Expression Cloning of RasGRP: a member of the CDC25 family of Ras exchangers

by

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Date  
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DE-6 (2/88)
Abstract

The fact that Ras mutations are found in approximately 30% of all human malignancies points towards a role for Ras signalling pathways in the process of malignant transformation. An expression cloning strategy was used to identify genes that were equal to Ras in their ability to transform cells. Upon screening a T hybridoma cell cDNA library we identified a novel cDNA whose overexpression in NIH 3T3 cells caused transformation phenotypically similar to that of Ras overexpression. RasGRP, the protein encoded by this cDNA, contained several unique structural features, the foremost being regions of high sequence similarity to the CDC25 family of guanine nucleotide exchange factors (GNEFs). RasGRP not only contained the three structurally conserved regions found in these domains but also contained a Ras exchange motif (REM) box that is found in GNEFs that are capable of exchange on true Ras proteins. Overexpression of RasGRP caused hyperactivation of MAP kinases, specifically ERK 1 and 2. This data is compatible with the idea RasGRP is capable of causing Ras activation. Furthermore, the ability of RasGRP to transform cells and activate MAP kinase was eliminated by mutations affecting the exchange factor domain or by deletions of the REM box. RasGRP also contained a C1 domain which was very similar in sequence to diacylglycerol- and phorbol ester-binding C1 domains of protein kinase Cs (PKCs). RasGRP’s C1 domain was found to be essential for fibroblast transformation and could be functionally replaced by a prenylation signal or by the C1 domain of PKCδ. RasGRP was found to localize to the ER as well as other membranes and this localization was dependent upon the C1 domain. The isolated C1 domain was also capable of re-localization in response to phorbol ester stimulation and to increases in diacylglycerol produced upon phosphatidylcholine-phospholipase C stimulation. These responses were identical to those of the PKCδ-C1 domain. Taken altogether, these results provide the evidence on which to base the following model of Ras activation via RasGRP. It states that RasGRP’s C1 domain facilitates the translocation of RasGRP to membranes enriched in diacylglycerol or
phorbol ester. Once at the membrane, RasGRP is able to interact with Ras proteins tethered there by prenylation. GDP exchange then takes place via RasGRP's REM/GEF domains and the Ras pathway is activated. RasGRP also contains a pair of calcium binding EF hands, a proline rich region, and an α-helical domain. These domains were found to be non-essential for fibroblast transformation and it will be of interest to discover what roles they play in RasGRP function. RasGRP exhibits a unique pattern of expression when compared to other GNEFs capable of exchange on Ras. It is expressed extensively in brain, thymus, spleen, and bone marrow, which points to possible roles in neuronal and lymphoid signalling.
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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>B23</td>
<td>Bosc 23</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CRD</td>
<td>cystein rich domain</td>
</tr>
<tr>
<td>CRU</td>
<td>competitive repopulating unit</td>
</tr>
<tr>
<td>CS</td>
<td>calf serum</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEP</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EPAC</td>
<td>exchange protein directly activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FC</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>full length</td>
</tr>
<tr>
<td>FTase</td>
<td>farnesyl transferase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GDS</td>
<td>guanine nucleotide dissociation stimulator</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<tr>
<td>GNEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S transferase</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<td>HBS</td>
<td>hepes buffered solution</td>
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<td>horse serum</td>
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<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KSR</td>
<td>kinase suppressor of Ras</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitate</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositoltriphosphate</td>
</tr>
<tr>
<td>LAT</td>
<td>lymphoid specific adapter protein</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP/ERK kinase</td>
</tr>
<tr>
<td>MMLV</td>
<td>moloney murine leukemia virus</td>
</tr>
<tr>
<td>MPSV</td>
<td>myeloproliferative sarcoma virus</td>
</tr>
<tr>
<td>Mt</td>
<td>mitochondria</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosyl binding</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>RasGRP</td>
<td>Ras guanine nucleotide releasing protein</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras binding domain</td>
</tr>
<tr>
<td>REM</td>
<td>Ras exchange motif box</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
</tr>
<tr>
<td>SCR</td>
<td>structurally conserved region</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STR</td>
<td>short term repopulating</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>
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It is good to have an end to journey towards,
but it is the journey that matters, in the end.
Ursala Le Guin
Chapter I: Introduction

Ras Proteins

The Ras proteins are three closely related GTPases: H-, K- and N-Ras. Ras proteins have been shown to bind guanine nucleotides and possess intrinsic GTPase activity. (Shih et al, 1980; Papageorge et al, 1982; Poe et al, 1985; Hara et al, 1988) H-Ras and K-Ras were first identified as oncogenes in the acutely transforming Harvey and Kirsten strains of rat sarcoma viruses (Reviewed by Barbacid, 1987; Young et al, 1979; Shih et al, 1979; Dhar et al, 1982; Shimizu et al, 1983) while N-Ras was identified as a dominant oncogene in a human neuroblastoma. (Ireland et al, 1989; Taparowsky, 1983; Ballas, 1988; Jouanneau et al, 1987) It was determined that the viral oncogenes were derived from cellular proto-oncogenes found in the host’s genome. (Langbeheim et al, 1980; McBride et al, 1983) The virally transduced H-Ras and K-Ras, and the tumour-derived N-Ras, were found to have activating point mutations that disabled their GTPase activity. In addition to causing sarcomas in vivo, activated Ras expression was found to induce oncogenic transformation in rodent cell lines. (Chang et al, 1982; Stacey and Kung, 1984; McKay et al, 1986) For example, fibroblast cells expressing activated Ras lost their wild type flattened morphology and exhibited an increase in their proliferative capacity. (VanRoy et al, 1986; Wilson et al, 1990). These phenotypic changes are characteristic of the phenomenon known as oncogenic transformation. It has now been determined that Ras proteins play a critical role in the control of normal and transformed cell growth (Shirasawa et al, 1993; Papageorge et al, 1986; Reviewed by Lowry et al,
and are highly conserved throughout evolution, having been identified in many diverse systems such as fungi, flies, nematodes and mammals.

**Basic Function of Ras GTPases**

Ras proteins are often described as molecular switches that are active in their GTP-bound state and inactive in their GDP-bound state. Conformationally distinct from the inactive form, the active GTP-bound Ras protein is capable of interacting with putative effector molecules and passes on a signal to downstream proteins before returning to its inactive state. (Reviewed by Campbell *et al.*, 1998; Vojtek *et al.*, 1998)

The GTP-bound form is slowly converted to the GDP-bound form by the protein’s intrinsic capacity to hydrolyze GTP. (Bourne *et al.*, 1991; Maegley *et al.*, 1996) Figure 1 illustrates the basic concepts of the GTPase cycle that have been described here.

Activating mutations can cause a reduction of the molecule’s GTPase activity which results in the accumulation of GTP-bound Ras. (Reviewed by Barbacid, 1993; Clark *et al.*, 1993; Segal *et al.*, 1993; Portier *et al.*, 1993; Dalley and Cannon, 1996)

Alternatively, some transforming mutations increase the rate at which Ras exchanges guanine nucleotide with the surrounding medium. This increase in exchange rate also leads to an accumulation of the active GTP-bound form of Ras.

**Structure of Ras GTPases**

Sequence comparisons of different Ras family members have helped to define five highly conserved domains which have been named G1 through G5. (Reviewed in Gomez *et al.*, 1998) Four of these regions (G1, G3, G4, and G5), mediate binding and hydrolysis of the guanine nucleotide. The effector loop, or region of the Ras molecule
Figure 1. The Ras GTPase Cycle
Ras and Ras-related proteins cycle between active GTP-bound and inactive GDP-bound states. The GDP/GTP balance of Ras molecules can be dictated by the rates of guanine nucleotide exchange controlled by Guanine Nucleotide Exchange Factors (GNEFs) and GTP hydrolysis which is controlled by the actions of GTPase Activating Proteins (GAPs).
that interacts with other proteins when in its GTP-bound state, is found in regions G2 and part of G3. (Reviewed: Campbell et al, 1998) The conformation of Ras-GTP differs from that of Ras-GDP in two distinct regions within the molecule and these have been designated the switch I (residues 30-37) and switch II regions (residues: 59-76).(See: Figure 2; Milburn et al, 1990; Schlichting et al, 1990)

Another common structural feature found in Ras molecules is the carboxyl (C)-terminal prenylation signal. Protein prenylation is a post-translational lipid modification which leads to plasma membrane association. It can be defined as the stable covalent modification of cellular proteins with isoprenoids of two types, either C15 farnesyl or the C20 geranylgeranyl, to carboxyl-terminal cysteine residues. (Reviewed by Seabra, 1998)

Newly synthesized Ras molecules are farnesylated by an enzyme known as farnesyl transferase (FTase) which recognizes a conserved motif found at the carboxyl-terminus known as a CAAX box (C=cysteine, A=aliphatic residue, X=any amino acid). After farnesylation, Ras proteins undergo proteolysis of the last three amino acids in the CAAX box via a CAAX protease.(Gutierrez et al, 1989) This event is followed by carboxyl methylation of the farnesylated cysteine by a specific prenylcysteine-dependent methyl transferase.(Clarke, 1992; Rando et al, 1996) The end result of this process is a Ras molecule which possesses a methylated farnesyl group attached to its very C-terminal cysteine. Farnesylation itself provides barely enough hydrophobicity for membrane association and methylation increases this membrane avidity by ten fold. In addition to prenylation/methylation, Ras molecules possess another signal which is necessary for stable membrane association. This second signal lies just upstream of the CAAX box and can be of two types: either a stretch of basic residues known as a polybasic region as is
Figure 2. Domains found within Ras proteins
The five domains found in Ras molecules that were identified by sequence comparison, are highlighted in the figure and are named G1 through G5. Amino acid numbering is found below the figure. Switch I and II regions are indicated by the arrow heads. The core effector domain is deliniated by the bracketted, dotted lines. The C terminal prenylation signal is signified by CAAX.
the case with K-Ras, or palmitoylation of upstream cysteines as is the case with H-Ras. (Glomset et al, 1990; Maltese, 1990; Seabra, 1998; Gomez et al, 1998; Gelb, 1998) Therefore two signals, prenylation/methylation and a polybasic region or palmitoylation, are required for stable membrane attachment. The importance of this process to the biological function of Ras has been revealed by studies showing that mutation of the C-terminal cysteine is able to inactivate oncogenic Ras by preventing membrane association. (Willumsen et al, 1984a/b) Therefore, targeting of Ras proteins to the plasma membrane concentrates and localizes these molecules in regions where they are able to come in contact with downstream effector molecules.

**The Ras Family of small GTPases**

H-, K- and N-Ras proteins have been classified as a subfamily known as “true” Ras proteins found within the Ras family of GTPases. The Ras family itself is part of a very broad superfamily of small GTPases known as the Ras superfamily. Table 1 outlines the classification of these GTPases. In addition to the Ras family, the Ras superfamily is comprised of five families that have been classified based upon their sequences and functional similarities, they are: Rho/Rac, Rab, Ran, Arf, and Rad. Further discussion will be limited to the Ras family of proteins as these are the most relevant for the understanding of this thesis project.

**Functions of Mammalian Ras Family Members**

The Ras family can be broken down into nine different subfamilies consisting of: the true Ras proteins, the Rap subfamily, R-Ras, TC21(R-Ras2), R-Ras3, RalA/B, Rheb,
The Ras Super Family of GTPases

I  Ras Family
   a  True Ras Protein Subfamily
      (N-, H-, K-Ras4A, K-Ras4B)
   b  Rap Subfamily
      (Rap1A/B, 2A/B)
   c  R-Ras
   d  TC21 (R-Ras2)
e  R-Ras3
   f  Ral Subfamily (A/B)
g  Rheb
   h  Rin
   i  Rit

II  Rho/Rac Family

III  Rab Family

IV  Ran Family

V  Arf Family

VI  Rad Family

Table 1. The Ras Super Family of GTPases
Rin, and Rit. (Reviewed by Campbell et al, 1998) Additional information about each member of the Ras family will be discussed in the next few sections.

**“True” Ras Proteins**

The true Ras protein subfamily consists of the four highly related proteins (H-Ras, N-Ras, K-Ras4A and K-Ras4B) and are often generically referred to as p21. The K-Ras4 gene can be alternatively spliced to generate the two C-terminally distinct gene products known as K-Ras4A and K-Ras4B. (Pells et al, 1997) Each of the three distinct Ras genes are found on different chromosomes and appear to originate from an ancestral gene. (Reviewed by Barbacid, 1987) These molecules can transmit signals from tyrosine kinases at the plasma membrane to a cascade of serine threonine kinases which deliver signals to the cell nucleus. (Reviewed by McCormick, 1995; Campbell et al, 1998) Signal transduction via true Ras molecules has been implicated in a variety of different functions such as proliferation, apoptosis, and differentiation, and often, the phenotypic response to Ras activation will depend upon the cell type and the nature of the stimulus. A number of proteins have been shown to be effectors for true Ras proteins, including Raf-1, PI3K, and three exchange factors known as RalGDS, RGL, and Rif. The role of these proteins in the Ras signalling pathway will be discussed in further detail later on in this chapter.

**The Rap Subfamily**

The Rap (Ras proximate) subfamily of small GTPases consists of the molecules: Rap1A, 1B, 2A and 2B. Rap1A (also known as k-rev1) was first identified because it possessed homology to true Ras family proteins and also because overexpression of this
protein was found to reverse cellular transformation induced by K-Ras. (Noda, 1993)

Rap1B differs from Rap1A only in nine amino acids in the C-terminal region of the protein and the functional differences between these two family members is unclear. (Bos et al., 1997; Reviewed by Bos, 1998) Very little is known about the protein function of Rap2A/B although two hybrid screens have recently identified an exchange factor named RPIP8 (Rap2-interacting protein 8) that binds to the GTP-bound form of Rap2A and is highly expressed in brain. (Janoueix-Lerosey et al., 1998) These proteins are 53% identical to H-Ras, share the same amino acid sequence as the effector loop of Ras, and many lines of evidence point towards a function of competing with Ras for binding partners. The formation of nonproductive complexes with key Ras effectors would then have an impact upon Ras transformation. (Franke et al., 1997) However, recent studies looking at the effects of Rap1 activation in vivo do not support this idea since activation of endogenous Rap1 fails to have any effect upon Ras-dependent ERK activation. (Zwartkruis et al., 1998) In contrast to the proposed anti-mitogenic action of Rap via downregulation of the Ras pathway, there are also some observations suggesting that Rap1 proteins might exert a positive role in the control of the cell cycle. (Yoshida et al., 1992) Experiments performed in cAMP responsive cells (Swiss 3T3) indicated that expression of wild-type Rap1B was sufficient to reveal the full oncogenic potential of Rap1. (Altschuller et al., 1998)

R-Ras

R-Ras is 55% identical to H-Ras and has an effector binding site identical to that of the true Ras subfamily of proteins. R-Ras transformed NIH 3T3 cells lack the strong
morphological changes as seen with Ras transformed cells which suggests that it may differ from true Ras proteins in its biological activity. (Huff et al, 1997) Also, unlike true Ras proteins, R-Ras is dispensable for the viability of NIH 3T3 cells and it has been suggested that it may regulate the processes of apoptosis and integrin-mediated cellular adhesion. (Zhang et al, 1996; Huff et al, 1997; Marte et al, 1997) R-Ras appears to be regulated by activators and effectors distinct from those that control true Ras subfamily proteins. (Huff et al, 1997) R-Ras does not appear to bind to the serine threonine kinase Raf-1 but has been shown to bind an anti-apoptotic protein known as bcl-2 via its C terminus. (Wang et al, 1996) It has also been shown to bind to phosphoinositol 3-kinase (PI3K) suggesting a potential role for R-Ras in activating the protein kinase B (PKB) pathway. (Marte et al, 1997)

**TC21**

TC21, also known as R-Ras2, is highly related (55% sequence homology) to the true Ras subfamily of small GTPases and has been shown to be oncogenic when mutationally activated, indicating a role in mitogenesis. (Chan et al, 1994; Graham et al, 1994) It has been suggested that TC21 utilizes some distinct signalling pathways from that of true Ras proteins since it interacts poorly with and cannot activate Raf’s serine/threonine kinase activity. (Graham et al, 1996) TC21 also has the unique property of being capable of becoming farnesylated and geranyl-geranylated with similar efficiency. (Carboni et al, 1995) TC21 has also been shown to bind to RalGDS (an exchange factor for Ral) suggesting a potential for TC21 to activate the Ral pathway. (Lopez-Barahona et al, 1996)
R-Ras3

R-Ras3 is a protein found highly expressed in brain and heart that shows high sequence similarity to TC21. (Kimmelman et al, 1997) This protein appears to have distinct biological activity when compared to other Ras Family members in that although it is capable of oncogenic transformation when mutated, it only weakly stimulates the MAPK pathway, it weakly interacts with known Ras effectors such as Raf and RalGDS, and does not interact at all with RglIII. In addition, the complement of transcription factors normally activated by the Ras pathway are only modestly stimulated by R-Ras3 overexpression. (Kimmelman et al, 1997)

The Ral Subfamily

The RalA and RalB proteins comprise a distinct family of ubiquitously expressed GTPases (Olofsson et al, 1988) Ral proteins are thought to act downstream of Ras since its guanine nucleotide exchange factor, RalGDS, has been found to be an effector of Ras. (Albright et al, 1993) Although the exact functions of Ral are still unknown, it has been shown that it is required for Src and Ras-dependent activation of phospholipase D. (Jiang et al, 1995) It has also been proposed that Ral provides a link between Ras and CDC42 and Rac since proteins identified as Ral binding proteins (i.e. RalBP1) have been shown to possess GAP function for CDC42 and Rac. (Cantor et al, 1995; Jullien-Floes et al, 1995; Park et al, 1995) Although non-transforming by itself, Ral has been reported to be involved in Ras-dependent transformation of NIH 3T3 cells. (Okazaki et al, 1996)
**Rheb, Rin and Rit**

