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Novel forms of Ras regulation and function in mammalian cells

by

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ABSTRACT

Ras is a low molecular weight guanyl nucleotide binding protein that alternates between GTP-bound-"on" and GDP-bound-"off" conformations. A number of extracellular stimuli alter the ratio of Ras-GTP to Ras-GDP in the cell through the action of guanyl nucleotide exchange factors (GEF's) and GTPase activating proteins (GAP's). Ras-GTP then elicits a number of downstream biochemical responses that include the activation of protein kinases, modulation of gene expression and reorganization of the actin cytoskeleton. In mammalian cells, Ras signaling regulates several biological processes such as proliferation and differentiation. Here, I investigated three novel forms of Ras regulation and function.

I discovered that Ras becomes activated in rat2 fibroblasts exposed to hypothermic stress. Upon rewarming of the cells, hypothermically-activated Ras stimulates downstream effector responses including the Raf-Mek-Erk protein kinase cascade. Studies were performed *in vitro* using purified Ras, Ras GEF's and Ras GAP's and in rat2 cells to gain insight into the mechanism of hypothermic Ras activation. Hypothermic activation of Ras-Erk signaling was observed after moderate and severe hypothermic stress and was conserved in many cultured cell types. Cultured cells show DNA fragmentation, a marker for apoptosis, and a loss of viabilty upon recovery from prolonged hypothermic stress. Pharmacological inhibition of Mek attenuated DNA fragmentation and increased cell viability after prolonged hypothermia. Previously, hypothermia has been shown to inhibit acute growth factor signaling to Erk. In this study, I also defined the point in the epidermal-growth factor signaling pathway that was inhibited by low temperature.

Our lab identified a novel Ras GEF, named RasGRP, from a rat brain cDNA library. RasGRP represents a new class of mammalian Ras GEF due to the presence of a diacylglycerol- (DAG) binding domain and a pair of calcium-binding EF-hand motifs. I showed here that RasGRP can activate Ras and signaling events downstream of Ras in response to exogenous and endogenous DAG signals in rat2 cells.

Ceramides are sphingolipid second messengers generated in response to a number of extracellular stimuli. Others have found that overexpression of dominant-negative N17 Ras in rat2 cells inhibited phosphatidylinositol 3-kinase activation in response to cell-permeable C2-ceramide. Here, I demonstrated that C2-ceramide can modestly activate Ras in rat2 cells.

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ABBREVIATIONS AND SYMBOLS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CaM kinase II	calmodulin-dependent protein kinase II
CAPK	ceramide-activated protein kinase
CAPP	ceramide-activated protein phosphatase
CDK	cyclin-dependent protein kinase
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CREB	cyclic AMP response element binding protein
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DOC	sodium deoxycholate
DTT	dithiothreitol
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl) ether
Et-1	Endothelin-1
FAK	Focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FPLC	fast performance liquid chromatography
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanyl nucleotide exchange factor
GPCR	G-protein coupled receptor
GRF	guanyl nucleotide releasing factor
GRP	guanyl nucleotide releasing protein
GSK3	glycogen synthase kinase 3
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HBS	Hepes-buffered saline
IgG	immunoglobulin G
IRS-1	insulin receptor substrate-1
JNK	c-jun N-terminal kinase
MBP	maltose binding protein

MDCK	Madin-Darby canine kidney cells
Mek	MAPK-Erk kinase
Mnk	MAPK interacting kinase
mRNA	messenger RNA
N17 Ras	Ras with a S17N substitution (dominant-negative allele)
NF1	product of the gene mutated in neurofibromatosis type-1
NGF	nerve growth factor
NP40	nonylphenoxy polyethoxy ethanol
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEI	polyethyleneimine
PH	Pleckstrin homology
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3 kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLaseA2	phospholipase A2
PMA	phorbol 12-myristate 13-acetate
PSD	post synaptic density
PTB	protein tyrosine binding (domain)
REC	rat embryo cell
RNA	ribonucleic acid
rsk	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SOS	son of sevenless protein
TAE	Tris-acetate-EDTA buffer
TNFα	Tumor necrosis factor-α

Single letter amino acid code

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Greek letters

α - alpha
β - beta
γ - gamma
δ - delta
κ - kappa
μ - mu (micro)
ζ - zeta

Chapter 1 - General introduction to Ras signaling

Ras is a low molecular weight guanyl-nucleotide binding protein that alternates between GDP-bound-"off" and GTP-bound-"on" conformations. A variety of extracellular stimuli alter the ratio of Ras-GDP to Ras-GTP in the cell through the action of Ras regulatory proteins. Ras-GTP then elicits a number of downstream biochemical responses that ultimately regulate cell biology. As such, Ras functions as a cellular signaling switch. This introduction will review the biochemistry, regulation and downstream signaling function of Ras in mammalian cells.

History of Ras as an oncogene

Although Ras is now understood to be important in the regulation of normal biological processes, initial excitement over Ras was due to its involvement in human cancer. Ras was first identified in the early 1960's as a potent dominant oncogene contained within several strains of retrovirus that caused tumor formation when injected into newborn mice (Harvey, 1964, Kirsten and Mayer, 1967). These strains, which spontaneously arose in rats or mice injected with murine leukemia virus, carried transduced versions of the rat or mouse cellular *Ras* gene, respectively, that were activated by point mutation (Ellis *et al.*, 1981, Capon *et al.*, 1983a). The involvement of Ras in human cancer became apparent when activated *Ras* genes were found in human tumor tissues (Parada *et al.*, 1982, Reddy *et al.*, 1982, Santos *et al.*, 1982, Tabin *et al.*, 1982, Capon *et al.*, 1983b, Shimizu *et al.*, 1983, Taparowsky *et al.*, 1983, Yuasa *et al.*, 1983). We now know that Ras mutations are present in 30% of all human tumors (Khosravi-Far and Der, 1994, Bos, 1989). The frequency of Ras mutation varies between tumor types and some human cancers show a very high incidence of Ras mutation (Table 1-1). For example, greater than 80% of pancreatic adenocarcinomas contain a mutated *Ras* gene.

The activated Ras proteins encoded by the retroviral oncogenes and the oncogenes derived from human tumors are mutated at similar residues. Activation of Ras in the viral oncogenes is due to mutation at residue glycine-12 (Taparowsky *et al.*, 1983). Oncogenic Ras proteins from human tumors are predominantly mutated at residues -12 and glutamine-61 (Reddy *et al.*, 1982, Taparowsky *et al.*, 1983, Tabin *et al.*, 1982, Bos, 1989). We now understand that mutation at position 12 or 61 gives rise to Ras proteins that fail to be inactivated by GTPase activating proteins (GAP's) (Gibbs *et al.*, 1984, Sweet *et al.*, 1984, Der *et al.*, 1986, Trahey and McCormick, 1987, Adari *et al.*, 1988, Krengel *et al.* 1990, Scheffzek *et al.*, 1997). Thus, oncogenic Ras proteins are constitutively active and are chronically stimulating downstream signaling pathways. Detailed molecular understanding of these mutations has been obtained from structural studies.

Oncogenic *Ras* genes, when overexpressed in certain cultured rodent fibroblast cell lines, produce a neoplastic transformed phenotype that is characterized by a loss of growth regulation (Ellis *et al*, 1981). For example, growth of Ras transformed cells is not inhibited by contact with neighboring cells in a confluent monolayer. Because of this lack of contact inhibition, a Ras transformed fibroblast will continue to grow and form a distinct, dense and raised lump of dividing cells, known as a focus. Each focus, in theory, originated from one transformed cell. Ras transformed cells also lack the requirement for anchorage to the culture substratum for growth and show a distinctive change in cell shape (Seeburg *et al.*, 1984, Der *et al.*, 1986, Kitayama *et al.*, 1989). Cellular transformation represents a cell culture model of tumorigenesis and provides a convenient bioassay for Ras function in mammalian cells.

Ras genes

The p21 Ras protein is highly conserved and ubiquitously expressed. In humans and rodents, Ras proteins are encoded by 3 genes, *H-Ras*, *N-Ras* and *K-Ras*, each located on different chromosomes (Lowy and Willumsen, 1993). *H-* and *K-Ras* are the cellular homologues of the *Ras* oncogenes found in the Harvey and Kirsten transforming retroviruses, respectively (Capon *et al.*, 1983a, 1983b, Shimizu *et al.*, 1983). *N-Ras* is the cellular homologue of the *Ras* oncogene found in the SK-N-SH human neuroblastoma cell line (Taparowsky *et al.*, 1983). All mammalian Ras proteins are encoded by 4 exons and the exon boundaries within the Ras primary structure are identical between all mammalian Ras proteins. As such, the 3 *Ras* genes appear to have arisen by gene duplication during evolution. The introns, however, differ widely in size and sequence (Lowy and Willumsen, 1993). In addition, 2 *K-Ras* isoforms (*K-RasA* and *K-RasB*) are produced by use of alternate 4th exons (Capon *et al.*, 1983a, McGrath *et al.*, 1983). Thus, 4 Ras proteins of 189 amino acids are encoded in mammals (except for K-Ras*B* which contains 188 amino acids). Tissue specific differences in expression between the 3 *Ras* genes can be detected at the mRNA level. For example, *H-Ras* expression is high in brain, skin and skeletal muscle, *K-Ras* expression is high in gut and thymus and *N-Ras* expression is high in testis and thymus (Leon *et al.*, 1987). However, it appears that at least one *Ras* gene type is expressed in every human cell type.

A comparison of the amino acid sequences of mammalian Ras proteins has led to the definition of 4 domains in Ras (Figure 1-1a) (Barbacid, 1987, Lowy and Willumsen, 1993). The N-terminal third of the protein (residues 1-85) comprises a region of very high conservation. For example, the amino acid sequences of the N-terminal domain are

identical between all human and rodent Ras proteins. Residues 86-166 define the second domain where the sequences diverge slightly from one another (79% identical between human Ras proteins). The remaining C-terminal sequences of Ras (167-185) are highly variable except for the last 4 amino acids (CAAX) which signal for critical post-translational modifications (described in detail below).

Besides mammals, *Ras* genes have been cloned from chicken, insects (*Drosophila*), worms (*C. elegans*), mollusks (*Aplysia*), frogs (*Xenopus*), molds (*Dicostellium* and *Neurospora*) and the yeasts *S. cerevisiae* and *S. pombe* (Barbacid, 1987, Lowy and Willumsen, 1993). The organization of 4 distinct regions in the primary structure is conserved between the Ras proteins encoded by all these *Ras* genes. Strikingly, there is at least 84% homology within the N-terminal domain between the mammalian and invertebrate Ras proteins (Barbacid, 1987).

The function of Ras has been generally conserved in evolution. For example, many characteristics are shared between the Ras proteins of *S. cerevisiae, S. pombe* and mammals (Barbacid, 1987). All yeast and mammalian Ras proteins cycle between GTP- and GDP- bound conformations and are regulated by 2 classes of regulatory proteins, guanyl nucleotide exchange factors (GEF's) and GAP's. Furthermore, both yeast and mammalian Ras proteins, when GTP-bound, activate downstream effector molecules to elicit biochemical and biological responses in the cell. Conservation is clearly demonstrated by the ability of mammalian and yeast *Ras* genes to function in heterologous systems. For instance, expression of a wildtype mammalian *Ras* gene rescued growth of a *S. cerevisiae* strain that contained a non-viable *Ras* mutation (DeFeo-Jones *et al.*, 1985, Kataoka *et al.*, 1985). On the other hand, chimeric Ras proteins comprised of yeast and mammalian Ras sequences were able to elicit a typical transformed phenotype when overexpressed in mouse fibroblasts (DeFeo-Jones *et al.*, 1985). However, the primary effector for the Ras2 protein of *S. cerevisiae* is adenylate cyclase which is not an effector for the *S. pombe* Ras protein or any mammalian Ras protein (Barbacid, 1987). Thus, not all aspects of Ras function are conserved between mammalian and yeast Ras proteins.

Characteristics of Ras as a G-protein

Ras is a member of the G-protein superfamily of signaling molecules. All G-proteins function as molecular switches by cycling between GDP-bound-"off" and GTP-"on" conformations. In general, G-proteins bind guanyl nucleotides with high affinity (Goody *et al.*, 1991). For example, the association constant of Ras for GDP or GTP has been measured to be 10^{11} - 10^{12} M^{-1} at 4°C (Neal *et al.*, 1988, John *et al.*, 1990, Goody *et*

al., 1991). This ensures that Ras is saturated with GDP or GTP at the cellular concentrations of nucleotide (approximately 10^{-5} M) (Bourne *et al.*, 1991). Also, all G-proteins possess intrinsic GTPase activity.

In resting mammalian cells, Ras is primarily GDP-bound (Gibbs *et al.*, 1990, Satoh *et al.*, 1990). The first step in the conversion of Ras-GDP to Ras-GTP is the dissociation of GDP from Ras (Figure 1-2). Nucleotide-free Ras then reassociates with free guanyl nucleotide. Because the concentration of GTP is approximately 10-fold greater than GDP in the cell, the product of reassociation is primarily Ras-GTP (Bourne *et al.*, 1991, Boriack-Sjodin *et al.*, 1998). The sum of guanyl nucleotide dissociation followed by reassociation constitutes nucleotide exchange. All G-proteins become GTP-bound through this general GDP dissociation-GTP reassociation mechanism (Bourne *et al.*, 1991). Through the intrinsic GTPase activity of Ras, the bound GTP is hydrolysed to form GDP, which remains complexed with Ras, and inorganic phosphate (P_i), which is released.

For G-proteins in general, the ratio of GTP- to GDP-bound forms is determined by the relative rates of nucleotide exchange and hydrolysis. In the cell, Ras GEF's positively regulate Ras by stimulating the rate of nucleotide dissociation which in turn leads to increased nucleotide exchange. Conversely, Ras GAP's negatively regulate Ras by stimulating the intrinsic GTPase activity. The regulation of Ras GEF's and Ras GAP's is described in detail later within this introduction.

G-protein superfamily

Nature has evolved a large number of guanyl nucleotide-binding proteins (G-proteins) which perform a wide range of functions in the cell. This G-protein superfamily can be divided into 3 major families: protein translation regulatory factors, heterotrimeric G-proteins , and low-molecular weight G-proteins (of which Ras is a member) (Figure 1-3). G-protein translation regulatory factors are found in all eukaryotes and prokaryotes. The best studied member of this family is EF-Tu from *Eschericia coli* which functions in a translation proof-reading mechanism (Bourne *et al.*, 1990, Pape, 1998). Other examples of this family are the prokaryotic EF-G which regulates ribosome translocation, prokaryotic initiation factor IF2 which regulates the formation of the 70S initiation complex and eukaryotic initiation factor eIF-2 which regulates the formation of the 43S preinitiation complex (Bourne *et al.*, 1990, Pain, 1996).

The second major G-protein family consists of the heterotrimeric G-proteins that are ubiquitous in eukaryotes. Heterotrimeric G-proteins are comprised of an α subunit which binds GDP or GTP and an associated $\beta\gamma$ dimer (Neer, 1995). Heterotrimeric G-proteins

relay signals from G-protein coupled receptors (GPCR) at the plasma membrane to intracellular biochemical responses. The mammalian heterotrimeric G-protein family is immense, containing at least 17 α, 5 β and 12 γ members (Dhanasekaran *et al.*, 1998). Furthermore, GPCR's are the largest group of cell surface receptors with more than 1000 members (Gutkind, 1998).

Low-molecular weight G-proteins are also ubiquitous in eukaryotes. Members of this family, which are all between 20-35 kDa, can be further divided into 5 sub-families based on primary structure: Ras, Rho, Rab, Arf and Ran (Kahn *et al.*, 1992).

Within the Ras family, there are the true Ras proteins (H-Ras, N-Ras, K-RasA and K-RasB) which are the focus of this thesis, and close Ras relatives. The relation between Ras family members currently known are illustrated by a dendogram in Figure 1-3. Rap1A, TC21 and R-Ras are 3 examples of close Ras relatives. The amino acid sequences of Rap1A, TC21 and R-Ras are all between 53-55% identical to H-Ras (Campbell *et al.*, 1998). In addition, the "switch 1" regions of Rap1A, TC21, R-Ras and the true Ras proteins are identical. The switch 1 region interacts with, and signals to, downstream molecules (see section discussing Ras structure below). The identity in the switch 1 region suggests that true Ras proteins, Rap1A, TC21 and R-Ras could interact with similar downstream molecules and trigger similar biological responses. Studies have shown however, that overexpression of Rap1A in certain circumstances can antagonize Ras signaling and reverse the cellular transformation phenotype produced by an activated Ras protein (Kitayama *et al.*, 1989, Cook *et al.*, 1993). Others have found that activated forms of TC21 and R-Ras bind, but do not activate Raf, a well characterized effector of true Ras-proteins (Graham *et al.*, 1996, Huff *et al.*, 1997). The *in vivo* signaling functions of close Ras relatives remains to be clarified. The true Ras proteins will simply be referred to as Ras in the following discussion.

The Rho family of GTPases currently contains at least 14 mammalian members, of which the most widely studied are Rac1, RhoA and Cdc42 (Zohn *et al.*, 1998). Rho proteins are best known for their role in regulating actin cytoskeleton rearrangements. Members of the Rho family can also activate the MEKK-SEK-JNK protein kinase cascade which in turn gives rise to phosphorylation of the c-jun transcription factor and a modulation of gene expression (Vojtek and Cooper, 1995). The function and regulation Rho family proteins appear interrelated with Ras signaling in mammalian cells. As such, Rho proteins will be mentioned several times in our discussion of Ras regulation and downstream signaling functions.

The mammalian family of Rab proteins is currently known to contain at least 30 members (Simons and Zerial, 1993, von Mollard *et al.*, 1994). All Rab proteins are

thought to be involved in membrane vesicle trafficking in the cell. Genetic and biochemical evidence has demonstrated that Rab proteins are essential for all stages of vesicle trafficking and each Rab protein is thought to regulate one particular step of vesicle trafficking.

Arf family proteins are known to specifically regulate membrane trafficking of the trans-Golgi network (Schekman and Orci, 1996). Arf proteins are known to activate phospholipase D, as well (Brown *et al.*, 1993). Originally, Arf was identified as a protein factor necessary for the ADP ribosylation of Gs, a heterotrimeric G-protein α subunit (Kahn and Gilman, 1984). At least 6 mammalian Arf members have been identified (Kahn *et al.*, 1992).

The mammalian Ran family currently contains Ran and related protein TC4 (Kahn *et al.*, 1992). Ran regulates nuclear import and export of proteins and RNA-protein complexes (Koepp and Silver, 1996, Goldfarb, 1997). These regulatory functions are carried out at nuclear pore complexes in the nuclear envelope. Ran may have other functions as well. Ran has been shown to regulate DNA synthesis and cell-cycle progression (Avis and Clarke, 1996). Recently, *in vitro* studies using Xenopus egg extracts were presented which directly implicated Ran in microtubule spindle formation (Ohba *et al.*, 1999, Wilde and Zheng, 1999).

Prokaryotic and eukaryotic cells have evolved to employ G-proteins as molecular switches in a variety of contexts to regulate a wide range of processes. Ras remains one of the best characterized G-proteins, genetically and biochemically. In addition, the strong involvement of Ras in human cancer is unmatched by any other G-protein.

3-D Structure of Ras

3-D structural analysis of Ras has led to a detailed understanding of nucleotide binding, nucleotide hydrolysis and signaling to downstream effector molecules. The crystal structures of Ras in GDP- and GTP-bound forms have been solved (de Vos *et al.*, 1988, Pai *et al.*, 1990). In both structures, Ras is a globular protein consisting of six β sheet strands connected by hydrophilic loops and α helices (Figure 1-4). The general 3-D structure of G-proteins is conserved. For example, the overall α/β topologies in the structures of Ras and EF-Tu are almost identical despite the fact that the primary sequences are only 30% identical (Bourne *et al.*, 1991).

3 loop regions of Ras are especially important for Ras function. Loop 1, which comprises residues 10-17 of Ras (as defined in Milburn *et al.*, 1990), is critical for nucleotide binding. The backbone chain of several residues within this region plus the sidechain of lysine-16 form hydrogen bonds with the α and β phosphates of bound GDP

or GTP. Loop 1 contains the $_{10}$GAGGVGKS$_{17}$ (one-letter amino acid code) sequence that represents the GX$_4$GK(S/T) motif found in many nucleotide triphosphate utilizing enzymes (Bourne *et al.*, 1991). In Ras, mutation of serine-17 to asparagine (N17) produces a mutant protein that has an abnormally low affinity for GTP (Feig and Cooper, 1988). As a result of this low affinity for GTP, N17 Ras is thought to form stable dead-end complexes with Ras GEF's. N17 Ras is frequently used as a dominant-negative allele of Ras. A detailed mechanism of N17 Ras will be presented in our discussion on GEF's. Also found in loop 1 is glycine-12, the residue frequently mutated in activated Ras proteins. Glycine-12, however, does not contact the bound nucleotide and the functional significance of mutation at position 12 involves the interaction of Ras with GAP's (described in detail within the discussion on GAP's).

Loop 2 (residues 27-37) and loop 4 (residues 59-66) of Ras are also in close contact with the nucleotide. These structures, found close together on the outer surface, are highly flexible and important for Ras to act as a molecular switch. By comparing the structures of Ras-GDP and Ras-GTP, 2 "switch" regions that change conformation were defined (Figure 1-4) (Milburn *et al.*, 1990, Pai *et al.*, 1990). Loop 2 comprises a large portion of switch 1 (residues 32-38) and loop 4 comprises a portion of switch 2 (60-76). These conformational changes are caused by 2 sets of interactions between the γ phosphate of GTP and Ras. The backbone of threonine-35 in the switch 1 region directly forms hydrogen bonds with the γ phosphate of GTP and the associated Mg^{2+} ion. This interaction gives rise to a conformational change in the entire switch 1 region. The sidechain of threonine-35 was observed to completely flip in orientation between the GDP- and GTP-bound Ras structures. In the second set of interactions, backbone atoms of residues 60 and 61 form hydrogen bonds with the γ phosphate of GTP. These contacts induce the conformational change observed in the switch 2 region. Ras-binding proteins that recognize only GTP-bound conformation rely on the conformational changes in the switch 1 and switch 2 regions (Nassar *et al.*, 1995, Druggan *et al*, 1996)

Several residues of loop 4 (switch 2) are critical for the intrinsic GTPase activity of Ras. The most important interaction is by residue glutamine-61 which comes in close contact with the γ phosphate of GTP (Pai *et al.*, 1990, Prive *et al.*, 1992). The carbamoyl oxygen of glutamine-61 is thought to form a hydrogen bond with a closely situated water molecule. This interaction activates the water molecule to undergo a nucleophilic attack on the γ phosphate moiety of GTP. Mutation of glutamine-61 reduces the intrinsic GTPase rate substantially (Der *et al.*, 1986).

Post-translational processing of Ras

Ras is localized to the inner surface of the plasma membrane as a result of post translational modification at its C-terminus (Hancock *et al.*, 1989, 1991, Fujiyama and Tamanoi, 1990). The signal for modification is the CAAX motif (cysteine-186 followed by 2 aliphatic residues followed by residue X, serine in the case of Ras) which comprises the last 4 C-terminal residues of all Ras proteins. The modification occurs through several steps which each increase the hydrophobicity of Ras. First, the isoprenoid 15 carbon farnesyl group is attached to cysteine-186. Residues 187-189 are then proteolytically cleaved and the carboxy-terminal group of cysteine-186 is methylated. Finally, multiple cysteines in the hypervariable region (residues 180-185) are reversibly palmitoylated. Inhibition of the initial farnesylation step blocks all further processing and disrupts membrane localization and function of Ras. Inhibition of Ras modification has been achieved through mutation of cysteine-186, interfering with isoprenoid lipid synthesis, and by drugs specifically targeting the farnesyl transferase (Jackson *et al*, 1990, Khol *et al.*, 1993, James *et al.*, 1993, Schafer *et al.*, 1989). Farnesyl transferase inhibitors have been proposed to be a possible anti-cancer strategy (Travis, 1993, Cox and Der, 1997). In cell culture, farnesyl transferase inhibitors can reverse the morphological changes and inhibit the anchorage- (attachment) independent growth of Ras transformed cells (James *et al.*, 1993, Khol *et al.*, 1993). Farnesyl transferase inhibitors have also been shown to dramatically reduce the sizes of tumors in mice injected with Ras transformed fibroblasts and transgenic mice carrying an activated *Ras* gene (Kohl *et al.*, 1994, 1995).

3 distinct classes of mammalian Ras GEF's

In vivo, a major mechanism of Ras activation in response to extracellular stimuli is through the action of GEF's. 3 families of Ras GEF's are known to regulate Ras in mammalian cells (Figure 1-1b). One family is comprised of SOS1 and SOS2 which function similarly and are widely expressed (Bowtell *et al.*, 1992). The second family is comprised of RasGRF1 and GRF2 which resemble the yeast CDC25 Ras GEF in domain structure (Shou *et al.*, 1992, Fam *et al.*, 1997). Both GRF1 and GRF2 appear to be expressed exclusively in brain. The third family contains only one member, RasGRP, which appears to be enriched in brain and in hematopoietic cells (Ebinu *et al.*, 1998, Kawasaki *et al.*, 1998, Tognon *et al.*, 1998). As described below, SOS and GRF proteins are regulated by distinct mechanisms. The structure and function of RasGRP is discussed in detail within chapter 4 of this thesis. All members of the SOS, GRF and GRP families carry the conserved Ras GEF catalytic domain.

Mechanism of Ras GEF's

Because of the high affinity of Ras for nucleotide, the rate of exchange arising from spontaneous GDP dissociation followed by GTP reassociation is low. For example, *in vitro*, the spontaneous dissociation rate is approximately 10^{-5} moles of nucleotide dissociating per second per mole of complex (John *et al.*, 1990). At this rate, it is estimated that nucleotide dissociation, and consequently nucleotide exchange, would require hours (Wittinghofer, 1998). In the cell, Ras becomes activated within minutes in response to growth factor stimulation due to the action of Ras GEF's. *In vitro*, Ras GEF's can stimulate nucleotide exchange by up to 5 orders of magnitude (Lenzen *et al.*, 1998).

The molecular mechanism of Ras GEF action was revealed from the crystal structure of Ras in complex with the catalytic domain of SOS1 (Boriack-Sjodin *et al.*, 1998). GEF's promote nucleotide exchange by binding to Ras-GDP, making nucleotide binding unfavorable and increasing the rate of GDP dissociation. GEF's do this through several mechanisms. A helical hairpin region of SOS inserts into Ras and displaces loop 1 of Ras that normally secures the nucleotide. In addition, residues leucine-938 and glutamine-942 of SOS form interactions with Ras close to the nucleotide binding pocket which blocks the sites where the α-phosphate and associated Mg^{2+} ion of GDP would bind. Binding to SOS also compresses loop 4 of Ras and this conformational change causes an interaction between the sidechain of glutamate-62 in loop 4 and the sidechain of lysine-16 in loop 2 of Ras. This interaction further disrupts binding between Ras and the phosphate moieties of GDP and associated Mg^{2+} ion. After promoting nucleotide dissociation, SOS forms a stable complex with nucleotide-free-Ras. In this complex, SOS does not completely block the guanyl nucleotide binding site of Ras. This allows GTP to reassociate with Ras which results in the dissociation of SOS from the Ras-GTP-SOS complex.

The N17 dominant-negative allele of Ras has an abnormally low affinity for GTP (Feig and Cooper, 1988). It is thought that SOS binds to N17 Ras, stimulates GDP dissociation and forms a stable complex with N17 Ras. In contrast to the situation with wildtype Ras, GTP fails to rebind N17 Ras to allow the release of SOS. As a result, Ras GEF's, such as SOS, remain bound in dead-end complexes with N17 Ras (Farnsworth and Feig, 1991). N17 Ras, when overexpressed in cells, is thought to complex with and inactivate all Ras GEF's thereby depriving endogenous Ras of positive regulatory proteins. In this way, N17 Ras acts as a dominant-negative protein for the Ras signaling pathway.

Regulation of SOS proteins

SOS proteins can be divided into 3 regions: an N-terminal region that contains a Dbl homology and a Pleckstrin homology (PH) domain, the GEF catalytic region and a C-terminal domain that contains the poly-proline sequences that interact with the adapter protein Grb2 (Figure 1-1b). PH domains are found in a variety of proteins and are involved in binding lipids whereas Dbl homology domains function as GEF's for Rho family GTPases (Lemmon *et al.*, 1996, Toker and Cantley, 1997, Zohn *et al.*, 1998).

The regulation of SOS1 by peptide growth factors in mammalian cells is particularly well understood (Figure 1-5a) (Buday and Downward, 1993, Chardin *et al.*, 1993, Egan *et al.*, 1993). For simplicity, SOS1 will be referred to as SOS. Epidermal growth factor (EGF) binds to the EGF receptor tyrosine kinase (RTK) on the cell surface and induces EGF RTK dimerization. Following, the EGF RTK's auto-trans-phosphorylate on a number of tyrosine residues in the intracellular domain (Cohen *et al.*, 1982, Downward *et al.*, 1984, Chen *et al.*, 1987, Bertics *et al.*, 1988). Activation and tyrosine phosphorylation of the RTK leads to membrane recruitment of SOS through several mechanisms.

SOS is constitutively bound to the adapter protein Grb2 through interaction of the N-terminal SH3 domain of Grb2 to the poly-proline region in the C-terminus of SOS (also see Figure 1-1b) (Buday and Downward, 1993, Chardin *et al.*, 1993, Egan *et al.*, 1993). Grb2 also contains a SH2 phosphotyrosine binding motif that directly interacts with phosphorylated residues on the EGF RTK. Thus, a complex of RTK-Grb2-SOS is formed in response to EGF treatment of cells. The formation of this complex recruits SOS to the plasma membrane where it can regulate Ras.

A variation of this theme has been demonstrated by Shc adapter proteins (Figure 1-5b). Shc proteins each contain one SH2 domain and one PTB domain (another protein-tyrosine-binding motif) which can interact with the phosphotyrosine residues on the RTK's after growth factor stimulation (Pelicci *et al.*, 1992, Rozakis-Adcock *et al.*, 1992, Bonfini *et al.*, 1996). In addition, Shc proteins are tyrosine phosphorylated on a number of residues by RTK's in response to growth factor signaling. Tyrosine phosphorylated Shc proteins, in turn, provide sites for attachment of the Grb2-SOS complex (Pelicci *et al.*, 1992, Ruff-Jamison *et al.*, 1993, Yokote *et al.*, 1994). This mechanism is involved in signaling downstream of a variety of extracellular signals such as EGF, nerve growth factor (NGF), platelet-derived growth factor (PDGF), insulin, interleukins and granulocyte macrophage colony-stimulating factor (Cutler Jr. *et al.*, 1993, Ruff-Jamison *et al.*, 1993, Obermeier *et al.*, 1994, Pronk *et al.*, 1994, Yokote *et al.*, 1994,). In many circumstances, complexes consisting of RTK-Shc-Grb2-SOS likely form simultaneously with RTK-Grb2-

SOS complexes. Shc-containing complexes could also perform Ras-independent signaling functions. For example, the *Drosophila* Shc homologue shares 46% amino acid similarity with the mammalian Shc proteins (Bonfini *et al.*, 1996). However, *Drosophila* Shc does not interact with the *Drosophila* Grb2 homologue *in vivo* or *in vitro* and therefore, likely does not regulate Ras. At least in *Drosophila*, Shc proteins appear to perform Ras-independent signaling functions downstream of RTK's.

Several other mechanisms can recruit the Grb2-SOS complex to the plasma membrane. For example, the fibroblast growth factor (FGF) RTK itself does not bind Grb2 (Figure 1-5c). Upon FGF stimulation, the FGF RTK instead phosphorylates FRS2, a docking protein that is constitutively membrane bound due to a myristate modification (Kouhara *et al.*, 1997). Tyrosine phosphorylated residues on FRS2 then provide sites for binding of the Grb2-SOS complex. The Grb2-SOS machinery can also be utilized by non-RTK signaling systems such as integrins to link cell contacts to Ras activation (Figure 1-5d). For example, the attachment of cells to the extracellular matrix (ECM) leads to integrin activation and the production of large protein complexes called focal adhesions at the plasma membrane (Clark and Brugge, 1995). Focal adhesion kinase (FAK), within this activated complex, autophosphorylates on tyrosine residues to produce binding sites for Grb2-SOS complexes (Schlaepfer *et al.*, 1994).

The primary mode of SOS regulation appears to be membrane recruitment which brings GEF activity into proximity with Ras. Accordingly, SOS can be constitutively targeted to the plasma membrane by attachment of the CAAX modification signal of H-Ras (Aronheim *et al.*, 1994). Overexpression of this construct in mouse fibroblasts leads to activation of Ras signaling and cellular transformation. However, the regulation of SOS is likely more complex than simple membrane recruitment. One study reported that deletion of either the N- or C-terminal non-catalytic sequences produces activated SOS proteins suggesting that non-catalytic sequences can regulate GEF activity via intramolecular mechanisms (Corbalan-Garcia *et al.*, 1998). A conflicting study found that deletion of the N-terminal region produced a dominant-inhibitory SOS protein (Karlovich *et al.*, 1995). In addition, mutation of critical residues in the Dbl-homology or PH domain impaired the ability of a membrane targeted SOS protein to transform mouse fibroblasts (Qian *et al.*, 1998). One study also found that phosphatidylinositol-4-5-bis-phosphate (PIP_2) specifically inhibited the Ras GEF activity of SOS *in vitro* (Jefferson *et al.*, 1998). Thus, the non-catalytic sequences of SOS appear to be functionally important although our understanding of these sequences is limited.

Regulation of RasGRF proteins

RasGRF1 and RasGRF2 are thought to link Ca^{2+} signals generated upon activation of GPCR's and heterotrimeric G-proteins to the activation of Ras. Both GRF proteins have a Calmodulin-binding IQ domain in the N-terminal region. Indeed, interactions between Calmodulin and both GRF proteins have been demonstrated in response to Ca^{2+} signals *in vivo* (Farnsworth *et al.* 1995, Fam *et al.*, 1997). Furthermore, activation of Ras and downstream signaling by Ca^{2+} ionophores in cultured cells was enhanced by the overexpression of either GRF1 or GRF2. There may also be Ca^{2+}-independent mechanisms to regulate GRF's. It has been shown that GRF1 becomes phosphorylated in response to signaling from heterotrimeric G-proteins and that this phosphorylation increases *in vitro* Ras GEF activity (Mattingly and Macara, 1996). In addition, GRF1 and GRF2 both have a Dbl-homology and 2 PH domains, although the function of these regions in GRF proteins has not been demonstrated.

The major pathway of Ras activation in response to extracellular stimuli is thought to be through the regulation of GEF's. SOS and GRF proteins each appear to be linked with distinct upstream signaling pathways. As such, Ras may act as a point of convergence for a diverse set of upstream stimuli. In addition, both SOS and GRF proteins contain substantial non-catalytic sequences whose function remain to be defined in detail. For example, the Dbl-homology domain could allow SOS and GRF proteins to coordinately regulate Ras and Rho family proteins.

4 families of mammalian Ras GAP's

In mammals, 4 distinct classes of Ras GAP's are currently known: p120GAP, NF1, the members of the GAP1 family, and SynGAP. As described below, these proteins may negatively regulate Ras in response to protein phosphorylation and lipid, inositol and Ca^{2+} second messengers. In addition, these proteins may provide another link between Ras and the regulation of Rho family proteins.

Mechanism of Ras GAP's

The intrinsic rate of GTP hydrolysis by Ras is low. For example, the half life of Ras-GTP *in vitro* is estimated to be approximately 2 hours at 25°C (Sweet *et al.*, 1984). In the cell, the rate of GTP hydrolysis is greatly enhanced by the action of Ras GAP's (Trahey and McCormick, 1988). *In vitro*, Ras GAP's have been shown to accelerate GTP hydrolysis by up to 5 orders of magnitude (Gideon *et al.*, 1992).

The crystal structure of Ras in complex with the catalytic domain of p120GAP (referred to here as the Ras-GAP complex) has provided much insight into the mechanism by which GAP's stimulate GTP hydrolysis (Scheffzek *et al.*, 1997). As discussed earlier, intrinsic hydrolysis involves the activation of a water molecule by residue glutamine-61 of Ras followed by nucleophilic attack on the gamma phosphate of GTP (Figure 1-4) (Pai *et al.*, 1990, Prive *et al.* 1992). During the reaction, a partial negative charge develops in the active site. From the structure of the Ras-GAP complex, 2 major mechanisms through which GAP's stimulate hydrolysis were observed. Loop $L1_c$ of GAP is inserted directly into the active site of Ras. It is thought that arginine-789 within this loop directly interacts with the γ phosphate moiety of GTP and neutralizes the partial negative charge in the transition state. The interaction of GAP with Ras, through hydrophobic interactions and a number of ionic interactions, also stabilizes the entire loop 4 region of Ras around residue glutamine-61 which is normally quite flexible (Milburn *et al.* 1990). This stabilizing effect helps to properly orient glutamine-61 in the hydrolysis transition state.

Ras proteins with substitutions at glycine-12 are insensitive to GAP regulation (Adari *et al.*, 1988, Trahey and McCormick, 1988). In the structure of the Ras-GAP complex, the Cα atom of glycine-12 participates in van der Waals contacts with the main chain carbonyl moiety of arginine-789. Even subtle substitutions such as glycine-12 to alanine are thought to produce unfavorable steric clashes with arginine-789 that would in turn, lead to steric clashes with the sidechain of Ras residue glutamine-61. In this way, mutation of glycine-12 perturbs the key catalytic interaction between arginine-789 of GAP and glutamine-61 of Ras.

p120GAP

p120GAP was the first Ras GAP to be discovered and is the best characterized Ras GAP (Trahey *et al.*, 1988, 1988, Vogel *et al.*, 1988). Human p120GAP is a widely expressed 1047 amino acid protein that contains several distinct domains (Figure 1-1c). The N-terminal region contains one SH3 domain flanked by 2 SH2 domains. The middle third of p120GAP contains a PH domain and a region similar to the C2 phospholipid-dependent Ca^{2+}-binding domain of PKCα (Luo and Weinstein, 1993, Maekawa *et al.*, 1994). The GAP catalytic domain is found in the C-terminal third of the protein.

The presence of several putative functional domains suggests that p120GAP activity can be regulated in response to cellular signaling. In support of an allosteric regulatory mechanism, deletion of the N-terminal non-catalytic sequences of p120GAP gives rise to a mutant protein with decreased Ras binding and GAP activity *in vitro* (Gideon *et al.*, 1992).

One study has also demonstrated translocation of p120GAP to a membrane-enriched particulate fraction in cells treated with Ca^{2+} ionophore (Gawler *et al.*, 1995). This translocation depended on the C2 domain of p120GAP. Thus, p120GAP may translocate to the plasma membrane in response to intracellular Ca^{2+} signals via its C2 domain.

The SH2 domains of p120GAP have been extensively demonstrated to interact with a number of tyrosine phosphorylated proteins. For example, upon treatment of canine kidney epithelial cells with PDGF, full-length p120GAP or the isolated SH2 domains of p120GAP bind to the PDGF RTK (Cooper and Kashishian, 1993). These *in vivo* interactions are ablated by mutation of tyrosine-771 in the intracellular domain of the PDGF RTK. In EGF-stimulated rat2 fibroblasts, p120GAP has also been shown to form distinct complexes with tyrosine phosphorylated p60dok, a protein of unknown function, and tyrosine phosphorylated p190, a GAP that negatively regulates Rho family proteins (Moran *et al.*, 1991, Settleman *et al.*, 1992, Carpino *et al.*, 1997, Yamanashi and Baltimore, 1997). The p120GAP-p190 complex appears predominantly cytosolic and it has been shown that the catalytic activity of p120GAP in complex with p190 is lower than that of monomeric p120GAP (Moran *et al.*, 1991). In addition, p120GAP itself is also tyrosine phosphorylated by a variety of protein kinases such as the EGF RTK (Ellis *et al.*, 1990, Liu and Pawson, 1991). Thus, the primary structure and existing biochemical data suggest the regulation of p120GAP by intramolecular interactions, protein-protein interactions through phosphotyrosine binding motifs, membrane translocation, and interactions with Ca^{2+} ions. p120GAP is also thought to be involved in signaling biochemical changes downstream of Ras (i.e. act as a Ras effector). The possible role of p120GAP in downstream signaling is discussed in a later section.

NF1

NF1 is the product of the gene mutated in von Recklinghausen disease also known as neurofibromatosis type-1 (Ballester, 1990, Xu *et al.*, 1990a). The encoded protein is 220 kDa and the Ras GAP catalytic domain is located in the middle of the protein (Figure 1-1d). Due to extensive homology in large regions flanking the catalytic domain, NF1 is classified as the mammalian homologue of the yeast Ras GAP's, ira1 and ira2. NF1 is expressed in most mammalian cell types but appears enriched in brain, white blood cells, spleen and skeletal muscle (Wallace *et al.*, 1990). The GAP catalytic domain of NF1 has been shown to act as a Ras GAP *in vitro* and *in vivo* (Ballester *et al.*, 1990, Martin *et al.*, 1990, Xu *et al.*, 1990b). However, the GAP catalytic sequences only comprise one eighth of the protein and the function of the remaining sequences is unknown.

Our understanding of the biological function of NF1 has been largely gleaned from analysis of the neurofibromatosis condition. Primary symptoms of the disease are benign and malignant tumors in multiple tissues derived from the neural crest such as neurofibromas and Schwannomas (Riccardi, 1981). The genetic basis behind neurofibromatosis appears to be the inheritance of a disrupted NF1 allele followed by somatic mutation of the remaining allele (Viskochil *et al.*, 1990, Wallace *et al.*, 1990). Thus, NF1 is classified as a tumor suppressor and indeed, loss of heterozygosity has been observed to proceed the formation of fibrosarcomas and other lesions (Serra *et al.*, 1997). Accordingly, analysis of Schwannoma cell lines derived from neurofibromatosis type-1 patients show low to undetectable levels of NF1 protein (Basu *et al.*, 1992, DeClue *et al.*, 1992). In these cells, lower total GAP activity and higher levels of Ras-GTP were also observed, even though p120GAP is clearly expressed. These findings suggest that: 1) NF1 is the major Ras negative regulator in these neural crest derived-cells, 2) p120GAP in these cells is inactivated, and 3) loss of NF1 Ras GAP function and the consequent rise in Ras-GTP levels comprise the molecular mechanism behind neurofibromatosis. In support of the latter conclusion, anchorage-independent growth of the 88-14 NF1-tumor derived cell line is inhibited by overexpression of the p120GAP catalytic domain (DeClue *et al.*, 1992). Mitotic division of 88-14 cells was also inhibited by microinjection of Y13-259 Ras monoclonal antibody which blocks signaling to downstream molecules (Basu *et al.*, 1992).

GAP1

GAP1 proteins are a recently identified family of Ras GAP's that may be a physiological receptor of IP_4, the orphan second messenger produced by phosphorylation of IP_3 (Irvine *et al.*, 1986). There are 2 members of the mammalian GAP1 family: GAP1m and GAP1(IP4BP) (Maekawa *et al.*, 1993, 1994, Cullen *et al.*, 1995). Both proteins are roughly 100 kDa and have similar domain structure (Figure 1-1c). The N-terminal region contains 2 C2 Ca^{2+}-binding motifs in tandem that are similar to the C2 regions of p120 GAP and PKC proteins. The GAP catalytic domain is located in the middle of the protein and a PH domain is found in the C-terminal region. Both GAP1 proteins are widely expressed but appear particularly enriched in brain, skeletal muscle, spleen and peripheral blood leukocytes (Lockyer *et al.* 1999a).

In vitro, both GAP1m and GAP1(IP4BP) bind IP_4 and act as GAP's for Ras (Maekawa *et al.*, 1994, Cullen *et al.*, 1995, Fukada and Mikoshiba, 1996, Bottomley *et al.*, 1998). GAP1 proteins have also been shown to function as Ras GAP's *in vivo*. For example, expression of mammalian GAP1m can rescue growth of a temperature-sensitive

ira2 yeast mutant (Maekawa *et al.*, 1994). There is also direct evidence that GAP1 proteins are regulated by inositol second messengers. Ras GAP activity of GAP1(IP4BP) *in vitro* is inhibited upon addition of liposomes with a lipid content mimicking the inner leaflet of the plasma membrane (Cullen *et al.*, 1995). This activity is then restored by the addition of IP_4 to the reaction. GAP1 proteins may be regulated by additional mechanisms. For example, GAP1m was observed to translocate from the cytosol to plasma membrane within 1 minute of EGF stimulation in PC12 (rat pheochromocytoma) cells (Lockyer *et al.*, 1999b). GAP1(IP4BP) on the other hand, is found constitutively at the plasma membrane in rat leukemia and COS-7 (monkey kidney) cells (Lockyer *et al.*, 1997, 1999a). A recent report has also shown that GAP1m interacted with α-12, an α subunit from the heterotrimeric G-protein family *in vitro* and in COS-7 cells (Jiang *et al.*, 1998). Furthermore, this interaction stimulated *in vitro* Ras GAP activity. Thus, GAP1 proteins may negatively regulate Ras in response to inositol phosphate second messengers and heterotrimeric-G proteins.

SynGAP

SynGAP was recently isolated in a search for proteins that interacted with the post synaptic density (PSD), a large cytoskeletal complex found on the cytoplasmic face of post synaptic membranes in the brain (Kim *et al.*, 1998). Rat SynGAP is a 1293 amino acid protein with a 135 kDa predicted molecular weight (Figure 1-1c). The N-terminal region of SynGAP contains a PH and a C2 domain. The GAP catalytic domain is situated in the middle of the primary structure and the C-terminal region contains a proline-rich region and the QTRV sequence. QTRV conforms to the (S/T)XV consensus motif which is involved in protein-protein interactions with PDZ domains. Several proteins that contain PDZ domains are enriched in the PSD (Gomperts, 1996).

SynGAP expression is primarily limited to the brain although a trace amount of SynGAP transcript has been observed in the lung (Chen *et al.*, 1998, Kim *et al.*, 1998). The major role of SynGAP is thought to be Ras regulation at synaptic junctions. SynGAP protein has been localized to excitatory synapses in dissociated hippocampal neurons by immunofluorescence (Kim *et al.*, 1998). A recombinant protein consisting of the catalytic domain of SynGAP fused to the glutathione S-transferase (GST) tag demonstrated *in vitro* Ras GAP activity (Kim *et al.*, 1998). Isolated PSD's from rat brain also exhibit Ras GAP activity *in vitro* and this activity is inhibited 75% by an antibody specific for the catalytic domain of SynGAP (Chen *et al.*, 1998). One *in vitro* study has suggested that SynGAP is phosphorylated by calmodulin-dependent kinase II (CaM kinase II), a serine/threonine

protein kinase that is enriched in the PSD (Chen *et al.*, 1998). It was also shown that pre-incubation of isolated PSD's in a buffer containing Ca^{2+} and ATP, a treatment that activates CaM kinase II, inhibited the PSD-associated *in vitro* Ras GAP activity by 80-90%. These findings suggest that the Ras GAP activity of SynGAP is regulated by Ca^{2+} signals through the action of CaM kinase II.

In considering the function of Ras GAP's, themes that were observed during the discussion of Ras GEF's arise again. Several different classes of Ras GAP's are involved in the negative regulation of Ras. Although modulation of Ras GAP activity in response to extracellular stimulation is not well understood, the primary structures of the various Ras GAP's and preliminary evidence suggest that Ras GAP's are regulated by a variety of intracellular signals. Thus, Ras again appears to be a convergence point for upstream signals in the cell.

