REGULATION OF THE RAS EXCHANGE FACTOR RAS-GRF2

by

Carmen Lenore de Hoog

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Molecular and Medical Genetics, University of Toronto

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ABSTRACT

The Ras GTPases play a pivotal role in cellular proliferation and differentiation and become activated in response to a variety of extracellular signals, including growth and differentiation factors, hormones, immune antigens and calcium influx. Ras requires the action of guanine nucleotide exchange factors (GEFs) in order to become GTP-bound, enabling it to transmit a signal to downstream signaling components. Ras-GRF2 (GRF2) is a calcium-responsive Ras exchange factor which contains many recognizable protein domains, including an ilimaquinone (IQ) motif and a destruction box (DB). The IQ motif of a homologous protein, Ras-GRF1, binds calmodulin (CaM) and it was postulated that this binding activated GRF1. I demonstrated that the role of calcium and CaM in GRF2 function is not the same as that proposed for GRF1. GRF2 does indeed bind calmodulin but the IQ motif is not the sole determinant of CaM binding, and mutant proteins that cannot bind CaM are still calcium-responsive. Interestingly, an IQ-deleted protein can activate Ras but cannot activate the effector molecular Raf. I postulate that exchange factors may play a role in determining effector interactions and therefore play a larger role than previously thought in Ras signaling. The varied responses to different extracellular signals that all activate Ras could be a result of the individual GEF that was activated upstream of Ras.
Because the DB is a motif that targets proteins for ubiquitin-mediated proteolysis, I examined the proteolysis of GRF2. I demonstrated that activated GRF2 is an unstable protein and that binding to Ras targets GRF2 for destruction. Deletion of the DB protects GRF2 from Ras-triggered degradation. I established that GRF2 is ubiquitinated \textit{in vivo} and that it becomes ubiquitinated only upon binding to Ras; downstream signaling is not required for this degradation, suggesting it is merely binding that triggers this degradation. Point mutants that cannot bind Ras are not ubiquitinated. GRF2 accumulates in a highly ubiquitinated state in the presence of proteasome inhibitors. I have reported the first evidence of \textit{in vivo} ubiquitination of a mammalian exchange factor and have provided a mechanism as to how the protein is targeted for proteolysis.
ACKNOWLEDGMENTS

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I wish to thank my parents, my brother and the rest of my family for the warmth and love that they show me every single day. I have only succeeded because of you. (I’m finally finished school, Mom, so a “real job” can’t be far away!)

And finally, I want to thank you, Leonard, for making me happy.
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LIST OF ABBREVIATIONS

AEBSF: 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride
Ab: antibody
AMP: adenosine monophosphate
ADP: adenosine diphosphate
Amp: ampicillin
APC: Anaphase Promoting Complex
ARF: ADP-ribosylation factor
ATP: adenosine triphosphate
BES: N-N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BLB: Bacterial Lysis Buffer
Boss: bride of sevenless
BSA: bovine serum albumin
C-: carboxyl-
Ca\(^{2+}\): calcium
CaM: calmodulin
CC: coiled-coil
cDNA: complementary deoxyribonucleic acid
CDK: cyclin dependent kinase
CNrasGEF: cyclic nucleotide ras guanine nucleotide exchange factor
CRI: conserved region 1
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DB</td>
<td>destruction box</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycholate</td>
</tr>
<tr>
<td>Drk</td>
<td>downstream of receptor kinase</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GDS</td>
<td>guanine nucleotide dissociation stimulator</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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GRF: guanine nucleotide releasing factor
GRP: guanine nucleotide releasing protein
GSH: glutathione
GST: glutathione S-transferase
GTP: guanosine triphosphate
GTPase: guanosine triphosphatase
GTPγS: guanosine 5'-O-(3-thiotriphosphate)
HA: hemagglutinin
HBS: HEPES-buffered saline
HECT: Homology to E6-AP C-terminus
HEK: human embryonic kidney
HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Hh: hedgehog
Ig: immunoglobulin
IgG: immunoglobulin G
IP: immunoprecipitation
IPTG: isopropylthio-β-D-galactoside
IQ: ilimaquinone
kb: kilobase
JNK: c-Jun N-terminal kinase
LLnL: N-acetyl-Leu-Leu-Norleucinal
LPA: lysophosphatidic acid
MAPK: mitogen-activated protein kinase
MBP: myelin basic protein
MG132: N-carbobenzoxy-L-Leu-Leu-Leucinal
MLB: Magnesium Lysis Buffer
MOPS: 3-(N-morpholino)propane-sulfonic acid
MS: mass spectrometer
m/z: mass to charge ratio
N-: amino-
NEB: Nucleotide Exchange Buffer
neoR: Neomycin-resistant
NF: nucleotide-free
NF1: Neurofibromatosis type 1
NP40: Nonidet P-40
nt: nucleotide
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PBST: phosphate-buffered saline with Tween
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
PDGFR: platelet-derived growth factor receptor
PEST: proline, glutamate, serine, threonine
PH: pleckstrin homology
Phyl: phyllopod
PI3K: phosphatidylinositol 3'-OH kinase
PKA: protein kinase A
PKC: protein kinase C
PS: phosphatidylserine
RA: Ras association
RACK1: Receptor for activated C-kinase 1
REM: Ras Exchanger Motif
RBD: Ras-binding domain
RTK: receptor tyrosine kinase
SAPK: stress-activated protein kinase
SCF: Skp1-cullin-F-box
SDS: sodium dodecyl sulphate
sev: sevenless
SH2/3: Src-homology 2 or 3
Sina: seven-in-absentia
Slimb: supernumary limbs
Sos: Son-of-sevenless
SRE: serum response element
SRF: serum response factor
TB: Terrific Broth
TBS: Tris-buffered saline
TCF: ternary complex factor
Tet: tetracycline
TPCK: N-tosyl-L-phenylalanine chloromethyl ketone
Tris: Tris(hydroxymethyl)aminomethane
Ttk: tramtrack
Tx-100: Triton X-100
Ub: ubiquitin
Ubp: ubiquitin-specific processing protease
w/v: weight/volume
WT: wild-type
CHAPTER 1

Introduction
RAS SIGNALING

General introduction to Ras signaling

The ras proto-oncogenes, H-, K- and N-ras, encode small, 21 kDa membrane-bound guanosine triphosphatases (GTPases) that play critical roles in various aspects of cellular regulation. The p21 Ras proteins are central players in the growth and differentiation of cells, evidenced by the fact that the H- and K-ras genes were originally identified as oncogenes of the acutely transforming Harvey and Kirsten sarcoma viruses (10). Later, it was recognized that these retroviral oncogenes were derived from normal cellular genes (126, 172, 202) and that they had been activated by point mutations affecting critical amino acids at position 12 and 59 (181, 216, 252). The ras genes are conserved throughout evolution and are present in all eukaryotes, including mammals, Drosophila, slime molds, nematodes and yeast (10). The importance of Ras regulation in cellular growth is supported by the finding that approximately 30% of human cancers contain oncogenic mutations in Ras at codons 12, 13 or 61 that cause the protein to be constitutively activated. In fact, in some cancer types, such as pancreatic cancer, the prevalence of Ras mutations is approximately 90% (18).

Ras functions downstream of a variety of extracellular signals that elicit mitogenic or cell differentiation responses. Signals leading to transient Ras activation include various hormones, growth and differentiation factors, immune antigens, extracellular matrix interactions and calcium influx (15, 134, 179). These signals activate one of numerous receptor systems, including receptor tyrosine kinases, G-protein coupled receptors, and integrins (50, 144, 229, 234).
The Ras protein acts as an intracellular switch, biologically inactive when bound to GDP and activated by the exchange of GDP for GTP (Fig. 1-1). These two forms of Ras are not only functionally different but structurally different as well (164). The capacity of Ras to act as a switch is a result of its ability to interact with upstream activators in its GDP-bound state and to transmit signals through a number of downstream effector pathways when in its GTP-bound conformation.

**Figure 1-1  Ras as a binary switch.**

The Ras protein acts as a binary switch, interacting with upstream activators when bound to GDP and with downstream effectors when bound to GTP. The activation of Ras occurs in response to various extracellular signals through the actions of guanine nucleotide exchange factors (GEFs), and activated Ras can interact with various downstream components to activate them, leading to a variety of outcomes. Ras is ultimately inactivated by GTPase activating proteins (GAPs).
Regulation of Ras

Ras binds guanine nucleotides with high affinity ($K_d \approx 10^{-11}$ M) which results in an intrinsic (spontaneous) rate of dissociation that is very low ($\sim 10^{-5}$ moles per second dissociating per mole of complex). Ras also possesses only weak intrinsic GTPase activity (88) with a rate of hydrolysis that gives Ras-GTP a half-life of at least 1-5 hours (134). In quiescent cells, more than 95% of Ras proteins are associated with GDP. These properties of Ras result in a requirement for specific guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) to modulate Ras in vivo. The concerted action of these two classes of proteins control the oscillation of Ras between GDP-bound and GTP-bound conformations (59) (Fig. 1-1).

GAPs bind to Ras-GTP and stimulate its weak GTPase activity, converting the protein to the inactive, GDP-bound state. Members of this family include p120 Ras-GAP (225) and neurofibromatosis type 1 (NF-1) (8, 141, 245); mutations in these proteins have been associated with a high frequency of tumour formation. Structural evidence suggests that p120 Ras-GAP contributes an essential arginine to stabilize the transition state formed during nucleotide hydrolysis (2, 195).

On the other hand, GEFs interact with Ras in its GDP-bound state, stimulate release of GDP and stabilize the nucleotide-free form of Ras, thus allowing for subsequent GTP binding. The release of GDP is the rate-limiting step in this reaction, and a nucleotide-free protein rapidly binds GTP, which is much more abundant than GDP within the cell (15, 71, 134). GEFs function to stabilize the very unstable nucleotide-free form of the GTPase and therefore have a high affinity for the nucleotide free form of the GTPase (91, 127). Members of the Ras GEF family include Cdc25p and Sdc25p in Saccharomyces cerevisiae (21, 23, 49).
104), the son-of-sevenless gene product (Sos) (20, 37, 205), the guanine nucleotide releasing factors, Ras-GRF1 (32, 140, 203, 240) and Ras-GRF2 (40, 66), the guanine nucleotide releasing protein RasGRP (62, 222), and the cyclic nucleotide ras GEF (CNrasGEF) (176).

The work described in this thesis relates exclusively to the characterization of Ras-GRF2 (GRF2). Given that constitutively active forms of Ras can lead to malignant transformation of numerous cell types, understanding the cellular processes which regulate normal Ras function will greatly enhance our understanding of oncogenesis.

The Ras proteins are expressed ubiquitously throughout all tissues but are coupled to different extracellular signals and downstream effects in different cell types and organisms (134). Ras regulation can be achieved by tissue-specific exchange factors and GTPase activating proteins which themselves are coupled to different upstream signals. Tissue-specific expression of GEFs, GAPs and effector molecules would enable tissue-specific responses to general extracellular signals that activate Ras. The regulation of Ras-GTP levels, and consequently its varied downstream effects, can therefore be controlled by regulating the expression and/or activity of GAPs and GEFs. Despite the important role of Ras in cellular regulation, little is known about how GEFs regulate its activity.

Members of the Ras superfamily and their functions

In 1980, two ras genes were known: the first, known as v-H-ras, is the transforming gene of Harvey, BALB and Rasheed sarcoma viruses, and the second, v-K-ras, is the transforming gene of Kirsten sarcoma virus (10). By 1990, a large number of distinct Ras-like proteins had been identified in many diverse organisms.

p21 Ras serves as the prototype of a superfamily of Ras-related GTPases which contains over 60 members. These have been classified into subfamilies based on their
sequences; these subfamilies include the Ras, Rho, Rab, Ran, Rad and ARF families (Table 1-1). The main branches of the Ras superfamily include the Ras, Rho and Rab families. The Ras family includes proteins that share the strongest homology (50-55% amino acid identity) with H-, K- and N-Ras. Members of the other branches of the Ras superfamily share between 30-35% amino acid identity with Ras.

The Ras subfamily includes four ‘true’ Ras proteins (H-Ras, N-Ras, K-RasA and K-RasB), members of the Rap GTPases (Rap1A, 1B, 2A and 2B), as well as R-Ras, RalA, RalB and TC21. Rap1A, also known as k-Rev1, is 53% identical to H-Ras. Rap1A was identified by its ability to reverse cellular transformation induced by K-Ras, and may function by competing with Ras proteins for interactions with their effectors (17). Both R-Ras and TC21 are 55% identical to H-Ras, and 70% to one another. Mutant forms of R-Ras have been shown to cause malignant transformation of NIH3T3 cells but do not produce a striking morphological change in cells, suggesting that R-Ras activates different molecules than H-Ras (47). The gene encoding TC21 was isolated from a human teratocarcinoma library. Activated mutants of TC21 were found to exhibit the same transforming activity as oncogenic Ras and apparently activate the same downstream signaling pathways (35, 82).

Rho GTPases (e.g. RhoA, RhoB, Rac1, Rac2 and Cdc42Hs) regulate many important processes in all eukaryotic cells, including organization of the actin cytoskeleton, oxidase regulation, cell cycle progression, membrane trafficking and gene transcription downstream of two MAPK cascades known as stress-activated protein kinase (SAPK, also known as c-Jun N-terminal kinase (JNK)) and p38 kinase (14, 121). Their activity is regulated by signals originating from different classes of surface receptors including G-protein-coupled receptors, tyrosine kinase receptors, cytokine receptors, and adhesion receptors (121).
The Rab GTPases regulate vesicular transport in endocytosis and exocytosis, as well as transport within the biosynthetic/secretory pathways (38). To date, ~40 different Rab proteins have been identified in mammalian cells, each of which may interact specifically with one type of membrane or vesicle (210).

The major role of the very abundant Ran proteins is in nucleocytoplasmic transport of proteins and RNA through the nuclear pore complex; several observations have also implicated Ran in regulation of the onset of mitosis (191).

Rad, Gem/Kir, mRem and Ges are members of a newly emerged subfamily of GTPases within the Ras superfamily. Their function at this point appears largely unknown; however, Ges has been shown to play a role in linking extracellular signals to cytoskeleton/morphological changes in endothelial cells (167).

Members of the ARF (ADP-ribosylation factor) subfamily regulate membrane traffic and organelle structure in eukaryotic cells (57). Class I ARFs are involved in trafficking in the ER-Golgi and endosomal systems; virtually nothing is known about class II ARFs whereas class III ARFs function exclusively in the endosomal-plasma membrane system (57). While it seems that Rabs and ARFs play a similar role, they actually are quite distinct: ARFs allow vesicles to bud off the donor membrane with its specific set of cargo molecules, while Rabs ensure the vesicle is targeted to the appropriate acceptor membrane (38).

Structure/Function of Ras

The three ras genes, H-, K-, and N-ras, have a common structure with a 5' non-coding exon and four coding exons. The introns of the three genes differ widely in size and sequence; therefore, the sizes of the genes are disparate, with the K-ras gene spanning more
than 35 kb, while H-ras spans only ~3 kb (134). There is a sequence of ~235 bp found in the first intron that appears to contribute to promoter activity, as deletion of this sequence results

Table 1-1  Ras superfamily members and their functions.

<table>
<thead>
<tr>
<th>SUBFAMILY</th>
<th>MEMBERS</th>
<th>FUNCTION OF SUBFAMILY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras</td>
<td>H-, K- &amp; N-Ras</td>
<td>Cellular proliferation, differentiation, transformation</td>
</tr>
<tr>
<td></td>
<td>R-Ras, M-Ras</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rin, Rit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RalA, RalB</td>
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<tr>
<td></td>
<td>Rap1A, Rap1B</td>
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<td></td>
<td>Rap2A, Rap2B</td>
<td></td>
</tr>
<tr>
<td>Rho</td>
<td>RhoA, RhoB, RhoC</td>
<td>Actin cytoskeletal organization, oxidase regulation, cell motility, cytokinesis</td>
</tr>
<tr>
<td></td>
<td>Rac1, Rac2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cdc42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RhoE, RhoG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC10</td>
<td></td>
</tr>
<tr>
<td>Rab</td>
<td>Rab4, Rab5, Rab17, Rab18, Rab20, Rab25, etc...</td>
<td>Regulation of vesicular transport in endocytosis and exocytosis; control of vesicle docking and fusion</td>
</tr>
<tr>
<td></td>
<td>(over 40 members)</td>
<td></td>
</tr>
<tr>
<td>Rad</td>
<td>Rad</td>
<td>??????</td>
</tr>
<tr>
<td></td>
<td>Gem/Kir</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mRem (RGK)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ges</td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>ARF1, ARF2, ARF3</td>
<td>Trafficking in the ER-Golgi and endosomal systems</td>
</tr>
<tr>
<td></td>
<td>ARF4, ARF5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARF6</td>
<td></td>
</tr>
<tr>
<td>Ran</td>
<td>Ran</td>
<td>nucleocytoplasmic transport of proteins and mRNA</td>
</tr>
</tbody>
</table>
in a 3-10 fold decrease in transcription (134). Two elements at the 3' end of H-ras may also contribute to its expression: a tandem repeat sequence downstream of the polyadenylation signal exhibits a weak enhancer activity and the last exon contains sequences which, when mutated, can result in a 10-fold higher level of p21 protein (43, 134). Transcriptional regulation of ras genes seems to occur as evidenced by the tissue-specific distribution of expression and by the several-fold increase in transcript levels in regenerating liver cells (134); the mechanism of this is poorly understood as the effect of serum and growth factors is delayed, suggesting an indirect effect (134). The K-ras gene encodes two p21 proteins, K-RasA and K-RasB, as a consequence of alternate fourth exon utilization (134).

The four Ras proteins, H-Ras, K-RasA, K-RasB, and N-Ras, share 98% amino acid identity in the residues between 5 and 120. There is more divergence at the C-terminus where they show only 62% amino acid identity. Comparison of all four proteins shows that in their first 164 amino acids they are strikingly homologous, with the 86 N-terminal residues being identical and 79% of the next 78 residues being identical. However, the last 25 amino acids of the proteins are divergent, except for an invariant cysteine residue at residue 186, four residues from the C-terminus (10). There appears to be strong selective pressure on this divergent region, as it is highly conserved in each protein between mouse and human, suggesting that this region may be associated with distinct functions in each protein (10).

The cysteine at position 186 is required for proper post-translational modification of Ras which is extensively modified at its C-terminus. Cys186 is first modified by the isoprenoid farnesyl; next, the three amino acids C-terminal to Cys186 are proteolytically cleaved and the carboxy-terminal group of Cys186 is methylated. The outcome of these two events is a more hydrophobic protein with higher affinity for membranes which results in the
association of Ras with the inner face of the plasma membrane (79). Finally, cysteine residues upstream of the farnesylated cysteine in the H-Ras, N-Ras and K-RasA proteins become reversibly palmitoylated (134). These modifications are required for full activity of the Ras proteins, as activated Ras proteins lose their transforming ability when additional mutations are introduced that inhibit these modifications (79, 134).

Three conserved sequences important for nucleotide interaction have been described. The first, GXXGXGKS (amino acids 10-17) is involved in binding to the α- and β-phosphates of GDP and GTP; in the second, DXXG (amino acids 57-60), the aspartate binds the Mg²⁺ ion and the glycine binds the γ-phosphate of GTP; and the third, NKXD (residues 116-119) is important for binding the guanine ring and therefore is called the guanine specificity region (19). Single point mutations of these residues have been found to increase the dissociation rate constant, thereby lowering GTP binding by 1-3 orders of magnitude (235).

Oncogenic Ras is associated with permutations in amino acids that are important for binding guanine nucleotides. The single amino acid substitutions at positions 12, 13 or 61 that unmask the transforming potential of Ras create mutant proteins that display a reduced intrinsic GTPase activity and an unresponsiveness to GAPs (18). Consequently, these oncogenic Ras mutants are locked in the active, GTP-bound state which leads to constitutive, deregulated activation of Ras function. The viral Ras proteins display amino acid substitutions at positions 12 (134). The activation of Ras that results from mutations at residues 116, 119 or 146 is associated with a substantial increase in the intrinsic nucleotide exchange rate as a result of an overall lower affinity for nucleotide (177).
Ras proteins containing a substitution of Asn for Ser at residue 17 are potent inhibitors of cell proliferation (73, 212). Ser17 is important for the binding of Mg2+ associated with bound nucleotide (148, 164, 165). The growth-inhibitory property of N17 Ras is a consequence of improper Mg2+ coordination which locks the mutant Ras protein in a constitutively inactive state (69). N17 Ras exhibits a reduced affinity for nucleotide (Kd for GTP ≈ 250 nM), thus guanine nucleotides are less likely to displace GEFs from this mutant in cells (72). However, this mutant fails to activate downstream effectors even when bound to GTP (69). Therefore, sufficient over-expression of N17 Ras in cells prevents activation of endogenous Ras by binding more tightly to Ras-specific exchange factors than does normal Ras and sequestering them in “dead-end” complexes.

There are two regions within the Ras proteins that differ in conformation between their GDP- and GTP-bound states; these are referred to as Switch 1 and Switch II. The Switch 1 region is comprised of residues 30-38 which constitute part of the L2 loop and part of the β2 β strand; the Switch 2 region is composed of residues 60-76 and is highly mobile, existing in multiple conformations (148, 164, 165). Extensive analysis of residues within the Switch 1 region has shown that certain mutations destroy the ability of oncogenic Ras to signal but do not compromise GTP binding or membrane association (138). These mutations, referred to as effector mutations, define key residues that are required for Ras-effector interaction and signaling.

Effectors of Ras

In order to determine if a molecule is a bona fide effector of Ras, the putative effector should meet several criteria. First, the protein should interact with Ras only in its GTP-
bound state rather than to GDP-bound Ras, and it should preferably bind to the core effector domain of Ras (residues 32-40 as determined by genetic analysis). Therefore, if there is little differential binding between inactive and active Ras, the protein is unlikely to be an effector.

Second, the interaction between Ras and the putative effector should result in the activation of the effector molecule or produce an effect on downstream targets. Finally, a functional knockout of the putative effector should abolish part of Ras-mediated signaling. The best indication of a true Ras effector is a demonstration in vitro of GTP-dependent activation of the effector by Ras; however, this will not be able to be demonstrated for those proteins for whom membrane recruitment of the effector is the only function of Ras. Various effector molecules have been identified that are activated downstream of Ras. These effector molecules include Raf (228, 233, 256), p120 GAP (147), NF-1 (141), phosphatidylinositol-3'-OH kinase (PI3K) (186), RalGDS (98, 211), and the zeta isoform of protein kinase C (PKCζ) (55).

PI3K is a lipid kinase that phosphorylates phosphoinositides at the 3' position of the inositol ring; it is composed of a p110 catalytic and a p85 regulatory subunit which are both members of a family of related proteins (31). Five isoforms of each type of subunit have been identified with the catalytic subunits differing in their substrate specificity and regulation (30). Multiple isoforms of PI3K can associate with Ras and Ras can activate both wortmannin-sensitive and -insensitive isoforms (187). Recombinant p110 (α and β) displays a high affinity interaction with GTP-bound Ras through the Ras effector domain (186, 188). In intact cells, activated Ras can stimulate PI3K activity and is required for optimal activation of PI3K in response to growth factor stimulation (122, 186). Moreover, dominant-negative Ras inhibits PI3K activation in response to platelet-derived growth factor (PDGF)
and activated Ras results in a significant induction of inositol phosphates (188). In total, these data strongly suggest the involvement of PI3K as a downstream effector of Ras.

The zeta isoform of protein kinase C (PKCζ) has also been implicated as a Ras effector. Firstly, PKCζ is required for Ras-induced maturation in Xenopus oocytes and for serum-stimulated mitogenic signaling in mammalian cells (27). Secondly, the regulatory fragment of PKCζ shows a preferential binding to GTP-bound Ras in vitro and a peptide corresponding to the effector domain of Ras blocked this interaction (55). Furthermore, PDGF stimulation promoted PKCζ association with Ras in vivo, and dominant-negative Ras blocked PDGF-stimulated activation of PKCζ. Taken together, these observations suggest that PKCζ may serve as a positive effector of Ras growth stimulation.

TheRal guanine nucleotide dissociation stimulator (RalGDS) and two closely related proteins (RGL and RGL2/Rlf) represent intriguing candidate effectors of Ras that may link Ras with other Ras-related proteins. The interaction of the RalGDS C-terminal segment with Ras is specific, dependent on activation of Ras by GTP, and blocked by a mutation that affects Ras effector function (98). RalGDS was found to inhibit the binding of Raf to Ras, suggesting that the two proteins share a binding site on Ras, presumably the effector domain (98). The Ras binding domain of RalGDS interacts with H- and K-Ras in two-hybrid assays, and with active but not with inactive point mutants of these GTPases (211). Moreover, using purified proteins, there is a direct GTP-dependent interaction of Ras with RalGDS in vitro. Taken together, these characteristics suggest that RalGDS may also be a Ras effector.

p120 GAP clearly functions as a negative regulator of Ras (15); however, there is also evidence that it serves an additional role as a Ras effector. The first piece of evidence came from studies measuring ionic currents through muscarinic receptor-activated potassium
channels. GAP was found to inhibit channel opening in isolated atrial cell membranes, but only in the presence of GTP-bound Ras suggesting an effector function for GAP (27). Furthermore, the GAP effect requires more than just simple binding to Ras, as the isolated catalytic domain is over 100 fold less effective in blocking channel opening. Ras activation induces germinal vesicle breakdown in *Xenopus* oocytes and this can be inhibited by injecting an antibody directed against the SH3 domain of GAP, suggesting that the SH3 domain of GAP is essential for Ras-mediated events in *Xenopus* oocyte maturation (60).

There exists a large body of evidence, both biochemical and genetic, that implicates the serine/threonine kinase Raf-1 as a critical effector of Ras. In 1993, a series of papers from independent investigations revealed that the kinases of the Raf family bind to Ras (150, 228, 233, 237, 256). These papers demonstrated that the interaction between the two proteins was GTP dependent and that it occurs at the effector-binding region. Dominant-negative Raf-1 mutants can impair Ras-transforming activity and constitutively activated Raf-1 mutants result in a phenotype indistinguishable from that of activated Ras (27). Interaction with Ras promotes a translocation of the normally cytoplasmic Raf protein to the plasma membrane and the Ras-Raf complex may facilitate subsequent events that lead to the activation of Raf's kinase function. These events are quite complex and remain to be fully elucidated. The N-terminus of Raf contains two Ras interaction domains, the Ras-binding domain (RBD) composed of residues 51-131 and the cysteine-rich domain (CRD) comprised of residues 139-184 (151). The CRD has also been demonstrated to bind to phosphatidylserine and addition of phosphatidylserine allows Ras to activate c-Raf-1 in vitro; mutants of CRD that cannot bind phosphatidylserine are defective in Ras-mediated activation (27).
The interaction between Ras and the RBD is a high-affinity interaction \( (K_d = 20 \text{ nM}) \) and this interaction appears to allow for a second interaction between the CRD and Ras (24, 151). When Raf is in an inactive state, members of the 14-3-3 protein family are bound to it through phosphoserine at position 259 and perhaps the CRD; this interaction obscures the CRD and stabilizes the inactive conformation (151). When Ras becomes GTP-loaded, the RBD of Raf binds to Ras, displacing 14-3-3 from the Ser259 site and unmasking the CRD which is now accessible for Ras binding. The binding of the CRD to Ras and phosphatidylserine results in a change in Raf-1 conformation that serves to expose the kinase domain. The 14-3-3 that was displaced from Ser259 is now free to bind to the higher affinity Ser621 site; this interaction may serve to stabilize an "open" Raf-1 conformation (155). This "open" Raf-1 may now be further stimulated by other modifications occurring at the membrane, such as tyrosine phosphorylation of residues 340 and 341 by Src family members. PKC and a ceramide-activated protein have also been reported to activate Raf at the membrane (151).