Rheb stands for Ras homologue enriched in brain and is another Ras subfamily protein that shows high sequence similarity in the area of the Ras effector domain. (Yamagata et al, 1994) Rheb appears to be more functionally related to Rap proteins as overexpression of Rheb fails to cause cellular growth or transformation. (Yee et al, 1997) It has been proposed that Rheb may act in a similar manner to Rap1A in antagonizing Ras signalling. (Clark et al, 1997c) It is also rapidly induced by receptor dependent synaptic activity, suggesting a role in long term activity-dependent neuronal responses. (Yamagata et al, 1994)

Rin (for Ras-like protein expressed in neurons; not to be confused with RIN1, a potential Ras effector) and Rit (Ras-like protein expressed in many tissues) show high sequence similarity to the Drosophila gene RIC and appear to be mammalian homologues of this protein. (Lee et al, 1996) RIC was identified in a screen for proteins that bound calmodulin and differs from true Ras proteins in that it diverges slightly in sequence within its G2 effector domain and does not possess a C-terminal prenylation signal. (Wes et al, 1996) Both Rin and Rit were identified via a PCR-based strategy that utilized degenerate primers to conserved regions of the Ras molecule. Both of these proteins are found at the plasma membrane despite the fact that they also lack a sequence for prenylation or palmitolation. They also exhibit some sequence similarity to the core Ras effector domain. Rin has also been shown to bind to calmodulin in a calcium dependent manner hinting towards a possible role in calcium responsive signalling in neurons. (Lee et al, 1996)
Regulation of Ras Activation

A large variety of extracellular signals such as growth factors, cytokines, hormones, and neurotransmitters have been shown to stimulate Ras activation. These ligands bind to cell surface receptors that contain their own intrinsic tyrosine kinase activity, as is the case with receptor tyrosine kinases (RTKs), or bind to non-receptor tyrosine kinase associated receptors or G protein-coupled seven transmembrane receptors (GPCRs). (Reviewed by Campbell et al, 1998) RTKs are able to activate Ras via adapter molecules which bind to specialized guanine nucleotide exchange factors. Details of these specific membrane proximal signalling events will be given in relevant sections describing particular guanine nucleotide exchange factor (See: below). Receptors not directly associated with tyrosine kinases, such as GPCRs, may activate Ras indirectly through src-like kinases (Park et al, 1998) or ligand-independent activation of receptor tyrosine kinases such as the EGF receptor. (Daub et al, 1996)

Ras proteins possess an intrinsic GTPase activity which, although slow and inefficient, is sufficient for self-inactivation. Whether or not it is GTP or GDP that is bound to Ras can be influenced by the action of two classes of regulatory proteins: the guanine nucleotide exchange factors (GNEFs) and the GTPase activating proteins (GAPs). (See: Figure 1)

Guanine Nucleotide Exchange Factors: Activators of Ras Signalling

GNEFs promote Ras activation by catalyzing exchange of GDP for GTP. (Boguski and McCormick, 1993; Overbeck et al, 1995; Quilliam et al, 1995) The steps involved in this molecular process have been fully studied. A ternary complex is formed
containing both the GNEF and the GDP-bound GTPase. The binding of the exchange factor helps to displace the GDP at an increased rate and once GDP is released, a binary complex containing only the GNEF and Ras molecule is left. GTP then binds this binary complex, which in turn, releases the GNEF from the resulting ternary complex. (Lenzen et al, 1998; Boriack-Sjodin et al, 1998; Wittinghofer, 1998) Empty GTPases do not have a preference for GTP over GDP but the high intracellular concentration of GTP versus GDP (approximately 10 fold higher) ensures that the released nucleotide is replaced with GTP rather than with GDP. (Lenzen et al, 1998; Lai et al, 1993) Akin to the GTPase’s lack of nucleotide preference, GNEFs have also been shown to associate with either the GTP or the GDP bound forms of GTPases but they seem to recognize the GDP bound forms more often in vivo, perhaps due to other cellular factors. (Munder and Furst, 1992) Once GTP loaded, the exchange factor dissociates and the now active GTPase can pass on its signal to downstream effector molecules. (Bourne et al., 1990a/b; Masters et al, 1990) Information about Ras specific GNEFs will be presented in detail as it is directly related to the main focus of this thesis. A list of all the Ras subfamily-specific GNEFs is found in Table 2 along with additional information detailing activating specificities. With the exception of SmgGDS, GNEFs found within this family are structurally similar in that they all possess a CDC25 homology domain. This is the minimal domain required for exchange activity. (See: below) Each GNEF also possesses a set of specialized structural domains capable of responding to various upstream signals. The unique combination of structural features found within each GNEF points towards the existence of different regulatory mechanisms which could control their activity.
## Guanine Nucleotide Exchange Factors (GNEFs) specific for Ras Subfamily Members

<table>
<thead>
<tr>
<th>GNEF</th>
<th>Organism</th>
<th>Tissue Distribution</th>
<th>Activity on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC25</td>
<td>yeast</td>
<td>N/A</td>
<td>Ras2</td>
</tr>
<tr>
<td>Ste6</td>
<td>yeast</td>
<td>N/A</td>
<td>Ras1</td>
</tr>
<tr>
<td>efe25+</td>
<td>yeast</td>
<td>N/A</td>
<td>Ras1(?)</td>
</tr>
<tr>
<td>SDC25</td>
<td>yeast</td>
<td>N/A</td>
<td>Ras1/2, H-Ras</td>
</tr>
<tr>
<td>Lte1</td>
<td>yeast</td>
<td>N/A</td>
<td>CDC42</td>
</tr>
<tr>
<td>BUD5</td>
<td>yeast</td>
<td>N/A</td>
<td>RSR/BUD1 (Rap homolog)</td>
</tr>
<tr>
<td>RasGRF1</td>
<td>mammalian</td>
<td>brain</td>
<td>H-Ras</td>
</tr>
<tr>
<td>RasGRF2</td>
<td>mammalian</td>
<td>ubiquitous, major species: brain</td>
<td>Ras and Rap</td>
</tr>
<tr>
<td>Sos1</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>H-Ras</td>
</tr>
<tr>
<td>Sos2</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>H-Ras</td>
</tr>
<tr>
<td>C3G</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>Rap1A</td>
</tr>
<tr>
<td>SmgGDS</td>
<td>mammalian</td>
<td>high: brain, less: other tissues</td>
<td>K-Ras4B, Rap1A/B RhoA, CDC42</td>
</tr>
<tr>
<td>RalGDS</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>Ral</td>
</tr>
<tr>
<td>RGL</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>Ral</td>
</tr>
<tr>
<td>Rlf (RGL2)</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>Ral</td>
</tr>
<tr>
<td>cAMP-GEFI</td>
<td>mammalian</td>
<td>ubiquitous</td>
<td>Rap1A, Rap1A</td>
</tr>
<tr>
<td>cAMP-GEFII</td>
<td>mammalian</td>
<td>brain, adrenal gland</td>
<td>Rap1A</td>
</tr>
</tbody>
</table>

Table 2. Ras Guanine Nucleotide Exchange Factors (GNEFs)
The CDC25 Family of Ras-specific GNEFs

**CDC25**

One of the first GNEFs identified was the CDC25 gene product found in *Saccharomyces cerevisiae.* (Robinson *et al*, 1987) It was genetically mapped upstream from Ras, it was found to be absolutely required for viability as well as for proper Ras activation, and loss of function of this protein led to cell death. (Broek *et al*, 1987) The minimum functional catalytic domain of CDC25 has been determined using deletion experiments and a portion of the exchange factor domain, containing 450 amino acids, is absolutely required. (Lai *et al*, 1993) Likewise, certain residues within the Ras molecules have been identified (residues 75, 76, 78) that are critical for Ras-GEF interactions. (Quilliam *et al*, 1994) A CDC25 homologue identified in *Schizosaccharomyces pombe* was called Ste6.

**SDC25, Lte1 and BUD5**

SDC25 was identified as another homologue of CDC25. Although it exhibited high sequence similarity to CDC25 it was found not to be essential for viability. (Boy-Marcotte *et al*, 1989) Part of SDC25 was shown to have activity towards mammalian Ras when overexpressed in murine fibroblasts. (Crechet *et al*, 1990a/b) Two additional yeast proteins, Lte1 (Keng *et al*, 1994; Shirayama *et al*, 1996) and BUD5 (Chant *et al*, 1991; Powers *et al*, 1991) have been identified as having CDC25 homology. The function of Lte1 is still not entirely clear, it appears to be required at low temperatures for cell cycle progression at the stage of the termination of M phase in *S. cerevisiae.* (Shirayama *et al*, 1996) Genetic analysis was able to determine that BUD5 is
an upstream activator (presumably an exchange factor) for the yeast Rap homologue, RSR/BUD1. (Chant et al, 1991)

**RasGRF1 and 2**

In 1992, the mammalian CDC25 homologue RasGRF1 (also known as CDC25\textsuperscript{Mm}) was isolated using degenerate PCR primers to conserved regions of CDC25 (Shou et al, 1992; Wei et al, 1992; Cen et al, 1993) or by complementation cloning of CDC25-defective yeast strains. (Martegani et al, 1992) RasGRF1, and a second member of this family of mammalian GNEFs called RasGRF2 (Fam et al, 1997), can couple H-Ras activation to G coupled receptors and calcium signals and contain both CDC25 and Dbl homology domains. (Jones and Jackson, 1998) It has also been demonstrated that RasGRF2 is a bifunctional protein that can bind and activate Ras via its CDC25 homologous GEF domain and Rac via its Dbl homology domain thereby regulating the signals being passed on by the extracellular-signal-related kinase (ERK) as well as the stress activated protein kinase (SAPK) pathways. (Fan et al, 1998) RasGRF also contains a domain known as an IQ motif. IQ motifs bind to calmodulin and/or related calcium binding proteins in a calcium dependent manner. (Cheney and Mooseker, 1992) Calcium, via binding to calmodulin can activate Ras directly by activation of RasGRF. (Farnsworth et al, 1995; Reviewed by Gawler, 1998) The mechanism by which this effect is achieved remains to be determined. RasGRF1 was found to be expressed primarily in the brain. (Martegani et al, 1992) A minor mRNA species of RasGRF2 was found to be ubiquitously expressed while its major species was found to be expressed in brain. (Fam et al, 1997)
In contrast to RasGRF, the GNEFs Sosl and Sos2 are found to be widely expressed in mouse and human tissues. (Botwell et al, 1992; Chardin et al, 1993) These two molecules are most closely related to the Drosophila son-of-sevenless gene product which functions upstream of Ras in R7 photoreceptor cell differentiation (Karlovich et al, 1995) and show a high degree of sequence similarity to each other. It has been determined that Sos2 has a significantly higher binding affinity for Grb2 relative to that of Sosl (Yang et al, 1995), that it is also much less stable then Sosl and is degraded by a ubiquitin-dependent process. (Nielsen et al, 1997) These unique characteristics of Sos2 suggest a differential contribution to receptor mediated Ras activation. Akin to RasGRFs, Sos proteins are capable of not only facilitating exchange on Ras but can also couple Ras to Rac through their Dbl and pleckstrin homology domains in a PI3K-dependent manner. (Nimnual et al, 1998)

Sos has been shown to be regulated by adapter molecules which provide the link between many types of activated cell surface receptors and Ras. (Ravichandran et al, 1995). When RTKs are activated by ligand, the receptors dimerize and specific tyrosines found in their cytoplasmic domains are phosphorylated by the receptor’s intrinsic kinase. These specific phosphotyrosines serve as docking sites for the src homology 2 (SH2) domain-containing adapter protein Grb2. (Simon et al, 1993) In some cases, alternative phosphotyrosines attract the phosphotyrosyl binding (PTB) domain-containing adapter molecule known as Shc. Upon binding to the receptor, Shc becomes autophosphorylated which attracts the SH2 domain of Grb2. (Reviewed by Bonfini et al, 1996) Grb2 stably associates with the GNEF Sos via its SH3 domain and the recruitment of this complex to
the plasma membrane is believed to bring Sos into close proximity to Ras, thus allowing it to activate Ras by catalyzing the exchange of GDP for GTP. Sos has also been shown to be bound by additional SH3 containing adapter molecules such as the lymphoid specific adapter proteins LAT and 3BP2 (Zhang et al, 1998; Finco et al, 1998) and the adapter protein known as Crk. (Greulich et al, 1996) Crk is thought to play an important role in signalling from the epidermal growth factor (EGF) receptor to Ras. (Kizaka-Kondoh et al, 1996) Adapter proteins do not contain any enzymatic activity but are attracted to activated receptors and promote the movement of GNEFs from the cytosol to the plasma membrane. Therefore, they provide an important link between many cell surface receptors and Ras. (See: Figure 3) In addition to the aforementioned mechanism, it has also been suggested that the pleckstrin homology (PH) domain of Sos may participate in regulating the inducible association of Sos with the membrane via binding to phosphatidylinositol (3,4,5)-trisphosphate (Pl(3,4,5)P3). (Reviewed: Lemmon et al, 1996; Chen et al, 1997; Kubiseski et al, 1997)

**C3G**

Another family of Ras GNEFs was identified in an expression cloning strategy using the SH3 domain of Crk as a probe and was called C3G (for Crk SH3 binding GEF). (Tanka et al, 1994) C3G is also ubiquitously expressed but appears to act on the Ras related protein, Rap1A. (Gotoh et al, 1995; van den Berghe et al, 1997)

**RalGDS, RGL, and Rif**

A new group of related GNEFs has been identified, it consists of three related proteins: Ral guanine dissociation stimulator (RalGDS), the RalGDS-like (RGL) protein, and the RalGDS-like factor (Rlf or also referred to as RGL2). Although all three proteins
have been shown to associate with Ral, Rap1A, Ras, and in some cases TC21 (Albright et al, 1993; Kikuchi et al, 1994; Spaaragaren and Bishoff, 1994; Peterson et al, 1996; Wolthuis et al, 1996; White et al, 1996; Lopez-Barahona et al, 1996; Murai et al, 1997; Kishida et al, 1997) they have only been shown to actively promote exchange on Rap. (Albright et al, 1993; Urano et al, 1996; Murai et al, 1997; Wolthuis et al, 1997) These proteins are interesting because they could provide a means of linking the Ras pathway with Rap. Additional information on these specific GNEFs will be presented in the section on Ras effectors. (See: below)

**cAMP regulated Rap exchangers**

Two new highly related exchange factors (cAMP-GEFI/EPAC [exchange protein directly activated by cAMP] and cAMP-GEFII) have been identified which possess exchange factor activity for Rap1A. (Rooij et al, 1998; Kawasaki et al, 1998) cAMP-GEFI also possesses some exchange activity for RalA and is widely expressed in human tissues. cAMP-GEFII is predominantly expressed in brain and adrenal glands. It was previously thought that cAMP induced effects were mediated solely through PKA. The identification of cAMP regulated GNEFs for Rap1A and RalA opens up the possibility for cAMP-mediated, PKA-independent signalling events.

**SmgGDS**

SmgGDS, although structurally dissimilar to other known RasGNEFs, has been shown to possess exchange activity for the Ras family members: K-Ras4B, Rap1A or B, as well as the Rho/Rac family members: RhoA, CDC42Hs and Rac1. (Mizuno et al, 1991)
GAPs: Inactivators of the Ras pathway

In contrast to GNEFs which promote the active state of Ras GTPases, GAPs control the conversion of Ras to the inactive state by stimulating the hydrolysis of GTP to GDP. (See: Figure 1; Wittinhofer et al, 1997; Sheffzek et al, 1997; Reviewed by Scheffzek et al, 1998) Ras molecules possess very low intrinsic GTPase activity and are dependent upon GAP proteins for efficient downregulation. The active site for efficient catalysis of GTP to GDP is shared by Ras and its RasGAP. These two separate proteins come together as a transient heterodimeric enzyme in order to catalyze GTP-hydrolysis and separate after the job is done. A recent model which attempts to describe the action of GAPs on Ras molecules has been called the arginine-finger hypothesis. It states that one of the primary elements required for proper GTPase enhancement is an invariant arginine found in the GAP. The model also predicts that there is an interaction or "docking" between the arginine finger of the GAP and the P loop and switch regions of Ras (Scheffzek et al, 1997; Reviewed by Noel, 1997; Scheffzek et al, 1998) There are certain Ras mutations (i.e. single amino acid substitutions at 12,13, or 61) which lock the GTP binding protein in its active conformation and these mutants are insensitive to GAP activation. (Bos, 1989; Clark and Der, 1993) GAP mutations also exist that increase its GTPase activity thereby constitutively inactivating the Ras pathway.

An increasing number of mammalian Ras family GAPs have been identified. These include: p120GAP, neurofibromatosis type 1 gene product (NF1; Reviewed by McCormick, 1995), GAP1m (Maekawa et al, 1994), GAP1IP4B (Cullen et al, 1995), IQGAP1 (Weissbach L et al, JBC 1994), and Rap1GAP (Rubinfeld et al, 1991).
GAPs within a subfamily share a high degree of sequence similarity but the degree of sequence similarity between subfamilies is low. Therefore, other members of the Ras superfamily of GTPases have their own GAPs (i.e. RhoGAPs, RacGAPs). Despite the fact that GAPs often contain a number of additional protein-protein interaction motifs such as SH3, SH2, and PH domains in the case of p120GAP, it is still inconclusive as to whether or not GAPs are involved in downstream signalling and whether or not they can be considered true Ras effectors. (Reviewed by Tocque et al, 1997)

**Ras Effectors**

**Domains Required for Effector Interaction**

Ras effectors bind preferentially to the GTP-bound form of Ras and require for their binding an intact effector-binding region of Ras. Mutations created in this area can destroy the transforming potential of Ras without affecting nucleotide binding, stability of the molecule, or its subcellular localization, suggesting that they only affect the interaction of Ras with its downstream targets. (Cales et al, 1988; Willumsen et al, 1986) This interaction requires an intact core Ras effector domain (residues 32-40) that spans part of the switch I region as well as sequences flanking this core region. This area has been shown to play a role in stabilizing the interaction with Ras effectors, providing specificity, and activating downstream signalling molecules. Other regions of Ras that appear to play a role in influencing the extent and specificity of downstream signalling are the switch II region (residues 59-76), a region spanning residues 92-106 and the lipid modified C-terminus. (See: Figure 2) Data obtained from three dimensional structural analysis of GTP and GDP bound forms of Ras indicate that the effector domain is one of
two regions whose conformation change upon GTP hydrolysis. (Milburn et al, 1990; Schlichting et al, 1990) This would be expected of an area of the protein that is involved in contacting target molecules since only the GTP bound form should be interacting with effectors.