Activation of Ras through modulation of Ras GEF and Ras GAP activity

In the cell, the ratio of Ras-GTP to Ras-GDP is determined by the relative activities of GEF's and GAP's. The accumulation of Ras-GTP (i.e. activation) through the recruitment of GEF activity in response to extracellular stimuli is well understood and widely appreciated. An inhibition of GAP's in response to extracellular stimuli could also activate Ras. Although the inhibition of GAP's in response to extracellular stimuli does not appear to be a common route of Ras activation in mammalian cells, there have been 2 reports to suggest such a mechanism. Peripheral blood lymphoblasts treated with phorbol ester, a diacyglycerol analog, and neutrophils stimulated with FMLP, a chemotactic signal, show decreased total cellular GAP activity that correlates with Ras activation (Downward *et al.*, 1990, Zheng *et al.*, 1997). However, in NIH3T3 mouse fibroblasts or PC12 cells, stimulation with peptide growth factors leads to no change or an increase in cellular GAP activity, respectively (Gibbs *et al.*, 1990, Li *et al.*, 1992). The role and molecular mechanism of GAP inhibition in response to extracellular stimuli remain to be clarified.

Ras effector proteins

Signals are propagated from Ras to downstream biochemical and biological responses by Ras effector molecules. The fundamental characteristic of all Ras effectors is specific binding to the GTP-bound conformation of Ras. As would be predicted from the GTP-dependent conformational changes of the switch regions, Ras effectors interact with the Ras switch 1 region (Nassar *et al.*, 1995). Furthermore, geneticists have isolated a

large number mutations in switch 1 that disrupt interaction with effectors and impair Ras function (Willumsen *et al.*, 1986, White *et al.*, 1995, Rodriguez-Viciana *et al.*, 1997, Stang *et al.*, 1997). There is also evidence that the switch 2 region is involved in effector interactions and signaling downstream of Ras (Nur-E-Kamal *et al.*, 1992, Moodie *et al.*, 1995, Druggan *et al.*, 1996)

A number of Ras effector proteins are now understood to be important in producing the biochemical responses downstream of Ras. Thus, Ras is a point of divergence for downstream signaling. We will discuss 3 Ras effectors that are particularly well understood: the protein kinase Raf, phosphatidylinositol-3 kinase (PI3K) and RalGDS, a GEF for Ral. p120GAP may also be considered a Ras effector for several reasons. The signaling pathways downstream of Ras are summarized in Figure 1-6.

Raf

One of the best characterized Ras effectors is the protein kinase Raf. The function of Raf downstream of Ras was initially described using activated and dominant-negative forms of Ras and Raf. For example, activated Ras leads to Raf phosphorylation in NIH3T3 and PC12 cells (Morrison *et al.*, 1988, Wood *et al.*, 1992). Dominant-negative mutants of Raf can inhibit transformation caused by activated Ras (Kolch *et al.*, 1991). In mammals, there are 3 members of the Raf family: Raf1, A-Raf and B-Raf (Lee *et al.*, 1996, Wojnowski *et al.*, 1997). The function and regulation of Raf1, herein referred to simply as Raf, is well characterized and will be discussed.

Biochemically, Raf behaves like a typical Ras effector. Raf has been shown to interact with Ras in a GTP dependent manner *in vitro* and in the yeast 2-hybrid system (Van Aelst *et al.*, 1993, Vojtek *et al.*, 1993, Warne *et al.*, 1993, Zhang *et al.*, 1993). The regions of Raf involved in binding Ras-GTP are well defined. The primary interaction takes place between a region of Raf called the Ras-binding-domain (RBD) that recognizes the switch 1 region of Ras (Chuang *et al.*, 1994, Druggan *et al.*, 1996). It is thought that secondary interactions take place between a cysteine-rich zinc-finger region in Raf and regions of Ras that include switch 2 (Brtva *et al.*, 1995, Druggan *et al.*, 1996, Cutler, Jr. *et al.*, 1998). The crystal structure of the Raf-RBD in complex with the GTP-bound form of Rap1A, a close Ras relative, has been solved (Nassar *et al.*, 1995). In the structure, an extensive network of hydrogen bonds and polar interactions between residues in the Rap1A switch 1 region and Raf-RBD are observed.

The binding of Ras to Raf leads to Raf activation by a complex mechanism that is not fully understood. Simple binding of activated Ras to Raf *in vitro* does not lead to

activation of Raf kinase activity (Zhang *et al.*, 1993). It is thought that the major role of Ras-GTP could be to translocate Raf to the plasma membrane where Raf is further activated by several mechanisms that involve serine and tyrosine phosphorylation on Raf and interaction with accessory factors such as 14-3-3 proteins and costimulatory lipids (Kolch *et al.*, 1993, Traverse *et al.*, 1993, Ghosh *et al.*, 1994, Leevers *et al.*, 1994, Stokoe *et al.*, 1994, Wartmann and Davis, 1994, Marias *et al*, 1995, Barnard *et al.*, 1998, King *et al.*, 1998, Tzivion *et al.*, 1998).

The activation of Raf leads to signaling down the Raf-Mek-Erk protein kinase cascade. Once activated, Raf directly binds and phosphorylates the protein kinase Mek on residues serine-218 and serine-222 (Howe *et al.*, 1992, Kyriakis *et al.*, 1992, Macdonald *et al.*, 1993, Alessi *et al.*, 1994, Zheng and Guan, 1994, Johnson *et al.*, 1996). Serine-218 and serine-222 lie in the activation loop of Mek and phosphorylation of these residues is thought to induce a conformational change that leads to improved alignment of catalytic residues giving rise to increased kinase activity (Johnson *et al.*, 1996). *In vitro*, a stable complex can be formed between Ras-GTP, Raf and Mek suggesting a translocation of Mek to the plasma membrane in response to Ras activation *in vivo* (Moodie *et al.*, 1993). In mammals, there are 2 Mek proteins Mek1 and Mek2, encoded by separate genes (Crews *et al.*, 1992, Zheng and Guan, 1993). The function of Mek1 has been more widely studied. Although Mek2 is thought to behave similarly to Mek1 in the cell, there may be one or two differences in the regulation of Mek1 and Mek2 (Wu *et al.*, 1993, Catling *et al.*, 1995). Both Mek proteins, once activated, go on to phosphorylate and activate the protein kinases Erk1 and Erk2. These phosphorylation events take place on the threonine and tyrosine residues of the TEY motif found within the activation loop of Erk1 and Erk2 (Crews and Erikson, 1992, Alessandrini *et al.*, 1992).

Once activated, both Erk proteins go on to phosphorylate a wide range of cellular targets leading to a variety of biochemical changes. For example, it is widely documented that activated Erk translocates to the nucleus where it regulates a number of transcription factors (Treisman, 1996). Elk-1, SAP1 and SAP2, members of the Ternary Complex Factor family, have all been shown to be phosphorylated and activated in response to Ras-Erk signaling (Marias *et al.*, 1993, Hipskind *et al.*, 1994, Price *et al.*, 1995, Gillie *et al.*, 1995). Erk can also phosphorylate several cytoplasmic substrates such as the protein kinases p90rsk (ribosomal S6 kinase) and Mnk (MAPK-interacting kinase) (Blenis, 1993, Fukunaga and Hunter, 1997, Waskiewicz *et al.*, 1997). The function of Mnk is unknown but p90rsk has been shown to phosphorylate and activate the transcription factor CREB (cyclic-AMP response element binding protein) in response to Erk signaling (Xing *et al.*, 1996). It has also been demonstrated that phospholipase A2 (PLaseA2) at the plasma

membrane is phosphorylated and activated by Erk *in vitro* and in COS cells (Lin *et al.*, 1993). *In vitro*, Erk has been shown to phosphorylate SOS and phosphorylated SOS is less efficient at forming an *in vitro* RTK-Grb2-SOS complex (Porfiri and McCormick, 1996). Thus, Erk may function in a negative-feedback mechanism that inhibits Ras activation *in vivo*. Through the Raf-Mek Erk kinase cascade, Ras signaling is linked to a variety of biochemical events that will alter gene expression, activate additional protein kinases, and result in the production of lipid signals destined to be exported from the cell.

PI3K

The function of PI3K downstream of Ras has recently gained much attention due to the implication of PI3K in anti-cell death signaling. It is now understood that a large family of PI3K proteins phosphorylate phosphatidylinositol substrates at the D-3 position in mammalian cells (Whitman *et al.*, 1988, Carpenter and Cantley, 1996a, 1996b, Domin and Waterfield, 1997). In general, PI3K proteins are comprised of a regulatory subunit that is thought to be constitutively bound to a catalytic subunit. To date, 5 types of PI3K regulatory subunits and 7 types of PI3K catalytic subunits have been identified (Domin and Waterfield, 1997). The multiple PI3K proteins arising from the various combinations of subunits are divided into 3 major classes, based on subunit composition and substrate preference (Domin and Waterfield, 1997, Carpenter and Cantley, 1996a, Rameh and Cantley, 1999).

Only class 1 PI3K proteins are effectors for Ras. Class 1 PI3K members are comprised of a p85 regulatory subunit (either p85α, β, or γ) and a p110 catalytic subunit (either p110α, β, δ, or γ) (Carpenter *et al.*, 1990, Otsu *et al.*, 1991, Domin and Waterfield, 1997). Class 1 PI3K proteins commonly phosphorylate phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bis-phosphate (PI-4,5-P_2) to generate the second messengers PI-3,4-P_2 and PI-3,4,5-P_3, respectively. For simplicity, class 1 PI3K proteins will be referred to as PI3K and the subunits will be referred to generically as p85 and p110.

PI3K associates with Ras *in vivo*. It has shown that PI3K activity co-immunoprecipitates with Ras from lysates of Ras transformed rat liver epithelial cells (Sjolander *et al.*, 1991). *In vitro*, it has been shown that Ras-GTP directly binds the p110 subunit of PI3K (Rodriguez-Viciana *et al.*, 1994, 1996). This interaction is thought to promote a conformational change in p110 that directly increases the specific activity of the enzyme (Rodriguez-Viciana *et al.*, 1996). The Ras-PI3K interaction also promotes translocation of PI3K to the plasma membrane. This translocation brings PI3K into proximity with its lipid substrates. In addition, PI3K is thought to be further activated at

the plasma membrane by mechanisms that involve tyrosine phosphorylation of p85 and interaction of tyrosine phosphorylated proteins to the SH2 domain of p85 (Backer *et al.*, 1992, Hayashi *et al.*, 1993, Rodriguez-Viciana *et al.*, 1996). The binding of tyrosine phosphorylated peptides to p85 has been shown to synergize with Ras-p110 interaction to activate PI3K (Rodriguez-Viciana *et al.*, 1996).

Overexpression of activated forms of PI3K can inhibit apoptosis (programmed cell death) induced in a variety of cellular contexts (Franke *et al.*, 1997a, Kauffmann-Zeh *et al.*, 1997, Khwaja *et al.*, 1997). It is now thought that PI3K mediates its anti-apoptotic signaling by activating the protein kinase Akt. *In vitro*, PI3K generated products bind to Akt, promote homodimerization of Akt, and stimulate Akt activity (Franke *et al.*, 1997b, Klippel *et al.*, 1997). In COS cells, overexpression of a constitutively activated version of PI3K leads to Akt activation (Franke *et al.*, 1997b). Akt is also phosphorylated on residues threonine-308 and serine-473 and it is thought that binding of PI3K generated products to Akt promotes a conformational change that results in increased accessibility of threonine-308 to protein kinases in the cell (Alessi *et al.*, 1997, Downward, 1998). Once activated, Akt goes on to phosphorylate a number of substrates that are implicated in the regulation of apoptosis. For example, BAD and caspase-9, both implicated in the intracellular machinery responsible for triggering apoptosis, are phosphorylated *in vitro* and *in vivo* by Akt in response to PI3K activation (Datta *et al.*, 1997 , del Peso *et al.*, 1997, Cardone *et al.*, 1998). In addition, the phosphorylation of both BAD and caspase-9 suppresses their pro-apoptotic function. In response to insulin-like growth factor stimulation of human kidney epithelial cells, Akt has also been shown to translocate to the nucleus where it phosphorylates FKHRL1, a member of the Forkhead transcription factors (Brunet *et al.*, 1999). Phosphorylated FKHRL1 then becomes excluded from the nucleus where it is unable to induce the expression of pro-apoptotic genes such as Fas ligand.

The phospholipid products generated by PI3K likely have other cellular targets besides Akt. Many proteins contain a PH domain and a subset of these proteins may bind the second messengers produced by PI3K. For example, the PH domains found in both SOS and Vav have been shown to bind PI-3,4,5-P_3 (Rameh *et al.*, 1997, Han *et al.*, 1998). Vav contains a Dbl-homology domain and *in vitro*, it has been demonstrated that PI3K products can stimulate the GEF activity of Vav on several Rho family proteins (Han *et al.*, 1998). *In vivo*, it has been shown that activation of Rho proteins downstream of PI3K is enhanced by the overexpression of SOS proteins containing only the Dbl-homology and PH domains (Nimnual *et al.*, 1998). In sum, PI3K appears to link activated Ras to anti-apoptotic signaling and regulation of Rho family proteins.

RalGDS

RalGDS functions as a GEF for Ral, a close Ras relative. Initially, RalGDS was isolated by degenerate PCR (polymerase-chain reaction) using primers complementary to a conserved region within the catalytic domain of the CDC25 Ras GEF (Albright *et al.*, 1993). RalGDS has been shown to interact with Ras *in vitro*, in COS-7 cells overexpressing Ras and RalGDS and in the yeast 2-hybrid systems (Hofer *et al.*, 1994, Kikuchi and Williams, 1996, Urano *et al.*, 1996). In addition, it has been demonstrated that the GEF activity of RalGDS is activated in response to Ras signaling *in vivo* (Urano *et al.*, 1996). Thus, Ras signaling may activate Ral via RalGDS. It is interesting that Ral itself, may regulate Rho family proteins. This link could take place through RalBP, an effector protein for Ral that contains a RhoGAP domain (Cantor *et al.*, 1995). As such, Ras may be at the top of a signaling cascade that takes place through several different low molecular weight G-proteins.

p120GAP as a Ras effector

p120GAP was originally considered a Ras effector for several reasons. p120GAP bound Ras in a manner expected for effectors. For example, p120GAP binds only to the GTP-bound form of Ras and this interaction depended on an intact switch 1 region (Adari *et al.*, 1988, Vogel *et al.*, 1988). p120GAP could also bind the activated oncogenic forms of Ras (Krengel *et al.*, 1990).

Numerous studies have suggested an involvement of p120GAP in the biological effects downstream of Ras. For example, activated Ras protein has been shown to inhibit the stimulation of atrial potassium channels by muscarininc GPCR's in an *in vitro* patch clamping assay (Yatani *et al.*, 1990). Purified p120GAP or the N-terminal region of p120GAP containing only the SH3 and SH2 domains were able to mimic the inhibitory effect of activated Ras in this system (Martin *et al.*, 1992). In a bioassay of Ras signaling in Xenopus oocytes (germinal vesicle breakdown), antibodies generated against the SH3 domain of p120GAP blocked the phenotype caused by activated Ras protein (Pomerance *et al.*, 1996). In NIH3T3 cells, overexpression of an N-terminal fragment of p120GAP also inhibited the transformation caused by an activated Ras gene (Clark *et al*, 1993).

In mammalian cells, activated Ras produces actin cytoskeletal changes (described in further detail below) and p120GAP also appears to be involved in this biological effect. Microinjection of full-length p120GAP into Swiss 3T3 mouse fibroblasts triggers actin stress fiber formation and overexpression of the N-terminal region of p120GAP in rat2 fibroblasts causes a disruption of the actin cytoskeleton and morphological changes

(Leblanc *et al.*, 1998, Mcglade *et al.*, 1993). In addition, microinjection of antibodies against p120GAP (the same antibodies that inhibited germinal vesicle breakdown) into Swiss 3T3 cells prevents actin stress fiber formation induced by activated Ras (Leblanc *et al.*, 1998).

Thus, several pieces of evidence suggest that p120GAP may be an effector for Ras. The precise mechanisms whereby p120GAP leads to biological responses is not known. One model describes the recruitment of p120GAP to the plasma membrane by interaction with Ras-GTP. Following, the SH2 and SH3 domains within the N-terminus of p120GAP interact with additional proteins such as p190 RhoGAP and p60dok. Clearly, the link with p190 suggests a possible mechanism for eliciting actin cytoskeletal changes.

Contrary to the idea of p120GAP as a Ras effector, signaling through p120GAP is apparently dispensable for transformation of some cell types. P34R-Ras, which contains a proline to arginine substitution in the effector (switch 1) domain, has dramatically reduced binding to p120GAP *in vitro* (Stone *et al.*, 1993). *In vivo*, P34R-Ras is GTP-bound at abnormally high levels, likely due to a lack of negative inhibition by p120GAP. Importantly, overexpression of P34R-Ras in rat2 fibroblasts can cause full transformation despite an apparent dissociation from p120GAP binding. The significance of p120GAP as a Ras effector remains unsettled since transformation may be quite different from the biological processes regulated by Ras in normal cells. Also, transformation appears to be a cell type specific process.

Biological effects of Ras signaling

The biochemical responses produced by effector pathways such as the Raf-Mek-Erk cascade, PI3K and RalGDS translate Ras signaling into biological changes. The best understood processes regulated by Ras are cell proliferation, cell differentiation and actin cytoskeleton rearrangements.

Ras and Proliferation

Ras signaling has been associated with cell proliferation since its discovery as a dominant oncogene. The transformation phenotype caused by activated *Ras* oncogenes is characterized by a lack of normal growth control and abnormally high levels of cell division. Indeed, microinjection of activated Ras protein into quiescent cultured mammalian cells of several types stimulated DNA synthesis and led to a transformed-like morphology within 8-12 hours (Feramisco *et al.*, 1984, Stacey and Kung, 1984).

Furthermore, microinjection of inhibitory Ras antibodies blocked DNA synthesis stimulated in quiescent NIH3T3 cells by serum (Mulcahy *et al.*, 1985). These results suggested that Ras is necessary and sufficient for re-entry into the cell-cycle from the quiescent Go-phase and progression into S-phase. Consistent with a role in cell-cycle regulation, Ras has been shown to be activated in mid-G1-phase of the cell-cycle (Taylor and Shalloway, 1996).

Ras, the cell-cycle and cell-cycle arrest

Pieces of the mechanism linking Ras signaling to the cell-cycle have been uncovered and the Raf-Mek-Erk kinase cascade appears central. Like Ras, Erk is activated in a cell-cycle manner (Tamemoto *et al.*, 1992). Erk activity is modestly elevated in G1-S-phase and increases in mitosis (M-phase). In addition, it has been shown that overexpression of activated Ras or wildtype Erk in JEG4 human trophoblasts stimulates transcription of cyclinD1 (Albanese *et al.*, 1995). CyclinD1 activates cyclin-dependent kinase 4 (CDK4) which is thought to drive cells through G1-phase into S-phase by phosphorylating the retinoblastoma protein (Rb) (Chen *et al.*, 1989, Ewen *et al.*, 1993). In its unphosphorylated form, Rb binds to and inhibits the transcription factor E2F which is responsible for inducing expression of genes required for proliferation and DNA synthesis such as c-myc, dihydrofolate reductase, thymidine kinase and DNA polymerase (Nevins, 1992, Johnson *et al.*, 1993). As such, activation of CDK4 enables E2F. In several cell types, inhibition of Ras-Erk signaling using the Mek specific drug PD098059, dominant-negative Ras (N17) or dominant-negative Erk prevented the accumulation of cyclinD1 mRNA and protein leading to cell-cycle arrest (Weber *et al.*, 1997, Peeper *et al.*, 1997). Thus, Ras-Erk signaling appears necessary and sufficient to drive some cell types through G1-phase into S-phase by promoting cyclinD1 expression, activation of CDK4, and phosphorylation of Rb. As expected, expression of N17 Ras in mouse fibroblasts that have a homozygous disruption of *Rb* does not lead to cell-cycle arrest (Peeper *et al.*, 1997).

In many cases, activated Ras promotes growth of mammalian cells but the picture now appears not so simple. Studies on activated *Ras* genes, the promotion of cell growth, and transformation have primarily been done in immortalized rodent fibroblasts such as NIH3T3, rat1 and rat2 cells. In one study, it was shown that overexpression of an activated *Ras* gene in primary (normal) cells such as IMR-90 human diploid fibroblasts, MEF (mouse embryo fibroblasts) and REF52 cells (normal-like rat fibroblasts) leads to an irreversible cell-cycle arrested phenotype called senescence (Serrano *et al.*, 1997). In Ras-induced senescent cells, there is an accumulation of p53 and p16INK4a, 2 proteins that

have been detected in other types of senescent cells (Kulju and Lehman, 1995, Hara *et al.*, 1996). p53 is a transcription factor that induces the expression of p21CIP, an inhibitor of several different classes of CDK's (Harper *et al.*, 1993, Xiong *et al.*, 1993). p16INK4a specifically inhibits CDK4. Thus, in normal mammalian cells, overexpression of activated *Ras* genes leads to permanent cell-cycle arrest though the action of p53 and CDK inhibitors such as p16INK4a and p21CIP. In line with this conclusion, "normal" cells with a homozygous disruption of either p53 or p16INK4a are not growth-arrested by activated Ras (Serrano *et al.*, 1997). Activated Ras appears to increase p53 and p16INK4a expression by signaling through the Raf-Mek-Erk kinase cascade. Overexpression of activated Raf and Mek proteins in IMR-90 cells also gives rise to cell senescence (Lin *et al.*, 1998, Zhu *et al.*, 1998).

In mammalian cells, we have seen that Ras signaling can lead to several different effects on the cell-cycle. In some contexts, Ras-Erk signaling drives cell-cycle progression by up-regulating cyclinD1 which causes the phosphorylation of Rb. In normal cells on the other hand, activated Ras leads to the production of CDK inhibitors that trigger permanent cell-cycle arrest. What determines the balance between these 2 outcomes? The most important factor is likely the presence of proteins such as p53, Rb, p16INK4a and p21CIP, which in normal cells, form a "growth alarm" system. Activated Ras genes in normal cells trigger this alarm leading to cell-cycle arrest. Immortalized cultured cells frequently have a loss of function in either p53, Rb, p16INK4a or p21CIP. Loss of one or more of these proteins essentially compromises the cell's alarm allowing activated Ras to stimulate proliferation and transformation. However, the effects of Ras on cell fate are still not that simple. In some other contexts, Ras signaling leads to apoptosis. For example, overexpression of activated Ras in cells detached from the substratum, cells expressing an inhibitor of NF-κB, and cells overexpressing c-myc triggers apoptosis (Kauffmann-Zeh *et al.*, 1997, Khwaja *et al.*, 1997, Mayo *et al.*, 1997). It is not fully understood why the cell undergoes apoptosis in these cases. It is possible that certain potentially dangerous situations, such as when the cell is detached from the substratum, stimulate a "cautionary" signaling pathway to alert the cell. Activated Ras signaling in conjunction with this cautionary signal may trigger a sensor in the apoptotic machinery of cell. This postulated mechanism could be involved in preventing cancer. For example, a Ras transformed cell that can survive without substratum attachment could potentially spread through the body and invade other tissues.

Some evidence has suggested that the mechanism linking Ras signaling to apoptosis involves the Raf-Mek-Erk kinase cascade and p53 (Fukasawa and Vande Woude, 1997). Mos is a protein kinase that has been shown to function in *Xenopus* oocyte meiotic

maturation (Choi *et al.*, 1996). Because Mos phosphorylates and activates Mek, Mos overexpression can be used to stimulate Mek and Erk mammalian experimental systems (Posada *et al.*, 1993, Fukasawa and Vande Woude, 1997). Overexpression of Mos in MEF's that express wildtype levels (+/+) of p53 led to a dramatic increase in the frequency of apoptosis (Fukasawa and Vande Woude, 1997). However, in MEF's lacking p53 (-/-), Mos-induced apoptosis was dramatically inhibited. These results are consistent with a model where Ras-induced signaling through the Raf-Mek-Erk kinase cascade leads to apoptosis via a p53-dependent mechanism.

Multiple pathways downstream of Ras for Transformation

Evidence has been gathered that argues for multiple Ras effectors, namely the Ras-Mek-Erk cascade, PI3K and RalGDS, in cellular transformation. Convincing evidence for the function of these 3 pathways was provided from studies of Ras effector mutants in NIH3T3 cells (Rodriguez-Viciana *et al.*, 1997). Point mutations in the switch 1 region of Ras can selectively disrupt interactions with each of the different Ras effectors. For example, an activated mutant of Ras (V12) with serine substituted at residue 35 (V12, S35) selectively binds to and activates Raf. Conversely V12, G37 and V12, C40 Ras alleles selectively activate RalGDS and PI3K, respectively. In NIH3T3 cells, these Ras effector mutants cooperate to cause cellular transformation. Overexpression of each allele alone gave rise to only low levels of transformation whereas overexpression of any 2 mutants together gave rise to dramatic increases in transformation efficiency. Thus, it appears that distinct signals from Ras, transmitted through Raf, PI3K and RalGDS, are involved in transformation, at least in NIH3T3 cells.

The effect of PI3K signaling on transformation may be due to its anti-apoptotic signaling. As mentioned above, co-expression of activated *Ras* gene with c-myc triggers apoptosis in rat1 fibroblasts (Kauffmann-Zeh *et al.*, 1997). Apoptosis was exacerbated when the S35 substitution was made in the activated Ras gene. This substitution effectively dissociates Ras from PI3K signaling. Taken together, the data suggest that Ras normally activates PI3K to inhibit apoptosis.

RalGDS may promote transformation through the function of Rho proteins. I described earlier how RalGDS, via Ral and RalBP, could activate Rho proteins. In Swiss 3T3 fibroblasts overexpressing activated Ras, inhibition of RhoA leads to the accumulation of p21CIP and inhibition of DNA synthesis (Olson *et al.*, 1998). This result suggests that Ras signals through a RhoA-dependent pathway to inhibit p21CIP-mediated cell-cycle arrest. Also, overexpression of dominant-negative Rac, a Rho family member, inhibited transformation induced by activated Ras in NIH3T3 cells (Qiu *et al.*, 1995).

Contrary to the idea of multiple transforming Ras effector pathways, some evidence argues that signaling through the Raf-Mek-Erk pathway is sufficient for transformation. First, overexpression of activated Raf and Mek proteins can transform NIH3T3 and rat2 cells (Feig and Cooper, 1988, Mansour *et al.*, 1994, Bottorff *et al.*, 1995). Second, a number of effector mutants in the v-Ras (activated) background have been isolated that bind Raf but not RalGDS in the yeast 2-hybrid system (Stang *et al.*, 1997). These effector alleles are distinct from the 3 effector mutants studied by Rodriguez-Viciana *et al.* (1997). Overexpression of the v-Ras effector mutants isolated by Stang *et al.* (1997) transformed rat2 cells as efficiently as v-Ras without a mutation in the effector domain. From their data, Stang *et al.* (1997) concluded that signaling through the Raf-Mek-Erk cascade was sufficient for full transformation, at least in rat2 cells. Also, Stang *et al.* (1997) failed to confirm cooperation between the S35, G37, and C40 effector mutants in the transformation of rat2 cells.

Can the data arguing for one transforming pathway be compatible with the observations of Rodriguez-Viciana *et al.* (1997) which argue for multiple transforming pathways? Perhaps a cell can be fully transformed if activated Raf and Mek proteins are overexpressed at high enough levels. In the study of Stang *et al.* (1997), the v-Ras effector mutants were overexpressed in rat2 cells using retroviral vectors. In contrast, Rodriguez-Viciana *et al.* (1997) overexpressed their effector mutants by transfecting expression plasmids into NIH3T3 cells using a lipid-based reagent. Thus, the effector mutants of Stang *et al.* (1997) may have been expressed at a higher level than the effector mutants in the study by Rodriguez-Viciana *et al.* (1997). However, higher expression levels in the rat2 cells studied by Stang *et al.* (1997) cannot explain why cooperation was not observed between the S35, G37, and C40 effector mutants. Rodriguez-Viciana *et al.* (1997) constructed these effector mutants in the V12 Ras background (glycine-12 mutated to valine-12) while Stang *et al.* (1997) constructed the same effector mutations in the v-Ras background (glycine-12 mutated to arginine and alanine-59 mutated to threonine). Perhaps the effector mutants in the context of V12 Ras function differently than in the context of v-Ras. Alternatively, sensitivity to transformation may be quite different between rat2 and NIH3T3 cells.

In a Ras transformed cell, all of the signals that are elicited through the many Ras effectors likely contribute to the overall transformed phenotype. Whether a cell needs activation of all effectors for full transformation probably depends on the amplitudes of the signals down the various pathways. For example, strong signaling down 1 pathway may be equivalent to weak signals down 3 effector pathways. In addition, the promotion of transformation can be very cell-type specific.

Ras and differentiation

Ras-Erk signaling often leads to cell proliferation but in some instances, Ras-Erk signaling can also promote cell differentiation. It was first noted that infection of PC12 cells with retroviruses containing activated *Ras* genes could induce neuronal differentiation (Noda *et al.*, 1985). A role of Erk signaling in the differentiation of hematopoietic cells is also documented. Dominant-negative and activated forms of Mek were used to demonstrate the involvement of Erk in the differentiation of immature CD4-8- to mature CD4+8+ thymocytes (Crompton *et al.*, 1996). Similar strategies showed that maturation of human megakaryocytes by stem cell factor depended on Erk signaling (Melemed *et al.*, 1997). A role of Ras-Erk signaling in the differentiation of 3T3-L1 fibroblasts into adipocytes has also been demonstrated (Benito *et al.*, 1991).

Ras-Erk signaling has been shown to relay both differentiation and proliferation messages within the same cell type. For example, stimulation of PC12 cells with EGF leads to Ras activation through a Shc-independent pathway and robust but transient activation of Erk (Traverse *et al.*, 1992, Nguyen *et al.*, 1993, Marshall, 1995). Transient activation of Erk signals for cell proliferation. On the other hand, stimulation of PC12 cells with NGF activates Ras through a Shc-dependent pathway that leads to sustained Erk activation. Sustained Erk activation signals for differentiation and neurite outgrowth. Thus, the duration of Erk signaling appears to determine cell fate. Tombes *et al.* (1998) have shown that NGF stimulation of PC12 cells leads to increased levels of p21CIP and an inhibition of DNA synthesis. As such, the mechanism that interprets the duration of Ras-Erk signaling appears to involve cell-cycle arrest. It remains unclear how the arrested cell goes on to initiate differentiation.

Ras and cytoskeletal rearrangements

Ras signaling has been well documented to induce actin cytoskeletal rearrangements. For example, Ras transformed cells have a distinct morphology as compared to non-transformed cells (Seeburg *et al.*, 1984, Huff *et al.*, 1997). Furthermore, it was observed that microinjection of activated Ras protein into rat embryo fibroblasts led to an actin cytoskeletal change termed membrane ruffling within 1-2 hours (Bar Sagi and Feramisco, 1986). Activated Ras likely elicits these effects through Rho proteins such as Rac1, RhoA and Cdc42 which are now well understood to regulate actin cytoskeletal changes (Ridley *et al.*, 1992, Ridley and Hall, 1992, Nobes and Hall, 1995). The function

of Rho proteins in actin cytoskeletal changes downstream of Ras is supported by the inhibition of Ras-induced membrane ruffling by dominant-negative mutants of Rac (Rodriguez-Viciana *et al.*, 1997). Thus, Rho proteins may be involved in 2 important aspects of Ras signaling: controlling cell shape changes and, as described earlier, preventing cell-cycle arrest.

Role of Ras in mammalian development

From studies using gene "knock-out" approaches, we now understand that Ras function is important for mammalian development. For example, homozygous disruption of the gene encoding p120GAP gave rise to mice that died approximately day 10 of embryogenesis (Henkemeyer *et al.*, 1995). These embryos showed defective organization of the vascular system and extensive neuronal death. Similarly, the *NF1* gene has been knocked-out and these mice die at day 13-14 of embryogenesis with defects in cardiac development (Jacks *et al.*, 1994). Both p120GAP and NF1 may have Ras-independent functions but the observed defects in both cases are thought to be though loss of Ras negative regulation. Indeed, mice with homozygous disruptions in both *p120GAP* and *NF1* have a more severe phenotype that included embryonic arrest at day 8. Others have attempted to knock out the gene encoding Grb2 (Cheng *et al.*, 1998). Embryos with homozygous disruptions could not be observed and are thought to have arrested very early, soon after the time of implantation in the uterus. Embryonic stem cells with homozygous *Grb2* disruption could be generated and these failed differentiate in cell culture. Thus, loss of Grb2, and consequently SOS function, severely impairs cell differentiation. Homozygous disruption of the gene encoding RasGRF1, the brain-specific Ras GEF, led to more subtle phenotypes (Brambilla *et al.*, 1997). These mice developed normally into adults but were behaviorally impaired at emotion conditional learning. The basolateral amygdala of these mice, a region involved in conditional memory also showed abnormal long-term plasticity in electophysiological tests. These gene knock-out strategies clearly show that Ras regulatory proteins, and therefore Ras itself, is important in the development of several different organ systems.

Future of Ras signaling research

At one time, Ras signaling was heralded for its simplicity in linking peptide growth factors through the linear Raf-Mek-Erk pathway to the modulation of nuclear transcription factors. Many signaling branches leading to, and stemming from Ras have since been

added and it is clear that many additional facets of Ras signaling remain to be uncovered. Powerful genetic schemes in model organisms are identifying novel Ras signaling proteins that add many interesting twists to the Ras field. For example, novel Ras effectors have been identified such as Rin1 which may link Ras with the signaling from the Abl tyrosine kinase (Han and Colicelli, 1995, Han *et al.*, 1997). AF6/Rsb1/canoe is a putative Ras effector that is associated with cellular junctions and Notch signaling (Hunter, 1997, Kuriyama *et al.*, 1996). A yeast 2-hybrid screen for Ras interacting proteins has identified from a *C. elegans* cDNA library, another putative effector, PLC210, that contains a phospholipase C domain and a region with homology to the CDC25 Ras GEF (Shibatohge *et al.*, 1998). Sur-8, isolated in a *C. elegans* genetic screen, interacts directly with Ras but does not appear to be a typical Ras effector since interaction is not GTP-dependent (Sieburth *et al.*, 1998). Sur-8 may be a scaffolding protein that is involved in modulating the interaction of effectors and regulatory proteins to Ras. Ksr and Cnk, 2 proteins isolated in *Drosophila* screens, are now understood to be involved in Ras-Raf signaling (Sundaram and Han, 1995, Therrien *et al.*, 1995, 1998, Sternberg and Alberola-Ila, 1998). Lastly, a genetic selection in *Drosophila* has recently identified Sprouty, a protein that can interact with Grb2 and GAP1 (Casci *et al.*, 1999). Thus, Sprouty may be an additional factor involved in Ras regulation. Ras signaling in the future will likely resemble a complex circuit board rather than a linear model and it appears this circuitry will involve additional players functioning via novel mechanisms.

Theses objectives

This thesis describes 3 projects that are each aimed at novel mechanisms of Ras regulation and function in mammalian cells.

1. We discovered that Ras becomes activated in rat2 cells that are exposed to hypothermic stress. A primary objective of this project was to characterize the mechanism behind this novel form of Ras activation through studies done *in vitro* and in rat2 cells. This project also aimed to determine the biochemical and physiological consequences of hypothermically-activated Ras.

2. Our lab has isolated a novel Ras GEF, named RasGRP, from a rat brain cDNA library. RasGRP represents a new class of Ras GEF due to the presence of a diacylglycerol binding domain and a pair of EF-hand Ca^{2+} binding motifs. My work in this project was aimed at the role of RasGRP in linking diacylglycerol signals to Ras activation in rat2 cells.

3. Dr. D.N. Brindley and co-workers (Department of Biochemistry, University of Alberta) observed PI3K activation in response to the sphingolipid second messenger, ceramide. In rat2 cells that I engineered to overexpress N17 Ras, ceramide-induced activation of PI3K was inhibited. In an effort to understand further the relationship between Ras and ceramide, I studied Ras activation in rat2 cells treated with cell-permeable C2-ceramide.

Tumor Type	No. samples (+)	No. samples Tested	Percentage
Acute myelogenous leukemia	14	52	27 %
Lung adenocarcinoma	15	45	33 %
Colon adenocarcinoma	64	158	40 %
Pancreas adenocarcinoma	53	63	84 %
Thyroid follicular carcinoma	8	15	53 %

Table 1-1. Frequency of a mutated *Ras* gene in various human cancers. Shown for each tumor type are the number of samples positive (+) for a mutated *Ras* gene, total number of samples studied, and the calculated percentage. The data for each tumor type were obtained from independent studies. Mutations in *Ras* were detected using either a oligodeoxynucleotide hybridization assay or a RNase mismatch cleavage assay. Since these techniques only test a portion of the *Ras* gene, the percentages reported here are minimum estimates. Adapted from Bos, 1989.

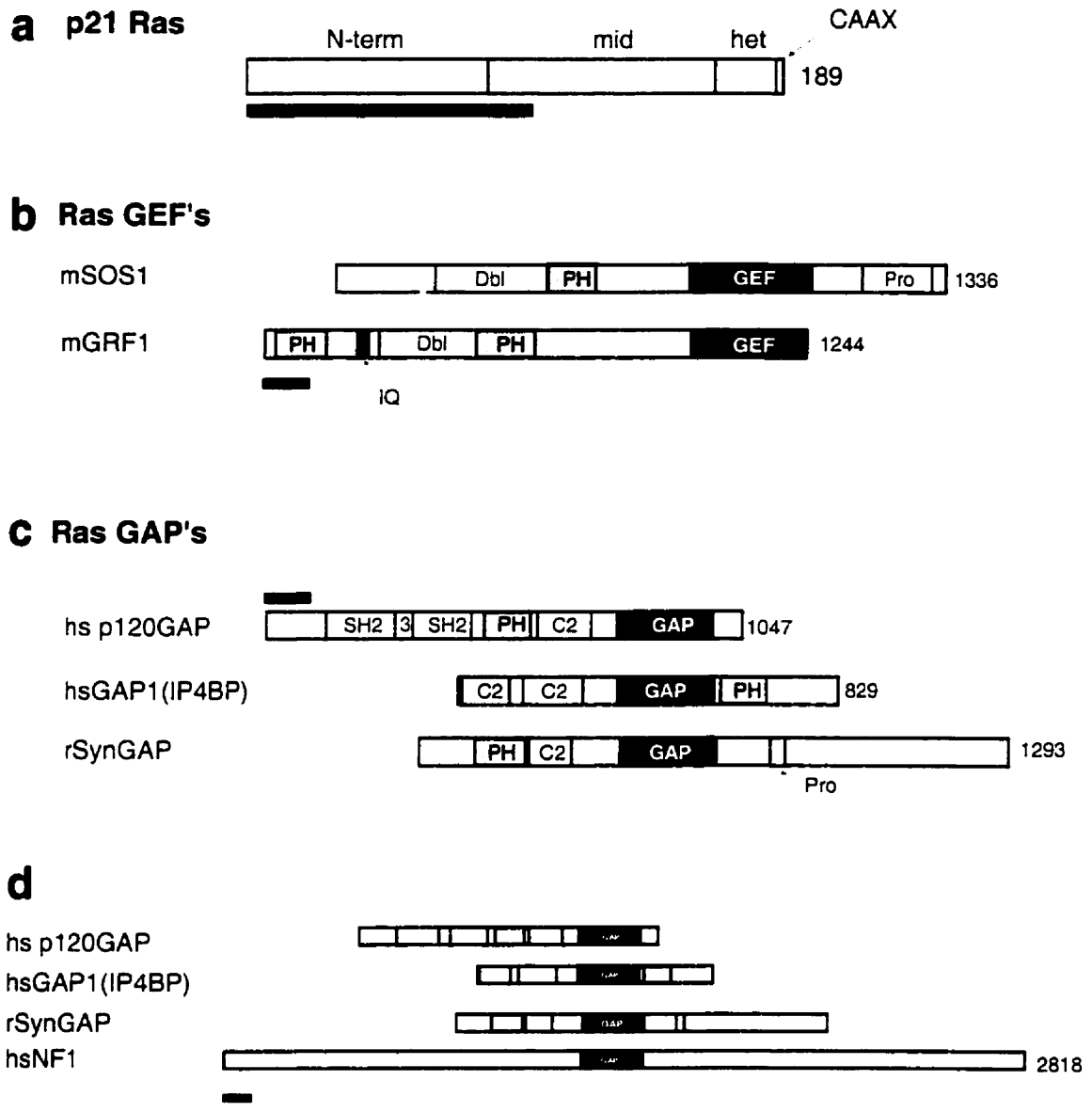

Figure 1-1. Domain structure schematics of p21 Ras, Ras GEF's, and Ras GAP's. a. Analysis of all mammalian Ras proteins has defined 4 domains: an N-terminal domain (residues 1-85) of very high conservation (N-term), a region in the middle of the protein that is slighty variable (residues 86-166) (mid), a C-terminal region of high heterogeneity (residues 167-185) (het), and the last 4 residues (186-189) which comprise the CAAX motif. **b.** There are 3 main classes of mammalian Ras GEF's: the SOS family, represented by murine SOS1, the GRF family, represented by murine GRF1, and RasGRP (domains defined as in Boguski and McCormick, 1993, Fam *et al.*, 1997). Details on RasGRP are presented in chapter 4. **c and d.** There are 4 main classes of mammalian Ras GAP's which are represented by: human p120GAP, human GAP1(IP4BP), rat SynGAP, and human NF1 (domains defined as in Boguski and McCormick, 1993, Cullen *et al.*, 1995, Scheffzek *et al.*, 1997, Chen *et al.*, 1998). Scale: black bar represents 100 amino acids. Domain labels: Pleckstrin-homology domain (PH), Dbl-homogy domain (Dbl), Calmodulin-binding IQ domain (IQ), GEF catalytic domain (GEF), poly-proline/proline-richsequence (Pro), SH2 domain (SH2), SH3 domain (3), C2 domain (C2), GAP catalytic domain (GAP).

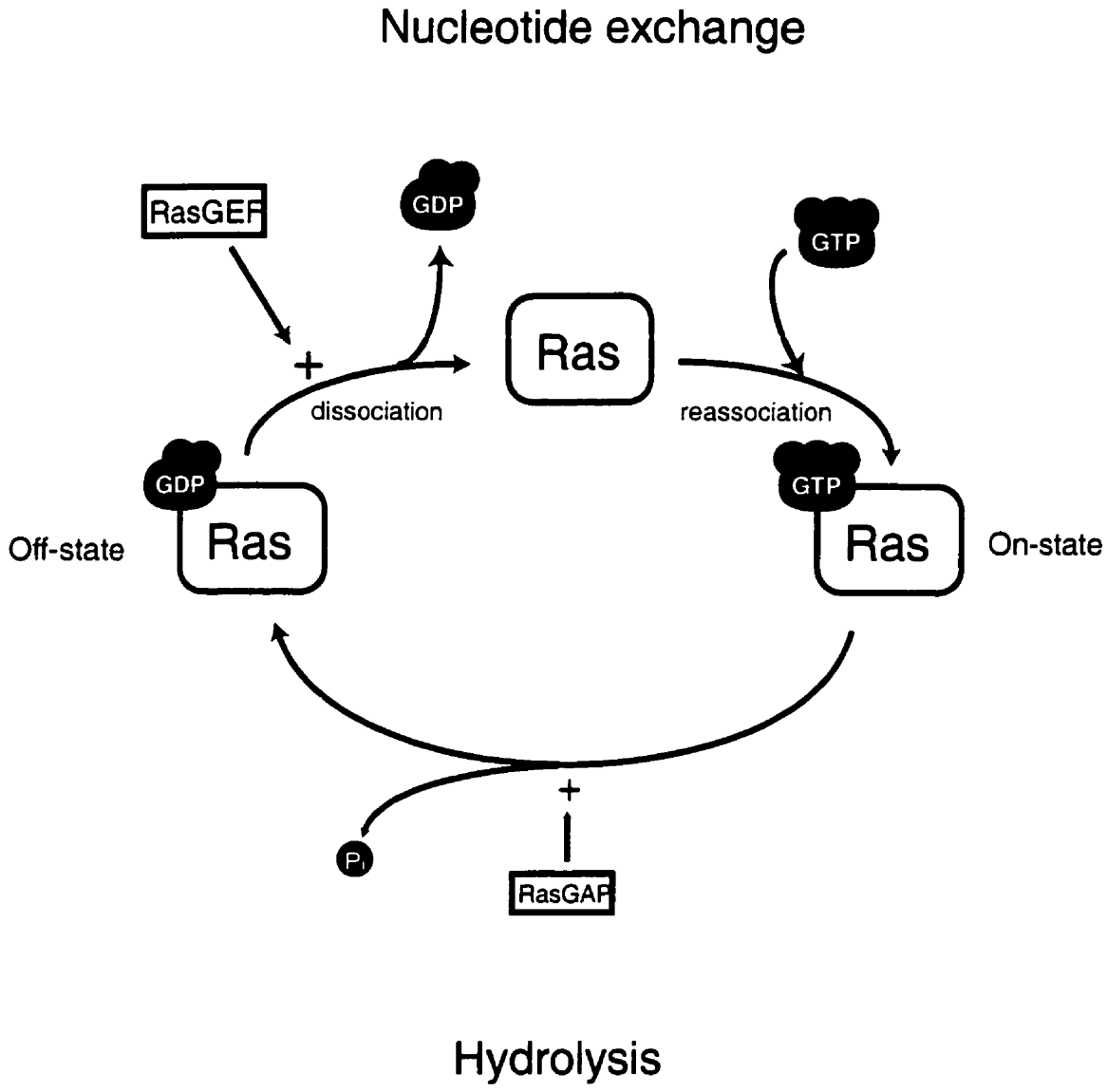

Figure 1-2. Ras GTP cycle - p21 Ras alternates between a GDP-bound-"Off" and a GTP-bound-"On" state. Ras-GDP is converted to Ras-GTP by dissociation of GDP followed by reassociation of GTP, a process known as nucleotide exchange. In the cell, nucleotide exchange is catalysed by Ras GEF's which increase the rate of nucleotide dissociation. Ras-GTP is converted to Ras-GDP by the intrinsic GTPase activity of Ras. The rate of this reaction is greatly enhanced in the cell by Ras GAP's. Note that the interaction between the Ras GEF and nucleotide-free Ras is not illustrated in this schematic.

