenesis but seems to be dispensable for fully differentiated cells in adult flies, indicating a role for Rap1 in cell differentiation and proliferation rather than cell survival (Asha et al., 1999).

Rap2 is 60% identical to Rap1, has the same effectors and is regulated by the same set of GEFs (C3G, Epac, RasGRP2, RA-GEF I) and RapGAPs (rap1GAPII and SPA-1). However, Rap2 is unique in its low sensitivity to GAPs and more than half of Rap2 remains GTP-bound, leading to the proposal that Rap1 and Rap2 function respectively as fast and slow molecular switches (Ohba et al., 2000a).

1.5 Function in hematopoietic cells

As noted above, the vast majority of data on Ras functions have been generated by the use of dominant inhibitory or active mutants of Ras proteins or cross-reactive monoclonal antibodies. Thus very little is known about the actual role of individual members of the Ras subfamily in functions of hematopoietic cells. In some cases, there is additional evidence of involvement of potential downstream pathways such as the MEK-Erk pathway. However, while it is often assumed that this implicates p21 Ras, Erk can also be activated downstream of Rap, and by mechanisms independent of activation of the Ras family. Thus, while such experiments suggest that collectively members of the Ras subfamily may have critical functions in many aspects of the development and functions of hematopoietic cells, they do not conclusively establish their roles.

1.5.1 T cells

Ras family proteins influence all stages in the life of a T cell, from development and antigenic activation, to anergy. Several mouse models have indicated that Ras plays a critical role in positive selection of thymocytes. These include transgenic mice with thymocyte-specific expression of H-Ras S17N or dominant negative MEK-1 (Alberola-Ila et al., 1995; Swan et al., 1995), as well as mice with ablation of genes for Erk1 and RasGRP1 (Dower et al., 2000; Molina et al., 1992; Pages et al., 1999). There is evidence that the strength of Erk signaling may be crucial for differentiation along the CD4 lineage (Sharp et al., 1997), although the role of Ras in activation of Erk in this commitment process is unclear as Erk can also be activated downstream of PKC (Puente et al., 2000). Transgenic mice expressing H-Ras S17N were also used to demonstrate the importance of Ras and Erk in differentiation of naive peripheral T cells into Th2 cells. Evidence was presented that weak antigenic stimulation may not activate the

Ras-MAPK pathway efficiently and may favor Th1 cell differentiation, whereas strong stimulation may be required to activate this pathway sufficiently to induce Th2 differentiation (Yamashita et al., 1999). Thus, the extent to which the Ras-MAPK pathway is activated seems to determine the lineage along which T cells differentiate.

Early studies on p21 Ras activation in lymphocytes suggested that 40-50% of p21 Ras is activated after ligation of the T cell receptor (Downward et al., 1990; Izquierdo et al., 1992). The accumulation of activated p21 Ras appeared to correlate with the inhibition of RasGAP activity. It was also noted that there were tyrosine kinase-dependent but PKC-independent pathways that led to Ras activation (Izquierdo et al., 1992). The ability of activators of PKC such as PMA to stimulate Ras activation in T cells has now been explained by discovery of the RasGRP family of exchange factors that exhibit DAG binding domains. The role of Ras in activation of Erk downstream of the TCR has been demonstrated. Thus, Jurkat T cells overexpressing RasGRP1 were hypersensitive to TCR-stimulated Erk activation whereas T cells with homozygous RasGRP1 null alleles were unable to activate Erk after TCR ligation (Dower et al., 2000; Ebinu et al., 1998). There appear to be at least two parallel mechanisms for Ras activation after T cell (or B cell) receptor ligation (reviewed in Altman and Deckert, 1999)
. Ligation of antigen receptors results in activation of tyrosine kinases - mainly Lck for the TCR, Lyn for the BCR - that phosphorylate the respective receptor chains and recruit kinases, ZAP70 or Syk to the receptor. Their activation leads to recruitment of adapter proteins, LAT and SLP76, or BLNK, that in turn bind the adapter proteins Shc and Grb2, which is constitutively associated with the exchange factor mSos. A second pathway to Ras activation occurs via recruitment of PLCγ to the activated receptor complex. This leads to production of IP_3 and DAG which provides a binding site at the membrane for RasGRPs. Whether the TCR signals through RasGRP1 or through mSos appears to be dependent on the strength of the signal (Priatel et al., 2002).

Rap1 (but not Rap2) is also activated following ligation of the TCR. Interestingly, co-stimulation of CD28 prevents this activation (Reedquist and Bos, 1998) raising the possibility that interference with the activation of Rap1 may be important for the normal response of T cells to antigen. Ligation of the TCR in the absence of costimulation of CD28 results in a state of long-term functional unresponsiveness termed anergy. Anergic T cells no longer respond to antigenic stimulation by activation of the Ras-MAP kinase pathway and fail to produce IL-2 (Fields et al., 1996). It has been suggested that this is accounted for by increased levels of GTP-bound Rap1 in anergic T cells. Jurkat T cells that over-express activated Rap1 do not produce IL-2, mimicking an anergic phenotype, and again suggesting a role for Rap1 in negative regulation of TCR-induced IL-2 induction in anergy (Boussiotis et al., 1997). However, as noted above, the competition for Raf-1 with Ras resulting from over-expression of activated Rap1 may be an experimental artifact.

1.5.2 B cells

The development, survival, activation, and apoptosis of B lymphocytes are regulated by signals from a variety of receptors, including the BCR and CD40. An important role for the Ras family in B cell development is suggested by several studies with transgenic mice. In mice expressing an H-Ras S17N transgene under the Lck promoter, B cell development is blocked at a very early stage (pre-proB to pro-B transition), a phenotype that is rescued by activated Raf (Iritani et al., 1997). In a second study, the H-Ras S17N transgene was driven by the V_H gene promoter. In these mice, early pre-B cells develop, but the numbers of late pre-B cells are reduced, suggesting that Ras is important for survival of pre-B cells (Nagaoka et al., 2000). Other evidence for a role for Ras in B cell development came from expression of an activated H-Ras on a $Rag^{-/-}$ background. This permits $Rag^{-/-}$ B cells to progress from the pro-B stage, where they are normally arrested, to late pre-B cells (Shaw et al., 1999).

Ligation of the mature B cell receptor can result in activation, apoptosis, or tolerance, depending on the presence or absence of co-stimulation through CD40. Ligation of the BCR with anti-IgM antibodies causes an elevation of Ras-GTP levels from about 15% to 25% and coincides with the formation of complexes containing phosphorylated Shc, Grb2 and mSos1 (Harwood and Cambier, 1993; Saxton et al., 1994). Whether BCR ligation results in DAG-dependent activation of Ras, analogous to that seen ligation of the TCR, has not been clear. However, in support of a role for DAG, in the chicken B cell-line DT40, the absence of Grb2 results in only a 50% reduction of Erk activation after ligation of the BCR, whereas Erk activation is almost completely abolished in the absence of PLC-γ2 (Hashimoto et al., 1998).

Signaling downstream of the BCR is negatively regulated by co-ligation of the FcγRIIB. One component of the inhibitory action on Erk activation may involve the recruitment of RasGAP to the FcγRIIB via its association with SHIP and $p62^{dok}$ (Tamir et al., 2000).

CD40 plays important roles in T cell-dependent B cell activation, survival of germinal centre B cells, differentiation to antibody-secreting cells, and isotype switching. In the mature B cell-line Daudi, ligation of CD40 was shown to result in activation of Ras proteins precipitated by the monoclonal antibody Y13-259, as well as of MEK (Gulbins et al., 1996). However, crosslinking of CD40 did not result in activation of Erk in immature WEHI231 B cells (Sutherland et al., 1996), suggesting that p21 Ras may not be activated in this system.

B cells undergo changes in their ability to respond to antigen after they have been exposed in the periphery to self-antigens, a state termed tolerance. Antigenic stimulation of B cells rendered tolerant by exposure to high levels of antigen during development no longer results in

activation of JNK and NFκB, although Erk and NFAT are still activated (Healy et al., 1997). Ligation of the BCR results in activation of Rap1 via PLC-dependent production of DAG (McLeod et al., 1998). However, a possible role for Ras or Rap in B cell tolerance has not yet been explored.

1.5.3 Macrophages

The macrophage growth factor CSF-1 is critical to macrophage survival and development. Binding of CSF-1 to its receptor, Fms, was shown to result in activation of Ras proteins, a result consistent with the recruitment of Shc, Grb2 and Sos to activated Fms (Lioubin et al., 1994). A recent study suggests that CSF-1-induced activation of Ras is dependent on both the presence of the Grb2 docking site on Fms, and on Src activity (Lee and States, 2000). Erk activation by CSF-1 appears to be partially mediated by A-Raf, but not by Raf-1, and another PI-3 kinase-dependent but Raf-independent pathway leading to Erk activation also exists (Lee and States, 2000).

The importance of Ras proteins for macrophage functions is documented in a study on transgenic mice in which the activity of Ras proteins or their downstream transcription factor targets was suppressed by expression of mutants of p120 RasGAP or Ets-2. This resulted in increased sensitivity of macrophages to the withdrawal of CSF-1 and an altered, spindle-shape morphology. The increase in expression of the protease uPA by CSF-1 was also severely reduced (Jin et al., 1995). Antisense experiments suggested that N-Ras is required for the development of macrophages, but not granulocytes (Skorski et al., 1992). Interestingly, several hematopoietic cell lines such as 32D, U937, and FDC-P1 undergo differentiation to monocytes in response to expression of v-H-Ras or H-Ras G12D (Hibi et al., 1993; Maher et al., 1996; Mavilio et al., 1989).

R-Ras, but not H-Ras, has been reported to have a role in adhesion and phagocytosis in macrophages through a mechanism involving activation of $\alpha_M\beta_2$ integrins via activation of Rap1 (Caron et al., 2000; Self et al., 2001).

A major function for macrophages in immune responses is to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), for example in response to bacterial lipopolysaccharide (LPS). LPS stimulation of human monocytes was shown to activate the Ras-Erk pathway. This was important for activation of Elk-1 and Egr-1 and efficient production of TNF (Geng et al., 1994; Guha et al., 2001). Activation of Ras induced by LPS was completely inhibited by pre-incubation with IL-10, but the underlying mechanism remains unclear (Geng et al., 1994). These observations contrast with older data from transgenic mice expressing v-H-Ras. Stimulation of splenocytes from this "oncomouse" with LPS resulted in the production of only about 50% of the amount of TNFα made by wild type cells (Chen et al., 1993).

The production of nitric oxide is also characteristic to activated macrophages. Ras may regulate the activation of nitric oxide synthase (NOS)-2 by TNFα, IL-1, or IFNγ, since overexpression of H-Ras Q61L blocks and H-Ras S17N enhances activation of this enzyme, at least in lung cancer cells (Delarue et al., 2001).

1.5.4 Mast Cells

The role of individual members of the Ras family in mast cell development and function has not yet been intensively explored. However, several studies suggest that the development of mast cells is critically dependent on the Ras-Erk pathway. Haploinsufficiency for the RasGAP Nf1 results in hyper-responsiveness to steel locus factor (SLF) and increased mast cell numbers

(Hiatt et al., 2001; Ingram et al., 2000). Also, loss of the transcription factors GATA-2 and microphthalmia (mi), which may be targets of Erk, results in absence of c-Kit expression and lack of mast cell development (Kitamura et al., 2000; Tsai and Orkin, 1997). Signaling through c-Kit and the IL-3 receptor promotes mast cell survival and proliferation. Both receptors, as well as the IL-5 receptor, were shown to activate Ras proteins (Duronio et al., 1992).

Little is known about the role of Ras in the release of granule-associated mediators of acute inflammation, such as histamine and cytokines, from mast cells. Microinjection of activated H-Ras into rat mast cells results in their degranulation (Bar-Sagi and Gomperts, 1988). Ligation of the FcεRI on mast cells triggers the synthesis of a variety of cytokines. Ras was shown to be important in the induction of the IL-5 gene after FcεRI stimulation, which is regulated by the transcription factors Elk-1 and NFAT (Prieschl et al., 1995). Experiments involving dominant active and negative H-Ras both suggest that these transcription factors were targets for Ras signals. Rac1 appears to be critical for NFAT activation (Turner and Cantrell, 1997). Whether the production of TNFα by mast cells involves the Ras-Erk pathway remains controversial. Using the MEK inhibitor PD098059, one group concluded that FcεRI-induced production of TNF was independent of MEK activation in the MC/9 cell line (Ishizuka et al., 1997), whereas another group using the CPII cell line found that MEK activation was required for TNF production (Csonga et al., 1998). In human mast cells, inhibition of Erk activation results in decreased production of arachadonic acid metabolites and GM-CSF (Kimata et al., 2000). Based on studies with dominant active mutants and inhibitors, the Ras-related GTPases Rac and Rho also appear to regulate secretion (Price et al., 1995).

1.6 Objectives

When the projects of this thesis were initiated, much was known about the general functions of Ras family members as a group, for example in the regulation of hematopoietic cells, where they have roles in growth, survival, differentiation, cytokine production, cell motility, vesicle-trafficking, and phagocytosis. However, because commonly used research tools fail to discriminate between the different family members, very little was known about the functions of individual isoforms of the Ras family. It was entirely unknown whether extracellular stimuli could activate these closely related proteins in parallel or differentially.

Our primary objective was to determine if three members of the Ras family, H-Ras, K-Ras 4B, and M-Ras, would be activated by the ligation of the antigen receptors of B and T lymphocytes or by stimulation with hematopoietic growth factors, and to compare the extent of activation. When the initial studies were underway, a seminal contribution on the differential localization of H-Ras and K-Ras 4B to lipid rafts was made by John Hancock's group (Prior et al., 2001). This work inspired us to test the hypothesis that localization to these membrane domains could affect the susceptibility to activation of Ras isoforms. When it appeared that signaling to Ras proteins initiated from the activated BCR and TCR were independent of the localization of Ras to rafts, we decided to investigate the possibility that the BCR, like the TCR, would operate through a select set of GEFs which in turn would be selective in their activation of Ras isoforms.

CHAPTER II Materials and Methods

All data shown in the present study with the exception of those shown in figure 3.4 are representative of at least three independent experiments. The experiments in figure 3.4 were conducted twice each in A20 cells and splenocytes.

2.1 Methods used for chapter III

2.1.1 Vector constructs

The cDNAs for K-Ras 4B (a gift from Janis Jackson, The Scripps Research Institute, La Jolla, USA), H-Ras and M-Ras were cloned into pEGFP-C1 (Clontech) for expression of these Ras proteins fused at their amino-terminus with GFP. Chimeric Ras proteins were generated in which the carboxy-terminal hypervariable regions following amino acids R164 (H-Ras and K-Ras 4B) or R176 (M-Ras) were exchanged using standard PCR techniques and cloned into pEGFP-C1 (Fig. 3.1). A construct for mammalian expression of a fusion protein of GST and the GEF domain of mSos1 was a gift from Larry Feig (Tufts University School of Medicine, Boston, USA).