Complexity of Ras Signalling

By 1993, a large amount of experimental evidence helped to create a model whereby Ras acted as a binary switch, relaying signals originating from cell surface receptors to downstream MAP kinase cascades, eventually affecting the activities of nuclear transcription factors. Since then, Ras signalling has emerged as a very complex process which utilizes many different effector molecules which are likely to transduce their signals through multiple pathways. There have been many well written reviews published within the last year that have detailed the growing number of potential Ras effectors. (Joneson and Bar-Sagi, 1997; Campbell et al, 1998; Vojtek and Der, 1998) This particular discussion will be limited to molecules that have been proven to be true Ras effectors. These include: Raf-1, PI3K, and Ral GNEFs. Further information detailing their involvement in the Ras signalling pathway will be given in the next few sections.

Raf: a critical effector for Ras

One of the most well characterized Ras mediated signalling pathways involves Raf. There are three mammalian Raf family members: c-Raf-1, A-Raf, and B-Raf. It was once thought that the sole purpose of Ras binding to Raf was to translocate Raf to the membrane where Ras would then activate it via phosphorylation at several sites. (Fabian
et al, 1993) However, additional data has indicated that interaction between Ras and Raf in vitro is insufficient to stimulate Raf kinase activity (Traverse et al, 1993; Dent et al, 1995a/b) suggesting that although Ras may promote events that eventually lead to full activation, other proteins are required in vivo for this to occur (Leevers et al, 1994) The role of protein phosphorylation in Raf activation is also unclear. Ras activation of Raf has been shown by some groups to be a phosphorylation-independent event (Stokoe et al, 1997) while other groups have suggested that phosphorylation is an essential component of the mechanism by which Ras activates Raf (Jelinek et al, 1996)

Two distinct sites of Raf bind to Ras. One area is known as the Ras Binding Domain (RBD) and it is required for Raf translocation. A second site, known as the cysteine rich domain (CRD) has been shown to be required for Raf activation (Roy et al, 1997; Clark et al, 1995; Mineo et al, 1997) Other proteins that are thought to play a role in Raf activation are the Hsp90 and p50 molecular chaperones, phospholipids (i.e. phosphatidylserine), serine/threonine and tyrosine kinases, the kinase suppressor of Ras (KSR) (Sugimoto et al, 1998), and 14-3-3 (Reviewed by Aiken, 1995; McCormick, 1995b) What was once thought of as a linear activation pathway linking Ras to Raf is now being thought of as a multi-step process that appears to involve a number of different proteins and the formation of a Raf activation complex.

Upon activation, Raf phosphorylates and activates two MAPK kinases known as MAP/ERK kinases (MEK1 and 2). The MEKs then go on to phosphorylate threonine and tyrosine residues found in two extracellular signal regulated kinases (ERK1 and 2) also known as mitogen activated protein kinases (MAPKs). MEKs also play a critical role in turning off the Ras signalling cascade. They are responsible for the phosphorylation of
Sos, which leads to its dissociation from Grb2 thereby interrupting its ability to catalyze nucleotide exchange on Ras. (See: Figure 3; Waters et al, 1995) The active, phosphorylated ERKs can activate cytoplasmic target proteins such as Rsk (ribosomal S6 kinase; Sturgill et al, 1988; Palmer et al, 1998) and Mnk (Waskiewicz et al, 1997; Wang et al, 1998). Once activated these proteins translocate to the nucleus where they can phosphorylate and activate a number of substrates including the Elk-1 transcription factor. Within the nucleus, activated Elk-1 forms a complex with the serum response factor (SRF) and binds to the serum response element (SRE) found in the promoters of many genes actively transcribed from in response to receptor stimulation, such as c-fos. (Marais et al, 1993) Figure 3 outlines this classic Ras/Raf/MAPK signal transduction pathway.

To complicate matters, the MAPK pathway can also be activated by Ras/Raf-independent mechanisms including PI3K (Grammer et al, 1997), PKCs (Minden et al, 1994; Cai et al, 1997), and integrin mediated signalling events (Schlaepfer et al, 1994). Experiments performed using Ras effector mutants defective in Raf binding have also illustrated that Ras can cause transformation in some strains of NIH 3T3 cells via coordinate activation of Raf-dependent and -independent pathways that involve Rho family proteins. (Khorsavi-Far et al, 1996) It has recently been shown that the Raf/MEK/ERK1/2 pathway is necessary for activity in experimental metastasis assays illustrating that the Ras effector pathways mediating the tumorigenic and metastatic activities can be segregated. (Webb et al, 1998) It is thought that different cell types and stimuli may use alternative cytoplasmically proximal signalling pathways and that many of these cascades eventually converge on the MAPK pathway.
Extracellular signals are transduced via RTKs and result in the activation of intracellular signalling cascades. The receptor’s intrinsic tyrosine kinase phosphorylates particular tyrosine residues within the cytoplasmic domain of the receptors. These phosphotyrosines attract SH3 containing molecules such as Shc and Grb2. In this particular diagram Shc attracts Grb2 via its SH2 domain and the GNEF Sos is covalently linked to Grb2. The end result of receptor activation is the translocation of the exchange factor to the plasma membrane. Sos interacts and exchanges on Ras thereby activating it. In its GTP bound form, Ras is capable of interacting with various downstream effectors such as Raf-1 (1: Stippled), RalGDS (2: Grey), and PI3K (3: White). Details on these effector signalling cascades are presented in the text.
**PI3K Signalling: parallel to the Raf/MAPK pathway?**

Phosphoinositol 3-kinase (PI3K) is a lipid kinase with specificity for the 3-position of the inositol ring and recent data has implicated this molecule as both an effector for Ras and its activator. (Carpenter and Cantley, 1996) As is the case for many of the Ras-mediated signalling events, this apparent functional contradiction has been explained by stating that the cellular context and the type of stimuli will dictate the function of PI3K. PI3K is made up of two different subunits, each of which have multiple isoforms. They are known as the catalytic p110 subunit and the p85 regulator subunit. PI3K has been shown to interact with GTP bound R-Ras and is activated both in vitro and in vivo as a result of this interaction. (Kodaki et al, 1994; Rodriguez-Viciana et al, 1994, 1996) PI3K also interacts with other Ras family members such as Rap1A/B and TC21 but it is still unknown as to whether or not binding to these molecules will activate PI3K. Activation of PI3K by a wide variety of different stimuli results in accumulation of the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), and a number of molecules have been implicated as being downstream of PI3K. These include: Rac (Hawkins et al, 1995; Rodriguez-Viciana et al, 1997), p70S6 kinase (Romanelli et al, 1999), PKB AKT (Burgering et al, 1995; Marte et al, 1997), and novel and atypical isoforms of PKC (Mendez et al, 1997). It has been suggested that a cell survival pathway exists, parallel to the Ras/Raf/MAPK pathway involving Ras/PI3K/PKB. (Franke et al, 1997; See: Figure 3) The signalling events connecting Ras and Rac have become better understood in the past year. PI3K and Sos have been identified as being key players in this pathway. PI3K stimulated PIP₃ production is thought to attract the PH domain of
Sos. The DH domain of Sos, in turn, binds to Rac thereby linking up Ras activation to Rac activation. (Nimnual et al, 1998)

**RalGDS, RGL, and Rlf: Linking Ras to other Ras-related proteins**

Experiments using yeast two hybrid screens to look for proteins that bind to Ras and Ras-related proteins have identified three new exchange factors: RalGDS, RGL, and Rlf. All three proteins have been shown to associate withRal, Rap1A, and Ras.(Albright *et al*, 1993; [Kikuchi et al, 1994; Spaaragaren and Bishoff, 1994; Peterson et al, 1996; Wolthuis et al, 1996; White et al, 1996; Murai et al, 1997; Kishida et al, 1997] In addition, RalGDS has also been shown to associate with TC21. (Lopez-Barahona *et al*, 1996) These molecules are also interesting because they interact with GTP-bound Ras and may provide a way for Ras activation to influence the activity of other Ras-related proteins. (Kikuchi *et al*, 1994; Wolthuis *et al*, 1998; Wolthuis *et al*, 1997) The exact function of Ral is still unknown but it has been demonstrated that in some cases, Ral activation is a downstream effect of growth-factor induced Ras activation (Wolthuis *et al*, 1998) and that constitutively active Rlf has an effect upon transcriptional activity and cell growth. (Wolthuis *et al*, 1997) It should also be noted that there have also been reports of Ras-independent activation of Ral via a calcium dependent pathway which may have an influence on how results can be interpreted. (Hofer *et al*, 1998) A number of Ral specific binding proteins have been identified, such as Ral binding protein 1 (RalBP1/RIP1). RalBP1 has been shown to possess potential GAP activity towards CDC42 and Rac *in vitro*. (Cantor *et al*, 1995; Jullien-Floes *et al*, 1995; Park *et al*, 1995; Hinoi *et al*, 1996) Ral has also been shown to bind to phospholipase D (PLD). (Jiang *et al*, 1995)
together, these results suggest that Ras signalling can influence Ral via its effectors RalGDS, RGL, and Rlf and this interaction may eventually regulate the actions of CDC42, Rac and PLD. (See: Figure 3) The Ras effector domains of these molecules are also capable of competing with Raf for Ras binding and could potentially have the ability to down regulate the MAPK pathway.

**Approaches utilized to Identify Ras Activators and Effectors**

A number of different techniques have been used to identify proteins that act as Ras activators or effectors. These include strategies such as using cDNA probes or sets of PCR primers to screen cDNA libraries, the yeast two hybrid system, and transformation based screens in yeast.

A mouse eye cDNA library was screened with a $^{32}$P-labeled fragment of the *Drosophila* Sos gene in order to identify the mammalian GNEFs, Sos 1 and 2. (Bowtell *et al*, 1992) Conserved sequence information has also been used to synthesize degenerate PCR primers in order to isolate homologous regions found in other proteins. RasGRF1 was isolated using primers to conserved regions of the yeast specific exchange factors, CDC25 and SDC25. (Shou *et al*, 1992; Wei *et al*, 1992) Similarly, the same sequence information can be used to screen databases.

A more function-dependent approach used to search for Ras effectors involves identifying proteins in cell extracts that bind specifically to GTP-bound Ras. This technique was used to implicate PI3K as being an effector for Ras. (Carpenter and Cantley, 1996) Raf was also found to interact with Ras using the yeast 2 hybrid system. (Vojtek *et al*, 1993) The Ras effectors, RalGDS, RGL, and Rlf were identified in
a yeast two hybrid screen by using the Ras effector domain as bait.(Kikuchi et al, 1994) Finally, other potential Ras effectors such as the protein RIN1 (Ras interaction/interference gene 1), have been identified because of their effects upon Ras induced phenotypic changes. RIN1 was identified in a library screen searching for proteins that could suppress Ras-induced cyclic AMP activation in yeast.(Han et al, 1997; Han and Colicelli, 1995)

**Cellular Processes Influenced by Ras**

**Morphological Transformation of Cell Lines**

*Ras Transformation of Primary and Immortalized Cell Lines*

Cancer arises through the accumulation of genetic changes which enhance the growth or survival of developing tumor cells. In an experimental setting, the most well established model of this phenomenon involves the transformation of primary cultures or immortal cell lines by oncogenes. Transformation in this global sense refers to an increase in the proliferative potential of cells which can be accompanied by distinct morphological changes. Oncogenic Ras transforms most immortal rodent cell lines to a tumorigenic state, whereas transformation of primary cells either requires a cooperating oncogene (i.e. E1A) or the inactivation of tumour suppressors such as p53 or p16\(^{INK4}\). (Weinberg, 1997) This difference stems from the fact that primary cells possess a mechanism for actively countering the transforming potential of Ras. Prolonged expression of oncogenic Ras induces a permanent cell-cycle arrest in primary rodent fibroblast cells that appears to be identical to cellular senescence.(Serrano et al, 1997) It was later determined that these cells exhibited increased p53 and p16 activity suggesting
that these tumour suppressors might act in a compensatory mechanism that suppresses Ras-induced transformation. (Lin et al, 1998) Conversely, immortalized cell lines may have lost aspects of the senescence program and can be easily transformed by oncogenic Ras alone. A large proportion of the studies examining Ras signalling in mammalian systems have utilized immortal or tumor-derived cell lines harboring unknown genetic alterations.

**Phenotypic Changes associated with Ras Transformation in Fibroblast cells**

The morphological and physiological changes associated with transformation are oncogene specific. For example, fibroblast cells transformed by activated forms of Ras exhibit a distinctive set of changes in their physical appearance, proliferative capacity and metastatic potential. (Reviewed by Zohn et al, 1998) Physically, Ras transformed NIH 3T3 cells appear elongated and highly refractile. This is in contrast to wild type cells which exhibit a flattened morphology and are non-refractile. The foci generated by cells overexpressing activated Ras are fairly flat and produce a swirling pattern when viewed under the microscope. Ras transformed cells proliferative at a faster rate in comparison to wild type cells and also possess a decreased requirement for serum. Ras transformed cells have also lost the ability to respond to contact-contact inhibition of growth and as a consequence of this they are able to grow in layers of more than one cell deep. Unlike wild type cells, transformed cells are able to grow in an anchorage independent manner and because of this they are able to form colonies when grown in soft agar. Lastly, Ras transformed fibroblast cells are able to form tumors in nude mice when injected subcutaneously.
Phenotypic changes Associated with Rho Transformation in Fibroblast cells

The physical changes induced in fibroblast cells by Ras transformation are different from those produced by the overexpression of Rho family member proteins. Rho transformed NIH 3T3 cells are able to pile up to a large extent and produce large, non-refractile, protruding foci. These distinct differences in morphology represent differences in the cytoskeleton of these transformed cells. Rho proteins are thought to actively promote the assembly of the actin cytoskeleton whereas activated forms of Ras are thought to actively promote the disassembly of actin stress fibers. (McCormick, 1998; Olson et al, 1998) The exact signalling mechanisms by which Rho relays these effects are not clear but it appears to be mediated by the activation of downstream kinase cascades which eventually leads to the activation of transcription factors.

Role for Rho and Rac in Ras Transformation

One theme that has emerged in the past few years is that Ras transformation is mediated by signaling activities that are much more complex than originally envisioned. This makes sense in light of the fact that transformation is itself a complex process involving changes in the rate of proliferation, cell cycle and cell shape. Although distinct phenotypic differences are observed when fibroblast cells overexpress Ras compared to when they overexpress Rho and Rac, a role for Rho and Rac in Ras transformation also exists. The Rho and Rac pathways appear to synergise with other Ras effector pathways in order to produce the distinctive morphological changes associated with Ras transformation. (Reviewed by Campbell et al, 1998; Vojtek and Der, 1998)

There are many instances where the Ras pathway can be coupled to Rho family proteins and it is thought that Rho proteins contribute significantly to the actions of
oncogenic Ras. (Reviewed by Joneson and Bar-Sagi, 1997; Zohn et al, 1998) Many different examples can be used to illustrate how Ras activation can lead to the activation of Rho GTPase. For example, as stated previously Ras/PI3K interactions have the potential to activate Rac via Sos (Nimnual et al, 1998) and Ras/RalGDS/Ral interactions have the potential to initiate a cascade of events which eventually influences the activity of Rac and CDC42. (Cantor et al, 1995; Jullien-Floes et al, 1995; Park et al, 1995; Hinoi et al, 1996) Another connection between Ras and Rho/Rac/CDC42 pathways was made when a p120 GAP binding protein, p190GAP, was found to be a GAP for RhoA and to some extent CDC42 and Rac. (Settleman et al, 1992).

In fibroblasts, Rho proteins help to control the organization of the actin cytoskeletal. More specifically, CDC42 is thought to be involved in the production of filopodia, Rac1 is thought to regulate the production of lamellipodia and membrane ruffling, and Rho proteins are thought to regulate the production of focal adhesions and stress fibers. (Reviewed by Ridley and Hall, 1992) It was found that activated Ras, when microinjected into serum starved Swiss 3T3 cells, caused membrane ruffling and stress fibers formation and that this effect could be blocked by dominant negative Rac (17N) and C3, an inhibitor of Rho proteins. (Ridley et al, 1992, Ridley and Hall, 1992) This data provided the impetus for further study into the ability of Ras to transiently stimulate Rac and Rho. It was later determined that overexpression of dominant negative versions of Rho and Rac had a negative effect upon Ras transformation in fibroblast cells. It was also determined that activated forms of Rho and Rac could synergize with activated Raf-CAAX to cause transformation. Constitutively activated mutants of Rac1 were also
found to cause transformation in NIH 3T3 or Rat-1 fibroblast cells. (Joneson et al, 1996a/b; Qui et al, 1995a/b)

Within the past couple of years researchers have been able to segregate the mitogenic and cytoskeletal signals transduced through Rho family proteins. Rac effector binding mutants have been isolated which failed to activate the Jun MAP kinase cascade (RacV12H40) but retained the ability to transform and stimulate membrane ruffling. (Joneson et al, 1996; Lamarche et al, 1996) These results suggest that Rac induced cytoskeletal changes are functionally relevant to Ras transformation. How the reorganization of the cellular actin cytoskeleton affects gene expression ultimately leading to transformation is still poorly understood. In contrast to this, studies performed using Rho effector domain mutants have determined that Rho induced cytoskeletal changes are not sufficient to cause transformation. (Qui et al, 1995) Rho signalling also appears to be able to induce the serum response element (SRE) (Zohar et al, 1998) and whether or not this is necessary for transformation remains to be elucidated.