**ERK kinase cascade**

Raf activation by Ras results in the activation of one of a number of mitogen-activated protein kinase (MAPK) cascades. MAPK pathways are activated in response to a diverse array of stimuli, including growth factors, cytokines, irradiation, changes in osmolarity and other stress factors (241). The first kinase of a MAPK cascade is a MAPK kinase kinase (MAPKKK), a serine/threonine kinase that functions to phosphorylate and activate the next kinase in the cascade, a MAPK kinase (MAPKK). The MAPKKs are dual-specificity kinases that phosphorylate a Thr-X-Tyr motif in MAPKs. Upon activation,
MAPKs phosphorylate and activate numerous substrates, including transcription factors, protein kinases, phospholipases and cytoskeleton-associated proteins (241).

**Figure 1-2** The Raf-MAPK cascade in mammalian cells.
Upon activation of Ras, the Ras binding domain (RBD) of Raf binds to Ras. This interaction displaces the 14-3-3 proteins that are bound to the N-terminus of Raf, exposing the cysteine-rich domain (CRD). CRD binding to Raf and to phosphoserine results in a conformational change in Raf that exposes the kinase domain. This conformation is stabilized by the binding of 14-3-3 proteins to a phosphoserine at position 621 of Raf. Activated Raf phosphorylates MEK, which in turn phosphorylates ERK on tandem threonine and tyrosine residues. ERK phosphorylates a number of substrates, including p90 RSK. ERK can also translocate to the nucleus where it phosphorylates a transcription factor, Elk-1, which drives transcription of proteins involved in growth and/or differentiation.

In mammals, Raf activation leads to the stimulation of a MAPK signaling pathway, comprised of Raf, MEK1 and MEK2, and the extracellular signal-regulated kinases 1 and 2.
(ERK1, ERK2) (Fig. 1-2). MEK1 and 2 directly associate with the C-terminal catalytic domain of Raf and are phosphorylated by Raf (27). Activated MEKs phosphorylate tandem threonine and tyrosine residues (TEY motifs) in ERK, activating it (27). Once activated, the ERKs translocate to the nucleus where they phosphorylate a variety of substrates, including the transcription factor Elk-1 (27). Elk-1 forms a ternary complex with serum response factor (SRF) at the serum response element (SRE), which is present in many promoters such as the c-fos promoter (227). The ERKs have been shown to phosphorylate other substrates, such as p90 RSK serine/threonine kinase, and Sos (27, 45).

**ACTIVATORS OF RAS**

The intrinsic rate at which guanine nucleotides dissociate from purified Ras proteins is very slow, with the half life for the occupancy of the nucleotide binding site estimated to be in the order of one hour under normal ionic conditions in the cell (88).

![GEF-stimulated release of GDP from Ras](image)

**Figure 1-3**  GEF-stimulated release of GDP from Ras.
First, the GEF binds Ras and GDP dissociates from this complex, leaving a high-affinity GEF-Ras complex. Ras then rapidly binds GTP, whereupon the GEF dissociates, resulting in an activated Ras protein.
GEFs are therefore essential because GTP binding to Ras proteins is limited by the slow intrinsic rate of GDP dissociation. As GTP is the predominant guanine nucleotide found in the cytosol, cellular factors that cause GDP release from Ras would result in Ras becoming GTP bound, or activated. The GEF-stimulated release of GDP from Ras occurs in several steps (Fig. 1-3). First, the GEF forms a low-affinity docking complex with GDP-bound Ras. GDP dissociates from this initial complex, which then becomes a high-affinity GEF-small GTPase complex. Ras now rapidly binds GTP, whereupon the GEF dissociates from the complex leaving Ras in an activated state. Thus, GEFs have a dual biochemical activity: they destabilize the strong interaction with GDP and stabilize the nucleotide-free GTPase.

Exchange factors can be grouped into various classes based upon the catalytic domain they contain, and hence, their GTPase specificity. For instance, molecules with a Cdc25 domain activate proteins within the Ras subfamily and proteins containing a Dbl homology (DH) domain activate GTPases within the Rho subfamily; it is possible for an exchange factor to have a dual-specificity, as many Cdc25-domain containing proteins also contain a DH domain. Proteins containing Cdc25 domains can be further categorized: those that contain a small domain called the REM for Ras Exchanger Motif specifically activate Ras. Within these Ras-specific exchange factors, GEFs can be even further subdivided, based on the type of signal to which they respond (Fig. 1-4). The Sos proteins respond to receptor tyrosine kinase-activating signals, the Ras-GRFs respond to calcium, RasGRP responds to Ca\(^{2+}\) and diacylglycerol, and finally CNrasGEF responds to cyclic AMP and cyclic GMP.

Cdc25p, Sdc25p

Functional homologs of the Ras proteins are found in lower eukaryotes and genetic studies of Ras function in those organisms, including *Saccharomyces cerevisiae*, *Drosophila*
melanogaster, and Caenorhabditis elegans, provided the first identification of Ras GEFs. *S. cerevisiae* RAS is required for activation of adenylate cyclase and for maintaining cell viability. The product of the *CDC25* gene in *Saccharomyces cerevisiae* was the first proposed GDP/GTP exchange factor of Ras proteins, regulating adenylate cyclase activity via the RAS proteins (23, 185). The cell division cycle mutant *cdc25-1* arrests at the restrictive temperature as unbudded cells with a G1 DNA content; the cells also show a growth lesion which resembles that seen in nutrient-deprived cells (139). Broek et al. cloned and sequenced *CDC25*, revealing an open reading frame encoding a protein of 1589 amino acids. The essential function of *CDC25* was shown to be bypassed by mutations in *RAS2* that stabilize or promote the GTP-bound form of the GTPase (23, 185), leading the authors to suggest that the major cellular function of *CDC25* is to activate normal Ras proteins. The protein product of the *CDC25* gene, Cdc25p, was shown to promote nucleotide exchange on Ras (104) and to bind specifically to catalytically inactive Ras proteins (154).

Another Ras GEF in budding yeast, *SDC25*, shares strong identity within the Cdc25 domain but is not an essential gene in budding yeast. It has been shown to promote mammalian Ras activity when expressed in murine fibroblasts (49, 182). Both *CDC25* and *SDC25* are regulated in response to extracellular influences such as the availability of nitrogen and carbon sources. *CDC25* is essential for growth on glucose, while *SDC25* is not transcribed under these conditions; *SDC25* begins to be transcribed in stationary phase, when nutrients become limited and is also expressed during growth on non-fermentable carbon sources such as ethanol, glycerol and acetate (21). Both Cdc25p and Sdc25p activate Ras but probably in different periods of cell life and possibly under different controls (21).
The Cdc25 protein contains an SH3 domain and a destruction box at its N-terminus and a Cdc25 catalytic domain at its C-terminus; the rest of the protein contains no homology.

**Figure 1-4**  Schematic of the various classes of Ras-specific exchange factors.

Schematic depiction of the domain structures of the Ras exchange factors. All of these GEFs contain the Ras Exchanger Motif (REM) and the catalytic Cdc25 domain. *Saccharomyces cerevisiae* Cdc25p also contains a Src homology 3 (SH3) domain and a cyclin destruction box (DB). Sos contains a tandem pleckstrin homology (PH) and Dbl homology (DH) domain, along with a polyproline-rich region that can bind SH3 domains of proteins such as Grb2. RasGRP contains Ca^{2+} and diacyl-glycerol binding sites. CNrasGEF possesses a PDZ domain, cNMP-binding motifs, a Ras association (RA) domain, a SAV motif which can bind PDZ domains and a PY motif which is known to bind a WW domain in another protein. Both GRF1 and GRF2 possess an N-terminal PH domain, a coiled-coil motif, an ilimaquinone (IQ) motif, and tandem DH/PH domains. GRF2 also contains a destruction box.
to any known proteins (see Fig. 1-4). Cdc25p is tightly bound to a membrane fraction even after treatment with reagents able to release peripheral membrane proteins and this membrane localization is not dependent upon the SH3 domain or upon the presence of RAS proteins or Ira2p (a Ras GAP) (78). The membrane anchoring region was localized to a hydrophobic stretch within the Cdc25 domain (78). However, phosphorylation of Cdc25p in response to glucose results in a redistribution to the cytosol (85). Freeman et al. have reported an interaction between adenylyl cyclase associated protein and the SH3 domain of Cdc25p (76). Therefore, yeast Ras activation is not controlled by transient membrane recruitment of its activator but by other mechanisms not yet determined, whereas it seems that relocalization of the GEF plays a role in down-regulation of the signal transduction pathway.

The C-terminal part of Cdc25p has also been shown to oligomerize, forming homodimers as well as heterodimers with Sdc25p, through two-hybrid interactions and co-immunoprecipitation experiments with differentially epitope-tagged proteins; the association could be elicited by a 100 residue peptide located within the Cdc25 domain (28). The 180 kDa Cdc25p from yeast extracts elutes from a gel filtration column with an apparent molecular mass of 500 kDa, indicating that the Cdc25p belongs to a multimolecular complex (28).

**Sos, Son-of-sevenless**

The identification of other mammalian Ras GEFs was prompted by genetic studies of Ras signaling in *Drosophila melanogaster* and *Caenorhabditis elegans*. In *Drosophila*, studies have shown that formation of the R7 photoreceptor cell during compound eye development requires a pathway involving Ras and the Sevenless receptor protein tyrosine
kinase (90). R7 differentiation is initiated by an interaction between the transmembrane Sevenless expressed in the R7 precursor cell and the Bride-of-sevenless (Boss) ligand expressed exclusively in the neighbouring R8 cell (238). Further genetic studies looking for mutations that affect signaling through the sevenless receptor identified a gene product, son-of-sevenless (Sos), that was downstream of Sevenless but upstream of Ras (205). The protein product of this gene was found to encode a protein that is homologous to the S. cerevisiae Cdc25 protein. Unlike Cdc25p, Sos is a cytosolic protein which must be recruited to the plasma membrane in order to activate Ras. A physical link between Sos and Sevenless is provided by Downstream of receptor kinase (Drk), an adapter protein which binds the autophosphorylated Sevenless receptor as well as the C-terminal proline-rich motifs in Sos through its SH2 and SH3 domains, respectively (163, 206). The consequence of Drk binding to the activated receptor and to Sos is a translocation of cytosolic Sos to the membrane where it is now able to activate Ras.

In Caenorhabditis elegans, LET-60 encodes a Ras homolog that has essential roles in several developmental pathways (110). Animals with mutations in LET-60 exhibit a range of phenotypes including larval lethality, sterility, irregular male spicule development, abnormal hermaphrodite vulval development, and mis-specification of a cell fate in the posterior ectoderm (110). Much of the research done on the Ras signaling in C. elegans has involved the vulval development pathway. The wildtype vulva is derived from three of six multipotential vulval precursor cells in response to an inductive signal from the anchor cell in the somatic gonad (110). The inductive signal of the vulval development pathway is LIN-3, a growth factor produced by the anchor cell. LIN-3 activates LET-23, a transmembrane receptor tyrosine kinase EGF receptor homolog in the vulval precursor cells (5, 95). LET-23
activation initiates a signaling cascade that involves SEM-5, an adapter protein that interacts with LET-23 and with SOS-1, recruiting SOS-1 to Ras at the membrane (36, 110). SEM-5 is a homolog of the Drosophila Drk protein; the two proteins can functionally substitute for each other (110).

Mammalian Sos proteins were cloned based on homology with the fruit fly gene: murine mSos1 and mSos2 (20) and human hSos1 and hSos2 (37). All are widely expressed in various tissues and in many established cell lines. The hSos1 and mSos1 proteins are 98% identical and are 45% homologous to Drosophila Sos. Mouse knockouts of mSos1 have revealed that it is essential for intrauterine development, with null animals dying in midgestation in association with yolk-sac defects and embryonic heart abnormalities (235). In contrast, mSos2 mouse knockouts result in viable mice with no apparent phenotype suggesting that Sos2 gene function is dispensable for normal mouse development, growth and fertility (64). This suggests that the role of Sos1 in development is distinct from that of Sos2.

As mentioned above, one distinguishing feature of Sos proteins is a C-terminal region rich in proline residues, which is not present in Cdc25p nor in other Ras GEFs (see Fig. 1-4). This proline-rich region allows Sos to interact with the SH3 domain of Grb2, a small adapter protein containing only SH2/SH3 domains that is homologous to SEM-5 and Drk in C. elegans and Drosophila (63). The SH2 domain of Grb2 can bind to phosphotyrosine residues found on activated receptor tyrosine kinases and on kinase substrates such as Shc, coupling Sos activity to receptor tyrosine kinase activation, which leads to Ras activation (26, 130, 189, 190). Hence, Grb2 acts to recruit Sos to the plasma membrane in response to RTK activation. In most cells, Grb2 and Sos are associated prior to receptor stimulation
through interactions between the SH3 domain of Grb2 and the proline-rich region in Sos (63, 130).

The activation of Ras by Sos is not mediated by a change in catalytic activity of Sos (163), but is thought to occur merely by the translocation of the protein to the membrane through the action of Grb2 binding to phosphotyrosine residues on RTKs (63). Membrane targeting of Sos, achieved by engineering farnesylation or myristoylation signals into the protein, is sufficient for activating the Ras pathway (6). Binding to Grb2 is not required for activation, as membrane-targeted mutants missing the proline-rich region of Sos are equally competent for Ras activation (6). Similar to phosphorylation causing a downregulation of Cdc25p signaling, downregulation of the Ras signaling pathway in mammalian cells is achieved by MAP kinase phosphorylation of Sos. The downregulation and phosphorylation has been shown by numerous groups, but the precise mechanism of this phosphorylation event still remains controversial. Buday et al. reported that in T-cells phosphorylated Sos no longer interacts with the activated receptor but that this phosphorylation does not affect the ability of Sos to interact with Grb2; this was also shown by Porfiri and McCormick in Cos-1 cells (26, 178). However, two other groups have reported that the phosphorylation event in 3T3L1 and Chinese hamster ovary (CHO) cells results in the dissociation of Grb2 and Sos (39, 239).

The N-terminus of Sos contains two protein motifs in tandem: a Dbl homology (DH) domain and a pleckstrin homology (PH) domain. PH domains are found in numerous signaling and cytoskeletal molecules and have been suggested to target proteins to membranes either by interacting with lipids or with proteins such as the βγ subunits of heterotrimeric G-proteins (199). The DH domain is the catalytic domain required for
nucleotide exchange on Rho family proteins and is therefore analogous to the Cdc25 domain (34). Early experiments designed to test the role of the DH and PH domains of Sos demonstrated that in the absence of the N-terminus of Sos, the Cdc25 domain was no longer capable of activating Ras. McCollam et al. showed that the DH and PH domains of Sos are necessary for Ras activation in Cos-1 cells and hypothesized that these domains are required for localizing the catalytic domain appropriately to operate in intact cells (146). Mutations in the DH or PH domains of Sos eliminate the transforming ability of the expressed protein, and this correlates with the ability of the protein to activate the Ras signaling cascade (180). The N-terminus of Sos expressed in cells acts as a dominant-negative protein, and this is dependent on a wildtype DH and PH domain (180). The conclusion reached from these studies is that the N-terminus of Sos is essential for Sos activity and that the DH and PH domains make critical contributions to the effects of the N-terminus.

Studies of the PH domain of Sos revealed that the isolated PH domain binds to acidic phospholipids with high affinity and is targeted to specific regions of the plasma membrane, particularly the leading edge of motile cells (41). Expression of the isolated PH domain was also shown to inhibit endogenous Sos, suggesting that the PH domain can compete with endogenous Sos for a cellular target that is critical for Sos activation (41). There is evidence suggesting that activation of Ras by Sos can occur even in the absence of an interaction with Grb2, but under these conditions, the N-terminus of Sos appears to be the critical determinant for activation (108, 146, 236). Also, the observation that the PH domain can target itself to the plasma membrane suggests a mechanism by which the N-terminus of Sos can gain access to the membrane.
Because DH domains are catalytic domains required for the activation of Rho family GTPases, Sos was tested for activity against these proteins and was found to promote nucleotide exchange on Rac, although these experiments were not performed with purified Sos (159). Microinjecting the DH domain of Sos along with Rac results in membrane ruffles which is a hallmark of activated Rac (159). The DH domain was also shown to activate SAPK, a MAPK downstream of Rac; the DH-PH fragment was dependent upon activated Ras, and perhaps PI3K, for activation of SAPK (159). The authors speculate that activation of PI3K results in production of lipids that bind to the PH domain and activate DH-Sos (159). The activity of the DH domain of Vav has been shown to be regulated in this manner (89). However, a subsequent study has shown that the PH domain of Sos binds PtdIns4,5P₂ which results in inhibition of Sos, but does not bind to PtdIns3,4,5P₃ (103). Activation of Sos requires removal of the PtdIns4,5P₂, which may result from the actions of PI3K.

Alternatively, binding of a protein to the DH or PH domain may relieve the inhibition; Chen et al. hypothesized the existence of this factor to explain the dominant-negative effects of the N-terminus of Sos (41). Recently, more evidence was published using isolated DH/PH fragments of Vav and Sos which indicated that PI3K substrates promote the binding of the PH domain to the DH domain, blocking Rac binding, whereas PI3K products disrupt the DH/PH interaction and permit Rac binding (51).

**Crystal Structure of a Sos-Ras complex.**

The recent elucidation of the crystal structure of Ras and the Cdc25 domain of Sos provided insight into the mechanism by which GEFs catalyze nucleotide exchange on Ras (16). The overall structure of the Cdc25 domain is that of an oblong bowl with Ras bound at the centre of the bowl. The structure revealed that Sos interacts extensively with Ras and
stabilizes it in a nucleotide-free state by displacing residues that coordinate the magnesium ion and the phosphate groups of the nucleotide and by partly occluding the magnesium-binding site. The GEF fragment of Sos is a mostly helical protein consisting of the Cdc25 domain and REM motif. The REM motif seems to orientate and/or stabilize a helical hairpin composed of two protruding helices.

The structure of Ras, as determined previously, showed that conserved regions retain the same conformation whether bound to GTP or GDP, but that there are two patches on the surface, called switch regions, that have different conformations in the active and inactive state. These regions are involved in interactions with upstream activators such as GEFs (Switch 2) and downstream effectors (Switch 1). Sos binds to the Switch 2 area, blocking the entry of the phosphate moiety of the nucleotide, including the magnesium ion which is required for tight binding of nucleotide into the binding site. At the same time, Sos uses the helical hairpin to flip away the Switch 1 region so that the base-binding part of the nucleotide-binding site is completely open. For both switch regions, the conformational changes extend beyond the regions that differ in the GDP- and GTP-bound forms of Ras. Sos does not block the guanine ring or the sugar-binding sites, but it inserts a glutamate residue (Glu942) and a leucine residue (Leu938) into the phosphate- and Mg$^{2+}$-binding sites. The glutamate residue interacts with Ser17, a residue in the P-loop that binds the $\beta$-phosphate of GDP and Mg$^{2+}$. As a result, the P-loop is distorted into a conformation that is not compatible with nucleotide binding.

For Ras to become activated, GTP must enter the nucleotide-binding site, consequently dissociating the GEF. In the Ras-Sos complex, the empty nucleotide site is open to the solvent. The GTP molecule enters into the nucleotide binding site with the base
and the ribose interacting with the part of the Ras binding site that is not occluded by Sos. This ternary complex of loosely bound nucleotide, Ras, Sos and Mg\(^{2+}\) undergoes a conformational change in the Switch 2 segment and release of Switch 1 from the GEF, resulting in the restructuring of a competent binding site for phosphate and Mg\(^{2+}\). This results in a tightly-bound nucleotide which has a low affinity for the GEF, resulting in the dissociation of Sos.

**RasGRP**

A member of a third class of Ras exchange factors, RasGRP, has been identified which contains calcium-binding EF hands and a diacylglycerol-binding C1 motif (see Fig. 1-4). RasGRP was cloned from a rat brain library as well as from a T-cell library and its transcript is detected in the brain and the hematopoietic system (62, 109, 222). RasGRP is capable of transforming fibroblasts and treatment with a DAG analog causes changes in cell morphology (62). GST fusion proteins containing the EF hands bind Ca\(^{2+}\) and GST fusion proteins containing the DAG-binding motif do indeed bind DAG (62, 133). RasGRP activates Ras *in vivo* and this is further stimulated by treating cells with agents such as PMA or endothelin-1; deleting the C1 motif eliminated this activation (62). RasGRP is recruited to membrane fractions of cells in a DAG-dependent manner which requires the C1 motif (62), suggesting that translocation plays a role in Ras activation through RasGRP as it does with Sos. The DAG-binding motif is required for transformation by RasGRP; transformation could be restored by membrane targeting RasGRP via the addition of a prenylation signal or by the C1 domain of protein kinase C (PKC) (222).
CNrasGEF

A fourth class of exchange factor is represented by CNrasGEF (for cyclic nucleotide Ras GEF), an exchange factor that activates Ras in response to cAMP and cGMP (176). CNrasGEF was isolated in a search for proteins that bind to the WW domains of a ubiquitin protein ligase, Nedd4 (176) (Fig. 1-4). CNrasGEF represents a novel class of Ras GEFs containing several modular domains including a cAMP/cGMP-binding domain (cNMP-BD), a REM motif, PDZ and Ras association (RA) domains, a Cdc25 domain, two PY motifs responsible for binding to the WW motifs of Nedd4, and a C-terminal SAV sequence conforming to PDZ-binding motif sequences. CNrasGEF can activate Ras in vitro and can bind cAMP directly via its cNMP-BD; in cells, it can activate Ras and is stimulated by the intracellular elevation of cAMP or cGMP (176). This activation is eliminated in a protein lacking either the Cdc25 domain or the cNMP binding motif. CNrasGEF is expressed in the brain and its localization at the plasma membrane is dependent upon the presence of an intact PDZ domain; thus, the PDZ domain may be involved in targeting or tethering CNrasGEF to a PDZ-binding protein associated with the plasma membrane (176). These data suggest that CNrasGEF may directly connect cAMP- or cGMP-generating pathways to Ras.

GRF1 and GRF2

Finally, the last class of exchange factors, those activated by increases in intracellular calcium levels, are represented by the homologs Ras-GRF1 (GRF1) and GRF2 (Fig. 1-4). GRF1, also known as CDC25Mm, was first cloned from a mouse brain library by its ability to complement the yeast cdc25-1 mutation (140) and from a rat brain library using degenerate primers based on the Cdc25 domain of yeast CDC25 and SDC25 (203). Both isoforms were used to detect transcript via a Northern blot where expression was detected
solely in the brain. In fact, GRF1 protein is present in neuronal cells throughout the brain, particularly concentrated in punctate structures (214). GRF1 is widely expressed in central nervous system structures, including the hippocampus, cerebral cortex and thalamus (22). Brain subcellular fractionations revealed that GRF1 is present in synaptosomal fractions, and especially enriched in post-synaptic densities (214).

The phenotype of mice disrupted for the GRF1 gene is consistent with this expression in the brain. GRF1−/− mice are viable and fertile with no major morphological defects in the brain (22). However, as Ras signaling is purported to play a role in synaptic transmission and plasticity, GRF1−/− mice were subjected to various behavioural tests. GRF1 mutants do not show major defects in standard tests of hippocampal function, but do demonstrate defects in amygdala-mediated fear conditioning (22). Specifically, GRF1−/− mice are impaired in process of memory consolidation during fear-related behavioural tasks (22). Both wildtype and mutant mice are initially able to learn a task with no detectable differences between them; however, 24 hours after the initial training, GRF1-deficient mice could no longer perform the task suggesting that they had forgotten what they had previously learned (22).

GRF2 was cloned based on homology to the Cdc25 domain of GRF1 (40, 66). The domain structure and amino acid sequence of GRF2 is very similar to that of its homolog, GRF1. Both cDNA sequences are predicted to contain a number of recognizable protein domains, including, in amino to carboxyl order, a plekstrin homology (PH) domain, a coiled-coil motif, an ilimaquinone (IQ) motif, a Dbl homology (DH) domain, a second PH domain, a Ras Exchanger motif (REM) and the catalytic Cdc25 domain. However, between the REM and Cdc25 domain, GRF2 contains a destruction box (DB) motif which has been shown in other proteins to target the protein for destruction via the ubiquitin-proteasome
pathway. There is a large stretch of sequence adjacent to the DB of GRF2 that is not conserved in the two proteins. Two inserts of 60 and 13 amino acids are found in GRF1 that are absent in GRF2 (66) and the function of these inserts is unknown. The region between the REM and the Cdc25 domain in both GRF2 and GRF1 is rich in proline, glutamic acid, serine and threonine residues, reminiscent of PEST sequences postulated to target proteins for proteolysis. GRF2 contains the destruction box motif in this region, whereas GRF1 contains a much more degenerate destruction box sequence. This region of GRF2 also contains eleven Ser/Thr-Pro motifs characteristic of cyclin-dependent kinase (CDK) and MAPK phosphorylation sites (66).

Much work has been done to elucidate the upstream signaling components that activate GRF1. The activation of Ras by GRF1 can be inhibited by pertussis toxin, a G_{i,o} inhibitor, but not by the tyrosine kinase inhibitor genistein, suggesting that GRF1 activates Ras in response to signals that activate the heterotrimeric G-proteins (204). This G-protein coupled receptor (GPCR)-mediated activation of Ras is also corroborated by a study establishing phosphorylation-dependent activation of GRF1 by muscarinic receptors upstream of G-protein βγ subunits in NIH 3T3 cells (144). Mattingly has also identified serine 916 of GRF1 as a target for phosphorylation in vivo and in vitro in COS-7 and NIH-3T3 cells in response to activation of both muscarinic receptors and of protein kinase A (PKA) (143). Interestingly, serine 916 occurs within one of the insert regions of GRF1 and so has no homologous residue in GRF2, perhaps indicating a differential method of activation for the two highly related proteins. Kiyono et al. have shown that GRF1 becomes tyrosine phosphorylated in response to EGF through the action of ACK1, a nonreceptor tyrosine kinase that is an effector of Cdc42, suggesting a signaling cascade leading from
Cdc42 to Ras through GRF1 (118). GRF1 can be activated through another GPCR, one that is activated by lysophosphatidic acid (LPA). LPA induces an increase in the serine phosphorylation state of GRF1 that correlates with an increase in GEF activity towards H-Ras (145). GRF1 has also been shown to enhance the activation of Ras induced by serum stimulation, even though GRF1 does not form a complex with the activated EGF receptor or with Grb2 (33, 204). In NIH 3T3 cells, this serum-stimulated activation of Ras only occurs if GRF1 is membrane-associated and only if the N-terminus is retained, suggesting the N-terminus is important for coupling GRF1 to the serum-responsive components involved in signaling through GRF1 (33).

Both GRF2 and GRF1 activate Ras in response to a calcium influx (66, 70). Both GEFs bind the calcium sensor calmodulin (CaM), and this interaction can be disrupted in vitro by treatment with calcium chelators such as EGTA: deleting the IQ motif of both proteins results in the loss of CaM binding and in the loss of calcium-responsive activation of the Raf-ERK MAPK cascade (66, 70).