The constructs described in section 3.2.8 (M-Ras P40D, M-Ras E79D, MH3, and HM3) were generated using standard PCR techniques using wild-type cDNAs for M-Ras or H-Ras as templates. Codon 40 of M-Ras was mutated from CCT to GAT to generate the P40D mutant. Codon 79 of M-Ras was mutated from GAA to GAT to generate the E79D mutant. The entire helix 3/loop 7 regions of H-Ras (codon 92 to 109, amino acids DIHQYREQIKRVKDSDDV) and M-Ras (codons 102 to 119, amino acids HVDRFHQLILRVKDRESF) were exchanged between the two wild-type constructs to generate "MH3" (M-Ras with H-Ras helix 3/loop7) and "HM3" (H-Ras with M-Ras helix 3/loop 7). The PCR fragments were cloned into pEGFP-C1 for expression

of GFP-fusion proteins of these mutants. The cDNA for M-Ras ΔN was also cloned into pEGFP-C1. This mutant lacks the first 13 residues of M-Ras, which were replaced with ATG ACC GAA (amino acids MTE) to generate an amino-terminus that is identical to that of H-Ras. The identities of all constructs were verified by DNA sequencing.

2.1.2 Cells

Murine A20 B cells, murine WEHI231 B cells, and human Jurkat T cells were maintained in RPMI medium with 10% fetal calf serum, 100 U/mL penicillin, 50 U/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. DT40 chicken B cells and a DT40 clone lacking functional PLC-γ2 (Takata et al., 1995) were grown in the above medium supplemented with 1% chicken serum. NIH3T3 cells were cultured in DMEM with 100 U/mL penicillin, 50 U/mL streptomycin, and 10% calf serum.

2.1.3 Golgi trafficking studies

NIH3T3 cells were plated onto glass coverslips and transfected with pEGFP-Ras constructs using lipofectamine (Invitrogen). One day post transfection, 20 mM HEPES was added to the medium and the cells were placed at 16°C for two hours. Subsequent staining with BODIPY TR-labeled ceramide was performed according to manufacturer's instructions (Molecular Probes). The cells were then fixed with 4% paraformaldehyde and imaged by laser-scanning confocal microscopy using a Zeiss Axiovert S100 TV microscope.

2.1.4 Transfections and stimulations

Cells were electroporated with 20 µg of pEGFP construct using a BioRad gene pulser. After 4-8 hours (A20 or Jurkat cells) or 16 hours (DT40 cells), dead cells were removed by density centrifugation with Ficoll-Paque (Amersham Pharmacia). Live cells were subjected to serum starvation (1.5-2 hours) followed by stimulation with either 100 µg/mL anti-mouse IgG ($F(ab')_2$, Jackson Immunoresearch), 10 µg/mL anti-human CD3, clone OKT3 (a gift from Maya Kotturi, The Biomedical Research Centre, Vancouver, Canada), 5 µg/mL anti-chicken IgM (Southern Biotechnology Associates), 50 µM phorbol dibutyrate (PdBu; Sigma), or varying concentrations of ionomycin (Sigma) as indicated. WEHI231 cells were stimulated with 25 µg/mL anti-IgM ($F(ab')_2$, Jackson Immunoresearch).

2.1.5 Ras activation assay

The cells were lysed in "pull-down" buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 15% glycerol) with protease and phosphatase inhibitors (40 µg/mL PMSF, 1 µg/mL aprotinin, 0.7 µg/mL pepstatin, 10 µg/mL soy bean trypsin inhibitor, 1 mM sodium vanadate). Approximately 20 µg of fusion proteins of GST with the Ras-binding domains of Raf-1 or Nore1 (see 2.1.7) bound to glutathione sepharose (Amersham/Pharmacia) were incubated with aliquots of lysates for 30 minutes at 4°C to precipitate activated H-Ras or K-Ras 4B (with Raf-1), or M-Ras (with Nore1). Pull-down samples were run in parallel with an aliquot of the cell lysate on SDS-PAGE to enable estimation of the percentage of total cellular Ras that was activated and thus comparison of the degrees of activation of the different Ras isoforms. After SDS-PAGE (see 2.1.5), proteins were transferred to nitrocellulose membranes that were immunoblotted with antibodies to GFP (Clontech or Santa Cruz Biotechnology) or p21 Ras (Upstate Biotechnology, clone Ras10) to visualize GFP-Ras fusion proteins.

2.1.6 Western blotting

Proteins were denatured by adding 5X SDS loading buffer (10% SDS, 50% glycerol, 200 mM Tris-HCl pH 6.8, bromphenol blue) and boiling for 5 minutes. Denatured proteins were subjected to SDS-PAGE in "running buffer" (25 mM Tris, 192 mM Glycine, 0.1 mM SDS) using BioRad Protean II equipment. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) in "transfer buffer" (25 mM Tris, 192 mM glycine, 20% methanol) using the BioRad wet transfer system. The membranes were stained with Ponceau S (Sigma) to visualize molecular weight markers, followed by blocking in 5% BSA (Sigma) in TBS-N (20 mM Tris, 150 mM NaCl, 0.05% NP-40, pH 7.5). For blots with 9E10 ascites, 1% ovalbumin (Sigma) was also added to the blocking buffer. Primary antibodies were diluted in TBS-N and incubated with the membranes for either 1 hour at room temperature or overnight at 4°C. Following three 10 minute washes with TBS-N, secondary antibodies coupled to HRP (DAKO) were added in TBS-N for 30 min. at room temperature. After three 5 minute washes with TBS-N, the HRP-signal was detected using enhanced chemiluminescence (ECL) reagents according to the manufacturer's instructions (Amersham/Pharmacia) and Kodak autorad film. The following primary antibodies were used: Anti-GFP (Clontech and Santa Cruz Biotechnology), anti-p21 Ras (Upstate Biotechnology, clone Ras 10), anti-phospho-Erk1/2 (Cell Signaling), and anti-PKCδ (BD Transduction Laboratories). Anti-Lyn, anti-RasGRP, and anti-mSos1 were from Santa Cruz Biotechnology.

2.1.7 Sucrose gradients

To assess the segregation of Ras isoforms and chimeras into different areas of the membrane, we used a modified version of the detergent-free method for purification of caveolin-rich membranes first employed by Song et al. (Song et al., 1996) to demonstrate an association of

H-Ras with these membranes. A20 B cells were electroporated, serum-starved, and stimulated as described above and then lysed in 0.5 M Na_2CO_3 in MBS-M (25 mM MES, 150 mM NaCl, 5 mM $MgCl_2$, pH 6.5) followed by three sonication bursts of 10-15 seconds at 6-7 Watts. 200 µL of lysate was mixed with 200 µL of 90% sucrose in MBS-M and placed at the bottom of a 2 mL ultracentrifuge tube. This was overlaid with 1.2 mL of 35% sucrose in MBS-M and 400 µL of 5% sucrose in MBS-M. Protease and phosphatase inhibitors (40 µg/mL PMSF, 0.7 µg/mL pepstatin, 10 µg/mL soy bean trypsin inhibitor, 1 mM sodium vanadate) were present throughout the gradient. The samples were centrifuged at 250,000xg in a Beckman TL-100 ultracentrifuge for 12-16 hours at 4°C. Thirteen fractions of 150 µL were collected from the top of the gradient. The first two fractions were discarded, as preliminary experiments showed that these did not contain detectable amounts of protein on Western blots. Fractions 3-13 were each diluted in 350 µL MBS-M and membranes pelleted at 100,000xg for 30 minutes. Membrane pellets were resuspended in 1X SDS-loading buffer (2% SDS, 10% glycerol, 40 mM Tris-HCl pH 6.8, bromphenol blue), boiled, and subjected to SDS-PAGE and Western blotting using anti-p21 Ras (Upstate Biotechnology, clone Ras10) or anti-GFP (Santa Cruz Biotechnology) antibodies to detect GFP-Ras fusion proteins.

2.1.8 Preparation of GST-fusion proteins

Constructs encoding for the Ras-binding domains of Raf-1 and Nore1 fused to glutathione S-transferase were expressed in the *E. coli* strain DH5α. A 50 ml culture was grown overnight in LB containing 100 µg/ml ampicillin. This culture was used to inoculate a 1 L culture of the same medium the next day. Following inoculation, this culture was grown until it reached an OD_{600} of 0.4-0.5. The culture was then placed on ice for 20 minutes before addition of IPTG to a final concentration of 0.1 mM, and further incubation at 26°C for 4 hours. The cells were pelleted at

4,000 rpm for 20 minutes and resuspended in 10 ml of resuspension buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol, 10 µg/mL soybean trypsin inhibitor, 40 µg/mL PMSF). The bacteria were then treated with 50 µl of 10 mg/mL lysozyme and incubated on ice for 30 min., followed by addition of 100 µL of NP-40 and three freeze-thaw cycles between liquid nitrogen and a 37°C water bath. The bacterial DNA was disrupted by four brief sonication pulses, the bacterial lysate aliquoted into Eppendorf tubes and centrifuged for 10 minutes at 14,000 rpm. The resulting supernatant was stored at -80 °C.

2.1.9 Calcium assay

Cells were washed twice with Hanks' balanced salt solution (HBSS, Sigma), resuspended in 1 mL HBSS at $5x10^6$ cells/mL, and left in HBSS for 30 minutes to starve. Two µL each of 1 mM Fluo-4 and 2 mM SNARF-1 (Molecular Probes) was added and incubated with the cells for 25 minutes at 37°C. After two washes with HBSS, cells were resuspended at $1x10^6$ cells/mL and analyzed by flow cytometry as follows: A baseline was obtained for 30 seconds, the stimulus was added, and the FACS analysis resumed at 45 seconds. SNARF-1 is a pH indicator and was used because the uptake of Fluo-4 was much less efficient in the absence of SNARF-1.

2.2 Methods used for chapter IV

2.2.1 Vector constructs

Retroviral vectors were constructed by cloning myc-epitope tagged cDNAs for K-Ras 4B, H-Ras and M-Ras into pMXpie (a gift from Alice Mui, Jack Bell Research Centre, Vancouver, Canada). This vector drives expression of EGFP from an internal ribosomal entry site (IRES) downstream

of the Ras cDNA and features a puromycin resistance gene driven by a separate promoter. Chimeric Ras constructs, in which the carboxy-terminal hypervariable regions following amino acids R164 (H-Ras and K-Ras 4B) or R176 (M-Ras) were exchanged, were generated using standard PCR techniques and cloned into pMXpie with amino-terminal myc tags. The identities of all constructs were verified by DNA sequencing.

2.2.2 Cells and retroviral infections

The human wild-type CSF-1 receptor (the cDNA was a gift from Martine Roussel, St. Jude Children's Research Hospital, Memphis, USA) was equipped with a carboxy-terminal HA tag, expressed in IL-3 dependent Ba/F3 cells, and a clone designated Ba/F3-Fms was established. Ba/F3-Fms cells were maintained in RPMI, 10 % fetal calf serum, 100 U/mL penicillin, 50 U/mL streptomycin, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 µM β-mercaptoethanol, and 3% of a 10X concentrated conditioned medium from WEHI-3B cells as a source of IL-3. BOSC23 were cultured in DMEM with 100 U/mL penicillin, 50 U/mL streptomycin, and 10% fetal calf serum.

Ba/F3-Fms cells were retrovirally transduced by incubation with supernatants from BOSC23 packaging cells that had been transiently transfected using Lipofectamine (Invitrogen) with pMXpie-Ras constructs in the presence of 10 µg/mL polybrene. Infected cells were selected in 2 µg/mL puromycin. These polyclonal cell populations were used for stimulations within 2 weeks post infection.

2.2.3 Sucrose gradients

To assess the segregation of Ras isoforms and chimeras into different areas of the membrane of Ba/F3-Fms cells, a slightly modified version of the detergent-free method described above (section 2.1.7) was used. The cell lysates were sonicated three times for 15-20 seconds at 6-7 Watts. Twelve fractions of 160 µL were collected from the top of the gradients. The first two fractions were discarded as preliminary experiments showed that these did not contain detectable amounts of protein on Western blots. Fractions 2-12 were each diluted in 370 µL MBS-M and membranes pelleted at 100,000xg for 30 minutes. Membrane pellets were resuspended in SDS-loading buffer, boiled, and subjected to SDS-PAGE and Western blotting (see section 2.1.6). 9E10 mouse ascites (a gift from Hermann Ziltener, The Biomedical Research Centre, Vancouver, Canada) was used to detect myc-tagged Ras proteins. The same protocol was also employed for the IL-3 and CSF-1 receptors, and the receptor-associated molecules Shc, Grb2, and mSos.

2.2.4 Cell stimulation, Ras activation assay, and Western blotting

Ba/F3-Fms cells were deprived of serum and IL-3 for 1.5-2 hours prior to stimulation with 15 µg/mL synthetic IL-3 peptide (a gift from Ian Clark-Lewis, The Biomedical Research Centre, Vancouver, Canada) or 100 ng/mL recombinant human CSF-1 (R&D Systems). The cells were lysed in "pull-down buffer" (1% NP-40, 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 15 % glycerol) with protease and phosphatase inhibitors (40 µg/mL PMSF, 1 µg/mL aprotinin, 0.7 µg/mL pepstatin, 10 µg/mL soy bean trypsin inhibitor, 1 mM sodium vanadate). Ras activation assays were performed as described in 2.1.5. After SDS-PAGE (see section 2.1.6), myc-tagged Ras proteins were detected by blotting with 9E10 monoclonal antibodies from mouse ascites. Stimulation of cells was checked by immunoblotting of lysates with antibodies specific for

phosphorylated Erk1/2 (Cell Signaling), and equivalency of loading was verified (these blots are not shown). Other primary antibodies used were from Covance (anti-HA) and Santa Cruz Biotechnology (anti-βc, anti-Shc, anti-Grb2, anti-mSos1/2).

CHAPTER III

Analysis of Ras activation by B and T cell antigen receptors

3.1 Introduction

Areas of the plasma membrane that are enriched in cholesterol and glycosphingolipids and are termed *lipid rafts* have been implicated in signaling events downstream of many cell-surface receptors, including the antigen receptors of B and T lymphocytes (Cheng et al., 1999; Xavier et al., 1998). Many proteins with important roles in signaling of cells of the immune system, such as Lyn, Lck, LAT, and some members of the Ras family, are constitutively present in rafts. The activated BCR or TCR also recruit other critical signaling molecules into rafts such as ZAP-70, BLNK, and phospholipase C (PLC)-γ (Cheng et al., 1999; Guo et al., 2000; Montixi et al., 1998; Xavier et al., 1998; Zhang et al., 1998). Thus, lipid rafts are thought to facilitate the assembly of signal transduction machinery by activated antigen receptors (Delon and Germain, 2000).

Activation of the p21 Ras proteins has been associated with the engagement of many types of cell-surface receptors and is known to regulate cellular proliferation and differentiation. Ligation of the B or T cell antigen receptors was also reported to result in activation of p21 Ras (Downward et al., 1990; Harwood and Cambier, 1993). However, it is now evident that cross-reactivity of the antibody used to measure p21 Ras activation means that the activation that was observed could have been due to activation of any of the isoforms of p21 Ras (H-Ras, N-Ras, K-Ras 4A, or K-Ras 4B), or even of other members of the Ras family such as M-Ras or TC21. Relatively little is known about the contribution of these different isoforms to functions that have been attributed to "p21 Ras". Genetic disruptions in the Ras-Erk pathway block the development of B and T lymphocytes, with strong evidence that this is due to perturbations of Ras signaling

downstream of the TCR in the case of thymocytes (Dower et al., 2000; Iritani et al., 1997; Pages et al., 1999; Swan et al., 1995). However, it remains unknown which members of the Ras family are the critical players. There is also no information available on whether different extracellular stimuli activate the closely related Ras proteins in parallel or differentially. One potential factor in the latter may be the co-localization of some Ras isoforms in lipid rafts with the activating receptor. There is compelling evidence that H-Ras occurs in rafts, whereas K-Ras 4B is excluded from rafts (Niv et al., 2002; Prior et al., 2001; Roy et al., 1999). This differential localization depends upon the nature of their carboxy-termini. Thus, the palmitoylated carboxy-terminus of H-Ras directs its localization to rafts. K-Ras 4B lacks palmitoylation sites at its carboxy-terminus but exhibits multiple basic residues that direct its localization to the disordered membrane. The localization of H-Ras to lipid rafts appears to be critical for its activation of Raf-1 and PI-3 kinase (Jaumot et al., 2001; Roy et al., 1999).