**Cell Cycle Progression**

Growth factors stimulate the entry of arrested cells into the cell cycle and Ras proteins play a critical role in transducing these signals. Cycling cells require growth factor stimulation in order to enter into G1 phase and to pass what is known as a restriction point. Once cells have gone beyond the restriction point they no long require growth factor signalling and start on a program to replicate their DNA and divide. The key event that is required to get beyond this restriction point is the phosphorylation of the Rb (retinoblastoma) protein. Cyclin dependent kinases (cdks) are responsible for Rb
phosphorylation. In order for these kinases to become active they must: interact with cyclins, become phosphorylated themselves at certain residues, become dephosphorylated at inhibitory residues within their N termini, and release cdk inhibitory proteins. (Sebastian et al, 1993; Sherr and Roberts, 1995; Draetta, 1997; Reviewed by Kerkoff et al, 1998)

Ras has been shown to be activated in HeLa cells following release from mitosis and in NIH 3T3 cells following serum induced cell cycle entry. This activation happens during mid-G1 phase and does not require tyrosine phosphorylation of Shc proteins and their binding to Grb2 but does require RNA and protein synthesis. Ras activation in this case does not function by signalling through ERKs suggesting that alternative effector pathways are triggered by Ras during G1-phase progression. (Taylor et al, 1996) Raf-1 has been shown to phosphorylate and physically interact with Rb which could provide a potential link between mitogenic signalling and cell cycle regulation. (Wang et al, 1998)

The Ras/Raf pathway is thought to be involved in regulating the expression of cyclin D1. (Filmus et al, 1994; Winston et al, 1996; Aktas et al, 1997) The expression of the cyclin D1 gene is induced in response to growth factor stimulation of cells as a delayed early gene. (Matsushima et al, 1991) It interacts with cdks and this interaction is required for kinase activation. (Sherr, 1995) The cyclin D1 promoter has potential binding sites for jun and Ets, two transcription factors induced by Ras/Raf signalling. (Herber et al, 1994; Albanese et al, 1995)

An additional link between Ras activation and the cell cycle has been identified by researchers studying anchorage dependent growth signalling events. (Kang et al, 1996; Kawada et al, 1997; Yang et al, 1998; Lin et al, 1997) In particular, cells overexpressing
oncogenic Ras were capable of growing in an anchorage independent manner and they also exhibited an accelerated degradation of p27\textsuperscript{kip1}. p27\textsuperscript{kip1} is a cdk inhibitor (Polyak \textit{et al}, 1994) and its levels appear to be regulated by the rate of translation and the rate of protein degradation (Pagano \textit{et al}, 1995; Hengst and Reed, 1996). The Ras signalling pathway appears to be central in controlling the expression of p27\textsuperscript{kip1} (Atkas \textit{et al}, 1997) and it appears as though it is facilitated by the phosphorylation of p27\textsuperscript{kip1} by MAPK. Phosphorylated p27\textsuperscript{kip1} protein cannot bind to and inhibit the kinase activity of cdks (Kawada \textit{et al}, 1997).

The Ras/Raf pathway also affects another cdk inhibitor known as p21\textsuperscript{cip1}. In this situation however, p21\textsuperscript{cip1} levels are increased when high intensity signals from the Ras/Raf pathway are transmitted (Kerkhoff and Rapp, 1998). This appears to be a mechanism for cells to inhibit cell cycle progression in the face of cellular stress in contrast to the normal levels of Ras/Raf induced by serum stimulation.

The Ras/Raf pathway is also involved in the induction of c-myc expression. c-myc has been shown to cooperate with oncogenic Ras and Raf in transformation (Rapp \textit{et al}, 1985; Cleveland \textit{et al}, 1994). How the Ras/Raf cascade leads to induction of the c-myc promoter has not yet been determined.

Raf has also been shown to be in a complex with a phosphatase known as cdc25 (Galaktionov \textit{et al}, 1995). cdc25 appears to dephosphorylate the aminoterminal phosphotyrosine and threonine residues which is a prerequisite for cdk activation. cdc25 has been shown to be itself phosphorylated and activated by Raf-1 and this is thought to be regulated via c-myc induction (Galaktionov \textit{et al}, 1996).
Metastasis

Tumorigenesis is the process by which a tumor is produced. Metastasis is a complex process involving changes in cell proliferation, survival, motility and invasion of extracellular matrices. It can be defined as the process by which additional tumorigenic lesions are produced outside of the area first identified by the initial tumor.

Tumorigenicity and metastasis have been shown to be two distinct processes. Experiments performed in NIH 3T3 cells have illustrated that Ras-mediated tumorigenic transformation can occur independently of Raf-MEK-ERK1/2 pathway (Khosravi-Far et al, 1996; Webb et al, 1998) and that metastasis requires Raf-MEK and as well as increased ERK activation. (Webb et al, 1998)

Cells that express the Met tyrosine kinase (also know as the hepatocyte growth factor (HGF) receptor) can be triggered by HGF to grow invasively. This system has also been utilized to study the role of Ras activation in the process of metastasis. In these experiments, cells capable of signalling only through PI3K were found to be motile. Cells capable of signalling only through Ras were motile and exhibited increased transformation but were impaired in their ability to cause invasion and metastasis. Therefore it appears that these cells require both Ras-mediated and PI3K-mediated signalling events in order to achieve their full metastatic potential.(Bardelli et al, 1999) Although Ras activation appears to play an important role in metastasis, the nature of these molecular events leading to the acquisition of the metastatic phenotype remain unclear.
Cytokine Gene Expression

Ras is activated in response to T and B cell receptor stimulation (Woodrow et al., 1993; Satoh et al., 1991; Graves et al., 1992; Downward et al., 1990; Saxton et al., 1994) and by antigen cross linking of the FcER1 in mast cells (Turner and Cantrell, 1997). Inhibition of Ras activation has been shown to prevent the induction of the IL-2 gene (Baldari et al., 1992; Rayter et al., 1992). The transcription factor, nuclear factor of activated T cells (NFAT), is a major target for the Ras signalling pathway. NFAT controls the receptor-induced activation of cytokine genes such as IL-2, IL-4, GM-CSF, and TNF-α (Woodrow et al., 1993b). It has been determined that both Ras and calcium/calcineurin signalling pathways are required for the activation of NFAT (Genot et al., 1996; Reviewed by Masuda et al., 1998). The importance of Ras activation in the process of cytokine production is well illustrated by studies performed on anergic CD4+ T cells. Ras activation is blocked in these cells and as a consequence of this they are incapable of producing IL-2 (Fields et al., 1996; Li et al., 1996).

Lymphocyte Selection

The Ras pathway is also important for the process of thymocyte development. Mice engineered to overexpress a dominant negative Ras in their thymocytes produced normal thymocytes but exhibited a reduced number of single positive T cells (Reviewed in Conroy and Alexander, 1996; Swan et al., 1995). It was concluded from this that Ras played a role in the events that take place during the process of positive selection in T cells. A role for Ras in B cell development was identified by the overexpression of Ras in mice carrying a germ line deletion of their recombinase-activating gene 1 (RAG1).
RAG1 deficient mice normally exhibit a block in B cell development at the progenitor (pro)-B cell stage. (Shinkai et al, 1992; Mombaerts et al, 1992) Overexpression of Ras caused progression of these RAG1-deficient pro-B cells to cells that exhibit many of the characteristics of pre-B cells. (Shaw et al, 1999a/b) Therefore, it appears as though Ras activation is capable of inducing developmental progression from the pro-B cell stage to the pre-B cell stage.

**Differentiation**

Ras has also been shown to play a role in the process of differentiation. The most well known example of this occurs in the phaeochromocytoma cell line, PC12. PC12 cells are capable of undergoing neuronal differentiation in response to nerve growth factor (NGF) stimulation. (Yamada et al, 1996) Expression of activated Ras in PC12 cells can induce differentiation and expression of dominant negative Ras mutants can block NGF-induced differentiation. (Szeberenyi et al, 1990) Ras appears to be signalling through Raf and PI3K in order to induce cell cycle arrest and differentiation in these cells. It has also been reported that Ras activation of Ral GEFs has the opposite effect and suppresses cell cycle arrest and inhibits NGF-induced neurite outgrowth. (Goi et al, 1999)

Ras has also been implicated in the differentiation of hematopoietic cells. For example, Ras has been shown to play a role in the regulation of the critical thymic checkpoint known as positive selection where double positive CD4⁺CD8⁺ thymocytes mature into CD4⁺ or CD8⁺ single positive T cells. More specifically, transgenic mice generated to express a dominant negative form of Ras in their T cell lineages were severely compromised in their ability to undergo the process of positive selection. (Swan...
et al, 1995) Ras also appears to play a role in regulating early development of B lymphocytes by a mechanism that involves activation of Raf. In this case, transgenic mice generated to overexpress a dominant negative form of Ras in B-lymphocyte progenitors were found to have B cell development arrested prior to the formation of the pre-B cell receptor. (Iritani et al, 1997)

Programmed Cell Death

Although Ras activation has been shown to be important for proliferation and suppression of apoptosis under many circumstances there is also evidence that activation of the Ras pathway can inhibit proliferation and promote apoptosis. (Reviewed by Gomez et al, 1998; Downward, 1998) The Ras/Raf signalling pathway has been shown to influence apoptosis either in a positive or negative way depending upon the cells being studied. On the other hand, the Ras/PI3K signalling pathway has been shown to activate the serine/threonine kinase, PKB (also known as AKT) and this pathway has been strongly connected with protection from apoptosis. (Kauffman-Zeh et al, 1997; Khwaja et al, 1997) The effects of Ras activation on the control of cell death or cell survival appears to depend on a number of variables such as the nature of downstream effector pathways, the type of cells being studied, as well as the influence of other regulatory signalling events.

Use of Transformation Assays to Identify Modulators of Ras Activity

Many groups have utilized transformation assays, particularly focus formation assays, to screen cDNA libraries for novel oncogenes. (See: Chapter III) The variety of oncogenes identified using this method indicate that it is possible to use fibroblast
transformation assays to identify many of the components making up the Ras pathway. For example, growth factors (i.e. platelet derived growth factor (PDGF-β) (C. Tognon, unpublished results; Stice et al, 1999) and receptors (i.e. keratinocyte growth factor receptor (KGFR) (Miki et al, 1991a) have been isolated using this method and it has been determined that their overexpression produces an autocrine loop which triggers constitutive activation of the Ras pathway. Components of G coupled receptors such as the Go12 subunit (Chan et al, 1993) and Ras effectors such as Raf kinases (Whitehead et al, 1994; Miki et al, 1991b) have also been identified. Rho/Rac specific oncogenes have also been identified using these assays. For example, a number of novel Rho-specific GEFs have been identified by our lab. These include lfc, lsc, and dbs.(Whitehead et al, 1995b/c and 1996) In addition, other oncogenes that have not been linked to either the Ras or Rho/Rac pathways have been identified by transformation assays, these include such things as E2F (H. Kirk, unpublished results) and the net GEF.(Chan et al, 1996)

Fibroblast transformation assays have also been utilized to identify suppressors of Ras transformation. For example, Rap1A was identified in a screen for cDNAs that reverted the morphology of v-Ki-Ras-transformed cells.(Kitayama et al, 1989) Rare, non-transformed colonies were identified within a background of transformed cells. A slightly different method was utilized by other groups to identify similar suppressors of Ras activation. They produced cells resistant to Ras transformation by treating a parental Ras transformed cell line with mutagens. In this case, a selective agent designed to eliminate the parental transformed population was used in order to enable the detection of rare, non-transformed revertants in the parental population.(Yanagihara et al, 1990)
Genetic and biochemical analyses were performed on the revertant cell lines in order to identify the cause of the reversion phenotype.

**Thesis Objectives and General Strategy**

The Ras pathway plays a critical role in many diverse cellular events such as proliferation, differentiation, and apoptosis. In order to more fully understand the complex signalling processes that activate Ras in response to different extracellular signalling events, it is important to identify all the potential molecular players involved in these pathways. Therefore, the first major aim of this project was to identify a novel gene that played a role in stimulating or regulating the activation of the Ras pathway. (Chapter III) Our approach utilized a retroviral vector based expression cloning strategy in order to screen cDNA libraries for clones which were equivalent to Ras in their ability to induce fibroblast transformation.

The second major objective of this thesis project was to then characterize the novel molecule in order to more fully understand its potential role in regulating the Ras pathway. This objective was met first by performing extensive sequence analysis upon the full length cDNA. (Chapter IV) This data provided insights into the potential mechanisms by which this novel cDNA might influence Ras activation. Deletions and mutations were created based upon this sequencing information. Data generated from the overexpression of these constructs in the fibroblast transformation assay identified regions of the cDNA that were indispensable for transformation and also provided clues as to how this molecule might be regulated. (Chapter V) Based upon this information, a model was created in an attempt to explain how overexpression of this cDNA could lead
to fibroblast transformation. (Chapter V) Three very different experimental systems were then used in order to test two aspects of the model which centered around the potential functions of two potentially important regions of the molecule. (Chapter VI and VII) Further implications of these results will be discussed in Chapter VIII.
Chapter II: Material and Methods

Cell Lines

T28 cells (were originally from Dr. Rock, Dana Farber Institute, Boston, MA; Pyszniak et al, 1994) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf (FC) serum. Media used for all cells was supplemented with 2mM L-glutamine and the antibiotics penicillin (100U/ml) and streptomycin (100μg/ml (Stemcell Technologies)). BOSC23 packaging cells (obtained from William Pear; Pear et al, 1993) were cultured in DMEM containing 10%FC. NIH 3T3 cells were obtained from American Type Culture Collection (ATCC) and cultured at low density in DMEM containing 9% calf serum (CS). Balb-c 3T3 cells were cultured in DMEM containing 9%CS. Other cell lines used for Northern blot analysis: B6SutA (generously provided by Dr. Gerry Krystal, Terry Fox Laboratory; 10%FC RPMI), P338D₁ (ATCC; 10%HS DMEM), A20 (ATCC; 10%FC RPMI, β-mercaptoethanol (Sigma)), ABE8 (ATCC; 10%FC DMEM, β-mercaptoethanol (Sigma)), Ba/F3 (Palicios et al, 1985; 10%FC DMEM, 10ng/ml IL-3), NSF-70 (ATCC; 10%FC RPMI, 10ng/ml IL-3), WEHI231 (ATCC; 9%FC DMEM, β-mercaptoethanol (Sigma)), 32D (Greenberger et al, 1983; 10%FC DMEM, 10ng/ml IL-3), MBL-2 (Takei et al, 1984; 9%FCS DMEM), R1.1 (ATCC; 10%HS DMEM), YAC-1 (ATCC; 10%FC RPMI), DA-3 (J. Ihle; 10%FC RPMI), GM979 (Greenberger et al, 1983; 10%FC RPMI). Fibroblast cell lines used in Northern blot analysis to check for RasGRP expression: NIH 3T3, C3H10T1/2 (ATCC, 10%FC DMEM), and C127 (ATCC, 10% FC DMEM). Cell lines used for cDNA library
construction: UM87G (human glioblastoma/astroblastoma cell line) and HS766T (human pancreatic carcinoma cell line).

**Vector Construction**

Specific details of retroviral vector construction are described elsewhere. (Whitehead *et al*, 1995a) TL37 libraries were made using murine T28 cell line cDNA as starting material and the pCTV1B retroviral vector. A schematic diagram illustrating the different components found in this vector are found in Figure 4. This vector has a 5' MMLV LTR and a composite MPSV/MMLV 3' LTR that provides promoter and enhancer regions which have been shown to efficiently drive expression of the inserted cDNA in murine cell lines. (Stocking *et al*, 1985) It also contains an extended gag region that has been shown by other groups to increase titre by promoting efficient packaging of the vectors into retroviral particles (Armentano *et al*, 1987; Bender *et al*, 1987; Morgenstern *et al*, 1990). This vector also lacks the normal splice donor site and the initiation codon in order to minimize splicing events and to allow for efficient translation from initiator codons within inserted cDNAs. It has a polylinker with a BstXI stuffer region which facilitates ligation of the BstXI linkered cDNAs into the vector during the making of the libraries. This BstXI stuffer is displaced by cDNA insertion. (Seed *et al*, 1987; Seed, 1987) It also contains a SupF gene which provides a compact marker for bacterial transformation (Seed, 1983) and can also be used as a marker for cDNA recovery (See: Southern Blot Analysis of Recovered cDNAs). Lastly, this vector contains the replication origin from pUC8 and an SV40 origin of replication (McKnight and Tjian, 1986) so that the libraries can be propagated in *E. coli* and also used for
Figure 4. Schematic diagram of the retroviral vector and PCR primer positions

a) Diagram of CTV1B vector containing MMLV 5'LTR and composite MPSV/MMLV 3' LTR. The BstXI cloning sites and stuffer fragments are present to allow efficient cloning of BstXI linker cDNAs. SupF fragment can be used as a marker for bacterial transformation and also as a probe in Southern Blot analysis. β globin and SV40 polyadenylation sites are present, as well as origins from SV40 and pUC8 for replication in COS cells and E. coli, respectively. pCTV3 vectors contain a selectable hygromycin resistance gene driven by a composite polyoma/thymidine kinase promoter. Use of this selectable marker aids in determining retroviral titres and can help in selecting infected, expressing clones.

b) Integrated provirus composite MPSV/MMLV LTRs. PCR can be performed using flanking CTV5’ and CTV3’C primers. Subsequent PCR products can be cut with restriction enzymes such as MluI/SunI and then cloned back into MluI/SunI cut CTV3K retroviral vector for retesting.
expression cloning strategies dependent on episomal replication in COS cells, respectively. Isolated PCR products were cloned into pCTV3K for retesting of the cDNA's ability to cause transformation in NIH 3T3 cells. It is derived from pCTV1, but lacks the SupF region. Instead, it contains a synthetic polyoma enhancer and a thymidine kinase (TK) promoter that drives expression of a hygromycin resistance gene which facilitates selection of infected cells. (Gritz and Davies, 1983; Magli et al, 1987) This selectable marker was most helpful for determining the viral titres achieved during transfection experiments. Once a cDNA has retested positive it is moved over from the large pCTV3K retroviral vector to a smaller vector for more efficient sequencing. The sequencing vector, called BSM2, is a modified version of pBS KS+ (Stratagene) and has an MluI site engineered into the multiple cloning site (MCS) between the XhoI and the SalI sites. This MluI site facilitates the cloning of the PCR products isolated from the library screens into a sequencing vector since they all contain MluI/SstI sites flanking the inserted cDNAs.