Structure/function relationships of GRF1 have also been studied in detail. The first paper to look at details of specific domains in GRF1 other than the IQ motif showed that the N-terminal PH domain (PHn), coiled-coil and IQ motif act cooperatively to respond to calcium (25). Buchsbaum et al. ascertained that the PHn domain plays multiple roles in GRF1 regulation. Deletion of PHn redistributes a large percentage of GRF1 to the cytosol and renders the protein unresponsive to calcium; substituting the PH domain of GAP restores the proper localization of GRF1 but does not restore calcium responsiveness (25). Wildtype GRF1 is constitutively associated with the particulate fraction of cells, likely associated with membranes as it can be solubilized with detergent (25). These findings suggest that the N-
terminal PH domain, the coiled-coil motif and the IQ motif of GRF1 function together to connect GRF1 to multiple components in the particulate fraction of cells that are required for responsiveness of the protein to calcium signaling. GRF1 has been shown to bind to the βγ subunits of a heterotrimeric G-protein in vitro, although the binding is quite weak (223). GRF2, on the other hand, is a cytosolic protein that does not redistribute to the particulate fraction upon calcium influx (66). However, using indirect immunofluorescence and confocal microscopy, Fam et al. established that GRF2 translocates to the cell periphery in response to calcium (66).

*S. cerevisiae* Cdc25p was determined to form oligomers in cells, as discussed previously, and Anborgh et al. established that the Ras-GRFs also form oligomers (4). In a two-hybrid assay looking for proteins that interact with the DH domain of GRF1, a fragment of human GRF2 containing the DH domain was isolated that specifically bound to the GRF1 DH domain (4). Further work demonstrated that the isolated DH domains of the two proteins interact in a two hybrid assay as well as by coimmunoprecipitation and that GRF2 and GRF1 are capable of oligomerizing, forming either homo- or hetero-oligomers (4). Mutants in the DH domain of GRF1 that impair the oligomerization are no longer transforming, suggesting that oligomerization may play an important role in the regulation of the GRFs (4).

As with Sos, the functions of the DH-PH domains of the GRFs have been extensively investigated. Freshney et al. have reported that the tandem DH/PH domains of GRF1 are required for Ras activation; point mutations in either domain result in an inability of GRF1 to respond to calcium (77). In contrast, a GRF2 protein that is deleted for the DH domain is fully capable of responding to a calcium signal and activating Ras (67). Full-length GRF1 or the isolated DH/PH domain fragment have recently been shown to activate SAPK upon
stimulation with LPA (101). Although GRF1 is unable to activate Rac on its own, when coexpressed with the G-protein βγ subunits it becomes capable of activating Rac and SAPK (120). Most recently, the Rac GEF activity of GRF1 was shown to require phosphorylation by the nonreceptor tyrosine kinase Src (119). This bi-functional activity of the GRF exchange factors was first demonstrated by us with GRF2, which is also capable of activating Rac in vitro and SAPK in vivo (67). As cellular growth requires coordination among copious numbers of signaling pathways, it is possible that the ability of Sos and the GRFs to activate more than one pathway is required for the formation of multimolecular signaling complexes that are necessary for proper cellular regulation.

BACKGROUND ON GRF2

Cloning

As GRF1 was shown to be expressed solely in the brain, it is reasonable to assume that other tissues would require a mechanism to activate Ras downstream of Ca2+ signals. Based on this assumption, Chen et al. designed degenerate primers based on the Cdc25 domain of GRF1 and amplified cDNA prepared from mouse embryonic stem cells (40). The amplified fragment was used to generate antibodies, which detected GRF2 protein in various murine tissues including brain, heart and liver. This fragment was then used to screen a mouse brain cDNA library, isolating a clone coding for a 1189 amino acid, 135 kDa protein (66).

The alignment of the Cdc25 domains of GRF2, GRF1, mSos1 and S. cerevisiae CDC25 displays a high degree of sequence similarity. The Cdc25 domain of GRF2 is 31% identical to that of CDC25, 30% to mSos1, and 75% identical to Ras-GRF1; in other words,
based on the sequence of the catalytic domain, GRF2 and GRF1 are as similar to budding yeast Cdc25p as they are to the murine Sos proteins. The DH domain sequence is 18% identical to the related region of mSos1, and shares 16% identity with Dbl itself. Of the 25 residues found in the IQ motif of GRF2, 22 residues are identical to those found in the IQ motif of GRF1; the IQ motif of GRF2 is also similar to those found in other calmodulin binding proteins such as IQGAP1 and neuromodulin (66).

By Northern analysis, a GRF2 transcript of approximately 9 kb is detected in total RNA derived from different rat tissues. GRF2 is expressed most highly in the brain, with lesser expression in heart, lung, skeletal muscle and liver. Using affinity purified GRF2 antibodies, full-length protein has been detected in brain and lung, with a smaller 75 kDa species being detected in several tissues including kidney, liver, spleen, skeletal muscle, pancreas, cardiac muscles, thymus, testis and embryonic stem cells (40). It is not known what portion of full-length GRF2 is present in this smaller species or if the N-termini are different due to alternative splicing of exons.

**Description of the signaling capabilities of GRF2**

Having identified GRF2 as a homolog of GRF1, Fam et al. first tested to see if the Cdc25 domain of GRF2 was capable of catalyzing nucleotide exchange on members of the Ras superfamily. The GST-Cdc25 domain displayed exchange activity towards H-Ras *in vitro*, but was not active towards GST, RalA, Rap2, Cdc42Hs or RhoA (66). In COS-1 cells transfected with vector Ras-GTP levels were shown to be 38%, while in the presence of GRF2 Ras-GTP levels increased to 72%, showing that GRF2 can activate Ras *in vivo* as well (66). A protein that functions as a GEF should display a higher affinity for the nucleotide-free (NF) form of the GTPase as the binding of the GEF to the NF GTPase stabilizes the
unstable NF protein (127). GST-Ras, either NF, bound to GDP or to the non-hydrolyzable GTP analog GTPγS, was incubated with lysates containing GRF2 and then the amount of GRF2 bound to Ras was assessed by Western blotting. GRF2 binds specifically to the NF form of Ras and this is a function of the Cdc25 domain, indicating that it functions as an exchange factor for Ras (66, 67). GRF2 also binds to NF Rac using the same type of assay and this is dependent upon the presence of the DH domain (67). GRF2's ability to act as a Rac GEF is further evidenced by the fact that GRF2 can activate Rac in vitro, but cannot activate Rho or Cdc42 (67).

As mentioned previously, GRF2 can increase the basal activity of ERK1 and can also potentiate calcium-induced ERK1 activation (66). Deletion of the IQ motif leaves a protein both incapable of increasing the basal activity of the ERK pathway and of being stimulated by calcium and the same statements hold true for the SAPK pathway (66, 67). Deleting the DH motif has no effect on ERK activation; however, it has a dramatic effect on activation of SAPK downstream of activated Rac in that the ΔDH protein displays no basal or calcium-stimulated SAPK activity (67). These data illustrate that the IQ motif is required for proper calcium-stimulation of GRF2's GEF activity towards Ras and Rac, the Cdc25 domain is required for ERK activation and the DH domain is required for SAPK activation.

Ras and Rac harbouring a mutation to asparagine at position 17 are exploited as dominant-negative inhibitors of GEFs, as I mentioned earlier. N17 Ras and N17 Rac, when expressed individually, are effective inhibitors of both ERK and SAPK activation by GRF2 (67). The ability of either protein to inhibit signaling suggests that GRF2 is a common target of both N17 Ras and N17 Rac and that only one of the GEF domains of GRF2 may function at a time.
293 epithelial cells stably expressing GRF2 exhibit altered growth properties (66). Unlike the parental cells which grow in clusters of tightly opposed cells, the transfected cells grow in a more dispersed, disorganized fashion with decreased cell-cell contacts. As the expression of oncogenic Ras and Src have been shown to disrupt such epithelial cell-cell contacts (12, 112), it may be that GRF2 has similar effects by activating endogenous Ras proteins. Alternatively, it is also possible that the phenotype observed results from the regulation of Rac by GRF2.

Much work in this thesis describes the regulated proteolysis of GRF2 through the action of the ubiquitin proteolysis pathway. As stated earlier, GRF2 contains a motif resembling that found in mitotic cyclins and this destruction box motif targets other proteins for ubiquitination. In Chapter Three, I introduce data to demonstrate that GRF2 is indeed regulated by its DB; therefore, I will now provide some background into the ubiquitin proteasome pathway.

THE UBIQUITIN/PROTEASOME PATHWAY

Most of the proteins degraded in the cytosol are delivered to the key enzyme complex of the ubiquitin/proteasome pathway, a self-compartmentalized protease particle called the 26S proteasome which accounts for about 1% of total cellular protein (48). It is a large molecular complex, consisting of a cylindrical core proteinase formed from multiple proteases whose active sites face an inner chamber. This core cylinder is known as the 20S proteasome. Two identical, 19S regulatory complexes known as PA700 (for 700 kDa proteasomal activator) are attached to either end of the core 20S proteasome to form the 26S
proteasome (11). 26S proteasomes act on proteins that have been specifically marked for destruction by the covalent attachment of a small protein called ubiquitin, which is a 76 amino acid, highly conserved protein. The PA700 components of the proteasome seem to confer both ubiquitin-dependent and ATP-dependent protein degradation. The 19S regulatory particles consists of a base complex and a lid complex. The base complex contains ATPase subunits presumed to unfold the substrate and thread it into the central lumen of the core particle, whereas the lid complex possesses polyubiquitin binding subunits and deubiquitinating enzymes (80, 128). It is hypothesized that the multi-ubiquitin chain on a substrate binds one of the PA700 subunits, ATP dependent events occur which unfold the chain, open the central channel of the 20S proteasome and feed the protein into this channel (129). The protein is then cleaved into small peptides, and the ubiquitin conjugates are removed from either the small peptides or from the walls of the proteasome by deubiquitinating enzymes, releasing free ubiquitin (97).

General description of the ubiquitin proteolytic pathway

The attachment of ubiquitin to a target protein occurs via a biochemical "bucket-brigade" of enzymes first resolved in a rabbit reticulocyte system by Hershko et al. (94) (Fig. 1-5). First, free ubiquitin from cytoplasmic and nuclear pools is activated for conjugation in an ATP-dependent manner by formation of a thiol-ester bond between the C-terminal glycine residue of ubiquitin and a cysteine residue on an E1 enzyme (ubiquitin-activating enzyme). E1-bound ubiquitin is then transferred to a cysteine residue of an E2 enzyme (ubiquitin-conjugating enzyme) again via a thioester linkage. The E2 enzyme in cooperation with an E3 (ubiquitin ligase) transfers ubiquitin to the target protein, where it is attached via an isopeptide bond between the C-terminal glycine of ubiquitin and the epsilon amino group of
a lysine residue on the target protein. In many cases, the E3 participates directly in the
transferase reaction, forming an intermediate thioester with ubiquitin (193); in other

**Figure 1-5  Ubiquitin conjugation pathway.**

Schematic of the ubiquitin conjugation pathway. Ubiquitin (Ub) is activated in an ATP-
dependent manner and attached via a thioester bond to an E1 (ubiquitin activating) enzyme.
It is then transferred to an E2 (ubiquitin conjugating) enzyme, where it is again attached via a
thioester bond. The E2 enzyme transfers the Ub to the target protein with the assistance of an
E3 (ubiquitin ligase) protein (or protein complex). The Ub
molecule may be
attached via a thioester bond to the E3 protein,
or the E3 may act
merely to juxtapose
the E2 and the target
proteins to facilitate
Ub transfer to the
target protein. The Ub
protein is attached via
its C-terminal glycine
residue to the epsilon
amino group of a
lysine residue on the
target protein, forming
an isopeptide bond.
The ubiquitin pathway
may act repetitively on
a target and add
subsequent Ub
molecules; these next
Ub molecules are often attached at Lys48 of Ub. Once a Ub chain has been formed, this
multi-ubiquitinated protein binds to the 26S proteasome, is unfolded and fed through the
central channel of the proteasome and degraded, with concomitant recycling of Ub
molecules. (Reproduced with permission from Dr. Mike Tyers.)

instances, the E3 merely juxtaposes the substrate and E2 enzymes allowing direct transfer of
ubiquitin from the E2 to the substrate, but the E3 does not itself form adducts with ubiquitin
The mono-ubiquitinated protein is then acted upon again and the same enzymes attach an additional ubiquitin to the previous one, often at Lys\textsuperscript{48} of ubiquitin. Ubiquitin conjugation continues resulting in a high molecular weight complex with a chain of ubiquitin molecules attached to the substrate. Recently, a fourth E4 activity was described which appears to act as a processivity factor for ubiquitin chain elongation on some substrates (123). This polyubiquitinated product then becomes a target for rapid degradation by the 26S proteasome with concomitant recycling of ubiquitin via deubiquitinating enzymes (see below).

In general, the diversity of enzymes increases down the ubiquitin conjugation cascade. Budding yeast contain only one E1 enzyme which activates multiple E2 enzymes (at least 13), which in turn couple to a large but unknown number of E3s (93). Therefore, it is the E2s and even more so the E3s which are thought to provide substrate specificity to this pathway (97, 242). Most of the substrate specificity is thought to be provided by the E3 enzymes, as there seem to be many different types of E3 proteins that bear little or no resemblance to each other, a characteristic that has hampered their identification in protein databases (231). To further complicate easy identification of E3 enzymes, they appear to be quite structurally diverse, ranging from single proteins to multisubunit complexes.

Ubiquitin chains are highly dynamic, with rapid addition and removal of ubiquitin units, which may be a proofreading mechanism to ensure that only specific proteins are marked for destruction. Consequently, many deubiquitinating enzymes have now been identified. As the number of deubiquitinating enzymes is also very high, it raises the possibility that each Ubp (ubiquitin-specific processing protease) has a considerable degree of substrate specificity. Some Ubps act on unanchored ubiquitin chains (i.e. chains not attached to substrate), others act to remove ubiquitinated proteolytic remnants that remain on
the 26S proteasome after protein degradation to maintain adequate pools of free ubiquitin, and yet others possess proofreading activity. In mammals, several proteins implicated in tumorigenesis have been shown to be deubiquitinating enzymes (83, 87, 156, 168). The yeast *DOA4* gene has been demonstrated to encode a deubiquitinating enzyme that functions late in the proteolytic pathway by cleaving ubiquitin from substrate remnants still bound to the protease (168). Without Doa4p, the proteasome slows down and substrates accumulate. The human homolog of Doa4p is the *tre-2* oncogene, and Papa & Hochstrasser (168) demonstrated that the oncogenic version of this protein is an inactive form of the deubiquitinating enzyme. These findings suggest the perturbation of ubiquitin-dependent proteolysis in mammalian cells can lead to tumorigenic growth.

**Classes of E3 enzymes and their targeting signals**

At this time, four unrelated classes of E3 enzymes have been reported, each participating in the ubiquitination of particular types of substrates. The archetypal E3 ubiquitin ligase, E3α, targets proteins by the N-end rule pathway in which the N-terminal amino acid of a protein dictates recognition by the E3; some amino acids are stabilizing when found at the N-terminus of protein whereas others are destabilizing causing a protein to have a short half-life (230).

A second family of E3 enzymes, the HECT domain proteins (for homology to E6-AP C-terminus) is represented by E6-AP which complexes with the human papillomavirus E6 protein to target the tumour suppressor p53 for degradation (192). Other HECT domain proteins include Rsp5, Nedd4 and Pub1, all of which are implicated in the degradation of specific membrane proteins and other targets (93). The specificity determinants found in
HECT domain substrates are not well defined. The WW domains of Nedd4 bind proline rich PY motifs in a mammalian membrane sodium channel (213); the WW domains of Rsp5 bind phosphoserine sequences in Ste2p, a yeast pheromone receptor (135, 221).

The remaining two classes of E3s were described recently and were identified because of their essential roles in the cell cycle (Fig. 1-6). The Anaphase Promoting Complex (APC), or cyclosome, is a multisubunit E3 complex that mediates degradation of mitotic cyclins and other regulators for destruction (224). Substrates of the APC contain one or both of two small motifs known as the destruction box (DB) and KEN motif (175). The final class of E3 ubiquitin ligases are referred to as SCF complexes (for Skp1, Cdc53, F-box protein) and they target G1 cyclins in yeast, CDK inhibitors and many other proteins (171). SCF complexes recognize and ubiquitinate only phosphorylated substrates (48). In many cases, the phosphorylated sites are within the PEST regions of the protein, a situation seen with G1 cyclins in yeast (246).

**SCF and the F-box hypothesis**

The SCF ubiquitin ligase complex was initially discovered through analysis of cyclin and CDK inhibitor degradation in yeast and is now found to be an activity required for many cell processes including cell cycle regulation. Analysis of several cell division cycle (cdc) mutants in yeast provided the first clues into the role of ubiquitination in cell cycle regulation. In yeast, mutants in Cdc4p, Cdc34p or Cdc53p causes a G1 arrest phenotype as a result of a failure to degrade Sic1p, an inhibitor of Cdc28-Clb kinases (197). In a similar vein, Grr1p, Cdc34p and Cdc53p are required for degradation of G1 cyclins, Cln1p and Cln2p (54, 171, 243).
SCF complexes and the Anaphase Promoting Complex

The most well characterized SCF complexes are those found in budding yeast. They are composed of a cullin family member such as Cdc53 containing a cullin homology domain (CH); an E2 enzyme, such as Cdc34; Rbx1; Skp1; Sgt1; and a protein containing an F-box which acts as the adapter which brings substrates into the complex. There are many identified F-box proteins in yeast and in mammalian cells. Cdc4 and Grr1 are both *S. cerevisiae* F-box proteins. The WD40 domains of Cdc4 can bind to Sic1p, Cdc6p, Gcn4p and Far1p targeting them for ubiquitination by the SCF complex. The leucine rich repeats of Grr1 can bind Cln1/2 and Gic1/2, targeting them for ubiquitination. In mammalian cells, the WD40 motifs of β-TrCP can target IκBα and β-catenin for ubiquitination. The SCF complexes recognize and target phosphoproteins.

The Anaphase Promoting Complex is a large megadalton complex composed of many subunits, a number of which are homologs of subunits in the SCF. Ubc10 is a ubiquitin conjugating enzyme that performs the same role as Cdc34 in the SCF. APC2 is a CH domain-containing component of the APC, homologous to Cdc53. APC11 shares homology with Rbx1 of the SCF. At this point, there are no identified homologs of the other important members of the SCF, such as Skp1. The APC also has adapters that ferry substrates to it in order to facilitate ubiquitination in a role analogous to F-box proteins. These adapter proteins are Cdc20, Cdh1 and Ama1, which are responsible for the targeting of many proteins as shown in the figure. Cdc20 and Cdh1 are found in mammalian cells as well as in budding yeast. Rather than recognizing phosphoproteins, the APC recognizes proteins containing destruction boxes or KEN motifs. (Figure adapted from (105))
Next it was found that Cdc34p, an E2 enzyme, is physically associated with the Cdc4p and Cdc53p (142, 243). The full elaboration of these degradation pathways came with the identification of Skp1p as a suppressor of cdc4 mutants; in a key insight, Bai et al. aligned the Skp1p interacting proteins and deduced the binding site, a degenerate 40 amino acid motif they named the F-box, after cyclin F (7). Since Sic1p and Cln1p degradation seemed to require the same core components (Cdc34p, Cdc53p and Skp1p) but different F-box proteins (Cdc4p versus Grr1p), they hypothesized that F-box proteins such as Cdc4p and Grr1p act as receptors that recruit substrates into a Skp1/Cdc53/Cdc34 complex for ubiquitination and that each F-box protein functions to recruit a different subset of proteins into the SCF complex (7, 74, 207).

The architecture of the SCF has been described in many papers, many of which described SCF$^{Cdc4}$ (7, 74, 106, 142, 162, 207, 208, 217, 243) (Fig. 1-6). A recombinant complex composed of Cdc4, Cdc34, Cdc53 and Skp1 is able to support the ubiquitination of phosphorylated Sic1 with high specificity (74, 207). Substrate recognition is mediated by the C-terminus of Cdc4 which contains seven protein-protein interaction motifs known as WD40 repeats. The association of Cdc4 with Skp1 enhances substrate binding, implying that Cdc4 and Skp1 together form the binding surface for substrate binding or that Skp1 allosterically regulates Cdc4. Skp1 binds to the N-terminal region of Cdc53, whereas Cdc34 binds to a C-terminal domain of Cdc53 (170). In this complex, only Cdc34 appears to form thioester linkages with ubiquitin and therefore the SCF complex of Skp1, Cdc53 and F-box protein acts to juxtapose the E2 enzyme and substrate, facilitating ubiquitination (170, 207). Another recently identified component of SCF complexes is Rbx1, a RING finger protein also known
as Roc1 or Hrt1, which appears to stabilize the E2/E3 complex through its ability to interact independently with Cdc4, Cdc53 and Cdc34 (106, 162, 208, 217). SCF-Rbx1 complexes appear to greatly stimulate Cdc34 activity, generating speculation that by restricting E2 activity to within fully assembled E3 complexes the cell may prevent free E2s from randomly ubiquitinating cellular proteins (105, 198, 208). Finally, a sixth constituent of SCF complexes, called Sgt1, has been described. It assembles into recombinant SCF complexes in a Skp1-dependent manner, but it does not dramatically affect in vitro ubiquitination activity (117).

In summary, F-box proteins contain protein-protein interaction motifs that bind substrates and target them for ubiquitination through the SCF; many proteins important for various cellular processes are targeted for ubiquitination through the many F-box proteins that have been identified thus far. The recent publication of the C. elegans genome sequence allowed for the identification of more than 100 putative F-box proteins, 10 Skp1 homologs and 5 Cdc53 homologs; the various combinations implicated here would allow for a huge number of specific substrates to be targeted for ubiquitination (48). To date, all known or suspected SCF substrates are recognized by the F-box protein in a strictly phosphorylation dependent manner, thus linking intracellular signaling pathways to the ubiquitin system. SCF complexes appear to be constitutively active throughout the cell cycle so that control of ubiquitination resides primarily at the level of substrate phosphorylation.

**Signaling pathways regulated by SCF complexes**

The SCF system is used in the proteolytic control of many important signaling pathways. In yeast, three major metabolic pathways are regulated by different SCF complexes: glucose induction is mediated by SCF^{Gnt1}, methionine repression by SCF^{Met30} and
in part repression of amino acid biosynthesis by SCF<sup>Cdc8</sup> (48). In each case, it is a transcriptional activator or repressor that is targeted for degradation through the SCF complex.

The LIN-12/Notch family of transmembrane receptors mediate cell-cell communication that induces metazoan cells to adopt different fates, a process known as lateral specification (84). The Notch receptor (LIN-12 in <i>C. elegans</i>) is activated by contact with the transmembrane proteins of the Delta/Serrate/LAG-2 (DSL) family in adjacent cells. During signaling, the Notch intracellular domain is liberated by a cleavage event at the juxtamembrane region and this intracellular domain translocates to the nucleus to activate transcription in complex with other transcription factors (48). The F-box protein SEL-10 represses the Notch/LIN-12 pathway, regulating signaling either by inhibiting release of the intracellular domain and/or by stimulating proteolysis of it (48).

The hedgehog pathway regulates limb development in <i>Drosophila</i> by controlling the expression of wingless and decapentaplegic in the anterior-posterior axis of wing and leg imaginal discs (48). The hedgehog pathway is also repressed by the action of an SCF complex; in this case, the F-box protein is Slimb (for supernumerary limbs) (48). In the absence of Hedgehog (Hh) signal, protein kinase A (PKA) is active and phosphorylates the full length form of Cubitis interruptus (Ci), thereby targeting the C-terminus of Ci for cleavage and then degradation via a Slimb-dependent mechanism (48). The truncated N-terminus of Ci translocates to the nucleus where it represses transcription of Hh among others (48).

The NF-κB transcription factor is important for inflammation, immune and stress responses (137). NF-κB is produced as a 105 kDa precursor protein which must be cleaved
to a 50 kDa N-terminal fragment in order to generate an active transcription factor; the cleavage of NF-κB occurs by limited ubiquitin-dependent proteolysis (166). The access of NF-κB to the nucleus is regulated by IκBα which masks a nuclear localization signal in NF-κB and inhibits its ability to bind DNA (48). Upon activation of the immune response, IκBα is phosphorylated on serine residues which triggers its recognition by the SCF complex through the F-box protein β-TrCP (a human homolog of Slimb), resulting in its degradation by the ubiquitin system which releases NF-κB, allowing it to translocate into the nucleus (249). Interestingly, human immunodeficiency virus (HIV) uses β-TrCP to degrade the CD4 membrane receptor, thereby reducing superinfection and exposure of infected cells to immune surveillance (48). CD4 is captured and retained in the ER membrane by HIV protein gp160 where another HIV ER protein, Vpu, binds CD4 as well (48). Two closely spaced serine residues are phosphorylated in Vpu and this mediates binding to the F-box protein β-TrCP, stimulating degradation of CD4. A number of F-box proteins and Skp1 homologs are encoded in viral genomes, so the exploitation of the SCF pathway may be a common theme in viral life cycles (7).

**Anaphase Promoting Complex**

Entry into mitosis in all organisms is initiated by cyclin B/Cdc2 or its homologs. The activation of this kinase activity results in the activation of the ubiquitin destruction system responsible for targeting mitotic cyclins (113), which recognizes the DB of cyclins. Cyclin B does not seem to become phosphorylated prior to being targeted for destruction; instead, the fluctuation in activity during the cell cycle of a multisubunit E3 complex, known as the cyclosome or anaphase-promoting complex (APC), underlies destruction of DB-containing
proteins. The multisubunit APC core particle is necessary for anaphase, exit from mitosis and maintenance of G1 phase (253). However, the persistence of the APC in fully differentiated quiescent cells suggests that its activity is not confined only to proteins involved in cell cycle progression (253).

Several independent genetic and biochemical approaches aimed at identifying the mitotic cyclin degradation machinery lead to the discovery of the APC in 1995 (102, 114, 215). The composition of the APC has been investigated by immunopurification. The *Xenopus* and human complexes contain ≥ 10 subunits and the clam complex contains ≥ 9, whereas the yeast particle contains ≥ 12 subunits; most yeast subunits have counterparts in vertebrates suggesting that the APC has a similar composition in all eukaryotes (86, 174, 250, 254, 255). Several protein subunits (Apc1, Cdc16, Cdc23, Apc10/Doc1 and Cdc26) were identified in yeast through the isolation of mutants that are defective in mitotic cyclin proteolysis (100, 102, 255). Previous work had shown that Cdc23, Cdc16 and Cdc27 form a complex that is required for initiation of anaphase in yeast (173).

The stoichiometric APC subunits described above are part of the complex during all phases of the cell cycle (173). In contrast to this, two proteins that regulate APC activity associate with the APC in a cell-cycle-regulated manner; these proteins have been identified in yeast, *Drosophila* and *Xenopus*. Cdc20 and Cdh1 (a.k.a. Hct1), known as Fzy and Fzr in *Drosophila* or Slp1 and Srwl/Ste9 in fission yeast, are related WD40 repeat-containing proteins that are presumed to recognize the destruction box, as well as the KEN motif for Cdh1. Cdc20 and Cdh1 have been shown to activate the APC in a substrate specific manner and essentially they act as adapter proteins that associate with the APC only at specific points during the cell cycle, bringing substrates to the APC for ubiquitination (196, 232) in a role
analogous to that of the F-box protein in the SCF complex. A recent member of this class of adapter proteins in budding yeast is Ama1p which is expressed only during sporulation where it is required for the proteolysis of Clb1 by the APC in meiosis (44).