Here we examined the activation of three Ras proteins, H-Ras, K-Ras 4B, and M-Ras, following ligation of the B and T cell antigen receptors. We have tested the hypothesis that raft-associated H-Ras would be more efficiently activated by ligation of these receptors than would be K-Ras 4B or M-Ras, which we show localized to the disordered membrane like K-Ras 4B. We observed differential activation of Ras isoforms, but found no correlation with their localization to rafts. Instead, it appeared that the differential activation of Ras isoforms was due to their differential sensitivity to the DAG-responsive GEFs downstream of the activated BCR and TCR.

3.2 Results

3.2.1 Trafficking of Ras proteins to the plasma membrane through the Golgi apparatus requires a palmitoylated carboxy-terminus

H-Ras, which exhibits a palmitoylated carboxy-terminus, was shown to localize to lipid rafts, whereas K-Ras 4B, which lacks sites for palmitoylation but exhibits a stretch of lysine residues at its carboxy-terminus similar to M-Ras, is excluded from rafts (Prior et al., 2001). Based on this observation, we generated a series of constructs to be used in this study where the carboxy-termini were exchanged between H-Ras and either K-Ras 4B or M-Ras (Fig. 3.1). The carboxy-terminal switch constructs were termed HM or HK (for H-Ras with the carboxy-terminus of either K-Ras 4B or M-Ras), or MH and KH (for M-Ras or K-Ras 4B with the carboxy-terminus of H-Ras).

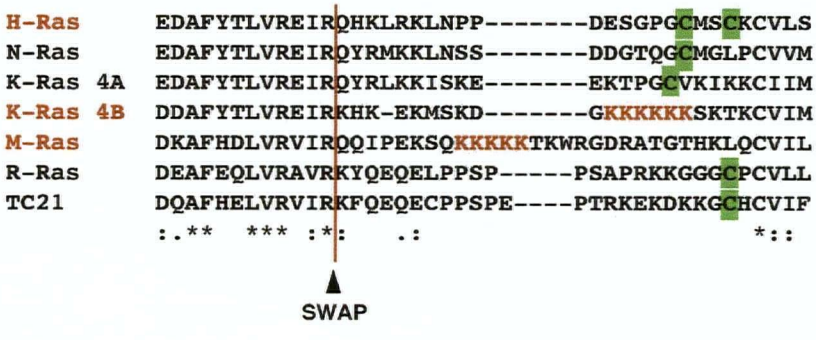

Figure 3.1 ***Alignment of the carboxy-termini of Ras proteins.*** The carboxy-terminal amino acids of the p21 Ras proteins H-Ras, N-Ras, K-Ras 4A and K-Ras 4B were aligned with those of the non-classical Ras proteins M-Ras, R-Ras, and TC21. Cysteines that are palmitoylated are highlighted in green. Stretches of multiple lysine residues appear in red. Ras constructs that were used in the present study are in red letters. The site of the carboxy-terminal swap is indicated.

We first established the trafficking patterns of all Ras constructs used in this study by expressing fusion proteins of GFP and the various isoforms and chimeras of Ras in NIH3T3 cells. We confirmed previous observations of differences in the trafficking of H-Ras and K-Ras 4B (Apolloni et al., 2000). Thus, GFP-H-Ras accumulated in a perinuclear region that co-stained for a marker of the Golgi, a fluorescently labeled ceramide. In contrast, GFP-K-Ras 4B was never seen in perinuclear areas (Fig. 3.2). M-Ras was also completely absent from areas that stained with ceramide (Fig. 3.2). This suggested that like K-Ras 4B, M-Ras takes a route to the plasma membrane that bypasses the Golgi apparatus.

To test whether the difference in trafficking pathways of H-Ras, M-Ras, and K-Ras 4B was due to differences in their carboxy-termini, we used the chimeric Ras proteins where the carboxy-terminal hypervariable regions were exchanged. Unlike wild-type H-Ras, the chimeras of H-Ras with the poly-basic carboxy-termini of either M-Ras or K-Ras 4B (HM, HK) were always absent from the Golgi region. However, expression of chimeras of either M-Ras or K-Ras 4B with the palmitoylated carboxy-terminus of H-Ras (MH, KH) produced accumulation of green fluorescence near the nucleus that had not been seen with their wild type counterparts (Fig. 3.2). These data suggest that the palmitoylated carboxy-terminus of H-Ras provides a necessary and sufficient signal to direct Ras proteins through the Golgi apparatus. Likewise, a carboxy-terminus lacking palmitoylation sites but exhibiting a stretch of basic residues directed Ras proteins to the plasma membrane via a route that did not involve the Golgi.

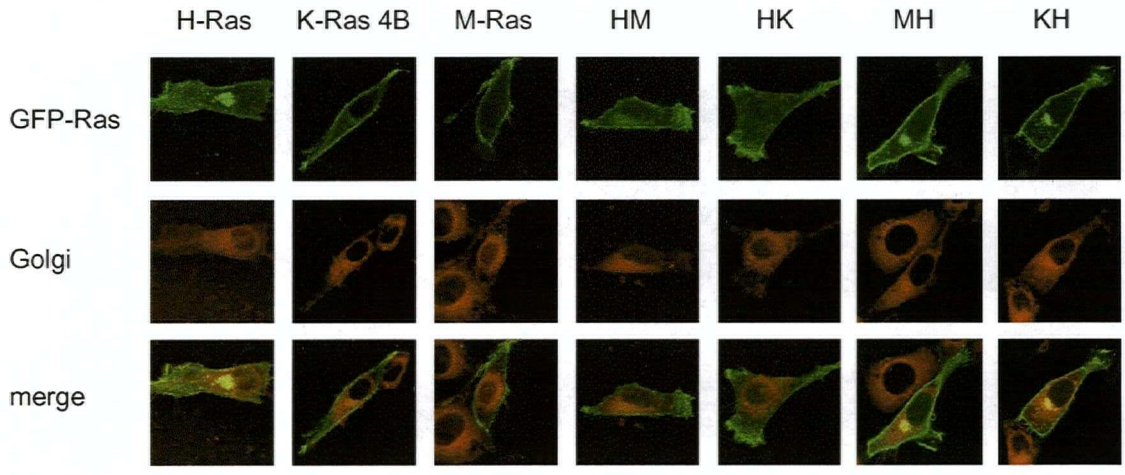

Figure 3.1 ***Ras proteins with palmitoylated carboxy-termini accumulate in the Golgi.*** NIH3T3 cells were transiently transfected with the indicated GFP-tagged Ras constructs. The cells were stained with a red fluorescent ceramide to mark the localization of the Golgi, fixed, and imaged by confocal microscopy. Box size: 63x63 μm. HM, HK: H-Ras with the carboxy-termini of K-Ras 4B or M-Ras, respectively. MH, KH: M-Ras or K-Ras 4B with the carboxy-terminus of H-Ras.

3.2.2 Localization of Ras proteins to lipid rafts in lymphocytes depends on the presence of a palmitoylated carboxy-terminus

Next, we determined whether the localization of H-Ras but not K-Ras 4B to lipid rafts that was previously reported in fibroblasts (Prior et al., 2001) also occurred in A20 B cells. We also determined whether M-Ras was localized to rafts or the disordered membrane. As in fibroblasts, most of the H-Ras accumulated in the low-density fractions of sucrose gradients, whereas K-Ras 4B was excluded from these fractions (Fig. 3.3). After pretreatment of cells with the cholesterol-depleting agent methyl-β cyclodextrin, much more of the H-Ras was present in higher-density fractions, consistent with the accumulation of cholesterol-rich membrane characteristic of lipid rafts in the low-density fractions. M-Ras was absent from low-density raft fractions, occurring in the high-density fractions where K-Ras 4B was found (Fig. 3.3). We also determined the localization of constitutively active mutants of these Ras proteins. K-Ras 4B

G12V and M-Ras Q71L were located in the same high-density fractions as their wild-type counterparts. However, unlike its wild-type counterpart, activated H-Ras G12V was not mainly in the-low density fractions but instead was distributed across most of the gradient (Fig. 3.3). This suggested that in A20 cells H-Ras leaves lipid rafts upon activation, which is consistent with observations made in fibroblasts (Prior et al., 2001). When sucrose gradients were performed with myc-tagged Ras proteins we observed the same partitioning as with the GFP-tagged proteins (not shown), indicating that the amino-terminal tag did not interfere with localization to

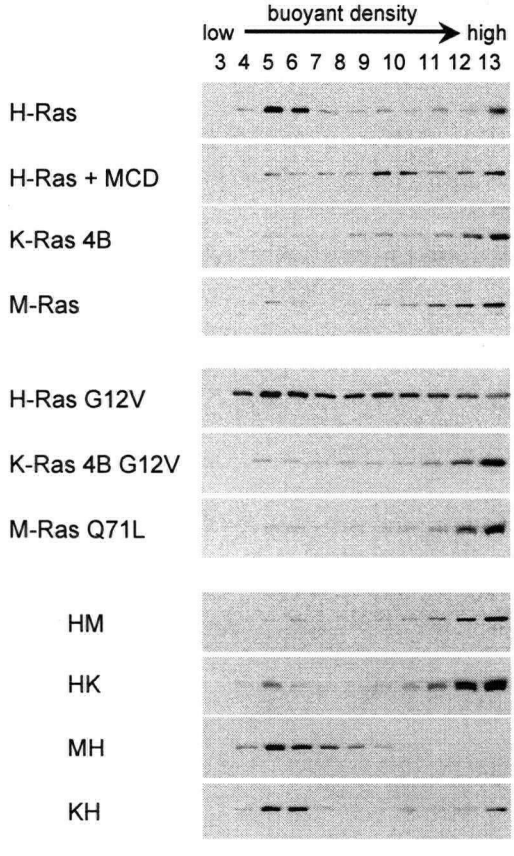

Figure 3.3 ***Location of H-Ras, K-Ras 4B, M-Ras, and chimeric Ras proteins in sucrose gradients.*** A20 cells were transiently transfected with the indicated GFP-tagged Ras constructs and lysates were fractionated according to buoyant densities over sucrose gradients. Thirteen fractions were collected from the top of the gradient (fractions 1 and 2 were discarded). After dilution, membrane preparations were obtained by further ultracentrifugation. The membrane pellets were resuspended in SDS loading buffer and subjected to SDS-PAGE and blotting with anti-GFP antibodies to visualize Ras proteins. Methyl-β cyclodextrin (MCD) treatment was for 20 minutes at 10 mM prior to cell lysis.

membrane domains. Thus, we concluded that in A20 B cells, H-Ras and K-Ras 4B localized respectively to rafts and disordered membranes. That M-Ras behaved like K-Ras 4B is consistent with the notion that polybasic carboxy-termini determine localization in the disordered plasma membrane.

To confirm whether localization of M-Ras in disordered membranes was due to the nature of its carboxy-terminus, we tested whether its localization would be affected by replacing its polybasic carboxy-terminus with the palmitoylated carboxy-terminus of H-Ras. We were also interested in whether the carboxy-termini of H-Ras and K-Ras 4B still dictated whether they localize in rafts in lymphocytes, which, unlike the fibroblasts used in other studies (Niv et al., 2002; Prior et al., 2001) lack caveolae. We observed that chimeras of M-Ras or K-Ras 4B in which the polybasic carboxy-termini were exchanged with the palmitoylated carboxy-terminus of H-Ras (MH, KH), occurred in the low-density raft fractions. Likewise, chimeras of H-Ras with polybasic carboxy-termini of M-Ras or K-Ras 4B (HM, HK) were found in the high-density, disordered membrane fractions (Fig. 3.3). These results indicate that the requirements for localization of Ras proteins to lipid rafts or the disordered membrane are identical in lymphocytes and fibroblasts.

We also investigated whether other proteins that are known to localize to rafts in sucrose gradients containing detergent would localize to rafts in detergent-free gradients used to assess the partitioning of Ras isoforms. Lyn is highly enriched in such detergent-resistant membranes (DRMs) in B cells, and the BCR was shown to translocate into DRMs after ligation of the receptor (Cheng et al., 1999; Petrie et al., 2000). However, under conditions used to separate Ras proteins in A20 cells only a relatively small fraction of Lyn was found in the low density fractions (Fig. 3.4). Much more Lyn was present in these fractions when the duration of sonication was slightly shortened. The same observation was made in mouse splenocytes (not shown). The location of the Igα chain of the BCR was also affected by the duration of sonication

and followed the pattern of Lyn. While Igα was enriched in low density fractions under conditions that also enriched for Lyn in these fractions, regardless of receptor stimulation (Fig. 3.4), much less Igα (~50%) was found in these fractions when the conditions for the separation of Ras isoforms were used in splenocytes (not shown). These observations suggested that the method to assess the association of Ras isoforms with rafts may not necessarily be useful to study the raft association of other proteins, at least not under conditions that produce optimal segregation of Ras isoforms. In Ba/F3 cells Lyn partitioned readily into rafts under the conditions used for Ras proteins (see chapter IV), suggesting that the membrane composition may be different in Ba/F3 cells and A20 cells or splenocytes. Thus, it is possible that in lymphocytes Lyn and the BCR might localize to a type of raft that is different from that which H-Ras is mainly localized in.

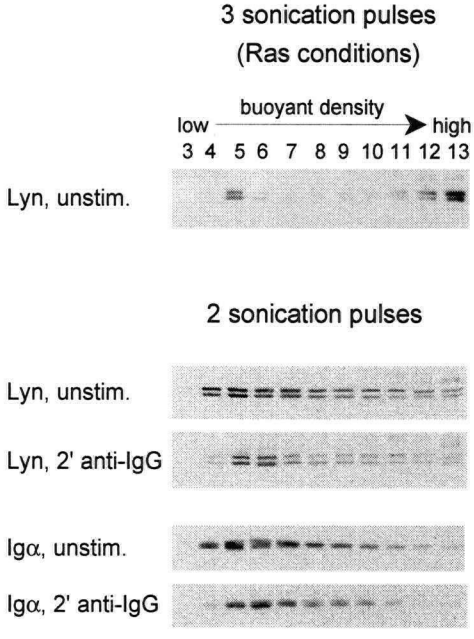

Figure 3.4 ***Conditions used to assess the association of Ras isoforms with rafts are inadequate for assessment of an association of Lyn or Igα with rafts.*** Lysates from A20 cells that had either been left untreated or had been stimulated with anti-IgG were subjected to either three or two sonication pulses and fractionated over sucrose gradients. Thirteen fractions were collected from the top of the gradient (fractions 1 and 2 were discarded). After dilution, membrane preparations were obtained by further ultracentrifugation. The membrane pellets were resuspended in SDS loading buffer and subjected to SDS-PAGE and blotting with anti-Lyn or anti-Igα antibodies.