**cDNA Library Construction**

mRNA was prepared from T28 cells by lysis in guanidinium isothiocyanate as described (Chomcynski and Sacchi, 1987), followed by binding to oligo dT cellulose (Jacobson, 1987). cDNA was synthesized with random sequence hexamers (Pharmacia) and Moloney murine leukemia virus reverse transcriptase using methods provided by the suppliers of the polymerase. (Life Technologies, Inc.) BstXI adapters (TCAGTTACTCAGG and CCTGAGTAACTGACACA; Seed and Aruffo, 1987; Seed, 1987) were added to the newly synthesized cDNA and this ligation product was run out
on a 1% agarose gel in order to remove excess adapters and to size fractionate the cDNAs. Three different size fractions were isolated from the gel and ligated with BstXI-cut pCTV1B retroviral vector. More specifically, 20ng of BstXI-digested and dephosphorylated pCTV1B plasmid along with equimolar amounts of the different size fractionated cDNAs were mixed together with a special ligation buffer consisting of: 25mM Tris.Cl (pH 7.8), 5mM MgCl₂, 1mM dithiothreitol (DTT), 1mM ATP, and 0.5 units of T4 DNA ligase (Life Technologies, Ltd.) for a final volume of 10ul. This ligation mixture was incubated at 16°C for 3 hours followed by 20 minutes at 72°C and then used to transform the highly electroporatable bacteria strain, E. coli MC1061/p3 (Seed et al, 1987) following the instructions provided by the operating manual of the GenePulser electroporation apparatus (BioRad). The transformed bacteria were then plated in soft agar and incubated at 37°C for 20 hours and then recovered as described by Vogeli et al (1985). Pooled plasmid DNA was purified from the transformed bacteria by alkaline lysis (Birnboim et al, 1983) followed by digestion with RNaseA and RNaseT1 and precipitation with ethanol. Libraries were constructed by Heather Kirk at the Terry Fox Laboratory, Vancouver, Canada.(1996)

Production of Viruses and Infection of NIH 3T3 cells

Plasmid DNA was introduced into BOSC23 (B23) packaging cells by calcium phosphate mediated transfection.(Pear et al., 1993; Loyter et al., 1982) A 10 cm dish of cells was grown to 85% confluence and the media was replaced with 8mls of 10FC/DMEM supplemented with 25μM chloroquine.(Sigma; Luthman et al, 1983) One millilitre of 2X HEPES Buffered Saline (HBS; Stem Cell Technologies; final
concentrations: 50mM HEPES, pH 7.05, 10mM KCl, 12mM dextrose, 280mM NaCl, 1.5 mM Na2HPO4) was added drop wise to a tube containing 0.5μg/ml plasmid cDNA library or control vector plasmid, and 25mM CaCl2 for a final volume of 2mls. This mixture was slowly added to the cells followed by a 10 hour incubation at 37°C in a humidified incubator with a 5% CO2 atmosphere. After the initial incubation period the media was replaced with chloroquine free medium and this was subsequently replaced every 12 hours with fresh media. At 48 hours post transfection the supernatant was collected and filtered through a 0.22 micron filter unit (Millipore) in order to remove BOSC23 cells from the supernatant. Two and a half millilitres of fresh media containing 9CS/DMEM, 2.5mls of filtered viral supernatant, and polybrene (Sigma; final concentration 10mg/ml; Chaney et al, 1986; Kawai et al, 1984) were mixed together and added to a 25cm dish containing 5 x 10^5 NIH 3T3 cells. The cells were then incubated at 37°C for 8 hours after which time the infecting media was replaced by fresh 9CS/DMEM. The cultures were fed every two days with 9CS/DMEM and scored within five to ten days post-confluence. The foci that were formed from the cDNA library infection were identified morphologically and then manually isolated by aspiration using a sterile pipette tip, placed in separate 6 well dishes with fresh 9CS/DMEM and grown to confluence.

Genomic DNA isolation (Blin and Stafford, 1976) was then performed for each well containing cells from an individual focus. (See: Recovery of cDNAs) Foci formed by overexpression of the control vector were not isolated in the same manner but were counted to determine the background levels of transformation of the target NIH 3T3 cell line used for the library screen. Alternatively, confluent 25 cm dishes infected with the
cDNA libraries or control vector alone could be stained with methylene blue (0.2% methylene blue in methanol) and scored for the number of foci formed per dish.

**Recovery of cDNAs from Infected NIH 3T3 cells**

Figure b illustrates the integrated provirus and the positioning of the PCR primers used to isolate the cDNA. Genomic DNA was isolated from infected NIH 3T3 cell clones by proteinase K digestion, phenol extraction and ethanol precipitation. (Blin and Stafford, 1976) Long range PCR conditions following the methods described by Cheng et al. (1994) were utilized. Twenty-five millilitre reactions contained the following components: 20mM Tris-Cl (pH 8.55), 8% glycerol, 2% DMSO, 1mM MgCl₂, 85mM KOAc, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia), 100ng each of 5' and 3' primers (CTV5'C: CTCACTCCTTCTCTAGCTC; CTV1-3'C: AACAAATTGGACTAATCGATACG), 100ng of template genomic DNA, 5 units of Taq DNA polymerase (GIBCO BRL) and 0.5 units of Pfu DNA polymerase (Stratagene). Amplification was performed with the following cycles: 95°C for 60s x 1; 95°C for 60s, 52°C for 30s, 72°C for 150s x 30. In order to reclone the PCR products back into the retroviral vectors for retesting, 20μl of the amplified DNA was extracted with phenol:chloroform (1:1), ethanol precipitated, and then cut with MluI and SphI to isolate the cDNA from flanking proviral regions. These restriction enzyme-cut PCR products were separated by size using agarose gel electrophoresis and each distinct band was manually excised from the gel. Glass wool spin columns were used to remove the DNA from the agarose gel and it was subsequently ethanol precipitated, cloned by ligation into pCTV3K and used to transform the *E. coli* strain, DH5αp3. Plasmid DNA was isolated
from the bacteria using an alkaline lysis miniprep procedure (Birnboim, 1983) with phenol:chloroform extraction. Each individual plasmid preparation was then ethanol precipitated a second time, washed with 80% ethanol, air dried, and resuspended in deionized water. Products that hybridized in Southern blot experiments to the SupF probe (See: Southern Blot Analysis) were then used in NIH 3T3 transfection assays (See: Production of Virus) in order to retest for transforming ability.

**Southern Blot Analysis of Recovered cDNAs**

In order to determine if the generated PCR products actually came from the transferred cDNA libraries, 5μl of the PCR product (See: Recovery of cDNAs) was run out on 1% agarose gels, denatured in gel denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 minutes, rinsed with water, neutralized with gel neutralization solution (1M Tris-HCl (pH 7.4), 1.5M NaCl) for 15 minutes and dry blotted onto Hybond N+ nylon membranes (Amersham) for a minimum of 5 hours.(Southern, 1975) The blot was then rinsed in 10X SSC (1.5M NaCl, 150mM Na-citrate, pH 7.0), auto cross linked using a UV Stratalinker 1800 (Stratagene) crosslinker for 90 seconds, placed in pre-hybridization solution (250mM Na-PO$_4$ (pH 7.2), 1mM Na.EDTA, 20% SDS, 5mM Na$_4$P$_2$O$_7$.10H$_2$O (subsequently referred to as NaPPi)), 0.5% BR (Blocking Reagent; Boehringer Mannheim) 10% stock dissolved in 100mM Na.maleate (pH 7.4), 150mM NaCl, DEP treated and autoclaved) and rotated at 68ºC in a hybridization oven (Hybaid Ltd.) for 1 hour. DNA probes containing the 600bp SupF region (EcoRI/SalI) of the SF183 vector were randomly primed using hexanucleotides (Pharmacia) and labelled with dCT$^{32}$P. (NEN Life Sciences Products; Hodgson and Fisk, 1987) Two hundred millimolar NaOH
was added to denature the probe. After five minutes, 100μl of this solution was added to 20mls of fresh pre-warmed hybridization solution and this was incubated with the blots overnight at 68°C with rotation in the hybridization ovens. The next morning, the blots were washed once for 30 minutes in a solution containing: 25mM NaPO₄, 500mM NaCl, 5mM NaPPi, 0.5% SDS, at 55°C in a shaking water bath. The blots were then transferred to a wash containing: 25mM NaPO₄, 150mM NaCl, 5mM NaPPi, 2% SDS and agitated for 30 minutes. The final 30 minute wash contained: 25mM NaPO₄, 50mM NaCl, 5mM NaPPi, and 2% SDS. The blots were removed from the washing solution, covered in plastic wrap, exposed overnight to Kodak (X-OMAT™) AR film using a ³²P intensifier screen (DuPont), and the film was developed.

**Isolation of 5' and 3' Extended cDNAs**

Primers matching a region near the 5' and 3' end of the TL37-2A cDNA clone were synthesized and used in conjunction with primers matching sites in the vector flanking the 5' or 3' end of the inserted cDNA.(CTV5’B: 5’CCTTCTCTAGCTCGAGACG3’; CTV5’C: CTCACTCCTTCTCTAGCTC; CTV3’B: GACCACCTGATATCCTGTC; CTV3’C: AACAAATTGGACTAATCGATACG)

Figure 8 illustrates the location of the different primers utilized for extension PCR. Nested PCR reactions were performed on plasmid DNA from the T28 and WEHI231 cDNA libraries using Taq DNA polymerase, the supplied buffer, and the following cycling parameters: 95°C for 30s x 1; 95°C for 40s, 57°C for 60s, 68°C for 60s x 30. Amplified cDNA products were gel purified and cloned into pBluescript KS+
(Stratagene, USA) and sequenced. Regions of overlap between 5' and 3' extension products and the original TL37-2A cDNA sequence showed no discrepancies.

**Sequence Analysis and Comparison**

cDNA sequences were determined by thermocycle sequencing on 373 and 377 DNA sequencers using FS Taq Dye Terminators (UBC, Nucleic Acid-Protein Service Unit, Vancouver, British Columbia, Canada). Continuous sequence was determined for both strands. Database comparisons were performed with the MPSearch program, using the BlastX server operated by the European Molecular Biology Laboratory (Heidelberg, Germany). Additional motif analysis was performed using the SMART (Simple modular architecture research tool) Program (Schultz *et al.*, 1998). Similarities between RasGRP and other sequences were identified using the Bestfit program of the Genetics Computer Group (Madison, WI, USA).

**Elk-1 Activation Assays**

NIH 3T3 cells were co-transfected with pGal4-LUC and pGal4-Elk-1, along with expression vectors expressing GST-tagged forms of RasGRP cDNAs, prenylated RasGRF-1, and wild type or dominant negative forms of the Ras family GTPases: H-, K-, N-, R-, and TC21. The data shown are representative of three separate experiments, with each point performed in triplicate in each experiment. This work was performed by Ian Whitehead at UNC in Chapel Hill (1998).

**ERK Phosphorylation Assays**

A ERK Assay Kit from New England Biolabs was utilized to determine the relative levels of ERK 1 and 2 activity in cells overexpressing various constructs.
Briefly, stable lines of NIH 3T3 cells overexpressing control or RasGRP derived constructs were grown to 90% confluence in 10cm dishes in 9CS/DMEM, switched to serum free DMEM for 2 hours, and then stimulated for 15 minutes with the following: serum free media, serum (9CS/DMEM), or 50ng/ml PMA/DMEM. Similarly, BOSC23 packaging cells transiently overexpressing different control or RasGRP constructs were grown in 10cm dishes to 90% confluence in 10FCS/DMEM. Thirty-two hours post transfection, the BOSC23 cells were washed three times with PBS and switched to serum free medium (RPMI, 4μg/ml insulin, 5ng/ml sodium selenite) for 19 hours and then treated with the following for 15 minutes: nil, 10FCS/DMEM, or 50ng/ml PMA/DMEM. All treated cells were rinsed once with ice-cold PBS and lysed directly on the plates with lysis buffer using the protocol described by the manufacturer.(New England Biolabs) The lysed cells were sonicated, centrifuged to remove cellular debris and the supernant was utilized in immunoprecipitations (IPs) with phospho-ERK1/2 antibodies. Equal protein amounts of the IPs were run out on 10% acrylamide gel and blotted onto Immobilon P membranes. The filters were then probed with phospho-ERK1/2 antibodies in order to detect the levels of phosphorylated ERK1/2 in each of the cell lines. The filters were then stripped and reprobed with ERK1/2-specific antibodies to determine the levels of total ERK1/2 protein in the samples.

**Construction of RasGRP Deletion and Mutant Constructs**

All deletion mutants were made by fusing natural or PCR-generated restriction enzyme sites to start codons, stop codons, green fluorescent protein (derived from pEGFP-C1; Clonetech) and/or hemagglutinin (HA) epitope tags, provided by pCTV.
derived vectors. A full description of each deletion and mutation construct along with their names can be found in Figures 11, 16a, and 21.

The R271E point mutation in the GEF domain (GEF\(\mu\)) was made by the fusion of two PCR fragments, introducing the base changes at the site of fusion. The resulting encoded peptide sequence is PTPQLEAEVF\(\overline{I}\)K, compared to the normal sequence PTPQLRAEVFIK. The deletion of the EF hands (E\(\Delta\)) was made by replacing the fragment of the TL37-2A cDNA lying between the \(\text{HindIII}\) and \(\text{StuI}\) sites with a synthetic oligonucleotide hybrid encoding the appropriate amino acids. The mutation affecting the proline cluster (Pro\(\mu\)) was made by replacing the fragment of the TL37-2A cDNA lying between the SstI and \(\text{AccI}\) sites with a synthetic oligonucleotide hybrid encoding the appropriate amino acids. The encoded peptide sequence of this mutant is RAQGLTLSKGGV\(\overline{V}\)V: the normal sequence is RAPPLTSPKPPVVV. The deletion of the REM domain (REMA) was made by replacing the fragment of the TL37-2A cDNA lying between the MscI and \(\text{BglII}\) sites with a synthetic oligonucleotide hybrid encoding the appropriate amion acids. The prenylation signal was made by inserting an oligonucleotide hybrid downstream of an HA tag in a pCTV retroviral vector. The resulting prenylation vector encodes the following amino acids from the C terminus of K-Ras: \(\text{xzGSRKHKEKMSKDGGKKSSTKCVIM#}\), where x is the site of insertion of the cDNA, z is the HA epitope tag, and # is the stop codon. RasGRP cDNAs were then inserted into this vector, resulting in in-frame fusion of the cDNA to the HA tag and prenylation signal.
The second PKCδ-C1 domain and the RasGRP-C1 domain were generated by PCR amplification from the T28 library and from the TL37-2A cDNA respectively, using primers which modified the N and C termini of the encoded peptides. The encoded peptides of the RasGRP-C1 domain is STFPHNF....LVVFECKKRIKPT. The murine PKCδ-C1 domain is stMPHRF....KVANLCkrikpt, with the uppercase letters indicating the PKCδ-C1 domain sequence and the lowercase letters indicating sequence identical to those at equivalent positions in the RasGRP-C1 domain sequence. The PCR generated C1 domains were inserted into a RasGRP cDNA, such that their N termini are fused to amino acid 539 of RasGRP and their C termini are fused to an HA tag and a stop codon. Thus, the PKCδ-C1 domain-encoding (RasGRP)CΔ1/PKCδ-C1) and the RasGRP C1 domain-encoding (RasGRP(CΔ1/C1) cDNAs are completely identical except for the C1 domain-encoding sequences themselves.

All HA tag and GFP fusions, mutations, and PCR-generated DNA fragments were sequenced to confirm specific mutations and reading frame preservation and to ensure that no secondary mutations had occurred. The levels of expression in cells engineered to overexpress the various GFP-tagged constructs was determined using FACs analysis.(See: Figure 16b)

**NIH 3T3 Transformation Assay: Testing RasGRP Deletion and Mutant Constructs**

Deletion and mutant constructs of RasGRP (See: above and Figure 16a) were converted into infectious retroviral particles and used to infect NIH 3T3 cells. The cells were grown to confluence and for an additional 5 to 10 days post confluence, then assayed
for transformation by comparison to cells overexpressing control constructs or known transforming oncogenes such as activated N-Ras. The wells of infected cells were fixed with methanol and stained with methylene blue.