The most remarkable finding to emerge from these studies is that two APC subunits, Apc2 and Apc11, are related to subunits from the SCF (Fig. 1-6). Apc2 is a member of the cullin family of proteins, which includes Cdc53 from the SCF (251, 254). Apc11 is closely related to Rbx1, the fourth essential component of the SCF and like its SCF counterpart, Apc11 interacts with the C-terminal region of its cullin, Apc2 (162). Taken together, these data suggest that the APC and SCF are members of a family of complex E3 ubiquitin protein ligases that share a cullin and a RING finger subunit and might originate from the same ancestral ubiquitin ligase complex (253).

**Pathways regulated by the Anaphase Promoting Complex**

The APC owes its name to the observation that its activity is essential for the initiation of sister chromatid separation in anaphase (102, 114). Anaphase is initiated upon the destruction of the anaphase inhibitor, Pds1p, through the action of APC^{Cdc20} which results in disintegration of a multisubunit complex called cohesin that binds sister chromatids together (247). Yeast cells that lack both APC and Pds1p function proceed normally through anaphase, releasing the cohesin protein Scc1p from chromatin and arrest in late anaphase, indicating that the only role of the APC in anaphase onset is to degrade Pds1p (42). Pds1p forms a stable complex with a 180 kDa protein called Esp1p, which is essential for the dissociation of Scc1p from sister chromatids and for their separation. APC^{Cdc20} promotes sister separation not by destroying cohesins but instead by liberating Esp1 protein from its inhibitor Pds1p (42).
The initiation of anaphase is essentially a point of no return in mitosis and the cell cycle. Initiating this complex series of events too early or too late will have drastic consequences with regards to the maintenance of genomic stability in proliferating cells. To avoid unequal separation of sister chromatids, anaphase must not be started until all chromosomes are connected to the mitotic spindle and aligned along the metaphase plate. Cells monitor this attachment through a surveillance mechanism called the spindle assembly checkpoint. Activation of this checkpoint inhibits APC-dependent degradation of Pds1p, thereby preventing inappropriate sister chromatid separation (68, 99, 111, 132).

In eukaryotes, the cell cycle is driven by alternating periods of low CDK activity in G1 phase and a high level of CDK activity in S, G2 and M phase (157). CDK activity is linked to replication and chromosome segregation: low CDK activity triggers exit from mitosis and establishment of functional origins of replication. whereas high CDK activity activates firing of the pre-replicative origins, prevents their re-firing and catalyzes entry into mitosis (157). The mammalian CDK cyclin B-Cdc2 complex plays a major role in progression through mitosis; its accumulation triggers the transition from G2 phase to mitosis (224). By destroying B-type cyclins, APC$^{Cdc20}$ promotes inactivation of mitotic CDKs (thereby facilitating cytokinesis) and removes a block to chromosome rereplication. By maintaining B-type cyclin degradation throughout G1 phase, APC$^{Cdh1}$ delays entry into S phase in cooperation with the B-type CDK inhibitors such as Sic1p (3, 196).
THESIS OVERVIEW

This thesis has focussed on the regulation of GRF2 through the function of the protein's non-catalytic regions. More specifically, I discuss the role of CaM binding to GRF2 and how my thesis work demonstrates that CaM binding does not correlate with activation of GRF2, contrary to what was previously theorized in the literature. The previous hypothesis was that upon calcium influx into the cell, CaM binds to the IQ motif and activates the GRFs, such that they can now activate Ras. In Chapter Two, I show that GRF2 mutants that no longer bind CaM are still calcium-responsive and activate Ras to equivalent levels as the wildtype protein. Also, an IQ deleted protein can activate Ras but cannot activate any of the downstream components in the Raf-ERK cascade, indicating that GRF2 can influence the activation state of at least one Ras effector. I also provide evidence that the N-terminus of GRF2 may have a modest inhibitory effect on the Cdc25 domain, as proteins deleted for this region are up to twice as active as full-length GRF2 in vitro and in vivo.

The crystal structure of the Cdc25 domain of Sos bound to Ras shows that the REM motif and the Cdc25 domain together bind to Ras and stimulate GDP release. The DB of GRF2 is situated between the REM and Cdc25 domain, tempting speculation that binding of GRF2 to Ras could expose the DB to the ubiquitination machinery. In Chapter Three, I provide evidence that this is indeed the case: GRF2 protein is destroyed after binding Ras and this destruction is prevented by the removal of the DB. GRF2 is ubiquitinated in vivo and the ubiquitination is severely decreased in point mutants that eliminate Ras binding. Experiments using proteasome inhibitors determined that GRF2 is destroyed by the proteasome. I present data showing that GRF2 is phosphorylated at multiple sites on two peptides located near the DB; these phosphorylation events may be involved in targeting
GRF2 for ubiquitination. Taken together, these data demonstrate that GRF2 is an unstable protein that is targeted for ubiquitination and destruction upon Ras binding.

The model that I propose for the regulation of GRF2 is as follows: GRF2 is activated by calcium, resulting in a relocation to the plasma membrane and an activation of Ras, with the GEF playing a role in determining which effector pathways are activated. The binding of GRF2 to Ras through the REM and Cdc25 domains results in the exposure of the DB, targeting GRF2 for ubiquitination and subsequent destruction by the 26S proteasome. Perhaps the protein must be destroyed after S phase in order to prevent activation of Ras during G2/M calcium waves.

In Chapter Four, I summarize the data presented above. Outstanding questions that have yet to be answered are discussed. Some of these questions include: a) what proteins are responsible for the ubiquitination of GRF2? b) what are the extracellular ligands and upstream signaling components that activate GRF2? c) what is the function of the oligomerization of GRF2? and d) what is the role of the IQ motif? Suggested experiments to address these issues are described, as are their expected outcomes and possible interpretations.
CHAPTER 2

Calmodulin-independent coordination of Ras and ERK activation by Ras-GRF2


I performed the experiments presented in this chapter except for the following: construction of the epitope-tagged Ras-GRF2 ΔPH mutants was done by C. Anne Koch and construction of the epitope-tagged GRF2 ΔDH mutant was done by Wing-Tze Fan, the in vitro exchange assays (Fig. 2-3 except for REM-Cdc25 and Fig. 2-9) were done by C. Anne Koch, and the ERK kinase assay (Fig. 2-5) was performed by Wing-Tze Fan.
ABSTRACT

GRF2 is a widely expressed, calcium-activated regulator of the small-type GTPases Ras and Rac. It is a multidomain protein composed of several recognizable sequence motifs: PH, coiled-coil, IQ, DH, PH, REM (Ras Exchanger Motif), PEST/destruction box, and Cdc25. The DH and Cdc25 domains possess guanine nucleotide exchange factor (GEF) activity, and interact with Rac and Ras, respectively. Herein I examine whether the other N-terminal domains of GRF2 regulate Ras activation and signaling. It has been suggested that the binding of calmodulin, which requires an intact IQ domain, is important for regulating calcium-induced signaling by GRF2. I show that calmodulin binding requires several amino-terminal domains of GRF2 in addition to the IQ sequence. No correlation was found between calmodulin binding by GRF2 and its ability to directly activate Ras and indirectly stimulate the MAP kinase ERK in response to calcium. The precise role of the GRF2-calmodulin association therefore remains to be determined. In addition, the REM-Cdc25 region is sufficient for maximal activation of Ras in vitro and in vivo causes Ras and ERK activation independent of calcium signals suggesting that, at least when overexpressed, it contains all of the determinants required to access and activate Ras signaling. Further mutational analysis of the N-terminal region of GRF2 indicates that the carboxyl PH domain imparts a modest inhibitory effect on Ras GEF activity and probably normally participates in intermolecular interactions. Interestingly, a GRF2 mutant missing the IQ sequence was competent for Ras activation, but failed to couple this to stimulation of the ERK pathway. This demonstrates that Ras-GTP formation is not sufficient for MAP kinase signaling. I
conclude that in addition to directly activating Ras, GRF2, and likely other GEFs, promotes the assembly of a protein network able to couple the GTPase with particular effectors.

INTRODUCTION

GRF2 is a widely expressed guanine nucleotide exchange factor that stimulates the release of bound guanine nucleotide by the low-molecular-weight G protein Ras (40, 66). GRF2 stimulates the conversion of Ras from its GDP-bound state into a GTP-bound activated conformation. The Ras-binding domain of GRF2 that catalyzes the activation of Ras is located in its carboxyl-terminal region, and is approximately 40% identical to the Ras GEF domain of the Saccharomyces cerevisiae Cdc25 gene product (66). GRF2 is a bifunctional GEF: in addition to its activity on Ras, it binds to the small G protein Rac through its Dbl homology (DH) domain (67).

By virtue of its two distinct GEF activities, GRF2 is a potent activator of two different MAP kinases which function downstream of Rac and Ras (67). They are, respectively, the stress-activated protein kinase (SAPK) and the extracellular signal-regulated protein kinase (ERK). The brain-specific protein GRF1 shares a similar domain structure to GRF2 (32, 203), and recently its DH domain was demonstrated to possess Rac GEF activity (120). The Son-of-sevenless gene product (Sos) also has been demonstrated to function as a Rac GEF (159). The frequent coupling of Ras and Rac GEF activities into a single polypeptide may reflect a strict requirement for the coordination of Ras and Rac effector pathways.

GRF2 has not been subjected to three dimensional structural analysis, but inspection of its primary sequence and functional studies suggest that it is a modular protein composed
of discrete functional domains (66, 67). It contains, in amino-to-carboxyl order, a pleckstrin homology (PH) domain, coiled-coil, illimaquinone (IQ) motif, DH domain, a second PH domain, a Ras exchanger motif (REM), a PEST-region (rich in the amino acids proline, glutamate, serine and threonine) that contains a candidate destruction box (DB), and, finally, the Cdc25 domain (66). Based on the solved structure of the REM and Cdc25 regions of the Son-of-sevenless (Sos) protein, it is likely the REM and Cdc25 regions of GRF2, and indeed of all Ras GEFs, interact to form a stable Ras-binding domain (16). DH domains, including that of GRF2, are flanked on their carboxyl side by a PH domain. In Sos, this neighboring PH domain may stabilize the Rac-binding region in the DH domain (209) and may be affected by the lipid products of phosphatidylinositol 3'-kinase (PI3K) (159). Hence, both the Cdc25 and DH classes of GEF domain are augmented and perhaps regulated by a neighboring non-catalytic domain. However, the intra- and inter-molecular interactions involving the various non-catalytic domains in GRF2 have not been determined.

Activation of the SAPK and ERK pathways by GRF2 is stimulated by calcium influx, and this requires the IQ motif of GRF2, which is required to maintain the calcium-dependent binding of calmodulin (CaM) to GRF2 (66, 67). Relative to vector conditions, cells expressing GRF2 exhibit a five- to six-fold increase in basal ERK1 activity. This increase is further enhanced an additional two- to three-fold when intracellular calcium levels are elevated by the addition of the calcium ionophore, ionomycin. The ΔIQ protein does not respond to calcium and does not increase basal activity of ERK1, nor is it calcium responsive. It has been shown to be defective in CaM binding (66) leading to the hypothesis that CaM binding is required for activation of GRF2.
GRF1-mediated activation of ERK is also regulated in a similar fashion (70). However, in contrast to the signaling data, GRF1 Ras-specific GEF activity does not appear to be stimulated by the binding of calmodulin in vitro. In fact, some studies have shown that this association inhibits the Cdc25 domain (9). Therefore, the precise role of CaM in the regulation of GRF2 and GRF1 and their CaM-binding site(s) remain to be determined. Activated GRF1 is phosphorylated on serine/threonine (144) and tyrosine (120) suggesting that phosphorylation may play a direct role in the regulation of the GRFs.

In this study, I addressed the structure-function relationships in GRF2 as well as the precise role of CaM binding in the regulation of GRF2. Deleted and truncated versions of GRF2 were constructed and analyzed for their ability to bind CaM, to activate Ras in vitro and in vivo, and to stimulate the ERK pathway. The data indicate that CaM binding does not correlate with Ras-ERK activation, which is contrary to the previously held belief that CaM binding activates GRF-mediated signaling. This analysis indicates that GRF2 is a modular protein subject to complex regulatory mechanisms involving its non-catalytic domains.

MATERIALS AND METHODS

Construction of Flag-tagged GRF2 deletions.

The ΔIQ protein was constructed by deleting codons 205-229 by PCR using the following primers: (1) a 5’ primer that contains the BamHI site that is adjacent to the nucleotides to be deleted followed by GRF2 codons 230-236 and (2) a 3’ primer containing primer sequences that flank the Apal site at the 3’ end of the GRF2 cDNA. The PCR product
was digested with BamHI and ApaI and subcloned into BamHI-ApaI digested pcDNA3-Flag-GRF2 cDNA.

The ΔDH mutant was constructed using two rounds of PCR. The ΔDH construct was obtained by deleting GRF2 codons 245-459 with the first two reactions using the following primers: (1) a 5' primer that deletes the above codons and a 3' flanking primer that anneals to codons 933-925; (2) a 3' primer that deletes the above codons and a 5' flanking primer that anneals to codons 124-131 of GRF2. These two PCR products were mixed and used as a template for a second round of PCR using the flanking primers to generate a PCR product deleted for codons 245-459. The PCR product was digested with BamHI and Ndel and the 608 bp fragment containing sequences deleted for codons 254-459 was isolated. The pcDNA3-Flag-GRF2 plasmid was digested with BamHI and XhoI and the 6021 bp fragment containing vector sequences and GRF2 sequences up to the unique BamHI site was isolated. The pcDNA3-Flag-GRF2 plasmid was also digested with Ndel and XhoI and the 1804 bp fragment that contains GRF2 sequences at the 3' end of the gene was isolated. The BamHI-XhoI and Ndel-XhoI-digested fragments were ligated together with the BamHI-Ndel-digested PCR product to produce the ΔDH mutant of GRF2.

The GRF2 PH deletion mutants were generated by doing two rounds of PCR. The ΔPHn construct was obtained by deleting GRF2 codons 23-135, with the first two reactions using the following primers: (1) a 5' primer flanking a unique SpeI site and a 3' primer that deletes the above codons; (2) a 5' primer that deletes the above codons and a 3' primer that flanks the unique BamHI site in GRF2. These two PCR products were mixed and used as a template for a second round of PCR using the BamHI and SpeI flanking primers, resulting in a PCR product deleted for codons 23-135. The PCR product was digested with BamHI and
$SpeI$ and subcloned into $SpeI$-$BamHI$-digested pcDNA3-Flag-GRF2. GRF2 codons 460-590 were deleted to generate the ΔPHc mutant, using the following primers: (1) a 5' primer flanking a unique $BamHI$ site and a 3' primer that deletes the above codons; (2) a 5' primer that deletes the above codons and a 3' primer that flanks the unique $KpnI$ site in GRF2. These two PCR products were mixed and used as a template for a second round of PCR using the $BamHI$ and $KpnI$ flanking primers, resulting in a PCR product deleted for codons 460-590. The PCR product was digested with $BamHI$ and $KpnI$ and subcloned into $BamHI$-$KpnI$-digested pcDNA3-Flag-GRF2. ΔPHn+c, which deletes both PH domains, was obtained by digesting the ΔPHn and ΔPHc constructs each with $BamHI$ and $KpnI$, and ligating together the 343 bp ΔPHn insert with the 8010 bp fragment containing pcDNA3-FlagGRF2ΔPHc.

The REM-Cdc25 construct was generated by PCR, amplifying codons 591-1189 of GRF2, using a 5' primer containing a $KpnI$ site, Kozak sequence and Flag sequence, and a 3' primer containing a stop codon and a $BamHI$ site. The PCR product was digested with $KpnI$ and $BamHI$ and subcloned into $KpnI$-$BamHI$-digested pcDNA3.

The ΔCdc25 construct was constructed by amplifying nt 1770-2799 by PCR using full-length GRF2 DNA as a template. This construct deletes codons 934-1189. A stop codon was added after nt 2799 by the addition of the sequence "tca" in the 3' primer used for PCR. The PCR product was cloned into the pCR-Blunt II-TOPO PCR vector using the ZeroBlunt TOPO PCR cloning kit (Invitrogen) and the construct verified by automated sequencing. Nucleotides 1807-3570 of full-length pcDNA3-Flag-GRF2 were replaced by nt 1807-2799 (plus a stop codon) by digesting the PCR product in pCR-Blunt II-TOPO with $EcoRI$ and the 1.1 kb fragment ligated to the 7 kb fragment produced by digestion of
pcDNA3-Flag-GRF2 with EcoRI. A variety of restriction digests were performed to verify correct orientation of the 1.1 kb fragment.

All constructs were confirmed by automated sequencing (York University Core Molecular Biology Facility) or enzymatic restriction analysis.

**Cell culture and transfections.**

293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 4.5 g/L L-glutamine, 10 μM nonessential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. 293T cells grown in 10 cm dishes were transiently transfected by calcium phosphate coprecipitation as described previously (115).

**Western blotting.**

After separation of samples by SDS-PAGE, proteins were electrophoretically transferred to 0.45 micron pore-size nitrocellulose using a semi-dry transfer apparatus (Bio-Rad). Western blots were blocked with 5% (w/v) non-fat dry milk in phosphate buffered saline containing 0.1% (v/v) Tween-20 (PBST) for one hour at room temperature, then probed with primary antibody diluted in 5% (w/v) milk/PBST for one hour at room temperature or overnight at 4°C. Blots were washed twice in PBST for 10 minutes each, once in phosphate buffered saline (PBS) for 10 minutes, and then probed with horseradish peroxidase-conjugated goat anti-mouse (or goat anti-rabbit) IgG (Bio-Rad) diluted 1:20,000 in 5% milk/PBST for one hour at room temperature. Blots were washed as described above and protein bands were visualized by enhanced chemiluminescence using SuperSignal Chemiluminescent Substrate (Pierce). Antibodies for MEK and phospho-MEK were
purchased from New England Biolabs, the Raf antibody was purchased from Transduction Labs and the phospho-Raf antibodies were purchased from Medicorp. The M2 anti-Flag antibody was purchased from Kodak (now from Sigma) and the CaM antibody from Upstate Biotechnology, Inc.

**In vitro guanine nucleotide exchange assays.**

293T cells expressing wildtype (WT) GRF2 or mutant proteins were rinsed with phosphate-buffered saline (PBS) and lysed with ice cold NP40 lysis buffer (20 mM Tris-HCl pH 7.5; 50 mM NaCl; 1% Nonidet P-40; 50 mM NaF; 10 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 10 μg/mL aprotinin; 0.1 mM AEBSF and 10 μg/mL leupeptin). Lysates were clarified and immunoprecipitated with 5 μg of anti-Flag antibody coupled to agarose (Kodak) for 90 minutes at 4°C. Immunoprecipitates were washed twice with lysis buffer and twice with exchange buffer (25 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.06% Nonidet P-40; 1 mM MgCl₂; 1 mM dithiothreitol (DTT); 1.25 mg/mL BSA). Exchange reactions (20 μL per reaction) were performed essentially as described in Downward (58) and contained 100 ng GTPase in solution, 500 ng GRF2, 10 μM GTP and 3.3 μCi [α-32P]GTP. After 0 or 20 minutes at 23°C, reactions were terminated by the addition of 5 mL ice-cold stop buffer (25 mM Tris-HCl, pH 7.5; 100 mM NaCl; 5 mM MgCl₂), and filtration over 0.45 micron pore-size nitrocellulose followed by five washes with stop buffer. Dried filters were dissolved in ethyleneglycol monomethylether and quantified by liquid scintillation counting. Values are ± s.e.m. and corrected by subtraction of spontaneous nucleotide exchange which occurred during the 20 minute incubation period. For exchange assays measuring the effect of bound calmodulin, the WT Flag immunoprecipitates were
washed in buffer without EGTA or with 1 mM EGTA to remove calmodulin as described previously (66) and then used in exchange assays as described above.

**ERK1 in vitro kinase assays.**

293T cells were transiently cotransfected with 4 μg of pJ3M-ERK1 (encoding myc epitope-tagged ERK1) and 4 μg of either pcDNA3 vector or pcDNA3-Flag-GRF2 constructs as indicated. After 48 hours, the cells were serum starved for 18 hours and then stimulated with 5 μM of ionomycin for 5 min at 37°C, washed in PBS, and then lysed in ice-cold NP40 lysis buffer. Clarified lysates were incubated with 1 μg of 9E10 Myc monoclonal antibody precoupled to 20 μL goat anti-mouse IgG-agarose beads (Sigma) for 2 h at 4°C with gentle rotation to immunoprecipitate myc-ERK1 proteins. The beads were then washed twice with lysis buffer and twice with kinase buffer (50 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 1 mM DTT). Kinase reactions were started by resuspending immunoprecipitates in 30 μL of kinase buffer containing 0.25 mg/mL of myelin basic protein (MBP), 25 μM ATP and 10 μCi [γ-³²P]ATP (6000 Ci/mmol; NEN) and incubated for 20 minutes at 30°C. Reactions were stopped by adding 15 μL of 3x SDS sample buffer; the mixtures were heated for 5 minutes at 95°C and then separated by SDS-PAGE. After Coomassie Blue staining, gels were dried and MBP phosphorylation was quantified using a Bio-Rad GS250 phosphorimager and Molecular Analyst software (Bio-Rad).

**Raf-1 kinase assay.**

293T cells were transiently transfected with GRF2 and mutant proteins. After 24 hours, cells were serum-starved for 18 hours and then either treated with 4 μM ionomycin for 5 min. at 36°C or left alone. Cells were rinsed in PBS and then lysed in ice-cold NP40 lysis
buffer. Clarified lysate was used to immunoprecipitate Raf-1 which was then used in the Raf-1 Kinase Cascade Assay Kit (Upstate Biotechnology). Briefly, immunoprecipitates were incubated with 35 μL of assay buffer containing GST-MEK1, GST-MAPK, ATP and magnesium for 30 minutes at 30°C. Beads were pelleted and 4 μL of the supernatant was removed and incubated in 35 μL of buffer containing MBP and [γ-32P]ATP. Reactions were incubated at 30°C for 10 minutes and then spotted onto phosphocellulose filters. Filters were washed and then phosphorylation levels measured by scintillation counting.

Calmodulin binding.

293T cells were transiently transfected with 8 μg of pcDNA3 or pcDNA3-Flag-GRF2 constructs as indicated. Forty-eight hours after transfection, the cells were washed in PBS and lysed in ice cold NP40 lysis buffer. The lysates were clarified and protein concentration was determined using a Bradford assay, Coomassie Plus Protein Assay Reagent (Pierce). Equal amounts of protein in lysates were precleared with goat anti-mouse IgG-agarose beads, then immunoprecipitated with 3 μg of anti-Flag (M2) monoclonal antibody in the presence of 20 μL goat anti-mouse IgG-agarose beads for 2 hours at 4°C with gentle rotation. The immunoprecipitates were washed 3 times in NP40 lysis buffer, and then resuspended and boiled in 30 μL of Laemmli loading buffer. The samples were then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Flag (M2) or anti-calmodulin antibodies.

Activated Ras pulldown assay.

GST-RBD (Ras binding domain) fusion protein containing the Ras-binding domain of Raf was prepared as follows: bacteria containing the plasmid pGEX2T-Raf (amino acids 1-
149) (kindly provided by Steve Taylor) were grown overnight at 37°C in 20 mL of Terrific Broth containing 100 μg/mL ampicillin (TB + Amp). Bacteria were diluted into 400 mL TB + Amp the next morning and grown until A₆₀₀ = 0.7. GST fusion protein expression was induced by the addition of 1 mM isopropylthio-β-D-galactoside (IPTG) at 37°C for 2 hours. Bacteria were pelleted by centrifugation at 4°C, washed in HEPES-buffered saline (HBS: 25 mM HEPES, pH 7.5; 150 mM NaCl) and either lysed directly or frozen at -70°C for later use. Bacteria were resuspended in 10 mL of ice cold bacterial lysis buffer (BLB: 20 mM HEPES, pH 7.5; 120 mM NaCl; 10% glycerol; 2 mM EDTA; 10 μg/mL leupeptin; 10 μg/mL aprotinin; 0.1 mM AEBSF) and sonicated to lyse cells. Nonidet P-40 (NP40) was added to a concentration of 0.5%, and lysate was clarified by centrifugation at 20,000 g, 15 minutes, 4°C. The supernatant was saved and incubated with 500 μL of washed glutathione-agarose beads (Sigma) for 15-30 minutes at 4°C with gentle rotation. Beads were collected by centrifugation and washed 8 times with BLB + 0.5% NP40, and stored in BLB + 0.5% NP40 + 1 mM DTT at 4°C.

293T cells were transiently transfected with 5 μg of pcDNA3 or pcDNA3-Flag-GRF2 constructs as indicated. Forty-eight hours after transfection, the cells were washed in HBS and lysed in ice cold MLB (25 mM HEPES, pH 7.5; 150 mM NaCl; 1% NP40; 0.25% NaDOC; 1 mM EDTA; 10% glycerol; 10 mM MgCl₂; 25 mM NaF; 10 μg/mL aprotinin; 10 μg/mL leupeptin; 1 mM sodium orthovanadate; 0.1 mM AEBSF). Clarified lysates were used in a Bradford assay to determine protein concentration. Levels of activated Ras in the lysate were determined as described below. 50 μg of GST-RBD (~20 μL of beads) was incubated with 2.5 mg of lysate for 30 minutes at 4°C with gentle rotation. Beads were collected by centrifugation and washed 3 times with MLB. 20 μL of 2x SDS sample buffer
was added, samples were heated to 95°C for 5 minutes and proteins were separated by SDS-PAGE. The level of activated Ras bound to the beads was detected by immunoblotting using an anti-Ras antibody (LA045; Quality Biotech).

Partial proteolysis.

GRF2 or deletion mutants were transiently transfected into 293T cells. After 48 hours, cells were rinsed in PBS, and lysed in ice cold NP40 lysis buffer. The lysate was clarified and then used in a Bradford assay. Using equal amounts of total protein, the lysates were precleared with 30 μL goat anti-mouse IgG-agarose (Sigma) for 45 minutes at 4°C with gentle rotation. Lysates were then incubated with 2 μg of M2 anti-Flag antibody in the presence of 20 μL goat anti-mouse IgG-agarose for 2 hours at 4°C with gentle rotation. Immune complexes were collected by centrifugation and washed 2 times with NP40 lysis buffer and 2 times with pH 7.5 buffer (10 mM Tris, pH 7.5; 100 mM NaCl; 5 mM KCl; 1 mM CaCl₂; 0.5 mM MgCl₂). Beads were divided into four tubes. To each tube, 30 μL of pH 7.5 buffer was added which contained from 0 to 10 μg of TPCK-treated trypsin (Worthington Diagnostics). Reactions were incubated on ice for 15 minutes and then stopped with bovine pancreas trypsin inhibitor at a 2 times molar concentration. Beads were collected and the supernatant, containing the released trypsin fragments, was collected and separated by SDS-PAGE. Samples were Western blotted with a GRF2 polyclonal antibody raised against the Cdc25 domain.

RESULTS

To investigate the function of the N-terminal region of GRF2, different deletion mutants, including one with a deletion of the entire N-terminus, were made (Fig. 2-1) and all
of these mutant proteins were expressed to similar levels in transiently transfected 293T cells (see below). To determine if these large deletions affected the folding of GRF2, wildtype

Figure 2-1  Schematic representation of the constructs used in this report.

The various abbreviations are described in the text. The constructs were made using murine GRF2: ΔCdc25 is missing codons 934-1189; ΔPHc, codons 460-590; ΔPHn, codons 23-135; ΔDH, codons 245-459; ΔIQ, codons 205-229; ΔPHn+c, codons 23-135 and codons 460-590; ΔDB, codons 742-751; ΔREM, codons 638-686; and REM-Cdc25 contains codons 591-1189.