3.2.3 Ligation of the BCR or TCR results in activation of a subset of Ras proteins which does not correlate with their localization to rafts

We next investigated whether ligation of the BCR or TCR, which when activated are known to reside in lipid rafts (Cheng et al., 1999; Xavier et al., 1998), would preferentially activate those Ras isoforms which are also localized in rafts. In keeping with this notion, ligation of either the BCR or the TCR resulted in activation of H-Ras but not of M-Ras (Figure 3.5A). However, K-Ras 4B was strongly activated following ligation of the BCR or TCR, despite its exclusion from rafts (Figure 3.5A). Similar results were obtained by ligation of the BCR on the immature B cell line

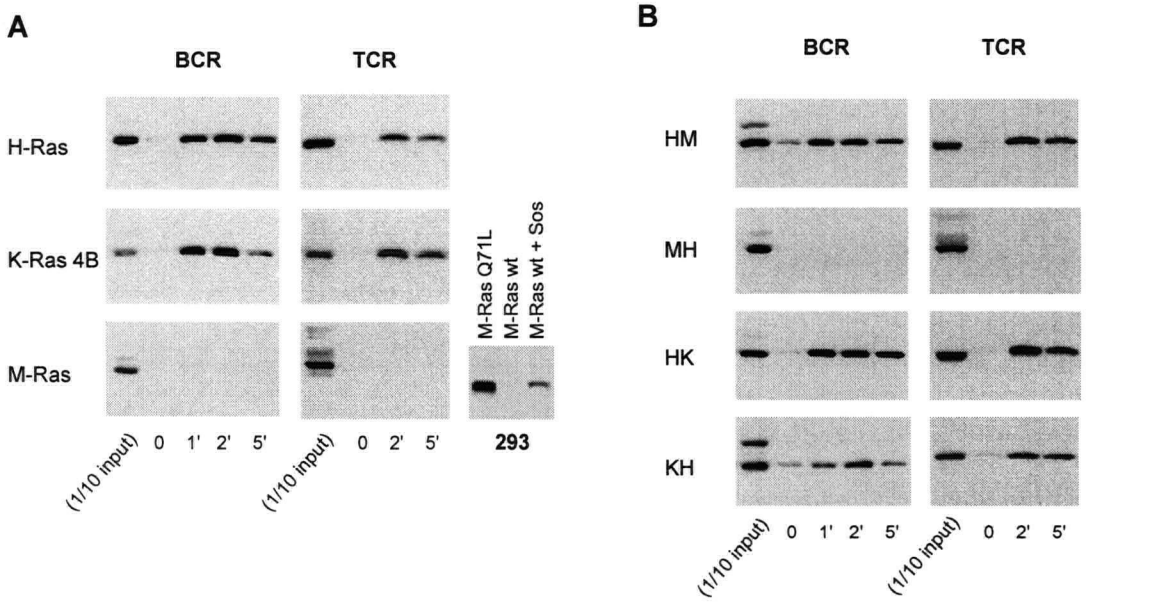

Figure 3.5 ***Ligation of the BCR or TCR induces activation of H-Ras and K-Ras 4B, but not M-Ras, and exchanging the carboxy-termini has no effect on the susceptibility to activation.*** A20 B cells and Jurkat T cells were transfected with wild-type (A) or chimeric (B) GFP-tagged Ras constructs as indicated and stimulated with either anti-IgG or anti-CD3 for the indicated times. Lysates were subjected to pull-down assays using either GST-Raf-1 RBD to precipitate activated, GTP-bound H-Ras and K-Ras 4B, or GST-Nore1 to precipitate active M-Ras. One tenth of the amount of lysate used for a pull-down was run in parallel with the pull-down samples ("1/10 input"). The last panel in the bottom row in (A) shows a control experiment where activated M-Ras was precipitated from HEK293 cells transfected with equal amounts of M-Ras constructs with or without mSos1. All cell lysate samples were also analyzed for phosphorylated Erk1/2 and equal loading was confirmed (not shown).

WEHI231 (not shown). Control experiments demonstrated that the GFP-M-Ras fusion protein was capable of activation by co-expression of mSos1 in HEK293 cells (Figure 3.5A). We also observed that a chimera of H-Ras with the polybasic carboxy-terminus of M-Ras (HM) that was not localized to rafts (Figure 3.3) was activated to the same extent as wild-type H-Ras following ligation of the BCR or TCR (Figure 3.5B). Conversely, a chimera of M-Ras and the palmitoylated carboxy-terminus of H-Ras (MH) was not activated (Figure 3.5B). These results suggest that ligation of BCR or TCR leads to activation of a subset of Ras proteins whether or not the latter are localized to rafts.

3.2.4 Activation of H-Ras and K-Ras 4B by the BCR is dependent on PLC-γ2

Since the ability of Ras isoforms to become activated by the BCR and TCR was not determined by their localization to rafts, we asked whether their differential activation resulted from differences in their susceptibility to activation by the relevant GEFs. Recent data suggest a critical role for members of the DAG-responsive RasGRP family of GEFs in TCR-mediated p21 Ras activation (Ebinu et al., 2000), although the role of RasGRPs in BCR-induced Ras activation is unclear. Therefore, we examined the activation of Ras proteins induced by ligation of the BCR in a clone of the chicken B cell line DT40 that lacks expression of PLC-γ2 (Takata et al., 1995). As shown in figure 3.6A, the absence of PLC-γ2 resulted in a dramatic reduction in activation of endogenous p21 Ras following ligation of the BCR. Phosphorylation of Erk was also severely reduced (Figure 3.6A), as had been reported before (Hashimoto et al., 1998). We next investigated whether BCR-mediated activation of specific Ras isoforms was dependent on the presence of PLC-γ2. In the parental DT40 cells, ligation of the BCR resulted in efficient activation of exogenous H-Ras or K-Ras 4B, but the activation of M-Ras was undetectable (Figure 3.6B), consistent with the results from the A20 mouse B cells. In the DT40 cells lacking

PLC-γ2, the activation of both H-Ras and K-Ras 4B was severely reduced (Figure 3.6B). These results indicate that the activation of H-Ras and K-Ras 4B induced by ligation of the BCR both occur through pathways that depend on PLC-γ2.

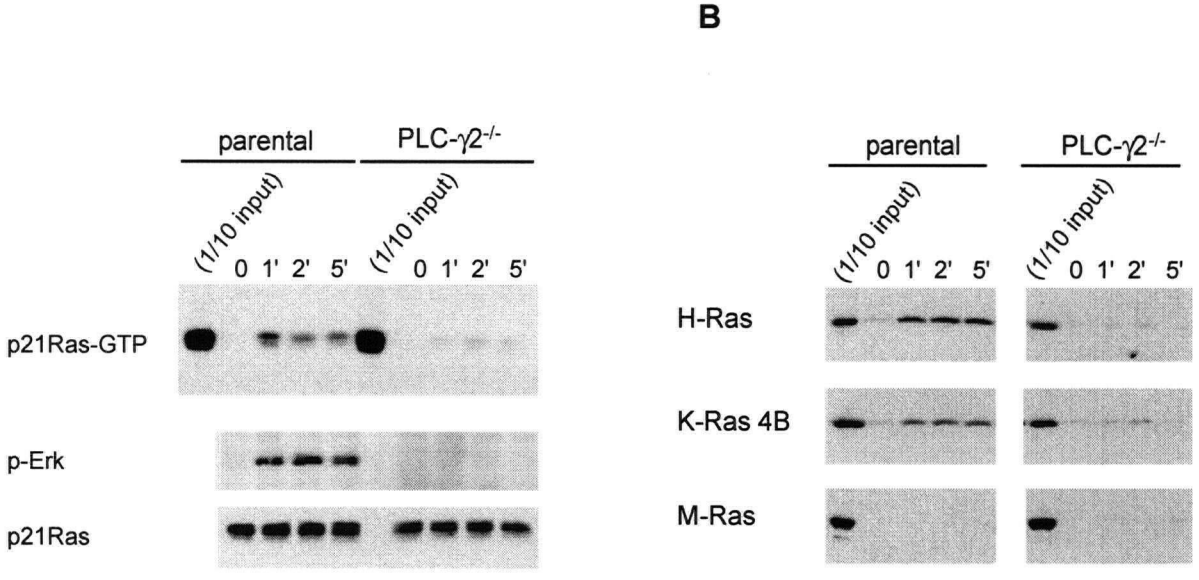

Figure 3.6. ***Reduced Ras activation in the absence of PLC-γ2.*** Parental or PLC-γ2 deficient DT40 B cells were stimulated with anti-IgM for the indicated times and activated Ras proteins were precipitated. One tenth of the amount of lysate used for a pull-down was run next to the pull-down samples ("labeled 1/10 input"). (A) Activated endogenous p21 Ras was precipitated with the Raf-1 RBD. The middle and lower panels show blots of the same lysates used for the pull-downs probed with anti-phospho Erk and anti-p21 Ras. (B) Activated exogenous Ras proteins were precipitated with the Raf-1-RBD (H-Ras and K-Ras 4B) or Nore1-RBD (M-Ras). The same lysates used for the pull-down assays were also probed with anti-phospho Erk and anti-p21 Ras to ensure effective BCR stimulation and equivalency of loading (not shown).

3.2.5 Increases in calcium levels alone do not induce the activation of H-Ras and K-Ras 4B

Two families of GEFs can be regulated by the products of PLC-γ activity, DAG and/or calcium fluxes. Calcium fluxes alone activate RasGRF1/2, while DAG enhances the GEF activity of all four RasGRP proteins. RasGRPs also exhibit two calcium-binding EF hands, but calcium

inhibited the GEF activity of RasGRP2 on N-Ras (Clyde-Smith et al., 2000). To investigate whether calcium fluxes alone could stimulate Ras activation in B cells, we first determined the concentration of the calcium ionophore ionomycin that stimulated a calcium flux comparable to that induced by anti-IgM stimulation of DT40 cells. This was between 5 and 10 nM (Figure 3.7).

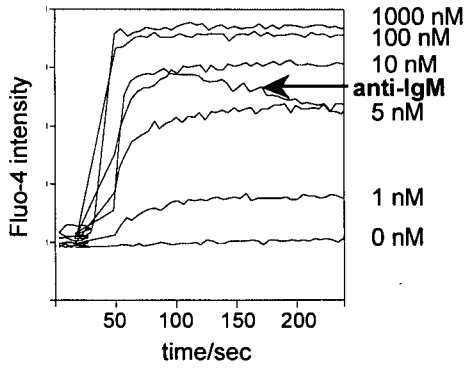

Figure 3.7 ***Calcium fluxes induced by 5-10 nM ionomycin are equivalent to calcium fluxes induced by anti-IgM stimulation of DT40 cells.*** Calcium fluxes induced by different concentrations of ionomycin or by 5 µg/mL anti-IgM in DT40 cells were assessed by flow cytometry using the calcium-activated dye, Fluo-4. The baseline was obtained for 30 seconds before addition of ionomycin or 5 µg/mL anti-IgM.

However, even when DT40 cells expressing H-Ras, K-Ras 4B, or M-Ras were stimulated with concentrations of ionomycin up to 100 nM, there was no detectable activation of any of the Ras proteins (Figure 3.8). At very high concentrations of ionomycin (1 µM), H-Ras and K-Ras 4B were weakly activated, which may have been caused by an activation of PLCs by a massive influx of calcium. However, our results indicate that calcium fluxes equivalent to those evoked by BCR ligation were insufficient for the activation of H-Ras and K-Ras 4B in B cells. Thus, the calcium-activated GEFs RasGRF1 and RasGRF2 are unlikely to be involved in BCR-induced activation of Ras.

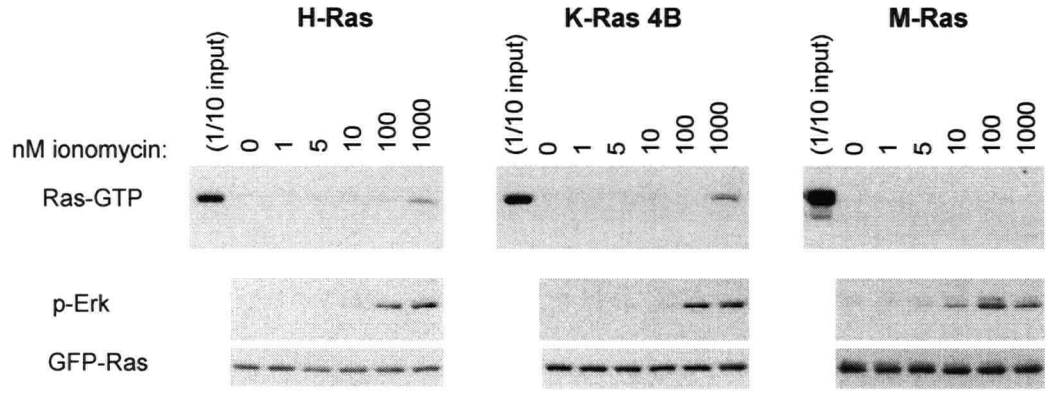

Figure 3.8 ***Failure of calcium fluxes alone to activate Ras proteins in DT40 cells.*** DT40 cells transfected with Ras constructs were stimulated with increasing concentrations of ionomycin as indicated, and activated, GTP-bound Ras proteins were precipitated (upper panels). The middle and lower panels show blots of the same lysates as were used for the pull-downs probed with anti-phospho Erk and anti-GFP.

3.2.6 The phorbol ester PdBu stimulates activation of H-Ras and K-Ras 4B but not of M-Ras

To assess the role of the second product of PLC-γ2 activity, DAG, we used the phorbol ester PdBu, which mimicks DAG in binding to the C1 domains of isoforms of PKC or RasGRP. We observed that stimulation of DT40 cells with PdBu alone was sufficient for activation of H-Ras and K-Ras 4B, but not of M-Ras (Fig. 3.9), paralleling the results obtained by ligation of the BCR or TCR. Collectively our results indicate that the production of DAG by PLC-γ2 is both necessary and sufficient for activation of H-Ras and K-Ras 4B, but not M-Ras, following ligation of the BCR.

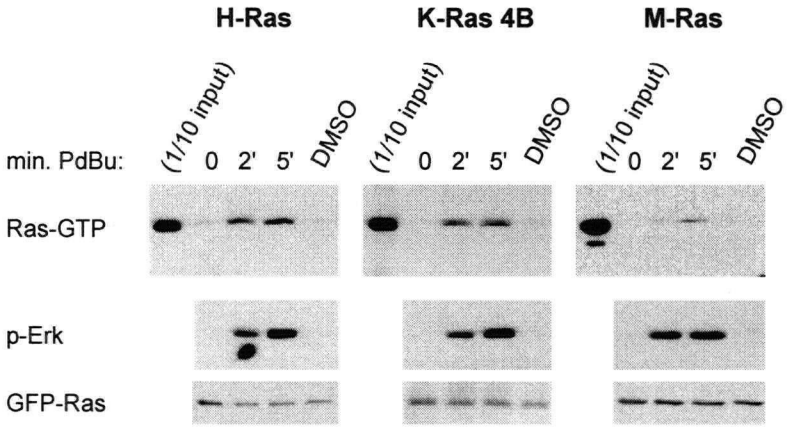

Figure 3.9 ***Activation of H-Ras and K-Ras 4B but not M-Ras by PdBu.*** DT40 cells were transiently transfected with Ras constructs and stimulated with PdBu for the indicated times, or stimulated with DMSO carrier for 5 minutes. Pull-downs of activated, GTP-bound Ras proteins were performed (upper panels). The middle and lower panels show blots of the same lysates as were used for the pull-downs probed with anti-phospho Erk and anti-GFP.