**RNA Isolation and Northern Blot Analysis**

Total RNA and mRNA were prepared using the following protocol, based upon a method described by Chomcynski et al (1987) using guanidinium isothiocyanate/phenol. Briefly, ten million non-adherent cells or a confluent 25 cm dish of adherent cells were pelleted by centrifugation. The pellet was resuspended in 5mls of GCS (Stock solution: 12.5g guanidinium isothiocyanate (Gibco BRL), 880ml 750mM Na.citrate (pH 7.0), 1.32ml 10% L-lauroyl-sarcosine (Sigma), 180ml β-mercaptoethanol, 14.6ml H₂O) and passed through an 18 gauge needle a number of times in order to break up any clumps of cells. One hundred microlitres of 2M sodium acetate (pH 4.0) and 200ul of CHCl₃: isoamylalcohol (49:1) were added, this mixture was extracted thoroughly and placed on ice for 15 minutes. The solution was centrifuged for 20 minutes at 4°C and the aqueous phase was removed and precipitated with one volume of isopropanol at -20°C for 1 hour. The RNA was pelleted out by centrifugation at 4°C and rinsed with 95% ethanol. The pellet was air dried and dissolved in 300ul of GCS solution and reprecipitated with two volumes of 95% ethanol at -20°C for 1 hour. The RNA was pelleted out by centrifugation at 4°C for 15 minutes, rinsed with 80% ethanol and centrifuged for 1 minute. The pellet was air dried once more and dissolved in a solution containing diethylpyrocarbonate (DEP) treated 10mM HEPES, 1mM EDTA (pH 7.5). The quality and amount of RNA was determined by running it out on an agarose gel containing 0.1% SDS. Samples were
prepared by adding 2.5 ul of RNA sample mixed with 7.5ul of dye/formamide solution. The dye/formamide solution was made up of 1ml of deionized formamide, 160ul 10X MOPS, 250ul formaldehyde and 90ul 5% bromophenol blue (in H₂O). The sample was then run out on 5% formaldehyde agarose gels and wick blotted onto Hybond N+ nylon membrane. (Southern, 1975; Amersham) Hybridization and high stringency washings were performed as described in the Southern Blotting protocol section. A MluI/BglII 5' end fragment of TL37-2A was utilized as a probe and labelled with ³²P by extension of random sequence primers. (Hodgson et al, 1987) This probe, lacking the highly conserved C1 domain or potential DAG binding region, was chosen in order to minimize cross hybridization with other unrelated C1 domain containing proteins. Hot blots were covered in plastic wrap, exposed to Kodak Biomax MS film using an intensifying screen (DuPont), stored at -70°C for one week and the exposed film was then developed.

**Localization of GFP-labelled Constructs in Serum Containing Media**

NIH 3T3 fibroblast cells overexpressing green fluorescent protein (GFP)-labelled constructs were plated onto circular glass cover slips (Fisher Scientific) and fixed with 4% paraformaldehyde/PBS (Stem Cell Technologies) for 20 minutes at room temperature. The coverslips were mounted onto glass slides using a mounting media/anti-fade containing the following chemicals: 200mM 1,4 diazabicyclo [2,2,2] octane (DABCO), 2mM Tris (pH 8.0), 90% glycerol, and visualized using a Zeiss fluorescent microscope equipped with filter set 02. Photomicrographs were taken using Fuji ASA 400 film.
Co-localization Experiments

NIH 3T3 cells expressing GFP-labelled constructs were grown on coverslips in serum-containing media. The cells were washed with PBS containing 0.5mM CaCl₂/1.0 mM MgCl₂ to help the cells maintain maximum adherence to the cover slips (all subsequent washes contained CaCl₂ and MgCl₂). In order to stain the endoplasmic reticulum (ER), the cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes and then washed three times with PBS. The fixed cells were then permeabilized with 0.1% Triton-X 100, washed three times with PBS, blocked with 2.5% BSA/PBS, and then washed three times with PBS. Finally, the cells were incubated with the anti-BiP monoclonal antibody SPA-827 (StressGen Biotechnologies, Inc.), washed three times with PBS, incubated with a Texas Red-conjugated secondary antibody (Jackson Labs) and then washed three times with PBS in order to get rid of any nonspecific antibody staining. ER staining and GFP fluorescence were detected in tandem since the Zeiss fluorescent microscope that was utilized was incapable of simultaneous detection of both signals. In order to visualize the mitochondria, live cells were incubated with 25nM MitoTracker Red CMXRos (Molecular Probes Inc.) for 5 minutes and fixed with 4% paraformaldehyde. Mitochondrial staining and GFP fluorescence were also visualized in tandem using the Zeiss fluorescent microscope. Photomicrographs were taken using Fuji ASA 400 colour film.

Localization of GFP-labelled constructs upon stimulation

NIH 3T3 cells overexpressing GFP-labelled constructs were grown on circular glass coverslips (Fisher Scientific). In order to serum starve the cells, the cells were
washed 3X with PBS (containing 0.5mM CaCl$_2$/1.0 mM MgCl$_2$) and then placed in
DMEM lacking serum for 3.5 hours. Serum starved cells were fixed with 4%
paraformaldehyde at this point, whereas stimulated cells were treated with one or a
combination (See: Figure legends for details) of the following: 10% serum, 50ng/ml
phorbol myristate acetate (PMA, Sigma), 4U/ml phosphatidylcholine specific PLC Type
IV from B. cereus (Sigma), or 100ng/ml ionomycin (Sigma). Treated cells were then
fixed with 4% paraformaldehyde and mounted using anti-fade mounting media and
visualized using a Zeiss fluorescent microscope equipped with filter set 02.
Photomicrographs were taken using Fuji ASA 400 colour film.

**Contribution of Others to this Thesis Project**

cDNA libraries were constructed by Heather Kirk (Terry Fox Lab). Isolation of a
portion of the 5' end of RasGRP was performed by Heather Kirk (Terry Fox Laboratory).
Many of the constructs (See: Construction of RasGRP Deletion and Mutant Constructs)
utilized in the transformation and localization experiments were made by Rob Kay (Terry
Fox Lab). The transformation assays and FACs analysis of GFP expression levels in the
NIH 3T3 cells were performed as a collaborative effort between Rob Kay and myself.
The ERK phosphorylation experiments were a collaborative effort between Heather Kirk
(Terry Fox Lab) and myself. The Elk-1 phosphorylation experiments were performed by
Ian Whitehead (Chapel Hill). All other work described in this thesis was performed by
myself.
Chapter III: Screening for Genes Involved in Growth Regulation

3.1 Introduction

Strategy for cloning cDNAs that activate the Ras pathway

In order to fulfill our main objective of identifying a novel growth controlling protein involved in the Ras pathway we utilized a retroviral vector based expression cloning strategy and performed large scale cDNA library screens in NIH 3T3 fibroblast cells. (See: Figure 5) The rationale for using this particular method will be given in the next few sections.

Signals transduced by growth factor stimulation and contact with other cells make their way to the nucleus by way of complex signalling cascades. Changes in the proliferative response of a cell can be attributed to alterations in the behavior or expression pattern of key proteins involved in growth control pathways. In the past, a number of genes encoding these types of molecules have been identified through the analysis of cells that exhibited abnormal patterns of growth. The murine fibroblast cell line, NIH 3T3, has been used by many groups to assay the oncogenic potential of certain genes and it has been shown to be highly susceptible to oncogenic transformation. It is speculated that NIH 3T3 cells may contain a number of intrinsic genetic alterations which have the effect of priming these cells for subsequent transformation by the overexpression of oncogenes. (See: Chapter I/Ras Transformation of Primary and Immortal cell lines)

This information provided the rationale for choosing NIH 3T3 cells as the target cell line for use in the transformation assay.
Figure 5. Protocol: Retroviral vector-based expression cloning strategy

a) cDNA libraries are converted into infectious retroviral particles using the ecotropic packaging cell line, BOSC23, via transient calcium phosphate-mediated transfection. b) Viral supernatants are used to infect target NIH 3T3 fibroblast cells. c) Infected cells are grown 5 to 10 days post-confluence in 25cm dishes and the number of foci per plate is determined. d) Each focus is phenotypically identified and manually isolated, trypsinized, replated into 6 well dishes, and grown to confluence. e) Genomic DNA is isolated from the cells produced from each focus. f) PCR, using vector specific primers, is performed on the isolated genomic DNA. g) Small portions of PCR products are run out on agarose gels, Southern blotted, and probed with vector-specific regions (SupF) in order to identify subvisual PCR products. h) Remaining volume of PCR products are cut with MluI/SunI and run out on agarose gels and the individual bands are isolated and recloned into the retroviral vectors. i) Recloned PCR products are retested for their ability to cause transformation in the fibroblast cells. j) Any clone retesting positive is subcloned into a sequencing vector and then sequenced.
a) cDNA library

Calcium phosphate precipitation

BOSC23 packaging cell line

b) Filter viral supernatant

Infect fibroblast cells

c) Grow to confluence + 5 to 10 days

d) Isolate foci

Plate cells in 6 well dishes

e) Isolate genomic DNA

f) Perform PCR with vector specific primers

Use portion of PCR product for:

a) Southern blot

b) Isolation of single bands from agarose gel

i) Reclone PCR product into retroviral vector

j) Retest for transformation

Sequence positive clones
Fibroblast transformation assays continue to be used to isolate genes involved in regulating cell growth and the potential for identifying novel proteins using this system remains. Many different Ras pathway components, from growth factors to transcription factors, have been identified. (See: Chapter I/Use of Transformation Assays to Identify Modulators of Ras Activity) In addition, distinct morphological differences produced by signalling pathways (See: Chapter I/Phenotypic Changes Associated with Ras/Rho/Rac Fibroblast Transformation) can be used to identify genes that fall into a particular subset of growth controlling signalling pathways. Furthermore, since all cells do not express exactly the same repertoire of signalling molecules the potential for identifying novel oncogenes by screening unique cDNA libraries also exists. This project made use of these distinctions in an attempt to identify growth controlling genes involved in the Ras pathway.

Our particular experimental system also made use of retroviral-based vectors in order to express the cDNA libraries in the target NIH 3T3 cells. Naturally occurring oncogenic retroviruses have been shown to overexpress cellular transcripts that were picked up by the retrovirus during its replication process. In essence, these events could be used to identify growth controlling genes. One advantage of using man-made retroviral vectors over naturally occurring oncogenic retroviruses is the ability to control the source of the transferred genetic material. Secondly, the isolation of the cDNA from the genomic DNA of the target cell is facilitated by the knowledge of the flanking vector sequences and this is not the case with naturally occurring events. Lastly, the occurrence of oncogenic retroviruses are rare whereas man made retroviral vectors can be easily utilized to transfer entire cDNA libraries into target cells. These advantages of retroviral
vectors make them useful tools for expression cloning strategies and increase the probability of identifying and isolating oncogenic cDNAs. A number of groups have also used transfection to express cDNA libraries in NIH 3T3 cells. (i.e. Miki et al, 1991b) This method is limited by the level of cDNA expression and the number of cDNA clones that can be expressed. Retroviral vectors can achieve a high level of expression of the introduced genetic material in infected cells which facilitates the isolation of rare transforming cDNAs. Retroviral vectors are also very efficient at transducing a large number of cells.

In our protocol, cDNA libraries were converted into infectious retroviral particles using the ecotropic packaging cell line, BOSC23 via transient calcium phosphate-mediated transfection. Filtered viral supernatants were then used to infect target NIH 3T3 fibroblast cells and these cells were grown for five to ten days post-confluence. Any foci produced during this time period were phenotyped and the genomic DNA was isolated from the cells making up each focus. PCR using vector specific primers facilitated the isolation of the cDNA from the genomic DNA. Each cDNA was re-ligated into a retroviral vector and then retested for its ability to cause transformation in the fibroblast cells. Any cDNAs retesting positive in the secondary transformation assay were sequenced at the 5’ and 3’ ends to determine their identity. Using this method, we attempted to isolate foci exhibiting a Ras-like transformed phenotype in the hopes of identifying novel genes involved in the Ras pathway. The results from cDNA library screens performed in NIH 3T3 cells will be discussed in this chapter.
3.2 Results

cDNA library screens in NIH 3T3 cells

A number of different cDNA libraries were screened in a focus forming assay in NIH 3T3 cells using the expression cloning strategy described in Figure 5. Three different cDNA libraries TL28/29, TL30/31, and TL35/36/37, originating from the human cells lines HS766T (epithelial) and U87MG (breast cell carcinoma), and the murine cell line T28 (T cell hybridoma) respectively were utilized in these experiments. For each cell line, several independent cDNA libraries were available, differing in the size of the cDNA clones. For example, the TL35 library was constructed to contain cDNAs primarily in the size range of 1.0-1.5 kb, the TL36 cDNA library contains cDNAs with the size range of 1.5-2.0 kb, and the TL37 cDNA library was selected to contain cDNAs over 2 kb in size. The number of clones in each library ranged from $4.2 \times 10^5$ to $2.4 \times 10^6$. The rationale behind choosing these particular cDNA libraries was simply their availability. These cDNA libraries were constructed by Heather Kirk (Terry Fox Lab, 1996).

cDNA libraries were packaged into infectious retroviral particles and used to infect $5 \times 10^5$ NIH 3T3 cells. Table 3 outlines the results of these screens performed on the aforementioned libraries. A cell culture infected with an empty retroviral vector (CTV3B) produced three foci. This represents the background of spontaneous transformation events. The number of foci produced by infecting the cells with the various cDNA libraries varied from 2 to 16 foci. The total number of foci produced by the library infected cells was 40. Each library-induced focus was scored by
### Table 3. Results of transforming cDNA library screens in NIH 3T3 cells

TL28A and 29A libraries are derived from the human cell line HS766T. TL30 and 31 libraries are derived from the human cell line U87MG. TL35, 36, and 37 libraries are derived from the murine T28 cell line.
transformation phenotype and then isolated. Of the 40 foci produced in the library screen, slightly less than 50% exhibited a refractile, swirling morphology with no distinct cell clumping, a phenotype more reminiscent of Ras transformation than of Rho transformation. Thirty-five foci were manually recovered and PCR was performed on the genomic DNA isolated from these foci. The PCR products were individually subcloned back into the retroviral vector. Each subcloned cDNA was overexpressed in the fibroblast cells in order to assay for its ability to cause transformation. A total of twelve positive transforming cDNAs were identified from these screens representing a 30% recovery rate. On the right hand side of the chart are the sequencing results of the clones that retested positive in the transformation assay.

Most of the cDNAs encoded oncoproteins already known to cause transformation in NIH 3T3 cells. These included: the growth factor, PDGF-β; the Rho exchange factors, Dbs, Lfc, and Lsc; the serine threonine kinase, Raf-1; and the polyoma viral oncprotein, Large/Middle T antigen. The transformation mechanisms of two other positive clones were not as obvious. One of the clones encoded an antisense cDNA to a putative ribosome binding protein known as Ribophorin II. The other clone encoded S5, a subunit of the 26S proteosome which is a multisubunit enzyme required for ubiquitin-mediated proteolysis. The exact mechanisms utilized by these molecules to cause transformation have not been determined and is beyond the scope of this thesis.

One novel transforming cDNA (TL37-2A) was identified out of the T28 library screen and overexpression of TL37-2A caused Ras-like transformation in the fibroblast cells. Sequencing data indicated that TL37-2A was a novel protein. The isolation of the
TL37-2A cDNA appeared to fulfill our primary objective of identifying a novel growth controlling protein that could potentially play a role in activating the Ras pathway. Therefore, this particular cDNA became the primary focus of my thesis project.

**Overexpression of TL37-2A causes Ras-like transformation**

Overexpression of the TL37-2A cDNA in NIH 3T3 cells was able to cause rapid changes in cellular morphology. When visualized under the microscope, TL37-2A transformed cells were refractile, elongated, and did not respond to normal cell-to-cell contact inhibition of growth. One hundred percent of TL37-2A infected fibroblast cells exhibited a transformed phenotype. (See: Figure 6, TL37-2A) Cells infected with virus containing an activated N-Ras cDNA exhibited similar changes in phenotype. (See: Figure 6, N-Ras) This is in contrast to control infected cells which, when visualized under the microscope grew in a cobble stone pattern of cell arrangement, were non-refractile and contact inhibited. Minimal background levels of transformation (depicted by darkly staining spots) were observed when the control construct was used to infect fibroblast cells. (See: Figure 6, Control)

**Expression patterns of TL37-2A**

Northern blot analysis of mRNA from isolated murine tissues revealed a 4.8kb transcript in brain, thymus, spleen and bone marrow which hybridized to the TL37-2A cDNA. Fainter bands of approximately 4 and 5kb could also be observed in thymus and brain. Highest expression was observed in thymus and brain with lower levels observed in spleen and bone marrow. (See: Figure 7a) No transcripts hybridizing to the TL37-2A were detectable in kidney, liver, lung, stomach, or muscle by Northern blot analysis.
Figure 6. Transforming activity of the TL37-2A cDNA in NIH 3T3 cells

NIH 3T3 cells overexpressing various constructs: control construct (empty retroviral vector), RasGRP (retroviral vector carrying the TL37-2A cDNA), or N-Ras (retroviral vector carrying a cDNA encoding an activated form of N-Ras). Cells were grown to confluence, maintained for an additional 5 days post-confluence, and then stained with methylene blue.
The TL37-2A-hybridizing 4.8kb transcript was highly expressed in the T cell lines MBL-2 and T28, with lower levels of expression observed in P338D1, which is a cell line derived from a B lymphoma that subsequently underwent monocytic differentiation. The 4.8kb transcript was also expressed in the B cell lines: WEHI231, ABE8, and A20, as well as the primitive hematopoietic cell line, B6SutA. Fainter bands of 4 and 5kb could also be observed in ABE8, WEHI231 and MBL-2. (See: Figure 7c) Transcripts hybridizing to the TL37-2A cDNA were not detectable in: the B cell lines Ba/F3 and NSF-70; the T cell lines R1.1 and YAC-1; the myeloid cell lines DA-3 and 32D; or the erythroid cell line GM979. Northern blot analysis revealed that no detectable transcripts capable of hybridizing to the TL37-2A probe were expressed in the fibroblast cell lines NIH 3T3 or C3H10T1/2, or in the breast derived cell line C127. (See: Figure 7b)

These results suggest that the gene encoding the transcripts that hybridized to the TL37-2A probe are expressed in some but not all murine lymphoid cell types and possibly some uncommitted hematopoietic precursors as represented by the B6SutA cell line. Two additional RNA species of 4 and 5 kb could be observed to hybridize to the TL37-2A cDNA in some cases indicating either the possibility of alternatively spliced forms or the existence of additional family members of this group of proteins which may cross hybridize with the TL37-2A probe.