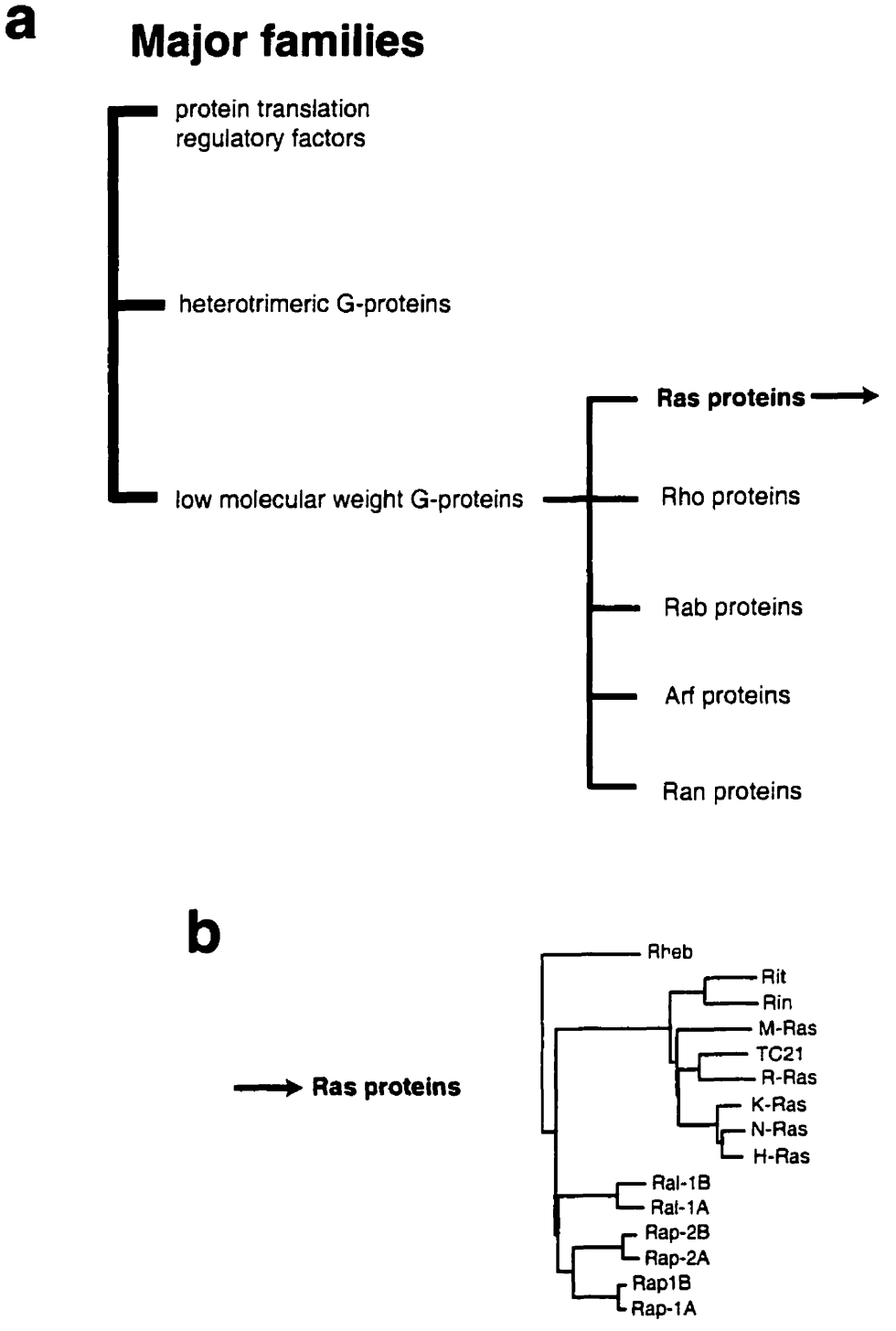

Figure 1-3. Map of the G-protein superfamily. a. There are 3 major families of G-proteins: the protein translation regulatory factors, the heterotrimeric G-proteins, and the low molecular weight G-proteins. The low molecular weight G-proteins can be further divided into 5 smaller families: Ras, Rho, Rab, Arf and Ran proteins. **b.** The members of the Ras protein family are shown in a phylogenetic tree (dendogram). The distance between any two family members is directly proportional to the homology in their primary sequences. Adapted from Campbell *et al.*, 1998.

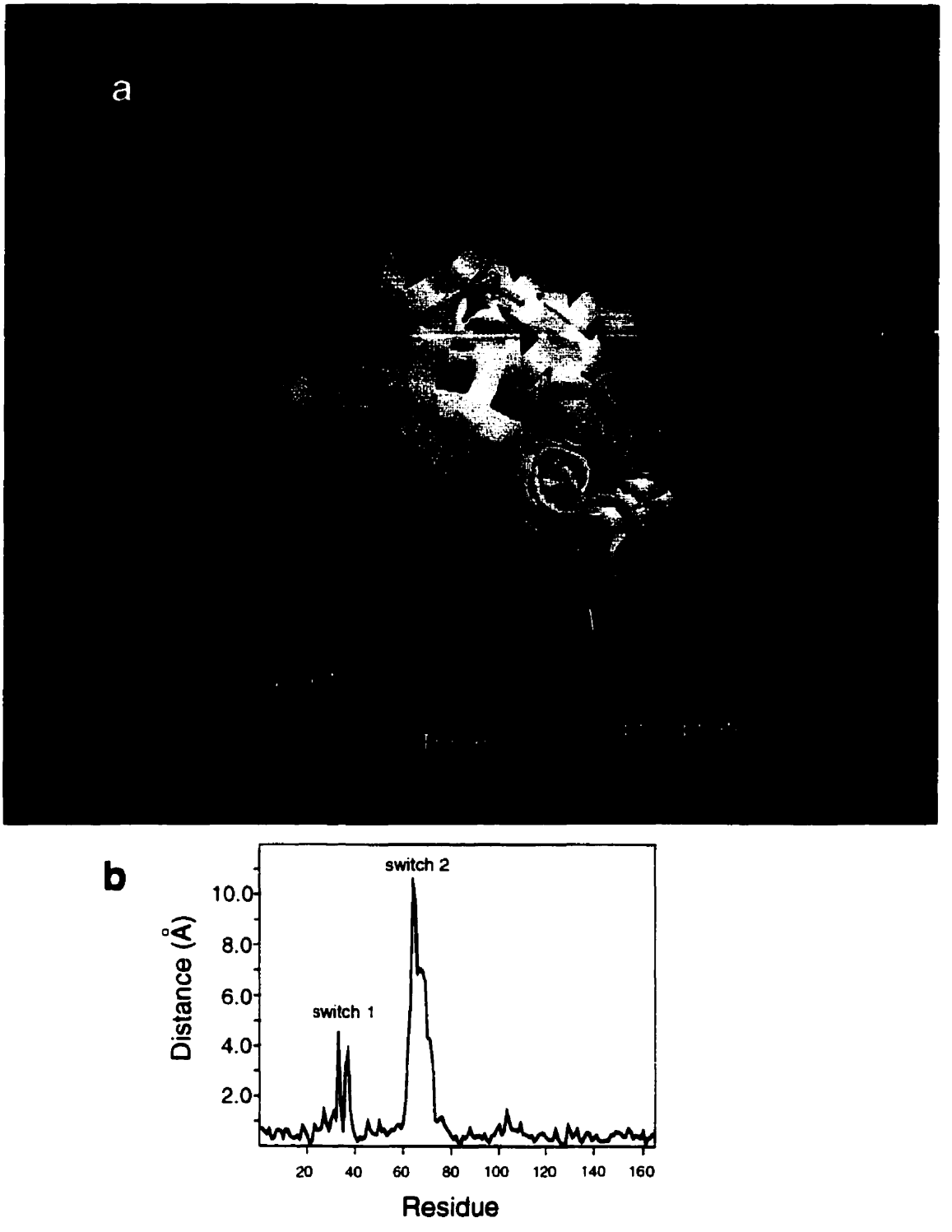

Figure 1-4. 3-D structure of Ras. a. 3-D structure of Ras in complex with GTP. The switch 1 (residues 32-38) and switch 2 (residues 60-76) regions are shown in green. The positions of residues threonine-35 and glutamine-61 and the γ-phosphate moiety of GTP are also indicated. Representation obtained using published structure coordinates of Pai *et al.*, 1990. **b.** Graph showing the movement of Ras amino acid residues observed when comparing the crystal structures of Ras-GTP and Ras-GDP. The switch 1 and switch 2 regions are defined by their high degree of conformational change. Adapted from Milburn *et al.*, 1990

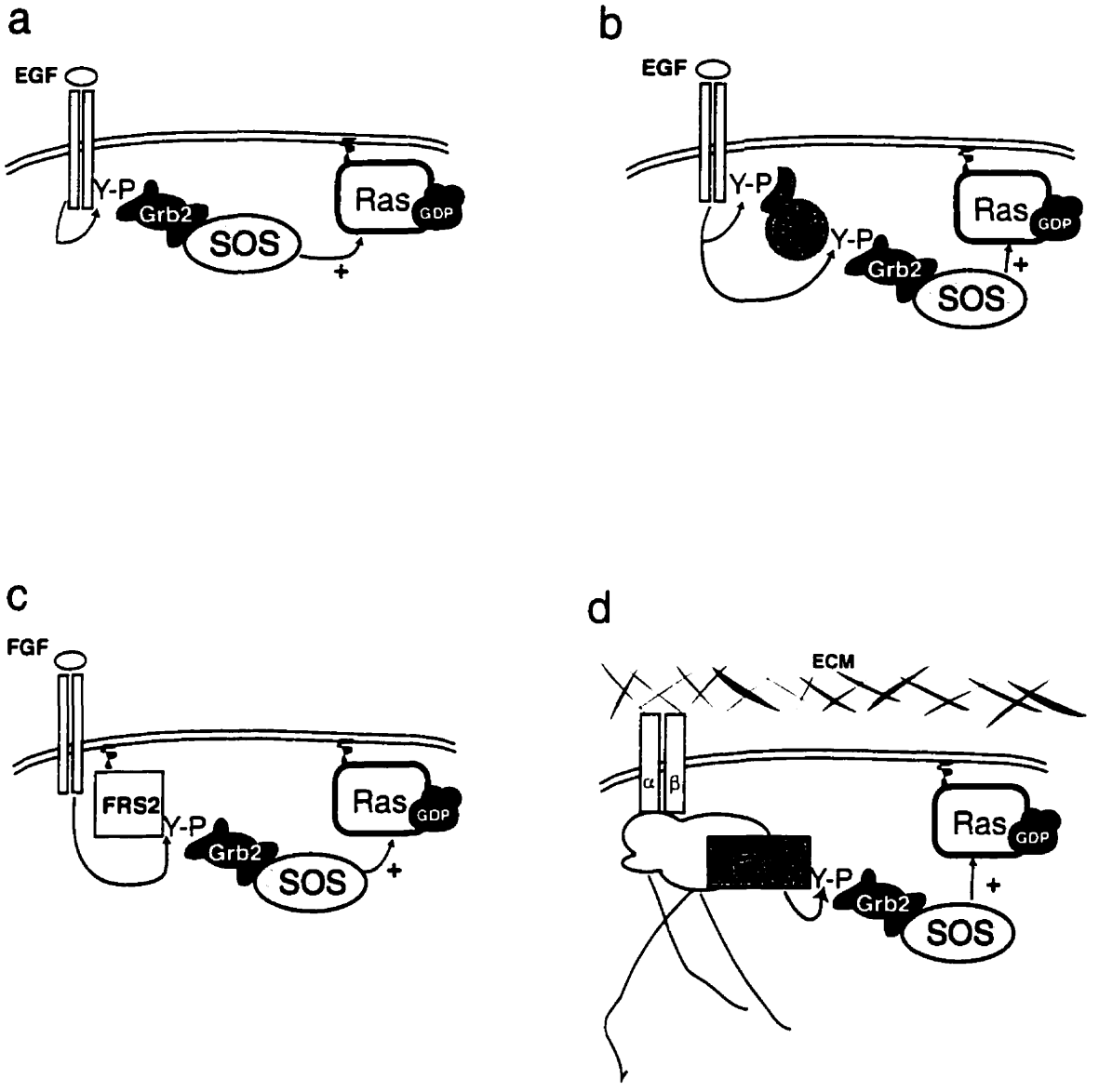

Figure 1-5. Recruitment of the Grb2-SOS complex to the plasma membrane in response to extracellular stimuli. a. Stimulation of mammalian cells with growth factors such as EGF result in dimerization of the RTK and autophosphorylation on tyrosine residues (Y-P) in the RTK intracellular domain. Complexes of Grb2-SOS are recruited to the plasma membrane through interaction of the SH2 domain of Grb2 with phosphotyrosine residues on the RTK . Once at the plasma membrane, SOS positively regulates Ras. **b.** In response to EGF stimulation, adapter proteins such as Shc can also bind to the RTK phosphotyrosine residues. RTK's can phosphorylate tyrosine residues in Shc which provides another group of ligands for the SH2 domain of Grb2. **c.** FGF can stimulate its RTK to phosphorylate tyrosine residues on the FSR2 docking protein which is anchored to the plasma membrane by a lipid modification. Grb2-SOS complex can be recruited to the plasma membrane by interacting with the phosphotyrosine residues on FSR2. **d.** Adhesion of mammalian cells to the extracellular matrix (ECM) can stimulate the formation of focal adhesions centered around the α and β integrin subunits. Focal adhesions are protein super-complexes that contain at least 7 different proteins including filamentous actin. FAK, a protein tyrosine kinase contained within focal adhesions, autophosphorylates on tyrosine residues to provide sites of attachment for Grb2-SOS complexes.

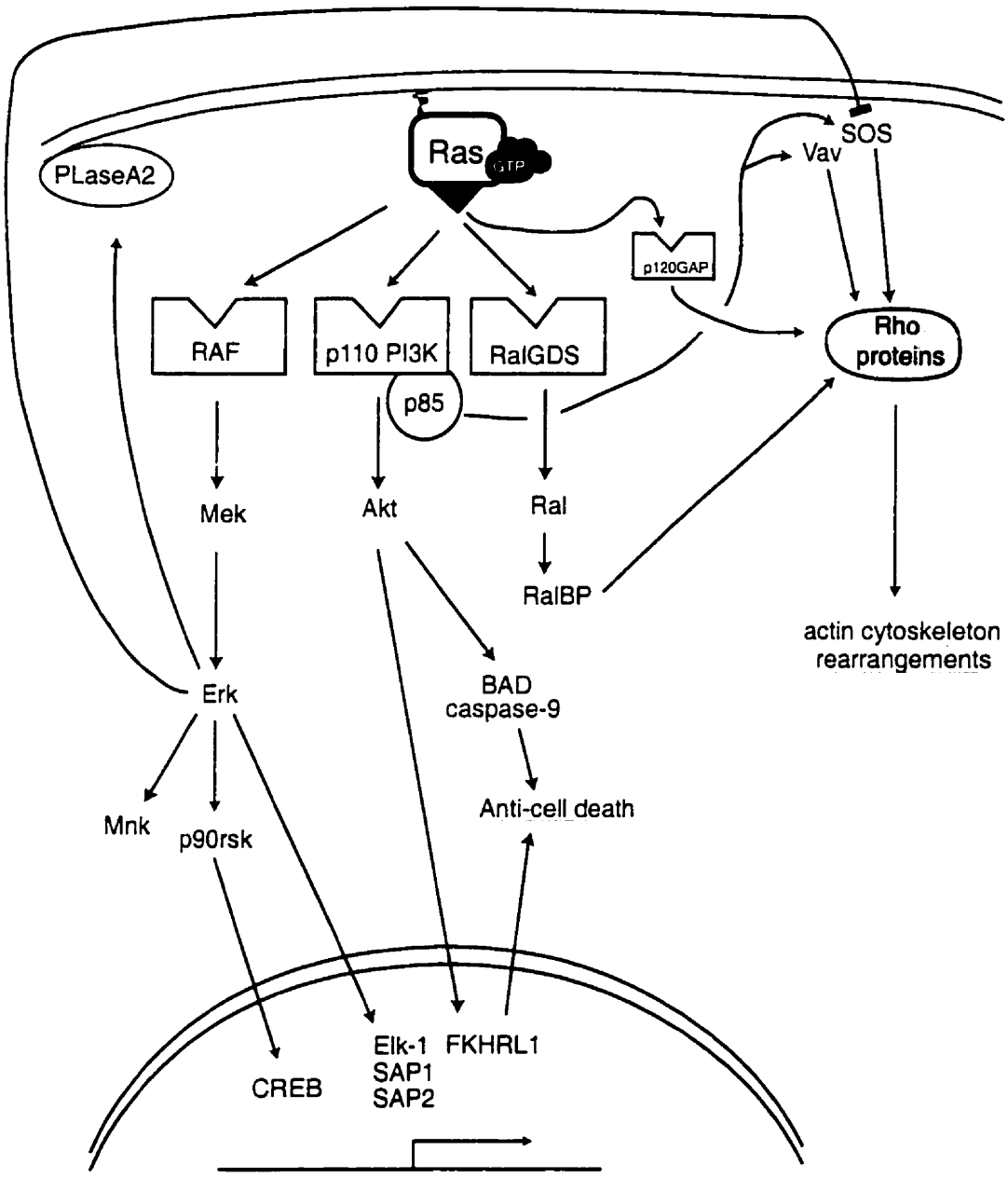

Figure 1-6. Biochemical responses downstream of Ras. The active GTP-bound form of Ras can signal to downstream effectors such as Raf, PI3K and RalGDS. p120GAP may also be a Ras effector. Through these effector molecules, Ras signaling gives rise to a number of events that include the activation of protein kinases, modulation of nuclear transcription factors, and actin cytoskeleton rearrangements. SOS and p120GAP are positive and negative regulators of Ras, respectively. However, this schematic only illustrates the signaling functions of SOS and p120GAP downstream of Ras. Details are within text.

References

Adari, H., Lowy, D.R., Willumsen, B.M., Der, C.J., and McCormick, F. (1988) Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. *Science* **240**:518-521.

Albanese, C., Johnson, J. Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R.G. (1995) Transforming p21 *ras* mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.* **270**:23589-23597.

Albright, C.F., Giddings, B.W., Liu, J., Vito, M., and Weinberg, R.A. (1993) Characterization of a guanine nucleotide dissociation stimulator for a *ras*-related GTPase. *EMBO J.* **12**:339-347.

Alessandrini, A., Crews, C.M., and Erikson, R.L. (1992) Phorbol ester stimulates a protein-tyrosine/threonine kinase that phosphorylates and activates the *Erk*-1 gene product. *Proc. Natl. Acad. Sci.* **89**:8200-8204.

Alessi, D.R., Saito, Y., Campbell, D.G., Cohen, P., Sithananandam, G., Rapp, U., Ashworth, A., Marshall, C.J., and Cowley, S. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by p74 $^{raf\text{-}1}$. *EMBO J.* **13**:1610-1619.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology* 7:261-269.

Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the r*as* signaling pathway. *Cell* **78**:949-961.

Avis, J.M., and Clarke, P.R. (1996) Ran, a GTPase involved in nuclear processes: its regulators and effectors. *J. of Cell Science* **109**:2423-2427.

Backer, J.M, Myers Jr, M.G., Shoelson, S.E., Chin, D.J., Sun, X., Margolis, B., Skolinik, E.Y., Schlessinger, J., and White, M.F. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* **11**:3469-3479.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990) The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. *Cell*:**63**:851-859.

Barbacid, M. (1987) *ras* Genes. *Ann. Rev. Biochem.* **56**:779-827.

Barnard, D., Diaz, B., Clawson, D., and Marshall, M. (1998) Oncogenes, growth factors and phorbol esters regulate *Raf*-1 through common mechanisms. *Oncogene* **17**:1539-1547.

Bar-Sagi, D., and Feramisco, J.R. (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibrobalsts by *ras* proteins. *Science* **233**:1061-1068.

Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S., and Downward, J. (1992) Aberrant regulation of *ras* proteins in malignant tumour cells from type 1 neurofibromatosis patients. *Nature* **356**:713-715.

Benito, M., Porras, Q., Nebreda, A.R., and Santos, E. (1991) Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of *ras* oncogenes. *Science* **253**:565-568.

Bertics, P.J., Chen, W.S., Hubler, L., Lazar, C.S., Rosenfeld, M.G., and Gill, G.N. (1988) Alteration of epidermal growth factor receptor activity by mutation of its primary carboxyl-terminal site of tyrosine self-phosphorylation. *J. Biol. Chem.* **263**:3610-3617.

Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci.* **90:**5889-5892.

Boguski, M.S., and McCormick, F. (1993) Proteins regulating Ras and its relatives. *Nature* **366**:643-654.

Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L., and Pelicci, P. (1996) Not all Shc's roads lead to *ras*. *TIBS* **21**:257-260.

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D., and Kuriyan, J. (1998) The structural basis of the activation of *ras* by *sos*. *Nature* **394**:337-343.

Bos, J. (1989) *Ras* oncogenes in human cancer: a review. *Cancer Research* **49**:4682-4689.

Bottomley, J.R., Reynolds, J.S., Lockyer, P.J., and Cullen, P.J. (1998) Structural and functional analysis of the putative inositol 1,3,4,5- tetrakisphophate receptors $GAP1^{IP4BP}$ and $GAP1^{m}$. *Biochemical and Biophysical Research Communications* **250**:143-149.

Bottorff, D., Stang, S., Agellon, S., and Stone, J.C. (1995) *Ras* signalling is abnormal in *c-raf1 MEK1* double mutant. *Mol. Cell. Biol.* **15**:5113-5122.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348:**125-132.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349:**117-127.

Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992) Identification of murine homologues of the *Drosophila* son of sevenless gene: potential activators of *ras*. *Proc. Natl. Acad. Sci.*. **89**:6511-6515.

Brambilia, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C.E., Ramsey, M., Wolfer, D.P., Cestari, V., Rossi-Arnaud, C., Grant, S.G.N., Chapman, P.F., Lipp, H., Sturani, E., and Klein, R. (1997) A role for the *ras* signalling pathway in synaptic transmission and long-term memory. *Nature* **390**:281-286.

Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C., and Sterweis, P.C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**:1137-1144.

Brtva, T.R., Drugan, J.K., Ghosh, S., Terrell, R.S>, Campbell-Burk, S., Bell, R.M., and Der, C.J. (1995) Two distinct *raf* domains mediate interaction with *ras. J. Biol. Chem.* **270**:9809-9812.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* **96**:857-868.

Buday, L.,and Downward, J. (1993) Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and *Sos* nucleotide exchange factor. *Cell* **73**:611-620.

Campbell, S.L., Khosravi, R., Rossman, K.L., Clark, G. J., and Der, C.J. (1998) Increasing complexity of *ras* signaling. *Oncogene* **17**:1395-1413.

Cantor, S., Urano, T., and Feig, L.A. (1995) Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.* **15**:4578-4584.

Capon, D.J., Seeburg, P.H., McGrath, J.P, Hayflick, J.S., Edman, U., Levinson, A.D., and Goeddel, D.V. (1983a) Activation of Ki-*ras* gene in human colon and lung carcinomas by two different point mutations. *Nature* **304**:507-513.

Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. and Goeddel, D.V. (1983b) Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its homologue. *Nature* **302**:33-37.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**:1318-1321.

Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S., and Cantley, L.C. (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.* **265**:19704-19711.

Carpenter, C.L., and Cantley, L.C. (1996a) Phosphoinositide 3-kinase and the regulation of cell growth. *Biochemica et Biophysica Acta* **1288**:M11-M16.

Carpenter, C.L., and Cantley, L.C. (1996b) Phosphoinositide kinases. *Current Opinion in Cell Biology* **8**:153-158.

Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., and Clarkson, B. (1997) $p62^{dok}$: a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell* **88**:197-204.

Casci, T., Vinos, J., and Freeman, M. (1999) Sprouty, an intracellular inhibitor of ras signalling. *Cell* **96**: 655-665.

Catling, A.D., Schaeffer, H.-J., Reuter, C.W.M., Reddy, G.R., and Weber, M.J. (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. *Mol. Cell. Biol.* **15**:5214-5225.

Chardin, P., Camonis, J.H., Gale, N.W., Van Aelst, l., Schlessinger, J., Wigler, M.H., and Bar-Sagi, D. (1993) Human *sos* 1: a guanine nucleotide exchange factor for *ras* that binds to GRB2. *Science* **260**:1338-1343.

Chen, H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M.B. (1998) A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron.* **20**:895-904.

Chen, P. Scully, P., Shew, J., Wang, J.Y.J., and Lee, W. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**:1193-1198.

Chen, W.S., Lazar, C.S., Poenie, M., Tsien, R.Y., Gill, G.N., and Rosenfeld, M.G. (1987) Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* **328**:820-823.

Cheng, A.M., Saxton, T.M., Sakai, R., Kulkarni, S., Mbamalu, G., Vogel, W., Tortorice, C.G., Cardiff, R.D., Cross, J.C., Muller, W.J., and Pawson, T. (1998) mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell*:**95**:793-803.

Choi, T., Rulong, S., Resau, J., Fukasawa, K., Matten, W., Kuriyama, R., Mansour, S., Ahn, N., and Vande Woude, G.F. (1996) Mos/mitogen-activated protein kinase can induce early meiotic phenotypes in the absence of maturation-promoting factor: A novel system for analysing spindle formation during meiosis I. *Proc. Nat. Acad. Sci.* **93**:4730-4735.

Chuang, E., Barnard, D., Hettich, L., Zhang, X., Avruch, J., and Marshall, M.S. (1994) Critical binding and regulatory interactions between *ras* and *raf* occur through a small, stable N-terminal domain of *raf* and specific *ras* effector residues. *Mol. Cell. Biol.* **14**:5318-5325.

Clark, E.A., and Brugge, J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science* **268**:233-239.

Clark, G.J., Quillam, L.A., Hisaka, M.M., and Der, C.J. (1993) Differential antagonism of *ras* biological activity by catalytic and *src* homology domains of *ras* GTPase activiation protein. *Proc. Natl. Acad. Sci.* **90**:4887-4891.

Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) A native 170, 000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. *J. Biol. Chem.* **257**:1523-1531.

Cook. S.J., Rubinfeld, B., Albert, I., and McCormick, F. (1993) Rap V12 antagonizes *ras*-dependent activation of ERK1 and ERK2 by LPA and EGF in rat-1 fibroblasts. *EMBO J.* **12**:3475-3485.

Cooper, J.A., and Kashishian, A. (1993) In vivo binding properties of SH2 domains from GTPase-activating protein and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **13**:1737-1745.

Corbalan-Garcia, S., Margarit, S.M., Galron, D., Yang, S., and Bar-Sagi, D. (1998) Regulation of *sos* activity by intramolecular interactions. *Mol. Cell. Biol.* **18**:880-886.

Cox, A. and Der, C.J. (1997) Farnesyltransferase inhibitors an cancer treatment: targeting simply *ras? Biochemica et Biophysica Acta* **1333**:F51-F71.

Crompton, T., Gilmour, K.G., and Owen, M.J. (1996) The MAP kinase pathway controls differentiation from double-negative to double positive thymocyte. *Cell* **86**:243-251.

Crews, C.M, Alessandrini, A., and Erikson, R.L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* **258**:478-480.

Crews, C.M. and Erikson, R.L. (1992) Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activiates the *Erk*-1 gene product: relationship to the fission yeast *byr*1 gene product. *Proc. Natl. Acad. Sci.* **89**:8205-8209.

Cullen, P.J., Hsuan, J.J., Truong, O., Letcher, A.J., jackson, T.R., Dawson, A.P., and Irvine, R.F. (1995) Identification of a specific ins (1,3,4,5)P_4-binding protein as a member of the GAP1 family. *Nature* **376**:527-530.

Cutler Jr., R.E., Stephens, R.M., Saracino, M.R., and Morrison, D. (1998) Autoregulation of the *raf*-1 serine/threonine kinase. *Proc. Natl. Acad. Sci.* **95**:9214-9219.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997) *Cell* **91**:231-241.

DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C., and Lowy, D.R. (1992) Abnormal regulation of mammalian $p21^{ras}$ contributes to malignant tumor growth in von Recklinghausen (type1) neurofibromatosis. *Cell* **69**:265-273.

DeFeo-Jones, D., Tatchell, K., Robinson, L.C., Sigal, I.S., Vass, W.C., Lowy, D.R., Scolnick, E.M. (1985) Mammalian and yeast ras gene products: biological function in their heterologous systems. *Science*. **228**:179-184.

Del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., Nunez, G. (1997) Interleuken-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**:687-689.

Der, C.J., Finkel, T., and Cooper, G.M. (1986) Biological and biochemical properties of human ras^H genes mutated at codon 61. *Cell* **44**:167-176.

de Vos, A.M., Tong, L., Milburn, M.V., Matias, P.M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., and Kim, S. (1988) Three-dimensional structure of an oncogene protein: catalytic domain of human c-H-*ras* p21. *Science* **239**:888-893.

Dhanasekaran, N., Tsim, S., Dermott, J.M., Onesime, D. (1998) Regulation of cell proliferation by G proteins. *Oncogene* **17**:1383-1394.

Domin, J., and Waterfield, M.D. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Letters*. **410**:91-95.

Downward, J., Parker, P. And Waterfield, M.D. (1984) Autophosphorylation sites on the epidermal growth factor receptor. *Nature* **311**: 483-385.

Downward, J., Graves, J., Warne, P.H., Rayter, S., and Cantrell, D.A. (1990) Stimulaiton of p21**ras** upon T-cell activation. *Nature* **346**:719-723.

Downward, J. (1995) KSR: a novel player in the *ras* pathway. *Cell* **83**:831-834.

Downward, J. (1998) Lipid-regulated kinases: some common themes at last. *Science* **279**:673-674.

Drugan, J.K., Khosravi-Far, R., White, M.A., Der, C.J., Sung, Y., Hwang, Y, and Campbell, S.L. (1996) *Ras* interaction with two distinct binding domains in *raf*-1 may be required for *ras* transformations. *J. Biol. Chem.* **271**:233-237.

Ebinu, J.O., Bottorff, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science*. **280**:1082-1086.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. and Weinberg, R.A. (1993) Association of *sos ras* exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**: 45-51.

Ellis, C., Moran, M., mcCormick, F., and Pawson, T. (1990) Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature* **343**:377-381.

Ellis, R.W., DeFeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R., and Scolnick, E.M. (1981) The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. *Nature* **292**:506-511.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J., and Livingston, D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**:487-497.

Fam, N.P., Fan, W., Wang, Z., Zhang, L., Chen, H., and Moran, M.F. (1997) Cloning and characterization of *ras*-GRF2, a novel guanine nucleotide exchange factor for *ras*. *Mol. Cell. Biol.* **17**:1396-1406.

Farnsworth, C.L., and Feig, L.A. (1991) Dominant inhibitory mutations in the Mg^{2+} binding site of ras^{H} prevent its activaton by GTP. *Mol. Cell. Biol.* **11**:4822-4829.

Farnsworth, C.L., Freshney, N.W., Rosen, L.B., Ghosh, A., Greenberg, M.E., and Feig, L.A. (1995) Calcium activation of *ras* mediated by neuronal exchange factor *ras*-GRF. *Nature* **376**:524-527.

Feig, L.A., and Cooper, G.M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. *Mol. Cell. Biol.* **8**:3235-3243.

Feramisco, J.R., Gross, M., Kamata, T., Rosenberg, M., and Sweet, R.W. (1984) microinjection of the oncogene form of the human H-*ras* (T-24) protein results in rapid proliferation of quiescent cells. *Cell* **38**:109-117.

Franke, T.F., kaplan,D.R., and Cantley, L.C. (1997a) PI3K: Downstream AKTion blocks apoptosis. *Cell* **88**:435-437.

Franke, T.F., Kaplan, D.R., Cantley, L.C., Toker, A. (1997b) Direct regualtion of the *Akt* proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**:665-668.

Fujiyama, A., and Tamanoi, F. (1990) RAS2 protein of *Saccharomyces cerevisiae* undergoes removal of methionine at N terminus an removal of three amino acids at C terminus. *J. Biol. Chem.* **265**:3362-3368.

Fukasawa, K., and Vande Woude, G.F. (1997) Synergy between Mos/Mitogen-activated protein kinase pathway and loss of p53 function in transformation and chromosome instability. *Mol. Cell. Biol.* **17**:506-518.

Fukuda, M., and Mikoshiba, K. (1996) Structure- function relationships of the mouse Gap 1^{m}. *J. Biol. Chem.* **271**:18838-18842.

Fukunaga, R., and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16**:1921-1933.

Gawler, D.J., Zhang, L.-J., Reedijk, M., Tung, P.S., and Moran, M.F. (1995) CaLB: a 43 amino acid calcium-dependent membrane/phospholipid binding domain in p120 GTPase-activating protein. *Oncogene*. **10**:817-825.

Ghosh, S., Xie, W.Q., Quest, A.F.G., mabrouk, G.M., Strum, J.C., and Bell, R.M. (1994) The cysteine-rich region of *raf*-1 kinase contains zinc, translocates to liposomes, and is adjacent to a segment that binds GTP-*ras*. *J. Biol. Chem.* **269**:10000-10007.

Gibbs, J.B., Sigal, I.S., Poe, M., and Scolnick, E.M. (1984) Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. *Proc. Natl. Acad. Sci.* **81**: 5704-5708.

Gibbs, J.B., Marshall, M.S., Scolnick, E.M., Dixon, R.A.F., and Vogel, U.S. (1990) Modulation of guanine nucleotides bound to *ras* in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). *J. Biol. Chem.* **265**: 20437-20442.

Gideon, P., John, J., Frech, M., Lautwein, A., Clark, R., Scheffler, J.E., and Wittinghofer, A. (1992) Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: the C-terminal domain of GAP is not sufficient for full activity. *Mol. Cell. Biol.* **12**:2050-2056.

Gille, H., kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, m.H., and Shaw, P.E. (1995) ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.* **14**:951-962.

Goldfarb, D. (1997) Whose finger is on the switch?. *Science* **276**:1814-1816.

Gomperts, S.N. (1996) Clustering membrane proteins: it's all coming together with the PSD-95/SAP90 protein family. *Cell* **84**:659-662.

Goody, R.S., Frech, M., and Wittinghofer, A. (1991) Affinity of guanine nucleotide binding proteins for their ligands: facts and artefacts. *TIBS*. **16**:327-328.

Graham, S.M., Vojtek, A.B., Huff, S.Y., Cox, A.D., Clark, G.J., Cooper, J.A., and Der, C.J. (1996) TC21 causes transformation by *Raf*-independent signaling pathways. *Mol. Cell. Biol.* **16**:6132-6140.

Gutkind, J.S. (1998) Cell growth control by G-protein coupled receptors: from signal transduction to signal integration. *Oncogene* **17**:1331-1342.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A., and Broek, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of *rac*-related guanosine triphosphatases by *vav*. *Science* **279**:558-560.

Han, L., and Colicelli, J. (1995) A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. *Mol. Cell. Biol.* **15**:1318-1323.

Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) *Proc. Nat. Acad. Sci.* **94**:4954-4959.

Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989) All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell **57**:1167-1177.

Hancock, J.F., Cadwallader, K., and Marshall, C.J. (1991) methylation and proteolysis are essential for efficient membrane binding of prenylated $p21^{K\text{-}ras\ (B)}$. *EMBO J.* **10**:641-646.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996) Regulation pf p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol. Cell. Biol.* **16**:859-867.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**:805-816.

Harvey, J.J. (1964) An unidentified virus which causes the rapid production of tumors in mice. *Nature*. **204**:1104-1105.

Hayashi, H., Nishioka, Y., Kamohara, S., Kanai, F., Ishii, K., Fukui, Y., Shibasak, F., Takenawa, T., Kido, H., Karsunuma, N., and Ebina, Y. (1993) The α-type 85-kDa subunit of phosphatidylinositol 3-kinase is phosphorylated at tyrosines 368, 580, and 607 by the insulin receptor. *J. Biol. Chem.* **268**: 7107-7117.

Henkemeyer, M., Rossi, D.J., Holmyard, D.P., Puri, M.C., Mbamalu, G., Harpal, K., Shih, T.S., Jacks, T., and Pawson, T. (1995) Vascular system defects and neuronal apoptosis in mice lacking *ras* GTPase-activating protein. *Nature* **377**:695-701.

Hipskind, R.A., Baccarini, M., and Nordheim, A. (1994) Transient activation of RAF-1, MEK, and ERK2 coincides kinetically with ternary complex factor phosphorylation and immediate-early gene promoter activity in vivo. *Mol. Cell. Biol.* **14**:6219-6231.

Hofer, F., Fields, S., Schneider, C., and Martin, G.S. (1994) Activated *ras* interacts with the *ral* guanine nucleotide dissociation stimulator. *Proc. Natl. Acad. Sci.* **91**:11089-11093.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. (1992) Activation of the MAP kinase pathway by the protein kinase *raf*. *Cell* **71**:335-342.

Huff, S.Y., Quillam, L.A., Cox, A.D., and Der, C.J. (1997) R-*ras* is regulated by activators and effectors distinct from those that control *ras* function. *Oncogene* **14**:133-143.

Hunter, T. (1997) Oncoprotein networks. Cell 88:333-346

Irvine, R.F., Letcher, A.J., Heslop, J.P., and Berridge, M.J. (1986) The inositol tris/tetrakisphosphate pathway-demonstration of Ins $(1,4,5)P_3$ 3-kinase activity in animal tissues. *Nature* **320**:631-634.

Jacks, T., Shih, T.S., Schmittt, E.M., Bronson, R.T., Bernards, A., and Weinberg, R.A. (1994) Tumour predisposition in mice heterozygous for a targeted mutation in *Nf1*. *Nature Genetics* **7**:353-361.

Jackson, J.H., Cochrane, C.G., Bourne, J.R., Solski, P.A., Buss, J.E., and Der, C.J. (1990) *Proc. Natl. Acad.Sci* **87**:3042-3046.

James, G.L., Goldstein, J.L., Brown, M.S., Rawson, T.E., Somers, T.C., McDowell, R.S., Crowley, C.W., Lucas, B.K., Levinson, A.D., and Marsters Jr., J.C. (1993) Benzodiazepine peptidomimetics: potent inhibitors of *ras* farnesylation in animal cells. *Science* **260**:1937-1942.

Jefferson, A.B., Klippel, A., and Williams, L.T. (1998) Inhibition of mSOS-activity by binding of phophatidylinositol 4,5-P_2 to the mSOS pleckstrin homology domain. *Oncogene* **16**:2303-2310.

Jiang, Y., Ma, W., Kozasa, T., Hattori, S., and Huang, X. (1998) The G protein Gα12 stimulates Bruton's tyrosine kinase and a *ras*GAP through a conserved PH/BM domain. *Nature* **395**:808-813.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., and Goody, R.S. (1990) Kinetics of interaction of nucleotides with nucleotide-free H-*ras* p21. *Biochemistry* **29**:6058-6065.

Johnson, D.G., Schwarz, J.K., Cress, W.D., and Nevins, J.R. (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**:349-352.

Johnson, L., Noble, M.E., and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85:**149-158.

Kahn, R.A., and Gilman, A.G. (1984) Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J. Biol. Chem.* **259**:6228-6234.

Kahn, R.A., Der, C.J., and Bokoch, G.M. (1992) The *ras* superfamily of GTP-binding proteins: guidelines on nomenclature. *NEWS* **6**: 2512-2513.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. (1985) Functional homology of mammalian and yeast *ras* genes. *Cell* **40**:19-26.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by *ras* signalling through PI(3)K and PKB. *Nature* **385**: 544-548.

Kawasaki, H., Springett, G.M., Toki, S., Canales, J.J., Harlan, P., Blumenstiel, J.P., Chen, E.J., Bany, I.A., Mochizuki, N., Ashbacher, A., *et al.*, (1998) A Rap nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Nat. Acad. Sci.* **95**:13278-13283.

Khosravi-Far, R., and Der, C.J. (1994) The *ras* signal transduction pathway. *Cancer and Metastasis Reviews* **13**:67-89.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1997) Matrix adhesion and *ras* transformation both activate a phophoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**:2783-2793.

Kikuchi, A., and Williams, L.T. (1996) Regulation of interaction of *ras* p21 with RalGDS and Raf-1 by cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **271**:588-594.

Kim, J.H., Liao, D., Lau, L.-F., and Huganir, R.L. (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron.* **20**:683-691.

King, A.J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M.S. (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phophorylation of serine 338. *Nature* **396**:180-183.

Kirsten, W.H., and Mayer, L.A. (1967) Morphologic responses to a murine erythroblastosis virus. *J. of the Nat. Cancer Institute* **39**:311-335.

Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) A *ras*-related gene with transformation supressor activity. *Cell* **56**:77-84.

Klippel, A., Kavanaugh, M., Pot, D., and Williams, L.T. (1997) A specific product of phosphatidylinositol 3-kinase directly activates the protein kinas Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* **17**:338-344.

Koepp, D.M., and Silver, P.A. (1996) A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? *Cell* **87**:1-4.

Kohl, N.E., Mosser, S.D., DeSolms, S.j., Giuliani, E.A., Pompliano, D.L., Graham, S.L., Smith, R.L., Scolnick, E.M., Oliff, A., and Gibbs, J.B. (1993) Selective inhibition of *ras*-dependent transformaiton by farnesyltransferase inhibitor. *Science* **260**:1934-1937.

Kohl, N.E., Wilson, F.R., Mosser, S.D., Giuliani, E., DeSolms, S.J., Conner, M.W., Anthony, N.J., Holtz, W.J., Gomez, R.P., Lee, T., Smith, R.L., Graham, S.L., Hartman, G.D., Gibbs, J.B. and Oliff, A. (1994) Protein farnesyltransferase inhibitors block the growth of *ras*-dependent tumors in nude mice. *Proc. Natl. Acad. Sci.* **91**:9141-9145.

Kohl, N.E., Omer, C.A., Conner, M.W., Anthony, N.J., Davide, J.P., DeSolmes, S.J., Giuliani, E.A., Gomez, R.P., Graham, S.L., Hamilton, K., handt, L.K., Hartman, G.D., O'Neill, T.J., Rands, E., Schaber, M.D., Gibbs, J.B. and Oliff, A. (1995) Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nature Medicine* **1**:792-797.

Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U.R. (1991) Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. *Nature* **349**:426-428.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H. Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U.R. (1993) Protein kinase Cα activates RAF-1 by direct phophorylation. *Nature* **364**: 249-252.

Kornfeld, K., Hom, D.B., and Horvitz, R.H. (1995) The *ksr*-1 gene encodes a novel protein kinase invovlved in *ras*-mediated signaling in *C. elegans. Cell* **83**:903-913.

Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the *ras*/MAPK signaling pathway. *Cell* **89**:693-702.

Krengel, U., Schlichting, I., Scherer, A., Schumann, R., Frech, M., John, J., Kabasch, W., Pai, E.F., and Wittinghofer, A. (1990) Three-dimensional structures of H-*ras* p21 mutants: molecular basis for their inablility to function as signal switch molecules. *Cell* **62**:539-548.

Kulju, K.S., and Lehman, J.M. (1995) Increased p53 protein associated with aging in human diploid fibroblasts. *Exp. Cell Res.* **217**:336-345.

Kuriyama, M., Harada, N., Kuroda, S., Yamamoto, T., Nakafuku, M., Iwamatsu, A., Yamamoto, D., Prasad, R., Croce, C., and Canaani, E., and Kaibuchi, K. (1996) Identification of AF-6 and canoe as putative targets for Ras. J. Biol. Chem. 271:607-610.

Kyriakis, J.M., App, H., Zhang, X., Banerjee, P., Brautigan, D.L., Rapp, U.R., and Avruch, J. (1992) Raf-1 activates MAP kinase-kinase. *Nature* **358**:417-421.

Leblanc V., Tocque, B., and Delumeau, I., (1998) *Ras*-GAP controls Rho-medicated cytoskeletal reorganization through its SH3 domain. *Mol. Cell. Biol.* **18**:5567-5578.

Lee, J., Beck, T.W., Wojnowski, L., and Rapp, U.R. (1996) Regulation of A-raf expression. *Oncogene* **12**:1669-1677.

Leevers, S.J., Paterson, H.F. and Marshall, C.J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* **369:**411-414.

Lemmon, M.A., Ferguson, K.M., and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85:**621-624.

Lenzen, C., Cool, R.H., Prinz, H., Kuhlmann, J., and Wittinhofer, A. (1998) Kinetic analysis by flourescence of the interaction between *ras* and the caalytic domain of the guanine nucleotide exchange factor $Cdc25^{Mm}$. *Biochemistry* **37**:7420-7430.

Leon, J., Guerrero, I., and Pellicer, A. (1987) Differential Expression of the *ras* gene family in mice. *Mol. Cell. Biol.* **7**:1535-1540.

Li, B.-Q, Kaplan, D., Kung, H. and Kamata, T. (1992) Nerve growth factor stimulation of the ras-guanine nucleotide exchange factor and GAP functions. *Science* **256:**1456-1459.

Lin, A.W., Barrada, M., Stone, J.C., Van Aelst, L., Serrano, M., and Lowe, S.W. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & Development* **12**:3008-3019.

Lin, L.-L, Wartman, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R.J. (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72:**269-278.

Liu, X. and Pawson, T. (1991) The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. *Mol. Cell. Biol.* **11**:2511-2516.

Lockyer, P.J., Bottomley, J.R., Reynolds, J.S., McNulty, T.J., Venkateswarlu, K., Potter. B.V.L., Dempsey, C.E., and Cullen, P.J. (1997) Distinct subcellular localisations of the putative inositol 1,3,4,5-tetrakisphophate receptors $GAP1^{IP4BP}$ and $GAP1^{m}$ result from the $GAP1^{IP4BP}$ PH domain directing plasma membrane targeting. *Current Biology* **7**:1007-1010.

Lockyer, P.J., Vanlingen, S., Reynolds, J.S., McNulty, T.J., Irvine, R.F., Parys and Cullen, P.J. (1999a) Tissue-specific expression and endogenous subcellar distribution of th inositol 1,3,4.5-tetrakisphosphate-binding proteins $GAP1^{IP4BP}$ and $GAP1^{m}$. *Biochemical and Biophysical Research Communications* **255**:421-426.

Lockyer, P.J., Wennstrom, S., Kupzig, S., Venkateswarlu, K., Downward, J., and Cullen, P.J. (1999b) Identification of the ras GTPase-activating protein GAP1(m) as a phosphatidylinositol-3,4,5-triphosphate-binding protein in vivo. *Current Biology*. **9**:265-268.

Lowy, D.R., and Willumsen, B.M. (1993) Function and regulation of Ras. *Annu. Rev. Biochem.* **62**:851-891.

Luo, J.-H., and Weinstein, I.B. (1993) Calcium-dependent activation of Protein kinase C *J. Biol. Chem.* **268**:23580-23584.

Macdonald, S.G., Crews, C.M., Wu, L., Driller, J., Clark, R., Erikson, R.L. and McCormick, F. (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. *Mol. Cell. Biol.* **13:**6615-6620.

Maekawa, M., Nakamura, S. and Hattori, S. (1993) Purification of a novel *ras* GTPase-activating protein from rat brain. *J. Biol. Chem.* **268:**22948-22952.

Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Imai, Y.Kohsaka, S., Nakamura, S. and Hattori, S. (1994) A novel mammalian Ras GTPase-activating protein which has phospholipid-binding and Btk homology regions. *Mol. Cell. Biol.* **14:**6879-6885.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F. and Ahn, N.G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265:**966-970.

Marais, R., Wynne, J. and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73:**381-393.

Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.* **14:**3136-3145.

Marshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80:**179-185.

Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthorn, R.M., Innis, M.A. and McCormick, F. (1990) The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. *Cell* **63:**843-849.

Martin, G.A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A.M. and McCormick, F. (1992) GAP domains responsible for Ras p21-dependent inhibition of muscarinic atrial K^+ channel currents. *Science* **255:**192-194.

Mattingly, R.R. and Macara, I.G. (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25^{Mm} exchange factor by muscarinic receptors and G-protein βγ subunits. *Nature* **382:**268-272.

Mayo, M.W., Wang, C.-Y. Cogswell, P.C., Rogers-Graham, K.S., Lowe, S.W., Der, C.J. and Baldwin, A.S., Jr. (1997) Requirement of NK-κB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **278:**1812-1815.

McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L.B. and Pawson, T. (1993) The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. *EMBO J.* **12:**3073-3081.

McGrath, J.P., Capon, D.J., Smith, D.H., Chen, E.Y., Seeburg, P.H., Goeddel, D.V., and Levinson, A.D. (1983) Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene. *Nature* **304**:501-506.

Melemed, A.S., Ryder J.W. and Vik, T.A. (1997) Activation of the mitogen-activated protein kinase pathway is involved in and sufficient for megakaryocytic differentiation of CMK cells. *Blood* **90:**3462-3470.

Milburn, M.V., Tong, L., de Vos, A.M., Brünger, A., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1990) Molecular switch for signal transduction: Structural differences between active and inactive forms of protooncogenic *ras* proteins. *Science* **247:**939-945.

Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Complexes of Ras•GTP with raf-1 and mitogen-activated protein kinase kinase. *Science* **260:**1658-1660.

Moodie, S.A., Paris, M., Villafranca, E., Kirshmeier, P., Willumsen, B.M. and Wolfman, A. (1995) Different structural requirements within the switch II region of

the Ras protein for interactions with specific downstream targets. *Oncogene* **11:**447-454.

Moran, M.F., Polakis, P., McCormick, F., Pawson, T. and Ellis, C. (1991) Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21^{ras} GTPase-activating protein. *Mol. Cell. Biol.* **11:**1804-1812.

Morrison, D.K., Kaplan, D.R., Rapp, U. and Roberts, T.M. (1988) Signal transduction from membrane to cytoplasm: Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. *Proc. Natl. Acad. Sci. USA* **85:**8855-8859.

Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985) Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature* **313:**241-243.

Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F. and Wittinghofer, A. (1995) The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. *Nature* **375:**554-560.

Neal, S.E., Eccleston, J.F., Hall, A. and Webb, M.R. (1988) Kinetic analysis of the hydrolysis of GTP by p21$^{N\text{-}ras}$. *J. Biol. Chem.* **263:**19718-19722.

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80:**249-257.

Nevins, J.R. (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258:**424-429.

Nguyen, T.T., Scimeca, J.-C., Filloux, C., Peraldi, P., Carpentier, J.-L., and Van Obberghen, E. (1993) Co-regulation of the mitogen activated protein kinase, extracellular signal-regulated kinase 1, anr the 90-kDa ribosomal S6 kinase in PC12 cells. *J. Biol. Chem.* **268**:9803-9810.

Nimnual, A.S., Yatsula, B.A. and Bar-Sagi, D. (1998) Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. *Science* **279:**560-563.

Nobes, C.D., and Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**:53-62.

Noda, M., Ko, M., Ogura, A., Liu, D.-G, Amano, T., Takano, T. and Ikawa, Y. (1985) Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature* **318:**73-75.

Nur-E-Kamal, M.S.A., Sizeland, A., D'Abaco, G. and Maruta, H. (1992) Asparagine 26, glutamic acid 31, valine 45, and tyrosine 64 of Ras proteins are required for their oncogenecity. *J. Biol. Chem.* **267:**1415-1418.

Obermeier, A., Bradshaw, R.A., Seedorf, K., Choidas, A., Schlessinger, J. and Ullrich, A. (1994) Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLCγ. *EMBO J.* **13:**1585-1590.

Ohba, T., Nakamura, M., Nishitani, H., and Nishimoto, T. (1999) Self-organization of the microtubule asters induced in Xenopus egg extracts by GTP-bound Ran. *Science*. **284**:1356-1358.

Olson, M.F., Paterson, H.F. and Marshall, C.J. (1998) Signals from Ras and Rho GTPases interact to regulate expression of $p21^{Waf1/Cip1}$. *Nature* **394:**295-299.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A.D., Morgan, S.J., Courtneidge, S.A., Parker, P.J. and Waterfield, M.D. (1991) Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/$pp60^{c\text{-}src}$ complexes, and PI3-kinase. *Cell* **65:**91-104.

Pai, E.-F., Krengel, U., Petsko, G.A., Goody, R.S., Kabsch, W., and wittinghofer, A. (1990) Refined crystal structure of the triphosphate conformation of H-ras at 1.35A resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* **9**:2351-2359.

Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* **236:**747-771.

Pape, T., Wintermeyer, W. and Rodnina, M.V. (1998) Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. *EMBO J.* **17:**7490-7497.

Parada, L.F., Tabin, C.J., Shih, C. and Weinberg, R.A. (1982) Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene. *Nature* **297:**474-478.

Peeper, D.S., Upton, T.M., Ladha, M.H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J.A., and Ewen, M.E. 1997. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* 386:177-181.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavalla, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P.G. (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70:**93-104.

Pomerance, M., Thang, M.N., Tocque, B. and Pierre, M. (1996) The Ras-GTPase-activating protein SH3 domain is required for Cdc2 activation and mos induction by oncogenic Ras in Xenopus oocytes independently of mitogen-activation protein kinase activation. *Mol. Cell. Biol.* **16:**3179-3186.

Porfiri, E. and McCormick, F. (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the Ras exchange factor hSOS1. *J. Biol. Chem.* **271:**5871-5877.

Posada, J., Yew, N., Ahn, N.G., Vande Woude, G.F., and Cooper, J.A. (1993) Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro. *Mol. Cell. Biol.* **13**:2546-2553.

Price, M.A., Rogers, A.E. and Treisman, R. (1995) Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *EMBO J.* **14:**2589-2601.

Privé, G.G., Milburn, M.V., Tong, L., deVos, A.M., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1992) X-ray crystal structures of transforming p21 ras mutants suggest a transition-state stabilization mechanism for GTP hydrolysis. *Proc. Natl. Acad. Sci. USA* **89:**3649-3653.

Pronk, G.J., de Vries-Smits, A.M.M., Buday, L., Downward, J., Maassen, J.A., Medema, R.H. and Bos, J.L. (1994) Involvement of Shc in insulin- and epidermal growth factor-induced activation of p21^{ras}. *Mol. Cell. Biol.* **14:**1575-1581.

Qian, X., Vass, W.C., Papageorge, A.G., Anborgh, P.H. and Lowy, D.R. (1998) N Terminus of Sos1 Ras exchange factor: critical roles for the Dbl and Pleckstrin homology domains. *Mol. Cell. Biol.* **18:**771-778.

Qiu, R.G., Chen, J., Kirn, D., McCormick, F. and Symons, M. (1995) An essential role for Rac in Ras transformation. *Nature* **374:**457-459.

Rameh, L.E., Arvidsson, A., Carraway, K.L., III, Couvillon, A.D., Rathburn, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D.-S, Chen, C.-S and Cantley, L.C. (1997) A comparative analysis of the phosphoinositide binding specificity of Pleckstrin homology domains. *J. Biol. Chem.* **272:**22059-22066.

Reddy, E.P., Reynolds, R.K., Santos, E. and Barbacid, M. (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature* **300:**149-152.

Riccardi, V.M. (1981) Von Recklinghausen neurofibromatosis. *Neurofibromatosis* **305:**1617-1627.

Ridley, A.J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70:**389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70:**401-410.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370:**527-532.

Rodriguez-Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D. and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15:**2442-2451.

Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D., Das, P., Waterfield, M.D., Ridley, A. and Downward, J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89:**457-467.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363:**83-85.

Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K. and Cohen, S. (1993) Epidermal growth factor stimulates the tyrosine phosphorylation of SHC in the mouse. *J. Biol. Chem.* **268:**7610-7612.

Santos, E., Tronick, S.R., Aaronson, S.A., Pulciani, S. and Barbacid, M. (1982) T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. *Nature* **298:**343-347.

Satoh, T., Endo, M., Nakafuku, M., Nakamura, S. and Kaziro, Y. (1990) Platelet-derived growth factor stimulates formation of active $p21^{ras}$•GT P complex in Swiss mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA* **87:**5993-5997.

Schafer, W.R., Kim, R., Sterne, R., Thorner, J., Kim, S.-H and Rine, J. (1989) Genetic and pharmacological suppression of oncogenic mutations in *RAS* genes of yeast and humans. *Science* **245:**379-385.

Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F. and Wittinghofer, A. (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* **277:**333-338.

Schekman, R. and Orci, L. (1996) Coat proteins and vesicle budding. *Science* **271:**1526-1533.

Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372:**786-791.

Seeburg, P.H., Colby, W.W., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984) Biological properties of human c-Ha-*ras*1 genes mutated at codon 12. *Nature* **312:**71-75.

Serra, E., Puig, S., Otero, D., Gaona, A., Kruyer, H., Ars, E., Estivill, X. and Lázaro, C. (1997) Confirmation of a double-hit model for the *NF1* gene in benign neurofibromas. *Am. J. Hum. Genet.* **61:**512-519.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. and Lowe, S.W. (1997) Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell* **88:**593-602.

Settleman, J., Albright, C.F., Foster, L.C. and Weinberg, R.A. (1992) Association between GTPase activators for Rho and Ras families. *Nature* **359:**153-154.

Shibatohge, M., Kariya, K., Liao, Y., Hu, C.-D, Watari, Y., Goshima, M., Shima, F. and Kataoka, T. (1998) Identification of PLC210, a *Caenorhabditis elegans* phospholipase C, as a putative effector of Ras. *J. Biol. Chem.* **273:**6218-6222.

Shimizu, K., Birnbaum, D., Ruley, M.A., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M. and Wigler, M. (1983) Structure of the Ki-*ras* gene of the human lung carcinoma cell line Calu-1. *Nature* **304:**497-500.

Shou, C., Farnsworth, C.L., Neel, B.G. and Feig, L.A. (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for ras p21. *Nature* **358:**351-354.

Sieburth, D.S., Sun, Q. and Han, M. (1988) SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling *C. elegans*. *Cell* **94:**119-130.

Simons, K. and Zerial, M. (1993) Rab proteins and the road maps for intracellular transport. *Neuron* **11:**789-799.

Sjölander, A., Yamamoto, K., Huber, B.E. and Lapentina, E.G. (1991) Association of $p21^{ras}$ with phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **88:**7908-7912.

Stacey, D.W. and Kung, H.-F. (1984) Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. *Nature* **310:**508-511.

Stang, S., Bottorff, D. and Stone, J.C. (1997) Interaction of activated Ras with Raf-1 alone may be sufficient for transformation of rat2 cells. *Mol. Cell. Biol.* **17:**3047-3055.

Sternberg, P.W., and Alberola-Ila, J. (1998) Conspiracy Theory: RAS and RAF do not act alone. Cell 95:447-450.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science* **264:**1463-1467.

Stone, J.C., Colleton, M. and Bottorff, D. (1993) Effector domain mutations dissociate $p21^{ras}$ effector function and GTPase-activating protein interaction. *Mol. Cell. Biol.* **13:**7311-7320.

Sundaram, M. and Han, M. (1995) The C. elegans *ksr-1* gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83:**889-901.

Sweet, R.W., Yokoyama, S., Kamata, T., Feramisco, J.R., Rosenberg, M. and Gross, M. (1984) The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. *Nature* **311:**273-275.

Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R. and Chang, E.H. (1982) Mechanism of activation of a human oncogene. *Nature* **300:**143-149.

Tamemoto, H., Kadowaki, T., Tobe, K., Ueki, K., Izumi, T., Chatani, Y., Kohno, M., Kasuga, M., Yazaki, Y. and Akanuma, Y. (1992) Biphasic activation of two mitogen-activated protein kinases during the cell cycle in mammalian cells. *J. Biol. Chem.* **267:**20293-20297.

Taparowsky, E., Shimizu, K., Goldfarb, M. and Wigler, M. (1983) Structure and activation of the human N-*ras* gene. *Cell* **34:**581-586.

Taylor, S.J. and Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Current Biology* **6:**1621-1627.

Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wassarman, D.A. and Rubin, G.M. (1995) KSR, a novel protein kinase required for RAS signal transduction. *Cell* **83:**879-888.

Therrien, M., Wong, A.M. and Rubin, G.M. (1998) CNK, a RAF-binding multidomain protein required for RAS signaling. *Cell* **95:**343-353.

Tognon, C.E., Kirk, H.E., Passmore, L.A., Whitehead, I.P., Der, C.J., and Kay, R.J. (1998) Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* **18**:6995-7008.

Toker, A. and Cantley, L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387:**673-676.

Tombes, R.M., Auer, K.L., Mikkelsen, R., Valerie, K., Wymann, M.P., Marshall, C.J., McMahon, M. and Dent, P. (1998) The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.* **330:**1451-1460.

Trahey, M. and McCormick, F. (1987) A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. *Science* **238:**542-545.

Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G.A., Ladner, M., Long, C.L., Crosier, W.J., Watt, K., Koths, K. and McCormick, F. (1988) Molecular cloning of two types of GAP complementary DNA from human placenta. *Science* **242:**1697-1700.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. *Biochem. J.* **288:**351-355.

Traverse, S., Cohen, P., Paterson, H., Marshall, C., Rapp, U. and Grand, R.J.A. (1993) Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of *ras*-transformed retinal cells. *Oncogene* **8:**3175-3181.

Travis, J. (1993) Novel anticancer agents move closer to reality. *Science.* **260**:1877-1878.

Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.* **8:**205-215.

Tzivion, G., Luo, Z. and Avruch, J. (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394:**88-92.

Urano, T., Emkey, R. and Feig, L.A. (1996) Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO J.* **15:**810-816.

Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993) Complex formation between RAS and RAF and other protein kinases. *Proc. Natl. Acad. Sci. USA* **90:**6213-6217.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R. and O'Connell, P. (1990) Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* **62:**187-192.

Vogel, U.S., Dixon, R.A.F., Schaber, M.D., Diehl, R.E., Marshall, M.S., Scolnick, E.M., Sigal, I.S. and Gibbs, J.B. (1988) Cloning of bovine gap and its interaction with oncogenic *ras* p21. *Nature* **335:**90-93.

Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian ras interacts directly with the serine/threonine kinase Raf. *Cell* **74:**205-214.

von Mollard, G.F., Stahl, B., Li, C., Südhof, T.C. and Jahn, R. (1994) Rab proteins in regulated exocytosis. *TIBS* **19:**164-168.

Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Brownstein, B.H. and Collins, F.S. (1990) Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science* **249:**181-186.

Warne, P.H., Rodriguez-Viciana, P. and Downward, J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature* **364:**352-355.

Wartmann, M. and Davis, R.J. (1994) The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.* **269:**6695-6701.

Waskiewicz, A.J., Flynn, A., Proud, C.G. and Cooper, J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16:**1909-1920.

Weber, J.D., Raben, D.M., Phillips, P.J. and Baldassare, J.J. (1997) Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G_1 phase. *Biochem. J.* **326:**61-68.

White, M.A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M. and Wigler, M.H. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell* **80:**533-541.

Whitman, M., Downes, C.P., Keeler, M., Keller, T. and Cantley, L. (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* **332:**644-646.

Wilde, A., and Zheng, Y. (1999) Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science.* **284**:1359-1362.

Willumsen, B.M., Papageorge, A.G., Kung, H.-F., Bekesi, E., Robins, T., Johnsen, M., Vass, W.C. and Lowy, D.R. (1986) Mutational analysis of a *ras* catalytic domain. *Mol. Cell. Biol.* **6:**2646-2654.

Wittinghofer, F. (1998) Caught in the act of the switch-on. *Nature* **394:**317-320.

Wojnowski, L., Zimmer, A.M., Beck, T.W., Hahn, H., Bernal, R., Rapp, U.R. and Zimmer, A. (1997) Endothelial apoptosis in Braf-deficient mice. *Nature Genet.* **16:**293-297.

Wood, K.W., Sarnecki, C., Roberts, T.M. and Blenis, J. (1992) *ras* Mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, raf-1, and RSK. *Cell* **68:**1041-1050.

Wu, J., Harrison, J.K., Dent, P., Lynch, K.R., Weber, M.J. and Sturgill, T.W. (1993) Identification and characterization of a new mammalian mitogen-activated protein kinase kinase, MKK2. *Mol. Cell. Biol.* **13:**4539-4548.

Xing, J., Ginty, D.D. and Greenberg, M.E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273:**959-963.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* **366:**701-704.

Xu, G., O'Connell, P., Viskochil, D., Cawthorn, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R. and Weiss, R. (1990a) The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* **62:**599-608.

Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R. and Tamanoi, F. (1990b) The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. *Cell* **63:**835-841.

Yamanashi, Y. and Baltimore, D. (1997) Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell* **88:**205-211.

Yatani, A., Okabe, K., Polakis, P., Halenbeck, R., McCormick, F. and Brown, A.M. (1990) *ras* p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. *Cell* **61:**769-776.

Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C.-H and Claesson-Welsh, L. (1994) Direct interaction between Shc and the platelet-derived growth factor β-receptor. *J. Biol. Chem.* **269:**15337-15343.

Yuasa, Y., Srivastava, S.K., Dunn, C.Y., Rhim, J.S., Reddy, E.P. and Aaronson, S.A. (1983) Acquisition of transforming properties by alternative point mutations within c-*bas/has* human proto-oncogene. *Nature* **303:**775-779.

Zhang, X., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. and Avruch, J. (1993) Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364:**308-313.

Zheng, C.-F and Guan, K.-L. (1993) Cloning and characterization of two distinct human extracellular sign-regulated kinase activator kinases, MEK1 and MEK2. *J. Biol. Chem.* **268:**11435-11439.

Zheng, C.-F and Guan, K.L. (1994) Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *EMBO J.* **13:**1123-1131.

Zheng, L., Eckerdal, J., Dimitrijevic, I. and Andersson, T. (1997) Chemotactic peptide-induced activation of Ras in human neutrophils is associated with inhibition of p120-GAP activity. *J. Biol. Chem.* **272:**23448-23454.

Zhu, J., Woods, D., McMahon, M. and Bishop, J.M. (1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12:**2997-3007.

Zohn, I.M., Campbell, S.L., Khosravi-Far, R., Rossman, K.L. and Der, C.J. (1998) Rho family proteins and Ras transformation: the RHOad less traveled gets congested. *Oncogene* **17:**1415-1438.

Chapter 2 - Experimental Methods

Sources of Recombinant Ras and Ras regulatory proteins

H-Ras was expressed in *Escherichia coli* and purified by gravity-driven G75 size-exclusion chromatography followed by FPLC anion-exchange chromatography on a Resource-Q column (Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada). H-Ras purified from *E. coli* was stored in 50 mM Tris pH 8.0, 5 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT at 4°C until use Preparation of H-Ras from *E. coli* was performed by the author. Since some exchange factors may work better with substrate Ras that has been fully processed, we also used 6x His-tagged-H-Ras that was expressed in Sf9 cells using the "Bac-to-Bac" bacculovirus system (Life Technologies-Gibco BRL, Inc., Burlington, Ontario, Canada) and purified by nickel chromatography. 6x His-tagged-H-Ras was stored in "buffer A + glycerol" [20 mM Tris pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol] at -80°C until use. Preparation of 6x His-tagged-H-Ras was performed by others within our lab (D. Bottorff). In both *E. coli* and Sf9 expression systems, Ras purifies as a complex with GDP in a 1:1 stoichiometric ratio (Poe *et al.*, 1985).

The catalytic domain of RasGRF1 (p30GRF1) was expressed as a 6x His-tagged protein in Sf9 cells and purified by nickel chromatography (Leonardsen *et al.*, 1996). Purified 6x His-p30GRF1 was stored in buffer A + glycerol at -80°C until use. Preparation of 6x His-p30GRF1 was performed by others within our lab (D. Bottorff, J. Ebinu). A recombinant protein consisting of maltose binding protein fused to residues 49-471 of RasGRP (MBP-cat) was expressed in *E. coli* and purified on amylose resin beads (New England Biolabs, Inc., Beverly, Massachusetts, USA) (Ebinu *et al.*, 1998). MBP-cat was stored in buffer A + glycerol at -80°C until use. MBP-cat was prepared by others within our lab (D. Bottorff and S. Stang). Purified p120GAP was a generous gift from Onyx pharmaceuticals (Richmond, California, USA).

Analysis of Ras and Ras regulatory proteins *in vitro*

To assess spontaneous association of guanyl nucleotide *in vitro* (chapter 3), bacterial Ras-GDP complex was incubated with a molar excess of [^{32}P-α]-GTP (ICN Biomedicals, Inc., Costa Mesa, California, USA) either at 0°C or 30°C. Each 0.2 ml reaction contained 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA, 1 μM Ras-GDP and 100 μM GTP including 40 μCi [^{32}P-α]-GTP. At various times, 0.05 ml aliquots were removed and Ras was immuno-precipitated for 1-2 hours at 0°C

using the rat monoclonal antibody Y13-259 (2 μg/sample) prebound to rabbit anti-rat IgG coupled protein-A Sepharose (Sigma Chemical Co., St. Louis, Missouri, USA). Immuno-precipitates were washed 8 times with OnyxA buffer [50 mM Tris pH 8.0, 5 mM $MgCl_2$, 5 mM NaCl] and precipitated guanyl nucleotides were dissociated and chromatographed on cellulose-polyethyleneimine (PEI) (J.T. Baker, Toronto, Ontario, Canada) in 1 M K_2HPO_4 pH 3.40. Radiolabeled GDP and GTP spots were quantified by phosphorimager analysis (MacBas 1000, Fuji Film Corp., Stamford, Connecticut, USA). Percent loading was calculated as (experimental amount of associated radiolabeled GDP + GTP / maximal association of radiolabeled GDP + GTP) x 100. To maximally load Ras with radiolabeled nucleotide, a parallel reaction was carried out at 0°C for 10 minutes in 7.5 mM EDTA followed by titration with excess $MgCl_2$. This procedure equilibrates Ras with the guanyl nucleotides contained in the reaction.

We also studied catalyzed association using 6x His-Ras purified from Sf9 cells and recombinant Ras GEF's (chapter 3). These reactions (0.05 ml) contained: 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 10% v/v glycerol, 0.2 mg/ml BSA, 0.2 μM 6x His-Ras-GDP, 1 μM GTP and 10 μCi [^{32}P-α]-GTP. Reactions also included 0.1 μg purified 6x His-p30GRF1. After incubation at 0°C or 30°C for various times, Ras was immuno-precipitated and analyzed as described for the spontaneous association reactions except that TMN [20 mM Tris 7.5, 100 mM NaCl, 5 mM $MgCl_2$] was used for washing beads 8 times instead of OnyxA buffer.

To assess guanyl-nucleotide hydrolysis (chapter 3), 6x His-Ras was prebound to [^{32}P-α]-GTP. A binding reaction that would yield enough Ras-[^{32}P-α]-GTP complex for 12 hydrolysis reactions would consist of 2.4 μg 6x His-Ras in a final volume of 100 μl in 200 μg/ml BSA, 20 mM Hepes pH 7.2, 5 mM DTT, 5 mM EDTA and 120 μCi [^{32}P-α]-GTP (1.5 μM final concentration). The binding reaction was incubated at room temperature for 5 minutes followed by incubation on ice for 5 minutes. $MgCl_2$ was then added to the binding reaction (10 mM final concentration). Following, unbound [^{32}P-α]-GTP was separated from the Ras-[^{32}P-α]-GTP complex using "Nick spin" desalting columns (Amersham Pharmacia Biotech, Inc.). Hydrolysis reactions (0.06 ml) contained 20 mM Hepes pH 7.2, 2 mM $MgCl_2$, 5 mM DTT, 0.5 mg/ml BSA and approximately 0.04 μg Ras-[^{32}P-α]-GTP complex. Where indicated, reactions also contained 0.3 μg purified p120GAP. After incubations, Ras-associated guanyl-nucleotides were immuno-precipitated and analyzed as described for the spontaneous association reactions except that TMN was used for washing beads 8x instead of OnyxA buffer.

Studies demonstrating an *in vitro* interaction between Ras and RasGRP utilized nucleotide-free (apo) Ras and MBP-cat (chapter 4). Apo-Ras was prepared by

equilibrating purified 6x His-Ras-GDP in 50 mM EDTA at 22°C for 30 minutes. 5 μg of purified of MBP-cat was prebound to 40 μl of amylose resin beads for each binding reaction. Beads prebound to MBP-cat or bare beads were incubated with 5 μg of apo-Ras in a final volume of 0.5 ml in GRF binding buffer [20 mM Tris pH 7.5, 50 mM NaCl, 25 mM imidazole, 50 mM EDTA, 1 mM DTT, 5% v/v glycerol, 0.1% v/v Triton X-100, 2 μg/ml leupeptin (Boehringer Mannheim, Laval, Quebec, Canada), 20 μg/ml aprotinin (Sigma Chemical Co.), 1 mM Pefa (Boehringer Mannheim)] for 2 hours at 4°C. Aprotinin, leupeptin and Pefa are protease inhibitors. After the incubation reactions, beads were washed 4x with GRF binding buffer and bound proteins were eluted off with SDS sample buffer. Proteins were resolved by standard 11% SDS- PAGE (polyacrylamide gel electrophoresis), transferred to nitrocellulose and immuno-blotted with anti-Ras monoclonal antibody (R02120, Transduction Laboratories, Lexington, Kentucky, USA).

Cell culture

Most experiments dealing with cultured cells were performed with the rat2 cell line. Rat2 cells are a thymidine-kinase deficient derivative of the rat1 embryonic fibroblast cell line (Botchan *et al.*, 1976, Topp, 1981). Our studies concerning hypothermia also employed MDCK cells (Madin-Darby canine kidney), KD cells (primary human lip fibroblasts), REC (primary rat embryo cells) and CEF (primary chicken embryo fibroblasts) (chapter 3). MDCK and KD cells were kindly provided by Drs. M. Pasdar and R. Day, respectively (both of the University of Alberta). CEF were purchased from Spafas, Inc. (Preston, Connecticut, USA) and REC were purchased from Bio-Whittaker (Walkersville, Maryland, USA). "Normal growth medium" for all cells was Dulbecco's modified Eagle medium (DMEM)/10% fetal bovine serum (FBS) except for CEF which were maintained in DMEM/10% tryptose phosphate broth/1.1% chicken serum/4.4% calf serum. DMEM, FBS, tryptose phosphate broth, chicken serum and calf serum were obtained from Life Technologies-Gibco BRL, Inc.

Several trans genes were overexpressed in rat2 cells using the retrovirus vectors pBabepuro and a helper-free retrovirus packaging system (Morgenstern and Land, 1990, Pear *et al.*, 1993). In some cases, wildtype H-Ras was overexpressed in rat2 cells using the retroviral vector pBW1631 which also carries the neomycin resistance gene. pBW1631 was developed by B. Willumsen (Willumsen *et al.*, 1986, 1991). In chapter 3, some cells overexpressed wildtype H-Ras, Y32H-H-Ras or Raf-1 using the pBabepuro vector (Stang *et al.*, 1996). In chapter 4, cells overexpressed full-length RasGRP, rbc7HA or ΔDG using the pBabepuro vector. Some cells overexpressed both RasGRP and wildtype H-Ras

together. In these cases, H-Ras was overexpressed using pBW1631. In chapter 5, all cells overexpressed H-Ras using pBW1631. To select for expression of trans genes carried in pBabepuro, cells were maintained in normal growth media supplemented with 2.5 μg/ml puromycin (Sigma Chemical Co.). To select for simultaneous expression of trans genes carried in pBabepuro and pBW1631, cells were maintained in normal growth media supplemented with 2.5 μg/ml puromycin and 0.4 mg/ml active G418 (Life Technologies-Gibco BRL, Inc). To select for expression of trans genes carried in pBW1631, cells were maintained in normal growth media supplemented with 0.6 mg/ml active G418.

Several different pretreatment and treatments were given to cells in the experiments of chapter 3. PD098059 (50 μM) (Calbiochem-Novabiochem Corp., La Jolla, California, USA) pretreatment was used for specific inhibition of Mek activation (Alessi *et al.*, 1995). Wortmannin (50 nM) and LY294002 (20 μM) (both from Calbiochem-Novabiochem Corp.) pretreatments were used to inhibit phosphatidylinositol 3-kinase (PI3K). Two calcium chelators were employed as pretreatments. In some experiments, EGTA was added to the cell culture medium (2-5 mM final concentration) for 15 minutes at 37°C before initiating the experiment to chelate extracellular calcium. Alternatively, cells were pretreated with the cell-permeable compound BAPTA/AM (25 μM) (Calbiochem-Novabiochem Corp.) for 15 minutes at 37°C to chelate intracellular calcium. In some cases, both EGTA and BAPTA/AM were used together. For positive control, cells were stimulated with epidermal growth factor (EGF) (100 ng/ml) (Sigma Chemical Co.). Other positive controls included anisomycin (10 μg/ml) (Sigma Chemical Co.) and staurosporine (2 μM) (Calbiochem-Novabiochem Corp.).

In chapter 4, cells were stimulated with phorbol ester (PMA) (100 nM) or endothelin-1 (Et-1) (100 nM) (both from Sigma Chemical Co.). Where indicated, cells were starved in DMEM/0.1% FBS or DMEM/0.5% FBS for 4-6 hours at 37°C before the start of the experiment.

In chapter 5, cells were stimulated with C2-ceramide or dihydro-C2-ceramide (both from Biomol Research Laboratories, Inc., Plymouth Meeting, Pennsylvania, USA) at 40 μM. For all studies concerning ceramide, cells were starved overnight at 37°C in DMEM/0.5% fatty-acid free BSA (Sigma Chemical Co.) before the start of the experiment. Where indicated, cells were treated with vehicle DMSO (0.1%) for negative control.

Exposure of cultured cells to hypothermic stress

For studies involving hypothermic Ras activation, rat2 cells were incubated on ice for various times in either ^{32}P labeling media [DMEM/0.5% dialyzed FBS] (chapter 3, Figure 3-1a and 3-3) or DMEM/10% FBS (chapter 3, Figure 3-1b and 3-8b). In some cases, the cell culture medium was changed to Hepes-buffered saline (HBS) [10 mM Hepes pH 7.0, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$] or DMEM containing various amounts of FBS before the start of the experiment (chapter 3, Figure 3-8).

To study biochemical events during the warming period, cells were incubated on ice in normal growth medium (unless indicated otherwise) for various amounts of time and then warmed using one of two protocols. To study early events after recovery from hypothermic stress, the temperature was raised quickly with an "add back" protocol. In this method, growth medium was removed from the culture during the last 15 minutes of incubation at 0°C. This medium was warmed to 37°C and then added back to the original culture at the start of the recovery period, as the culture was placed in a 37°C water bath. In the second, "shift" protocol, cultures were simply transferred from ice to a warm metal tray in a 37°C CO_2 incubator. The shift protocol was used in several experiments (see chapter, Figures 3-7, 3-10a, 3-11 and 3-12). Using this method, the temperature takes several minutes to rise. Thus, the kinetics of reactions are not directly comparable between different Figures.

Assessment of cell death after prolonged hypothermic stress

To assay cell viability after prolonged hypothermia, rat2, REC and MDCK cells were seeded at $0.5x10^6$ - $1x10^6$ / 25 cm^2 tissue culture flask and incubated overnight at 37°C in a 10% CO_2 incubator. Cells were pretreated with the Mek inhibitor PD098059 (50 μM) or DMSO vehicle control for 90 minutes at 37°C and then incubated in a ice-water bath for 0-72 hours in tightly sealed culture flasks containing a 10% CO_2 environment. Following, cultures were unsealed and incubated for 4 hours in a 37°C CO_2 incubator. Both substrate-attached and detached cells were pooled and plated at low density to determine the number of viable colony forming units. The remainder of each sample was plated in a new 25 cm^2 tissue culture flask and incubated for 4 hours in a 37°C CO_2 incubator to allow reattachment. Reattached (i.e. viable) cells were then trypsinized and counted with a Coulter counter (Coulter Electronics Ltd., Hialeah, Florida, USA). Counting colonies from the low density cultures after 12 days of incubation provided an alternative index of cell survival with similar results.

DNA fragmentation was measured as described previously (Lenahan and Ozer, 1996). $1x10^6$ rat2 cells were seeded in 25 cm^2 flasks and incubated overnight at 37°C in a 10% CO_2 incubator. Cells were pretreated with PD098059 and exposed to hypothermic stress as described in the cell viability assay. After rewarming, genomic DNA from both loose and attached cells was extracted in a buffer that contained 10 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% w/v SDS, and 0.5 mg/ml proteinase K. Extracts were allowed to digest for 24 hours at 37°C. Following, the concentration of NaCl was increased to 1 M and high-molecular weight DNA was precipitated at 4°C for 24 hours. After centrifugation at 14000 x g for 30 minutes at 4°C, the supernatant was extracted 2x with phenol/chloroform/isoamyl alcohol and DNA was precipitated with 70% final concentration of ethanol. DNA samples were analyzed on a 1% TAE-agarose gel.

Biochemical analysis of signaling proteins in cultured cells

Measurement of Ras activation *in vivo*

To measure Ras activation, cultures of rat2 cells ($1.0x10^6$ cells/sample) were labeled with $^{32}P_i$ (0.5 mCi/sample) in phosphate-free DMEM/0.5% dialyzed FBS for 4 hours at 37°C. After various treatments, cells were lysed on ice in p21 buffer [50 mM Tris pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% v/v NP40, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa] containing rat monoclonal antibody Y13-259 (0.5 μg/sample). Cell lysates were precleared by centrifugation at 14000 x g for 3 minutes and supernatants were incubated with activated charcoal (0.1 g/sample) for 1 hour at 0°C. Following, activated charcoal was precipitated by brief centrifugation and Ras was immuno-precipitated from supernatants for 1-2 hours at 0°C using rabbit anti-rat IgG coupled protein-A Sepharose. Ras immuno-precipitates were washed 8 times with p21 buffer and 1 time with Dulbecco's phosphate buffered saline (PBS) containing 20 mM $MgCl_2$ (product 14190, Life Technologies-Gibco BRL, Inc.). Ras-associated guanyl-nucleotides were analysed by chromatography on cellulose-PEI as described in the *in vitro* guanyl nucleotide association reactions. In some cases, GDP and GTP spots were quantified by phosphorimager.

Alternatively, Ras activation was assayed by a non-isotopic method that exploits the specific binding of Ras-GTP to the Ras-binding domain of Raf (Taylor and Shalloway, 1996). A recombinant protein consisting of the glutathione-S-transferase (GST) tag fused to residues 1-149 of wildtype Raf-1 (GST-Raf) was expressed in *E. coli* and bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech, Inc.). GST-Raf bound to glutathione-Sepharose beads was prepared fresh for each experiment. All rat2 cells used

for this assay overexpressed wildtype H-Ras. After various treatments, cells (1 x 10^6 / sample) were lysed in rat2 binding buffer [25 mM Hepes pH7.5, 150 mM NaCl, 1% NP-40, 0.25% DOC, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa]. Lysates were precleared by centrifugation at 14000 x g for 5 minutes. Supernatants were then incubated for 30 minutes at 4°C with 30-50 μg GST-RBD bound to glutathione-Sepharose beads. After the binding reaction, beads were washed 3 times with rat2 binding buffer. Precipitated Ras was resolved on standard 11% SDS-PAGE, transferred to nitrocellulose and detected by immuno-blotting with anti-Ras monoclonal antibody (R02120, Transduction Laboratories).

Immune-complex kinase assays

To measure Raf-1 activity (chapter 3), rat2 cells overexpressing wildtype Raf-1 ($3x10^6$ cells/sample) were lysed in ice-cold RIPA buffer [20 mM Tris pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% v/v glycerol, 1% v/v NP40, 0.1% w/v SDS, 0.5% w/v DOC, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa, 100 μM Na-vanadate] after various treatments. Cell lysates were cleared by centrifugation at 14000 x g for 5 minutes. Following, Raf-1 was immuno-precipitated from 1/2 of the cell lysate with 1 μg anti-Raf-1 polyclonal antibody (C-12, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) bound to protein-A-Sepharose for 2-3 hours at 0°C. Immuno-precipitates were washed 3 times with DOC- and SDS-free RIPA buffer and 1 time with Raf kinase buffer [30 mM Hepes pH 7.4, 10 mM $MgCl_2$, 7 mM $MnCl_2$, 2 mM DTT]. Raf immuno-precipitates were then incubated at 30°C (60 μl final volume) in Raf kinase buffer containing 0.5 μg purified GST-MEK, 2 μg purified kinase-defective Erk, 20 μM ATP and 10 μCi [^{32}P-γ]-ATP (Amersham Pharmacia Biotech, Inc.). After 30 minutes, the reaction was stopped with SDS sample buffer and the products were resolved on standard 10% SDS-PAGE. Proteins were transferred to nitrocellulose and radiolabeled Erk was quantified by phosphorimager analysis. Mek1 was immuno-precipitated from 1/10 of the same precleared cell lysate with 1 μg Mek1 monoclonal antibody (M17020, Transduction Laboratories) bound to rabbit-anti mouse IgG-coupled protein A Sepharose (Sigma Chemical Co.). Immuno-precipitated Mek was washed 3 times with DOC- and SDS-free RIPA lysis buffer and 1 time with Mek kinase buffer [25 mM Hepes pH 7.4, 10 mM Mg-$(H_3C_2OO)_2$, 2 mM DTT]. Washed immuno-precipitates were incubated at 30°C (60 μl final volume) in Mek kinase buffer containing 2 μg purified kinase-defective Erk, 20 μM ATP, and 10 μCi [^{32}P-γ]-ATP for 30 minutes. Phosphorylated Erk substrate was analyzed as described in the Raf immune-complex kinase assay.

Akt immune-complex kinase assay

To assay Akt activity (chapter 3), $5x10^6$ rat2 cells/sample were lysed in Akt buffer [20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol v/v, 1% NP40 v/v, 10 mM DTT, 10 mM EDTA, 10 mM NaF, 40 mM β-glycerophosphate, 100 μM Na-vanadate, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa] after various treatments. Lysates were precleared by centrifugation at 14000 x g for 5 minutes and Akt was immuno-precipitated using 2 μg Akt polyclonal antibody (PKB-PH, Upstate Biotechnology, Inc., Lake Placid, New York, USA) bound to protein A-Sepharose for 16 hours at 0°C. Akt immuno-precipitates were washed 2 times with Akt buffer, 2 times with high-salt buffer [25 mM Hepes 7.2, 1 M NaCl, 1% NP40 v/v, 0.1% w/v BSA, 10 mM DTT, 100 μM Na-vanadate, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa], and 1 time with Akt kinase buffer [20 mM Hepes pH 7.2, 10 mM $MgCl_2$, 10 mM DTT]. Washed immuno-precipitates were incubated at 30°C (30 μl final volume) in Akt kinase buffer containing 2 μg PKI, 10 U purified glycogen synthase kinase 3 (GSK3) (New England Biolabs, Inc.), 1.3 mM EGTA, 66 μM ATP, and 10 μCi [^{32}P-γ]-ATP. After 30 minutes, the reaction was stopped with SDS sample buffer and the products were resolved on standard 10% SDS-PAGE. Proteins were transferred to nitrocellulose and radiolabeled GSK3 was quantified by phosphorimager analysis.

***In vitro* assay of JNK activity**

JNK activity was measured using an assay that employs a GST-jun recombinant protein (GST fused to residues 1-79 of c-jun) (chapter 3) (Hibi *et al.*,1993). GST-jun was expressed in *E. coli* and bound to glutathione-Sepharose beads. Aliquots of GST-jun bound to glutathione-Sepharose were stored at -80°C until use.

2 x 10^6 rat2 cells were lysed in WCE (whole cell extract) buffer [25 mM Hepes 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% v/v Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 100 μM Na-vanadate, 2 μg/ml leupeptin, 1 mM Pefa] after various treatments. Cell lysates were precleared by centrifugation at 14000 x g at 4°C for 10 minutes. Aliquots of precleared lysate containing 500 μg total protein content were diluted to 20 mM Hepes pH 7.7, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% v/v Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 100 μM Na-vanadate, 2 μg/ml leupeptin, 1 mM Pefa and incubated with 10 μg of GST-jun bound to glutathione-Sepharose for 1 hour at 4°C. Precipitated JNK was washed 3 times with WB (wash

buffer) [20 mM Hepes 7.7, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% v/v Triton X-100] and 1 time with JNK kinase buffer [20 mM Hepes pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 100 μM Na-vanadate, 1.9 mM para-nitrophenyl-phosphate, 1 mM DTT]. Washed precipitates were incubated at 30°C (30 μl final volume) in JNK kinase buffer containing 20 μM ATP and 5 μCi [^{32}P-γ]-ATP. After 20 minutes, the reaction was stopped with SDS sample buffer and the products were resolved on standard 10% SDS-PAGE. Proteins were transferred to nitrocellulose and radiolabeled GST-jun was quantified by phosphorimager analysis.

Assessment of protein phosphorylation by immuno-blotting procedures

In chapters 3 and 4, Erk activation was monitored using a gel mobility shift assay. This assay employs customized gel conditions designed to separate the phosphorylated and unphosphorylated forms of Erk. Proteins in standard SDS-PAGE sample buffer were separated on 15% SDS-free gels [stacker gel: 5% acrylamide, 0.13% bis- acrylamide, 125 mM Tris pH 6.8; resolving gel: 15% acrylamide, 0.086% bis-acrylamide, 375 mM Tris pH 8.8] using SDS-containing running buffer [384 mM glycine, 50 mM Tris, 0.1% w/v SDS]. After transfer to nitrocellulose, resolved proteins were immuno-blotted with anti-MAPK monoclonal antibody (13-6200, Zymed, Inc. Camarillo, California, USA).

Erk activation was also monitored by resolving proteins on 15% mobility shift type gels followed by immuno-blotting with an antibody specific for the phosphorylated forms of Erk (9105, New England Biolabs, Inc.). An aliquot of each lysate was analysed using standard 10% SDS-PAGE followed by immuno-blotting with anti-Erk polyclonal antibody (sc-94, Santa Cruz Biotechnology, Inc.) to demonstrate equivalent amounts of total Erk protein per sample.

Activation of SEK (chapter 3) was monitored by immuno-blotting proteins resolved on 15% mobility-shift gels with an antibody that specifically recognizes the phosphorylated form of the protein (9151, New England Biolabs, Inc.)

To assess tyrosine phosphorylation of Erk and Shc (chapter 3), cell lysates were resolved using 12.5% mobility shift type gels [stacker gel: same as in 15% gel; resolving gel: 12.5% acrylamide, 0.106% bis-acrylamide, 375 mM Tris pH 8.8] and immuno-blotted with a cocktail of two different monoclonal anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology, Inc. and PY69, Santa Cruz Biotechnology, Inc.). The identities of Erk and Shc were confirmed by immuno-blotting lysates with antibodies that specifically recognize these two proteins.

For all the protein phosphorylation immuno-blotting procedures described above, approximately 20-100 μg of protein was loaded in each lane. I confirmed that each lane within each experiment contained an equivalent amount of protein by Ponceau S (Sigma Chemical Co.) staining the nitrocellulose filters.

Co-immunoprecipitation of Ras and PI3K

Rat2 cells overexpressing H-Ras were plated in 10 cm tissue culture dishes and allowed to grow to confluence over 4-5 days at 37°C with frequent changing of the culture media (chapter 5). Cultures were then starved overnight in DMEM/0.5% fatty-acid free BSA. After various treatments, cells were lysed in PI3K binding buffer [50 mM Hepes, 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na-vanadate, 100 mM NaF, 10 mM Na-pyrophosphate, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged at 14000 x g for 5 minutes. After centrifugation, supernatants were precleared by incubation with protein A Sepharose for 1 hour at 4°C. The final binding reactions contained 100 μg total cellular protein from precleared lysates and 2 μg anti- p85α monoclonal antibody (Santa Cruz Biotechnology, Inc.) in a final volume of at least 0.5 ml in PI3K binding buffer. These binding reactions were incubated for 4-6 hours at 4°C. Following, protein A-Sepharose beads were added and binding reactions were incubated overnight at 4°C. PI3K immuno-precipitates were washed 3x with PI3K binding buffer and 1x with PBS. Proteins were eluted off the beads with SDS-sample buffer, resolved on standard 11% SDS-PAGE, and transferred to nitrocellulose. Co-immunoprecipitating Ras was visualized by immuno-blotting with anti-Ras monoclonal antibody (R02120, Transduction Laboratories).

Subcellular fractionation

After various treatments, rat2 cells overexpressing Raf-1 ($2x10^6$ / sample) were scraped in Homogenization buffer [10 mM Tris pH 7.5, 25 mM NaF, 5 mM MgCl2, 1 mM EGTA, 100 μM Na-vanadate, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefa] (chapter 3). Cells were homogenized by 50 strokes in a small sized Dounce apparatus at 0°C and homogenates were centrifuged at 1000 x g for 5 minutes at 4°C. Precleared homogenates were then centrifuged at 100,000 x g for 1 hour at 4°C in a fixed angle rotor. Pellets (particulate fraction) were gently washed 3 times with Homogenization buffer and then resuspended in RIPA buffer. 50% of each pellet was analyzed by standard 10% SDS-

PAGE followed by immuno-blotting with Raf-1 monoclonal antibody (R19120, Transduction Laboratories).

Animal studies

To prevent extraneous temperature fluctuations, neonatal Sprague Dawley rats were maintained for one hour at 33-35°C in a cardboard grid that prevented contact between individuals (chapter 3). One set of animals was maintained at this temperature for a further 2 to 35 minutes. Two sets of animals were transferred to ice-cold containers that prevented contact between individuals for 20 minutes. This treatment deceased core body temperatures to 10-12°C. Core body temperature was monitored with a thermocoupler using a rectal probe. One set of cold animals was transferred back to the 33-35°C grid for 12-14 minutes. After warming, the animals regained muscle movement and core body temperature rose to 28-31°C. At the ends of these treatments, animals were sacrificed by decapitation and hind limbs were rapidly removed and homogenized in Erk lysis buffer [20 mM Tris pH 8.0, 10 mM EGTA, 5 mM $MgCl_2$, 1% Triton-X100, 0.5% w/v DOC, 100 μM Na-vanadate, 40 mM Na-pyrophosphate, 50 mM NaF, 2 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM Pefa]. Tissue homogenates were precleared by centrifugation (14000 x g, 5 minutes) and Erk was immuno-precipitated with anti-MAPK R2 polyclonal antibody (Erk1-CT, Upstate Biotechnology, Inc.) bound to protein-A-Sepharose. Immuno-precipitates were washed 3x with Erk kinase wash buffer [20 mM Tris pH 8.0, 1% v/v Triton X-100, 12 mM DOC, 10 mM EGTA, 100 μM Na-vanadate, 5 mM $MgCl_2$, 40 mM Na-pyrophosphate, 50 mM NaF], 1x with 30 mM Tris pH 8.0, and 1x with Erk kinase reaction buffer [30 mM Tris pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$]. Erk immuno-precipitates were then incubated at 30°C (60 μl final volume) in Erk kinase buffer containing 7.2 μg myelin basic protein, 10 μM ATP and 5 μCi [^{32}P-γ]-ATP. After 30 minutes the reaction was spotted on P81 cation-exchange paper (Whatman International, Ltd., Springfield Mill, Maidstone, Kent, England). After extensive washing in 0.5% phosphoric acid, radioactivity bound to filter was quantitated by scintillation counting.

References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. & Saltiel, A.R. (1995) PD098059 is a specific inhibitor of the activation of the mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**:27489-27494.

Botchan, M., Topp, W., and Sambrook, J. (1976) The arrangement of Simian virus 40 sequences in the DNA of transformed cells. *Cell.* **9**:269-287.

Ebinu, J.O., Bottorff, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science.* **280**:1082-1086.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV- responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes and Development.* **7**:2135-2148.

Lenahan, M.K. and Ozer, H.L. (1996) Induction of c-myc mediated apoptosis in SV40-transformed rat fibroblasts. *Oncogene.* **12**:1847-1854.

Leonardsen, L., DeClue, J.E., Lybæk, H., Lowy, D.R., and Willumsen, B.M. (1996). Rasp21 sequences opposite the nucleotide binding pocket are required for GRF-mediated nucleotide release. *Oncogene.* **13**:2177-2187.

Morgenstern, J.P., and Land, H. (1990) Advanced mammalian gene transfer: high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nuc. Acid Res.* **18**:3587-3596.

Pear, W.S., Nolan, G.P., and Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Nat. Acad. Sci.* USA. **90**:8392-8396.

Poe, M., Scolnick, E.M., and Stein, R.B. (1985). Viral Harvey ras p21 expressed in Escherichia coli purifies as a binary one-to-one complex with GDP. *J. Biol. Chem.* **260**:3906-3909.

Stang, S., Bottorff, D., and Stone, J.C. (1996) ras Effector loop mutations that dissociate p120GAP neurofibromin interactions. *Molecular Carcinogenesis.* **15**:64-69.

Taylor, S.J. & Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Curr. Biol.* **6**:1621-1627.

Topp, W.C. (1981) Normal rat cell lines deficient in nuclear thymidine kinase. *Virol.* 113:408-411.

Willumsen, B.M., Papageorge, A.G., Kung, H.-F., Bekesi, E., Robins, T., Johnsen, M., Vass, W.C. and Lowy, D.R. (1986) Mutational analysis of a *ras* catalytic domain. *Mol. Cell. Biol.* **6**:2646-2654.

Willumsen, B.M., Vass, W.C., Velu, T.J., Papageorge, A.G., Schiller, J.T., and Lowy, D.R. (1991) The bovine papillomavirus E5 oncogene can cooperate with *ras*: indentification of p21 amino acids critical for transformation by *c-rasH* but not *v-rasH*. Mol. Cell. Biol. 11:6026-6033.