(WT) and deletion mutant proteins were subjected to limited proteolysis with trypsin (Fig. 2-2). Specifically, GRF2 proteins in the form of anti-Flag immunoprecipitates were incubated with a range of concentrations of trypsin, followed by separation of the digestion products by...
SDS-PAGE, and analysis and imaging of the protease-resistant domains by using purified polyclonal antibodies directed against the Cdc25 domain (Fig. 2-2). The results for the WT and each of the proteins were essentially equivalent, giving rise to similar series of trypsin-resistant domains derived from the Cdc25 regions of the protein, indicating that the structure of the deletion mutants was not grossly altered.

Figure 2-2 Partial trypsin proteolysis of GRF2 and the various deletion mutants.
Assays were carried out 2 days after transfection of 293T cells with the indicated construct. GRF2 or the deletion mutants were isolated by anti-Flag immunoprecipitation and the immune complexes were treated with increasing amounts of trypsin (as indicated above each lane) on ice for 15 minutes. The supernatant containing released fragments was separated by SDS-PAGE and blotted with purified anti-Cdc25 domain polyclonal antibodies. The data shown are representative of three experiments.
**In vitro and in vivo activity of GRF2 mutants.**

To determine the effect of deleting various portions of the N-terminus on Ras activation, WT GRF2 and the various mutants were compared for their abilities to catalyze *in vitro* nucleotide exchange of bound GDP for $^{32}$P-labeled GTP on purified, recombinant Ras. GRF2 constructs expressed in 293T cells were immunoprecipitated with anti-Flag antibodies and the extensively washed immunoprecipitates were then tested for guanine nucleotide exchange activity *in vitro* with GST-H-Ras.

**Figure 2-3  In vitro exchange activity of GRF2 and mutants.**

Assays were carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. GRF2 was immunoprecipitated and used in an *in vitro* Ras exchange assay as described in Materials and Methods. Each bar shows the mean exchange activity and standard error of at least three experiments (duplicate readings in each experiment).
Figure 2-3 shows that all the deletion mutants retained Ras GEF activity approximately equivalent to WT GRF2, with the exception of REM-Cdc25, whose activity was increased approximately 1.5-fold. The fact that the activity of the N-terminal deleted protein was increased suggests that the N-terminus may in fact impart a modest inhibitory effect on the Cdc25 domain. The activity of the mutant missing the IQ motif (ΔIQ) was equivalent to wildtype, showing that unlike GRF1 the binding of CaM to GRF2 *in vitro* does not inhibit the activity of the Cdc25 domain. These results indicate that GRF2 is a modular protein and that the amino-terminal domains of GRF2 are not required for *in vitro* nucleotide exchange on Ras by the Cdc25 domain.

**Figure 2-4  GTP-Ras in 293T cells expressing GRF and mutants.**

Assays were carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. 2.5 mg of lysate was incubated with 50 μg of GST-RBD beads for 30 minutes at 4°C to pull down activated Ras. In the upper panel, the samples were Western blotted for Ras using the LA045 pan-Ras monoclonal antibody, thereby assessing levels of GTP-bound Ras within the cells. In the lower panel, the presence of GRF2 proteins was verified by Western blotting of lysates with Flag antibody. The data shown are representative of four experiments.
To test if the REM-Cdc25 protein was functional in vivo and to measure the activity of the various GRF2 deletion mutants, cell lysates expressing these proteins were analyzed for Ras-GTP levels using the pulldown assay of Taylor and Shalloway (219). All of the Flag epitope-tagged proteins were comparably expressed in transfected 293T cells and migrated at their expected molecular masses relative to WT GRF2 (Fig. 2-4, lower panel). Consistent with Figure 2-3, the N-terminal truncation mutant containing the REM and Cdc25 domains was approximately twice as efficient at activating Ras in vivo compared to WT GRF2 (Fig. 2-4, upper panel). The Cdc25-deleted protein (ΔCdc25) and a negative-control mutant missing residues 687 to 933 which are located immediately amino-terminal to and perhaps infringing on the Cdc25 domain, were inactive towards Ras in vivo. The deletion mutant missing the amino-terminal PH domain (ΔPHn) was as active as WT GRF2, whereas the mutants missing the carboxyl PH domain (ΔPHc) or both PH domains (ΔPHn+c) were more active (1.5-fold) than WT GRF2. Activated Ras was not detected in lysates from cells transfected with the empty expression vector (Fig. 2-4, Lane 1). It was shown previously that the mutant missing the DH domain (ΔDH) possesses a WT level of activity in terms of ERK activation (67). As expected, this protein was able to cause activation of Ras in vivo that was similar to activation by WT GRF2 (Fig. 2-4, Lane 7). Unexpectedly, the mutant missing the IQ motif, previously found to be defective in basal and calcium-stimulated ERK activation (67), was still able to cause activation of Ras in vivo. These results confirm that expression of GRF2 in 293T cells causes activation of endogenous Ras proteins and that domains other than REM-Cdc25 are dispensable for this activity.
Effect of GRF2 deletion mutants on ERK1 signaling.

To investigate whether the N-terminal region of GRF2 plays a role in ERK1 signaling, *in vitro* kinase assays were performed. 293T cells were cotransfected with the GRF2 constructs and myc-tagged ERK1 mitogen-activated protein kinase (MAPK).

Figure 2-5  **ERK1 in vitro kinase assay in 293T cells.**
Assays were carried out 3 days after transfection of 293T cells with the indicated GRF2 construct and myc-ERK1. Cells were serum starved for 18 h and then either left untreated (−) or treated with 5 μM ionomycin for 5 min at 37°C (+). In the lower panel, ERK activity was determined with an immune complex kinase assay using MBP as substrate. Equal precipitation of myc-ERK1 was confirmed by Western blotting (middle panel). In the upper panel, GRF2 expression was verified by Western blotting of the lysates with anti-Flag antibody. The data shown are representative of three experiments.

After serum deprivation for approximately 18 hours, the cells were treated for 5 minutes with the calcium ionophore, ionomycin, or left untreated. The cells were then lysed immediately, and ERK1 was immunoprecipitated with anti-myc antibodies. The immunoprecipitates were analyzed and quantified for kinase activity using myelin basic protein (MBP) as a substrate.
WT GRF2 and ΔPHn required stimulation with ionomycin to maximally activate ERK1 (Fig. 2-5, lanes 3 to 6), whereas the mutants missing the PH domain adjacent to the DH domain (ΔPHc and ΔPHn+c) were maximally activated under basal conditions (Fig. 2-5, lanes 9-10 and 11-12). Therefore, the PH domains of GRF2 are dispensable for ERK1 activation but are required for proper calcium stimulated activation of GRF2. The REM-Cdc25 protein was a potent activator of ERK1 and was more active (approximately 1.5-fold) than maximally stimulated WT GRF2 (Fig. 2-5, lanes 13-14). Activation of ERK1 by REM-Cdc25 was not stimulated by calcium and was maximal even under conditions of serum deprivation (Fig. 2-5). This suggests that the N-terminus of GRF2 plays an small inhibitory role in GRF2 regulation. Despite its ability to efficiently activate Ras (Fig. 2-4, (66)), the IQ-deleted GRF2 protein was defective for basal and calcium-stimulated ERK activation (Fig. 2-5, lanes 7-8).

ΔIQ does not activate Raf or MEK.

As demonstrated in Fig. 2-4 and 2-5, the ΔIQ protein can activate Ras but not ERK1, so I decided to examine the activation state of Raf and MEK, two kinases upstream of ERK in this Ras signaling cascade. MEK activity was assayed by using a phosphorylation specific antibody as phosphorylation at Ser 217/221 correlates with activity (Fig. 2-6). EGF, as a positive control, activates MEK strongly (lane 3). GRF2 expression increased the basal activation of MEK and this activation was further potentiated upon ionomycin stimulation, similar to that seen with ERK. The ΔCdc25 protein did not activate MEK, as expected, because it cannot activate Ras. The ΔDH protein activated MEK to levels equivalent to wildtype GRF2; however, the ΔIQ protein did not activate MEK over what was seen in vector conditions.
Figure 2-6 MEK activity induced by GRF2 in 293T cells.
Assays were carried out 3 days after transfection of 293T cells with the indicated GRF2 construct. Cells were serum starved for 18 hours and then either left untreated (−) or treated with 4 μM ionomycin for 5 minutes at 37°C (+) or 100 ng/mL EGF for 10 minutes at 37°C. In the lower panel, MEK activity was determined by blotting lysates with a polyclonal phospho-specific MEK antibody. Equal expression of MEK was confirmed by Western blotting of the lysate with a polyclonal MEK antibody (middle panel). In the upper panel, GRF2 protein expression was verified by Western blotting of the lysates with M2 anti-Flag antibody. The data shown are representative of three experiments.

Raf-1 activity was measured by incubating Raf-1 immunoprecipitates with GST-MEK1 and GST-MAPK and then assessing the activation state of MAPK by assaying its phosphorylation of MBP (Fig. 2-7). EGF stimulation of 293T cells transfected with vector alone was also performed as a positive control. Again, wildtype GRF2 and ΔDH activated Raf to a level similar to that of EGF, while the ΔCdc25 and ΔIQ proteins did not activate Raf significantly over vector conditions. These data suggest that the IQ motif plays a role in the activation of Raf downstream of Ras, but is dispensable for activation of Ras itself.
c-Raf-1 in vitro kinase assay in 293T cells.

Assays were carried out 3 days after transfection of 293T cells with the indicated GRF2 construct. Cells were serum starved for 18 hours and then either left untreated (−) or treated with 4 μM ionomycin for 5 minutes at 37°C (+), or with 100 ng/mL EGF for 10 minutes at 37°C. c-Raf-1 was immuno-precipitated from cell lysate and used with an in vitro kinase assay kit from Upstate Biotechnology. GRF2 protein expression was verified by Western blotting of the lysates with M2 anti-Flag antibody. The data shown are representative of three experiments.

When Raf is in an inactive state, members of the 14-3-3 protein family are bound to it through phosphoserine at position 259; this interaction obscures the cysteine rich domain (CRD) and also serves to stabilize the inactive conformation (151). When Ras becomes GTP-loaded, the Ras binding domain (RBD) of Raf binds to Ras, displacing 14-3-3 from the Ser259 site and unmasking the CRD which is now accessible for Ras binding. The binding
of the CRD to Ras and phosphatidylserine results in a change in Raf-1 conformation that serves to expose the kinase domain. The 14-3-3 that was displaced from Ser259 is now free to bind to the higher affinity Ser621 site; this interaction may serve to stabilize an "open" Raf-1 conformation (155). This "open" Raf-1 may now be further stimulated by other modifications occurring at the membrane, such as tyrosine phosphorylation of residues 340 and 341 by Src family members. If Ser259 or Ser621 are aberrantly phosphorylated in the presence of the ΔIQ protein, this could perhaps have an effect on Raf activation.

Figure 2-8  Phosphorylation of Raf at Ser259 and Ser621.
Assays were carried out 3 days after transfection of 293T cells with the indicated GRF2 construct. Cells were serum starved for 18 hours and then either left untreated (−) or treated with 4 μM ionomycin for 5 minutes at 37°C (+), or with 100 ng/mL EGF for 10 minutes at 37°C. GRF2 protein expression was verified by Western blotting of lysate with M2 anti-Flag antibody (upper panel). Equal expression of Raf was confirmed by Western blotting lysates with a polyclonal antibody against Raf (second panel). The phosphorylation state of Ser259 was assessed by Western blotting lysate with a phospho-specific antibody directed against Ser259 of Raf (third panel). The phosphorylation state of Ser621 was assessed by Western blotting lysate with a phospho-specific antibody directed against Ser621 of Raf (fourth panel). The data shown are representative of three experiments.
To test this, Western blots were performed using lysates from 293T cells transiently transfected with GRF2 and mutants (Fig. 2-8). There was no detectable differences between wildtype GRF2 and any of the mutants with respect to phosphorylation of S259 and the same statement holds true for the phosphorylation of Ser621 (Fig. 2-8, third and fourth panels, respectively). However, the antibodies detected no differences in the phosphorylation state of Raf after stimulation with ionomycin or EGF. This suggests either that the antibodies are not able to detect differences in phosphorylation or that the theories mentioned above regarding the regulation of Raf activity are not entirely correct; it is more likely that the commercially available antibodies to look at the phosphorylation state of these two serine residues are not properly recognizing the phosphorylated residues in c-Raf-1 in this experiment. Therefore, I cannot conclude whether a difference in 14-3-3 binding to either phosphoserine residue manifests itself as an inability of Ras to activate ERK1 in the presence of the ΔIQ protein.

**Role of calmodulin association in GRF2 activity.**

It was reported previously that the association of calmodulin with GRF2 is dependent on an intact IQ domain and is calcium-dependent (66). Since deletion of this domain impaired GRF2-mediated activation of ERK1, but not Ras, I sought to further examine the correlation of the GRF2-CaM complex with GRF2 activity. First, the effect of CaM on the *in vitro* GEF activity of GRF2 was examined. Second, the association of CaM with the various GRF2 variants was measured.

GRF2 isolated by immunoprecipitation was treated without or with EGTA to remove CaM as reported previously (66), and the presence or absence of CaM was verified by anti-
CaM immunoblotting (Fig 2-9a). GRF2 was then tested for Ras GEF activity. As shown in Fig. 2-9B, there was no effect of associated calmodulin on GRF2 catalytic activity measured by the *in vitro* GEF assay. Therefore, loss of CaM association either by deletion of the IQ motif or by calcium chelation with EGTA does not affect the Ras GEF activity of GRF2.

GRF2 immunoprecipitates containing approximately equivalent quantities of the indicated WT and mutant GRF2 proteins were analyzed for CaM by immunoblotting (Fig. 2-10a).

**Figure 2-9** *In vitro* exchange activity of GRF2 +/- calmodulin. Assays were carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. (A) Wild type GRF2 IPs were washed in buffer without or with 1 mM EGTA to remove bound calmodulin which was confirmed by Western blotting the IPs with a monoclonal calmodulin antibody. (B) The washed immune complexes were used in an *in vitro* Ras exchange assay as in Fig. 2-3. Each bar shows the mean exchange activity and standard error of two experiments (duplicate readings in each experiment).
Surprisingly, all of the mutants tested were impaired to some extent in their association with CaM. In addition, the extent of CaM association did not change with ionomycin treatment (52). GRF2 with either or both PH domains or the Cdc25 domain deleted still retained detectable CaM association, but at a level at least 10-fold reduced compared to WT GRF2. The REM-Cdc25 immunoprecipitate did not contain detectable CaM. As expected, the IQ-deleted GRF2 mutant did not bind CaM, which had been shown previously (66). The mutant lacking the destruction box motif (ΔDB) retained a WT level of associated CaM, whereas the DH-deleted protein (ΔDH) was devoid of associated CaM which is a surprise as this mutant is calcium responsive. The associated CaM could also be detected by silver staining an SDS-PAGE gel of the immunoprecipitates (Fig.2-10b).

Figure 2-10  Calmodulin binding to GRF2 or mutants in 293T cells.
Assays were carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. GRF2 and mutants were immunoprecipitated from 293T cells lysates using M2 anti-Flag antibodies. (A) The immune complexes were Western blotted using a monoclonal antibody against calmodulin. (B) The immune complexes were separated by SDS-PAGE and the gel was silver stained to visualize protein.
Figure 2-11  \( \Delta \text{Cdc25} \) protein acts as a dominant inhibitor.

Assays were carried out 2 days after transfection of 293T cells with the indicated GRF2 constructs. (A) Ras-GTP levels in 293T cells. 2.5 mg of lysate was incubated with 50 \( \mu \)g of GST-RBD beads for 30 minutes to pull down activated Ras. In the upper panel, the presence of GRF2 proteins was verified by Western blotting of lysates with anti-Flag antibody. In the lower panel, the samples were Western blotted for Ras, thereby assessing levels of GTP-bound Ras within the cells. In the middle panel, lysates were blotted for Ras to ensure equivalent levels of endogenous Ras between samples. (B) Quantitation of Ras-GTP levels in panel A. The amount of activated Ras was quantitated using a phosphor-imager. (C) Ras-GTP levels in 293T cells. In the upper panel, the presence of GRF2 proteins was verified by Western blotting of lysates with anti-Flag antibody. 2.5 mg of lysate was incubated with 50 \( \mu \)g of GST-RBD beads for 30 minutes to pull down activated Ras. In the middle panel, lysates were blotted for Ras to ensure equivalent levels of endogenous Ras between samples. In the lower panel, the complexes were Western blotted for Ras, thereby assessing levels of GTP-bound Ras within the cells.

The above results indicate that the interaction with CaM is not essential for calcium-induced signaling by GRF2. This suggests that the amino-terminal region of GRF2 interacts
with targets other than CaM that are important for GRF2 regulation. In support of this postulate, expression of the ΔCdc25 protein inhibited Ras activation mediated by WT GRF2 (Fig. 2-11a). The first two lanes of Fig. 2-11a are negative and positive controls, respectively, similar to the corresponding lanes in Fig. 2-4, and demonstrate the in vivo formation of Ras-GTP in cells expressing ectopic GRF2. Co-expression of ΔDH and WT GRF2 caused Ras-GTP formation to the levels expected based on the ability of both these proteins to activate Ras as shown in Fig. 2-4. Co-expression of ΔCdc25 and WT GRF2, however, interfered with Ras activation by WT GRF2 since Ras-GTP levels were only approximately 10% that expected based on the amount of WT GRF2 expression (Fig. 2-11b, right bar). Since the ΔCdc25 protein is unable to interact with Ras (67), and has only minimal interaction with CaM (see Fig. 2-10), it does not inhibit GRF2 by competing with GRF2 for interactions with these proteins. Titrating the expression of a mutant GRF2 protein missing the Cdc25 domain revealed that inhibition of WT GRF2 becomes more efficient as the level of mutant protein expression exceeds that of the WT protein (Fig. 2-11c). This suggests it functions as a competitive inhibitor, also referred to as dominant-negative inhibition. In this last experiment, the variant protein (ΔPHcΔCdc25) was missing both the PHc and Cdc25 domains, indicating that the carboxyl PH domain and any interactions it participates in are dispensable for the inhibitory activity.

The ability of Cdc25 domain-deleted GRF2 proteins to function as negative inhibitors would be explained by their direct binding to and inhibition of GRF2, by saturating putative membrane binding sites for GRF2 and/or by competing with GRF2 for interaction with other molecules required for GRF2 regulation. Self-association of GRF1 has been reported (4), and I have also detected intermolecular interactions between GRF2 molecules in reciprocal
co-immunoprecipitation experiments employing two differentially epitope-tagged GRF2 proteins (Fig. 2-12). Anborgh et al. (4) published that the oligomerization interface is within the DH domain of GRF1 and GRF2; however, I can still detect oligomer formation between wildtype GRF2 and the ΔDH protein (data not shown), suggesting either that the DH domain is not the oligomerization interface for GRF2 oligomers or that there is more than one self-association domain in GRF2.

Figure 2-12  GRF2 self-associates in 293T cells.
Assays were carried out 2 days after transient transfection of 293T cells with either Flag-GRF2, myc-GRF2 or both. GRF2 was immuno-precipitated from lysates and Western blotted for Flag-tagged GRF2 and myc-tagged GRF2 using M2 anti-Flag and 9E10 anti-myc monoclonal antibodies. The upper panel shows the western blots indicating that the two differentially tagged proteins interact. The middle panel demonstrates equal capture of proteins in the IPs. Equal expression of the constructs was confirmed by Western blotting of lysates (lower panel).
DISCUSSION

The primary sequence of GRF2 is indicative of a modular, multidomain protein, and even suggests some of the protein-protein interactions in which it may participate (40, 66). Indeed, the physical interaction of GRF2 with CaM and Ras is not surprising given its IQ and Cdc25 domains. Furthermore, since the GRF2-CaM interaction is calcium-dependent in vitro, it was logical to suggest that it accounts for the calcium-mediated activation of the ERK pathway by the Ras-GRF proteins. However, my findings indicate the regulation of GRF2 and its function in calcium-mediated stimulation of Ras and ERK are complex and are yet to be fully defined.

GRF2 is certainly a modular protein, and its carboxyl-terminal GEF region is remarkably tolerant of the various domain deletions analyzed here. The Ras-binding and Ras GEF activities of GRF2 reside in the carboxyl REM-Cdc25 region (40, 66, 67). The PH domains, DH domain, IQ sequence, and associated CaM are not required for these activities, and therefore do not obviously contribute to the stabilization of the functional Ras GEF domain. The REM-Cdc25 fragment was consistently up to twice as active as WT GRF2, which was also seen in vitro with a comparable GRF1 fragment (9). However, in vivo results with a similar GRF1 construct were not consistent with the in vitro results, in that MAPK activity was comparable to vector controls (101). While I cannot conclude this two-fold difference in GRF2 activity is biologically significant, it may indicate that GRF2 is capable of modest activation through relief of inhibition caused by intramolecular determinants that lie outside the REM-Cdc25 region. Similar conclusions have also been made for Sos (45).

The in vivo assay of GRF2-induced Ras-GTP formation gave results consistent with
those obtained in vitro, indicating that the PH domains, IQ sequence, DB, and DH domain are dispensable for the constitutive activation of Ras which accompanies GRF2 expression in 293T cells. Activation of Ras by the ΔDH mutant was expected since this protein is not diminished in its ability to stimulate ERK (67). This indicates that an ability to interact with Rac through the DH domain is not necessary for Ras-ERK signaling by GRF2. The PH domains of GRF2 are also not essential for GRF2 to access Ras and ERK. The three PH mutants (ΔPHn, ΔPHc, ΔPHn+c) were each active in the Ras and ERK assays, but the two mutants missing the PHc domain were more active than WT GRF2 for Ras activation (1.5-fold) and were fully active for ERK stimulation in the absence of Ca\(^{2+}\) treatment. One possible explanation for these differences is that the carboxyl Cdc25 domain might be directly affected by these mutations. However, the partial proteolysis results for the PH-deleted proteins were equivalent to wildtype GRF2. The proteins gave rise to similar series of trypsin-resistant domains derived from the Cdc25 regions of the protein, indicating that the PH domain deletions do not affect the intrinsic structure, and hence function, of their cognate Cdc25 domains. This favours the interpretation that these deletions affect protein function through their effects on intermolecular interactions. Since the ΔPHc mutants were competent for Ras-ERK signaling and activated Ras to a greater extent than WT GRF2 in vivo, I conclude that the REM-Cdc25 portion of GRF2 may be repressed in a manner dependent on the PHc domain in unstimulated cells.

The effects of deleting the DH and PHc domains in GRF2 contrast with those observed as a consequence of point mutations in the corresponding domains of GRF1 (77). In addition, deletion of the N-terminal PH domain in GRF2 and GRF1 has very different effects: GRF1 calcium-stimulated ERK activity is abolished, but there is little effect on
GRF2 signaling ((25), Fig. 2-5). For GRF1, the effect of deleting the N-terminal PH domain results in a substantial redistribution of the protein from the particulate to the cytosolic fraction of cells (25); therefore, this domain appears to be involved in targeting the protein to the membrane and appears to be required for maximal activation of Ras-ERK signaling. WT GRF2, on the other hand, is found predominantly localized in the cytosol of unstimulated cells (66) and further studies are aimed at identifying what factor(s) enable it to translocate to the cell periphery in response to calcium stimulation. My findings indicate that the REM-Cdc25 fragment contains the necessary localization determinants to access and activate the Ras-ERK pathway, at least when ectopically expressed in 293T cells.

Activation of Ras and activation of ERK are separable effects of GRF2, as seen with the ΔIQ protein which activates Ras but not Raf, MEK or ERK. A similar resolution of these two activities has been observed for GRF1 (4). Two models, not mutually exclusive, are apparent. One, as proposed by Anborgh et al. (4) for GRF1, suggests that calcium-stimulated ERK activation by GRF1 is Ras independent. I suggest that in the case of GRF2 it is Ras dependent as both basal and calcium-stimulated modes of ERK activation by GRF2 are fully inhibited by N17 Ras which targets the Cdc25 domains of Ras GEFs (67).

The role of calcium and CaM in GRF2 regulation.

The model in the literature regarding the role of calmodulin in activation of the GRFs is based on work done with GRF1. The model states that upon an increase in intracellular calcium, CaM binds to the GEF and this activates the protein, allowing for nucleotide exchange on Ras. It is apparent that this model is not correct when referring specifically to GRF2. It is obvious that calcium does indeed affect activity of GRF2 as its activity is clearly calcium responsive. It is possible that the role of calcium is to activate membrane binding
sites for GRF2, such that in the absence of a calcium signal GRF2 cannot translocate to the membrane and activate Ras. Or, instead of (or along with) affecting CaM binding, calcium may promote the assembly or proper orientation of a protein network able to couple with the ERK pathway. Other evidence in support of such a scaffolding or anchoring role for GRF2 in coupling Rac activation with stimulation of the SAPK pathway in 293T cells has been reported (67). In this model, GRF2 and perhaps other GEFs are not simply upstream activators of their target GTPases but may also determine effector interactions. This may provide some insight into the observation that oncogenic Ras is also inhibited by dominant-negative N17 Ras, indicating that activated Ras retains some requirement for exchange factors (73, 212). This result could be explained if interaction with an exchange factor is required for proper activation of downstream signals. It has also been observed that although several members of the Ras branch of the Ras superfamily share complete identity with the effector domain of Ras, they exhibit distinct biochemical and biological properties (27). If GEFs play a role in determining which effectors are activated, then this observation could be explained if different GEFs are activating these Ras subfamily members.

For GRF2, the IQ sequence is required for the interaction of Ras-GTP with effectors ultimately required for activation of ERK. An alternative scheme, which does not exclude this kind of scaffolding model, is that in the absence of the IQ motif, GRF2 is able to bind to and activate Ras in a futile manner such that GRF2 and the Ras-GTP produced are not properly localized, anchored, or oriented at the plasma membrane to physically couple to components of the ERK pathway. Yet another explanation for the ability of ΔIQ to activate Ras but not Raf may be that in the absence of the IQ motif, a Raf phosphatase is activated
and turns Raf off before the MAPK pathway is activated. Alternatively, the absence of the IQ motif may result in the absence of a kinase activity required for Raf activation.

Calmodulin binding to GRF2 is clearly not required for Ras GEF activity in vitro (Fig. 2-9) or in vivo (Fig. 2-4). Since both the IQ and DH deletion mutants no longer associate with CaM and because I have not generated a minimal CaM-binding fragment of GRF2, I cannot conclude that the IQ motif is the sole binding site for CaM. Indeed, since the PH mutants and the Cdc25-deleted protein were severely impaired in their association with CaM, it is clear that optimal CaM association with GRF2 requires an intact amino-terminal region of GRF2. In contrast, the association of CaM with GRF1 is not perturbed by similar deletions in the amino-terminus (25). I did not detect any interaction of CaM with the REM-Cdc25 protein, but this does not eliminate the possibility that in the context of native GRF2 such an interaction may occur as suggested for Ras-GRF1 (9).

There is no clear correlation between CaM association and Ras or ERK activation by GRF2. The ΔDH mutant remains responsive to calcium signals for ERK activation in the absence of CaM interaction. This indicates that calcium can affect GRF2 in a CaM-binding independent manner. For example, calcium may induce the phosphorylation of GRF2, and/or activate plasma membrane binding sites for GRF2 required for its activation. Furthermore, since the IQ motif is necessary for ERK activation by WT GRF2, I conclude that a function of the IQ motif, and possibly of the CaM interaction, is to overcome a constraint on GRF2-ERK signaling. The precise function of CaM association with GRF2 therefore remains to be determined.