3.2.7 Down-regulation of RasGRP by prolonged exposure to PMA results in reduced activation of p21 Ras by ligation of the BCR

To gain more direct evidence for an important role for RasGRP proteins in BCR-mediated activation of Ras proteins, the effects of reduced levels of endogenous RasGRPs on p21 Ras activation were tested. Since the classical and novel isoforms of PKC which exhibit DAG-binding C1 domains are known to be down-regulated by chronic exposure to phorbol esters such as PMA or PdBu, we speculated that this might also be the case for RasGRP proteins, which also exhibit a C1 domain. As shown in figure 3.10, an overnight exposure of WEHI231 B cells to PMA did result in down-regulation of RasGRP. At the same time, PKCδ was also down-regulated. The same observation was made in A20 B cells (not shown). When WEHI231 cells were stimulated with anti-IgM antibodies, the activation of endogenous p21 Ras was significantly reduced in cells that had been treated with PMA (Fig. 3.10).

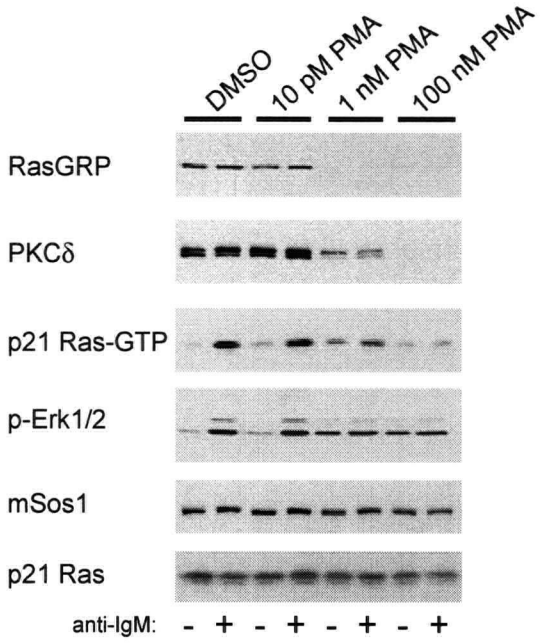

Figure 3.10 ***Prolonged exposure to PMA results in down-regulation of RasGRP, and reduced levels of RasGRP correlate with reduced activation of p21 Ras following ligation of the BCR.*** WEHI231 cells were exposed to either DMSO or different concentrations of PMA overnight and subsequently stimulated with 25 µg/mL anti-IgM. Levels of activated endogenous p21 Ras were determined by pull-downs with Raf-1 RBD as described. Cell lysates were also analyzed for RasGRP, PKCδ, phosphorylated Erk1/2, p21 Ras, and mSos1.

The expression levels of p21 Ras expression were unchanged by exposure to PMA, as were the levels of mSos1 (Fig. 3.10). These results suggest that prolonged exposure to PMA does not only lead to the down-regulation of PKC isoforms but other proteins with C1 domains as well. Of the known DAG-regulated non-PKC proteins (Brose and Rosenmund, 2002), DAG kinases, PKD1/PKCµ, as well as the β2 isoform of chimaerin, a protein with GAP activity towards Rac, could be expressed in hematopoietic cells and could have been down-regulated by phorbol ester treatment of WEHI231 cells together with RasGRPs. Although we cannot rule out a possible involvement of any of these proteins in BCR-induced activation of p21 Ras, this seems unlikely (see discussion) and the most straight-forward conclusion is that BCR-induced

activation of p21 Ras was reduced due to a depletion of RasGRP. This would implicate the RasGRP proteins as the critical RasGEFs that are activated by the BCR.

3.2.8 Failure to rescue the lack of activation of M-Ras

Because the differential activation of Ras isoforms appeared to result from differential sensitivity to a specific set of GEFs, the RasGRP proteins, we asked if there were specific residues present in M-Ras that could potentially interfere with efficient activation by RasGRPs. Based on

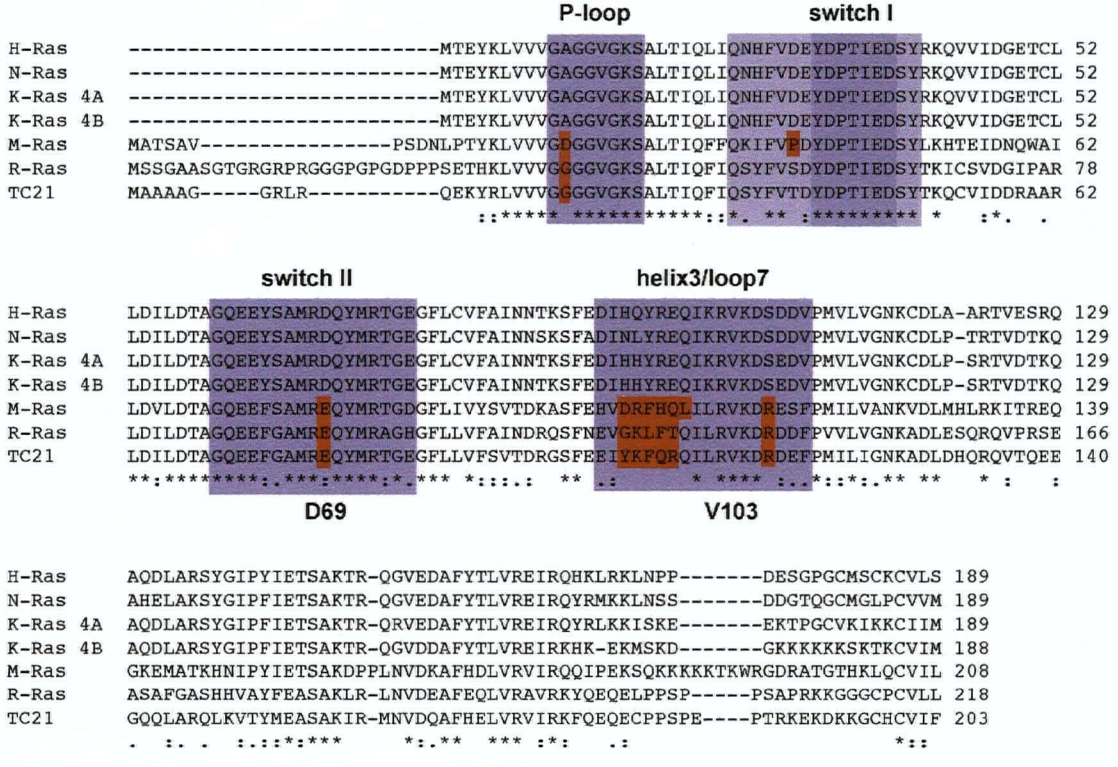

Figure 3.11 ***Alignment of classical and non-classical Ras proteins.*** Areas shaded in blue represent regions in H-Ras that are known to make contact with mSos (Boriack-Sjodin et al., 1998). Light blue areas represent the extended switch I region. Residues highlighted in red represent residues within regions of contact with GEFs of M-Ras, R-Ras, and TC21 that are not conserved in the corresponding residues in p21 Ras proteins. E79 in switch II is highlighted because the corresponding D69 in p21 Ras is known to be critical for interaction of H-Ras with RasGRP (Nielsen et al., 2001). V103 in H-Ras is also critical for interaction of H-Ras with RasGRP (Nielsen et al., 2001), but since this residue is conserved in non-classical Ras proteins it is not highlighted.

the crystal structure of H-Ras in complex with mSos1, four regions are possibly involved in the interaction of a Ras protein with a GEF: the P-loop, switch I, switch II, and helix 3/loop 7 (Fig. 3.11) (Boriack-Sjodin et al., 1998). A comparison with p21 Ras revealed that especially the helix 3/loop 7 region is quite different in M-Ras, and that P40 in the extended switch I could theoretically misposition the conserved YDPTIED core. In addition, D69 of H-Ras was shown to be critical for its interaction with RasGRP (Nielsen et al., 2001). The corresponding residue 79 in M-Ras is also negatively charged, but is an E. Another prominent difference between M-Ras and p21 Ras is the presence of 10 extra amino acids at the amino-terminus of M-Ras. Thus, M-Ras constructs were generated in which P40, E79, and the entire helix 3/loop 7 region were replaced with the corresponding residues of H-Ras. Another mutant, MΔN, was constructed according to the amino-terminus of H-Ras and thus lacked the ten extra amino-terminal amino acids present in M-Ras. Neither of those mutants were more susceptible to activation by ligation of the BCR or by stimulation with PdBu (Fig. 3.12). A mutant of H-Ras with the helix 3/loop 7 region of M-Ras was activated to a similar extent as wild-type H-Ras in a control experiment. The failure to rescue the activation of M-Ras could reflect the fact that multiple residues on Ras isoforms could be required in combination to establish efficient activation by GEFs.

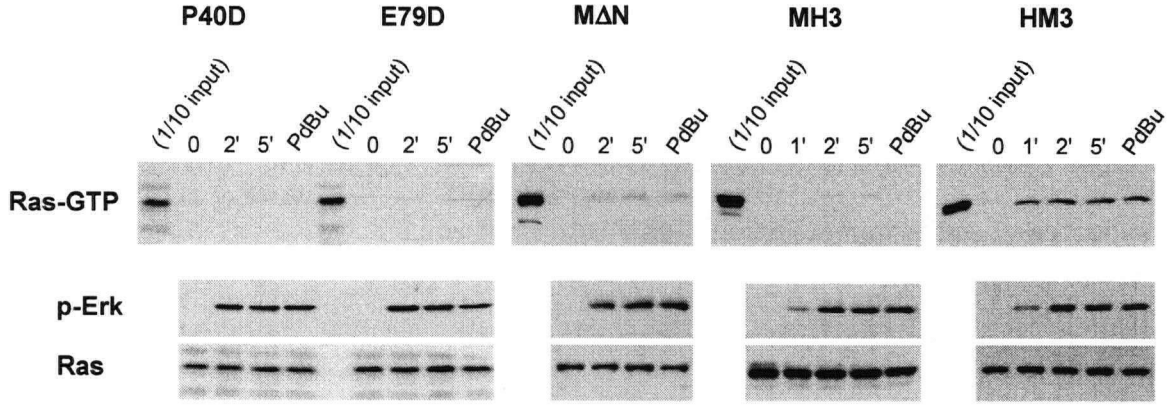

Figure 3.12 ***Failure to rescue the lack of activation of M-Ras.*** DT40 cells were transiently transfected with M-Ras P40D, M-Ras E79D, M-Ras ΔN, MH3, and HM3 constructs, and stimulated with anti-IgM or PdBu. Ras activation assays were performed as described above. MH3: M-Ras with the helix 3/loop 7 region of H-Ras. HM3: H-Ras with the helix 3/loop 7 region of M-Ras.

3.3 Discussion of Chapter III

We show here for the first time that Ras isoforms are activated differentially following ligation of the BCR or TCR. That both H-Ras and K-Ras 4B were strongly activated despite the former being in lipid rafts and the latter in the disordered membrane, suggests that BCR- and TCR-mediated signals occurred both inside and outside rafts. Whether or not the BCR and TCR are entirely localized to lipid rafts is a matter of debate which reflects the fact that the results obtained from experiments using different techniques to study rafts, each with their own caveats, can hardly be compared (Simons and Toomre, 2000). Certainly our own difficulties in trying to apply the methodology that is useful to study an association of Ras proteins with rafts to the BCR and Lyn reflects the limitations of this particular technique and will not allow conclusions about the relative *quantities* of Lyn and Igα in raft membranes in our cells. While the low density fractions obtained from our gradients clearly contain cholesterol-rich membranes, it remains uncertain what the high density fractions are composed of. This means that we cannot draw conclusions about the quantities of Ras proteins in raft membranes either, although our results in combination with other data obtained by electron microscopy or fluorescence recovery after photobleaching (FRAP) (Prior et al., 2001; Niv et al., 2002) do suggest that palmitoylated Ras proteins are predominantly in rafts and polybasic ones are predominantly excluded in lymphocytes. It has been suggested that only a fraction of the BCR moves into rafts after receptor ligation (Cheng et al., 1999; Petrie et al., 2000). This conclusion comes from experiments where detergent-resistant membranes (DRMs) were isolated. However, we found that at the concentration of Triton X-100 that was used in these studies, the Igα chain of the BCR is almost completely solubilized. This again makes it difficult to quantitate the proportion of membrane-associated BCR partitioned into DRMs. All of the BCR moves into patches and "caps" upon ligation of the receptor with antibodies (Graziadei et al., 1990), and such structures have been described as a type of raft (Harder et al., 1998). Thus, it seems more likely that the

BCR resides in small rafts or "lipid shells" (Anderson and Jacobson, 2002) in resting cells that coalesce into large rafts following ligation of the receptors. These larger rafts may be more readily isolated by detergent and biochemical fractionation. The same is likely the case for the TCR, as all of the TCR caps after engagement of the receptor (Viola et al., 1999), whereas only a portion was found in DRMs (Drevot et al., 2002).

Other evidence indicates that signaling events downstream of immunoreceptors are not restricted to rafts. Kovarova et al. demonstrated that phosphorylation of the raft-associated FcεRI by Lyn does not depend on the localization of Lyn to lipid rafts (Kovarova et al., 2001). The strong activation of K-Ras 4B that we observed, despite its absence from raft fractions, is consistent with the notion that important signaling events can occur outside rafts. This could result from movement of signaling complexes out of rafts where the activated receptors are located, as phosphorylation events occur in areas of the immunological synapse where the TCR is not found (Lee et al., 2002). Likewise, PI-3 kinase is active outside the T cell-APC contact site where TCR signaling is initiated (Costello et al., 2002; Harriague and Bismuth, 2002). Alternatively, our results could indicate that the BCR and TCR can also be activated outside rafts, as has been shown for stimulation of antigen-specific Th2 cells with high-affinity peptides (Balamuth et al., 2001). Certainly our data showing activation of both K-Ras 4B and H-Ras suggest that the GEFs activated downstream of the BCR and TCR can be recruited to both raft and non-raft areas of the membrane.

The pattern of activation of different Ras isoforms is not always independent of their localization to membrane domains. Thus, growth factors acting on receptor-tyrosine kinases or receptors of the hemopoietin family preferentially activate Ras proteins that have polybasic carboxy-termini and localize outside rafts (see chapter IV). It will be important to investigate multiple receptor systems and determine the basis for these differences. While the discovery of rafts has changed

our view of cell signaling, it is evident that the notion of a membrane divided into "raft" and "non-raft" domains is too simplistic and that different biochemical and microscopic techniques are indicating far more heterogeneity in subdomains of the membrane (Schade and Levine, 2002; Petrie and Deans, 2002, Wilson et al., 2002).

The differential activation of Ras isoforms following BCR or TCR ligation correlated with their differential sensitivity to signaling paths downstream of PLC-γ and DAG in B cells, implicating RasGRP proteins as the critical GEFs activated by the BCR. Although only the analysis of B cells deficient for isoforms of RasGRP will definitively establish their role in BCR-induced activation of Ras proteins, the fact that reduced levels of RasGRP expression correlate with reduced activation of p21 Ras is strong evidence for a critical involvement of these GEFs. RasGRPs are the only GEFs known to be regulated by DAG. Moreover, RasGRP1 has been clearly implicated in the activation of p21 Ras induced by ligation of the TCR (Ebinu et al., 2000). Our data imply that RasGRPs do not efficiently activate M-Ras. While over-expression of RasGRP1 can lead to activation of M-Ras (Kimmelman et al., 2002; Ohba et al., 2000b) (and our own observations), *in vitro* experiments showed that the activity of RasGRP1 on M-Ras is relatively weak and that of RasGRP2 and RasGRP3 is undetectable (Ohba et al., 2000b). Consistent with this, stimulation of PC12 neuronal cells with another phorbol ester, PMA, only resulted in activation of M-Ras in cells in which RasGRP1 was over-expressed, despite the fact that these cells appear to express endogenous RasGRP1 (Kimmelman et al., 2002). We conclude that RasGRPs are much weaker activators of M-Ras than of H-Ras or K-Ras 4B. That we were unable to establish the activation of M-Ras by the introduction of mutations of by deletions that made this protein more similar to H-Ras, could indicate that a combination of several of such changes may be required to achieve its activation following ligation of the BCR.