Chapter 3 - Activation of Ras-Erk signaling in response to hypothermic stress

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Introduction

In the course of our studies on Ras signaling, I discovered that Ras becomes activated in rat fibroblasts that are exposed to hypothermic stress. Hypothermia is a common environmental stress sustained by many species. Furthermore, hypothermia is often used to preserve human cells, tissues and organs for transplantation. In this latter situation, hypothermia is thought to protect cells by slowing metabolism during the anoxic storage period (Belzer and Southard, 1988). Hypothermic stress can also cause cell damage, however. This damage could stem from decreased ATP synthesis, failure to maintain cellular membrane Na^+ and Ca^{2+} ion gradients, cell swelling and activation of phospholipases (Hochachka, 1986, Belzer and Southard, 1988, Liu *et al.*, 1991, Mcanulty *et al.*, 1996). Hypothermia is also a recognized trigger of apoptosis (programmed cell death) (Perotti *et al.*, 1990, Kruman *et al.*, 1992, Gregory and Milner, 1994). Little is known about specific biochemical pathways that are activated in response to hypothermic stress. Furthermore, the relationship between known biochemical changes in hypothermic cells and cell damage is not understood. In this study, I set out to investigate: 1) the mechanism of hypothermically-induced Ras activation and 2) the biochemical and physiological consequences of hypothermically-activated Ras.

Previously, it had been found that low temperatures block acute growth factor signaling (Campos-González and Glenney, Jr., 1991, Okuda *et al.*, 1992). When fibroblasts are exposed to epidermal growth factor (EGF) at 4°C, Erk is not activated (Campos-González and Glenney, Jr., 1991). This temperature effect is not due to trivial effects on ATP levels, for example, since autophosphorylation of the receptor at low temperature is readily detectable. In parallel with our studies on the activation of Ras by hypothermic stress, I also aimed to define the level of the signaling pathway at which low temperature blocks acute EGF signaling.

Results

Activation of Ras in rat2 fibroblasts exposed to hypothermic stress

Ras activation in rat2 fibroblasts was assessed by measuring the ^{32}P-labeled guanyl nucleotides associated with Ras (Figure 3 -1a). Alternatively, I measured Ras activation in rat2 cells overexpressing H-Ras using a non-isotopic method that employs GST-Raf fusion protein to precipitate Ras-GTP from cell lysates (Figure 3-1b) (Taylor and Shalloway,

1996). Following precipitation, Ras is detected by an immuno-blot method. In both these experimental systems, Ras was primarily GDP-bound in untreated cells. Ras-GTP slowly accumulated in cells exposed to 0°C. Within 4 hours of 0°C incubation, the amount of Ras-GTP (33%) approached levels obtained after acute EGF stimulation at 37°C (44%). Remarkably, this activated state could persist for at least 32 hours. Within 1 minute of rewarming, Ras-GTP levels decreased and Ras-GDP levels increased (Figure 3-1a).

Ras guanyl nucleotide association and hydrolysis at 0°C

In the cell, Ras-GDP and Ras-GTP levels are determined by the relative rates of GTP hydrolysis and guanyl nucleotide exchange. To understand how Ras might be activated in hypothermic cells, spontaneous and catalyzed GTP hydrolysis and guanyl nucleotide exchange reactions were studied *in vitro*, using recombinant proteins. Since *in vivo* Ras activation at 0°C was slow and increased over a 4 hour period, these reactions were studied over this time period. Recombinant Ras co-purifies with GDP (Poe *et al.*, 1985). After incubating Ras-GDP complex with an excess of radiolabeled GTP for 4 hours at 30°C, 53% of Ras was associated with labeled guanyl nucleotide and 75% of this was hydrolyzed to GDP. By contrast, after incubation at 0°C for 4 hours, only 3% of Ras was associated with exogenous nucleotide (Figure 3-2a). In this case about 15% was hydrolyzed to GDP while the remaining 85% was present as GTP. Thus, spontaneous dissociation of Ras-GDP followed by GTP association (i.e. spontaneous nucleotide exchange) is very slow at 0°C. In addition, intrinsic GTP hydrolysis is substantially inhibited at 0°C.

In the cell, Ras is negatively regulated by GTPase activating proteins (GAP's). p120GAP is a major RasGAP in fibroblasts and this enzyme retained considerable activity at 0°C when assayed over a 4 hour period (Figure 3-2b). Similar results were obtained with the catalytic domain of NF1, another Ras GAP (Stang and Stone, unpublished data). Ras is positively regulated in the cell by guanyl nucleotide exchange factors (GEF's). The ability of two Ras GEF's to function at 0°C was examined. The catalytic domain of RasGRF1 caused a 10-fold increase in the formation of Ras-GTP relative to the non-catalyzed GTP association reaction (Figure 3-2c). The catalytic domain of RasGRP, another mammalian Ras GEF (Ebinu *et al.*, 1998), similarly displayed considerable activity at 0°C (Chan and Stone, unpublished data). Thus, Ras GEF's and Ras GAP's both appear to retain significant activity at low temperatures. These *in vitro* data suggest that Ras GAP's and GEF's may have the ability to regulate Ras *in vivo* at 0°C.

Inhibition of hypothermic Ras activation by mutation of the effector domain

In X-ray crystallographic studies, the interaction of Ras with both Ras GEF's and Ras GAP's was observed to involve the Ras effector (switch 1) region (Scheffzek *et al.*, 1997, Boriack-Sjodin *et al.*, 1998). Furthermore, subtle amino acid substitutions in the effector region can effectively dissociate Ras from Ras GEF and Ras GAP regulation (Adari *et al.*, 1988, Mistou *et al.*, 1992, Stang *et al.*, 1996). I hypothesized that certain Ras effector mutants would fail to be activated by hypothermic stress *in vivo* if Ras GEF's or Ras GAP's were involved in the mechanism. Indeed, the Y32H Ras allele (tyrosine-32 to histidine) did not become associated with GTP in rat2 cells after 4 hours of 0°C incubation (Figure 3-3). However, association of Y32H Ras with GTP could be stimulated by EGF treatment at 37°C. Thus, mutation of the effector domain inhibits hypothermic Ras activation but does not inhibit growth factor stimulated Ras activation at 37°C.

Activation of the Raf-Mek-Erk kinase cascade during recovery from hypothermic stress

To address the biochemical consequences of hypothermically activated Ras, the status of the Raf-Mek-Erk protein kinase cascade was assessed in hypothermic rat2 cells after various times of rewarming. These rat2 cells overexpressed wildtype Raf-1. Using an immune-complex kinase assay, Raf-1 was found to be maximally activated 1 minute after rewarming to 37°C (Figure 3-4a) while Mek1 was maximally activated after 2 minutes (Figure 3-4b). Tyrosine phosphorylation of Erk, a marker for activation by Mek, was maximal at 3 minutes after rewarming (Figure 3-4c). The activation of Raf, Mek and Erk during the recovery period was comparable in amplitude to the activation levels observed after EGF treatment of cells. A protein kinase downstream of Erk, p90rsk (ribosomal S6 kinase), was also activated after recovery from hypothermic stress (Bottorff and Stone, unpublished data). No activation of the Raf-Mek-Erk kinase cascade was evident without rewarming. Thus, Ras activated by hypothermic stress leads to a robust Raf-Mek-Erk effector response during the recovery period.

Activation of PI3K signaling by hypothermic stress followed by rewarming

In addition to the Raf-Mek-Erk kinase cascade, Ras is also thought to activate phosphatidylinositol-3 kinase (PI3K) *in vivo* (Rodriguez-Viciana *et al.*, 1994, 1996). Once activated, PI3K generates a series of phosphatydylinositol second messengers

phosphorylated at the D3 position (Carpenter and Cantley, 1996). PI3K generated phosphatidylinositol second messengers have been shown to activate the protein kinase Akt in NIH3T3 cells and *in vitro* (Franke *et al.*, 1997). Here, PI3K activation *in vivo* was monitored indirectly using an Akt immune-complex kinase assay. Treatment of rat2 cells with EGF gave rise to a 2-fold increase in Akt activity within 5 minutes (Figure 3-5). EGF-induced Akt activation was absent in cells pretreated with either Wortmannin or LY294002, two inhibitors of PI3K. Pretreatment of cells with Wortmannin or LY294002 appeared to decrease Akt activity to levels below untreated cells suggesting that a basal PI3K activity was present in untreated cells. Akt was activated upon rewarming after a 4 hour 0°C incubation. This activation was comparable in amplitude with that of EGF stimulated cells and could be detected beginning at 5 minutes after rewarming. The activation of Akt peaked between 10-20 minutes of rewarming. These observations are consistent with an activation of PI3K in response to hypothermic stress followed by rewarming.

Absence of robust JNK activation in response to hypothermic stress followed by rewarming

c-jun N-terminal kinase (JNK) is known to be activated by a number of cellular stresses such as UV radiation, pharmacological inhibition of protein synthesis and heat shock (Kyriakis *et al.*, 1994). In addition, it has been shown that activated Ras can stimulate c-jun-dependent transcription in several cell types suggesting that Ras signaling activates JNK (Binetruy *et al.*, 1991). JNK signals in a kinase cascade that is analogous to the Raf-Mek Erk kinase cascade. The protein kinase MEKK1 phosphorylates and activates SEK (MKK4) which in turn, phosphorylates and activates JNK (Yan *et al.*, 1994, Sanchez *et al.*, 1994, Lin *et al.*, 1995).

It was asked whether JNK signaling *in vivo* was activated in response to hypothermic stress followed by rewarming. SEK was not phosphorylated in rat2 cells that were incubated at 0°C for 4 hours followed by rewarming for up to 5 minutes (Figure 3-6a). Treatment of cells with anisomycin, an inhibitor of protein synthesis did stimulate SEK phosphorylation. The specific kinase activity of JNK precipitated from rat2 cell lysates was also measured. In this assay, anisomycin treatment led to a 300-fold increase in JNK activity (Figure 3-6b). Incubation of cells at 0°C for 4 hours followed by rewarming for 5 minutes did detectably activate JNK. However, the amplitude of this activation was only 3% of that obtained from the anisomycin positive control. EGF stimulation also did not lead to robust activation of SEK and JNK in rat2 cells. It is

concluded that hypothermic stress followed by rewarming does not lead to robust activation of JNK signaling.

Inhibition of hypothermic Ras-Erk signaling by Ca^{2+} chelators

Hypothermic stress is thought to increase cytosolic Ca^{2+} levels, in part by inhibiting Ca^{2+} active transport from the cytosolic compartment to the cell exterior and lumen of the endoplasmic reticulum (ER) (Hochachka, 1986, Perotti *et al.*, 1990, Liu *et al.*, 1991, Mcanulty *et al.*, 1996). Driven by a large concentration gradient, Ca^{2+} ions from the cell exterior and ER constantly leak into the cytosol through Ca^{2+} channels (Clapham, 1995). It was asked whether the activation of Ras-Erk signaling during recovery from hypothermic stress in rat2 cells involved Ca^{2+}. For these experiments, Erk phosphorylation was monitored using a gel mobility shift assay. Sequestration of extracellular Ca^{2+} in the growth medium with EGTA partially inhibited the phosphorylation of Erk during the recovery period following a 4 hour 0°C incubation (Figure 3-7). Intracellular Ca^{2+} can be sequestered using the cell-permeable chelator BAPTA/AM and pretreatment of cells with this reagent partially inhibited Erk phosphorylation during the rewarming period. Pretreated of cells with both EGTA and BAPTA/AM blocked the phosphorylation of Erk following rewarming. Neither EGTA, BAPTA/AM, nor the combination of both chelators affected the phosphorylation of Erk by EGF stimulation, however. The accumulation of Ras-GTP induced by 0°C exposure of rat2 cells was also inhibited by pretreatment with EGTA together with BAPTA/AM (Figure 3-1a, lane 7). These results suggest that Ca^{2+} ions are involved in the molecular mechanism of hypothermic Ras activation. The experiments have not addressed whether hypothermia-induced Ca^{2+} ion fluxes are responsible for hypothermic Ras activation, however.

Inhibition of hypothermic Ras-Erk signaling by removal of serum from the cell culture medium

I hypothesized that Ca^{2+} ions were the only component of the extracellular medium critical for hypothermic activation of Ras-Erk signaling. Therefore, I replaced the normal cell culture growth medium (DMEM/10 % fetal bovine serum (FBS)) with Hepes buffered saline (HBS) containing 20 mM Ca^{2+} and attempted to activate Ras-Erk signaling in rat2 cells by hypothermic stress followed by rewarming. To my surprise, the incubation of cells in HBS/20 mM Ca^{2+} inhibited hypothermia-induced Erk activation. This result prompted further investigation into the components of the cellular medium necessary for

hypothermic Ras-Erk activation. Incubation of rat2 cells in either DMEM or HBS inhibited the phosphorylation of Erk after a 4 hour 0°C exposure followed by rewarming (Figure 3-8a). EGF treatment of cells incubated in either DMEM or HBS at 37°C did stimulate Erk phosphorylation indicating that the EGF signaling pathway was functional. Importantly, addition of 0.5% FBS to DMEM restored the activation of Erk by hypothermic stress followed by rewarming. Incubation of cells in DMEM also inhibited the hypothermic-activation of Ras in rat2 cells overexpressing H-Ras (Figure 3-8b). The addition of serum to DMEM restored hypothermia-induced Ras-GTP accumulation in a concentration dependent manner. Thus, hypothermic Ras activation appears to be dependent on the amount of some unknown serum factor(s) in the growth media.

Inhibition of acute EGF signaling by hypothermia

In parallel with the above experiments, the mechanism underlying the previously documented inhibitory effect of low temperature on EGF signaling was investigated (Campos-González and Glenney, Jr., 1991). When rat2 cells were exposed to EGF at 0°C, Ras-GTP increased rapidly (Figure 3-1a, lane 2). Thus, low temperature does not appear to inhibit EGF activation of Ras.

In response to EGF stimulation of rat1 cells overexpressing the EGF receptor tyrosine kinase (RTK), Shc adapter proteins promptly bind the EGF RTK and become tyrosine phosphorylation on a number residues (Pelicci *et al.*, 1992). Phosphotyrosine residues on Shc, in turn, provide binding sites for the Grb2 adapter protein (Pelicci *et al.*, 1992, Rozakis-Adcock *et al.*, 1992, Ruff-Jamison *et al.*, 1993, Yokote *et al.*, 1994). As a result, the Grb2-SOS complex is recruited to the plasma membrane into proximity with Ras. As such, Shc proteins function between the RTK and Ras in the EGF signaling pathway.

Using an anti-phosphotyrosine immuno-blotting method, tyrosine phosphorylation of the Shc proteins and Erk in response to EGF stimulation at 37°C was confirmed in rat2 cells (Figure 3-9a). In agreement with previous work, it was found that 0°C blocks EGF signaling to Erk. However, 0°C did not inhibit EGF-induced tyrosine phosphorylation of Shc proteins, as expected from our results with EGF activation of Ras at 0°C.

Using an immune-complex kinase assay, it was shown within our lab that 0°C largely blocked EGF-stimulated Raf activation in rat2 cells (Chan *et al.*, 1999). Therefore, acute EGF signaling at 0°C is efficient to the point where Ras is activated but Raf activation is largely blocked.

Activated Ras directly binds to the N-terminal regulatory domain of Raf. This interaction is thought to recruit Raf to the plasma membrane for further activating events (Warne *et al.*, 1993, Zhang *et al.*, 1993, Traverse *et al.*, 1993, Marias *et al.*, 1995). I questioned whether cold temperatures inhibited Raf activation by preventing membrane recruitment of Raf. After stimulation of rat2 cells overexpressing wildtype Raf-1 with EGF at 37°C or 0°C, the particulate fraction enriched in plasma membrane was isolated by subcellular fractionation. EGF stimulation at 37°C lead to a substantial increase of Raf in the particulate fraction (Figure 3-9c). At 0°C, the EGF-stimulated increase of Raf in the particulate fraction was largely inhibited. In control experiments that involved rat2 cells co-expressing wildtype Raf-1 and Ras, it was confirmed that Ras remains associated with the particulate fraction after incubation at 0°C. In sum, the inhibition of Raf activation in response to acute EGF stimulation by hypothermia appears to correlate with an inhibition of EGF-induced membrane recruitment of Raf.

Conservation of hypothermic Erk activation and hypothermic inhibition of acute EGF signaling in vertebrate cells.

Erk phosphorylation assays were used to explore the circumstances where Ras-Erk signaling is activated in response to hypothermic stress followed by rewarming. The degree of Erk activation in rat2 cells is proportional to the duration of hypothermia for up to four hours, at which point Erk is fully activated (Figure 3-10a). As expected, hypothermic Erk activation was blocked by PD098059, a specific inhibitor of Mek (Alessi *et al.*, 1995). Rat2 cells were exposed to various temperatures for a 4 hour period and then examined for Erk activation after rewarming to 37°C (Figure 3-10b). 4°C hypothermic stress followed by rewarming was sufficient to elicit robust Erk activation while 10°C hypothermic stress followed by rewarming led to modest Erk activation. Hypothermic Ras-Erk activation is not an idiosyncrasy of rat2 cells. MDCK cells (canine kidney epithelial cell line), KD cells (primary human fibroblasts), CEF (primary chicken embryonic fibroblasts) and REC (primary rat embryo cells) all showed Erk activation after rewarming from 0°C hypothermic stress (Figure 3-10c). The inhibition of acute growth factor signaling at 0°C was also conserved in each of the cultured cell types.

Hypothermically-induced activation of Ras-Erk signaling is not limited to cultured cells. Activation of Erk was observed in hind-leg tissues of neonatal rats that were subjected to sub-lethal hypothermic stress followed by rewarming (Figure 3-10d). However, I was unable to confirm an accumulation of Ras-GTP in hypothermic neonatal rat tissues.

Negative-feedback inhibition of EGF signaling after recovery from hypothermic stress

In rat2 cells, the activation of Erk in response to hypothermic stress followed by rewarming was robust but transient. It was questioned whether such a biochemical event would produce changes in the biology of the cell. Erk has been show to phosphorylate SOS, the Ras GEF, and this phosphorylation may constitute a negative-feedback mechanism *in vivo* (Porfiri and McCormick, 1996). After a 4 hour 0°C incubation, Erk is quantitatively phosphorylated during the initial recovery period but returns to the unphosphorylated state within 30 minutes of rewarming (Figure 3-11). Cells that were given a pretreatment of hypothermic stress followed by 30 minutes of rewarming were considerably less responsive to subsequent stimulation with low concentrations of EGF. Thus, hypothermically-induced Ras-Erk signaling appears to elicit a negative-feedback mechanism in rat2 cells that inhibits subsequent acute EGF stimulation.

Improved cell viability after prolonged hypothermic stress by pharmacological inhibition of Mek

Cultured cells exposed to prolonged hypothermia were studied to determine whether hypothermically-induced Ras signaling influences viability. Hypothermic exposure times that resulted in a substantial loss of viability were first determined for various cells types. It was then confirmed in REC and rat2 cells that Erk can be activated after recovery from prolonged hypothermia and that this biochemical response is blocked by PD098059 pretreatment (Figure 3-12).

During the period of prolonged 0°C incubation, REC and rat2 cells remain attached and have a near-normal morphology. Immediately after warming, the cells become rounded and detach from the substratum (Figure 3-13a). When both the detached and attached cells are pooled and replated, a substantial loss of viability is evident from the failure of cells to reattach and to form colonies (Figure 3-13b). Similar results were obtained with the MDCK epithelial cell line (Figure 3-13c) and KD human fibroblasts. Pretreatment of cells with the Mek inhibitor PD098059 prevented the change in REC and rat2 cell morphology and substantially increased viability in all cells tested. The Mek inhibitor U0126 (Favata *et al.*, 1998) similarly protected rat2 cells from a loss of cell viability after prolonged hypothermic stress.

In rat2 cells, prolonged hypothermic stress followed by rewarming also leads to DNA fragmentation, a late cellular marker for apoptosis (Figure 3-14). PD098059 pretreatment decreased the amount of fragmented DNA produced during recovery from prolonged hypothermia.

Discussion

Ras is activated in rat2 fibroblasts exposed to hypothermic stress, as assessed by two different methods. The activation of Ras occurs over a period of several hours, persists for many hours and is comparable in magnitude to that achieved by acute growth factor signaling.

Guanyl-nucleotide exchange and hydrolysis reactions were studied *in vitro* to gain insight into the *in vivo* mechanism of hypothermic Ras activation. Intrinsic nucleotide exchange *in vitro* was very slow at 0°C and we hypothesized that this slow rate was insufficient to explain the Ras activation observed in fibroblasts at 0°C. Intrinsic GTP hydrolysis was also markedly inhibited at 0°C. Ras regulatory proteins do appear to retain some function at 0°C *in vitro*. This was demonstrated with two purified Ras GEF's and two well-characterized Ras GAP's. The amounts of Ras GAP's and Ras GEF's used in these *in vitro* experiments were arbitrary and likely do not reflect the relative activities of these factors in cells. As such, our studies do not provide a quantitative account of how temperature would affect the equilibrium between Ras-GDP and Ras-GTP *in vivo*. However, these *in vitro* data suggest that GEF's and GAP's may have the ability to regulate Ras in hypothermic rat2 cells.

Several observations from rat2 cells suggest that a GEF is involved in hypothemic Ras activation. Ras with a tyrosine to histidine substitution (Y32H) in the effector switch I region of Ras fails to be activated in cells exposed to hypothermic stress. In crystallographic structural studies, interaction of both Ras GEF's and Ras GAP's with Ras has been observed to involve the effector region (Scheffzek *et al.*, 1997, Boriack-Sjodin *et al.*, 1998). Thus, the Y32H mutation may be inhibiting hypothermic activation by dissociating the Ras protein from the positive regulation of a GEF. The effect of the Y32H mutation on interaction with GEF's is subtle however since acute growth factor stimulation at 37°C was able to efficiently activate the mutant Ras protein. In yeast 2-hybrid analysis, Y32H Ras has been documented to have normal affinity for CDC25, the yeast Ras GEF, and Raf1 (Stang *et al.*, 1997). *In vitro*, Y32H Ras can be negatively regulated by p120GAP but not the catalytic domain of NF1 (Stang *et al.*, 1996).

Activation of Ras in hypothermic rat2 cells appears dependent upon some serum factor(s) in the cell culture media. In addition, the degree of hypothermic Ras activation was proportional to the amount of serum in the media. Hypothermic activation of Ras in rat2 cells was also inhibited by extracellular and intracellular Ca^{2+} chelators.

Based on the studies performed *in vitro* and in rat2 cells, a model has been formulated that may explain *in vivo* hypothermic Ras activation. In this model, Ras activation in hypothermically stressed cells involves an inhibition of intrinsic GTP hydrolysis coupled with a low rate of guanyl-nucleotide exchange, in part catalyzed by a serum-dependent basal GEF activity. However, hypothermic Ras activation is slow and the unknown serum factor(s) do not lead to overt stimulation of the signaling components upstream of Ras.

There are at least two possible explanations for the inhibitory effects of the Ca^{2+} chelators. The unknown serum factor may directly stimulate a weak Ca^{2+} current through a plasma membrane channel and this weak Ca^{2+} current may regulate a Ca^{2+}-dependent GEF or a Ca^{2+}-dependent GEF regulatory protein. Two Ca^{2+}-responsive Ras GEF's, RasGRF1 and GRF2, have been described (Shou *et al.*, 1992, Farnsworth *et al.*, 1995, Fam *et al.*, 1997). The involvement of RasGRF1 or GRF2 in hypothermic Ras activation in rat2 cells is speculative since both GRF1 and GRF2 are thought to be expressed only in brain. Furthermore, increased levels of cytosolic Ca^{2+} in rat2 cells exposed to hypothermic stress remains undemonstrated. Alternatively, there may not be a rise in cellular Ca^{2+}. The GEF may simply require a basal amount of Ca^{2+} to function. The GEF responsible for hypothermic Ras activation remains unidentified.

Previously, others have described an inhibition of EGF signaling to Erk at low temperature (Campos-González and Glenney, Jr., 1991). Here, I have defined the level of the EGF signaling cascade in rat2 cells that is blocked by hypothermia. EGF signaling at 0°C is efficient to the level of Ras activation. Indeed, slightly more Ras-GTP was formed within 5 minutes of EGF treatment at 0°C compared to that seen at 37°C. This activation of Ras effectively demonstrates that Ras GEF's can function *in vivo* at 0°C. Furthermore, the unknown serum factor necessary for hypothermic Ras activation could be EGF or an EGF-like factor. As such, Ras activation by acute EGF stimulation at 0°C may be simply a robust version of slow Ras activation in hypothermic rat2 cells. In normal serum-containing growth media, dilute concentrations of EGF may be weakly stimulating a Ras GEF that slowly leads to Ras-GTP accumulation.

In contrast to EGF-stimulated Ras activation at 0°C, it has been shown within our lab that EGF-stimulated Raf activation is inhibited at 0°C in rat2 cells (Chan *et al.*, 1999). Raf activation involves recruitment to the plasma membrane, interaction with Ras-GTP,

and a complex series of events involving protein kinases and probably lipid regulators (Traverse *et al.*, 1993, Zhang *et al.*, 1993, Ghosh *et al.*, 1994, Leevers *et al.*, 1994, Stokoe *et al.*, 1994, Marias *et al.*, 1995, Barnard *et al.*, 1998). Subcellular fractionation studies in rat2 cells suggest that hypothermia inhibits membrane translocation of Raf in response to EGF stimulation. The mechanism behind this inhibition is unknown. Ras-GTP and the isolated Ras-binding domain of Raf interact with high affinity at low temperature *in vitro* but the protein complex containing full-length Raf *in vivo* might behave differently (Wartmann and Davis, 1994). Alternatively, full-length Raf may bind Ras-GTP at 0°C *in vivo*, but further regulatory events that are required for stable Ras-Raf interaction, such as interaction with accessory proteins, may be blocked. In this case, the interaction between Raf with the particulate fraction is not preserved during the experimental protocol.

Upon rewarming after hypothermic stress, Ras-GTP levels promptly decrease and Ras-GDP levels increase, probably due to a return of GTPase activity. However, activation of two Ras effector systems was observed during the recovery period. Activation of the Raf-Mek-Erk kinase cascade upon rewarming was transient but robust. During rewarming, we also demonstrated significant activation of the protein kinsase Akt which signals downstream of the Ras effector, PI3K (Franke *et al.*, 1997).

Hypothermic stress followed by rewarming did not lead to robust activation of JNK, also known as stress activated protein kinase (SAPK), or its upstream activating kinase, SEK (Sanchez *et al.*, 1994, Yan *et al.*, 1994) in rat2 cells. Another kinase generally associated with cellular stress, p38HOG, was also not activated by hypothermic stress followed by rewarming (Stang and Stone, unpublished data) (Han *et al.*, 1994). Thus, hypothermically-activated Ras does not signal to JNK nor p38HOG upon rewarming. In agreement with the lack of hypothermically-induced JNK signaling, EGF treatment of rat2 cells failed to strongly activate SEK and JNK. These results suggest that the JNK signaling pathway is not regulated by Ras, at least in rat2 cells. Although many other biochemical changes no doubt occur, hypothermic stress followed by warming appears to specifically and strongly activate Ras effectors such as the Raf-Mek-Erk cascade and PI3K.

Erk activation occurs after recovery from moderate, brief and prolonged hypothermic stress. Both hypothermic-activation of Ras-Erk signaling and hypothermic-inhibition of acute growth factor signaling are conserved in several primary cell populations and immortalized cell lines. We speculate that both temperature effects are relevant to situations encountered by animal and human tissues. In support of hypothermic Ras-Erk activation in animals, Erk activation was observed in hind legs of neonatal rats that were exposed to hypothermic stress and then rewarmed. However, Ras activation in tissues of

hypothermic neonatal rats has yet to be demonstrated. Thus, it remains unclear whether the hypothermic Erk activation observed in these hind legs was caused by hypothermically-activated Ras.

Although Erk activation upon recovery from hypothermic stress is transient, further downstream biochemical events are initiated. p90rsk, a known substrate of Erk, was activated in rat2 cells after hypothermic stress followed by rewarming (Bottorff and Stone, unpublished data) (Blenis, 1993). Erk has been proposed to phosphorylate SOS *in vivo* and this event may inhibit the ability of SOS to signal downstream of the EGF receptor (Porfiri and McCormick, 1996). Indeed, we found that rat2 cells that had been given a pretreatment of hypothermic stress followed by rewarming were less sensitive to subsequent stimulation with dilute concentrations of EGF. Thus, the activation of Erk upon recovery after hypothermic stress appears to trigger a negative-feedback loop that affects the subsequent signaling properties of the cell.

In whole organ tissues and tissue culture systems, prolonged hypothermic stress eventually causes damage and cell death (Belzer and Southard, 1988, Eberl *et al.*, 1996). We initially hypothesized that Ras-Erk activation during recovery from hypothermia is part of a protective mechanism and we predicted that pharmacological inhibition of this pathway in cultured cells would exacerbate hypothermic damage and reduce cell viability. To our surprise, Mek inhibitors increased cell viability after prolonged hypothermic stress. This effect is completely reproducible and it has been seen with four cultured cell types. The enhanced viability offered by the Mek inhibitor occurs over a range of hypothermic stress periods and the magnitude of this effect can be up to 40-fold (viability increased from 0.5% to 20%, Figure 3-13c). These observations suggest that Erk activation upon recovery from prolonged hypothermic stress promotes cell death.

Prolonged hypothermic stress is a well documented trigger of apoptosis in a number of cell types (Perotti *et al.*, 1990, Kruman *et al.*, 1992, Gregory and Milner, 1994). In some cellular contexts, Ras-Erk signaling has also been shown to promote apoptosis (Fukasawa and Vande Woude, 1997, Kauffmann-Zeh *et al.*, 1997, Khwaja *et al.*, 1997, Mayo *et al.*, 1997). Indeed, when rat2 cells were rewarmed after prolonged hypothermia, DNA fragmentation, a late biochemical marker for apoptosis, was observed. When cells were pretreated with the Mek inhibitor, the amount of fragmented DNA observed after hypothermic stress was significantly reduced. Thus, Erk activation upon recovery from prolonged hypothermic stress appears to stimulate apoptosis. Possibly, the hypothermic Erk response functions in a suicide mechanism that rids damaged cells from the organism.

The biochemical relationship between Ras-Erk activation, apoptosis, and cell death is unclear. Even if hypothermic Ras-Erk signaling does function to promote cell death, other factors must be important in determining the overall response to prolonged hypothermia since shorter periods of hypothermic stress lead to strong Erk signaling without the detrimental effects. Signaling through PI3K and Akt have been shown to be anti-apoptotic in rat1 fibroblasts and MDCK cells (Kauffmann-Zeh *et al.*, 1997, Khwaja *et al.*, 1997). In one model, biochemical responses that promote cell viability, such as Akt stimulation, may come into play after shorter periods of hypothermic stress, while the Ras-Erk pathway dominates the response after prolonged hypothermia. According to this model, I would predict that Akt is not activated upon rewarming after prolonged hypothermic incubation. Hypothermic stress can also lead to activation of NF-κB and NF-κB can reduce Ras-induced apoptosis in rodent fibroblasts (Rosette and Karin, 1995, Mayo *et al.*, 1997). According to the proposed model, short periods hypothermic stress, but not prolonged hypothermic stress, would be predicted to activate NF-κB. The involvement of NF-κB in hypothermia-induced apoptosis remains undemonstrated.

Hypothermia is thought to cause microtubule disruption in mammalian cells (Ostlund Jr. *et al.*, 1980, Rosette and Karin, 1995). It is also thought that prolonged hypothermic stress leads to a number of physiological changes including a disruption of the plasma membrane Na^+ ion gradient which in turn, leads to cell swelling (Belzer and Southard, 1988). As such, prolonged hypothermic stress may give rise to fragile, swollen cells that have a disrupted microtubule cytoskeleton. In primary rat hepatocytes, Erk activation leads to the appearance of biochemical markers of cell-cycle progression such as cyclin-dependent kinase 2 activation and increased DNA synthesis (Tombes *et al.*, 1998). Activation of Erk upon recovery from hypothermia, although transient, may similarly stimulate cell-cycle progression. Possibly, entry into mitosis or cytokinesis in the context of a cell structurally damaged by prolonged hypothermic stress may trigger a sensor that signals to the apoptotic regulatory machinery. Pharmacological inhibition of Erk activation may prevent apoptosis by slowing cell-cycle progression thereby allowing time for the cell to re-establish the microtubule cytoskeleton and osmotic homeostasis.

How inhibition of Erk activation protects cells after prolonged hypothermic stress remains to be elucidated. Even if the protective effect is not fully understood, our findings with the PD098059 Mek inhibitor may be useful for improving the survival of clinically useful cells in cold storage. Our findings also demonstrate the importance of strict temperature control when studying cultured cells.

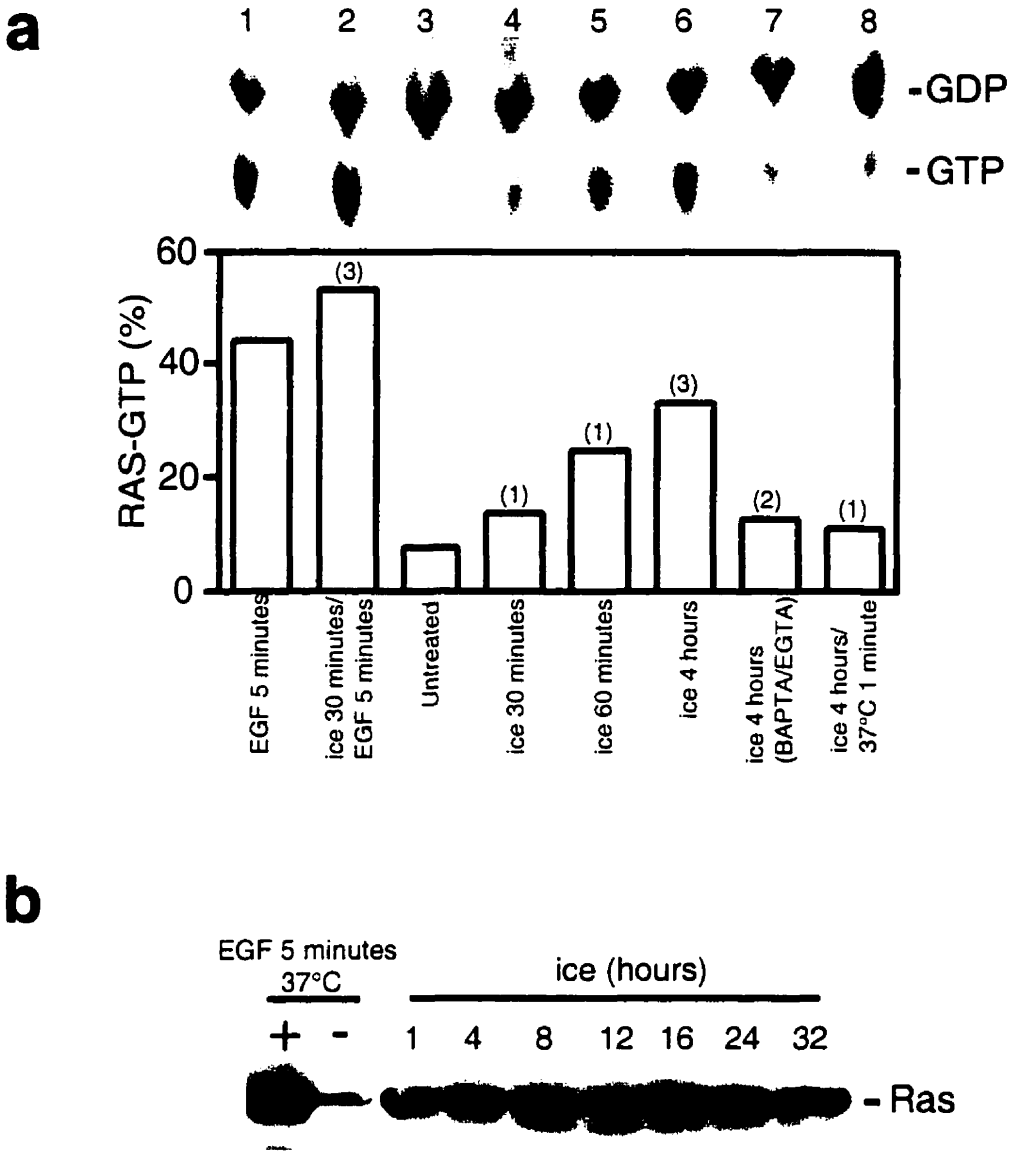


Figure 3-1. Activation of Ras in response to hypothermic stress *in vivo*. **a.** Ras-GTP levels were measured in rat2 cells using a ^{32}Pi-labeling method. For control, cells were stimulated with EGF (100 ng/ml) at 37°C (lane 1). In lanes 4-6, cells were incubated on ice for the indicated times. In lane 7, cells were given a pretreatment of BAPTA/AM (25 μM) and EGTA (2 mM final in cell medium) for 15 minutes at 37°C before a 4 hour incubation on ice. In lane 8, cells were incubated on ice for 4 hours and then warmed to 37°C for 1 minute. After cell lysis and immunoprecipitation of Ras, the levels of associated GTP and GDP were determined. Top panel: autoradiogram showing radiolabeled GDP and GTP after separation by chromatography. Bottom panel: the results in the top panel have been plotted as percent GTP relative to GTP+GDP. The results shown in lanes 1 and 3 have been observed numerous times. Shown above other bars is the number of independent times such observations were made. Note that the data in lane 2 (cells incubated on ice for 30 minutes and then stimulated with EGF for 5 minutes on ice) concern the low temperature block to acute EGF signaling). **b.** Rat2 cells overexpressing H-Ras were incubated on ice for 1 to 32 hours. Ras-GTP was precipitated from cell lysates with a GST-Raf fusion protein and precipitated Ras was detected with an anti-Ras immuno-blot method.

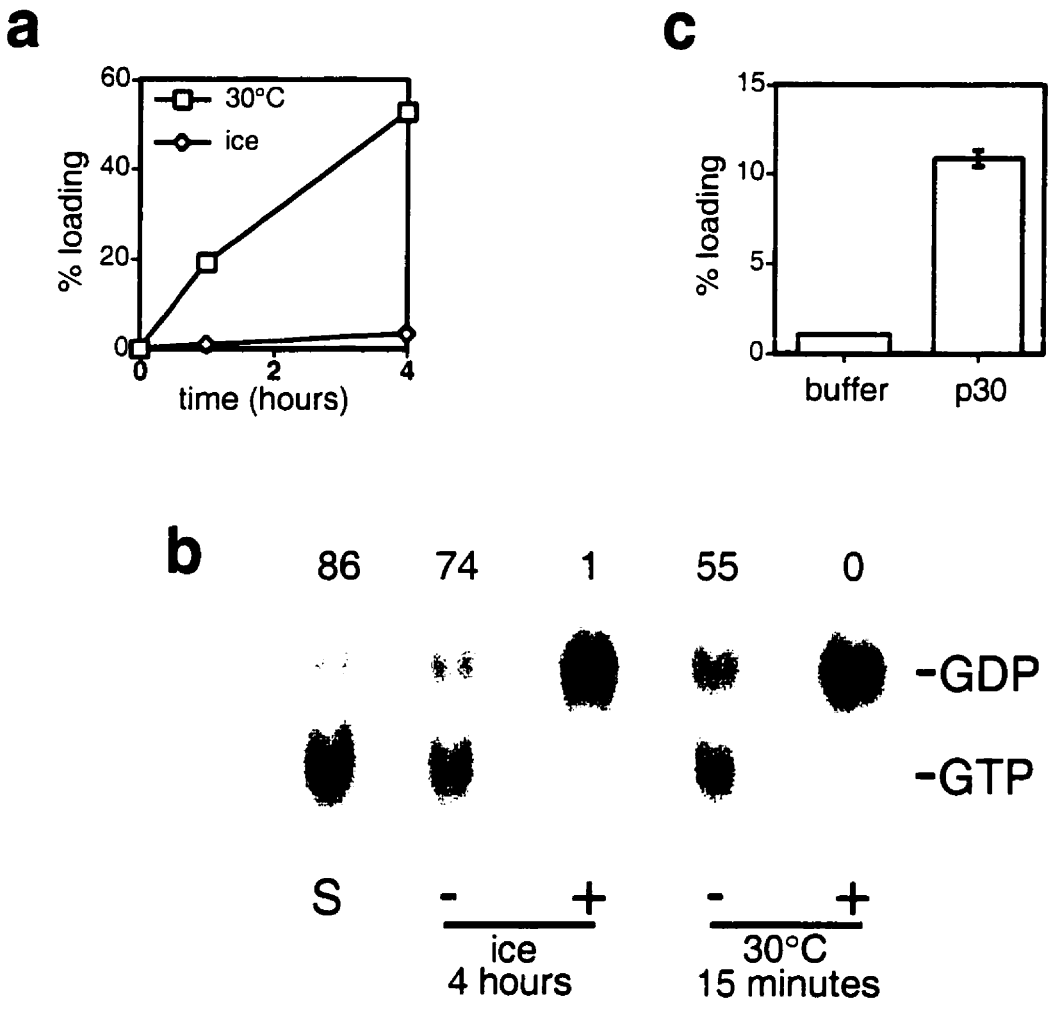


Figure 3-2. ***In vitro*** **GTP association and hydrolysis at 0°C. a.** Ras-GDP complex from *E. coli* was incubated with a molar excess of [^{32}P-α]-GTP at 30°C or on ice for up to 4 hours followed by immuno-precipitation of Ras and quantification of Ras-associated radiolabel. Values are expressed as percent of maximal association as determined by equilibration in magnesium-free conditions. Values shown are averages of duplicate data points. **b.** Ras from Sf9 cells was complexed with [^{32}P-α]-GTP and then incubated either with (+) or without (-) recombinant p120GAP on ice for 4 hours or at 30°C for 15 minutes. Ras was immuno-precipitated and associated radiolabeled nucleotides were separated by chromatography. Lane S represents the starting material, substrate Ras-[^{32}P-α]-GTP complex that was precipitated immediately. The percent GTP / GTP+GDP is shown above each lane. Chromatographic positions of GDP and GTP are shown on the right. **c.** Ras-GDP from Sf9 cells was incubated on ice with a molar excess of [^{32}P-α]-GTP in the presence (p30) or absence (buffer) of the catalytic domain of RasGRF1 for 4 hours. Ras was immuno-precipitated and associated radiolabeled nucleotide was quantified. Values are expressed as percent of maximal association and are the averages of triplicate data points with the standard deviation indicated. Note that when parallel reactions were performed at 30°C for 15 minutes, association values of 4% (buffer) and 43% (p30) were observed.

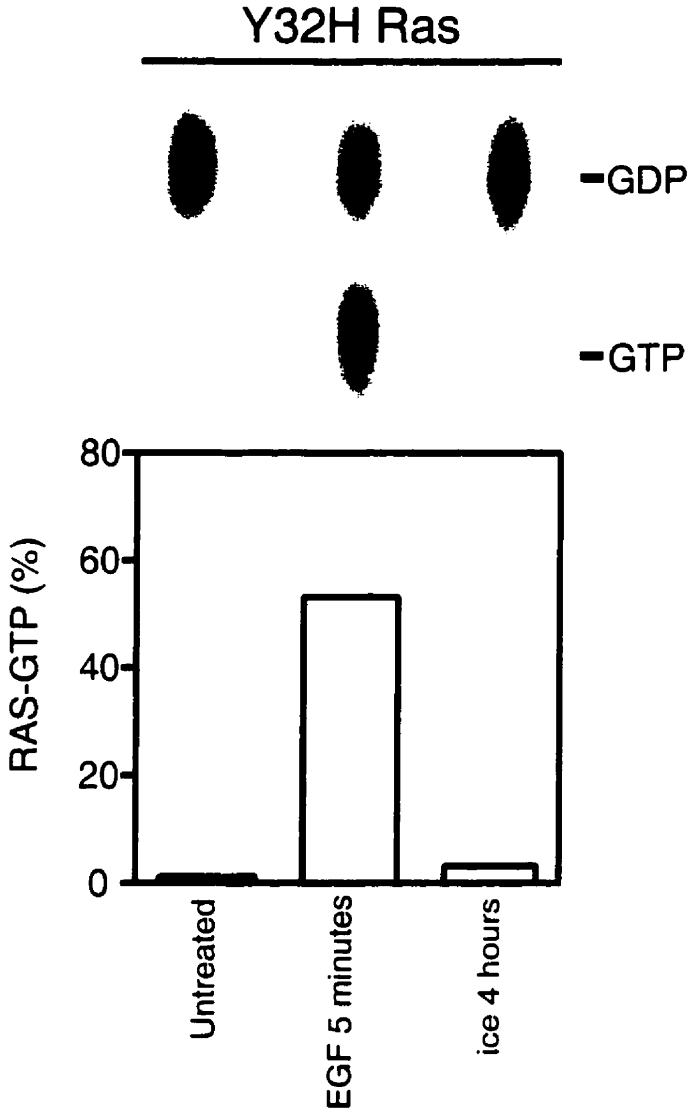


Figure 3-3. Inhibition of hypothermic Ras activation by a Y32H mutation in the effector domain. Ras-GTP levels in rat2 cells overexpressing Y32H Ras were measured by *in vivo* $^{32}P_i$-labeling as described in the legend of Figure 1a. Cells were either left untreated, stimulated with EGF (100 ng/ml) for 5 minutes at 37°C, or incubated on ice for 4 hours. Shown are the averages of duplicate data points. Y32H Ras protein is overexpressed at levels approximately 20-50 times that of endogenous Ras (Stang *et al.*, 1996). Thus, the observed signal, which represents total cellular Ras, is predominantly from the trans gene.

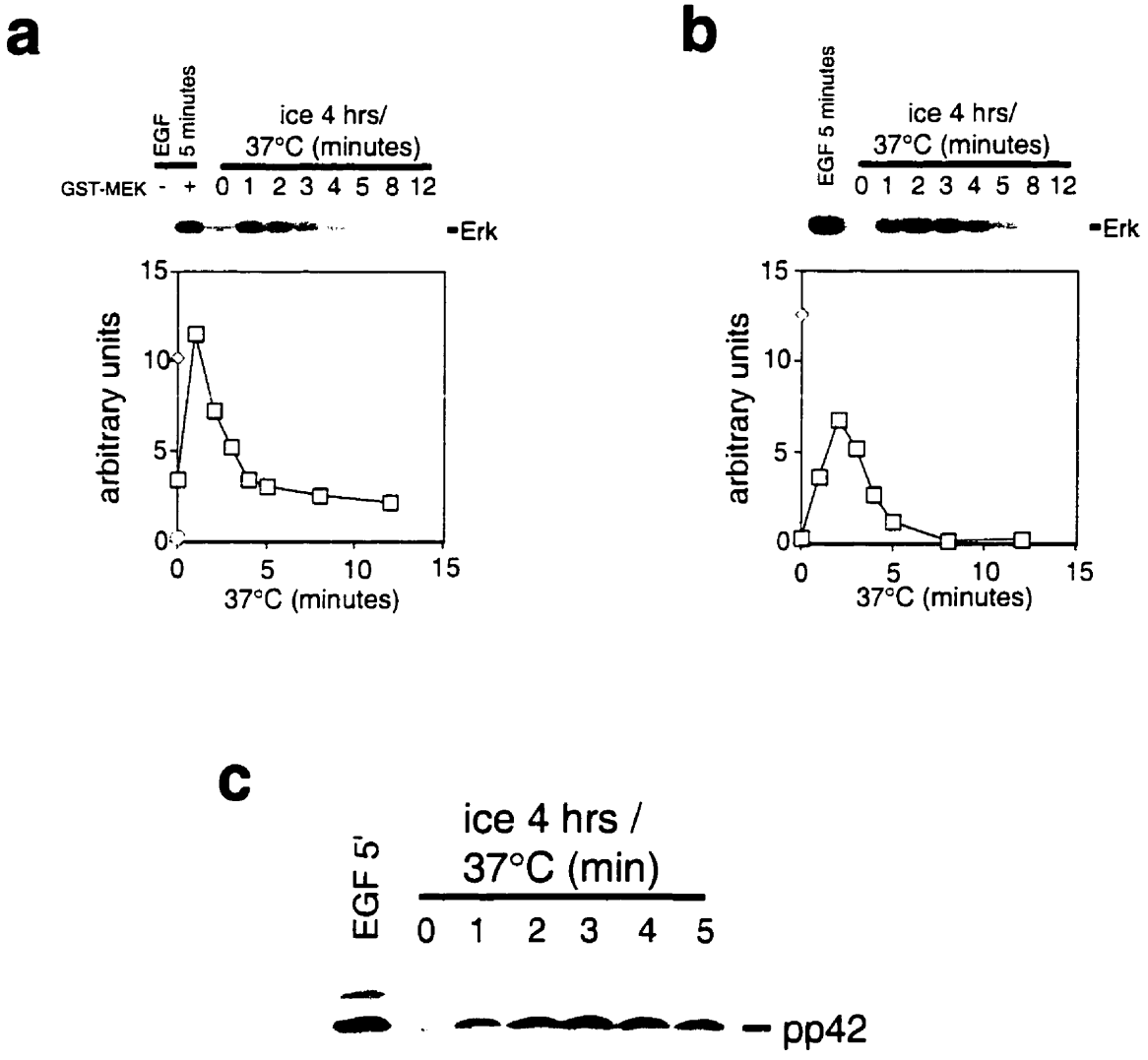


Figure 3-4. Activation of the Raf-Mek-Erk protein kinase cascade during recovery from hypothermic stress. a-c. Rat2 cells overexpressing wildtype Raf-1 were incubated on ice for 4 hours and then warmed to 37°C for 1 to 12 minutes. Samples of each lysate were assayed for Raf-1 or Mek-1 activity using immune-complex kinase assays, or for tyrosine phosphorylation of Erk using an immuno-blot method. **a.** In this assay, Raf activates GST-MEK1 to incorporate radioactivity onto a kinase-defective Erk substrate. As controls, EGF (100 ng/ml) treated cells were assayed for Raf-1 activity either without or with GST-MEK1. Top panel: autoradiogram. Bottom panel: Raf activity is plotted quantitatively. Circle - EGF treatment without GST-MEK1; Diamond - EGF-treated positive control; Squares - time course of Raf-1 activation. **b.** In this assay, immuno-precipitated Mek1 incorporates radioactivity onto a kinase defective Erk substrate. Top panel: autoradiogram. Bottom panel: Mek activity is plotted quantitatively. Diamond - EGF-treated positive control; Squares - time course of Mek-1 activation. **c.** The levels of Erk tyrosine phosphorylation were determined by immuno-blotting with anti-phosphotyrosine antibodies. The position of pp42 Erk is shown on the right.