The ability of the Ras-GRF proteins to respond to calcium signals distinguishes them from other Ras GEFs, but the physical association of GRF2 with CaM does not explain
GRF2’s ability to respond to calcium influx, as was previously thought. The ability of the ΔIQ mutant to activate Ras but not to couple it to the ERK pathway suggests that in addition to functioning as an upstream activator of Ras, GRF2, and interactions involving the IQ motif in particular, may serve to couple Ras with its effectors.

The yeast exchange factor, Cdc25p, forms oligomers and in this case the interaction interface is within its Cdc25 domain (28). With respect to the ΔCdc25 protein’s ability to act as a negative inhibitor, preliminary results show that ΔCdc25 and WT GRF2 do form oligomers, suggesting that inactive oligomers may explain why the ΔCdc25 protein acts as a negative inhibitor. It would also explain why the ΔCdc25 protein suppresses WT GRF2 function only when present in greater amounts than the WT protein: this would ensure that all oligomers contain at least one ΔCdc25 molecule, creating an inactive complex. Dimerization of exchange factors provides for a combinatorial possibility for Ras activation. If the GEFs are activated or produced in response to different signals, this allows for a more complex integration of signaling. The composition of the GEF dimer could also play a role in determining which effectors are activated downstream of Ras.

I conclude that GRF2 is a remarkably modular protein and that the interactions of its non-catalytic domains control its signaling functions. Ras, like the many other small GTPases, has several known effector proteins with which it can engage. I suggest a model wherein these interactions are determined by the GEF responsible for its activation. In this model, GEFs coordinate both the input and output signals of their target GTPase.
CHAPTER 3

Ras binding triggers ubiquitination of Ras-GRF2


I performed the experiments presented in this chapter except for the following: construction of the epitope-tagged Ras-GRF2ΔDB which was done by Li-Jia Zhang, the construction of Flag-RACK1 and GAP-KT3 was performed by Jackie A. Koehler and the mass spectroscopy (Fig. 3-5 and Fig. 3-10) was performed by Paul Taylor. pCMV-HA-Ubiquitin was provided by Dr. Dirk Bohmann (EMBL, Heidelberg).
GRF2 is a widely expressed multidomain protein composed of several recognizable sequence motifs in amino to carboxyl order: PH domain, coiled-coil, IQ, DH domain, a second PH domain, REM (Ras Exchanger Motif), PEST region containing a destruction box (DB), and finally a Cdc25 domain. The DH and Cdc25 domains possess guanine nucleotide exchange factor (GEF) activity, and interact with Rac and Ras, respectively. Herein, I examine whether the presence of a destruction box motif in GRF2 results in its proteolysis being regulated by the ubiquitin pathway. Based on the solved structure of the REM and Cdc25 regions of the Son-of-sevenless (Sos) protein, the REM may stabilize the Cdc25 domain during Ras binding. The DB motif is situated between the REM and the Cdc25 domain, suggesting that it may be exposed to ubiquitination machinery upon Ras binding. GRF2 protein levels were shown to decrease dramatically upon activation of GRF2. N17 Ras induces degradation of GRF2 demonstrating that signaling downstream of Ras is not required for the destruction of GRF2 and that binding to Ras is sufficient for degradation. GRF2 is ubiquitinated in vivo and the ubiquitin conjugates can be detected using mass spectroscopy. In the presence of proteasome inhibitors, Ras-GRF2 accumulates as a high-molecular weight conjugate suggesting that it is destroyed by the 26S proteasome. Deleting the DB reduces the ubiquitination of GRF2; however, there may still be a contribution from the PEST sequences. ΔCdc25 is not ubiquitinated, suggesting that a protein that cannot bind Ras cannot be properly targeted for destruction. Point mutants within the Cdc25 domain that eliminate Ras binding also eliminate ubiquitination, demonstrating that binding to Ras is
necessary for ubiquitination of GRF2. I conclude that conformational changes induced by GTPase binding expose the DB and thereby target GRF2 for destruction.

INTRODUCTION

The Ras proto-oncogenes encode small molecular weight, membrane-bound GTPases that play a central role in ensuring an appropriate cellular response to growth and differentiation factors by transducing and integrating extracellular signals (15, 134). Despite this pivotal role, little is known about how Ras is regulated. Ras acts as a critical intermediate in the transduction of signals from membrane receptors by acting as a molecular switch, transmitting signals to downstream components only when in an active GTP-bound form. Cycling of Ras between the inactive GDP-bound form and the active GTP-bound conformation is regulated by the opposing actions of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

GRF2 is a widely expressed GEF which catalyzes nucleotide exchange on Ras through its Cdc25 domain (40, 66). GRF2 is a bifunctional GEF; in addition to its activity on Ras, GRF2 is capable of binding to another small G-protein, Rac1, through its Dbl homology (DH) domain. Through its interaction with Ras and Rac, GRF2 is capable of activating both the ERK (extracellular signal-regulated kinase) and SAPK (stress activated protein kinase) MAP kinase cascades (66, 67). GRF2 is a modular protein containing a number of protein motifs in addition to the Cdc25 and DH domains. It contains, in amino-to-carboxyl order, a pleckstrin homology (PH) domain, coiled-coil motif, ilimaquinone (IQ) motif, DH domain, a second PH domain, a Ras exchanger motif (REM), a PEST-like region (rich in proline, glutamic acid, serine and threonine) that contains a candidate destruction box (DB), and,
finally, the Cdc25 domain (66). PH domains in other proteins are involved in protein-protein or protein-lipid interactions; the IQ motif in GRF2 appears to be important for allowing activated Ras to couple to the MAPK pathway (52); the REM in a related exchange factor, Sos, has been implicated in stabilizing the structure of the Cdc25 domain (16). Between the REM and the Cdc25 domain is a motif similar to the destruction box of B-type mitotic cyclins, as well as a stretch of amino acids C-terminal to the DB that is rich in proline, glutamate, serine and threonine (PEST sequences). Both motifs have been implicated in targeting proteins for ubiquitination and subsequent degradation via the 26S proteasome.

The ubiquitin system is a highly conserved method of protein degradation which involves the post-translational modification of proteins by the small protein ubiquitin and delivery of these modified proteins to the 26S proteasome for degradation (reviewed in (124)). The attachment of ubiquitin to a protein occurs via a biochemical "bucket-brigade" of enzyme activity. First, free ubiquitin is activated by an E1 enzyme and is then transferred to an E2 enzyme which, in cooperation with an E3 ubiquitin ligase protein (or protein complex), covalently links ubiquitin to a lysine residue on the target protein. The process can be repeated to add an additional ubiquitin to the previous one, commonly on Lys48 of ubiquitin. Ubiquitin conjugation continues, resulting in a high molecular weight complex containing a polyubiquitin chain that is essential for recognition and degradation by the 26S proteasome with concomitant recycling of ubiquitin. Recently, a fourth component called E4 was cloned that is required for efficient ubiquitin chain elongation (123).

Various signals can target proteins for ubiquitination. The DB, first found in mitotic cyclins, is a nine amino acid motif that targets proteins for ubiquitination usually in a cell cycle-specific manner, through the anaphase promoting complex (APC), an E3 ligase (81,
Another signal, the KEN box, targets a different subset of proteins to the APC (175). A third putative signal is a sequence rich in proline, glutamate, serine and threonine (PEST sequence): G1 cyclins are an example of proteins that contain this signal (246). The E3 involved in degrading these substrates is the SCF protein complex, which consists of the following proteins: a cullin family member, Skp1, and Rbx1/Roc1, and an F-box protein which binds the targeted substrate (7, 74, 106, 142, 162, 207, 208, 217, 243). A requirement for phosphorylation of the substrate prior to recognition by the SCF complex appears to be common to all SCF-substrate interactions.

Given the presence of the putative ubiquitination signals in GRF2, I chose to study the targeting of GRF2 by the ubiquitination system. The location of the REM in the crystal structure of Sos bound to Ras suggests that the two domains of Sos, REM and Cdc25, interact with each other during binding to Ras (16). In Sos, the REM and Cdc25 domain are situated in close proximity to one another, whereas in GRF2 there is a large block of intervening sequence. Interestingly, it is this stretch of amino acids in GRF2 that contains the PEST region and the DB. In this Chapter, I test the hypothesis that upon binding Ras, the DB is exposed to the ubiquitination machinery resulting in the ubiquitination of GRF2. I show that GRF2 contains a targeting signal for ubiquitination and that GRF2 is ubiquitinated following binding to Ras.

MATERIALS AND METHODS

Construction of GRF2 deletions and mutations.

The cloning of the ΔDH and ΔCdc25 deletions was described in the previous chapter.
The additional GRF2 mutants used in this Chapter were generated by PCR. The ΔREM construct, deleting GRF2 codons 638-686, was obtained by doing two rounds of PCR. The first two reactions used the following primers: (1) a 5' primer flanking the unique BamHI site in GRF2 and a 3' primer that deletes the above codons; (2) a 5' primer that deletes the above codons and a 3' primer that flanks the unique XhoI site in GRF2. These two PCR products were mixed and used as a template for a second round of PCR using the BamHI and XhoI flanking primers, resulting in a PCR product deleted for codons 638-686. The PCR product was digested with BamHI and XhoI and subcloned into BamHI-XhoI-digested pcDNA3-Flag-GRF2.

The ΔDB construct, deleting codons 742-751, was obtained using the same method. Reaction 1 used a 5' primer starting at codon 541 and a 3' primer that deletes the above codons. Reaction 2 used a 5' primer that deletes the above codons with a 3' primer that flanks the SacI site in the pcDNA3 multiple cloning site. These two PCR products were mixed and used as a template for a second round of PCR using the outside flanking primers, resulting in a PCR product deleted for codons 742-751. This PCR product was digested with EcoRI and the 1763 bp fragment was used to replace the 1793 bp fragment from pcDNA3-Flag-GRF2.

The point mutations R1022E and R1092A were constructed using the Transformer Site-directed Mutagenesis kit (Stratagene). Amino acid 1022 was changed from arginine to glutamic acid by changing the codon from “ctg” to “gag” using the following primer: 5' gccgacatcagctccgagcccaacgccattgagaag 3'. Changing amino acid 1092 GRF2 from arginine to alanine was performed in the same manner, changing the codon from “tgc” to “gcc” using the following primer: 5' ggaagatttaaaacctcgccgagactctcaaaaac 3'. pcDNA3-Flag-Cdc25 was
used as a template for both reactions. Automated cycle sequencing was used to confirm the codon changes and an EcoRV fragment containing the mutation was isolated and used to replace the wildtype EcoRV fragment in full length pcDNA3-Flag-GRF2.

Construction of pcDNA3-GAP KT3 (human p120GAP) was performed as follows: pECE-GAP KT3 was digested with SacI and SmaI and treated with Klenow. pcDNA3 was digested with EcoRI and treated with Klenow followed by calf intestinal phosphatase. The two products were then ligated together using T4 DNA ligase.

pMAL-Flag-RACK1 was digested with EcoRI and HindIII, treated with Klenow to produce blunt ends and then subcloned into pcDNA3 previously digested with NorI and XbaI, treated with Klenow and calf intestinal phosphatase. A Kozak sequence and start codon was introduced by PCR using the upstream primer of 5' cccgaattcgcggcccgccacatggactacaaggacgatg 3' and a downstream primer of 5' aagagcagcttgtggtatgc 3', using pMAL-FlagRACK1 as a template. The PCR product was digested with EcoRI and XhoI and used to replace the EcoRI-NorI fragment in the previously constructed pcDNA3-Flag-RACK1.

Sequencing of all the above constructs was performed (York University Core Molecular Biology Facility) to verify the integrity of the final product.

**Cell culture and transfections.**

HEK 293T (293T) and HEK 293 (293) cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, 4.5 g/L L-glutamine, 10 μM nonessential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All supplements were purchased from Gibco/BRL. 293 or 293T cells grown in 10 cm dishes
were transiently transfected by calcium phosphate precipitation as described previously (115).

Stimulation/Destruction.

293 cells (clone 13) stably expressing low levels of Flag-tagged Ras-GRF2 (66) were serum starved for 20 hours and then stimulated with 4 μM ionomycin (Calbiochem) for 5 min. at 37°C; the ionomycin media was removed and serum-free DMEM was added. Cells were placed at 37°C and harvested either immediately (5 min. sample), or 25, 55 and 175 min. later. Cells were washed in phosphate-buffered saline (PBS) and then lysed in NP40 lysis buffer (20 mM Tris-HCl pH 7.5; 50 mM NaCl; 1% NP40; 50 mM NaF; 10 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 10 μg/mL aprotinin; 0.1 mM AEBSF and 10 μg/mL leupeptin). Lysates were clarified and a Coomassie Plus Bradford assay (Pierce) performed to determine protein concentration. 60 μg of total protein was separated by SDS-PAGE and immunoblotted with anti-Flag (M2) monoclonal antibody (Kodak) and anti-actin monoclonal antibody (Oncogene Sciences). The Western blot was quantified using a Bio-Rad GS-250 Phosphorimager.

N17-induced destruction.

293 cells were transiently co-transfected with 8 μg of pcDNA3-Flag-GRF2 or pcDNA3-Flag-GRF2ΔDB, as well as 3 μg pcDNA3-N17 Ras and 1 μg pCMVβ (encoding β-galactosidase, from Clontech) as indicated. After 48 hours, the cells were serum starved in DMEM for 18 hours, washed in PBS and then lysed in ice cold NP40 lysis buffer. Lysates were clarified and a Bradford assay was performed to determine protein concentration. 30 μg of total protein was separated by SDS-PAGE, transferred to nitrocellulose and
immunoblotted with anti-Flag monoclonal antibody, anti-Ras (LAO45, Quality Biotech) monoclonal antibody and an anti-β-gal polyclonal antibody (Cortex). The protein levels were quantified using a Bio-Rad GS-250 Phosphorimager.

Northern analysis.

RNA extraction was performed as described previously (116). Briefly, cells were transfected in 15 cm dishes as described for N17-induced destruction. After serum starvation, cells were lysed directly in denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.1 M 2-mercaptoethanol; 0.5% N-lauroylsarcosine (Sarkosyl)), extracted with phenol/chloroform and the aqueous phase precipitated with isopropanol. The pellet was dissolved in 300 μL denaturing solution and precipitated again with isopropanol. The pellet was washed in ethanol, dried and the RNA pellet was dissolved in 200 μL DEPC-treated water. Northern analysis was performed as follows: 20 μg of total RNA was separated on an agarose/formaldehyde gel and transferred to a nylon filter. The filter was pre-hybridized in FSB (100 mM NaH2PO4, 50 mM sodium pyrophosphate, 7% SDS, 1 mM EDTA, 100 μg/mL denatured salmon sperm DNA) for 5 hours at 68°C and then hybridized with a random-prime synthesized probe overnight at 68°C. The probe used was DNA corresponding to codons 686-933 of GRF2. The filter was washed twice for 45 minutes each in FSB with SDS lowered to 1% and then exposed to X-ray film. The blot was stripped and re-probed with a random-prime synthesized probe corresponding to an internal fragment of a housekeeping gene, β-actin. The Northern was quantified using a Bio-Rad GS-250 Phosphorimager.
**ERKI assay.**

293T cells were transiently cotransfected with 3 µg of pJ3M-ERK1 (encoding myc epitope-tagged ERK1) and 5 µg of either pcDNA3 vector or pcDNA3-Flag-GRF2 constructs as indicated. After 48 hours, the cells were serum starved for 18 hours, and then stimulated with 4 µM of ionomycin for 5 min. at 37°C, washed in PBS, and then lysed in NP40 lysis buffer. A Bradford assay was performed using clarified lysates. 10 µg of lysate was separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Flag antibody, anti-myc monoclonal antibody (9E10), anti-MAPK polyclonal antibody (New England Biolabs, Inc.) and anti-phospho-MAPK polyclonal antibody (New England Biolabs, Inc.).

**Ubiquitination assays.**

293T cells were transiently transfected with 4 µg of pcDNA3-Flag-GRF2 constructs as indicated, with or without 1 µg of pCMV-HA-Ubiquitin (226). After 48 hours, cells were rinsed in PBS, lysed in NP40 lysis buffer and the lysates were clarified. Lysates were precleared with anti-mouse agarose beads (Sigma), then equal amounts of total protein were used to immunoprecipitate GRF2 using 2 µg of anti-Flag (M2) monoclonal antibody in the presence of 20 µL anti-mouse agarose beads for 2 hours at 4°C with gentle rotation. The immunoprecipitates were washed 3 times in NP40 lysis buffer, and then resuspended and boiled in 20 µL of Laemmli loading buffer. The samples were then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Flag to detect GRF2 and with anti-HA (12CA5; Boehringer) to detect ubiquitin. For Fig. 4b, 293T cells were transiently transfected with pECE-GAP-KT3 and HA-Ubiquitin, and GAP was immunoprecipitated with anti-KT3 ascites. For Fig. 4c, pcDNA3-Flag-RACK1 was
expressed in 293T cells along with HA-Ubiquitin, and RACK1 was immunoprecipitated using anti-Flag monoclonal antibody.

To test if GRF2 is covalently linked to ubiquitin, IPs were performed as described above, resuspended in 100 μL SDS buffer (20 mM Tris, pH 7.5; 50 mM NaCl, 1% SDS) and heated to 95°C for 10 minutes. The IP was allowed to cool to room temperature and any precipitated protein was spun out. The supernatant was diluted to 1.1 mL with Tx-100 buffer (20 mM Tris, pH 7.5; 50 mM NaCl, 1% Triton X-100) and GRF2 was immunoprecipitated as above. These IPs were washed 3 times in Tx-100 buffer, and then resuspended and boiled in 20 μL of Laemmli loading buffer. The samples were then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Flag (M2) to detect GRF2 and with 12CA5 anti-HA to detect ubiquitin.

For proteasome inhibitor experiments, 293 cells stably expressing HA-ubiquitin and Flag-GRF2 at 85% confluency were treated for indicated times with carrier (0.02% DMSO), 50 μM MG-132 (Calbiochem), 50 μM LLnL (Sigma) and 10 μM lactacystin (Calbiochem) before lysing and processing as described above.

GTPase interaction.

H-Ras, prepared as a GST fusion protein (92), immobilized on GSH-agarose beads (~1 mg protein/mL resin) was rendered free of nucleotide by incubation for 20 min. at 23°C in nucleotide exchange buffer (NEB: 20 mM Tris, pH 7.5; 50 mM NaCl; 5% glycerol; 1 mM dithiothreitol (DTT); 0.1% TX-100) supplemented with 10 mM EDTA. Nucleotide-bound Ras was prepared by incubating nucleotide-free protein for 15 min. at 23°C in NEB containing 10 mM MgCl₂ plus 200 μM GDP or 20 μM GTPγS. The proteins were
resuspended in 500 μL of the same NEB buffer used in their preparation, then combined with 2.5 mg of cell lysate. Lysates were prepared from 293T cells transfected with the appropriate GRF2 protein and lysed in ice cold NP40 lysis buffer. Following incubation for 2 hours at 4°C, beads were washed extensively with their respective nucleotide depleting/binding buffer followed by immunoblotting with anti-Flag antibody to determine levels of bound GRF2.

**Mass spectrometry.**

Lysates from control and GRF2-transfected 293T cells were immunoprecipitated with M2 anti-Flag antibody and the isolated complexes were separated by SDS-PAGE as described above. The gel-separated proteins were visualized by silver staining and the GRF2-specific bands were excised. The proteins were reduced, the free cysteine residues alkylated with iodoacetamide, then subjected to digestion by trypsin (Boehringer Mannheim) using the method of Shevchenko et al. (201). The extracted peptides were purified by C18 reverse phase chromatography and resuspended in 50% methanol/50% formic acid prior to analysis. Mass spectrometry was carried out on a prototype quadrupole-time-of-flight hybrid mass spectrometer (Sciex) (200) equipped with a nanospray ion source (MDS Protana). Each sample was introduced into a nanospray needle installed in front of the ms orifice and continuously electrosprayed at a low flow rate as previously described (244). MS spectra were acquired to determine the m/z ratio of the peptides present in the proteolytic digest. Individual peptides were selected and fragmented by collision-induced dissociation and the resulting fragments separated, generating a MS/MS spectrum. For every MS/MS spectrum, a small stretch of the amino acid sequence was manually determined generating a “sequence tag” which was fed into a search engine (PeptideScan), which was used to identify the
provenance of the peptide by protein/DNA database searches. Every peptide identified was confirmed manually.

**Cell synchronization.**

All media used in these protocols, unless indicated otherwise, is DMEM supplemented with serum, L-glutamine and non-essential amino acids as described above.

**Thymidine/Thymidine:** A confluent plate of 293 cl. 13 cells expressing GRF2 were split 1:5 and allowed to attach to 10 cm plates. Media containing 2 mM thymidine was added for 16 hours to arrest cells in S phase. Cells were rinsed with PBS and media was added for nine hours to allow the cells to progress through S phase, into G2/M or G1. Media containing 2 mM thymidine was added again for 15-16 hours to synchronize the cells at the beginning of S phase. Cells were then rinsed with PBS prior to the addition of media lacking thymidine. Time points were taken every two hours by rinsing cells once in PBS and lysing in ice cold NP40 lysis buffer. Lysates were processed and GRF2 protein levels assessed by Western blotting.

**Thymidine/Nocodazole:** 293 cl. 13 cells expressing GRF2 were transferred to a T-175 flask (2 confluent 10 cm dishes per flask) and allowed to attach. Media containing 2 mM thymidine was added for 16 hours to arrest cells in S phase. Cells were then rinsed once with PBS and media containing 0.5 μg/mL nocodazole was added for 14-15 hours to synchronize the cells in metaphase. Cells were then dislodged from the flasks and transferred into centrifuge tubes, collected by centrifugation, rinsed with PBS and then transferred into 10 cm plates at 30% confluency. Time points were taken every two hours by
rinsing cells once in PBS and lysing in ice cold NP40 lysis buffer. Lysates were processed and GRF2 protein levels assessed by Western blotting.

Flow cytometry.

Synchronized cells from the cell cycle experiments were also processed for flow cytometry so as to assess the quality of the synchronization by following the amount of DNA present in cells. Approximately $2.5 \times 10^6$ (40-50% confluent plate) of 293 cells were lifted from plates using PBS, collected by centrifugation and then rinsed once with PBS. Cells were fixed in 80% ice cold ethanol on ice for 60 minutes, then rinsed with PBS, washed once in PIB (0.12% Triton, 0.12 mM EDTA in PBS). The cell pellet was resuspended in 500 µL of PIB containing 10 µg/mL RNase A and incubated at 37°C for 30-45 minutes. 25 µg of propidium iodide in PIB was added and cells were incubated in the dark for 60 minutes. Samples were then processed on a Becton Dickinson FACSCalibur Flow Cytometry System and analyzed by CellQuest software.

RESULTS

GRF2 contains a candidate destruction box (DB).

Sequence analysis revealed that GRF2 contains a sequence with some similarity to one found in mitotic cyclins and in other unstable proteins (Fig. 3-1). The region in mitotic cyclins required for ubiquitin-mediated proteolysis contains an amino acid motif called the destruction box (81, 248). The consensus for this motif is RXALGXIXN; the Arg and Leu residues are conserved in all the destruction boxes of A- and B-type cyclins, while the Asn residue is only conserved in B-type cyclins (81). The PEST-rich region of GRF2 contains a motif very similar to the destruction box of A-type cyclins. The Ras exchange factor in
yeast, Cdc25p, has been shown to contain a functional destruction box of the sequence RSSLNSLGN (107) It is interesting to note that GRF2’s DB, KLSLTSSLN, resembles the yeast exchange factor’s DB more closely than the destruction boxes of mitotic cyclins.

**Figure 3-1   Destruction boxes.**

Schematic representation of the domain structure of murine GRF2 with a sequence alignment of the destruction box motif of GRF2 (codons 743-751); the Ras exchange factor in *Saccharomyces cerevisiae*, Cdc25p (codons 148-156); and the consensus sequence of mitotic A-type cyclins. The boxed residues are those that are conserved in known destruction boxes; the R and L residues are conserved in A- and B-type cyclins, whereas the N residue is conserved only in B-type cyclins.

**GRF2 is an unstable protein in stimulated cells.**

If GRF2 is a protein that is targeted for destruction upon Ras binding, then one would predict that activating GRF2 and therefore activating Ras would result in a decrease in GRF2 protein levels. To test this, a 293 cell line stably transfected with GRF2 and expressing low levels of the protein was grown to 90% confluency and serum-starved for 20 hours. Cells were then stimulated with ionomycin, a calcium ionophore that raises intracellular calcium levels, leading to the activation of GRF2 (52, 66, 67). Cells were harvested at various time
points and equal amounts of total protein were resolved by SDS-PAGE. GRF2 was detected by Western blotting with M2 anti-Flag antibody and the amount of GRF2 present in each condition was quantified (Fig. 3-2). The steady-state levels of GRF2 decreased dramatically in stimulated cells, and it appears that the total amount of GRF2 in the cells dropped by approximately 50% within 5 minutes (Fig. 3-2, Lane 2). The steady-state levels of GRF2 continued to drop until one hour after stimulation and then began to rise again. As a control, actin protein levels were assessed to ensure that the decline in protein is specific to GRF2. As shown in the lower panel of Fig. 2, actin protein levels remained relatively constant throughout the experiment. This decline in GRF2 levels suggests that the disappearance of GRF2 is a signal-triggered event.

Figure 3-2  Ras-GRF2 decreases in response to Ca^{2+}. Serum-starved 293 cells stably expressing Flag-tagged GRF2 were stimulated for 5 minutes at 37°C with 4 μM ionomycin and harvested at the indicated periods of time. GRF2 protein levels in 60 μg of lysate were assessed by Western blotting with the M2 monoclonal anti-Flag antibody to detect GRF2; actin levels were assessed by blotting with an anti-actin monoclonal antibody. The data shown are representative of four experiments.
**GRF2 destruction depends upon its DB motif.**

In order to determine if the DB motif is responsible for the instability of GRF2 in lysates, a construct of GRF2 was used in which the DB was deleted (ΔDB). 293 cells were transiently transfected with GRF2 or ΔDB, with or without N17 Ras (Fig. 3-3). N17 Ras displays a dominant-negative behaviour as a consequence of its inability to coordinate magnesium properly; it prevents activation of endogenous Ras by sequestering exchange factors into dead-end complexes (69). It has been shown previously that this Ras mutation prevents GRF2 from signaling to the MAPK pathway (67). By sequestering exchange factors in this method, N17 Ras results in a prolonged interaction between GRF2 and Ras rather than the usual, presumably transient, interaction. As a control, the cells were also co-transfected with a construct encoding β-galactosidase to ensure that any effects seen were specific to GRF2 and not to other transfected proteins. After loading equal amounts of protein on a gel and immunoblotting for Flag-GRF2, the level of GRF2 protein was found to be reduced approximately 90 percent in the presence of N17 Ras, while the levels of the ΔDB construct were reduced only slightly (Fig. 3-3a). The levels of β-galactosidase did not change.

Northern blot analysis was performed to determine if this observed effect was at the protein level or whether it was a result of much lower transcript levels in the WT samples. A histogram of the results of the Northern (Fig. 3-3b) confirms that the protective effect of deleting the DB was at the protein level. The Northern shows that the introduction of N17 Ras resulted in a decrease in transcript levels; however, the decrease was the same for WT and ΔDB. This was not surprising, as N17 Ras has been shown previously to globally reduce transcription (1).
Figure 3-3  Deleting the DB protects GRF2 from N17 Ras-induced degradation.