Our results also preclude a major role for the exchange factors mSos1 and -2 in BCR-induced activation of H-Ras and K-Ras 4B as their activation depended on the presence of PLC-γ2 and mSos is not known to function downstream of this enzyme. The fact that M-Ras is readily activated both *in vitro* and *in vivo* by mSos1 and RasGRF (Quilliam et al., 1999; Ohba et al., 2000b) provides more evidence that the BCR and TCR may not primarily signal through these GEFs. Although mSos1/Grb2/Shc complexes are recruited to the activated BCR and complexes of mSos1 with p36LAT and Grb2 are rapidly formed after TCR ligation (Nunes et al., 1994; Saxton et al., 1994), the functional significance of these complexes is unclear. In favour of a role for mSos in Ras activation by the BCR is evidence that the activation of Erk was reduced by over-expression of dominant-negative Shc or dominant-negative Grb2 (Jacob et al., 2002). Moreover, p21 Ras was activated normally after ligation of the BCR in DT40 cells lacking expression of BLNK, despite the fact that PLC-γ was not activated (Ishiai et al., 1999). Thymocytes of mice with haplo-insufficiency for Grb2 show reduced, but not absent activation of p21 Ras after TCR ligation (Gong et al., 2001). This discrepancy remains to be resolved. The strength of signals may be one important factor. Thus, RasGRP1 is critical for Erk activation by weak but not strong TCR signals (Priatel et al., 2002). It is conceivable that a similar mechanism could be in place for the BCR, although the stimuli that was used in this study - ligation by polyclonal antibodies - should probably be seen as a "strong" signal.

Chronic exposure to PMA has only been reported to lead to the down-regulation of isoforms of PKC. The observation that RasGRP can also be down-regulated by this treatment means that other phorbol ester receptors, such as chimaerins, the Munc-13 family of proteins, DAG kinases, and PKD1/PKCμ (Brose and Rosenmund, 2002), may also be down-regulated. Consequently, careful re-evaluation of data obtained by prolonged exposure of cells to phorbol esters is required to establish a role for any one of these proteins. For example, activation-induced cell death (AICD) by exposure of T cells to anti-CD3 was prevented by pre-treatment

with PMA (Jin et al., 1992; Yahata et al., 1999) and it was concluded that PMA-sensitive PKCs were involved in this process. It is now clear that both PKCs and RasGRPs could have been involved. The expression of Munc-13 and chimaerin proteins is restricted to the neuronal system and testis, with the notable exception of β2 chimaerin which may be expressed by hematopoietic cells and may have been down-regulated together with RasGRP and PKCs in WEHI231 cells. However, it is unclear how reduced expression of a RacGAP could inhibit the activation of Ras proteins by ligation of the BCR. DAG kinases convert DAG to phosphatidic acid. Depletion of DAG kinases would therefore increase the pool of DAG, which in turn would be predicted to aid in Ras activation rather than lead to an inhibition. PKD1/PKCμ is thought to be involved in cell proliferation, apoptosis, transport vesicle formation and transport of proteins from the Golgi to the plasma membrane (Brose and Rosenmund, 2002). How reduced expression levels of this enzyme could affect the activation of Ras proteins by the BCR is also not clear. Thus, while we cannot rule out a possible involvement of other C1 domain-containing proteins, including as yet unidentified ones, the most likely explanation for our observation regarding the reduced activation of Ras by the BCR after chronic exposure to PMA would be that this was a result of a depletion of GEFs of the RasGRP family.

In summary, we show that H-Ras, K-Ras 4B, and M-Ras were activated differentially by ligation of the BCR and TCR, and that the preferential activation of H-Ras and K-Ras 4B was independent of their localization to different areas of the plasma membrane. That M-Ras was not activated was shown to correlate with its deficiency in susceptibility to activation by PLC-γ and DAG-dependent pathways used by the BCR to induce activation of Ras proteins. Finally, we present evidence that, similar to the TCR system, the DAG-responsive RasGRP family of GEFs is most likely involved in BCR-mediated activation of Ras proteins.

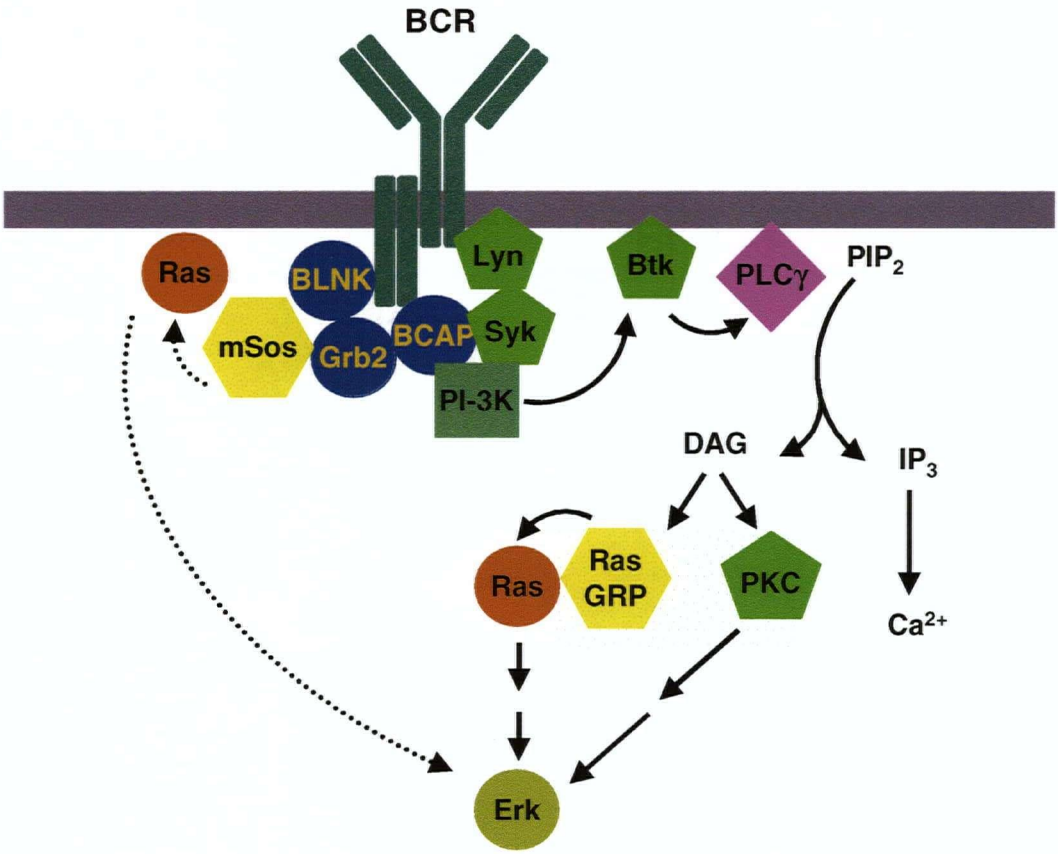

Figure 3.13 ***Model for BCR-mediated activation of Ras proteins.*** Ligation of the BCR induces the activation of p21 Ras proteins primarily via a PLC-γ and DAG-dependent mechanism that most likely involves exchange factors of the RasGRP family. PLC-γ may be activated by a PI-3K-dependent pathway and/or may be recruited to BLNK. A second, but minor pathway to BCR-induced activation of p21 Ras may exist (dashed arrows) that involves the recruitment of Grb2/mSos complexes to the activated receptor. Note that although depicted differently, DAG, Ras, RasGRP, and PKC are also at the plasma membrane (purple bar). BCR-induced activation of Erk lies downstream of both Ras and isoforms of PKC (Hashimoto et al., 1998; Martin et al., 2002). TCR-mediated activation of Ras proteins proceeds in a similar fashion (Dower et al., 2000; Priatel et al., 2002).

CHAPTER IV

Analysis of Ras activation by hematopoietic growth factors

4.1 Introduction

The differentiation of hematopoietic stem cells into cells of the various hematopoietic lineages is regulated by the action of growth factors and cytokines. For example, interleukin-3 (IL-3), IL-5, and GM-CSF, regulate the proliferation, differentiation and survival of many hematopoietic cell lineages such as macrophages, mast cells, megakaryocytes, eosinophils, granulocytes and early hematopoietic progenitors (Schrader et al., 1988). IL-3 acts through binding and activation of the IL-3 receptor, which consists of two subunits, α and β. IL-3Rα binds IL-3 with low affinity. The β chain is also a component of the receptors for IL-5 and GM-CSF and thus is referred to as β common (βc). By interaction of βc with any of the three IL-3R-, IL-5R-, or GM-CSFR-α chains, three specific, high-affinity receptors are formed (Kitamura et al., 1991). Mice express an additional β chain, β_{IL-3} (Itoh et al., 1990), which only associates with IL-3Rα, and therefore mice express two types of high affinity IL-3 receptors. Stimulation of the IL-3R results in activation of multiple cellular signaling pathways, including those activated by JAK/STAT molecules, PI-3 kinase, and MAP kinases (de Groot et al., 1998). IL-3 and other hematopoietic growth factors were reported to activate p21 Ras on the basis of assays using the anti-p21 Ras antibody Y13-259 (Duronio et al., 1992; Satoh et al., 1991). However, this antibody also precipitates other Ras family members including M-Ras (Ehrhardt et al., 1999; Feig, 1999), so it is not clear whether these stimuli measured activation of p21 Ras or other Y13-259-reactive isoforms. There is evidence that expression of dominant negative p21 Ras blocks IL-3 dependent proliferation and Erk activation (Okuda et al., 1994), however, since GEFs are

shared, the conclusion that p21 Ras was involved is not valid. Activation of Ras proteins is thought to be achieved through direct binding of Shc and/or SHP-2, both of which recruit Grb2 and mSos to the activated, phosphorylated βc (Pratt et al., 1996; Bone et al., 1997). Since expression of a dominant negative mSos1 ablates IL-3 induced activation of p21 Ras and Erk2 in Ba/F3 cells, Grb2-dependent GEFs, hence most likely mSos1/2, appear to be the critical RasGEFs activated by IL-3 (Tago et al., 1998).

The development of macrophages from pluripotent bone marrow stem cells is regulated by macrophage-colony stimulating factor (M-CSF or CSF-1), which promotes the survival, differentiation, and proliferation of these cells. The importance of CSF-1 for the development and functions of mononuclear phagocytes is evident in mice carrying the *osteopetrotic* (*op/op*) mutation. These mice lack functional CSF-1 and are severely deficient in macrophages and osteoclasts (Wiktor-Jedrzejczak et al., 1990). The CSF-1 protein exists as a homodimer and exerts its biological effects through binding to a cell surface receptor encoded by the proto-oncogene *c-fms*. Upon ligand binding, the receptor homodimerizes and autophosphorylates, thereby creating binding sites for various docking proteins such as Grb2 and PI-3 kinase. CSF-1 was reported to activate Ras (Gibbs et al., 1990), and complexes of Shc, Grb2 and mSos are recruited to the activated CSF-1R (Lioubin et al., 1994). The activation of Ras family members in monocytes was shown to be necessary for terminal differentiation into macrophages and contributes to their survival (Jin et al., 1995). Grb2, and thus presumably mSos, are important mediators of CSF-1-induced activation of Ras, since a mutant CSF-1R lacking the Grb2 binding site is unable to efficiently activate Ras (Lee and States, 2000). However, other pathways activated by the CSF-1R may also contribute to the activation of Ras proteins, including pathways dependent on the activity of Src family kinases or, perhaps, PLC-γ (Bourette et al., 1997; Ebinu et al., 1998; Lee and States, 2000).

Although it has been well established that Ras proteins are activated by most, if not all cell surface receptors, including receptors for growth factors, it has largely remained unknown whether isoforms of p21 Ras (H-Ras, N-Ras, K-Ras 4A, and K-Ras 4B) or other members of the Ras family are activated, and if these proteins may be activated to the same extent. This uncertainty stems from the fact that the tools that had typically been used to study p21 Ras - antibodies and dominant negative mutants of p21 Ras proteins - cross-react with all these proteins (see 1.4.2). Moreover, these tools also do not distinguish between p21 Ras proteins and other members of the Ras family, such as M-Ras or TC21 (Ehrhardt et al., 1999; Feig, 1999).

Since growth factor receptors appear to mainly utilize a Grb2/mSos-dependent mechanism to activate Ras, and since p21 Ras proteins and M-Ras appear to be activated by common GEFs, including mSos (Ohba et al., 2000b), it is possible that p21 Ras proteins and M-Ras would be activated in parallel by these receptors. However, other factors may also contribute to the activation of Ras proteins. For example, it has been reported that H-Ras and K-Ras 4B localize to lipid rafts and the disordered plasma membrane, respectively (Prior et al., 2001; Roy et al., 1999). This differential localization is a result of the post-translational modifications of their different carboxy-termini. That of H-Ras exhibits sites for palmitoylation and is transported through the Golgi apparatus to the plasma membrane where it localizes to lipid rafts. In contrast, K-Ras 4B lacks palmitoylation sites and instead exhibits a stretch of multiple basic residues at its carboxy-terminus. It is excluded from the Golgi and takes a different, largely undefined route to the plasma membrane and localizes outside rafts (Apolloni et al., 2000). The differential localization to membrane domains was shown to affect their downstream signaling (Jaumot et al., 2001; Roy et al., 1999). Thus, it is conceivable that the localization to membrane domains also affects their susceptibility to activation by cell surface receptors and GEFs that may be

recruited to them. Whether or not the receptors for IL-3 or CSF-1 localize to lipid rafts has not yet been explored and was addressed in the present study.

We chose two p21 Ras proteins, H-Ras and K-Ras 4B, as well as M-Ras, all of which may be activated by mSos, to study their activation by IL-3 and CSF-1 in the context of raft localization of the Ras proteins. We observed that H-Ras, which was present in lipid rafts, was not as efficiently activated as were K-Ras 4B and M-Ras, which localized outside rafts. The preferential activation of K-Ras 4B and M-Ras was dependent on their association with non-raft membranes. The activated IL-3 and CSF-1 receptors, as well as Shc, Grb2, and mSos, were also excluded from rafts. Thus, these proteins may have co-localized with K-Ras 4B and M-Ras in the disordered membrane which could have aided in their preferential activation. This is the first report of a differential activation of two p21 Ras proteins, H-Ras and K-Ras 4B, and demonstrates that localization outside of lipid rafts is important for activation of Ras proteins by the hematopoietic growth factors IL-3 and CSF-1.

4.2 Results

4.2.1 Localization of Ras proteins to lipid rafts depends on the presence of a palmitoylated carboxy-terminus

In fibroblasts, Prior et al. demonstrated that H-Ras localizes to lipid rafts whereas K-Ras 4B is excluded from these membrane domains (Prior et al., 2001). We investigated whether this was also the case in Ba/F3-Fms cells, which lack caveolae. We saw that in these cells, H-Ras accumulated in low density fractions of sucrose gradients. In contrast, K-Ras 4B was exclusively found in the high density fractions in Ba/F3-Fms cells (Fig. 4.1). We observed that M-Ras was restricted to the same fractions with high sucrose concentrations (Fig. 4.1). Thus, M-Ras resembled K-Ras 4B both in its exclusion from the Golgi and its exclusion from rafts.