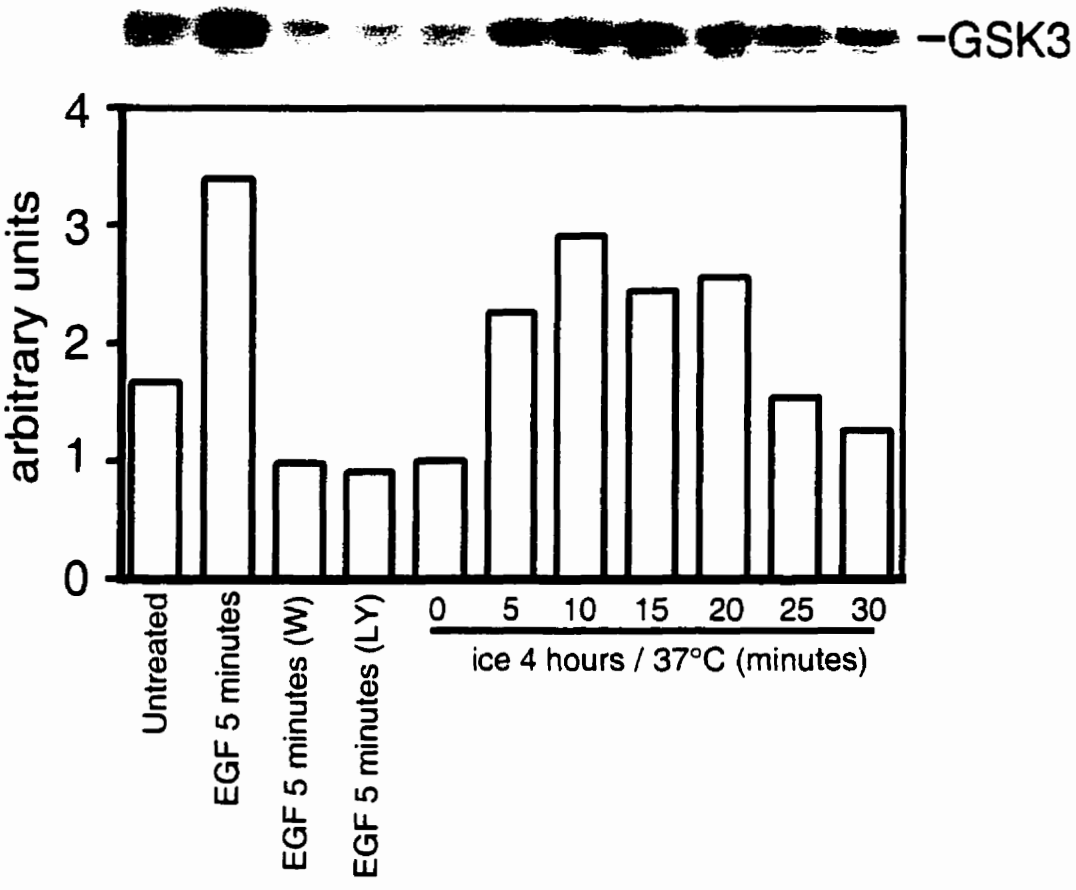
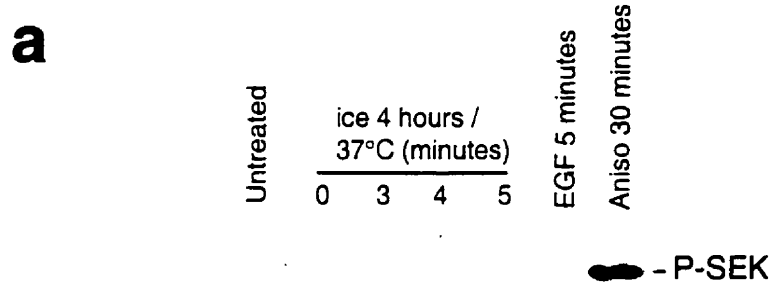


Figure 3-5. Activation of Akt during recovery from hypothermic stress. Rat2 cells were left untreated or treated with EGF (100 ng/ml) for 5 minutes at 37°C. For control, some cells were given a pretreatment of Wortmannin (W) (50 nM) or LY294002 (LY) (20 μM) for 30 minutes at 37°C prior to EGF treatment. Alternatively, cells were incubated on ice for 4 hours followed by warming to 37°C for various amounts of time. Akt activty in cell lysates was measured in a immune-complex kinase assay that employs GSK3 as substrate. Top panel: representative autoradiogram showing phosphorylated GSK3. Bottom panel: Akt activity is plotted quantitatively. Shown is a representative of two experiments.



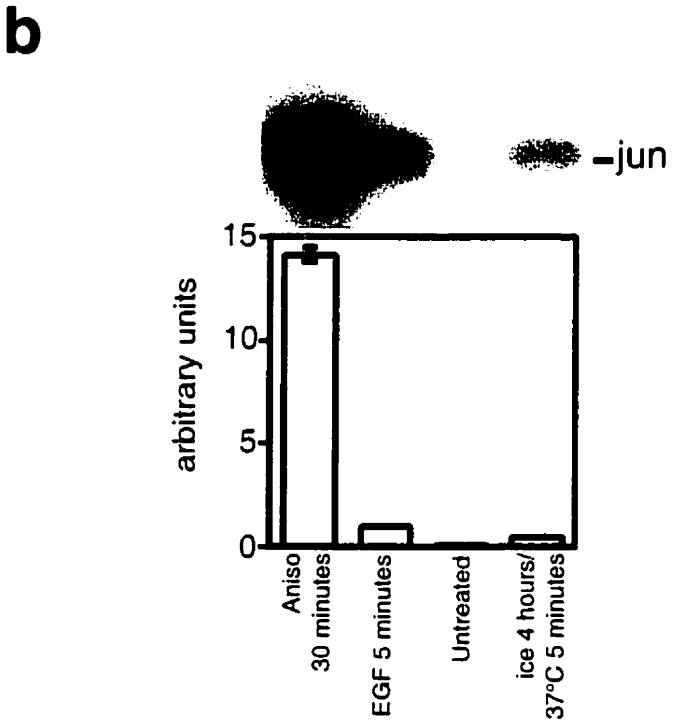


Figure 3-6. Lack of robust SEK-JNK activation during recovery from hypothermic stress. a. Rat2 cells were left untreated or incubated on ice for 4 hours followed by rewarming for various amounts of time. For control, cells were treated with EGF (100 ng/ml) for 5 minutes or anisomycin (10 μg/ml) for 30 minutes at 37°C. Cell lysates were analysed by immuno-blotting with an antibody that specifically recognizes the phosphorylated form of SEK. The position of phosphorylated SEK is shown on the right. **b.** Rat2 cells were treated with anisomycin (30 minutes) or EGF (5 minutes) at 37°C, left untreated, or incubated on ice for 4 hours followed by rewarming at 37°C for 5 minutes. JNK activity was measured in an *in vitro* kinase assay that employs a GST-jun fusion protein as substrate. Top panel: representative autoradiogram. The position of GST-jun is shown on the right. Bottom panel: JNK activity is plotted quantitatively. Data points are the averages of triplicate values with the standard deviation indicated.

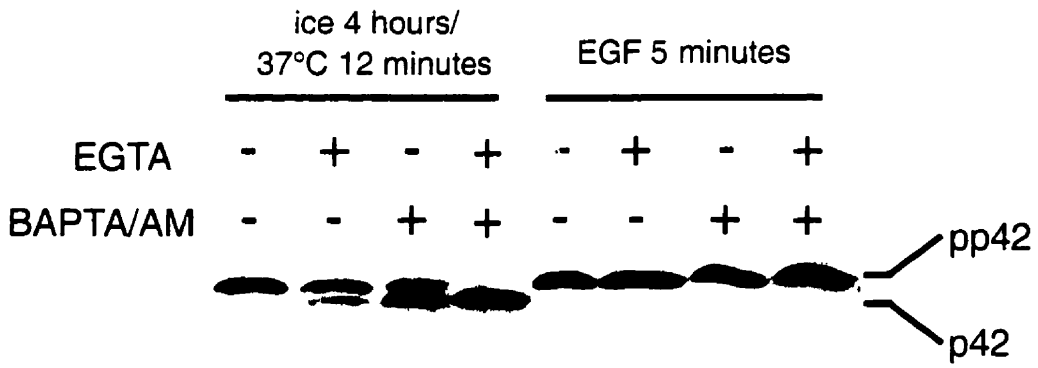


Figure 3-7. Inhibition of hypothermic Erk activation by Ca^{2+} chelators. Rat2 cells were given a pretreatment of EGTA (5 mM final concentration in cell medium), BAPTA/AM (25 μM), or both at 37°C for 15 minutes. Cells were then incubated on ice for 4 hours followed by warming to 37°C for 12 minutes. Alternatively, cells were stimulated with EGF (100 ng/ml) for 5 minutes at 37°C after pretreatment. Erk activation was measured in cell lysates using a electrophoretic mobility shift assay. The positions of the unphosphorylated p42 and phosphorylated pp42 Erk species are indicated on the right.

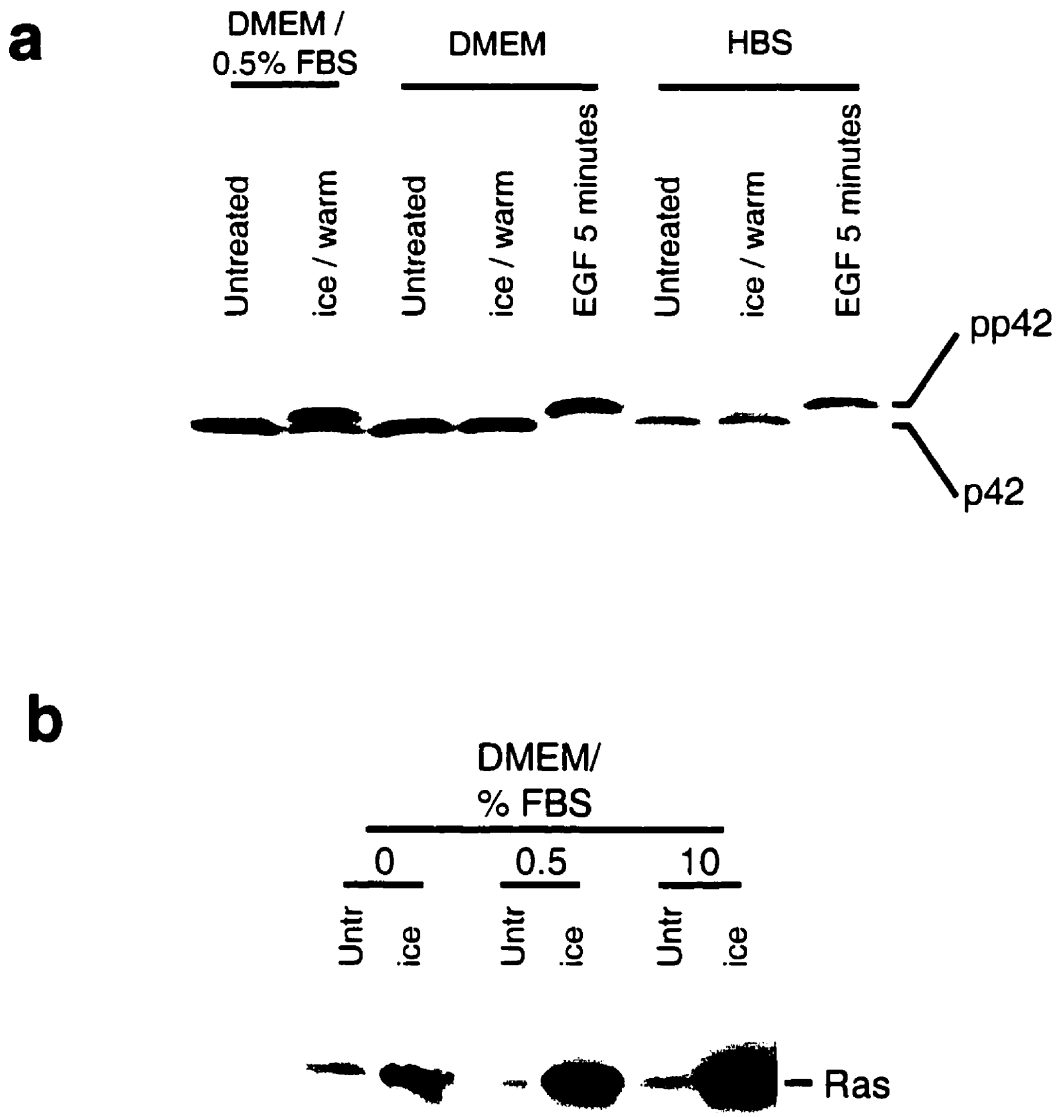


Figure 3-8. Inhibition of hypothermia-induced Ras and Erk activation by removal of serum from the cell culture media. a. The media for cultures of rat2 cells were changed to DMEM/0.5% FBS, DMEM, or Hepes-buffered saline (HBS). Cultures were then allowed to equilibrate for 15 minutes at 37°C. After equilibration, cells were left untreated, incubated on ice for 4 hours followed by rewarming to 37°C for 5 minutes (ice/warm), or stimulated with EGF (100 ng/ml) for 5 minutes at 37°C. Erk activation was measured by the electrophoretic mobility shift assay. **b.** The media for cultures of rat2 cells overexpressing H-Ras were changed to DMEM containing 0, 0.5 or 10% FBS. Cultures were then allowed to equilibrate for 4 hours at 37°C. After equilibration, cultures were left untreated (Untr) or incubated on ice for 4 hours. Ras-GTP levels in cell lysates were measured using the non-isotopic method described in the legend of Figure 3-1b.

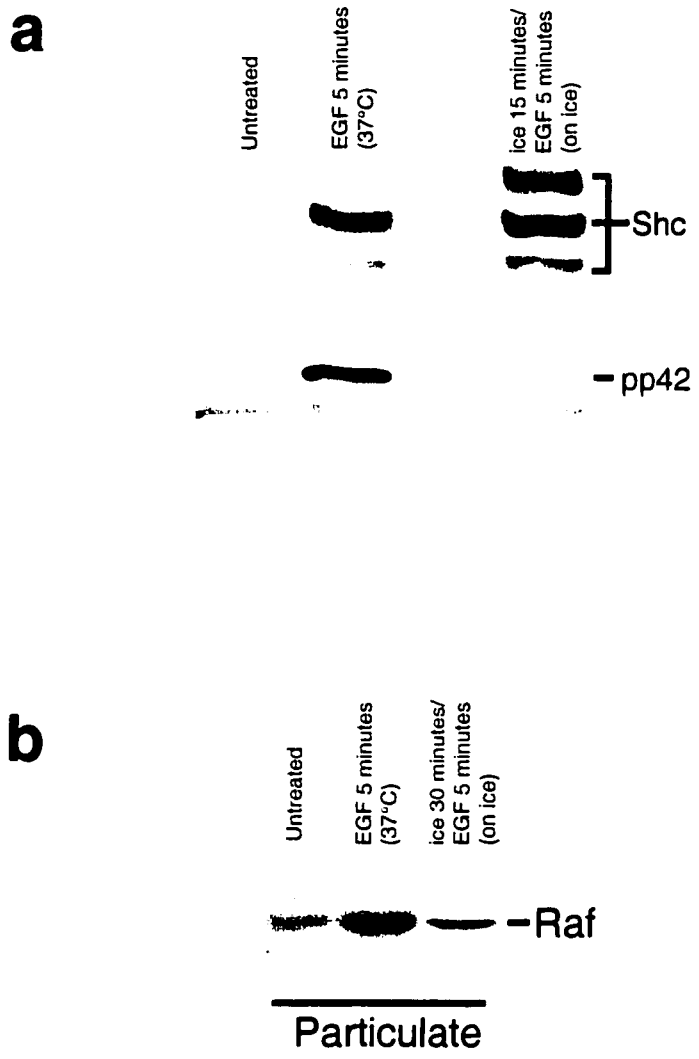


Figure 3-9. Effect of hypothermia on protein-tyrosine phosphorylation and membrane translocation on Raf. a. Rat2 cells were left untreated, stimulated with EGF (100 ng/ml) at 37°C or incubated on ice for 15 minutes followed by EGF stimulation on ice. Cellular proteins were analyzed using an anti-phosphotyrosine immuno-blot method. The positions of pp42 Erk and the different tyrosine phosphorylated Shc species are shown on the right. **b.** Rat2 cells overexpressing Raf-1 were left untreated, stimulated with EGF for 5 minutes at 37°C, or incubated on ice for 30 minutes followed by EGF stimulation on ice for 5 minutes. Cells were then lysed and subjected to subcellular fractionation. Raf in the particulate fraction was analysed by an immuno-blot method. The position of Raf is shown on the right.

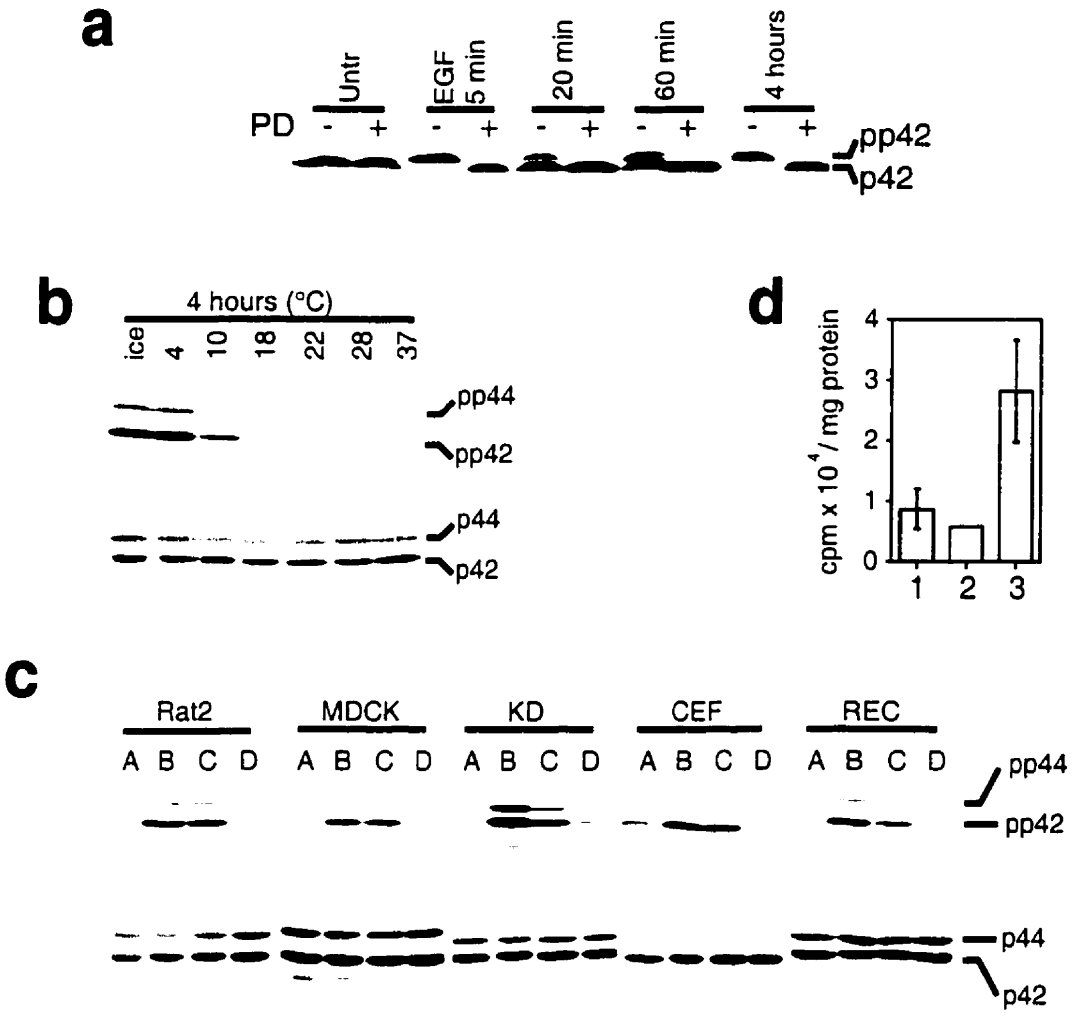


Figure 3-10. Activation of Erk in response to various hypothermic conditions and in different vertebrate cells. a. Where indicated, rat2 cells were given a pretreatment of PD098059 (50 μM) for 90 minutes at 37°C. Cells were then left untreated, stimulated with EGF (100 ng/ml) at 37°C, or incubated on ice for 20 minutes, 60 minutes or 4 hours followed by warming to 37°C for 12 minutes. Erk activation was measured by the electrophoretic mobility shift assay. **b.** Rat2 cells were exposed to various temperatures for 4 hours and then warmed to 37°C for 5 minutes. Top panel: Erk activation was determined with an immuno-blot method that employs a primary antibody specific for the dually phosphorylated active forms of Erk. Bottom panel: Equivalence of protein loading was demonstrated by immuno-blotting a parallel blot with an antibody that recognizes total Erk. SDS-PAGE conditions here do not resolve the phosphorylated forms of Erk. The positions of doubly phosphorylated pp42 and pp44 Erk and total p42 and p44 Erk are shown on the right. **c.** Rat2 , MDCK, KD, CEF and REC cells were treated as described below and Erk activation was assessed using the immuno-blotting assay described in b. A-untreated; B-acute growth factor treatment for 5 minutes at 37°C; C-incubation on ice for 4 hours followed by warming to 37°C for 5 minutes; D-incubation on ice for 30 minutes followed by acute growth factor treatment on ice for 5 minutes. Acute growth factor stimulation was EGF (100 ng/ml) for all cell types except CEF which were stimulated with 10% FBS. **d.** Neonatal Sprague-Dawley rats were (1) left untreated, (2) incubated at ice-cold temperature for 20 minutes, or (3) incubated at ice-cold temperature for 20 minutes and rewarmed by 36°C ambient temperature for 12-14 minutes. Hind limbs were removed from sacrificed animals and homogenized. Erk activity in homogenates was measured with an immune-complex kinase assay. Each value is the average derived from analyzing 4 animals with the standard deviation indicated.

Figure 3-11. Negative-feedback inhibition of EGF signaling after recovery from hypothermic stress. For control, rat2 cells were incubated on ice for 4 hours followed by rewarming to 37°C for 12 or 30 minutes. Alternatively, cells were left untreated or given a pretreatment of ice incubation for 4 hours followed by rewarming to 37°C for 30 minutes. After pretreatment, cells were stimulated with dilute concentrations of EGF for 5 minutes at 37°C. EGF stimulation was at either 1/1024 (98 pg/ml) or 1/4096 (24 pg/ml) of normal concentrations. Erk activation was measured by the electrophoretic mobility shift assay.

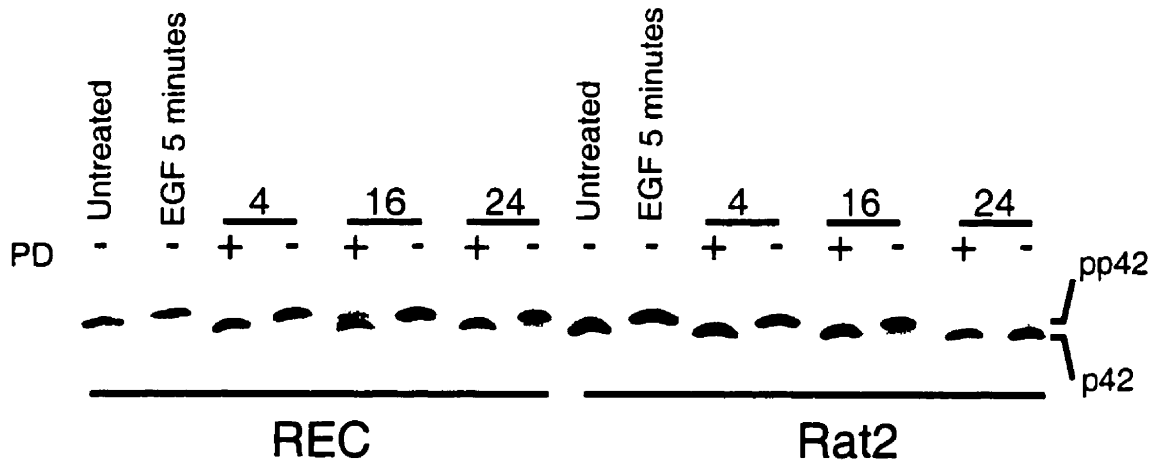


Figure 3-12. Activation of Erk after recovery from prolonged hypothermic stress. REC and rat2 cells were left untreated, stimulated with EGF (100 ng/ml) for 5 minutes at 37 °C, or incubated on ice for 4, 16 or 24 hours and then warmed to 37°C for 10 minutes. Where indicated, cells were given a pretreatment of PD098059 (50 μM) for 90 minutes at 37°C. Erk activation was assessed using the mobility shift assay.

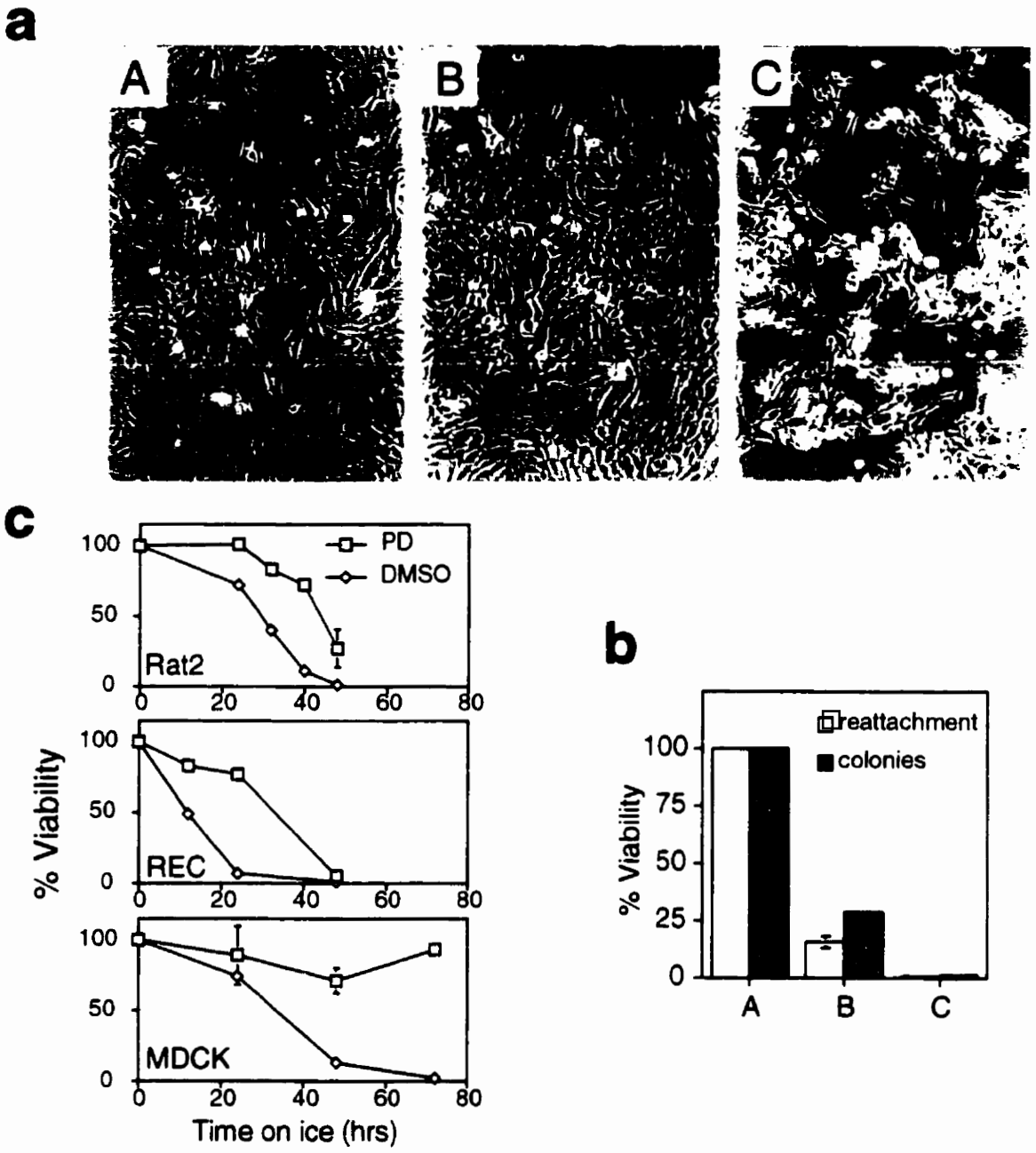


Figure 3-13. Enhanced cell viability after prolonged hypothermic stress using the Mek inhibitor PD098059. a-b. REC were untreated (A) or given a prolonged incubation on ice followed by warming to 37°C for 4 hours ((B) and (C)). Cells were given a pretreatment of 50 μM PD098059 (B) or DMSO vehicle (C) for 90 minutes at 37°C before the hypothermic incubation. The duration of the ice incubation was either 16 hours (a) or 24 hours (b). **a.** Cell morphology 4 hours after rewarming. **b.** Cell viability as determined by a reattachment assay and by the formation of colonies. **c.** Cells of the indicated type were given a pretreatment of PD098059 or DMSO vehicle as in a and b. Following, cells were incubated on ice for various times and warmed to 37°C for 4 hours. Cell viability was assessed using the reattachment assay. For rat2 and MDCK cells, each value is the average from three independent samples with the standard deviation indicated.

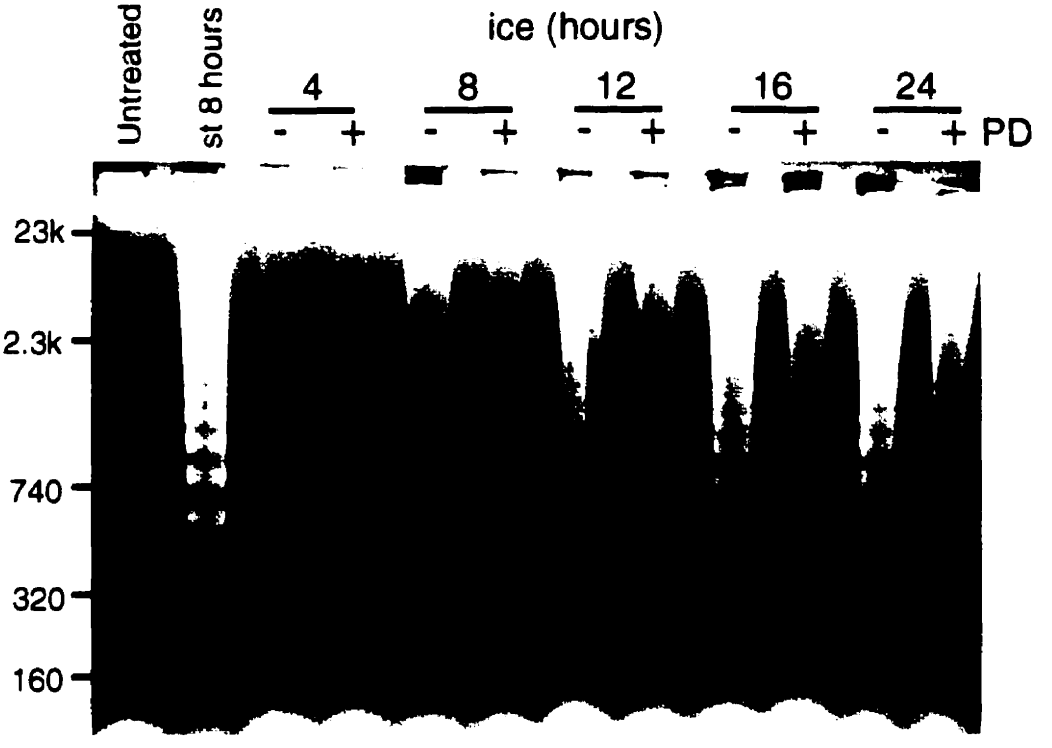


Figure 3-14. Attenuation of hypothermia-induced DNA fragmentation using the Mek inhibitor PD098059. For controls, rat2 cells were left untreated or treated with staurosporin (st) (2 μM) for 8 hours at 37°C. Alternatively, cells were incubated on ice for various times from 4 to 24 hours and rewarmed to 37°C for 4 hours. Where indicated, cells were given a pretreatment of PD098059 (50 μM) for 90 minutes at 37°C. DNA was extracted from cells and analyzed by electrophoresis in agarose. The positions of DNA size markers are indicated on the left.

References

Adari, H., Lowy, D.R., Willumsen, B.M., Der, C.J., and McCormick, F. (1988) Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. *Science* **240**:518-521.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. & Saltiel, A.R. (1995) PD098059 is a specific inhibitor of the activation of the mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**:27489-27494.

Barnard, D., Diaz, B., Clawson, D., and Marshall, M. (1998) Oncogenes, growth factors and phorbol esters regulate *Raf*-1 through common mechanisms. *Oncogene* **17**:1539-1547.

Belzer, F.O., and Southard, J.H. (1988) Principles of solid-organ preservation by cold storage. *Transplantation* **45**:673-676.

Binetruy, B., Smeal, T., and Karin, M. (1991) Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature* **351**:122-127.

Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci.* **90**:5889-5892.

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D., and Kuriyan, J. (1998) The structural basis of the activation of *ras* by *sos*. *Nature* **394**:337-343.

Campos-González, R., and Glenney, J.R., Jr. (1991) Temperature-dependent tyrosine phosphorylation of microtubule-associated protein kinase in epidermal growth factor-stimulated human fibroblasts. *Cell Regulation* **2**:663-673.

Carpenter, C.L., and Cantley, L.C. (1996) Phosphoinositide kinases. *Current Opinion in Cell Biology* **8**:153-158.

Chan, E.Y.W., Stang, S.L., Bottorff, D.A., and Stone, J.C. (1999) Hypothermic stress leads to activation of Ras-Erk signaling. *J. Clin. Invest.* **103**:1337-1344.

Clapham, D.E. (1995) Calcium signaling. *Cell* **80**:259-268.

Eberl, T., Steinlechner, R., Hengster, P., Herold, M., Schrocksnadel, H., Salvenmoser, W., Rhomberg, M., Gnaiger, E. and Margreiter, R. (1996) Assessment of endothelial preservation in human cell cultures. *Ann. Thorac. Surg.* **62**:526-532.

Ebinu, J.O., Bottorff, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science*. **280**:1082-1086.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., *et al.* (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273**:18623-18632.

Franke, T.F., Kaplan, D.R., Cantley, L.C., Toker, A. (1997) Direct regualtion of the *Akt* proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**:665-668.

Fukasawa, K., and Vande Woude, G.F. (1997) Synergy between Mos/Mitogen-activated protein kinase pathway and loss of p53 function in transformation and chromosome instability. *Mol. Cell. Biol.* **17**:506-518.

Ghosh, S., Xie, W.Q., Quest, A.F.G., mabrouk, G.M., Strum, J.C., and Bell, R.M. (1994) The cysteine-rich region of *raf*-1 kinase contains zinc, translocates to liposomes, and is adjacent to a segment that binds GTP-*ras*. *J. Biol. Chem.* **269**:10000-10007.

Gregory, C.D., and Milner, A.E. (1994) Regulation of cell survival in Burkitt lymphoma: Implications from studies of apoptosis following cold-shock treatment. *Int. J. Cancer* 57:419-426.

Han, J., Lee, J.-D., Bibbs, L., and Ulevitch. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science. 265:808-811.

Hochachka, P.W. (1986) Defence strategies against hypoxia and hypothermia. *Science* **231**:234-241.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by *ras* signalling through PI(3)K and PKB. *Nature* **385**: 544-548.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1997) Matrix adhesion and *ras* transformation both activate a phophoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**:2783-2793.

Kruman, I.I., Gukovskaya, A.S., Petrunyaka, V.V., Beletsky, I.P., and Trepakova, E.S. (1992) Apoptosis of murine BW 5147 thymoma cells induced by cold shock. *J. Cell. Physiol.* **153**:112-117.

Kyriakis, J.M., Banerjee, P., Nikoakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994) The stress-activated protein kinase subfamily of c-jun kinases. *Nature*. **369**:156-160.

Lin, A., Minden. A., Martinetto, H., Claret, F.-X, Lange-Carter, C., Mercurio, F., Johnson, G.L. and Karin, M. (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science* **268:**286-290.

Liu, B., Wang, L.C.H., and Belke, D.D. (1991) Effect of low temperature on the cytosolic free Ca^{2+} in rat ventricular myocytes. *Cell Calcium* **12**:11-18.

Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.* **14:**3136-3145.

Mayo, M.W., Wang C-Y., Cogswell P.C., Rogers-Graham, K.S., Lowe, S.W., Der, C.J., and Baldwin Jr., A.S. (1997) Requirement of NF-kB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **278**:1812-1815.

Mcanulty, J.F., Ametani, M.S., Southard, J.H., and Belzer, F.O. (1996) Effect of hypothermia on intracellular Ca^{2+} in rabbit renal tubules suspended in UW-gluconate preservation solution. *Cryobiology* **33**:196-204.

Mistou, M-Y, Jacquet, E., Poullet, P., Rensland, H., Gideon, P., Schlichting, I., Wittinghofer, A., and Parmeggianni, A. (1992) Mutations of Ha-ras p21 that define important regions for the molecular mechanism of the SDC25 C-domain, a guanine nucleotide dissociation stimulator. *EMBO J.* **11**:2391-2397.

Okuda, K., Sanghera, J.S., Pelech S.L., Kanakura, Y., Hallek, M., Griffin, J.D., and Druker, B.J. (1992) Granulocyte-macrophage colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. *Blood* **79**:2880-2887.

Ostlund Jr., R.E., Leung, J.T., and Hajek, S.V. (1980) Regulation of microtubule assembly in cultured fibroblasts. *J. Cell Biol.* **85**:386-391.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavalla, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P.G. (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**:93-104.

Perotti, M., Toddei, F., Mirabelli, F., Vairetti, M., Bellomo, G., McConkey, D.J., and Orrenius, S. (1990) Calcium-dependent DNA fragmentation in human synovial cells exposed to cold shock. *FEBS Lett.* **259**:331-334.

Poe, M., Scolnick, E.M., and Stein, R.B. (1985) Viral Harvey ras p21 expressed in Escherichia coli purifies as a binary one-to-one complex with GDP. *J. Biol. Chem.* **260**:3906-3909.

Porfiri, E. and McCormick, F. (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the Ras exchange factor hSOS1. *J. Biol. Chem.* **271**:5871-5877.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**:527-532.

Rodriguez-Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D. and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15**:2442-2451.

Rosette, C. and Karin, M. (1995) Cytoskeletal control of gene expression: Depolymerization of microtubules activates NF-kB. *J. Cell. Biol.* **128**:1111-1119.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363**:83-85.

Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K. and Cohen, S. (1993) Epidermal growth factor stimulates the tyrosine phosphorylation of SHC in the mouse. *J. Biol. Chem.* **268**:7610-7612.

Sánchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. and Zon, L.I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* **372:**794-798.

Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F. and Wittinghofer, A. (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* **277:**333-338.

Stang, S., Bottorff, D., and Stone, J.C. (1996). Ras Effector loop mutations that dissociate p120GAP and Neurofibromin interactions. *Mol. Carcinogenesis* **15**:64-69.

Stang, S., Bottorff, D. and Stone, J.C. (1997) Interaction of activated Ras with Raf-1 alone may be sufficient for transformation of rat2 cells. *Mol. Cell. Biol.* **17:**3047-3055.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science* **264:**1463-1467.

Taylor, S.J. and Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Current Biology* **6:**1621-1627.

Traverse, S., Cohen, P., Paterson, H., Marshall, C., Rapp, U. and Grand, R.J.A. (1993) Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of *ras*-transformed retinal cells. *Oncogene* **8:**3175-3181.

Warne, P.H., Rodriguez-Viciana, P., and Downward, J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature* **364**:352-355.

Wartmann, M., and Davis. R.J. (1994). The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.* **269**:6695-6701.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R. and Templeton, D.J. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372:**798-800.

Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C.-H and Claesson-Welsh, L. (1994) Direct interaction between Shc and the platelet-derived growth factor β-receptor. *J. Biol. Chem.* **269:**15337-15343.

Zhang, X., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. and Avruch, J. (1993) Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364:**308-313.

Chapter 4 - Activation of Ras-Erk signaling *in vivo* by RasGRP in response to diacylglycerol signals

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Introduction

Previously, two distinct classes of guanyl nucleotide exchange factors (GEF's) were known to regulate Ras in mammalian cells. SOS1 and SOS2, which comprise one class, activate Ras in response to extracellular signals that stimulate receptor tyrosine kinases (Bowtell *et al.*, 1992, Chardin *et al.*, 1993, Egan *et al.*, 1993, Buday and Downward, 1993). The second class is comprised of RasGRF1 and GRF2 which appear to activate Ras in response to Ca^{2+} second messengers generated downstream of heterotrimeric G-protein coupled receptors (GPCR) (Farnsworth *et al.*, 1995, Fam *et al.*, 1997). Our lab recently identified RasGRP (Ras guanyl nucleotide releasing protein), a novel Ras GEF which appears to be distinct from SOS and GRF proteins.

RasGRP was isolated in a genetic scheme that selected for transforming cDNA sequences (Ebinu *et al.*, 1998). Using retroviral vectors, a rat brain cDNA library was introduced into rat2 fibroblasts that expressed the Y32R-vH-Ras allele (oncogenic H-Ras with a tyrosine to arginine substitution at residue 32 within the switch 1 Ras effector domain). The expression of Y32R-vH-Ras does not transform cells on its own but makes them hypersensitive to additional transforming signals in the Ras pathway. Transformed foci were isolated and cDNA sequences were retrieved from individual clones. One identified cDNA, *rbc7*, encoded a protein that showed homology to the GEF catalytic domains of SOS1 and RasGRF1. By rescreening a rat brain cDNA library carried in a phage vector, *rbc7* was shown to be a 5'- and 3'-truncated version of a longer full-length transcript which encodes RasGRP (Figure 4-1).

Full-length RasGRP from rat is a 795 residue protein with a 90.3 kDa predicted molecular weight. From the primary structure, RasGRP appeared to form a novel class of mammalian Ras GEF. The N-terminal half of the protein contains the conserved GEF catalytic domain which can be further divided into the Ras-exchanger motif (REM), characteristic of Ras-specific GEF's, and the CDC25 box which forms the primary catalytic domain (Lai *et al.*, 1993, Fam *et al.*, 1997). The C-terminal half of RasGRP contains a structure resembling a pair of EF hand motifs. EF hands are motifs that are observed in a number of Ca^{2+}-binding proteins. In general, each EF hand forms a helix-loop-helix structure that binds a Ca^{2+} ion via interactions involving residues within the loop. Also within the C-terminal region is a structure similar to the diacylglycerol (DAG) binding domain of PKC's. The rbc7 protein retains the full GEF catalytic domain, both EF-hands and the DAG-binding domain.

Others within our lab have begun to characterize the different functional domains of RasGRP, *in vitro*. A 6x-His tagged recombinant protein that contained the catalytic domain of RasGRP (region "cat" in figure 4-1) was shown to stimulate nucleotide exchange with purified H-Ras substrate. This RasGRP protein, however, failed to stimulate nucleotide exchange on either RhoA, the GTPase that regulates actin cytoskeleton rearrangements, or R-Ras, a close relative of the true mammalian Ras proteins (55% identical to H-Ras). A recombinant protein containing the DAG-binding domain of RasGRP fused to the glutathione-S-transferase (GST) tag was shown to bind a radiolabeled DAG analogue *in vitro*. The binding of Ca^{2+} to RasGRP has also been demonstrated. The truncated rbc7 version of RasGRP fused to GST was expressed in *E. coli*. This recombinant protein, blotted on nitrocellulose, bound $^{45}Ca^{2+}$ in an overlay assay. This binding was ablated by mutation of critical Ca^{2+}-binding residues in the C-terminal EF hand motif. Thus, RasGRP showed *in vitro* properties of a Ras GEF that is regulated by DAG and Ca^{2+} second messengers.

This project aimed to: 1) further characterize the *in vitro* properties of RasGRP and 2) demonstrate the role of RasGRP in linking diacylglycerol second messengers to the activation of Ras signaling in rat2 fibroblasts.

Results and Discussion

Stable interaction between RasGRP and Ras *in vitro*

A stable interaction between purified yeast Ras2 protein and the CDC25 yeast Ras GEF has been demonstrated with *in vitro* binding assays (Lai *et al.*, 1993). Similarly, I asked whether RasGRP could stably interact with Ras *in vitro*. A recombinant protein consisting of maltose binding protein fused to the catalytic domain of RasGRP (MBP-cat) was expressed in *E coli.* and purified (Figure 4-1). MBP-cat contains the identical region of RasGRP that was included in the 6x-His tagged protein demonstrated for *in vitro* Ras GEF catalytic activity. MBP-cat was immobilized on amylose beads and then incubated with H-Ras purified from an Sf9 cell expression system. After extensive washing of the beads, precipitated Ras was detected by an immuno-blot method. Ras was found stably associated with MBP-cat containing beads but not amylose beads alone (Figure 4-2). Two bands were detected by Ras immunoblotting to precipitate with MBP-cat. We interpret the major band to be Ras protein that has undergone complete post-translational processing in the Sf9 cell expression system. The minor band is interpreted to be partially processed

Ras. The *in vitro* association of Ras with the catalytic domain of RasGRP, together with the demonstration of *in vitro* Ras specific GEF activity, provide evidence supporting the function of RasGRP as a Ras GEF *in vivo*.