(A) The assays were carried out 2 days after transfection of 293 cells with the indicated GRF2 construct and 30 μg of lysate was separated by SDS-PAGE. In the upper panel, the samples were Western blotted for GRF2 using M2 anti-Flag monoclonal antibody. In the middle panel, the presence of N17 Ras was verified by Western blotting of lysates with the LAO45 anti-Ras monoclonal antibody. In the lower panel, the lysate was Western blotted for β-galactosidase using an anti-β-galactosidase monoclonal antibody. The bar graph shows the quantitated data as relative protein levels. The data shown are representative of four experiments. (B) N17 Ras expression does not differentially affect transcript levels. Northern analysis was performed as described in Materials and Methods. A random-primed probe of DNA corresponding to codons 686-933 of GRF2 was used to probe RNA extracted from transiently transfected 293 cells. The data shown are representative of two experiments. (C) ERK1 activity in 293T cells. The assay was carried out 3 days after transfection of 293T cells with the indicated GRF2 construct and myc-ERK1. Cells were serum starved for 20 hours and then either left untreated (−) or treated with 4 μM ionomycin for 5 minutes at 37°C (+). In the upper panel, GRF2 protein expression was verified by Western blotting of the lysates with M2 anti-Flag antibody. In the lower panel, activated ERK was detected by Western blotting with a polyclonal anti-phospho-ERK1/2 antibody. Equal expression of myc-ERK1 was confirmed by Western blotting of the lysate with 9E10 anti-myc monoclonal antibody (middle panel). The data shown are representative of three experiments.
To address the possibility that the ΔDB protein was more stable as a result of its being misfolded or improperly localized, GRF2 or ΔDB was co-transfected with myc-ERK and levels of MAPK activity were assessed by using a phospho-specific antibody to MAPK (Fig. 3-3c). Both wild-type GRF2 and ΔDB signal efficiently to the MAPK cascade, suggesting that the differences seen in destruction are not because the deletion of the DB resulted in an unfolded protein that is not targeted properly. These findings imply that the destruction of GRF2 is dependent upon the presence of the DB and that the observed difference in protein levels is not a result of lower transcript levels. These data also suggest that signaling is not required for the destruction of GRF2, as downstream signaling is blocked in the presence of N17 Ras.

**GRF2 is ubiquitinated in vivo.**

Because destruction boxes in other proteins target those proteins for destruction via the ubiquitin degradation pathway, I wanted to determine if GRF2's DB motif targets it for ubiquitination. To do this, GRF2 was transiently co-transfected with an HA-tagged ubiquitin construct into 293T cells. GRF2 was immunoprecipitated from cell lysates prepared from these cells and blotted with 12CA5 anti-HA antibody to detect ubiquitin-conjugated GRF2 (Fig. 3-4a). A ladder of high molecular weight ubiquitinated products is seen at even intervals, starting slightly higher than the size of GRF2 (Fig. 3-4a, Lane 5). This is not seen in vector-transfected control cells, nor in cells not expressing the ubiquitin construct (Lane 4 and Lanes 1-3, respectively). ΔDH (Lanes 2 and 6) was used as a control in this experiment to show that an irrelevant deletion does not cause a decrease in GRF2's ubiquitination state. The ΔDH construct was ubiquitinated to a similar level as wild-type GRF2 whereas ubiquitination of the ΔDB construct was compromised.
Figure 3-4  GRF2 ubiquitination in vivo.
The assays were carried out 2 days after transfection of 293T cells with the indicated construct. (A) Flag-GRF2 or the deletion mutants were isolated by anti-Flag immunoprecipitation. The washed immune complexes were Western blotted with M2 anti-Flag antibody for GRF (lower panel) and with 12CA5 anti-HA antibody for ubiquitin (upper panel). The data shown are representative of five experiments. (B) p120 Ras-GAP (GAP) was isolated by anti-KT3 immunoprecipitation. The washed immune complexes were Western blotted with anti-KT3 ascites for GAP (lower panel) and with 12CA5 anti-HA antibody for ubiquitin (upper panel). The data shown are representative of two experiments. (C) Flag-RACK1 was isolated by anti-Flag immunoprecipitation. The washed immune complexes were Western blotted with M2 anti-Flag antibody for RACK1 (right panel) and with 12CA5 anti-HA antibody for ubiquitin (left panel). The data shown are representative of two experiments. (D) GRF2 was isolated by anti-Flag immunoprecipitation. Two samples were then boiled in 1% SDS buffer for 5 minutes, allowed to cool and then diluted in 1% Triton X-100 buffer. GRF2 was again isolated from these samples by anti-Flag immunoprecipitation. All of the washed immune complexes were Western blotted with M2 anti-Flag antibody for GRF2 (upper panel) and with 12CA5 anti-HA antibody for ubiquitin (lower panel). The bands seen in Lanes 1 and 4 are the result of the 12CA5 antibody cross-reacting with the large amount of GRF2 present in the gel; there are no HA-tagged proteins present in these lanes. The data shown are representative of three experiments.
The possibility exists that merely overexpressing a protein can result in its ubiquitination due to large amounts of misfolded polypeptides. However, this does not appear to be a factor in this assay as other proteins such as p120 Ras-GAP and RACK1 are not ubiquitinated when overexpressed under these conditions (Fig. 3-4b&c, respectively). These data provide further evidence that the DB of GRF2 is important for targeting the protein for ubiquitination.

The ubiquitin moiety is covalently attached to the GRF2 protein and the ubiquitin conjugates seen in the GRF2 immunoprecipitates are not the result of an unknown ubiquitinated protein co-immunoprecipitating with GRF2. This was tested by boiling the immune complex in a buffer containing 1% SDS, thereby disrupting all protein-protein interactions. Triton X-100 buffer was added to dilute the SDS to allow a second immunoprecipitation to be performed. GRF2 was immunoprecipitated again, and this sample tested for the presence of ubiquitin conjugates of the appropriate size (Fig. 3-4d, Lane 5). The bands seen in Lanes 1 and 4 are the result of the 12CA5 antibody cross-reacting with the large amount of GRF2 present in the immunoprecipitate, as these anti-HA-reactive bands are present in immunoprecipitates from samples not expressing an HA-tagged protein. The presence of conjugates larger than GRF2 in the second immunoprecipitate suggest that the ubiquitin moiety is directly attached to GRF2 and the observed conjugates are not the product of an interacting protein or a non-specific co-precipitating protein.

**GRF2-Ubiquitin conjugates can be detected by mass spectrometry.**

Mass spectrometric analysis detected peptides derived from GRF2 in tryptic digests of 3 different bands present on a gel of GRF2 immunoprecipitates. In particular, along with the predicted location of GRF2 at 135 kDa (apparent molecular weight), two larger
Figure 3-5  Mass spectrometric analysis of peptides from tryptic digest of GRF2. Bands were excised from a gel at 135 kDa (the molecular weight of GRF2), 175 kDa, and 200 kDa. These bands were analyzed by a quadrapole time-of-flight mass spectrometer to detect the peptides generated. Individual peptides were used to generate a MS/MS spectrum and the amino acid sequence was manually determined. The upper panel shows a peptide detected in the 135 kDa GRF2 digest that corresponds to residues 463-476. The lower panel shows a peptide in the GRF2 tryptic digest that corresponds to ubiquitin (residues 12-27). (B) The panels on the right show the time-of-flight MS for the GRF2 peptide in gel slices from 135, 175 and 200 kDa and the panels on the left show the spectra for the ubiquitin peptide.

bands were identified at 175 kDa and 200 kDa. All peptides located in these bands were identified as originating from GRF2 with the exception of a doubly charged peptide ion
Located at M/Z=894.3. A MS/MS spectrum of this ion revealed it to be the tryptic peptide TITLEVEPSDTIENVK found only in ubiquitin (Fig. 3-5a, lower panel). An MS/MS spectrum of a diagnostic peptide derived from GRF2 is shown in Fig. 3-5a, upper panel. The abundance of the ubiquitin ion increased with increasing molecular weight of the GRF2-containing bands (Fig. 3-5b), and this effect is more pronounced when its signal is normalized to one of the GRF2 peptide signals in the same band. This evidence suggests that the increasing apparent molecular weight of the GRF2 protein is caused by an increasing load of conjugated ubiquitin.

**Ubiquitinated GRF2 accumulates with proteasome inhibitor treatment.**

In order to determine if the proteasome plays a role in the destruction of GRF2, I looked at the ubiquitination state of GRF2 in the presence of two proteasome inhibitors, LLnL and MG-132. An 293 cell line stably expressing Flag-GRF2 and HA-ubiquitin was grown to 85% confluency and then treated for various times with carrier (0.02% DMSO), 50 μM LLnL or 50 μM MG-132. Cells were then lysed, GRF2 was immunoprecipitated and its ubiquitination state assessed by immunoblotting for HA-tagged ubiquitin (Fig. 3-6). As length of treatment with the inhibitors increases, large molecular weight complexes of GRF2-Ub began to accumulate (Lanes 7-9, 12-14). These complexes had barely exited the stacking gel and were therefore very large (>200 kDa). Similar results were seen with lactacystin, another proteasome inhibitor (53). As the 26S proteasome is known to degrade ubiquitinated proteins, this indicates that GRF2 is likely destroyed by the 26S proteasome.
Figure 3-6  Treatment with 26S proteasome inhibitors.

A stable 293 cell line expressing Flag-tagged GRF2 and HA-tagged ubiquitin was treated for indicated amounts of time with 50 μM LLnL or 50 μM MG-132, two potent 26S proteasome inhibitors, or with 0.02% DMSO alone as a control. GRF2 was isolated by anti-Flag immunoprecipitation; the washed immune complexes were Western blotted with M2 Flag antibody for GRF2 (lower panel) and with 12CA5 antibody for ubiquitin (upper panel). The data shown are representative of three experiments.

Mutants that cannot bind Ras are not ubiquitinated.

Deletion of the Cdc25 domain severely reduces the susceptibility of GRF2 to ubiquitination (Fig. 3-7c, Lane 3). The Cdc25 domain has been shown to be required for interaction with Ras (67) so these data suggest that the exchange factor's interaction with Ras is important for targeting GRF2 for ubiquitination. However, as deleting the Cdc25 domain removes 18 lysines from the protein, I could not exclude the possibility that the reduced ubiquitination of the ΔCdc25 protein is a result of the fewer number of lysines available for attachment of ubiquitin moieties. To address this, point mutants were generated at conserved arginine residues in the Cdc25 domain at positions 1022 and 1092 (R1022E and R1092A) which have been shown in other Ras GEF proteins to abrogate binding to Ras (29, 152, 169). These mutants should, therefore, be unable to be ubiquitinated as a consequence of this diminished binding if Ras binding is indeed important for targeting GRF2 for ubiquitination.
**Figure 3-7  Cdc25 domain point mutants are not ubiquitinated.**

(A) The in vitro association of GRF2, R1022E, and R1092A with GST-Ras. GST-Ras complexed with agarose beads was incubated with lysate from 293T cells expressing Flag-GRF2 or the indicated mutant. Bound GRF2 proteins were detected by Western blotting with anti-Flag antibody. GRF2 protein expression in lysate was also detected using anti-Flag antibody (Lanes 1-3). The GST fusion protein had been prepared in either its nucleotide-free (NF), GDP-bound (GDP) or GTPγS-bound (GTPγS) form. The (−) and (+) refer to the absence or presence of cell lysate during the incubation. In Lane 5, lysate containing wild-type GRF2 was added. (B) ERK1 activity in 293T cells. The assay was carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. In the upper panel, GRF2 expression was verified by Western blotting of the lysates. In the lower panel, activated ERK was detected by Western blotting with a polyclonal anti-phospho-MAPK antibody. Equal expression of ERK1/2 was confirmed by Western blotting of the lysate with a polyclonal anti-MAPK antibody (middle panel). (C) Ubiquitination of point mutants. The assays were carried out 2 days after transfection of 293T cells with the indicated construct. Flag-GRF2 or the deletion mutants were immunoprecipitated and the immune complexes were Western blotted with anti-Flag antibody for GRF (lower panel) and with 12CA5 anti-HA antibody for ubiquitin (upper panel). The asterisk indicates a degradation product of R1092A in Lane 5. All data shown in this figure are representative of four experiments.
The mutants were tested for their ability to bind Ras in a pulldown assay (Fig. 3-7a). Bacterially produced, purified GST-Ras, either nucleotide-free (NF), bound to GDP or to GTPγS, was incubated with lysate containing the indicated GRF2 protein and the amount of GRF2 bound to the fusion protein was assessed by Western blotting. A common property of Cdc25 domains is their relatively high affinity for the nucleotide-free form of Ras, likely reflecting their catalytic mechanism of stabilizing an otherwise unfavourable nucleotide-free intermediate state (127). Wildtype GRF2 (Lanes 7-9) was able to bind specifically to nucleotide-free Ras, as previously shown (67). The binding of R1022E was barely detectable (Lanes 10-12) and the binding of R1092A was severely reduced to approximately 20% that of wild-type GRF2 (Lanes 13-15). To test whether this impaired binding translates into an inability to signal to the MAPK cascade, phospho-MAPK immunoblots were performed (Fig. 3-7b). Lysates from cells expressing the indicated GRF2 protein were immunoblotted with anti-Flag to detect GRF2, anti-MAPK to detect ERK 1/2 and anti-phospho-MAPK to detect phosphorylated, activated ERK 1/2. GRF2 (Lane 2) significantly activated ERK compared to the vector-alone control (Lane 1). R1022E activated ERK to a similar level as vector-alone, and R1092A activated ERK slightly, both correlating with their limited abilities to bind Ras.

In addition, the effect of reduced Ras binding and signaling on GRF2 ubiquitination was tested (Fig. 3-7c). Again, ΔCdc25 was remarkably decreased in its ability to be ubiquitinated, and R1022E and R1092A were also severely impaired (Lanes 4-5). This strongly supports the notion that GRF2 must bind Ras in order to be targeted for ubiquitination.
Figure 3-8 Ability of ΔREM to bind Ras and become ubiquitinated.

(A) The in vitro association of GRF2 and ΔREM with GST-Ras. GST-Ras complexed with agarose beads was incubated with lysate from transiently transfected 293T cells expressing Flag-GRF2 or ΔREM. Bound GRF2 proteins were detected by Western blotting with anti-Flag antibody. Protein expression in lysate was detected using anti-Flag antibody (lanes 1-2). The GST fusion protein had been prepared in either its nucleotide-free (NF), GDP-bound (GDP) or GTPγS-bound (GTPγS) form. The (−) and (+) refer to the absence or presence of cell lysate during the incubation. In Lane 4, lysate from cells expressing wild-type GRF2 was added. The data shown are representative of two experiments. (B) ERK1 activity in 293T cells. The assay was carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. In the upper panel, GRF2 protein expression was verified by Western blotting of the lysates. In the lower panel, activated ERK was detected by Western blotting with a polyclonal anti-phospho-MAPK antibody. Equal expression of ERK1/2 was confirmed by Western blotting of the lysate with a polyclonal anti-MAPK antibody (middle panel). The data shown are representative of three experiments. (C) Ubiquitination of ΔREM. The assays were carried out 2 days after transfection of 293T cells with the indicated construct. Flag-GRF2 or the deletion mutant was isolated by anti-Flag immunoprecipitation. The washed immune complexes were Western blotted with M2 anti-Flag antibody for GRF2 (lower panel) and with 12CA5 anti-HA antibody for ubiquitin (upper panel). The data shown are representative of two experiments.
The Ras Exchanger Motif appears to be involved in stabilizing the structure of the Cdc25 domain; it may orientate or stabilize a helical hairpin of the Cdc25 domain so that upon binding, the hairpin can alter the structure of Ras to allow for nucleotide release (16). After deleting the REM, GRF2 lost its ability to bind to Ras as shown in Fig. 3-8a (Lanes 9-11). Concomitant with this, the ΔREM protein was severely impaired in ERK activation (Fig. 3-8b). Along with this inability to bind Ras, ΔREM was also no longer ubiquitinated (Fig. 3-8c), again implying that binding to Ras is a necessary event in targeting GRF2 for ubiquitination.

**Overexpressing Ras increases ubiquitination of GRF2.**

If binding to Ras is important for ubiquitination, then one would predict that overexpressing Ras would have an effect on the ubiquitination of GRF2. In Fig. 3-9, H-Ras was overexpressed in 293 cells and the ubiquitination of GRF2 was assessed. Increasing cellular Ras levels increased the ubiquitination of wildtype GRF2 (Fig. 3-9, Lanes 2-3), as predicted. Interestingly, it also increased the amount of ubiquitination of R1022E and R1092A (Fig. 3-9, Lanes 4-5 and 6-7). Without overexpressing Ras, R1022E and R1092A had barely detectable levels of ubiquitination; increasing Ras levels presumably shifts the equilibrium to increase binding between Ras and the point mutants, thereby increasing ubiquitination.

**GRF2 is phosphorylated near the DB.**

GRF2 was analyzed for the presence of phosphorylated residues by deconvolution of the mass spectrum of a tryptic digest of the protein and searching for peptides differing by a
Figure 3-9  Overexpressing Ras affects ubiquitination levels of GRF2.

The assay was carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. 293T cells were transiently transfected with Flag-GRF2, HA-Ubiquitin, and either pcDNA3 (lanes 2, 4 and 6) or H-Ras (lanes 3, 5 and 7). Equal amounts of protein in lysates were separated by SDS-PAGE and western blotted as follows: ERK1 expression was detected with a polyclonal MAPK antibody (third panel); activated ERK was detected with a polyclonal phospho-MAPK antibody (fourth panel); in the second panel, the presence of overexpressed H-Ras was verified with LAO45 Ras monoclonal antibody (on a much longer exposure, endogenous Ras can be detected as well). GRF2 or the point mutants were isolated by anti-Flag immunoprecipitation. The washed immune complexes were Western blotted with M2 Flag antibody for GRF (bottom panel) and with 12CA5 antibody for ubiquitin (top panel). The data shown are representative of two experiments.

mass of 80 Da (neutral mass of HPO₄²⁻) (Fig. 3-10a). Two phosphate-containing peptides were located and their sequences were confirmed by MS/MS (Fig. 3-10d). Both peptides are located close to the DB and are within the PEST region of GRF2. The peptide KFSSPPPLAVSR (residues 723-734 of GRF2), located on the N-terminal side of the DB, was found to contain a single phosphorylation site (Fig. 3-10b). The peptide IGALDLTNSSSSSPTTTTHSPAASPPHTAVLESAPDK (residues 754-793), located on the C-terminal side of the DB, was found to have 4 phosphorylation sites (Fig. 3-10c).
Figure 3-10  Mass spectra of peptides generated from a trypsin digest of GRF2.

(A) GRF2 was immunoprecipitated from a 293 cell line, separated by SDS-PAGE and the GRF2-specific band was analyzed by mass spectrometry as described in Materials and Methods. Two peptides are shown that differ in size by the molecular weight of a phosphate group, indicating that this peptide is phosphorylated. (B). Both peptides from (A) were isolated and sequenced by MS/MS. The peptide is located at amino acids 723-734 of GRF2. The upper panel shows the spectrum derived from the 643.3 Da peptide; the lower panel shows that from the 683.3 Da peptide. The arrow depicts the loss of the phosphate group from the larger ion.

For both peptides, the mass spectra did not provide enough information to determine the exact amino acid(s) that is phosphorylated.
Cell cycle regulation of GRF2 protein levels.

The presence of a DB in GRF2 may predict that it is regulated in a cell cycle-specific manner as are other proteins containing a DB. In order to determine if there is any cell cycle regulation of GRF2 protein levels, I synchronized a GRF2 stable cell line at the S and M phases of the cell cycle using thymidine and nocodazole, then released them from the drugs and allowed them to progress through the cell cycle, taking time points every two hours for 15 hours and assessing GRF2 protein levels by Western blot. Figure 3-11a depicts a typical result from one of these experiments. In the thymidine/thymidine double block where cells are synchronized at the beginning of S phase and then released, GRF2 proteins levels were at their highest and began to fall shortly thereafter, while cells were still in S-phase as determined by flow cytometry. When cells were released from a metaphase arrest induced by treating with nocodazole, GRF2 protein levels rose throughout M and G1 phase. Figure 3-11b shows representative flow cytometry profiles from the thymidine/nocodazole block, taken every five hours after release. The first panel are the results obtained from an asynchronous culture of 293 cells, with the G1, S and G2/M peaks labeled. The profile at time=0 shows that the culture was indeed arrested in the M phase of the cell cycle, as would be expected when treating with nocodazole. The profile from time=5 hours shows that the cells were moving into G1 phase and had moved into S-phase at 15 hours after release. These data demonstrate that GRF2 protein levels fluctuate during the cell cycle with a decrease in protein level occurring through S phase.
Figure 3-II  Cell cycle regulation of GRF2 protein levels.
293 cells stably expressing Flag-GRF2 were synchronized using a thymidine/thymidine block or a thymidine/nocodazole block and then released from the drugs and allowed to progress through the cell cycle. (A) Time points were taken every two hours after release. Cells were lysed and GRF2 protein levels assessed by Western blotting. The graph shows relative GRF2 protein levels during cell cycle progression. The thymidine/thymidine treated samples (diamonds) were synchronized using a thymidine block, released for 9 hours to allow progression through S phase and then treated with thymidine again. The thymidine/nocodazole treated samples (squares) were synchronized with a thymidine block, released and then treated with nocodazole to block cells at metaphase. (B) Samples were processed every five hours for analysis by flow cytometry. Cells were fixed with ethanol, stained with propidium iodide and the DNA content of samples was measured. Asynchronous cells show a G1, S and G2/M phase peak, and the thymidine/nocodazole samples taken at 5 hour intervals show cells progressing through the cell cycle and reaching S phase by 15 hours.

DISCUSSION

Here I have tested the role of the DB motif in the ubiquitination of GRF2 and whether conformational changes induced by GTPase binding expose the DB and thereby target GRF2
for destruction. In yeast, Cdc25p has been shown to have a functional destruction box that confers instability on the exchange factor; however, there does not appear to be any cell cycle regulation of this destruction and direct involvement of ubiquitin was not demonstrated (107). The authors suggest that this indicates that regulation of Ras in yeast may be directly modulated by the cellular content of the exchange factor rather than variations in cellular localization or activity. *In vitro*-translated mouse Sos2 has been shown to be ubiquitinated, but the ubiquitination of Sos *in vivo* has not been explored (158). The data in this Chapter provide the first demonstration of the *in vivo* ubiquitination of an activator of Ras as well as a model to explain how it is targeted for destruction.

I show that GRF2 is an unstable protein and that its destruction is dependent upon the presence of its DB. The deletion of the DB did not appear to result in a mislocalized or misfolded protein, as the ΔDB is still fully functional in terms of its ability to activate the MAPK pathway. N17 Ras induces degradation of GRF2, demonstrating that signaling downstream of Ras is not required for the destruction of GRF2. This strongly indicates that binding to Ras is necessary for degradation.

GRF2 is ubiquitinated *in vivo* but it is not possible to see the GRF2-ubiquitin conjugates by Western blotting for GRF2, suggesting that only a small portion of GRF2 is becoming ubiquitinated. The experiments assaying ubiquitination were done in exponentially growing cell cultures, so if ubiquitination of GRF2 is linked to cell cycle events, then only a small portion of the total cell culture would be in the correct phase. This may explain why I did not find a larger population of GRF2 becoming ubiquitinated. An equally plausible explanation could just be that the sensitivities of the antibodies used are not
sufficient to detect the portion of GRF2 protein that is modified with ubiquitin or that the ubiquitin moieties prevent antibody recognition. However, in a GRF2 immunoprecipitate from unsynchronized cells, ubiquitin peptides can be detected using mass spectrometry techniques. Ubiquitin sequences can be found associated with GRF2 sequences in the absence of other proteins at apparent molecular weights much larger that GRF2 or ubiquitin alone. This is highly suggestive of a covalent interaction between the two proteins.

Furthermore, if the mass spectrometer detector response of the identified ubiquitin peptide is normalized to any peptide from GRF2, a stoichiometric estimate can be made. From this, it is apparent that on an SDS-PAGE gel the slower migrating GRF2 is more highly ubiquitinated.

ΔCdc25 is not ubiquitinated, suggesting that a protein that cannot bind Ras cannot be properly targeted for degradation. To test this further, and to ensure that this effect was not due to the removal of a large number of lysines, point mutants were made that are severely impaired in their ability to bind Ras. These point mutants within the Cdc25 domain also eliminated ubiquitination, demonstrating that binding to Ras is required for ubiquitination of GRF2.

While the ubiquitination of ΔDB is impaired, it is not eliminated. It is possible that the DB is not the sole determinant for ubiquitination of GRF2. As mentioned above, GRF2 also contains PEST sequences that are thought to be signals for ubiquitination, and perhaps these PEST sequences are cooperating with the DB in targeting GRF2 for destruction. Preliminary observations suggest that this may be the case, but further work is required.

The destruction box, first found in mitotic cyclins, is a nine amino acid motif that targets proteins for ubiquitination through the E3 ligase called the anaphase promoting
complex (APC) usually in a cell-cycle specific manner (248). Substrate recognition by the APC is thought to require one of several adapter proteins containing WD40 motifs. The known adapter proteins responsible for degrading various proteins such as the mitotic cyclins are Cdh1 and Cdc20, each of which appears to be responsible for specific substrates (232). All known Cdc20 substrates contain a DB, while Cdh1 substrates are recognized by the presence of a DB or a KEN box (175). It is unknown at this point if GRF2 is ubiquitinated by the APC pathway with a specific adapter protein that links it to the APC.

The APC is active from the end of mitosis and throughout G1 phase, at least when considering the proteolysis of G2 cyclins (3). My results suggest that GRF2 begins to be degraded in S phase (Fig. 3-11), implying that if the APC is degrading GRF2 it must be doing so through the use of a novel adapter. The difference in protein levels seen here is not extreme, as might be expected based on the expression levels of cyclins which are also targeted through a DB. However, cell cycle-regulated proteins often have an additional layer of control that helps to regulate the expression levels of proteins and that control is transcriptional. The transcripts of many of cell cycle proteins are also cell cycle regulated. In the cell cycle experiment with GRF2, I am driving expression from the strong cytomegalovirus promoter, which is likely active in all stages of the cell cycle; therefore, a level of regulation of GRF2 has been lost. It is possible that the difference in GRF2 protein levels throughout the cell cycle is much more dramatic when GRF2 is expressed from its endogenous promoter.

The degradation of GRF2 during S phase when the APC is not active towards cyclins suggests either that an unknown adapter protein for the APC is regulating its destruction or that GRF2’s ubiquitination is actually regulated by another E3, perhaps the SCF complex
through recognition of PEST sequences. To date, all known SCF substrates are recognized in a strictly phosphorylation dependent manner (48). Interestingly, GRF2 is phosphorylated on multiple residues within its PEST region, as shown by mass spectrometry. I do not know what the role these phosphorylation events play, but it is interesting that they fall within the PEST region of GRF2 as it is the PEST region in G1 cyclins that must be phosphorylated in order to target them for ubiquitination (48). It is also possible that these phosphorylation events are important in the activation of GRF2, as is the case for GRF1 (143-145).

The precedent has been set for proteins in the Ras pathway being destroyed by ubiquitin-mediated proteolysis, as evidenced by the destruction of tramtrack (Ttk), a transcriptional repressor in the Drosophila Ras signaling pathway that is required to specify R7 cell fate in the Drosophila eye (131, 218). The destruction of Ttk is dependent upon the presence of phyllopod (Phyl), which is induced by the Ras pathway downstream of the sevenless receptor tyrosine kinase. Phyl binds to a nuclear protein, seven in absentia (Sina), and this complex then binds Ttk, stimulating its ubiquitination and destruction. The Sina protein also binds to a ubiquitin conjugating enzyme, Ubc9, which presumably contributes to the ubiquitination of Ttk (218).