3.3 Discussion

Historically, fibroblast transformation assays have been very successful at identifying genes involved in growth control. This approach was also successful in this
Figure 7. Expression of TL37-2A in murine tissues, hematopoietic and fibroblast cell lines
Northern blots of total RNA from: a) the indicated tissues from a 6 week-old C57BL/6J mouse; or b) murine fibroblast derived cell lines were probed with TL37-2A cDNA; or c) murine hematopoietic cell lines. Marker sizes are indicated in kb. Note: Original autoradiogram shows a faint band in marrow lane which is not visible in the secondary photograph. Larger 5kb and smaller 4kb hybridizing transcripts were also observed in the original autoradiogram in brain, thymus, and the cell lines: ABE8, WEHI231, MBL-2.
case since eleven different transforming cDNA were isolated out of these screens. Eight of these clones were found to encode previously known oncogenes. Out of these eight, three were known to play a role in the Ras pathway (i.e. Raf-1, two cDNAs encoding PDGF-β), four were known to play a role in the Rho pathway (i.e. two cDNAs encoding Dbs, Lsc, and Lfc) and one encoded a known viral oncoprotein (i.e. polyoma Mid/Lg T Ag). Two additional transforming cDNAs isolated in these screens were found to encode previously known genes that were not known to be oncogenic (i.e. Ribophorin II, 26S5b proteosome subunit). One novel oncogene (TL37-2A) was also isolated whose overexpression causes Ras-like transformation in the fibroblast cells. Our primary objective of identifying a novel cDNA potentially involved in the Ras pathway was fulfilled by the identification of this oncoprotein and because of this, TL37-2A was chosen as the focus of this thesis project.

TL37-2A is a novel transforming cDNA isolated from the murine T28 T hybridoma cell line-derived cDNA library. When compared to fibroblast cells expressing oncogenic N-Ras, TL37-2A exhibited similar morphological changes (See: Figure 6) indicating that perhaps this novel cDNA might play a role in Ras signalling in the fibroblast cells. It should be noted that endogenous expression of this protein is not normally observed in any of the fibroblast cell lines tested (See: Figure 7b; NIH 3T3, C3H10T1/2, C127) and that overexpression of this particular cDNA in fibroblast cells represents an unnatural situation. Transcripts hybridizing to the TL37-2A probe were observed in hematopoietic tissues and these results correlated with those obtained from cell line northern blot analysis which indicated similar sized transcripts hybridizing to the
TL37-2A probe in some but not all lymphoid derived cell lines. In addition to hematopoietic tissues, a high level of expression was also observed in brain. This pattern of expression differs from that of other known Ras GNEFs. For example, Sos proteins are expressed ubiquitously (Botwell et al., 1992) and RasGRF proteins are mainly expressed in the brain (Fam et al., 1997; Shou et al., 1992; Wei et al., 1992) RasGRP represents the first Ras exchange factor that is selectively expressed in brain and hematopoietic organs and may provide a unique way to control Ras activation in these cells. Our goal now will be to discover how TL37-2A causes transformation in NIH 3T3 cells, to determine what pathway(s) it might activate and how its activity might be regulated.
Chapter IV: Structure of RasGRP, a new member of the CDC25 Family of GNEFs

The cloning of TL37-2A fulfilled our first objective. (See: Chapter III) This chapter will focus on our next set of objectives which were: 1) To determine the sequence of the protein encoded by TL37-2A; 2) To identify potential functional domains by sequence comparison and motif analysis; and 3) To develop hypotheses about how TL37-2A transforms cells based upon potential functions suggested by the sequence analysis.

4.1 Sequence of RasGRP

The TL37-2A cDNA was fully sequenced in both directions. It was 1878bp in length, included a continuous open reading frame (ORF) of 550 amino acids, possessed a stop codon upstream from the first potential initiator methionine but lacked an inframe stop codon at the 3’ end indicating that the coding region was definitely truncated at the 3’ end. Additional 3’ sequence was generated from the T28 cDNA library using vector and TL37-2A specific primers. (See: Figure 8) Addition of this sequence to TL37-2A resulted in the production of a 747aa ORF with an in frame stop codon. Additional 5’ end sequence was generated from the WEHI231 cDNA library using TL37-2A specific and vector specific primers. (See: Figure 8) Comparison of the original 5’ end sequence isolated from the T28 cDNA library with the 5’ extension generated from the WEHI231 library revealed that these sequences diverged from each other at one particular point. (See: Figure 9) A potential splice acceptor site consensus sequence, comprised of a polypyrimidine tract followed by the sequence NCAG (where N is any nucleotide), was
**Figure 8. Isolation of full length RasGRP**

Generation of 5' and 3' PCR extension products: the 5' extension product was isolated from the WEHI231 library and 3' extension product was isolated from the T28 library. TL37-2A is represented as the upper line in the diagram, arrows represent the placement of RasGRP specific primers and the library vector specific primers are found to the outer right and left underneath the figure. Extension products are represented as dotted lines underneath the original TL37-2A clone. Both downstream and upstream stop codons were identified in RasGRP.
### Figure 9. Comparison of alternative RasGRP 5' sequences

Comparison of differing RasGRP 5’ sequences obtained from the T28 library (*italics*) and the WEHI231 library (*bold*). Straight lines highlight conserved nucleotides between the two sequences. Nucleotide numbering is found to the left of the sequence. Splice acceptor (SA) site consensus is underlined. The vertical dotted line indicates the divergence point between the T28 and WEHI231 sequences.
identified at this point of divergence in the T28 derived 5' cDNA. (Dominski and Kole, 1994) It was hypothesized that the T28 5' end sequence was an incompletely spliced form of the cDNA and that the 5' extension generated from the WEHI231 library represented the properly spliced version of this cDNA. Subsequent descriptions of rat homologues by two other groups (Ebinu et al, 1998; Kawasaki et al, 1998) showed the same coding region structure confirming that our deduced coding region for this cDNA is correct.

The full length sequence, generated from the original cDNA isolated from the T28 library screen, the 3' extension obtained from the T28 library, and the 5' extension obtained from the WEHI231 library produced a 795aa ORF containing a downstream stop codon but lacking an upstream stop. Attempts were made to generate further 5' end sequence but they were not successful. Sequencing errors were corrected for by generating and sequencing individually isolated PCR products from either the T28 or WEHI231 cDNA libraries. This full length molecule will be called RasGRP for Ras Guanyl Nucleotide Releasing Protein for the remainder of this manuscript to coincide with the naming of the rat homologue identified by Ebinu et al. (1998). The rat homologue has also been cloned by an additional group and they chose to name this molecule CalDAG-GEFII. (Kawasaki et al, 1998) The name TL37-2A will be used to refer to the original 5' and 3' truncated cDNA isolated from the library screen. The full length nucleotide sequence and amino acid translation of RasGRP are found in Figure 10.
Figure 10. Full length nucleotide sequence and amino acid translation of RasGRP

The full length nucleotide sequence was obtained from the combined sequences generated from TL37-2A, the 3' PCR extension cDNA isolated from the T28 cDNA library and the 5' extension isolated from the WEHI231 cDNA library. The translated amino acid sequence is found below the corresponding nucleotide sequence. Initiator methionine of this cDNA is identified in bold. Stop codons are represented by *. The original TL37-2A clone encompassed nucleotides 204-2028. Nucleotide numbering is found to the right of the diagram.
Figure 11. Domain structure and amino acid translation of RasGRP

a) The line diagram depicts the various domain found within RasGRP. Each domain is labelled: REM, GEF, proline rich region (P), EF hands (EFH), C1, and leucine zipper (LZ). b) In the translated, full length amino acid sequence the REM and GEF domains are indicated by bold type, while the EF hands and C1 domain are indicated by the boxes and dotted underline, respectively. Potential MAP kinase phosphorylation sites are underlined. The potential amphipathic alpha helix is double-underlined, with bars under the aliphatic residues at 7th positions in the sequence. The open and filled dots indicate positions affected by point mutations in the GEF domain and proline rich region, respectively. The boundaries of deletion mutants are indicated by the arrows found above the amino acid sequence.
### 4.2 Motif and Sequence Analysis of RasGRP

**a) Ras Exchange Motif (REM) Box and the GNEF domain**

Blast queries performed with the RasGRP peptide sequence identified a region that exhibited high sequence similarity to the CDC25 family of GNEFs. (See: Chapter I/The CDC25 Family of Ras-specific GNEFs) Figure 12 illustrates RasGRP’s similarity to Sos1, RasGRFl, and CDC25, which are all Ras-specific exchange factors and to C3G which is an exchange factor specific for Rap1A. RasGRP is also compared to RalGDS which has been shown to interact with Rap1A, RalA/B, R-Ras, H-Ras, and TC21. Four distinct regions within RasGRP can be identified that contain significant expanses of sequence similarity to regions found in the other CDC25 family GNEFs. These are the Ras Exchange Motif (REM) box (Fam *et al*, 1997) and the three structurally conserved regions (SCR) found in the GNEF domain which were first described by Boguski and McCormick in 1993. The REM box is found in proteins that are known to exchange on true Ras proteins such as Sos 1 and 2 as well as other GNEFs such as C3G and RalGDS. (Lai *et al*, 1993) The three SCRs are found in all GNEFs making up the CDC25 family. RasGRP also contains three conserved arginines (R) within the GEF domain that have been identified as being absolutely necessary for proper exchange on Ras. (Asterixed in Figure 12; Fam *et al*, 1997; Lai *et al*, 1993) The question of whether RasGRP is capable of exchange on true Ras proteins will be addressed in Chapter VI.

**b) RasGRP contains a C1 domain**

An additional region of RasGRP, downstream of the GNEF domain, was identified during Blast searches as having high sequence similarity to C1 domains. C1
Figure 12. Comparison of REM box sequences and sequences of Structurally Conserved Regions (SCR) found within GEF domains

Comparison of RasGRP in a Pileup (GCG program) to proteins containing REM motifs and GEF domains, including Sos1, RasGRF-1, C3G, RaLGDS, and the yeast exchange factor, CDC25. Highlighting indicates conserved residues, defined as identical or structurally related residues occurring in at least four of the six sequences. Dots indicate gaps required for optimum alignment. * indicate conserved arginines required for Ras exchange activity. (Fam et al, 97)
domains are found in most PKC isoforms as well as other proteins such as N-chimerin.

The C1 domain itself can be defined as a 50 amino acid-long lipid interaction motif that
binds to two Zn\(^{2+}\) atoms. (Reviewed by Newton, 1997; and Hurley et al, 1997) The two
zinc-binding sites are formed by noncontiguous sections of primary sequence. Although
these domains are sometimes referred to as zinc fingers they are structurally unrelated to
any of the nucleic acid-binding zinc finger proteins.

These motifs are capable of binding diacylglycerol (DAG) with moderate affinity
and to other PKC activators such as phorbol esters (PE) with high affinity. Studies
performed on isolated PKC-C1 domains have shown that binding to DAG or PE does not
significantly alter the conformation of the protein in this particular region but does
change its surface by forming a hydrophobic cap allowing the new hydrophobic exterior
surface to embed itself in the membrane. Phorbol esters are potent tumor promoters and
function as ultrapotent analogs of DAG. (Reviewed by Baatout, 1998) They have also
been shown to activate C1 domain-containing proteins by altering the nature of the
protein surface and by stabilizing interaction with membranes. (Zhang et al, 1995) DAG
is produced in cellular membranes as a consequence of phosphatidylcholine (PC)
hydrolysis and by the phospholipase C (PLC)-catalyzed hydrolysis of
phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). (Reviewed by Wakelam, 1998)

Proteins possessing C1 domains can be classified into one of two classes. The
terms "typical" and "atypical" are used to refer to the classes of C1 domains that do and
do not bind PE, respectively. Proteins containing typical C1 domains are predicted to be
regulated by DAG, whereas those containing atypical domains are not. The conventional
(α,β,γ) and novel (δ,ε,η,θ) PKC isoforms possess typical C1 domains where as atypical (ζ,τ,λ) PKC isoforms and proteins such as Raf and vav possess atypical C1 domains. (Hurley et al, 1997) The atypical C1 domain-containing proteins may be capable of binding to other lipids and other proteins. For example, the C1 domain of Raf is capable of binding to phosphatidylycerine as well as Ras, although Ras binding to Raf also requires a separate domain of Raf. (Ghosh et al, 1996)

The crystal structure of the second C1 domain (cys2) found in PKCδ has been resolved. (Zhang et al, 1995) The structure of the C1 domain consists of two small β sheets and a short C-terminal α helix. Phorbol ester (PE) appears to bind into a groove made when two of the β strands are pulled apart at the tip of the domain. In the absence of phorbol binding, the gap between the strands contains bound water molecules that form bridging hydrogen bonds like those at the ends of β sheets in other proteins.

Figure 13 compares RasGRP's C1 domain to the typical C1 domains of molecules known to bind PE (PKC-α2, PKC-δ2, PKC-θ2, and N-chimerin) and to atypical C1 domains that do not bind PE (PKCζ, Raf-1, vav). The C1 domain is characterized by a consensus pattern of 6 cysteine and 2 histidine residues which are required for zinc coordination (highlighted in gray stippling). Typical PE binding C1 domains are also conserved at 11 positions that have been shown to participate in the formation of the PE-binding pocket (highlighted in black), whereas C1 domains that do not bind to PE are divergent at these particular residues. (Zhang et al, 1995) More notably, C1 domain-containing proteins that do not detectably bind PE lack a specific conserved proline (asterixed in the Figure). (Kazanietz et al, 1994) The C1 domain found in RasGRP
Figure 13. **Comparison of C1 domain sequences**

The C1 domain of RasGRP is aligned with C1 domains that bind phorbol ester (PKC-α, -δ, -θ, N-chimerin) and those that do not bind phorbol ester (the single C1 domains of PKC-ζ, Raf, Vav). Residues predicted to be capable of participating in phorbol ester binding have been highlighted in black. Conserved histidines and cysteines involved in zinc binding have been highlighted by grey stippling. * highlights a conserved proline thought to be important for phorbol ester binding.
contains all of the cysteines, histidines, and the 11 conserved amino acids including the proline residue thought to be required for PE and/or DAG binding. Therefore, based solely on sequence data, RasGRP appears to contain a potentially functional C1 domain and may be capable of binding to PE and/or DAG. It should also be noted that RasGRP also contains a number of basic residues (KKRIK) downstream from the C1 domain. This basic cluster could play a potential role in increasing the affinity of the C1 domain for membranes by interacting with negatively charged phospholipid head groups. One of the major questions that is raised by the presence of the C1 domain in RasGRP is whether or not it can bind to PE and what consequences this binding will have on the activation state of this molecule. The relevance of PE-RasGRP/C1 interaction will be discussed in further detail in Chapter VII.

c) RasGRP contains a pair of EF hands

Motif analysis performed on RasGRP identified a pair of EF hands present upstream from the C1 domain. EF hands are the most common intracellular Ca\(^{2+}\)-binding domain found in proteins. They were first identified in the crystal structure of parvalbumin (Kretsinger and Kockolds, 1973) and are found in many calcium sensing molecules such as calmodulin and troponin C. EF hands normally occur in pairs and high affinity binding of calcium is usually a result of cooperative binding to both sites. (Ikura, 1996) Upon calcium binding, the EF hands undergo conformational changes which create hydrophobic surfaces that can participate in intra- or inter-protein interactions. (Evenas et al, 1998) EF hand-containing proteins that undergo these large conformational changes upon binding to calcium are all known to have a trigger function
in the activation of target proteins and are often referred to as “regulatory” domains. EF hand-containing proteins which undergo small scale conformational changes in response to calcium binding often function in buffering intracellular calcium. This type of EF hand domain is often referred to as a “structural” or “buffer” domain.