Overexpression of rbc7 and full-length RasGRP sequences in rat fibroblasts

RasGRP sequences were overexpressed in rat2 fibroblasts using the pBabepuro retroviral vector (Morgenstern and Land, 1990). Others within our group have found that overexpression of rbc7 was weakly transforming in the absence of the Y32R-vH-Ras allele (Ebinu *et al.*, 1998). More pronounced transformation was observed in rat2 cells that overexpressed both rbc7 and wildtype H-Ras. The overexpression of full-length RasGRP, however, failed to show any significant transforming properties. Thus, deletion of N- and C-terminal RasGRP sequences increases the transforming activity of the protein. The molecular basis of this activation is unclear since no obvious functional motifs are present within the deleted sequences. There is some evidence suggesting that the RasGRP N- and C-terminal sequences destabilize the protein. In stably overexpressing cell lines, the steady state level of HA epitope tagged rbc7 protein was substantially higher than full-length HA-tagged RasGRP (Ebinu and Stone, unpublished results). It is not known how the deletion of N- and C-terminal sequences affects the nucleotide exchange activity of rbc7. I studied both the full-length and rbc7HA version of RasGRP in rat2 fibroblasts.

Activation of Ras by RasGRP *in vivo*

I measured the levels of GTP and GDP associated with Ras using an *in vivo* ^{32}P labeling assay. Rat2 cells overexpressed H-Ras alone or in combination with full-length RasGRP (Figure 4-3a). Low amounts of Ras-GTP were observed in H-Ras-overexpressing cells that were left untreated. Stimulation of these cells with phorbol ester (PMA), a cell-permeable DAG analogue, for 2 minutes did not affect Ras-GTP levels. Cells that overexpressed H-Ras and RasGRP showed a slight increase in the basal level of Ras-GTP. PMA treatment of these cells led to a marked increase in the levels of Ras-GTP. Thus, RasGRP can activate Ras *in vivo* in response to PMA stimulation.

The peptide growth factor endothelin-1 was used to determine if RasGRP could activate Ras in response to physiologic stimulation. In rat2 cells, endothelin-1 (Et-1) stimulates hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to generate DAG and inositol triphosphate (IP_3) through a heterotrimeric G-protein coupled receptor (Ambar and

Sokolovsky, 1993). The IP_3 second messenger subsequently stimulates Ca^{2+} ion currents from the endoplasmic reticulum.

A non-isotopic assay was used to measure Ras activation in response to Et-1 stimulation (Taylor and Shalloway, 1996). Immobilized GST-Raf fusion protein precipitates only the GTP-bound form of Ras from cell lysates. Precipitated Ras is subsequently detected by an immuno-blot method. Rat2 cells overexpressed H-Ras alone or in combination with full-length RasGRP. Stimulation of cells overexpressing only H-Ras with Et-1 for 10 minutes did not increase Ras-GTP levels (Figure 4-3b). Overexpression of H-Ras in combination with full-length RasGRP increased the basal amount of Ras-GTP. Stimulation of these cells with Et-1 for 10 minutes increased the amount of Ras-GTP further. These results show that RasGRP can activate Ras in response to endogenous DAG and possibly Ca^{2+} signals in rat2 cells.

Activation of Erk signaling by RasGRP *in vivo*

Activated Ras signals to several different effector systems including the Raf-Mek-Erk kinase cascade (Moodie *et al.*, 1993, Rodriguez-Viciana *et al.*, 1997). To study signaling events downstream of Ras, I assayed Erk phosphorylation in response to PMA treatment using a gel mobility shift assay (Figure 4-4a). In rat2 cells expressing empty vector, PMA stimulation gave rise to transient and partial phosphorylation of Erk that could be observed for up to 1 hour after stimulation. Within 4 hours, Erk returned to its basal unphosphorylated state. This response is probably due to direct phosphorylation of Raf by protein kinase C (Kolch *et al.*, 1993). PMA treatment of cells overexpressing rbc7HA gave rise to full phosphorylation of Erk that peaked 10 minutes to 1 hour after stimulation. In addition, significant phosphorylation of Erk could be observed up to 8 hours after PMA treatment. This enhanced and prolonged response to PMA was not observed in cells overexpressing a mutant rbc7HA protein that lacked the DAG-binding domain (ΔDAG) (Figure 4-4a, also see Figure 4-1). Thus, rbc7HA can stimulate strong and sustained Erk activation in response to PMA treatment through a mechanism dependent on the DAG-binding domain.

I confirmed that PMA treatment could stimulate sustained Erk activation in rat2 cells overexpressing full-length RasGRP (Figure 4-4b). In this experiment, I also observed that full-length RasGRP was not as potent as rbc7HA (compare rbc7HA and RasGRP after 4 hours of PMA treatment). One explanation for this difference could be the higher steady state protein level of rbc7HA as compared to full-length RasGRP (Ebinu and Stone, unpublished results). I also confirmed that Et-1 stimulation could lead to Erk activation in

rat2 cells overexpressing full-length RasGRP (Figure 4-4c). Thus, RasGRP can link exogenous and endogenous DAG signals to robust and sustained activation of Erk.

I also helped demonstrate the ability of RasGRP to elicit long-term biological changes. Rat2 cells overexpressing rbc7HA have a normal morphology very similar to parental cells (Ebinu *et al.*, 1998). Incubation of rbc7HA cells with PMA lead to a distinct transformed-like morphology within 40 hours. The effect of PMA was not observed in parental rat2 cells or in cells expressing ΔDAG. Furthermore, the morphological changes induced by PMA were more dramatic when the experiment was performed in low (0.5%) serum conditions. In sum, chronic PMA stimulation of rat2 cells overexpressing the rbc7 version of RasGRP leads to a transformed-like phenotype, likely due to sustained activation of Erk. In rat2 cells, stimulation of the Raf-Mek-Erk kinase cascade is sufficient to elicit cellular transformation (Bottorff *et al.*, 1995, Stang *et al.*, 1997).

Conclusions

The data presented here, along with previous findings from our lab, support the hypothesis that RasGRP is a DAG-responsive Ras GEF. *In vitro* biochemical studies have shown that various portions of RasGRP can catalyze guanyl-nucleotide exchange on Ras and bind Ras, Ca^{2+}, and a diacylglycerol analogue. In rat2 fibroblasts, RasGRP linked DAG signals to the activation of Ras, further downstream signaling and biological effects.

Through several different assays, the function of RasGRP in response to PMA was shown to be dependent upon the DAG-binding domain. A mechanism explaining how PMA regulates RasGRP was suggested by subcellular fractionation studies in rat2 cells (Ebinu *et al.*, 1998). In resting cells, rbc7HA was found roughly 50% in a cytosolic fraction and 50% in a particulate fraction that is enriched in plasma membrane. Upon treatment with PMA for 2 minutes, a higher amount of rbc7HA in the particulate fraction and a lower amount of rbc7HA in the cytosolic fraction were observed. This effect was not seen with the ΔDAG protein. These results suggest that PMA binds to the DAG-binding domain of RasGRP and promotes membrane translocation.

The association of RasGRP with cellular membranes has been demonstrated by an independent study that used green fluorescent protein (GFP) tagged constructs (Tognon *et al.*, 1998). In their study, GFP-RasGRP was observed to be evenly distributed in resting NIH3T3 mouse fibroblasts. In response to PMA treatment, GFP-RasGRP was observed in a punctate pattern localized mainly to the endoplasmic reticulum and nuclear periphery. These data suggest that the PMA-promoted translocation of RasGRP may largely be to structures other than the plasma membrane. It is possible that *in vivo*, a fraction of

RasGRP is localized to intracellular membranes. In the study by Ebinu *et al.* (1998), the particulate fraction may have contained some intracellular membranes. Also, a fraction of Ras in the cell has recently been localized to intracellular membranes (Choy *et al.*, 1999). As such, treatment of rodent fibroblasts with PMA may stimulate an association between RasGRP and Ras on intracellular membranes. Alternatively, the PMA-induced localization of GFP-RasGRP to intracellular membranes may be an artefact caused by abnormally high expression levels of GFP-RasGRP.

Other mechanisms of RasGRP regulation are possible. For example, our lab has observed phosphorylation of rbc7 on serine and threonine residues in response to PMA treatment (Ebinu and Stone, unpublished data). In addition, others have documented a small effect of Ca^{2+} ionophores on Ras activation by RasGRP in 293 human kidney cells (Kawasaki *et al.*, 1998).

By Northern analysis of human, mouse and rat tissues, RasGRP transcripts have been shown to be predominantly expressed in the hematopoietic system and brain (Ebinu *et al.*, 1998, Kawasaki *et al.*, 1998, Tognon *et al.*, 1998). Within the brain, Northern analysis has shown that RasGRP transcripts were particularly enriched in the cerebellum, cerebral cortex and amygdala (Kawasaki *et al.*, 1998). By *in situ* hybridization studies, RasGRP transcript was also shown to be localized to regions of the hippocampus (Ebinu *et al.*, 1998). In the hippocampus, DAG and IP_3 second messengers are thought to be generated by the action of phospholipase C downstream of metabotropic glutamate receptors (Bliss and Collingridge, 1993). Furthermore, this process is thought to be involved in the formation of long-term memory. We speculate that RasGRP links DAG signals to Ras signaling and memory production but the function of RasGRP in neuronal tissues remains to be defined more critically.

RasGRP may be a major regulator of Ras in T-cells. The activation of T-cells by antigen stimulation is thought to generate DAG and IP_3 second messengers through the action of phospholipase C (Cantrell, 1996). Stimulation of T-cells is also known to activate Ras which in turn, leads to increased expression of cytokines such as interleukin-2 by regulating nuclear factor of activated T-cells (NF-AT) (Downward *et al.*, 1990, Rayter *et al.*, 1992, Woodrow *et al.*, 1993, Cantrell, 1996). Currently, the mechanism of Ras activation in T-cells is thought to involve both membrane recruitment of SOS and an inhibition of Ras GAP's (GTPase-activating proteins) (Downward *et al.*, 1990, Nel *et al*, 1995, Cantrell, 1996). Our lab has preliminary evidence to suggest that RasGRP is a major Ras regulator in Jurkat cells, a human T-cell leukemia derived cell line. Radiolabeled GTP, when incubated *in vitro* with membrane fractions prepared from activated Jurkat cells, readily associates with the endogenous Ras within the membrane fraction. This

nucleotide exchange activity is attributed to a GEF that is associated with the membrane fraction. In this *in vitro* system, addition of an antibody specific for the catalytic domain of RasGRP inhibited the association of labeled GTP by approximately 50% (Ebinu and Stone, unpublished data). This result suggests that RasGRP comprises half of the GEF activity that regulates Ras in stimulated T-cells. RasGRP may normally function in T-cells to link DAG and possibly Ca^{2+} second messengers to Ras activation.

Our studies here demonstrated that RasGRP can regulate Ras *in vivo*. In addition, our findings provide some insight into the regulation of RasGRP by DAG that may be relevant to the function of the endogenous protein in neuronal and hematopoietic cells.

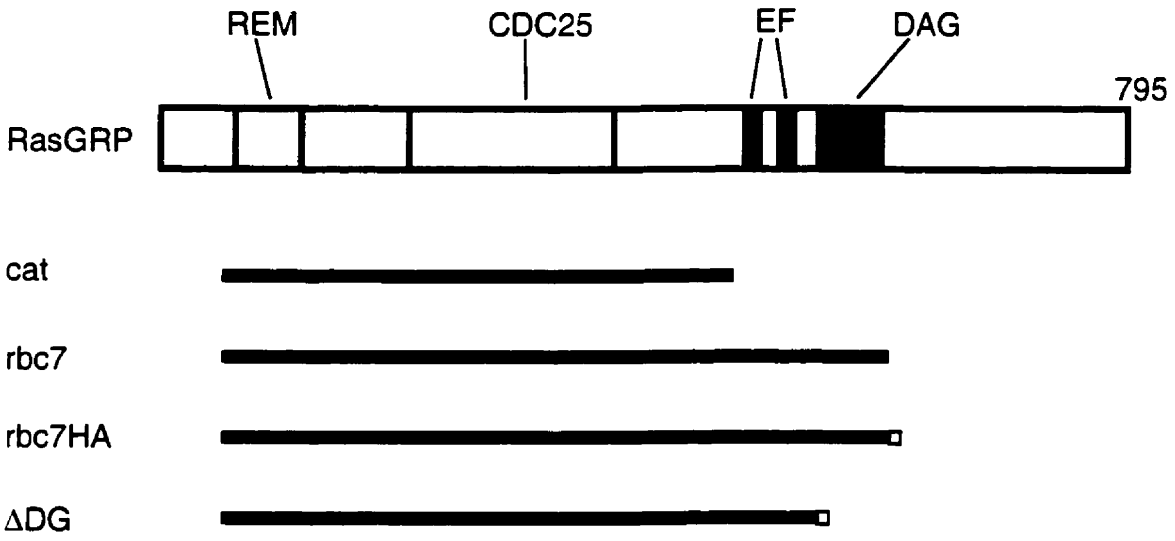


Figure 4-1. Domain structure schematic of RasGRP. Top: domain structure of the 795 amino acid full-length RasGRP from rat. The catalytic domain can be further divided into the REM domain and the minimal CDC25 GEF catalytic domain. A pair of atypical EF-hand calcium binding motifs and a diacylglycerol (DAG) binding domain are present in the C-terminal half of RasGRP. Bottom: schematic showing regions included in various RasGRP constructs. cat - catalytic domain used for *in vitro* work; rbc7 - N- and C-terminal truncated version of RasGRP initially isolated as transforming sequence; rbc7HA - rbc7 fused to an HA epitope (open box) at C-terminus; ΔDG - version of rbc7HA with an in-frame deletion of the DG-binding domain.

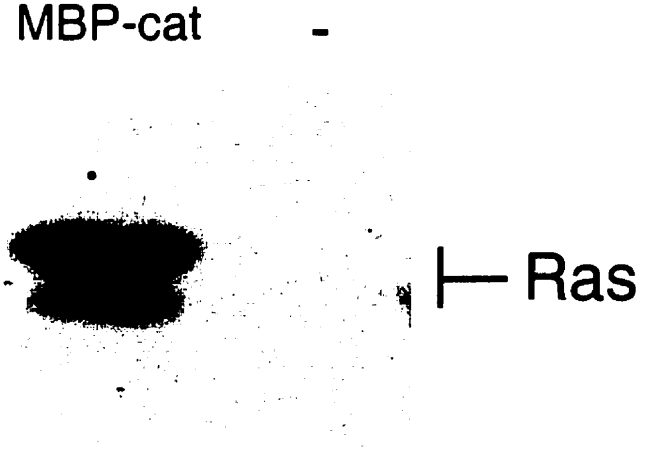


Figure 4-2. Stable interaction between RasGRP and Ras *in vitro*. A recombinant protein consisting of maltose binding protein fused to the catalytic domain of RasGRP (MBP-cat) was bound to amylose beads. Ras purified from Sf9 cells was then incubated with MBP-cat containing beads or bare beads (-). After extensive washing, bound Ras was analyzed by SDS-PAGE followed by an anti-Ras immuno-blot method. The position of Ras is indicated on the right. The smaller band that migrates faster is thought to be Ras protein that was partially processed in Sf9 cells.

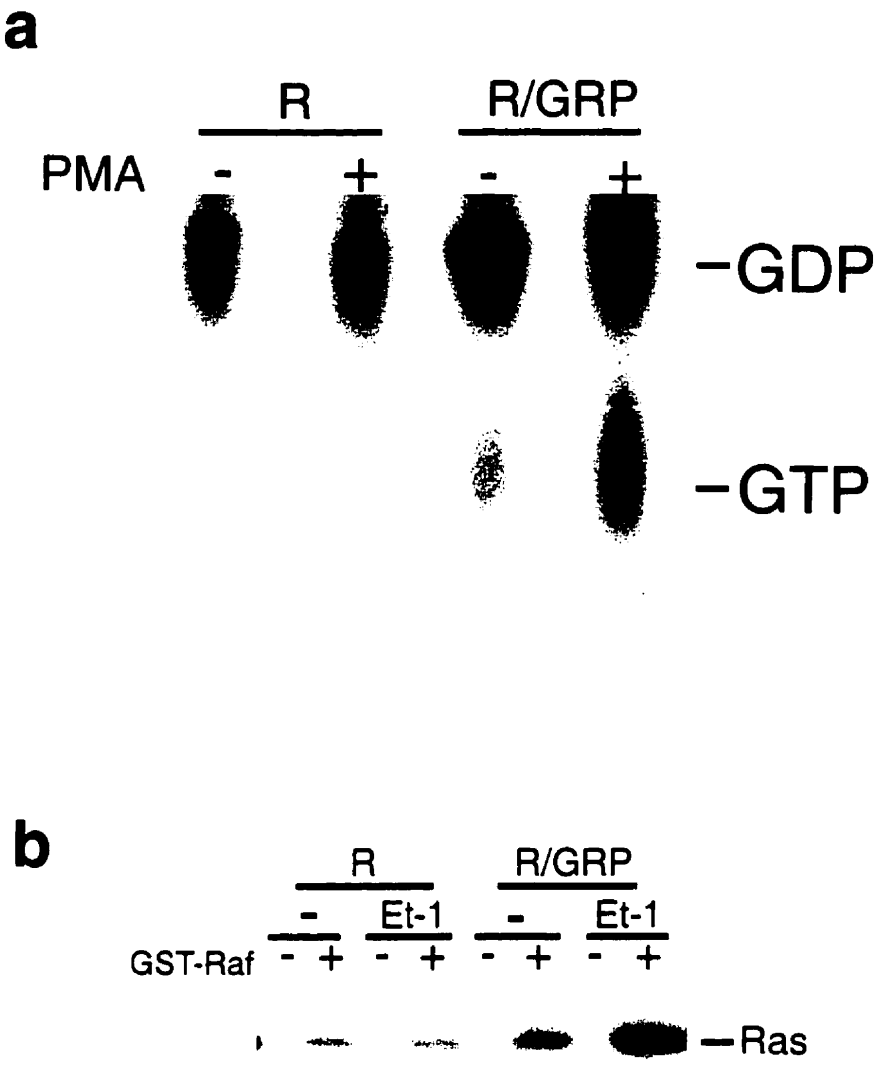


Figure 4-3. Activation of Ras by RasGRP *in vivo*. **a.** Ras-GTP levels were measured in rat2 cells that overexpressed H-Ras alone (R) or in combination with RasGRP (R/GRP) using an *in vivo* ^{32}Pi-labeling method. Cells were left untreated or treated with PMA (100 nM) for 2 minutes. After cell lysis and immuno-precipitation of Ras, associated radiolabeled GDP and GTP were separated by chromatography. and visualized by autoradiography. **b.** Rat2 cells that overexpressed H-Ras alone (R) or in combination with full-length RasGRP (R/GRP) were starved for 4 hours at 37°C in DMEM/0.1% FBS. Cells were then left untreated or treated with endothelin-1 (Et-1) (100 nM) for 10 minutes. Ras-GTP was precipitated from cell lysates using a GST-Raf fusion protein and precipitated Ras was detected with an anti-Ras immuno-blot method. The position of Ras is indicated on the right.

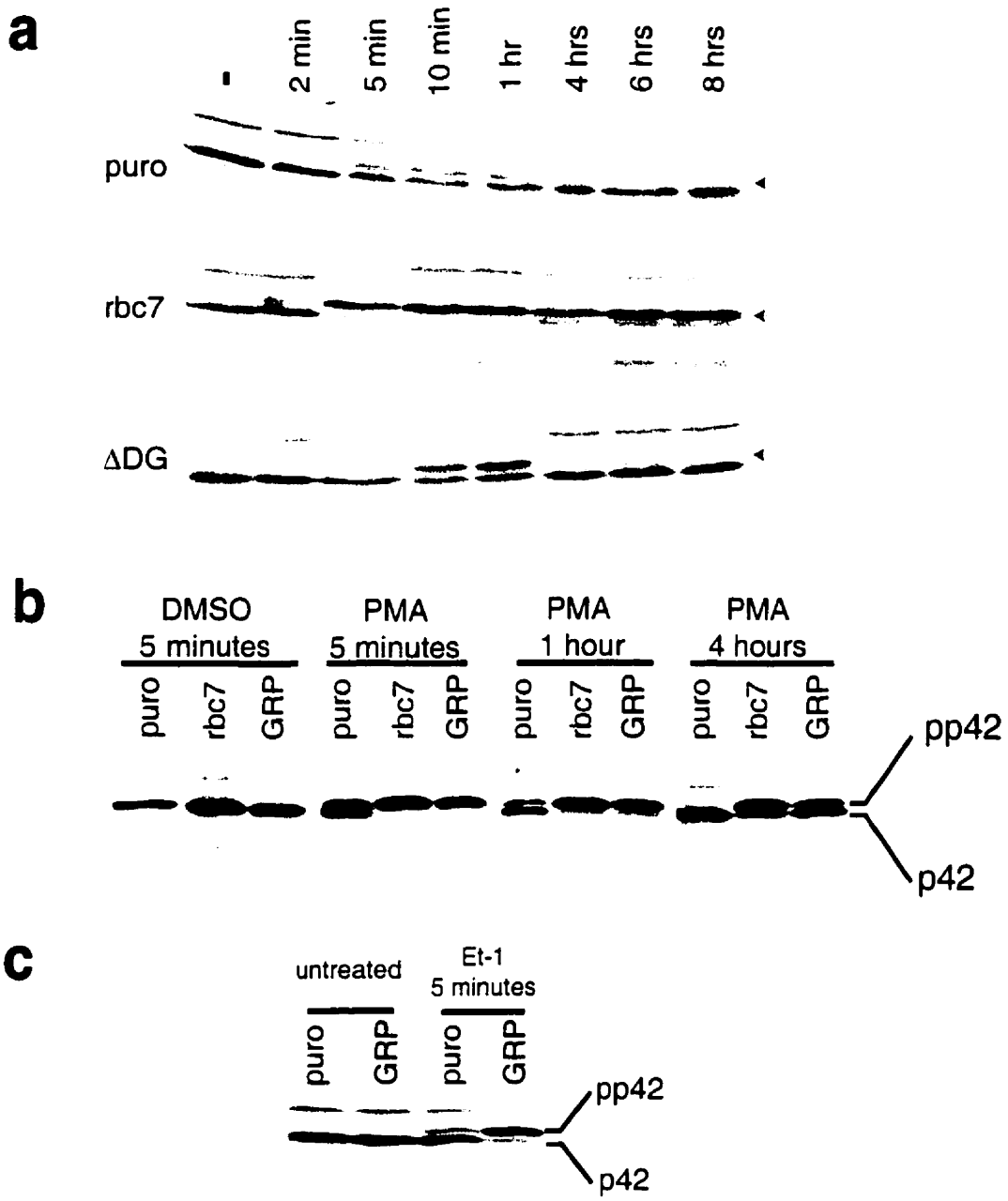


Figure 4-4. Activation of Erk by RasGRP *in vivo* **a.** Rat2 cells expressing empty pBabepuro vector (puro), rbc7HA (rbc7) or ΔDG were left untreated (-) or treated with PMA (100 nM) for various amounts of time. Erk activation was monitored by a gel-mobility shift assay. The phosphorylated, activated form of p42 Erk is indicated by the arrowhead on the right. **b.** Rat2 cells expressing empty vector (puro), rbc7HA (rbc7) or full-length RasGRP (GRP) were treated with DMSO vehicle control (0.1%) for 5 minutes or treated with PMA for various amounts of time. Erk activation was monitored by the gel mobility shift assay as above. The positions of unphosphorylated (p42) and phosphorylated (pp42) Erk forms are indicated on the right. **c.** Rat2 cells expressing empty vector (puro) or full-length RasGRP (GRP) were left untreated or treated with endothelin-1 (Et-1) (100 nM) for 5 minutes. Erk activation was monitored as in b. The cells in a and b were starved for 4 hours at 37°C in DMEM/0.5% FBS before the start of the experiment. The cells in c were starved for 6 hours at 37°C in DMEM/0.1% FBS before the start of the experiment.

References

Ambar, I., and Sokolovsky, M. (1993) Endothelin receptors stimulate both phospholipase C and phospholipase D activities in different cell lines. Eur. J. of Pharmacol. 245:31-41.

Bliss, T.V.P. and Collingridge, G.L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**:31-39.

Bottorff, D., Stang, S., Agellon, S., and Stone, J.C. (1995) *Ras* signalling is abnormal in *c-raf1 MEK1* double mutant. *Mol. Cell. Biol.* **15**:5113-5122

Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992) Identification of murine homologues of the *Drosophila* son of sevenless gene: potential activators of *ras*. *Proc. Natl. Acad. Sci.*. **89**:6511-6515.

Buday, L.,and Downward, J. (1993) Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and *Sos* nucleotide exchange factor. *Cell* **73**:611-620.

Cantrell, D. (1996) T-cell antigen receptor signal transduction pathways. *Ann. Rev. Immunol.* **14**:259-274.

Chardin, P., Camonis, J.H., Gale, N.W., Van Aelst, l., Schlessinger, J., Wigler, M.H., and Bar-Sagi, D. (1993) Human *sos* 1: a guanine nucleotide exchange factor for *ras* that binds to GRB2. *Science* **260**:1338-1343.

Choy, E., Chiu, V.K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I.E., and Philips, M.R. (1999) Endomembrane trafficking of Ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **98**:69-80.

Downward, J., Graves, J., Warne, P.H., Rayter, S., and Cantrell, D.A. (1990) Stimulaiton of p21**ras** upon T-cell activation. *Nature* **346**:719-723.

Ebinu, J.O., Bottorff, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science*. **280**:1082-1086.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. and Weinberg, R.A. (1993) Association of *sos ras* exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**: 45-51.

Fam, N.P., Fan, W., Wang, Z., Zhang, L., Chen, H., and Moran, M.F. (1997) Cloning and characterization of *ras*-GRF2, a novel guanine nucleotide exchange factor for *ras*. *Mol. Cell. Biol.* **17**:1396-1406.

Farnsworth, C.L., freshney, N.W., Rosen, L.B., Ghosh, A., Greenberg, M.E., and Feig, L.A. (1995) Calcium activation of *ras* mediated by neuronal exchange factor *ras*-GRF. *Nature* **376**:524-527.

Kawasaki, H., Springett, G.M., Toki, S., Canales, J.J., Harlan, P., Blumenstiel, J.P., Chen, E.J., Bany, I.A., Mochizuki, N., Ashbacher, A., *et al.*, (1998) A Rap

nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Nat. Acad. Sci.* **95**:13278-13283.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H. Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U.R. (1993) Protein kinase Cα activates RAF-1 by direct phophorylation. *Nature* **364**: 249-252.

Lai, C.-C., Boguski, M., Broek, D., and Powers, S. (1993) Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins. *Mol. Cell. Biol.* **13**:1345-1352.

Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Complexes of Ras•GTP with raf-1 and mitogen-activated protein kinase kinase. *Science* **260:**1658-1660.

Morgenstern, J.P., and Land, H. (1990) Advanced mammalian gene transfer: high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nuc. Acid Res.* **18**:3587-3596.

Nel, A.E., Gupta, S., Lee, L., Ledbetter, J.A. and Kanner, S.B. (1995) Ligation of the T-cell antigen receptor (TCR) induces association of hSos1, ZAP-70, phospholipase C-γ1, and other phosphoproteins with Grb2 and the ζ-chain of the TCR. *J. Biol. Chem.* **270:**18428-18436.

Rayter, S.I., Woodrow, M., Lucas, S.C., Cantrell, D.A. and Downward, J. (1992) p21ras mediates control of the Il-2 gene promoter function in T-cell activation. *EMBO J.* **11**:4549-4556.

Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D., Das, P., Waterfield, M.D., Ridley, A. and Downward, J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89:**457-467.

Stang, S., Bottorff, D. and Stone, J.C. (1997) Interaction of activated Ras with Raf-1 alone may be sufficient for transformation of rat2 cells. *Mol. Cell. Biol.* **17:**3047-3055.

Taylor, S.J. and Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Current Biology* **6:**1621-1627.

Tognon, C.E., Kirk, H.E., Passmore, L.A., Whitehead, I.P., Der, C.J., and Kay, R.J. (1998) Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* **18**:6995-7008.

Woodrow, M., Clipstone, N.A. and Cantrell, D. (1993) $p21^{ras}$ and Calcineurin synergize to regulate the nuclear factor of activated T cells. *J. Exp. Med.* **178:**1517-1522.

Chapter 5 - Activation of Ras in rat2 fibroblasts by C2-ceramide

Introduction

Ceramides are potent sphingolipid second messengers that are generated in the cell through the hydrolysis of sphingomyelin (Figure 5-1a). A number of extracellular stimuli such as tumor necrosis factor-α (TNFα), interleukin-1β, nerve growth factor (NGF), interferon -γ, and vitamin D3 have been shown to produce an accumulation of ceramides in various types of mammalian cells (Okazaki *et al.*, 1989, 1990, Ballou *et al.*, 1992, Hannun and Bell, 1993, Mathias *et al.*, 1993, Dobrowsky *et al.*, 1994). The mechanism whereby ceramides are generated in response to stimulation of cells by TNFα has been fairly well characterized. Stimulation of murine 70Z/3 pre-B-cells overexpressing the human 55 kDa-type TNFα receptor (TNF-R55) with TNFα leads to the activation of neutral sphingomyelinase (nSMase) at the plasma membrane and acidic sphingomyelinase (aSMase) which is localized to endosomal compartments (Weigmann *et al.*, 1994). The activation of nSMase and aSMase are dependent on distinct regions of the TNF-R55 intracellular domain. A nine amino-acid region of the TNF-R55 intracellular domain is thought to direct activation of nSMase via interactions with FAN (factor associated with nSMase activation) (Adam-Klages *et al.*, 1996). The mechanism linking FAN to nSMase activation remains unclear. Activation of aSMase has been shown to involve the "death domain" in the intracellular domain of TNF-R55 (Weigmann *et al.*, 1994). The death domain of TNF-R55 is associated with the cytotoxic effects of TNFα (Tartaglia *et al.*, 1993) and has been shown to interact with TRADD (TNF receptor associated protein with death domain) *in vitro,* in the yeast 2-hybrid system, and in transiently transfected 293 cells (Hsu *et al.*, 1995). TRADD, in turn, has been to interact with FADD (Fas-associated protein with death domain), again *in vitro,* in the yeast 2-hybrid system, and in transiently transfected 293 cells (Hsu *et al.*, 1996). TRADD and FADD may form a signal transduction pathway downstream of the TNF-R55 that leads to aSMase activation in response to TNFα stimulation. Overexpression of TRADD and FADD in 293 cells enhanced the TNFα-induced activation of aSMase (Schwander *et al.*, 1998).

The importance of ceramides in cellular signaling is demonstrated by their ability to mimic the biological effects elicited by a variety of extracellular agonists. For example, treatment of HL-60 leukemia cells with TNFα, interferon-γ or vitamin D3 leads to an accumulation of ceramides and differentiation into monocytes (Hannun and Bell, 1993). Treatment of HL-60 cells with cell-permeable ceramide alone can induce monocyte differentiation. In T9 glioma cells, NGF stimulation leads to ceramide accumulation, inhibition of DNA synthesis and neurite outgrowth (Dobrowsky *et al.*, 1994). Both

inhibition of DNA-synthesis and neurite outgrowth are mimicked by treatment of T9 cells with cell-permeable ceramide. In human gut lamina propria T lymphocytes (T-LPL), activation of the Fas-receptor generates ceramide and triggers apoptosis (De Maria *et al.*, 1996). Treatment with cell-permeable ceramide can induce apoptosis in T-LPL's as well as HL-60 cells, U937 monoblast leukemia cells, Jurkat cells (T-cell leukemia), HMN1 motor neuron cells and murine fibrosarcoma cell lines such as L929/LM and WEHI-164/13 (Cifone *et al.*, 1993, Obeid *et al.*, 1993, De Maria *et al.*, 1996, Brenner *et al.*, 1997, Zhou *et al.*, 1998). In Swiss 3T3 fibroblasts, cell-permeable ceramide has also been shown to stimulate proliferation (Olivera *et al.*, 1992, Hauser *et al.*, 1994). Based on their ability to mimic biological effects, ceramides have been proposed to be an important part of the signaling downstream of several extracellular agonists. In addition, cell-permeable ceramide clearly has the ability to initiate a number of biological changes, depending on the cell type.

Several proteins that are directly regulated by ceramides have been identified. Both ceramide-activated protein kinase (CAPK) and protein kinase C-ζ (PKCζ) are activated by ceramides *in vitro* and *in vivo* (Mathias *et al.*, 1991, Liu *et al.*, 1994, Lozano *et al.*, 1994, Muller *et al.*, 1995). CAPK has been reported to phosphorylate Raf and a peptide containing sequences of the epidermal growth factor (EGF) receptor *in vitro* (Mathias *et al.*, 1991, Yao *et al.*, 1995). One study has suggested that CAPK may be identical to Ksr, a protein isolated in several genetic schemes that is thought to be involved in Ras signaling (Sundaram and Han, 1995, Therrien *et al.*, 1995, Zhang *et al.*, 1997). Ceramides have also been shown to bind the catalytic domain of Raf (Muller *et al.*, 1998). Although the binding of ceramides to Raf does not affect kinase activity, the interaction of Raf to Ras is markedly enhanced *in vitro* and in Kym-1 (rhabdomyosarcoma) cells. Ceramides can also regulate protein phosphatases (ceramide-activated protein phosphatase (CAPP)) and activate the *in vitro* nucleotide exchange activity of Vav with a Ras substrate (Gulbins *et al.*, 1993, 1994, Wolff *et al.*, 1994). Since Vav may actually be a guanyl nucleotide exchange factor (GEF) for Rho family GTPases, the functional significance of Ras regulation *in vitro* by Vav is not clear (Han *et al.*, 1998). Currently, CAPK(Ksr), PKCζ, Raf, CAPP, and Vav are the putative cellular receptors for ceramide. The relationship between these proteins and the biological effects of ceramides is not understood.

In mammalian cells, a family of phosphatidylinositol-3 kinases (PI3K) phosphorylate phosphatidylinositol substrates at the D-3 position to generate a number of lipid second messengers (Carpenter and Cantley, 1996, Domin and Waterfield, 1997, Rameh and Cantley, 1999). Class 1 type PI3K proteins are composed of a p110 catalytic subunit that is stably bound to a p85 regulatory subunit (Carpenter *et al.*, 1990, Rodriguez-

Viciana *et al.*, 1994, Carpenter and Cantley, 1996, Domin and Waterfield, 1997, Rameh and Cantley, 1999). Previously, it has been shown that incubation of 3T3-L1 adipocytes with TNFα for 15 minutes stimulated tyrosine phosphorylation of p85 and the insulin receptor substrate-1 (IRS-1) (Guo and Donner, 1996). In these cells, TNFα treatment also promoted a stable association between the IRS-1 and p85. Interaction of IRS-1 with the SH2 domains of p85 has been shown to stimulate PI3K activity (Backer *et al.*, 1992). The TNFα-induced interaction between p85 and IRS observed in 3T3-L1 cells prompted Brindley and co-workers to ask whether ceramides stimulate PI3K activity. Their work, in rat2 fibroblasts, indeed showed that PI3K was activated in response to cell-permeable C2-ceramide (Hanna *et al.*, 1999) (Figure 5-1a). Within 10-15 minutes of treatment, catalytic activity of PI3K immunoprecipitated from cells increased 3-6 fold, PI3K was observed to associate with a membrane fraction by subcellular fractionation methods, and tyrosine phosphorylation of p85 increased. These effects of C2-ceramide were dose-dependent and dihyro-C2-ceramide (Figure 5-1b), a relatively inert analog, failed to elicit any effects. Recently, two other groups have demonstrated PI3K activation in response to cell-permeable ceramide treatment of Jurkat cells and rabbit colon smooth muscle cells (Gulbins *et al.*, 1998, Su *et al.*, 1999).

An involvement of Ras in ceramide-induced PI3K activation was suggested by several observations. The activated GTP-bound form of Ras is known to directly regulate PI3K activity via interaction with the p110 catalytic subunit (Rodriguez-Viciana *et al.*, 1994, 1996). In L929 fibroblasts, Ras activation has been observed upon TNFα treatment and apoptosis triggered by TNFα treatment is attenuated by overexpression of the N17 dominant-negative form of Ras (Trent, II *et al.*, 1996). Indeed, overexpression of N17 Ras in rat2 cells inhibited the activation of PI3K by C2-ceramide (Hanna *et al.*, 1999). This result implicated Ras in the activation of PI3K by C2-ceramide and suggested that C2-ceramide may regulate Ras. My studies here investigated Ras activation by C2-ceramide in rat2 fibroblasts. This work was done in collaboration with Dr. D.N. Brindley (Department of Biochemistry, Signal Transduction Laboratories, University of Alberta) and members of his lab.

Results

Modest activation of Ras in rat2 cells by C2-ceramide

A non-isotopic assay was used to measure Ras activation in response to C2-ceramide. Immobilized GST-Raf fusion protein precipitates only the GTP-bound form of

Ras from cell lysates. Precipitated Ras is subsequently detected by an immuno-blot method. The rat2 cells used in this experiment overexpressed H-Ras. In cells treated with DMSO vehicle control, low amounts of precipitated Ras were observed (Figure 5-2). Untreated cells also showed low amounts of precipitated Ras. Stimulation of cells with C2-ceramide led to modest but significant increases in precipitated Ras. By quantification of bands representing Ras in the Western-blot, this activation was estimated to be at maximum 2.5-fold within 10 minutes of C2-ceramide treatment as compared to untreated cells. Cells treated with dihydro-C2-ceramide did not show increased amounts of precipitated Ras. By comparison, stimulation of cells with EGF (100 ng/ml) for 5 minutes gave rise to a 10-fold increase in precipitated Ras.

The p110 subunit of PI3K interacts only with the GTP-bound form Ras *in vitro* (Rodriguez-Viciana *et al.*, 1994). As an alternative measure of Ras activation, we monitored the amount of Ras that co-immunoprecipitated with PI3K. Rat2 overexpressing H-Ras were treated with C2-ceramide and PI3K was immuno-precipitated from cell lysates using an antibody against p85α. The amount of Ras co-immunoprecipitating with PI3K increased within 5 minutes of treatment with C2-ceramide (Figure 5-3). This increase was quantitated from the Western blot to be approximately 8-fold as compared to untreated cells or cells treated with DMSO vehicle control. Treatment of cells with dihydro-C2-ceramide for 5 minutes did not increase the amount of Ras associated with PI3K. For comparison, treatment of cells with EGF (100 ng/ml) for 5 minutes produced a 27-fold increase in the amount of Ras that co-immunoprecipitated with PI3K.

Discussion

My results here demonstrate that cell-permeable C2-ceramide can activate Ras in rat2 fibroblasts. The activation of Ras was demonstrated by 2 non-isotopic assays that use immobilized Ras effector molecules to specifically bind to and precipitate the active GTP-bound form of Ras. These interactions can be formed *in vitro*, in the case with GST-Raf, or *in vivo* as observed in the stable complex between Ras and PI3K. The association of Ras with PI3K is confirmed by the demonstration of PI3K catalytic activity co-immunoprecipitating with Ras from rat2 cell lysates (Hanna *et al.*, 1999). Importantly, Ras-associated PI3K activity from C2-ceramide-treated cells was 3-fold higher than from untreated cells. This observation is consistent with increased Ras-GTP levels and increased PI3K-Ras-GTP binding in ceramide treated cells. The levels of C2-ceramide-induced Ras activation are modest but significant. In both assays, the levels of Ras

activation in C2-ceramide stimulated cells were approximately one quarter of the levels observed in cells treated with EGF.

It was shown previously that C2-ceramide treatment of rat2 cells stimulates total PI3K catalytic activity 3 to 6-fold (Hanna *et al.*, 1999). The modest activation of Ras by ceramide may be responsible for part of this activation of PI3K. Ras-GTP directly binds the p110 catalytic subunit of PI3K *in vitro* and this interaction is thought to induce a conformational change in p110 that gives rise to increased PI3K catalytic activity (Rodriguez-Viciana *et al.*, 1994, 1996). Also, the interaction of p110 with Ras likely promotes recruitment of PI3K to the plasma membrane bringing the enzyme into proximity with its substrates. Hanna *et al.* (1999) have found that overexpression of N17 Ras in rat2 cells completely inhibits the activation of PI3K by ceramides. Overexpression of N17 Ras is predicted to inhibit the modest ceramide-induced activation of Ras in rat2 cells. As such, modest activation of Ras may be necessary for ceramide-induced PI3K activation. An inhibition of PI3K by N17-Ras overexpression has been demonstrated in several other experimental systems (Rodriguez-Viciana *et al*, 1994, Hu *et al.*, 1995, Lopez-Ilasaca *et al.*, 1997, Gulbins *et al.*, 1998).

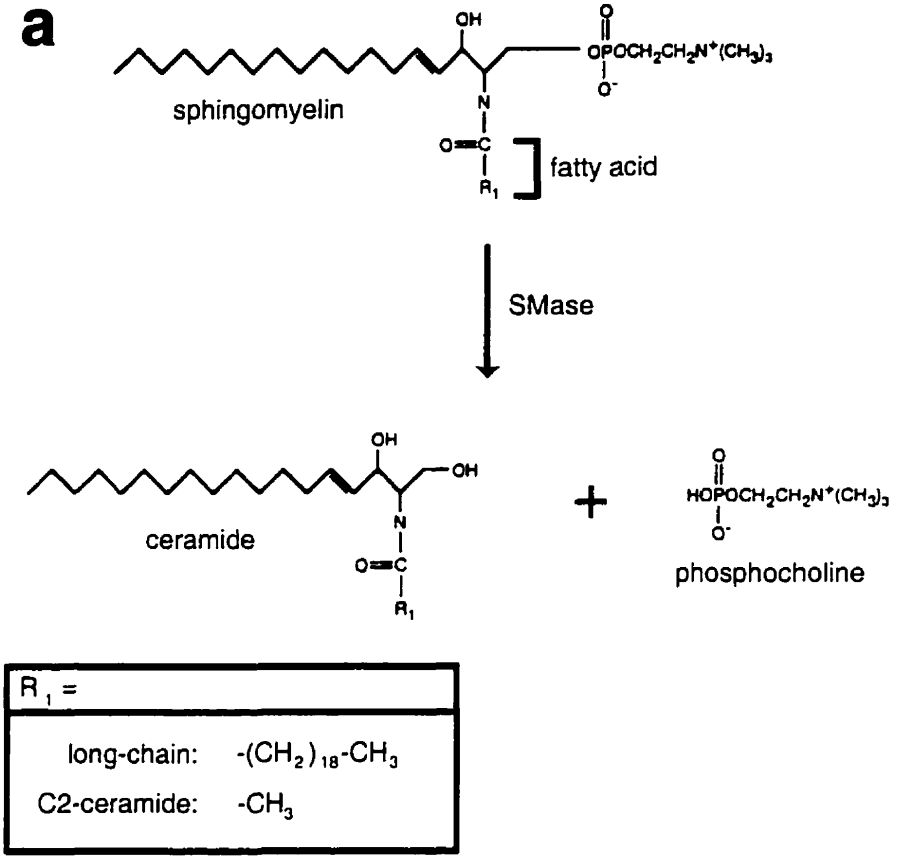
The mechanism whereby C2-ceramide activates Ras in rat2 cells is currently unknown. Ceramides have been shown to activate the GEF activity of Vav with Ras substrate *in vitro* (Gulbins *et al.*, 1993, 1994). The significance of Ras regulation by Vav is unclear since the GEF catalytic domain of Vav shares homology with the Dbl family of GEF's that are implicated in the regulation of Rho GTPases (Adams *et al.*, 1992, Han *et al.*, 1998). Furthermore, Vav appears to be exclusively expressed in hematopoietic cell types (Adams *et al.*, 1992). As such, expression of Vav in rat2 cells is not expected.

The activation of Ras by ceramide may involve protein tyrosine phosphorylation events. In rat2 cells, C2-ceramide treatment stimulated significant tyrosine phosphorylation of focal adhesion kinase (FAK) (Hanna *et al.*, 1999). Tyrosine phosphorylation of FAK could activate Ras by recruiting the Grb2-SOS complex to the plasma membrane (Schlaepfer and Hunter, 1996). Hanna *et al.* (1999) has also demonstrated that C2-ceramide-induced activation of PI3K in rat2 cells was completely ablated by 2 different non-specific tyrosine kinase inhibitors. Possibly, pharmacologic inhibition of protein tyrosine phosphorylation may block PI3K activation by interfering with Ras activation. The effect of protein tyrosine kinase inhibitors on C2-ceramide-induced Ras-activation in rat2 cells has not been investigated.

The inhibition of ceramide-induced PI3K activation by the tyrosine kinase inhibitors could also be through a Ras-independent mechanism. Tyrosine phosphorylated IRS-1 and tyrosine phosphorylated peptides with sequences derived from IRS-1 and the platelet-

derived growth factor (PDGF) receptor directly activate PI3K catalytic activity *in vitro* by binding to the SH2 domains of p85 (Rodriguez-Viciana *et al.*, 1996, Backer *et al.*, 1992). This *in vitro* system is thought to model the *in vivo* regulation of PI3K by interactions with tyrosine phosphorylated proteins. Although not demonstrated, C2-ceramide may activate PI3K in rat2 cells by stimulating tyrosine phosphorylation of the IRS-1 or PDGF receptor. These putative protein tyrosine phosphorylation events, which would be targeted by the tyrosine kinase inhibitors, may be necessary for ceramide-induced PI3K activation.

Incubation of rat2 cells with C2-ceramide for 24 hours led to modest elevations of DNA synthesis (150%), as measured by thymidine incorporation, and proliferation (30%), as measured by the number of cells per culture (Hanna *et al.*, 1999). These two proliferative effects were inhibited by pretreatment with LY294002, a specific inhibitor of PI3K. As such, prolonged incubation of rat2 cells with C2-ceramide appears to stimulate proliferation via a PI3K-dependent pathway. Although prolonged and acute C2-ceramide treatment may elicit very different signaling pathways in rat2 cells, it is speculated that the modest activation of Ras described here is involved in ceramide-induced proliferation.



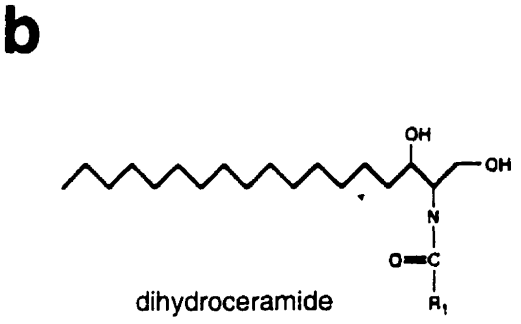


Figure 5-1. Structures of ceramide and precursors. **a.** In the cell, sphingomyelin is hydrolyzed by sphingomyelinase (SMase) to generate ceramide and phosphocholine. Inset: At the R_1 position, natural (long-chain) ceramides have a long aliphatic chain whereas cell-permeable C2-ceramide has only a methyl group. **b.** Dihydroceramide, an analog of ceramide that is relatively inert in biological systems, lacks the trans double bond in the backbone chain (arrow).