There are other examples in the literature of a binding-triggered signal for ubiquitination. Human papillomavirus protein E6 binds the cellular factor, E6-AP, and this pair associates with p53, whereupon p53 is targeted for destruction via the ubiquitin-mediated proteolytic pathway (194). An example of activation-triggered ubiquitination is found in a report regarding protein kinase C (PKC) (136). Treatment of cells with phorbol esters activates and then depletes some PKC isoforms; this depletion is a result of ubiquitination that is stimulated upon activation of PKC. Blocking activation of these
isoforms blocks ubiquitination and destruction (136). The authors speculate that activation of the ubiquitin-conjugating system is likely stimulated by a conformational change in PKC that occurs upon ATP binding or hydrolysis, resulting in a suicide model for PKC regulation.

**Figure 3-12 Model.**

When GRF2 becomes activated by upstream signals, the REM (Ras Exchanger Motif) assists in stabilizing the Cdc25 domain to allow for efficient exchange. This interaction and subsequent binding to Ras causes the DB to be looped out, creating a structure that is recognized by the ubiquitination machinery. This results in ubiquitination of GRF2, followed by degradation.

I propose that in an unstimulated cell GRF2 is in an inactive complex or conformation, perhaps involving intramolecular interactions or an interaction with an unknown negative regulator. Upon stimulation of the cell with an agent that causes an increase in intracellular calcium levels, GRF2 is activated such that it is now capable of binding to Ras. If the REM of GRF2 is involved in the stabilization of the Cdc25 domain as appears to be the case with Sos (16), this binding may “loop” out the stretch of amino acids containing the DB. As a consequence of the interaction with Ras, the intervening DB is
placed into an active state or conformation, causing the protein to be targeted for destruction (Fig. 3-12). Another Ras exchange factor, CNrasGEF, contains a PDZ domain between its REM and Cdc25 domains (176) and it is tempting to speculate that the activity of the PDZ domain is regulated by Ras binding in a similar manner as the DB of GRF2.

One possible explanation for the existence of multiple Ras-dependent signaling systems is that different signals are required at specific stages of the cell cycle. In addition to its ability to transform cells, Ras has been established as an important cell-cycle regulator. Microinjection of activated Ras into quiescent fibroblasts drives entry into S-phase. Moreover, in some cell types, injection of neutralizing antibodies to endogenous Ras results in the cell-cycle arrest of cells growing in serum and the inability to progress through to S phase (56, 61, 153). More recently, use of a novel method for detecting Ras-GTP, which involves affinity precipitation of activated Ras using its binding partner Raf, allowed the activation state of Ras to be followed throughout the cell cycle (219). Using this method, Taylor & Shaloway demonstrated that in quiescent HeLa cells treated with serum, activation of Ras is achieved immediately after serum addition. Four to five hours later in mid G1 phase, there is a second, much stronger activation of Ras, which does not appear to involve tyrosine phosphorylation (and therefore Grb2-Sos complexes). The pattern of Ras activation is the same when cells are grown in the presence or absence of serum, or in suspension or attached to a substratum. These results point to a mechanism of Ras activation that is integral to the cell-cycle machinery and is not solely linked to receptor-tyrosine kinase activation. It is possible that this mid-G1 activation of Ras is stimulated by GRF2, making this event calcium-dependent. I have shown that GRF2 protein is present at this time during the cell cycle and shortly thereafter GRF2 protein levels begin to decline, perhaps because
after this putative activation of GRF2 which leads to Ras binding, GRF2 is targeted for destruction.

As other DB-containing proteins are regulated in a cell-cycle dependent manner, perhaps GRF2 is also regulated in this way. Protein destruction is an excellent way to drive a pathway in one direction, as evidenced by the numerous cell-cycle regulatory proteins whose levels or activities are controlled in this manner. It is also a mechanism that can be utilized to prevent an event from occurring at an inopportune time, and it may be for this reason that GRF2 is destroyed: in order to prevent activation of Ras at an inappropriate time in the cell cycle, perhaps in G2/M phase when calcium oscillations are observed (13). I note that in rat fibroblasts, a high level of Ras activity in G\textsubscript{2} results in a G\textsubscript{2} arrest (96). An equally plausible explanation for the destruction of GRF2 is that this is a method of "turning off" the signal following Ras activation. This downregulation presumably would be part of a complex series of signaling events occurring following stimulation of a cell in order to elicit the desired response, be it progression through the cell cycle or differentiation. In either case, the regulated destruction of an exchange factor is a unique method of regulation in the Ras pathway in mammalian cells.
CHAPTER 4

Summary and Future Directions
SUMMARY

GRF2 is a widely expressed protein whose primary structure reveals the presence of multiple modular domains (66). These domains include (in N- to C-terminal order): an N-terminal PH domain, a coiled-coil motif, an IQ motif, a DH domain, another PH domain, a destruction box, a Ras Exchanger Motif and a C-terminal Cdc25 catalytic domain. By virtue of its multi-domain structure, GRF2 functions to regulate numerous signaling pathways inside the cell. More specifically, GRF2 is able to activate both the Ras-ERK pathway as well as the Rac-SAPK cascade (67). In addition to its role in coupling the Ras and Rac signaling pathways, GRF2 also regulates the activation of their respective MAPK cascades in response to changes in intracellular calcium levels (66, 67).

When expressed ectopically in 293T cells, GRF2 co-immunoprecipitates with the calcium sensor calmodulin (52, 66). This interaction is calcium-dependent in vitro as the addition of a calcium chelator such as EGTA abolishes the association of GRF2 with calmodulin. The interaction between GRF2 and calmodulin requires the IQ motif as a deletion mutant missing this region can no longer bind to calmodulin; this lead to the hypothesis that CaM binding to the IQ motif activates GRF2 and this is the reason that GRF2 is calcium-responsive. More recently, I have shown that CaM binding is not required for Ras or ERK activation or for calcium responsiveness, as the only GRF2 deletion mutant protein studied that binds CaM like the wildtype protein is ADB (52). Indeed, since the PH mutants and the Cdc25-deleted protein were severely impaired in their association with CaM, it is clear that optimal CaM association with GRF2 requires an intact amino-terminal region of GRF2 (52). There is no clear correlation between CaM association and Ras or ERK
activation by GRF2. The ΔDH mutant remains responsive to calcium signals for ERK activation in the absence of CaM interaction (52). This indicates that calcium can affect GRF2 in a CaM binding-independent manner.

However, the IQ motif does play an important role in GRF2 signaling in response to calcium, as abolishing the IQ motif results in a protein that is not calcium responsive and cannot activate ERK or SAPK (52, 67). ΔIQ can, however, activate Ras; therefore, I set out to determine at what point between Ras and ERK the MAPK cascade was downregulated. I examined the activation state of Ras, Raf, MEK and ERK and discovered that although Ras was activated by ΔIQ, Raf was not (52). I can predict various means by which this could occur. The first is that GRF2 is able to bind to and activate Ras in a futile manner such that GRF2 and the Ras-GTP produced are not properly localized, anchored, or oriented at the plasma membrane to physically couple to components of the ERK pathway. Yet another explanation may be that in the absence of the IQ motif, a Raf phosphatase is activated and turns Raf off before the MAPK pathway is activated. Alternatively, the IQ motif may play a role in recruiting or activating a kinase required for Raf activation such that in the absence of the IQ motif this kinase activity is absent. Since the IQ motif is necessary for ERK activation by WT GRF2, I conclude that a function of the IQ motif, and possibly of the CaM interaction, is to overcome a constraint on GRF2-ERK signaling. The precise function of CaM association with GRF2 therefore remains to be determined.

The role of calcium in activating GRF2 is, therefore, still unresolved. It is possible that calcium may induce the phosphorylation of GRF2, and/or activate plasma membrane binding sites for GRF2 required for its activation such that in the absence of a calcium signal GRF2 cannot translocate to the membrane and activate Ras (Fig. 4-1).
Figure 4-1  Model of calcium activation of membrane binding sites.

In the absence of Ca\(^{2+}\) GRF2 cannot access the membrane or Ras because its membrane binding site is either not exposed or is not present at the membrane. When calcium levels rise, the calcium activates membrane binding sites for GRF2, either by allowing exposure of a GRF2 binding protein or by recruiting this unknown protein to the membrane, allowing GRF2 access to Ras and activation of Ras effector pathways such as the Raf-ERK kinase cascade.

**WITHOUT CALCIUM**

**WITH CALCIUM**

Or, instead of (or along with) affecting CaM binding, calcium may promote the assembly or proper orientation of a protein network able to couple with the ERK pathway. Other evidence in support of such a scaffolding or anchoring role for GRF2 in coupling Rac activation with stimulation of the SAPK pathway in 293T cells has been reported (67). In this model, GRF2 and perhaps other GEFs are not simply upstream activators of their target GTPases but may also determine effector interactions (Fig. 4-2).

I have also shown that deleting the N-terminus of GRF2 results in a protein that is slightly more active than full-length GRF2 when assayed for Ras activation *in vitro* and *in vivo*. I conclude that one function of the N-terminus is to restrain GRF2 signaling in unstimulated cells. Since the ΔPHc mutants were competent for Ras-ERK signaling and activated Ras to a greater extent than wildtype GRF2 *in vivo*, I conclude that the REM-Cdc25
portion of GRF2 may be repressed in a manner dependent on the PHc domain in unstimulated cells.

**Figure 4-2** GEFs play a role in determining which effectors are activated.

It is possible that the panel of effectors that are activated in response to a specific Ras-activating signal are specified by the identity of the exchange factor responsible for activating Ras. Light blue box, IQ motif; dark blue box, Cdc25 domain.

I have also shown that the ΔCdc25 protein acts as a dominant-negative and inhibits activation of wildtype GRF2 when overexpressed. This could be occurring in one of three ways: 1) ΔCdc25 could be saturating membrane binding sites for GRF2; 2) it could be titrating out upstream activators; or 3) oligomers between WT GRF2 and ΔCdc25 could be inactive (Fig. 4-3). These possibilities need not be mutually exclusive. There is evidence that GRF2 forms oligomers both from my work and from Anborgh et al. (4) and I believe that this is a likely explanation for the dominant-negative phenotype. Dimerization of exchange factors provides for a combinatorial possibility for Ras activation. Various Ras GEFs are activated or produced in response to different signals and oligomerization would allow for a more
complex integration of signaling. The composition of the putative GEF dimer could also play a role in determining which effectors are activated downstream of Ras.

![Diagram](image)

**Figure 4-3**  Possible explanations for the dominant-negative behaviour of ΔCdc25.
One explanation is that the excess ΔCdc25 protein saturates the membrane binding sites (dark blue shapes) that GRF2 requires in order to activate Ras, thereby preventing access by WT GRF2. It is also possible that dimer formation is required for Ras activation by GRF2 and that the ΔCdc25/GRF2 dimer is inactive towards Ras. A third possibility is that the ΔCdc25 protein merely titrates out upstream activators (purple box), thereby preventing activation of WT GRF2. These are not, however, mutually exclusive possibilities.

I have also demonstrated that regulation of GRF2 is also controlled by ubiquitin mediated proteolysis. GRF2 contains a motif called a destruction box that is found in unstable proteins such as mitotic cyclins and the anaphase inhibitor, Pds1p. GRF2 is an unstable protein once it becomes activated and binds Ras, but it is protected from degradation
if the destruction box is deleted. GRF2 is ubiquitinated *in vivo*, but ubiquitin conjugates are severely reduced in the absence of Ras binding. The protein is still destroyed in the absence of Ras-ERK signaling which suggests that it is not a feedback loop that results in the downregulation of GRF2. Using proteasome inhibitors, I demonstrated that GRF2 is likely destroyed by the 26S proteasome.

**Figure 4-4** The role of the IQ motif.

In the presence of the IQ motif, activated Ras can activate Raf and the MAPK cascade. Without the IQ motif, Raf is not capable of becoming activated. The model as drawn depicts a direct interaction between Raf and the IQ motif, but it is equally possible that there are other proteins involved. Light blue box, IQ motif; dark blue box, Cdc25 domain.

In summary, I have demonstrated that the role of CaM and calcium in GRF2 signaling is significantly more complex than was previously thought. One major conclusion from my research is that GRF2, and perhaps other exchange factors, are capable of determining which effectors of their cognate GTPase become activated in response to an activated GTPase. The ability of the ΔIQ mutant to activate Ras but not to couple it to the ERK pathway suggests that in addition to functioning as an upstream activator of Ras, GRF2,
and interactions involving the IQ motif in particular, may serve to couple Ras with its effectors (Fig. 4-4). I have also provided the first evidence of \textit{in vivo} ubiquitin-mediated proteolysis of a Ras exchange factor. This work provides clues that the endogenous role of GRF2 is to activate Ras in a cell cycle dependent manner.

**FUTURE DIRECTIONS**

There are many aspects of GRF2 regulation and function that remain to be investigated. The challenge for the future is to determine the roles played by other domains of GRF2, how each of the domains interact with one another and how this contributes to the overall function of GRF2. Most importantly, we need to uncover the physiological role played by GRF2 which may provide important insight into how cells normally divide and differentiate and how they become neoplastic and metastatic in cancer.

**What is the physiological role of GRF2?**

To truly determine the function(s) of GRF2, the protein needs to be examined in its native setting. In this way, we will be better able to determine the signaling pathways that GRF2 participates in and the physiological role that GRF2 plays in the context of a particular cell type. The one thing that will advance GRF2 research in many areas is the discovery of a cell line that expresses detectable levels of GRF2. Alternatively, the production of better antibodies to detect the GRF2 that may be unknowingly present in many cell lines commonly in use in the laboratory would be of great benefit. Once a cell line is identified, we can introduce into it the dominant-negative version of GRF2 that I have identified, using a Tet-inducible system. Various experiments could then be performed to see if any responses of
the cell have changed in the absence of GRF2, whether it be mitogenic or differentiation
responses. Mouse knockouts of the brain specific GRF1 have been generated; phenotypes
noted were that memory consolidation is impaired, especially fear responses, while learning
and short-term memory are intact (22, 75). Generation of a knockout mouse for GRF2,
which is a much more widely expressed protein, would provide us with interesting
information regarding the role of GRF2 and Ras activation during development. It would be
interesting to see if a GRF2 knockout has overlapping phenotypes with a GRF1 knockout; a
double knockout would be exciting in that we could see the effects on the brain of missing
two Ras exchange factors that are normally expressed there. Given the more varied
expression pattern of GRF2 (as determined by Northern blot analysis) which includes
skeletal muscle, lungs and spleen, I predict that a GRF2 knockout would have more severe
effects than a GRF1 knockout.

The overexpression of GRF2 in NIH 3T3 and Rat2 cells fails to induce foci formation
(CdH, unpublished observations); similar negative results have also been reported for the
mammalian Sos1 protein (236). Despite this lack of transforming ability in fibroblasts,
epithelial cells (293 cells) stably expressing GRF2 exhibit altered growth properties (66).
Unlike the parental cells which grow in a typical epithelial organization of clusters of tightly
opposed cells, the GRF2-expressing cells grow in a more dispersed, disorganized fashion
with decreased cell-cell contacts. As the expression of oncogenic ras and src have been
shown to disrupt such epithelial cell-cell contacts (12, 112), it may be that GRF2 has similar
effects by activating endogenous Ras proteins.

Members of the Rho family of small G-proteins are effectors of actin organization
and focal complexes in fibroblasts (125, 160, 183, 184) and are known to affect cell adhesion
and the cytoskeleton in epithelial cells (161, 184). Given that GRF2 can activate Rac and SAPK (67), it is possible that it may also play a role in regulating changes in cytoskeletal structures, cell morphology and cell adhesion. These potential activities of GRF2 could be studied by transfecting or microinjecting GFP-tagged GRF2 expression plasmids into fibroblasts or epithelial cells, immunostaining the cells with rhodamine-phalloidin which detects polymerized actin, and comparing cells which express GRF2 to those which do not. In particular, we could look at changes in the actin cytoskeleton (i.e. appearance of ruffles, filopodia or stress fibres), variations in the components of focal adhesion complexes such as whether GRF2 localizes with cadherins, catenins, or vinculin, among others. Alterations in cell shape, growth rate and other morphological changes could be examined.

I have determined that GRF2 contains a DB similar to that of mitotic cyclins, suggesting that GRF2 may be regulated in a cell-cycle specific manner. My studies are limited as I am restricted to using a stable cell line overexpressing GRF2 from a cytomegalovirus promoter which appears to be active throughout the cell cycle. However, I have determined that GRF2 protein levels begin to decline at the beginning of S-phase. Often the levels of cell cycle regulated proteins are controlled at the transcriptional as well as post-translational level; therefore, I am missing an important level of regulation in my studies of GRF2. Once we have identified an endogenous source of protein, the cell cycle regulation of GRF2 protein levels can be studied in much greater detail. Once again, a Tet-inducible dominant-negative approach could be used to determine if there are any cell cycle phenotypes associated with the removal of GRF2 activity. Also, it would be useful to create a ΔDB stable line and use it to determine what effect lack of the DB has on the stability of the protein throughout the cell cycle.
What proteins regulate ubiquitination of GRF2?

One of the outstanding questions from my work relates to the identities of the proteins involved in the ubiquitination of GRF2. Because GRF2 contains ubiquitination signals that could target it through either the APC or the SCF ubiquitin ligase complexes, it is important to determine which of these important regulatory pathways contributes to the downregulation of GRF2. To elucidate which complex is contributing to the ubiquitination of GRF2, immunoprecipitates of GRF2 could be tested to see if GRF2 interacts with any of the known core components of the APC or SCF. If any interaction is seen, the next step would be to identify the adapter molecule that brings GRF2 to the ubiquitin ligase complex, whether that be an F-box protein for the SCF or a Cdh1-like adapter molecule for the APC. This experiment could also be done using mass spectrometry to identify these interacting proteins.

Along the same lines, it would also be useful to identify which E2 (ubiquitin conjugating) enzyme is responsible for ubiquitinating GRF2. If GRF2 is ubiquitinated by the SCF that would imply that Cdc34 is the E2 responsible for ubiquitinating GRF2, but if the APC is targeting GRF2, then the identity of the E2 is not as obvious.

GRF2-interacting proteins.

At this point, CaM, the small GTPases Ras and Rac, and other GRF2 molecules are the only proteins known to interact with GRF2. It would be interesting to know what other proteins interact with GRF2 and what effect these proteins have on the regulation and function of GRF2. As mentioned above, presumably GRF2 interacts with proteins capable of targeting it for ubiquitination. As I proposed in Chapter 2, I believe that GRF2 is capable of determining effector interactions; this in itself requires that GRF2 interact with proteins other than Ras and CaM but it is unknown at this point which proteins these are. GRF2 is capable
of activating the MAPK cascade only in the presence of the IQ motif indicating that perhaps the IQ motif either binds Raf directly or binds an unknown protein that is capable of affecting Raf activation downstream of GRF2.

The search for interacting proteins could be done either with mass spectrometry or with alternative methods for detecting protein-protein interactions, such as a two hybrid assay. These interacting proteins can then be characterized in terms of how they interact with GRF2 (i.e. which domains of GRF2 are required for binding?) and how the interaction affects GRF2 activity (i.e. do they negatively or positively regulate GRF2?).

What are the extracellular ligands and upstream signals that activate GRF2?

In order to fully understand the function of GRF2 in growth or differentiation, it is not sufficient to understand only the downstream pathways that GRF2 is capable of activating. Thus far, we have studied only these downstream effects of GRF2. It is also important to appreciate what extracellular ligands, receptor systems and upstream activators lead to the activation of GRF2. The best way to study this aspect of GRF2 function would be to use a cell line endogenously expressing GRF2. If the studies on GRF2 interacting proteins did not reveal any hints as to what the upstream activators of GRF2 are, then we could start with a candidate approach. Because GRF2 is a calcium responsive exchange factor, cell receptors for ligands such as lysophosphatidic acid, bombesin, and peptide growth factors that lead to increases in intracellular calcium upon interaction with their ligands would be an excellent place to begin. With GRF1, for example, LPA- or carbachol-stimulation of muscarinic receptors increases exchange factor activity and phosphorylation state (143-145). Given the similarities in primary structure between GRF1 and GRF2 and that they both are calcium responsive, GRF2 may also be activated by the stimulation of a G-protein-coupled receptor.
Cell responses to various receptors could be tested in the presence or absence of GRF2 (if it must be done with transfected GRF2) or in the presence or absence of the GRF2 dominant-negative protein with endogenously expressed GRF2.

Another approach to identifying the upstream signals that regulate GRF2 would be to examine the phosphorylation state of GRF2 and identify putative kinases. GRF2 was shown to be serine phosphorylated in response to ionomycin treatment (65). More recently, GRF2 was found to be tyrosine phosphorylated in cells transiently transfected with v-src (W.-T. Fan and M.F. Moran, unpublished results). Also, I have shown through mass spectrometry that GRF2 is phosphorylated near the DB on serine/threonine residues. It is not known what role these phosphorylation events play, but it is conceivable that they are regulatory phosphorylation events, leading to the activation of GRF2. The phosphorylation of GRF2 in response to stimulation by different ligands can be examined by anti-phosphoserine/threonine Western blotting, and the phosphorylated residues can be identified by tryptic peptide mapping or by mass spectrometry. Once the phosphorylated site is identified, putative kinases can be identified based on consensus sequence and their ability to phosphorylate GRF2 can be analyzed by performing kinase assays using GRF2 as a substrate. The role of the phosphorylation events in GRF2 regulation can be studied by mutating the site to a residue that cannot be phosphorylated and studying the effect that has on GRF2 function.

What is the function of the oligomerization of GRF2?

The solved structure of the REM-Cdc25 region of Sos bound to Ras does not suggest that Sos acts as a dimer (16), yet it has been published that GRF1 and GRF2 form homo- and hetero-oligomers through their DH domains (4). Anborgh et al. also investigated whether this interaction between DH domains in exchange factors is universal by examining whether
or not GRF1 can oligomerize with Sos and Dbl, two proteins containing DH domains (4). They detected no interaction between GRF1 and the other DH domain proteins and concluded that only the DH domains of the GRFs, which are the most similar to one another, are capable of forming this kind of interaction. Thus, the function of the oligomerization remains elusive. If the Cdc25 domain of Sos is capable of activating Ras without the added complexity of oligomer formation then what is the role of this oligomer formation in GRF2, which contains a Cdc25 domain homologous to that of Sos? Firstly, it would be useful to determine if GRF2 is forming dimers or higher order oligomers. This can be determined by separating lysate from GRF2-expressing cells on a size-exclusion column and comparing the position of the GRF2 elution peak to molecular weight standards. The size at which GRF2 elutes will provide clues as to whether GRF2 is part of a complex larger than the predicted size of a GRF2 dimer. Also, it is important to determine if GRF2 can form oligomers independent of other proteins, or if there is an unknown protein bridging the two (or more) molecules. This possibility could be tested by performing affinity chromatography or gel overlay assays with recombinant, purified GRF2 molecules. If the interaction is not direct, the molecule that is bridging the two proteins must be highly conserved, as the interaction between GRF1 and GRF2 can be detected in yeast (4).

If the recombinant proteins do interact with each other, then it would be possible to use these polypeptides in an in vitro exchange reaction to test their activity. This could be compared to a ΔCdc25 and WT oligomer, testing the hypothesis that it is a defect here that results in the ΔCdc25 protein behaving as a negative inhibitor.
How does GRF2 affect cell morphology?

293 epithelial cells stably expressing high levels of GRF2 exhibit altered growth properties (66). Unlike the parental cells which grow in clusters of tightly opposed cells, the GRF2 expressing cells grow in a more dispersed, disorganized fashion with decreased cell-cell contacts and look more similar to fibroblasts than epithelial cells. As the expression of oncogenic src has been shown to disrupt such epithelial cell-cell contacts (12), it may be that GRF2 has similar effects by activating endogenous Ras proteins. To discern whether tight and adherens junctions are disrupted in the GRF2 expressing cells, these structures can be visualized by electron microscopy (12). It would also be interesting to microinject GRF2 plasmids into cell nuclei and then follow the structure of the junctions over time as the concentration of GRF2 increases. The cells could be observed to examine whether GRF2 can stimulate dissociation and they could be subjected to indirect immunofluorescence to investigate whether there is a redistribution of any components of the junctions as GRF2 proteins are expressed. Alternatively, it is also possible that the phenotype observed in GRF2 expressing cells results from the regulation of the Rho family proteins by GRF2.

Rac, as a member of the Rho family of small G-proteins, is an effector of actin organization; its activation promotes actin polymerization at the cell periphery to form lamellipodia and membrane ruffles which can also be seen after stimulation of cells with PDGF (184). Significant cross-talk between Rho, Rac and Cdc42 has also been observed (160). Constitutively activated Cdc42 causes induction of filopodia as well as the activation of Rac, while activated Rac causes the formation of lamellipodia and membrane ruffling and the activation of Rho (160, 184). Activated forms of Rac1 and Cdc42 have also been shown to potently activate SAPK (46, 149, 220). Given that GRF2 can activate Rac and SAPK
through its DH domain, it is possible that it may also play a role in regulating changes in cytoskeletal structures, cell morphology and cell adhesion. These functions can be investigated by microinjecting GRF2 plasmid (WT and the isolated DH or DH/PH domains) into fibroblast or epithelial cells and examining the cells for any changes in actin morphology in cells expressing GRF2.

What is the role of the IQ motif?

I have shown that the ΔIQ protein activates Ras but not the downstream Raf-MAPK cascade and have proposed the following hypotheses to explain my results: 1) without the IQ motif of GRF2, Raf cannot relocalize to the membrane, suggesting the Raf either binds to the IQ motif or to a ligand of the IQ motif; 2) in the absence of the IQ motif, activated Raf is downregulated by a phosphatase; 3) in the absence of the IQ motif, Raf is not capable of being activated by a kinase, either because the kinase cannot be recruited to the complex, or because Raf is not oriented properly to become phosphorylated; and 4) Raf does translocate to the plasma membrane, forms a complex with Ras and is activated properly, but the complex isn't oriented properly and thus cannot access the rest of the MAPK cascade.

The first possibility can be pursued with immunocytochemistry: using anti-Raf antibodies, determine if Raf relocalizes to the membrane when GRF2 and ΔIQ are present. If the ΔIQ protein is preventing Raf from accessing Ras at the membrane, then perhaps we would see Raf being excluding from the plasma membrane after stimulation in the presence of the IQ motif. To determine if Raf is being shut off by a phosphatase that is activated in the absence of the IQ motif, we can co-transfect ΔIQ and activated Raf. If ΔIQ is activating a phosphatase, then the activated Raf should be inhibited by it. Using phospho-specific antibodies, I have tested the phosphorylation state of Raf at Ser259 and Ser621 and have
detected no differences between WT and ΔIQ, suggesting that the kinases responsible for these phosphorylations are capable of phosphorylating Raf in both cases.

What are the roles of the PH domains of GRF2?

The roles of the PH domains of GRF2 have yet to be thoroughly examined. While it has been demonstrated that the PHn domain may act to negatively regulate GRF2 function, the precise mechanism by which this is achieved has not been explored. PH domains are important in targeting GRF1 and Sos to specific subcellular compartments (25, 41). Although it has been shown that GRF2 translocates to the cell periphery in response to an increase in calcium levels (66), we have not shown that this response is mediated by the PH domains. To examine the putative role of the PH domains in regulating the subcellular localization of GRF2, cell biology experiments using EGFP-tagged proteins (wildtype GRF2 and individual PH domain) may be performed. The subcellular localization of GRF2 and its domains can be visualized by confocal microscopy. Lipid binding experiments and subcellular fractionation can also be used to determine whether the PH domains associate with components of the plasma membrane.
CHAPTER 5

References