Replacement of the polybasic carboxy-termini of M-Ras or K-Ras 4B with the palmitoylated carboxy-terminus of H-Ras resulted in localization of these MH or KH chimeras to the low density raft fractions. Conversely, chimeras of H-Ras with polybasic carboxy-termini of M-Ras or K-Ras 4B (HM, HK) were excluded from low density fractions (Fig. 4.1). These results indicate that in Ba/F3-Fms cells, the nature of the carboxy-termini of H-Ras, K-Ras 4B, and M-Ras direct their localization to either lipid rafts or disordered membrane, as is the case for A20 B cells (Fig. 3.3) and fibroblasts (Prior et al., 2001).

Under the same conditions used to assess the association of the different Ras proteins to lipid rafts we also found the majority of the Lyn, which is known to localize rafts (Cheng et al., 1999), in the same low density fractions that were enriched for palmitoylated Ras proteins. Thus, in Ba/F3-Fms cells it seems possible to study an association with rafts of proteins other than Ras

under identical conditions, which is different from A20 cells and splenocytes (see chapter III, Figs. 3.3 and 3.4).

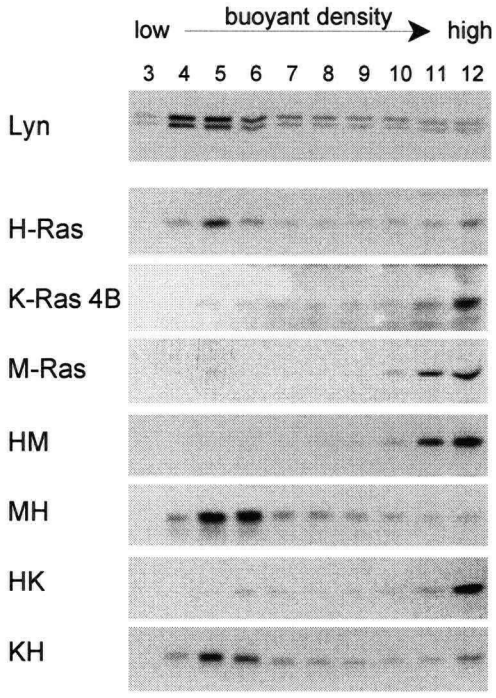

Figure 4.1 ***Location of wild-type and chimeric Ras proteins expressed in Ba/F3-Fms cells in sucrose gradients.*** Lysates from Ba/F3-Fms cells expressing the indicated myc-tagged Ras constructs were fractionated according to buoyant densities over sucrose gradients. Twelve fractions were collected from the top of the gradient (fractions 1 and 2 were discarded). After dilution, membrane pellets were obtained by further ultracentrifugation. The pellets were resuspended in SDS loading buffer and subjected to SDS-PAGE and blotting with anti-myc antibodies to visualize Ras proteins. The top panel indicates the location of Lyn, a marker for rafts, in these gradients.

4.2.2 The activated receptors for IL-3 and CSF-1 localize to non-raft membranes

We next asked whether or not the receptors for IL-3 and CSF-1 were present in lipid rafts. In unstimulated Ba/F3-Fms cells, the βc chain of the IL-3 receptor, which is the signal transducing part of the α/β heterodimeric receptor, was detected in the high density, bottom fractions of the sucrose gradients. After stimulation with IL-3, the location of βc was unchanged, being still present in high density fractions. In contrast, in unstimulated cells, the CSF-1 receptor was

found across the gradient in most fractions. However, upon stimulation with CSF-1, the CSF-1 receptor accumulated in high density fractions (Fig. 4.2). These data indicate that the IL-3 receptor βc chain localized outside lipid rafts independent of activation, whereas the CSF-1 receptor was present in rafts and other membrane areas in unstimulated cells, but moved out of rafts upon its activation.

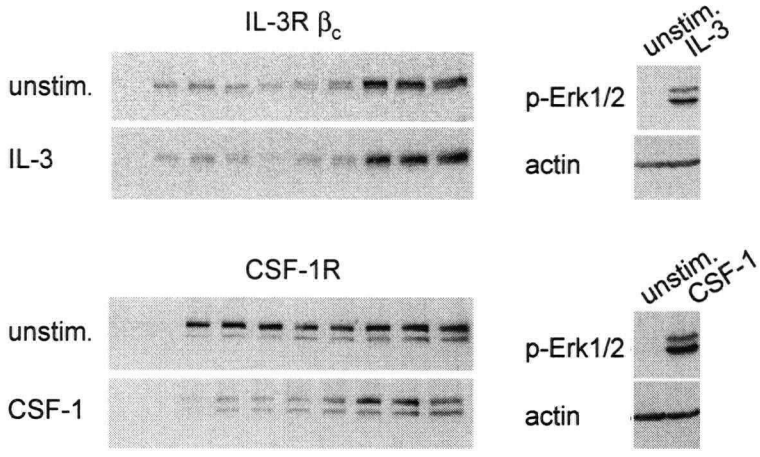

Figure 4.2 ***Location of the IL-3 and CSF-1 receptors in sucrose gradients with and without stimulation***. Lysates from unstimulated Ba/F3-Fms cells or from cells that had been stimulated with IL-3 for 5 min. or with CSF-1 for 2 min. were fractionated over sucrose gradients. The location of the receptors in fractions from the gradients was determined by blotting with anti-βc or anti-HA antibodies (left panels). Aliquots from the same cell lysates that were used for sucrose gradients were also analyzed for phosphorylated Erk1/2 to assess the efficiency of growth factor stimulation (right panels).

4.2.3 IL-3 and CSF-1 preferentially activate K-Ras 4B and M-Ras

To compare the activation of H-Ras, K-Ras 4B and M-Ras by IL-3 and CSF-1, these Ras proteins were expressed in Ba/F3-Fms cells. Stimulation with either IL-3 or CSF-1 resulted in efficient activation of both K-Ras 4B and M-Ras to approximately 10% of the total cellular K-Ras 4B or M-Ras as estimated by eye. In contrast, H-Ras was much less efficiently activated by these growth factors (about 2%) (Fig. 4.3).

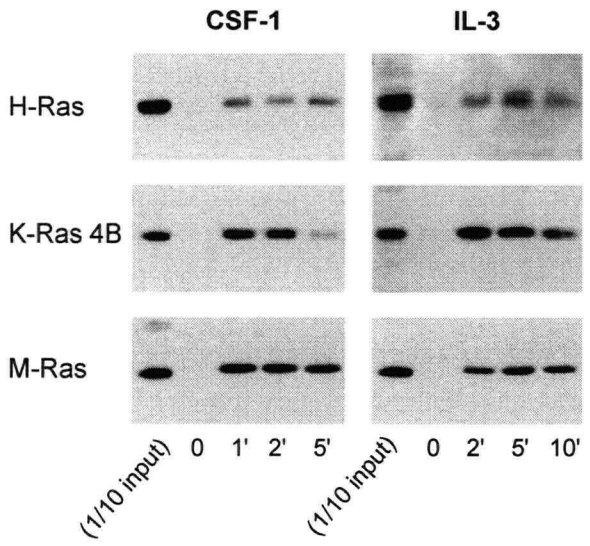

Figure 4.3 ***Ras proteins with polybasic carboxy-termini are preferentially activated by IL-3 and CSF-1.*** Ba/F3-Fms cells expressing the indicated Ras constructs were stimulated with either CSF-1 or IL-3 for the indicated times. Lysates were subjected to pull-down assays to precipitate activated, GTP-bound Ras proteins. Raf-1 RBD was used to precipitate activated H-Ras and K-Ras 4B, and Nore1 RBD was used precipitate activated M-Ras. One tenth of the amount of lysate used for a pull-down was run in parallel with the pull-down samples ("1/10 input"). All cell lysate samples were also analyzed for phosphorylated Erk1/2 and equal loading was confirmed (not shown).

To test whether this difference in degree of activation was due to differential localization in the plasma membrane, we investigated the activation of the chimeric Ras proteins. We observed that chimeras of M-Ras or K-Ras 4B with the carboxy-terminus of H-Ras (MH, KH) were not as efficiently activated as well as their wild-type counterparts (Fig. 4.4). Conversely, chimeras of H-Ras with a poly-basic carboxy-terminus of either K-Ras 4B or M-Ras (HK and HM) were activated much more efficiently than wild-type H-Ras (Fig. 4.4). These results indicate that Ras proteins that localize outside lipid rafts are preferentially activated by the hematopoietic growth factors IL-3 and CSF-1, the activated receptors of which are also excluded from rafts.

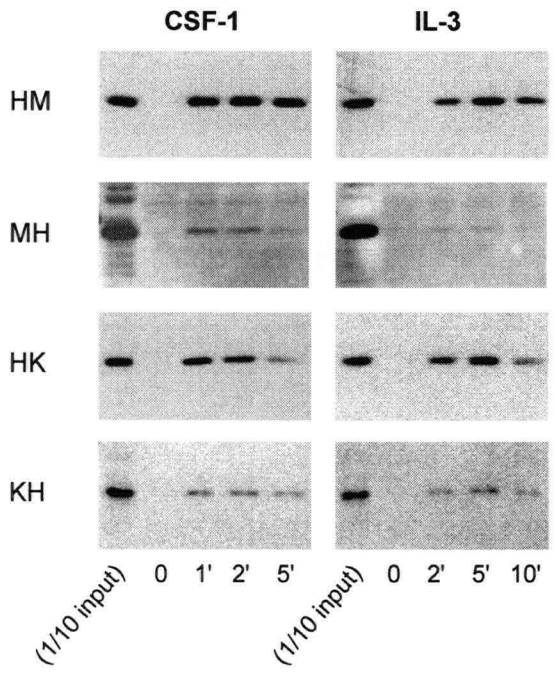

Figure 4.4 ***Exchanging polybasic for palmitoylated carboxy-termini on Ras proteins results in less efficient activation by IL-3 and CSF-1.*** Activation of chimeric Ras proteins expressed by Ba/F3-Fms cells was assessed as described in figure 4.3. HM, HK: H-Ras with the carboxy-terminus of M-Ras or K-Ras 4B, respectively. KH, MH: K-Ras 4B or M-Ras with the carboxy-terminus of H-Ras. All cell lysate samples were also analyzed for phosphorylated Erk1/2 and equal loading was confirmed (not shown).

4.2.4 Shc, Grb2, and mSos localize outside lipid rafts

There is evidence for a critical role of mSos in activation of p21 Ras induced by IL-3, and for a partial role at least in that induced by CSF-1 (Tago et al., 1998; Lee and States, 2000). Therefore, we tested whether mSos co-localized with the activated receptors and those Ras proteins that are preferentially activated in membrane fractions of sucrose gradients. We observed that mSos localized exclusively to high density non-raft fractions (Fig. 4.5). Its localization did not change upon stimulation with either IL-3 or CSF-1. Moreover, the adapter proteins Shc and Grb2 that are known to link mSos to the activated IL-3 and CSF-1 receptors were also present in the same high density fractions, regardless of stimulation of the receptors

(Fig. 4.5). Thus, all components of a signaling complex comprised of activated receptors, Shc, Grb2, mSos, and those Ras isoforms which were preferentially activated by these receptors, K-Ras 4B and M-Ras, were excluded from lipid rafts. While these results do not firmly establish co-localization of these proteins in certain non-raft areas of the plasma membrane, it is conceivable that co-localization may have aided in activation of K-Ras 4B and M-Ras by the IL-3 and CSF-1 receptors.

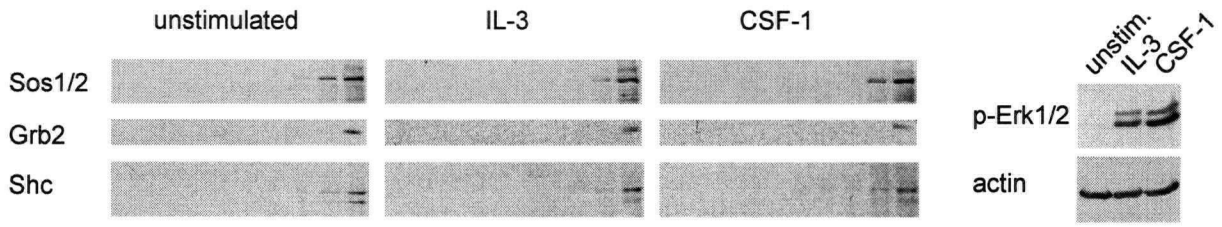

Figure 4.5 ***mSos1/2, Grb2, and Shc, occur in high density fractions in sucrose gradients.*** Lysates from Ba/F3-Fms cells that had been either left untreated or stimulated with IL-3 for 5 min. or CSF-1 for 2 min. were fractionated over sucrose gradients. The location of mSos1/2, Grb2, and Shc in membrane fractions obtained from the gradients was determined by Western blotting using the corresponding antibodies (left panels). Aliquots from the same cell lysates that were used for sucrose gradients were also analyzed for phosphorylated Erk1/2 to assess the efficiency of growth factor stimulation (right panels).

It is possible that other exchange factors besides mSos are involved in CSF-1 mediated activation of Ras proteins. GEFs such as RasGRF and RasGRP proteins exist that can be activated by the products of PLC-γ activity, calcium and DAG. Since there is conflicting data on whether calcium fluxes are evoked by stimulation with CSF-1 (Bourette et al., 1997; Qiu et al., 1998), we investigated whether calcium fluxes are elicited by stimulation of Ba/F3-Fms cells with CSF-1. As shown in Fig. 4.6, stimulation with CSF-1 resulted in a small, transient rise in intracellular calcium, whereas stimulation with IL-3 did not. This indicated that PLC-γ was activated in response to stimulation with CSF-1 in our system and suggests that the CSF-1

receptor could utilize additional pathways to activate Ras proteins in addition to direct binding of Shc/Grb2/mSos complexes to the activated receptor.

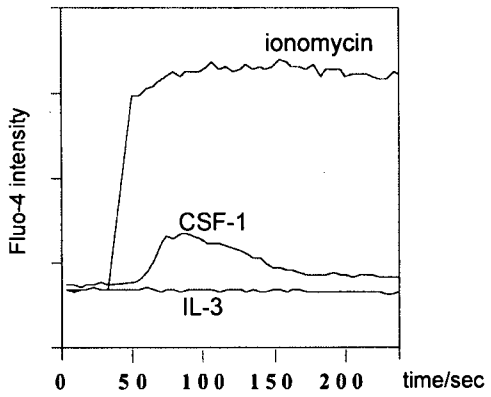

Figure 4.6 ***CSF-1, but not IL-3, induces a calcium flux in Ba/F3-Fms cells.*** Ba/F3-Fms cells were washed and starved for 30 min. before loading with the calcium-activated fluorescent dye, Fluo-4 (Molecular Probes). Calcium fluxes induced by stimulation with 15 μg/mL IL-3, or 100 ng/mL CSF-1, or 100 nM ionomycin were assessed by flow cytometry. The baseline was obtained for 30 seconds before addition of the stimuli.

4.3 Discussion of Chapter IV

The p21 Ras proteins H-, K-, and N-Ras, have been at the centre of intense investigations for almost 20 years. However, there is relatively little known about differences in function of the different p21 Ras isoforms. Even less is known about the functions of non-classical Ras proteins, such as M-Ras, which cross-react with experimental tools that are commonly used to study p21 Ras. There is evidence for differences in downstream signaling, and for differential activation by GFEs. However, it has remained unknown whether physiological stimuli, such as stimulation with cytokines and growth factors, activate Ras proteins in parallel or differentially. The differential association of palmitoylated H-Ras or polybasic K-Ras 4B with lipid rafts or the disordered membrane, respectively, may also govern activation of Ras proteins, as different receptors may localize to specific membrane subdomains. We addressed these questions in the present study for Ras activation by the hematopoietic growth factors IL-3 and CSF-1.