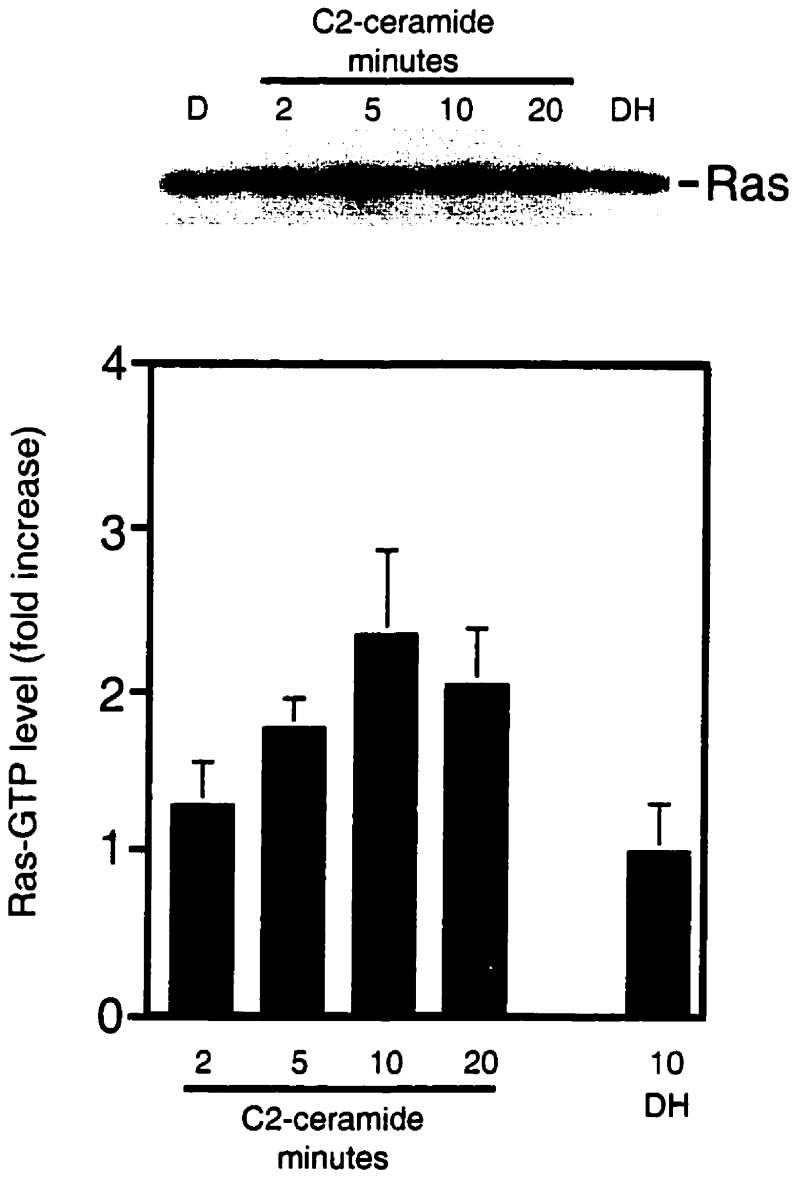


Figure 5-2. Activation of Ras by C2-ceramide *in vivo*. Rat2 cells overexpressing H-Ras were treated with DMSO (D) vehicle control (0.1% final concentration) for 5 minutes, treated with C2-ceramide (40 μM) for 2-20 minutes or treated with dihydro-C2-ceramide (DH) (40 μM) for 10 minutes. Ras-GTP was precipitated from cell lysates using a GST-Raf fusion protein and precipitated Ras was detected with an anti-Ras immuno-blot method. Top panel: representative Western blot showing levels of precipitated Ras. The position of Ras is indicated on the right. Bottom panel: Bands in Western blots representing Ras were scanned densitometry and plotted quantitatively. Shown are the averages and standard deviations derived from 3 independent experiments. Values are expressed as fold increase over levels observed in untreated cells.

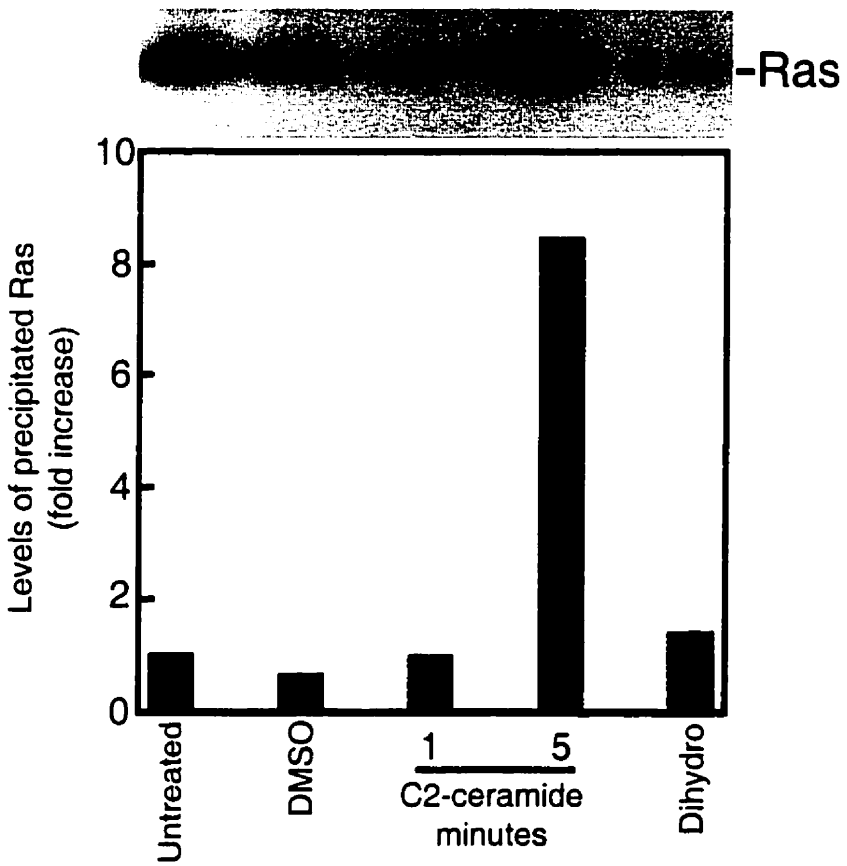


Figure 5-3. Interaction of Ras and PI3K induced by C2-ceramide *in vivo*. Rat2 cells overexpressing H-Ras were left untreated, treated with DMSO vehicle control (0.1% final concentration) for 5 minutes, treated with C2-ceramide (40 μM) for 1 or 5 minutes or treated with dihydro-C2-ceramide (DH) (40 μM) for 10 minutes. Phosphatidylinositol-3 kinase was immuno-precipitated from cell lysates using an antibody against p85α. Top panel: representative Western blot showing levels of Ras co-immunoprecipitating with PI3K. The position of Ras is indicated on the right. Bottom panel: Bands in Western blots representing Ras were scanned densitometry and plotted quantitatively. Values are expressed as fold increase over levels observed in untreated cells.

References

Adam-Klages, S., Adam, D., Wiegmann, K., Struve, S., Kolanus, W., Schneider-Mergener, J., and Kronke, M. (1996) FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. *Cell* **86**:937-947.

Adams, J.M., Houston, H., Allen, J., Lints, T., and Harvey, R. (1992) The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. *Oncogene*. 7:611-618.

Backer, J.M, Myers Jr, M.G., Shoelson, S.E., Chin, D.J., Sun, X., Margolis, B., Skolinik, E.Y., Schlessinger, J., and White, M.F. (1992) Phosphatidylinositol 3`-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* **11**:3469-3479.

Ballou, L.R., Chao, C.P., Holness, M.A., Barker, S.C., and Raghow, R. (1992) Interleukin-1-mediated PGE_2 production and sphingomyelin metabolism. *J. Biol. Chem.* **267**:20044-20050.

Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., and Gulbins, E. (1997) Fas-or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J. Biol. Chem.* **272**:22173-22181.

Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S>, and Cantley, L.C. (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.* **265**:19704-19711.

Carpenter, C.L., and Cantley, L.C. (1996) Phosphoinositide 3-kinase and the regulation of cell growth. *Biochemica et Biophysica Acta* **1288**:M11-M16.

Cifone, M.G., De Maria, R., Roncaioli, P., Rippo, M.R., Azuma, M., Lanier, L.L., Santoni, A., Testi, R. (1993) Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. *J. Exp. Med.* **177**:1547-1552.

De Maria, R., Boirivant, M., Cifone, M.G., Roncaioli, P., Hahne, M., Tschopp, J., Pallone, F., Santoni, A., Testi, R. (1996) Functional expression of Fas and Fas ligand on human gut lamina propria T lymphocytes. *J. Clin Invest.* **97**:316-322.

Dobrowsky, R.T., Werner, M.H., Castellino, A.M., Chao, M.V., and Hannun, Y.A. (1994) Activation of the sphingomyelin cycle through the low affinity neurotrophin receptor. *Science*. **265**:1596-1599.

Domin, J., and Waterfield, M.D. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Letters*. **410**:91-95.

Gulbins, E., Coggeshall, M.K., Baier, G., Katzav, S., Burn, P., and Altman, A. (1993) Tyrosine kinase-stimulated guanine nucleotide exchange activity of Vav in T-cell activation. *Science*. **260**:822-825.

Gulbins, E., Coggeshall, M., Baier, G., Telford, D., Langlet, C., Baier-Bitterlich, G., Bonnefoy-Berard, N., Burn, P., Wittinghofer, A., and Altman, A. (1994) Direct

stimulation of Vav guaning nucleotide exchange activity for Ras by phorbol esters and diglycerides. *Mol. Cell. Biol.* **14**:4749-4758.

Gulbins, E., Brenner, B., Koppenhoefer, U., Linderkamp, O., and Lang, F. (1998) Fas or ceramide induce apoptosis by Ras-regulated phosphoinoside 3-kinase. *J. Leukocyte Biol.* **63**:253-263.

Guo, D. and Donner, D.B. (1996) Tumor necrosis factor promotes phosphorylation and binding of insulin receptor substrate 1 to phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. *J. Biol. Chem.* **271**:615-618.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A., and Broek, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of *rac*-related guanosine triphosphatases by *vav*. *Science* **279**:558-560.

Hanna, A.N., Chan, E.Y.W., Xu, J., Stone, J.C., and Brindley, D.N. (1999) A novel pathway for tumor necrosis factor-a and ceramide signaling involving sequential activation of tyrosine kinase, p21ras, and phosphatidylinositol 3-kinase. *J. Biol. Chem.* **274**:12722-12729.

Hannun, Y.A. and Bell, R.M. (1993) The sphingomyelin cycle: a prototypic sphingolipid signaling pathway. *Adv. Lip. Res.* **25**:27-41.

Hauser, J.M.L., Buehrer, B.M., and Bell, R.M. (1994) Role of ceramide in mitogenesis induced by exogenous sphingoid bases. *J. Biol. Chem.* **269**:6803-6809.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell* **81**:495-504.

Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D.V. (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**:299-308.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1997) Matrix adhesion and *ras* transformation both activate a phophoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**:2783-2793.

Liu, J., Mathias, S., Yang, Z., and Kolesnick, R.N. (1994) Renaturation and tumor necrosis factor-α stimulation of a 97 kDa ceramide-activated protein kinase. *J. Biol. Chem.* **269**:3047-3052.

Lopez-Ilasaca, M., Crespo, P., Pellici, P.G., Gutkind, J.S., and Wetzker, R. (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ. *Science.* **275**:394-397.

Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L., and Moscat, J. (1994) Protein kinase C ζ isoform is critical for kB-dependent promoter activation by sphingomyelinase. *J. Biol. Chem.* **269**:19200-19202.

Mathias, S., Dressler, K.A., and Kolesnick, R.N. (1991) Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor α. *Proc. Nat. Acad. Sci.* **88**:10009-10013.

Mathias, S., Younes, A., Kan, C.-C., Orlow, I., Joseph, C., and Kolesnick, R.N. (1993) Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by Il-1β. *Science.* **259**:519-522.

Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995) PKC ζ is a molecular switch in signal transduction of TNF-α bifunctionally regulated by ceramide and arachidonic acid. *EMBO J.* **14**:1961-1969.

Muller, G., Storz, P., Bourteele, S., Doppler, H., Pfizenmaier, K., Mischak, H., Philipp, A., Kaiser, C., and Kolch, W. (1998) Regulation of Raf-1 kinase by TNF via its second messenger ceramide and cross-talk with mitogenic signaling. *EMBO J.* **17**:732-742.

Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993) Programmed cell death induced by ceramide. *Science.* **259**:1769-1771.

Okazaki, T., Bell, R.M., and Hannun, Y.A. (1989) Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. *J. Biol. Chem.* **264**:19076-19080.

Okazaki, T., Bielawska, A., Bell, R.M., and Hannun, Y.A. (1990) Role of ceramide as a lipid mediator of 1α, 25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J. Biol. Chem.* **265**:15823-15831.

Olivera, A., Buckley, N.E., and Spiegel, S. (1992) Sphingomyelinase and cell-permeable ceramide analogs stimulate cellular proliferation in quiescent Swiss 3T3 fibroblasts. *J. Biol. Chem.* **267**:26121-26127.

Rameh, L.E, and Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* **274**:8347-8350.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370:**527-532.

Rodriguez-Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D. and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15:**2442-2451.

Schlaepfer, D.D. and Hunter, T. (1996) Evidence for in vivo phosphorylation of the Grb2 SH2 domain binding site on Focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol. Cell. Biol.* **16**:5623-5633.

Schwander, R., Wiegmann, K., Bernardo, K., Kreder, D., and Kronke, M. (1998) TNF receptor death-domain-associated proteins TRADD and FADD signal activation of acid sphingomyelinase. *J. Biol. Chem.* **273**:5916-5922.

Su, X., Wang, P., Ibitayo, A., and Bitar, K.N. (1999) Differential activation of phosphoinositide 3-kinase by endothelin and ceramide in colonic smooth muscle cells. *Am. J. Physiol.* **276**:G853-G861.

Sundaram, M. and Han, M. (1995) The C. elegans *ksr-1* gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83:**889-901.

Tartaglia, L.A., Ayres, T.M., Wong, G.H.W., and Goeddel, D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**:845-853.

Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wassarman, D.A. and Rubin, G.M. (1995) KSR, a novel protein kinase required for RAS signal transduction. *Cell* **83:**879-888.

Trent, II, J.C., McConkey, D.J., Loughlin, S.M., Harbison, M.T., Fernandez, A., and Ananthaswamy, H.N. (1996) Ras signaling in tumor necrosis factor-induced apoptosis. *EMBO J.* **15**:4497-4505.

Weigmann, K., Schutze, S., Machleidt, T., Witte, D., and Kronke, M. (1994) Functional dichotomy of neutral and acidic sphingomyelinases in Tumor Necrosis Factor signaling. *Cell.* **78**:1005-1015.

Wolff, R.A., Dobrowsky, R.T., Bielawska, A., Obeid, L.M., and Hannun, Y.A. (1994) Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. *J. Biol. Chem.* **269**:19605-19609.

Yao, B., Zhang, Y., Delikat, S., Mathias, S., Basu, S., and Kolesnick, R. (1995) Phosphorylation of Raf by ceramide-activated protein kinase. *Nature.* **378**:307-310.

Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X.-H., Basu, S., McGinley, M., Chan-Hui, P.-Y., Lichenstein, H., and Kolesnick, R. (1997) Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell.* **89**:63-72.

Zhou, H., Summers, S.A., Birnbaum, M.J., and Pittman, R.N. (1998) Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *J. Biol. Chem.* **273**:16568-16575.

Chapter 6 - Summary and future directions

Summary of Ras activation in hypothermic cells

In 1995, our lab serendipitously found that Erk was activated in cells that had been chilled and then rewarmed. A sample culture of cells destined to be the untreated negative control was placed on ice for lysis. At this stage, it was realized that the lysis buffer was incorrect and the cells were returned to the 37°C incubator after sitting approximately 15 minutes on ice. When the experiment was reinitiated, the activity of Erk from this sample was unexpectedly high. The studies described in chapter 3 have followed up this initial observation.

I have shown that Ras becomes slowly activated in rat2 cells exposed to hypothermic stress. When these hypothermic cells are rewarmed to physiologic temperature, the Raf-Mek-Erk protein kinase cascade is activated in a transient but robust manner. Activation of Erk was observed upon rewarming after moderate and severe hypothermic stress. In addition, Erk activation by hypothermic stress followed by rewarming was conserved in many cultured cell types. I also gathered evidence showing that other Ras downstream effector systems, namely phosphatidylinositol-3 kinase (PI3K), may be activated by hypothermic stress followed by rewarming. Insight into the *in vivo* mechanism of Ras activation was obtained from *in vitro* studies using purified Ras and Ras regulatory proteins. I showed that a Ras effector mutant failed to be activated in hypothermic rat2 cells. Treatment of cells with Ca^{2+} chelators and the removal of serum from the growth media also inhibited hypothermic Ras activation. These *in vitro* and cell culture data helped formulate a model for *in vivo* hypothermic Ras activation.

Previously, it was shown that hypothermia blocked acute growth factor signaling to Erk. Along with others in our lab, I have shown that hypothermia does not inhibit epidermal growth factor- (EGF) stimulated Ras activation in rat2 cells. In contrast, hypothermia inhibits EGF-stimulated activation of Raf.

After prolonged hypothermic stress, cultured cells show a loss of viability and increased amounts of apoptosis. Pharmacological inhibition of Mek increased the survival of several cultured cell types and attenuated apoptosis in one cell type. Thus, it appears that Erk activation after prolonged hypothermic stress may decrease cell viability and trigger apoptosis. We speculate that the protective effect of the Mek inhibitor will be useful for improving the storage of clinically relevant cells and organs for medical procedures.

Model for *in vivo* Ras activation

In resting cells at 37°C, Ras-GTP levels are low because the basal rate of GTP hydrolysis exceeds the basal rate of catalyzed nucleotide exchange (Figure 6-1a). Our working model for *in vivo* hypothermic Ras activation involves an inhibition of basal GTP hydrolysis by cold temperature (Figure 6-1b). We have shown that catalysed nucleotide exchange is relatively independent of cold temperature. As a result, the basal rate of catalysed nucleotide exchange exceeds basal hydrolysis in hypothermic cells leading to a slow accumulation of Ras-GTP. The guanyl nucleotide exchange factor (GEF) that catalyses the basal nucleotide exchange remains unidentified.

In vivo hypothermic Ras activation is blocked by the removal of serum from the tissue culture growth media. Hypothermic Ras activation is also blocked by Ca^{2+} chelators. There are 3 possible scenarios that could explain these inhibitory effects. In the first 2 scenarios, hypothermia triggers a Ca^{2+} flux into the cytosolic compartment of the cell. In scenario 1, Ca^{2+} flux is serum-dependent. Some factor in serum may stimulate the opening of a Ca^{2+} channel in the plasma membrane that gives rise to basal amounts of Ca^{2+} currents which, in turn, leads to basal stimulation of a Ca^{2+}-dependent GEF. This Ca^{2+} flux could also regulate the GEF through interactions with a Ca^{2+}-binding accessory protein. In scenario 2, the Ca^{2+} flux is serum-independent. The Ca^{2+} flux occurs in hypothermic cells due to an inhibition of Ca^{2+} ATPase pumps in the endoplasmic reticulum membrane and plasma membrane. Inhibited Ca^{2+} extrusion coupled with a slow leak of Ca^{2+} into the cytosol leads to a slow Ca^{2+} influx. Some factor in serum leads to the basal GEF activity only in increased levels of cytosolic Ca^{2+}. As follows, the Ca^{2+} flux and stimulation from the serum factor are both necessary for the basal GEF activity. In scenarios 1 and 2, chelation of Ca^{2+} would directly target the mechanism of GEF regulation. These hypotheses are based on studies in other cell types which described increased cytosolic Ca^{2+} levels in hypothermic cells (Perotti *et al.*, 1990, Liu *et al.*, 1991, Mcanulty *et al.*, 1996). However, a Ca^{2+} flux has not yet been confirmed in hypothermic rat2 cells.

In scenario 3, there is no Ca^{2+} flux. Some serum factor(s) may stimulate the basal catalyzed nucleotide exchange via a Ca^{2+} independent-pathway. Chelation of Ca^{2+} may inhibit the GEF because trace amounts of Ca^{2+} are needed for its activity. This proposed inhibitory effect would have to be subtle, however, since Ca^{2+} chelators do not inhibit EGF-stimulated Erk activation at 37°C.

Future studies on the biochemistry of hypothermic-Ras signaling

Possible inhibition of GEF activity by chelation of Ca^{2+}

We postulated above (in scenario 3) that total Ca^{2+} chelation may create a cellular environment that inhibits GEF activity. To test this hypothesis, *in vitro* catalyzed nucleotide exchange reactions can be performed with recombinant GEF's such as SOS, RasGRF1 and RasGRP, in the presence of EGTA. A block of *in vitro* catalyzed exchange by EGTA would be consistent with the idea that GEF's require at least a trace amount of Ca^{2+} in the cell to function.

Identification of the GEF involved in hypothermic Ras activation

Y32H Ras was not activated in hypothermic cells and I hypothesized that the Y32H mutation in the effector (switch 1) region disrupted interactions with the weak GEF activity postulated in the model. Purified Y32H Ras protein is available and it would be informative to compare *in vitro* catalyzed nucleotide exchange at 0°C and 37°C on Y32H and wildtype Ras substrate using purified GEF's. The prediction is that catalyzed nucleotide exchange at 0°C on Y32H Ras will be inefficient. Since Y32H Ras can be activated by EGF *in vivo* at 37°C, catalyzed nucleotide exchange on Y32H Ras should be relatively intact at 37°C. This approach may also help us identify the protein responsible for the postulated weak GEF activity. For example, a GEF that is inefficient at catalyzing nucleotide exchange at 0°C on Y32H as compared to wildtype Ras would be a candidate for the GEF involved in hypothermic Ras activation. On the other hand, GEF's that work equally well at 0°C on Y32H and wildtype Ras could be ruled out of the mechanism for hypothermic Ras activation. As an alternative approach, Ras effector mutants can be tested for interaction with various GEF's in the yeast 2-hybrid system.

A large number of Ras effector mutants have been generated and characterized by our lab (Stang *et al*, 1997). It would be useful to identify other effector mutants that fail to be activated in hypothermic cells. A number of Ras effector mutants could be classified as hypothermia responders and non-responders. Following, several members of each class can be studied biochemically and in the 2-hybrid system. In this way, a correlation can be built that may enable the identification of the GEF postulated in the model.

Possible effects of hypothermia on G-proteins in general

Our working model for hypothermic Ras activation involves hypothermic inhibition of GTP hydrolysis coupled with basal catalysed nucleotide exchange that is relatively temperature-independent. As follows, the balance between GTP hydrolysis and nucleotide exchange is tipped slightly in favor of nucleotide exchange giving rise to a slow accumulation of Ras-GTP. All G-proteins in the cell are regulated by the balance of GTP hydrolysis and nucleotide exchange. Thus, it is reasonable to speculate that low temperature may affect other G-proteins besides Ras. A first experiment in this direction would be to examine the activation of close Ras relatives like Rap1A, Ral-1A and R-Ras in hypothermic cells. Second, investigations can be carried out with G-proteins more distantly related to Ras such as RhoA and Rac1 which are understood to regulate important cellular processes such as actin cytoskeleton rearrangements (Zohn *et al*, 1998). These studies could reveal a number of G-protein signaling events that are initiated by hypothermic stress.

Further studies on biochemical events downstream of hypothermic Ras-Erk signaling

During the rewarming period after hypothermic incubation, the Raf-Mek-Erk cascade was activated in a robust but transient manner in rat2 cells. Hypothermically-activated Erk can elicit further downstream biochemical changes. We have shown that p90 ribosomal S6 kinase, a signaling molecule that functions downstream of Erk, is activated upon rewarming of hypothermic cells (Bottorff and Stone, unpublished data). In addition, a pretreatment of hypothermic stress followed by rewarming rendered rat2 cells less-sensitive to subsequent EGF stimulation. We interpret this effect to be a negative feedback loop that involves the phosphorylation of SOS by Erk.

An important consequence of Erk signaling is the phosphorylation of transcription factors such as Elk-1 and the modulation of gene expression (Marias *et al.*, 1993, Price *et al.*, 1995). The modulation of gene expression after rewarming from hypothermic stress would provide additional evidence that hypothermic Ras-Erk signaling can alter the overall biology of the cell. In cells treated with hypothermic stress followed by rewarming, activation of Elk1 can be measured using an assay that employs transient transfection of a reporter construct (see Kawasaki *et al*, 1998). Erk activation is also understood to regulate the cell-cycle by promoting expression of cyclinD1 which in turn regulates cyclin-dependent kinase 4 (cdk4) (Weber *et al.*, 1997). Experiments should be performed to monitor cyclinD1 message levels in the recovery period after hypothermic stress. An

upregulation of cyclin D would suggest that hypothermic Ras-Erk signaling directly modulates the cell-cycle.

Further studies on the inhibition of hypothermic cell death by PD098059

The ability of the Mek inhibitor PD098059 to improve cell viability after prolonged hypothermic stress could be useful for the storage of human organs destined for transplantation. Others have studied vascular endothelial cells, such as primary human umbilical vein endothelial cells (HUV), as a cell culture model for organ storage (Eberl *et al.*, 1996). The vascular endothelium forms the lining of blood vessels, directly contacts the hypothermic storage solution and is the first site of damage in the organ when the storage solution is removed by reperfusion. Biochemical studies should be performed in HUV cells to confirm Erk activation after recovery from hypothermic stress. It will be important to ask whether HUV cells show morphology change, detachment from the culture substratum and a loss of viability after prolonged hypothermic stress in our system and whether PD098059 pretreatment shows a protective effect. In addition, others have developed assays to quantitate the viability and function of HUV cells after hypothermic storage such as prostaglandin release into the culture medium (Eberl *et al.*, 1996). It can be asked whether PD098059 can improve the function of HUV cells after hypothermic stress using these assays. Even if hypothermic Erk activation cannot be demonstrated in HUV cells, a protective effect of PD098059 alone would have practical implications for organ storage technology.

How PD098059 pretreatment enhances survival of rat2, REC and MDCK cells after prolonged hypothermia is not known. In other cell types, prolonged hypothermia is thought to cause damage primarily by inhibiting the Na^+-K^+ ATPase pump which in turn, leads to a rise in cytosolic Na^+ levels and cell swelling (Belzer and Southard, 1988). Intracellular acidosis is another important damaging effect of prolonged hypothermic stress. The link between these cellular changes and Erk signaling is not understood. Preliminary evidence from our lab has indicated that other factors are likely involved in the loss of cell viability observed after prolonged hypothermic stress. For example, I observed once that addition of EGTA to the medium could inhibit the morphological changes and detachment of cells during recovery after prolonged hypothermic stress. It has been observed several times that pretreatment of rat2 cells with LY294002, Wortmannin, or SB202190 can modestly enhance cell viability after prolonged hypothermic stress. The presence of DMSO vehicle control in the cell media also had a slight protective effect. These protective effects were unexpected. LY294002 and Wortmannin are inhibitors of PI3K which normally

gives rise to anti-cell death signaling. Inhibition of PI3K signaling would be predicted to decrease cell viability. SB202190 is an inhibitor of p38HOG, a stress-related protein kinase, which was not strongly activated during recovery after a 4 hour hypothermic stress (Stang and Stone, unpublished data).

We also predicted that expression of dominant-negative Ras and Mek would provide a protective effect. Dominant-negative Ras and Mek could be stably expressed at moderate levels in rat2 cells and their expression did influence cell viability after prolonged hypothermic stress. Overexpression of wildtype Ras also influenced cell viability after hypothermic stress. However, the effects of dominant-negative Ras or Mek or wildtype Ras overexpression were very inconsistent. Overexpression of N17 Ras and wildtype Ras has been shown to both inhibit and exacerbate rat2 cell death after prolonged hypothermia, depending on the experiment. Based on the protective effect of the PD098059 Mek inhibitor, dominant-negative Mek was predicted to also protect cells from hypothermic cell death. However, in one experiment overexpression of dominant-negative Mek rendered rat2 cells more sensitive to hypothermic death. In sum, our understanding of cell death after prolonged hypothermic stress is very limited. Due to the potential implications for organ storage technology, I feel that it is worthwhile to clarify some of these protective effects with careful studies. For example, it would be valuable to know if the protective effect of PD098059 in rat2 cells can be improved by use in combination with either LY294002 or SB202190. Other methods of optimizing protection such as varying the drug concentration have not been explored.

Studies on hypothermic Ras-Erk signaling and organ preservation techniques

The dependence on serum in the growth media suggests that hypothermic Ras activation may be limited to tissue culture. We have shown that Erk does become activated in hind limbs of neonatal rats that were rewarmed after hypothermic incubation. This suggests that hypothermic activation of Ras-Erk signaling is conserved in animal tissues. Preliminary studies with neonatal rats have also demonstrated modest Erk activation in hypothermically stressed heart, liver and lung that were rewarmed. In spite of this demonstration of Erk activation, I could not demonstrate an accumulation of Ras-GTP in heart, liver, lung or brain of neotatal rats that had been incubated at 0°C for up to 3 hours using the non-isotopic assay that involves the GST-Raf fusion protein (see Chapter 2 for more details on this method). However, measuring Ras-GTP levels in whole tissues is not straightforward and those experiments did not have a positive control to show that the

protocol was sound. It remains unclear whether the observed Erk activation in animal tissues stems from hypothermically activated Ras.

I propose that studies on hypothermic Ras-Erk signaling be performed in perfused heart and liver of adult rats. Perfusion offers clear advantage in the precise control of temperature and delivery of solution to almost every cell of the organ. The large number of studies on hypothermic storage of perfused organs also provides a valuable source of background information. A simple experiment is to ask whether Erk is activated in perfused heart and liver that have been stored under hypothermic conditions and then rewarmed. An important question is whether Ras-GTP accumulates in these hearts and livers during hypothermic storage. In perfused organs, we can also test our current model for hypothermic Ras activation by supplementing organ storage solutions with serum. The function of perfused hearts after recovery from hypothermic storage can be tested by measuring left ventricular minute work, an index of pumping efficiency (Ali *et al.*, 1998). It should be asked directly if PD098059 preincubation can affect the function of perfused hearts after hypothermic storage. In addition, drugs such as LY294002, Wortmannin or SB202190 can be tested for protective effects on heart function. Even if biochemical studies prove inconclusive, the identification of drugs such as kinase inhibitors that can protect heart function after hypothermic storage would be important.

Summary of RasGRP function in rat2 cells

The studies within chapter 4 were aimed at the function of RasGRP, a newly identified Ras GEF, in rat2 fibroblasts. RasGRP is interesting due to the presence of a diacylglycerol- (DAG) binding domain and a pair of Ca^{2+}-binding EF hand motifs in the primary structure. My work showed that RasGRP can activate Ras in response to exogenously added phorbol ester (PMA), a DAG analog, and endogenously generated DAG signals. I also showed that RasGRP can stimulate prolonged and strong activation of Erk in response to exogenous and endogenous DAG signals. Lastly, I helped show that RasGRP could cause a transformed-like cell morphology in response to treatment with PMA. The observed responses to DAG signals depended on the DAG-binding domain of RasGRP.

The role of Ca^{2+} in RasGRP regulation remains unclear. Another group has documented a small level of Ras activation in response to Ca^{2+} ionophore treatment of RasGRP overexpressing COS cells (Kawasaki *et al.*, 1998). However, I have failed to show RasGRP-dependent activation of Ras-Erk signaling in response to Ca^{2+} ionophores in rat2 cells. In addition, our lab found that point mutation of critical Ca^{2+}-binding residues

in RasGRP gives rise to proteins that are unstable in rat2 cells (Stang and Stone, unpublished data).

Based on work in rat2 cells from others within our lab, a model has been proposed where DAG binds to the DAG-binding domain of RasGRP and promotes translocation of RasGRP to the plasma membrane where it will be in proximity with Ras. Our studies on RasGRP in rat fibroblasts may provide insight into the function of the endogenous protein which is thought to be primarily expressed in hematopoietic and neuronal cells.

Future studies on RasGRP

RasGRP function in T-cells and neuronal cells

The major focus of future studies on RasGRP should be the biological function of the endogenous protein. Expression of RasGRP protein has been detected in Jurkat cells, a human T-cell leukemia derived cell line (Ebinu, Stang and Stone, unpublished data). In addition, there is preliminary evidence suggesting that RasGRP activates Ras in Jurkat cells. Radiolabeled GTP, when incubated *in vitro* with membrane fractions prepared from activated Jurkat cells, readily associates with the endogenous Ras within the membrane fractions. This nucleotide exchange activity is attributed to Ras GEF's that are associated with the membrane fraction. In this *in vitro* system, antibodies specific for the catalytic domain of RasGRP can inhibit the association of labeled GTP by approximately 50% suggesting that RasGRP is a major Ras GEF in activated Jurkat cells. Jurkat cells overexpressing RasGRP are also hypersensitive to T-cell receptor stimulation as measured by Erk activation and interleukin–2 secretion.

The function of RasGRP in neuronal cells is unexplored. By Northern analysis, RasGRP transcripts were shown to be enriched in the cerebellum, cerebral cortex and amygdala regions of rat brain (Kawasaki *et al.*, 1998). By *in situ* hybridization studies, RasGRP transcript was shown to be localized to regions of the rat hippocampus (Ebinu *et al.*, 1998). First, endogenous RasGRP protein expression needs to be characterized in brain tissues and neuronal cultured cells. Immunofluoresence studies should also be performed to document the localization of RasGRP within a neuronal cell.

As a first step in demonstrating Ras regulation by RasGRP in neuronal cells, I propose studies in PC12 cells, a rat pheochromocytoma cell line that has retained many neuronal properties (Shafer and Atchison, 1991). PC12 cells, under certain conditions, can synthesize and secrete neurotransmitters such as acetylcholine, norepinephrine and dopamine. PC12 cells also express several neurotransmitter cell surface receptors such as

nicotinic and muscarinic acetylcholine type receptors. Stimulation of PC12 with a variety of agonists can lead to an accumulation of DAG and Ca^{2+} second messengers. For example, stimulation of PC12 cells with moxonidine, an agonist for the I1-imidazoline receptor, leads to an accumulation of DAG via a phosphatidylcholine specific phospholipase C (Separovic *et al.*, 1997). Treatment of PC12 cells with chemical agents such as trimethyltin also leads to the accumulation of IP_3, and likely DAG (Kane *et al.*, 1998).

By Northern blot analysis, RasGRP transcript was undetectable in PC12 cell total RNA (Bottorff and Stone, unpublished data). By overexpression of RasGRP cDNA's in PC12 cells, Ras regulation can essentially be studied in the presence or absence of RasGRP protein. PC12 cell lines expressing other genes expressed under the control of a dexamethasone-inducible promoter have been successfully generated (Thomas *et al.*, 1992). Biochemical studies on the activation of Ras and downstream signaling can be performed in PC12 cells that inducibly express wildtype RasGRP or the mutant RasGRP protein lacking the DAG-binding domain. The mutant RasGRP proteins containing substitutions in the Ca^{2+} binding domains can also be expressed in PC12 cells. Perhaps these mutant proteins overexpressed in PC12 cells will be more stable than in rat2 cells. DAG and Ca^{2+} signals can be generated in these PC12 cell lines by the methods listed above. Sustained activation of Ras-Erk signaling is also well understood to promote differentiation of PC12 cells (Traverse *et al.*, 1992, Marshall, 1995). As such, PC12 cell differentiation provides a potential neurological bioassay to study the regulation of Ras-Erk signaling by RasGRP.

Long term goal: Knock-out studies on RasGRP

The mouse gene encoding RasGRP has been described (Bottorff *et al.*, 1999). This characterization has led to a strategy for engineering a homozygous disruption of the *RasGRP* gene in mice (Dower and Stone, unpublished data). RasGRP "knock-out" mice could potentially provide strong evidence for the biological role for RasGRP in mammalian hematopoietic or neural development. Investigation of RasGRP homologs in *C. elegans*, a model genetic organism, may also be fruitful. The ultimate goal is to study *C. elegans* animals that have their RasGRP gene "knocked-out". A *C. elegans* predicted open reading frame (ORF), F25B3.3, shows homology to the sequences of RasGRP. The putative full-length version of F25B3.3 is a candidate *C. elegans* RasGRP homolog. Green fluorescent protein expressed under the control of the predicted transcriptional regulatory sequences of F25B3.3 was observed predominantly in the nervous system of *C. elegans* (Urban, Stone,

Pilgrim, unpublished data). As follows, the putative *C. elegans* RasGRP homolog may be a neuronal regulator of Ras.

Knock-out strategies in *C. elegans* via homologous recombination have generally not been successful. Currently, the best approach for isolating a *C. elegans* gene knock-out animal is to locate the gene of interest on the *C. elegans* genetic map and then, based on this position, identified candidate genetic loci. Further studies need to be performed to localize the F25B3.3 ORF on the physical and genetic map of *C. elegans*. Strategies have been developed that can effectively eliminate a transcript of choice by microinjection of double-stranded RNA (dsRNA) molecules into adult *C. elegans*.(Fire *et al.*, 1998) These dsRNA molecules are to contain sequences that correspond to the targeted gene and are thought to promote degredation of the endogenously produced mRNA (Montgomery *et al.*, 1998). This technique may be able to generate *C. elegans* animals that lack the transcript encoding the putative RasGRP homolog and may provide a more accessible approach to study knock-out *C.elegans* animals.

Summary of Ras activation by ceramide

Ceramide is a ubiquitous sphingolipid second messenger that can elicit a variety of biological effects in mammalian cells. Previous studies in rat2 cells have shown that PI3K activation in response to ceramide is inhibited by overexpression of dominant-negative N17 Ras suggesting an involvement of Ras in this signaling pathway. In chapter 5, I showed that ceramide can lead to a modest activation of Ras in rat2 cells. The mechanism whereby ceramide leads to Ras activation has not been studied, however.

Future studies on Ras activation by ceramide

N17 Ras overexpression has been demonstrated to inhibit ceramide-induced activation of JNK, p38HOG and apoptosis in Jurkat cells (Brenner *et al.*, 1997, Basu *et al.*, 1998). This suggests that Ras activation by ceramide may be conserved in Jurkat cells. Vav is a GEF for Rho family proteins that is expressed primarily in hematopoietic cells (Adams *et al.*, 1994, Han *et al.*, 1998). However, Vav precipitated from Jurkat cells and Vav prepared by *in vitro* translation can act as a GEF for Ras *in vitro* (Gulbins *et al.*, 1993, 1994). Furthermore, the addition of ceramide increases the *in vitro* Ras GEF activity of Vav. Thus, Vav may be a ceramide-dependent Ras GEF in Jurkat cells. Studies should be performed to determine if ceramide can stimulate Ras activation in Jurkat and possibly, other T-cell populations. In rat2 cells, the activation of Ras in response to cell-permeable ceramide was modest compared to the levels of Ras activation typically observed from

peptide growth factor stimulation. Ras activation in hematopoietic cells may be more profound. A cell type showing higher amounts of ceramide-induced Ras activation would be more ideal for studying the mechanism of ceramide signaling.

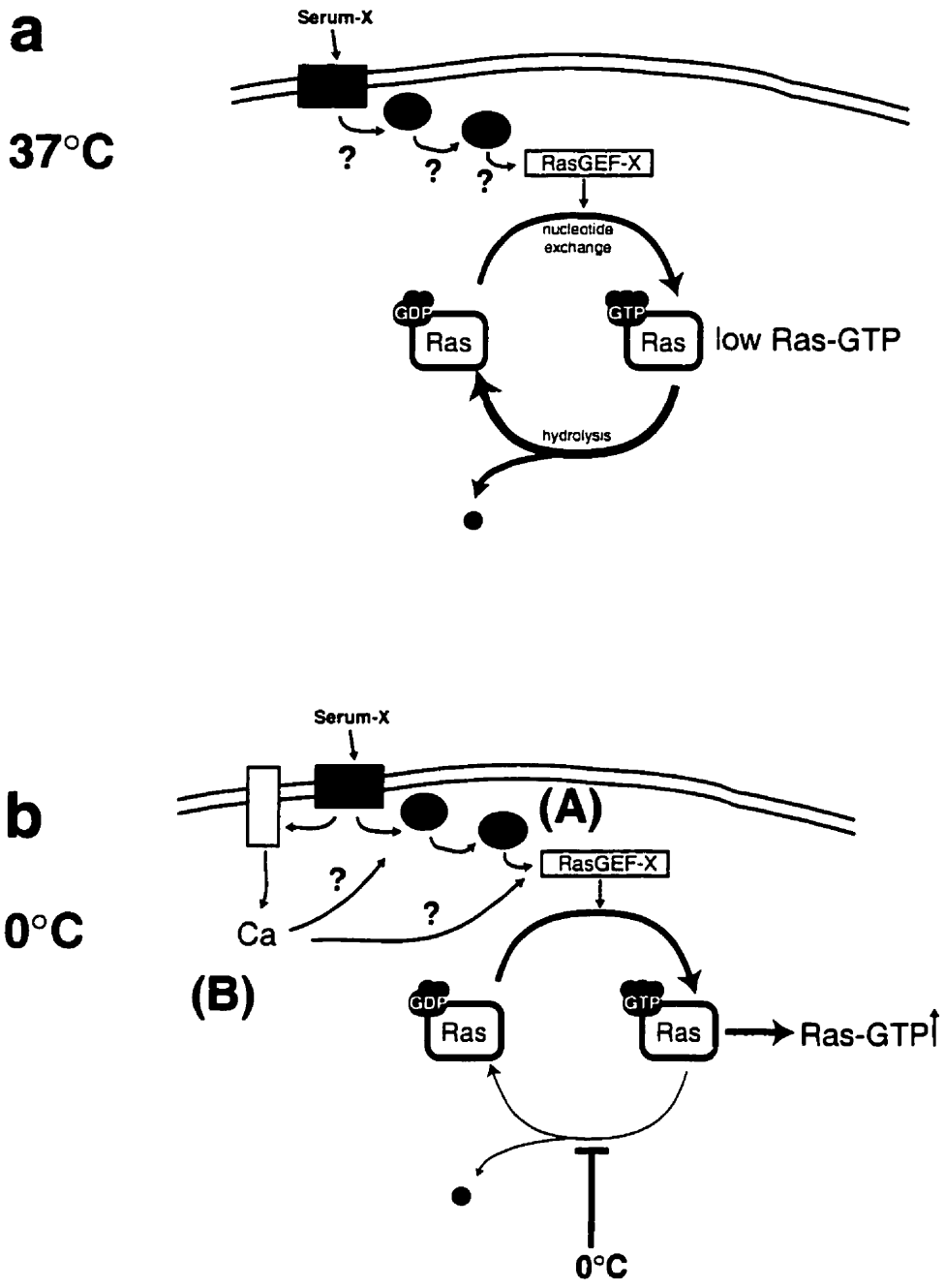


Figure 6-1. Model for *in vivo* Ras activation in hypothermic cells. a. In resting cells at 37°C, the level Ras-GTP is low because the rate of basal GTP hydrolysis exceeds the rate of basal nucleotide exchange. The basal nucleotide exchange activity is catalysed by an unknown RasGEF-X that is serum dependent. The factor in serum that stimulates basal nucleotide exchange is unknown. **b.** Under hypothermic conditions (0°C), basal GTP hydrolysis is inhibited. Because the serum-dependent basal exchange activity is temperature-independent, an imbalance ensues and Ras-GTP slowly accumulates. Serum factor X could regulate RasGEF-X through a calcium independent mechanism (A). Alternatively, serum factor X could stimulate a basal calcium signal through a plasma membrane channel (B). The low amplitude calcium signal could regulate RasGEF-X directly or through RasGEF-X interacting proteins. Pathway A in this schematic corresponds to scenarios 2 and 3 in the text. Pathway B corresponds to scenario 1 in text.

References

Adams, J.M., Houston, H., Allen, J., Lints, T., and Harvey, R. (1992) The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. *Oncogene.* **7**:611-618.

Ali, I.S., Gandhi, M., Finegan, B.A., Koshal, A., Clanachan, A.S. (1998) Cardioprotection by activation of NO/cGMP pathway cardioplegic arrest and 8-hour storage. *Ann. Thorac. Surg.* **65**:1303-1309.

Basu, S., Bayoumy, S., Zhang, Y, Lozano, J., and Kolesnick, R. (1998) BAD enables ceramide to signal apoptosis via Ras and Raf-1. *J. Biol. Chem.* **273**:30419-30426.

Belzer, F.O., and Southard, J.H. (1988) Principles of solid-organ preservation by cold storage. *Transplantation* **45**:673-676.

Bottorff, D., Ebinu, J., and Stone, J.C. (1999) RasGRP, a Ras activator: moue and human cDNA sequences and chromosomal positions. *Mammalian Genome* **10**:358-361.

Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., and Gulbins, E. (1997) Fas-or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J. Biol. Chem.* **272**:22173-22181.

Eberl, T., Steinlechner, R., Hengster, P., Herold, M., Schrocksnadel, H., Salvenmoser, W., Rhomberg, M., Gnaiger, E. and Margreiter, R. (1996) Assessment of endothelial preservation in human cell cultures. *Ann. Thorac. Surg.* **62**:526-532.

Ebinu, J.O., Bottorff, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with Ca^{2+}- and diacylglycerol-binding motifs. *Science.* **280**:1082-1086.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**:806-811.

Gulbins, E., Coggeshall, M.K., Baier, G., Katzav, S., Burn, P., and Altman, A. (1993) Tyrosine kinase-stimulated guanine nucleotide exchange activity of Vav in T-cell activation. *Science.* **260**:822-825.

Gulbins, E., Coggeshall, M., Baier, G., Telford, D., Langlet, C., Baier-Bitterlich, G., Bonnefoy-Berard, N., Burn, P., Wittinghofer, A., and Altman, A. (1994) Direct stimulation of Vav guaning nucleotide exchange activity for Ras by phorbol esters and diglycerides. *Mol. Cell. Biol.* **14**:4749-4758.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A., and Broek, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of *rac*-related guanosine triphosphatases by *vav*. *Science* **279**:558-560.

Kawasaki, H., Springett, G.M., Toki, S., Canales, J.J., Harlan, P., Blumenstiel, J.P., Chen, E.J., Bany, I.A., Mochizuki, N., Ashbacher, A., *et al.*, (1998) A Rap nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Nat. Acad. Sci.* **95**:13278-13283.

Kane, M.D., Yang, C.-W., Gunasekar, P.G., and Isom, G.E. (1998b) Trimethyltin stimulates protein kinase C translocation through receptor-mediated phospholipase C activation in PC12 cells. *J. Neurochem.* **70**:509-514.

Liu, B., Wang, L.C.H., and Belke, D.D. (1991) Effect of low temperature on the cytosolic free Ca^{2+} in rat ventricular myocytes. *Cell Calcium* **12**:11-18.

Marais, R., Wynne, J. and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**:381-393.

Marshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**:179-185.

Mcanulty, J.F., Ametani, M.S., Southard, J.H., and Belzer, F.O. (1996) Effect of hypothermia on intracellular Ca^{2+} in rabbit renal tubules suspended in UW-gluconate preservation solution. *Cryobiology* **33**:196-204.

Montgomery, M.K., Xu, S., and Fire, A. (1998) RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditus elegans. *Proc. Nat. Acad. Sci.* **95**:15502-15507.

Perotti, M., Toddei, F., Mirabelli, F., Vairetti, M., Bellomo, G., McConkey, D.J., and Orrenius, S. (1990) Calcium-dependent DNA fragmentation in human synovial cells exposed to cold shock. *FEBS Lett.* **259**:331-334.

Price, M.A., Rogers, A.E. and Treisman, R. (1995) Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *EMBO J.* **14**:2589-2601.

Separovic, D., Kester, M., Haxhiu, M.A., and Ernsberger, P. (1997) Activation of phosphatidylcholine-selective phospholipase C by I1-imidazoline receptors in PC12 cells and rostral ventrolateral medulla. *Brain Res.* **749**:335-339.

Shafer, T.J., and Atchison, W.D. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *NeuroToxicology.* **12**:473-492.

Stang, S., Bottorff, D. and Stone, J.C. (1997) Interaction of activated Ras with Raf-1 alone may be sufficient for transformation of rat2 cells. *Mol. Cell. Biol.* **17**:3047-3055.

Thomas, S.M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J.S. (1992) Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**:1031-1040.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. *Biochem. J.* **288**:351-355.

Weber, J.D., Raben, D.M., Phillips, P.J. and Baldassare, J.J. (1997) Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G_1 phase. *Biochem. J.* **326:**61-68.

Zohn, I.M., Campbell, S.L., Khosravi-Far, R., Rossman, K.L. and Der, C.J. (1998) Rho family proteins and Ras transformation: the RHOad less traveled gets congested. *Oncogene* **17:**1415-1438.