Three major conclusions can be drawn from our data. Our studies provide the first direct evidence that hematopoietic growth factors activate members of the p21 Ras proteins. This was not clear from previous experiments where the cross-reactive monoclonal antibody Y13-259 was used to precipitate Ras proteins (Satoh et al., 1991; Duronio et al., 1992). They also demonstrate that M-Ras can be activated by IL-3 and CSF-1, and that M-Ras is more efficiently activated than H-Ras. Given that M-Ras was also efficiently activated by nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) (Kimmelman et al., 2002), it seems possible that growth factors in general could preferentially use M-Ras over some p21 Ras isoforms to transduce signals. The significance of this remains to be established.

Second, we show that two p21 Ras proteins can be activated differentially by hematopoietic growth factors. To our knowledge, p21 Ras proteins have not been directly compared in their

ability to become activated by growth factors, although it has been reported that both H-Ras and K-Ras 4B can be activated by EGF (Niv et al., 2002). However, the extent of activation was not compared in this study. That two p21 Ras isoforms are activated differentially is not always the case, as there is evidence for parallel activation of H-Ras and K-Ras 4B by the B and T cell antigen receptors (chapter III). The preferential activation of K-Ras 4B and M-Ras by IL-3 and CSF-1 raises the question of the significance of this activation. Both K-Ras 4B and M-Ras are expressed in hematopoietic cells, including myeloid progenitor cells and macrophages (Ehrhardt et al., 1999; Reuter et al., 2000). A more detailed analysis of mast cells and macrophages, two cell types that IL-3 and CSF-1 can act on, derived from mice lacking functional K-Ras 4B and M-Ras would be required. Mice deficient for K-Ras 4B only (and not for both K-Ras 4A and 4B) have not been established, and hematopoietic cells from mice lacking M-Ras have not yet been analyzed in detail.

Third, we also present evidence that the hematopoietic growth factors IL-3 and CSF-1 preferentially activate Ras proteins that are not associated with rafts. This correlated with the presence of activated receptors and mSos, a GEF that is utilized by these receptors to activate Ras protein, in non-raft membranes. The presence in non-raft membranes isolated by gradient centrifugation does not necessarily mean that these proteins actually co-localize. Confocal or electron microscopy would be required to provide direct evidence for co-localization. However, there seems to be a very close association between the activated receptors and Ras in that the CSF-1R may directly bind to Grb2/mSos, and the IL-3R may directly bind to Shc and SHP-2 and recruit Grb2/mSos this way. Thus, it seems plausible that all of these proteins could have co-localized in the plasma membrane.

The finding that the CSF-1R moved out of lipid rafts upon stimulation with CSF-1 was unexpected, since the closely related PDGF receptor has been shown to localize to caveolae,

which represent a subset of rafts (Liu et al., 1996). There are very few reports on whether cytokine receptors are associated with raft- or non-raft membranes. In one study, the IFNγRα chain and gp130, a cytokine receptor chain shared by IL-6R, IL-11R, LIFR, CNTFR, OSMR, and CT-1R, were found in raft fractions (Sehgal et al., 2002). Two other groups have investigated the possible association of the IL-2R with rafts. While both agree that the IL-2Rα chain is constitutively present in rafts, they differ on whether the β and γ chains of this receptor is in rafts or outside and whether disruption of rafts affects downstream phosphorylation events (Marmor and Julius, 2001; Matko et al., 2002; Vereb et al., 2000). The differences could stem from the different cells that had been used, or from the different techniques that had been chosen to study rafts. Clearly, the choice of technique critically influences the type of raft that is studied (Simons and Toomre, 2000), and since there appear to be various different classes of rafts (Schade and Levine, 2002; Wilson et al., 2002, Petrie and Deans, 2002), it is possible that the above observations were all valid; however, more detailed knowledge about the type of membrane domain that was actually studied would be required to draw conclusions from these data. Many characteristics of native rafts, including lipid and protein composition, remain unresolved (Horejsi, 2002).

Our data show a correlation between the localization of the Ras isoforms that are preferentially activated by growth factors, that of growth factor receptors, and that of a signaling module comprised of Shc, Grb2 and the exchange factor mSos. However, although Grb2/mSos are clearly important (Lee and States, 2000), we cannot rule out a possible involvement of other GEFs besides mSos in the CSF-1-mediated activation of Ras proteins. Since CSF-1 appears to stimulate the activity of PLC-γ at least in some cells of the monocyte/macrophage lineage (Bourette et al., 1997; Qiu et al., 1998), it is conceivable that GEFs that are activated by the products of PLC-γ activity, calcium and DAG, could also contribute to the efficient activation of

Ras proteins. These GEFs include the calcium-activated RasGRF1/2 and the DAG-activated GEFs of the RasGRP family. PLC-γ appears to have a critical role in the differentiation of promyeloid cells into macrophages in response to CSF-1 (Bourette et al., 1997), a function that correlates with the function of Ras in these cells (Jin et al., 1995). However, it is unlikely that RasGRP proteins are involved in CSF-1-mediated activation of Ras isoforms. These appear to have low GEF activity towards M-Ras (see chapter III), which was efficiently activated by CSF-1. Moreover, it was shown that prolonged exposure to PMA, which as we have shown in chapter III will lead to down-regulation of RasGRP, had no influence on CSF-1-induced phosphorylation of Elk-1 in another study (Hipskind et al., 1994), a pathway which depends on p21 Ras. A possible role for RasGRF1/2 remains to be established.

In summary, we show that Ras isoforms can be activated differentially by the hematopoietic growth factors IL-3 and CSF-1, and that the preferential activation of K-Ras 4B and M-Ras correlates with their localization to non-raft membranes, where the activating receptors and GEFs that are involved in Ras activation are also localized.

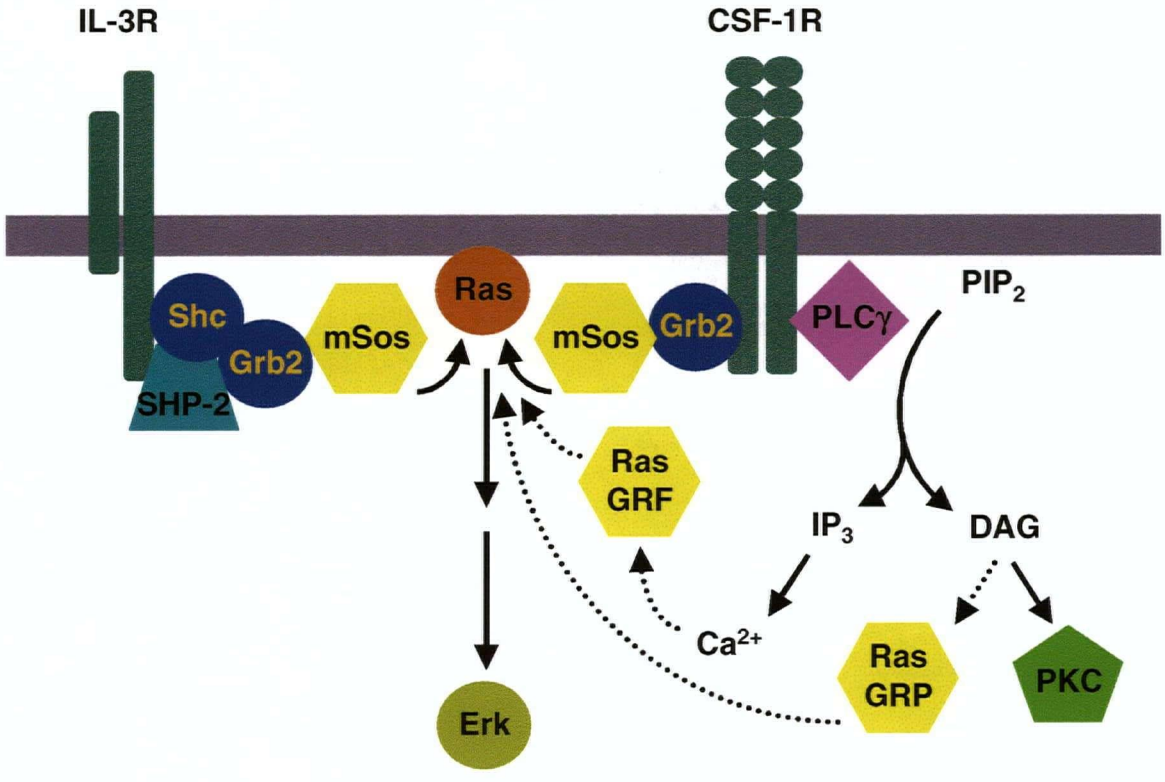

Figure 4.7 ***A model for IL-3 and CSF-1 receptor-mediated induction of Ras activation.*** Both receptors appear to mainly use a Grb2-dependent pathway to activate Ras proteins. In some cells of the monocyte/macrophage lineage, the CSF-1R may also use paths downstream of PLC-γ, calcium, and DAG (dashed arrows). DAG, RasGRP, and PKC are also at the plasma membrane (purple bar).

CHAPTER V Conclusions and recommendations for further work

Ras proteins have been subjects of intense investigation for the last two decades. It has become increasingly evident however that these structural similarities have clouded the interpretation of many experiments designed to investigate the function of individual members of the family, using monoclonal antibodies and dominant active or negative mutants. Nevertheless, much was learned about the Ras proteins as a family. Yet, individual members of p21 Ras, and other Ras family members, can be different in their susceptibility to activation by GEFs, in their downstream signaling, and in their functions. Thus, current work is primarily concerned with the elucidation of the functions of individual Ras isoforms.

The present study describes differences in the susceptibility to activation of three Ras proteins, H-Ras, K-Ras 4B, and M-Ras, to external cellular stimuli. In the first part we show that ligation of the antigen receptors of B and T cells results in the activation of a preferred set of Ras proteins, H-Ras and K-Ras 4B, which is dependent on DAG-responsive pathways. These results were unexpected because both receptors are known to localize to lipid rafts when activated, and these specialized membrane areas had been thought of as signal transduction platforms from which an activated receptor assembles its signaling machinery. The most obvious question that arises from these data is how an activated BCR or TCR can activate K-Ras 4B, which does not co-localize with these receptors in the same area of the membrane. Other recent studies presented data raising the same problem. For example, during formation of the immunological synapse, $PI(3,4,5)P_3$ accumulates not only at the contact site of the APC with the T cell, but also at the pole of the T cell opposite the contact site. It is not clear how signaling is maintained in an area that is so far away from the stimulus, but it was speculated that $PI(3,4,5)P_3$ may be able to diffuse out of the synapse (Costello et al., 2002; Harriague and Bismuth, 2002). Thus, it is possible that DAG could also diffuse away from the activating BCR or TCR to activate RasGRP.

It was also suggested that about 50% of $PI(4,5)P_2$ was localized to DRMs and 50% was excluded (Pike and Casey, 1996), which means that the same would be the case for DAG, which is generated from $PI(4,5)P_2$. In this case, RasGRP would be recruited to both raft and non-raft membranes. Moreover, the relationship of proteins with rafts can be dynamic, with evidence for the movement of activated H-Ras out of rafts or of the activated BCR into rafts (Cheng et al., 1999; Prior et al., 2001). One important caveat to conclusions based on purported differences in localization of proteins in rafts relates to the limitations and variations intrinsic to the variety of methods used to define and study rafts (Simons and Toomre, 2000). There is evidence that various different types of rafts exist (Schade and Levine, 2002; Wilson et al., 2002, Petrie and Deans, 2002). Moreover, if a protein is associated with the cytoskeleton, it will not float in a sucrose gradient (Simons and Toomre, 2000). This is an important consideration for our observations on M-Ras and K-Ras 4B, since their poly-basic tail may mediate an interaction with tubulin (Chen et al., 2000). Treatment of cells with disruptors of the cytoskeleton, cytochalasin or colchicine, and subsequent density gradient centrifugation could be performed to investigate the possibility that M-Ras and K-Ras 4B might actually localize to lipid rafts that contain cytoskeleton. However, it is still clear that H-Ras localizes to an area of the plasma membrane which is different from that which polybasic Ras proteins are located in. Clearly, there is an urgent need for a better understanding of the characteristics of the different classes of rafts, and for sensitive techniques to study native rafts.

In the second part of this work the activation of Ras isoforms that were activated by the hematopoietic growth factor receptors IL-3 and CSF-1 correlated with the localization of these isoforms with the activated receptors in non-raft areas of the membrane. It will be important to determine the significance of the preferential activation of M-Ras and K-Ras 4B using cells lacking expression of these proteins. The *K-Ras* gene has been knocked out in mice, but this means that these mice lack expression of both K-Ras 4B and K-Ras 4A, so that K-Ras 4A

would have to be re-introduced first. Mice lacking expression of M-Ras show no obvious phenotypes in the development of their hematopoietic system (Wang and Schrader, unpublished observations), although a more detailed analysis will be required to accurately identify its physiological roles.

It will also be important to develop specific and sensitive antibodies to isoforms of Ras. While some isoform-"specific" antibodies are commercially available, they lack sensitivity and specificity in that they recognize multiple proteins on Western blots. However, such tools would be invaluable for studies on endogenous protein.

It has become evident that cell surface receptors can primarily signal through one family of GEFs, despite the fact that there may be several different GEFs from different families expressed in the cell. Thus, mSos1/2 and RasGRPs may be the GEFs of choice for growth factor receptors and antigen receptors, respectively. Many more GEFs exist and their precise roles remains to be established. In particular, the various GEFs for Rap have not yet specifically been connected to a certain external stimulus. We and others have recently identified a pair of novel GEFs with no obvious regulatory elements. One of these GEFs may be regulated on a transcriptional level (Ferreira et al., 2002).The possibility of such regulation has so far been largely neglected.

Our data strongly implicate RasGRP proteins as the critical family of GEFs that are used by the BCR to mediate activation of Ras proteins. Thus the reduction in levels of expression of RasGRP correlated with a reduction in BCR-mediated p21 Ras activation. However, since RasGRP was down-regulated by prolonged treatment with PMA, and since PMA also down-regulated PKCs and potentially other proteins, it will be necessary to generate cells lacking expression of RasGRP isoforms to definitively establish their role in activation of Ras proteins

induced by signaling through the BCR. RasGRP1 has been deleted in mice (Dower et al., 2000), but no B cell defects have been reported yet. Other strategies such as siRNA could also be employed to achieve specific down-regulation of isoforms of RasGRP.

The observation that chronic exposure to PMA leads to a reduction of RasGRP expression levels is intriguing and raises the question whether other phorbol ester receptor proteins such as the chimaerins, which act as GAPs for the small GTPase Rac, or Munc-13 proteins, which have functions in synaptic vesicle fusion, can be down-regulated by treatment with PMA. If that is the case, careful re-evaluation of data obtained from experiments in which chronic exposure to PMA was used to down-regulate PKCs will be required. How exposure to PMA leads to the depletion of RasGRP remains to be established. The precise mechanism of how PKC is down-regulated by PMA is not completely understood. One model is based on the conformation-dependent sensitivity to proteases by exposure of a linker domain and subsequent proteolytic degradation. Alternatively, increased vesicle trafficking at the membrane induced by activation of PKC may lead to the association of PKC with these vesicles, resulting in its degradation (Parker et al., 1995). The DAG-binding C1-domain could be attached to another protein such as GFP to test the possibility that the susceptibility to degradation is intrinsic to this domain.

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