The calcium binding loop is 12 residues long and has a conserved glycine at position 6. The last three residues form the trailing helix and the 12th residue in the loop is a conserved glutamate residue that provides two calcium ligands. The binding of calcium to EF hands has the effect of neutralizing the positive charge of the protein and produces a hydrophobic structure. The first residues following the EF hand motif is always hydrophobic and these motifs are usually flanked on either side by a 12 base pair alpha helical region.(Evenas et al, 1998)

The two EF hands found in RasGRP match the consensus sequence for the calcium binding region.(See: Figure 14) One of the questions that arises is whether RasGRP’s EF hands are capable of binding to calcium, and if so, what effects this would have on the activity of the protein. EF hand-containing proteins that are though to be involved in “buffering” are usually comprised of these domains and very little else. It is unlikely that RasGRP is functioning in this capacity as it contains many additional structural features. If RasGRP’s EF hands function as “regulatory” domains then binding of calcium could positively or negatively regulate the activity of the molecule. For example, calcium-induced conformational changes in RasGRP could produce a hydrophobic surface in the protein that is capable of binding to membranes. These events could potentially synergise with RasGRP-C1 domain/DAG interactions thereby positively regulating RasGRP GEF activity. Alternatively, calcium-induced
Figure 14. Comparison of RasGRP to the consensus sequence for calcium binding EF hands
The position of the 13 residues found in the EF hand motif consensus sequence is depicted above the consensus sequence obtained from the Prosite Motif Database. The residues corresponding to the two EF hands found in RasGRP are aligned underneath the consensus sequence. The amino acid at position 6 is a conserved glycine and the amino acid in position 13 is always hydrophobic.
conformational changes could potentially disrupt the C1 domain or other domains thereby negatively regulating RasGRP activity. It should be noted that in most cases, calcium binding to EF hands helps to facilitate interactions and therefore it is unlikely that the EF hands in RasGRP act as negative regulators. The consequences of calcium-RasGRP/EF hand interaction will be discussed in further detail in Chapter VII.

d) Proline rich region

Visual searching of the RasGRP sequence identified a proline rich region located between the GNEF domain and the EF hands. (See: Figure 11b) This proline cluster delineated by RapPLtPsKppvvv (where upper case letters indicate residues that could participate in SH3 binding) has characteristics of both Class I and Class II SH3 (Src Homology) binding domains. (Alexandropoulos et al, 1995; Mayer and Eck, 1995; Sparks et al, 1996) Within this region is a match to the strong consensus sequence (Px[S/T]P) for a phosphorylation site recognized by MAP kinases. (Davis, 1993; Songyang et al, 1996) A potential interaction between SH3 domain-containing proteins and RasGRP could serve to potentiate exchange factor activation by recruiting RasGRP to plasma membrane localized signalling complexes. This potential mechanism can be thought of as analogous to the way Grb2 binding recruits the exchange factor Sos to the membrane. (Simons et al, 1993) The potential MAP kinase phosphorylation site suggests the possibility of regulating RasGRP activity via MAP kinases, perhaps by a negative feedback loop that could downregulate the activity of RasGRP after it had accomplished its job of activating the MAP kinase pathway via Ras. A similar MAP kinase dependent feedback loop is used to downregulate the activity of Sos. In this situation, MAP kinases
phosphorylate the C-terminal region of Sos so that its ability to interact with Grb2 is reduced. (Waters et al, 1995a/b and 1996; Porfiri et al, 1996) The contribution of the proline rich region to RasGRP activity is explored further in Chapter V.

e) Leucine zipper or α-helical motif

Additional motif analysis performed on the RasGRP sequence utilized the simple modular architecture tool (SMART) program (Schultz et al, 1998). In conjunction with visual searching, the coils2 module embedded within the SMART program identified a potential α-helical region spanning amino acids 744 to 779 which contained a series of repeated leucine residues at every seventh position. This type of structure is called a leucine zipper (LZ) and it is thought to be involved in mediating protein dimerization. The LZ consensus sequence is based upon an a to g labeling of the amino acids with the leucines occurring in the d position. Amino acids found in the a and d positions are mostly hydrophobic or an asparagine. e and g positioned amino acids are usually charged and solvent accessible (i.e. R/D/E/H/K) and b, c, or f positioned amino acids are solvent exposed and mostly hydrophilic (i.e. N/Q/S/T/Y/K). (Zeng et al, 1997)

Figure 15 compares RasGRP's LZ to that of other molecules containing this motif. RasGRP's leucine zipper has the following features: the leucine zipper pattern LLLAL at position d, 10/10 hydrophobic or asparagine residues at positions a and d, 7/10 charged residues at position e and g, and 11/15 hydrophilic residues at positions b, c, and f. RasGRP also has a conserved asparagine (N) that is thought to be involved in determining dimerization specificity of other known LZ containing proteins such as jun and GCN4. (Zeng et al, 1997) If these types of structures are coupled with an upstream
**Comparison of RasGRP α-helix to Consensus**

<table>
<thead>
<tr>
<th></th>
<th>b/c/f Hydrophilic?</th>
<th>e/g Charged?</th>
<th>a/d Hydrophobic?</th>
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<td>+</td>
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</tr>
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<td>N T L K A</td>
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<tr>
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<td>L K Q E N S S</td>
<td>E L K Q E</td>
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**Figure 15. Comparison of Leucine Zipper or α-helical motif sequences**

Comparison of RasGRP's LZ or α-helical region to the consensus lettering (Helix lettering) using information known about the characteristics of each residue found in the different positions of the helix. (Zeng et al, 1997) Hydrophobic residues found in position a or d are either present (+) or absent (-). Charged residues normally found in positions e and g are counted as present (+) or absent (-). Hydrophobic residues thought to occur in positions b, e, or f are either present (+) or absent (-) in RasGRP. Conserved leucine positions are highlighted by a stippled box and the asparagine found in the third position of RasGRP is boxed. RasGRP is compared, in a pileup figure (GCG program), to other α-helical containing proteins such as jun, fos, GCN4, and HBP1.
conserved basic region, as seen in many DNA binding transcription factors such as *fos* and *jun*, they comprise a basic leucine zipper (bZIP) motif. The sequence upstream from RasGRP’s leucine zipper does contain a number of basic residues but it does not match this specific bZIP transcription factors basic domain signature (Prosite: PDOC00036; consensus pattern: [KR]-x-(1,3)-[RKSAQ]-N-x(2)-[SAQ]-x-(2)-[RKTAENQ]-x-R-x-[RK]; where x is any amino acid). The presence of the LZ motif in RasGRP suggests that homodimerization or heterodimerization may take place. Protein dimerization could work to bring RasGRP into specific protein complexes or to subcellular locations where it would be in the correct context for activation. Alternatively, protein dimerization could also be a way to sequester RasGRP in its inactive form away from activating signals and would be a way of negatively regulating RasGRP activation. The contribution made by RasGRP’s LZ or α-helical domain to fibroblast transformation will be discussed in Chapter V and its effect on protein localization will be discussed in Chapter VII.

**f) RasGRP has a potential myristoylation signal**

A potential myristoylation signal was found at the N terminus of RasGRP. N-myristoylation is an acylation process absolutely specific to the N-terminal amino acid glycine in proteins with the initiator methionine (M) being removed. (Reviewed by J. A. Boutin, 1997) Approximately 70% of proteins containing N-terminal myristoylation sequences contain the consensus sequence G¹X²X³X⁴S/T⁵ where the fifth residue following the glycine is either a serine or threonine. The remaining 30% of myristoylated proteins present another amino acid at this position. The serine or threonine at position 5 promotes high affinity interactions with the acyltransferase. Basic residues in positions
7 and 8 result in better substrates than the corresponding neutral residue-substituted peptides which, in turn, are better substrates than those with acidic residues in these positions. (Reviewed by Gordon et al, 1991) RasGRP contains a glycine at its very N-terminus but does not possess a serine or threonine at the fifth position but does possess a basic residue at position 8. Myristoylation affects the overall hydrophobicity of the N-terminus thereby permitting the protein a better association with the membrane lipid bilayer. Myristoylation by itself is insufficient for stable membrane binding and additional factors are required for the actual binding of the protein to the membrane. Therefore, the potential myristoylation signal found in RasGRP may afford the protein preliminary plasma membrane association and this could be further strengthened by a RasGRP-C1/DAG interaction. The effect of this potential myristoylation event on the activity of RasGRP will be discussed in Chapter V.

4.3 Potential Roles for the Domains Identified in RasGRP

Potential Role of REM and GEF domains

The presence of the REM and GEF domains which exhibit sequence similarity to the CDC25 family of Ras exchange factors suggests the possibility that RasGRP is an activator of Ras-like GTPases. From this, we hypothesized that RasGRP induced transformation is mediated by Ras activation and that both the REM and GEF domain would be required for proper exchange activity of RasGRP. Data generated from the testing of this hypothesis will be discussed in Chapters V and VI.
Potential Role of the C1 Domain

The consequences of DAG binding are most well understood in the context of the PKC-C1 domain, with one of the most significant being translocation of the protein to membranes. The second consequence of DAG binding, which synergises with PKC phosphorylation, is the activation of the kinase domain. This occurs through a series of events beginning with the interruption of hydrophobic interactions occurring in another part of the molecule mediated by DAG binding to the C1 domain. This results in a conformational change in the hinge region of the protein and leads to the removal of the inhibitory pseudosubstrate domain from the kinase pocket. (Orr et al, 1992) Once the kinase pocket is unblocked it is active.

RasGRP was also found to contain a C1 domain which suggests that DAG or PE may potentially regulate the activity of RasGRP in some manner. Based upon this knowledge we hypothesized that the RasGRP/C1 domain could function in a similar manner to PKC/C1 domains in responding to increases in DAG or PE concentration by causing RasGRP to translocate to membranes. We also hypothesized that this event is required for Ras activation in vivo by bringing RasGRP in contact with its membrane localized Ras substrate or by causing conformational changes that somehow liberate the exchange factor domain thereby allowing it to become active. Experimental data generated by the testing of this hypothesis will be discussed in Chapters V and VII.

Potential Role of the EF hands

The EF hands identified in RasGRP suggest a role for calcium signals to mediated RasGRP function. Based upon this, we hypothesized that either the EF hands could be
functioning as alternates to the C1 domain or that they could be required in combination with the C1 domain for full activity of RasGRP. Data generated by the testing of these hypotheses will be discussed in Chapter V and VII.

**Potential Role of the Myristoylation Signal**

Based upon the fact that the original transforming protein was truncated at its N terminus, it was concluded that the potential myristoylation signal was not required for RasGRP function. However, if RasGRP were to be myristoylated we hypothesized that perhaps this potential membrane localizing modification may be capable of substituting for the C1 domain. The results generated by the testing of this hypothesis will be described in Chapter V.

**Potential Role of the Leucine Zipper or α-Helical motif**

Based upon similar logic, the leucine zipper was also concluded to be non-essential for RasGRP function since the original clone was truncated at its C terminus. However, it was hypothesized that this domain could potentially serve as a negative regulatory domain or as a potential alternative to the C1 domain. Data generated by the testing of this hypothesis will be discussed in Chapter V.

**Potential Novel Mechanisms for Regulating GNEF Activity**

When compared to other members of the CDC25 family, RasGRP’s unique combination of domains suggests the potential for an unusual mechanism of activation. For example, Sos is regulated by the binding of SH3-containing adapter molecules such as Grb2 in order for it to translocate to the plasma membrane where it can exchange on Ras. (Ravichandran et al, 1995) RasGRF1 and 2 use a region known as a pleckstrin
homology (PH) domain to bind to phosphoinositides found in membranes and are not dependent upon adapter proteins for this localization event. In addition, RasGRF molecules are responsive to calcium signalling via their IQ motifs. (Farnsworth et al, 1995) In both of these cases, membrane localization is a requirement for exchange factor activity. Like Sos, RasGRP possesses a potential SH3 binding site in its proline rich region which suggests the potential for regulation by adapter proteins. However, RasGRP may also be capable of responding to a unique set of signalling events such as calcium and DAG second messengers via its pair of EF hands and C1 domain respectively. In addition to this, the α-helical region and the potential myristoylation signal may also be able to affect the localization of RasGRP and therefore may function to regulate the activity of the molecule. The contribution made by each of these domains to RasGRP-induced fibroblast transformation will be discussed in the next chapter.
Chapter V: Contribution of individual domains of RasGRP to transformation of NIH 3T3 cells

5.1 Introduction

The objective of this chapter was to create and test hypotheses based upon the potential roles for each domain presented in Chapter 4.3. One advantage of using an expression cloning system to identify novel molecules is the fact that a cellular phenotype caused by cDNA overexpression is known from the beginning. RasGRP was originally identified on the basis that its overexpression in NIH 3T3 cells caused cellular transformation as measured by focus formation. The NIH 3T3 focus formation assay provided a system for determining the contribution made by each protein domain towards RasGRP function. cDNAs with mutations affecting specific domains of RasGRP were expressed in NIH 3T3 cells and the ability of these mutants to induce transformation were determined. Figure 16a illustrates the constructs that were created and whether they did (YES) or did not (NO) cause transformation in the fibroblast cells. This approach was used to test the following hypotheses concerning the functions of the individual domains of RasGRP. The level of expression of each GFP-tagged construct was determined using FACs analysis and these results are depicted in Figure 16b.

5.2 Testing the Hypotheses: Deletion and Mutant Analysis of RasGRP utilizing an NIH 3T3 Transformation Assay

Hypothesis 1 and Prediction: If the REM/GEF domains were essential for RasGRP activity then deletion or mutation of these domains would abolish transformation
Figure 16. Fibroblast transformation assay using RasGRP deletion and mutant constructs

a) A line diagram depicting RasGRP’s motif structure is found at the top of the figure. Underneath, lines have been used to represent the various deletion constructs. V shaped regions within a line represents the deletion of a particular domain from the construct. Filled triangles represent mutations created within a construct. Filled circles represent the addition of a prenylation signal. Filled rectangles represent the C1 domain of RasGRP. Open rectangles represent the C1 domain of PKC8. Whether or not overexpression caused transformation (Yes) or did not cause transformation (No) in the fibroblast cells is listed to the right of the line diagram. (C. Tognon and R. Kay, 1998)

b) Expression levels of GFP tagged constructs as assayed by FACs analysis.
a

<table>
<thead>
<tr>
<th>Construct</th>
<th>Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL37-2A</td>
<td>Yes</td>
</tr>
<tr>
<td>Gefm</td>
<td>No</td>
</tr>
<tr>
<td>REMΔ</td>
<td>No</td>
</tr>
<tr>
<td>CA1</td>
<td>No</td>
</tr>
<tr>
<td>RasGRP(CA1/C1)</td>
<td>Yes</td>
</tr>
<tr>
<td>RasGRP(CA1/PKC6-C1)</td>
<td>Yes</td>
</tr>
<tr>
<td>CA1/pr</td>
<td>Yes</td>
</tr>
<tr>
<td>X</td>
<td>Yes</td>
</tr>
<tr>
<td>CA4</td>
<td>No</td>
</tr>
<tr>
<td>CA1Δ +</td>
<td>Yes</td>
</tr>
<tr>
<td>EFΔ</td>
<td>Yes</td>
</tr>
<tr>
<td>CA2</td>
<td>No</td>
</tr>
<tr>
<td>CA2/C1</td>
<td>Yes</td>
</tr>
<tr>
<td>CA2/pr</td>
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</tr>
<tr>
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<td>CA3</td>
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</tr>
<tr>
<td>CA3/pr</td>
<td>Yes</td>
</tr>
<tr>
<td>FL</td>
<td>Yes</td>
</tr>
</tbody>
</table>

b

Relative Fluorescent Intensity per Cell
In order to test this first hypothesis two different RasGRP constructs were created. One construct possessed a mutation of a consensus arginine to a glutamine (position 271: R to G) found within the structurally conserved region 2 (SCR2) that has been shown to be required for interaction with Ras. (Lai et al, 1993; Fam et al, 1997) This mutant was called the GEFp. The other construct had a deletion of the REM box (REMA). Both of them were unable to cause transformation in the fibroblast cells which supported the first hypothesis and led to the conclusion that both of these motifs were absolutely required for proper RasGRP activity in the transformation assay.

**Hypothesis 2 and Prediction: If the C1 domain was required for transformation by serving as a membrane localizing signal then deletion of this domain would abolish transformation. If this were true, then it should also be possible to replace the RasGRP C1 domain with the C1 domain of PKCδ or a membrane prenylation signal without affecting its ability to transform.**

A number of different constructs were created in order to test this second hypothesis. A construct lacking the C1 domain was constructed (CA1) and was found to be unable to cause fibroblast transformation thereby supporting the first part of the second hypothesis. Re-addition of the C1 domain from RasGRP onto this construct (CA1/C1) was able to restore transformation. Furthermore, constructs that replaced the C1 domain with the C1 domain of PKCδ (CA1/PKC), or a prenylation signal (CA1/pr) were also able to cause transformation. The fact that RasGRP’s C1 domain was functionally replaceable by the PKCδ-C1 domain or by a prenylation signal supported the
second part of the hypothesis. It was concluded that RasGRP's C1 domain was required for transformation and that it could potentially function as a membrane localizing signal.

Hypothesis 3 and Prediction: *If a myristoylation signal exists in RasGRP and it can serve as a membrane targeting signal on its own, then removal of the C1 domain would have no effect upon transformation*

Addition of the 5' PCR extension product isolated from the WEHI231 library to the FL construct and expression of this construct (X) in fibroblast cells was also able to cause transformation. Therefore, it was concluded that this region of RasGRP also had no repressive effect on RasGRP induced transformation. In order to test the third hypothesis, the C1 domain was deleted from this full length construct (CA4). The presence of the potential myristoylation signal was unable to substitute for the C1 domain and these cells were observed to be non-transformed. These results contradicted the proposed hypothesis and it was concluded that the potential myristoylation signal was unable to substitute for the C1 domain in permitting transformation *via* RasGRP.

Hypothesis 4 and Prediction: *If the basic (KKRIK) sequence found downstream of the C1 domain was essential for the proper functioning of the C1 domain then deletion of this region would abolish transformation.*

In order to test this fourth hypothesis a deletion construct lacking this region was created (C1Δ+). Removal of this basic amino acid cluster had no effect upon transformation which contradicted the hypothesis. It was concluded that this basic sequence was not required for proper C1 domain functioning.

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Hypothesis 5 and Prediction: If the EF hands were essential for transformation then deletion of these motifs would abolish transformation. Or alternatively, if the EF hands worked in partnership with the C1 domain then deletion of just the EF hands would abolish transformation. In addition, if the EF hands truly served as alternates to the C1 domain then deletion of the C1 domain alone should have no effect upon transformation.

To test this fifth hypothesis a construct containing a deletion of the EF hands alone (EFA) was made. This construct was also able to address the question of whether the EF hands worked in partnership with the C1 domain in the process of transformation. Both of these hypotheses were contradicted since overexpression of this construct was still able to cause transformation. Constructs containing deletions of both the C1 domain and the EF hands (CΔ2) were unable to cause transformation. However, readdition of RasGRP’s C1 domain (CΔ2/C1) or a prenylation signal (CΔ2/pr) to this construct was able to restore transformation. This data further supports the conclusion that RasGRP’s EF hand is not required for transformation. It also supports that hypothesis that RasGRP’s C1 domain is required for transformation and that it could potentially function as a membrane localizing signal. As described above, a deletion construct lacking the C1 domain (CΔ1) but retaining the EF hands was unable to cause transformation. It was therefore concluded that the EF hands were not required for RasGRP induced transformation, that the EF hands alone were insufficient for transformation and that they were not necessary for C1-mediated transformation.
Hypothesis 6 and Prediction: *If the proline rich region was essential for transformation, then deletion or mutation of this region would abolish transformation.*

In order to test this sixth hypothesis a construct was created that contained a cluster of point mutations which eliminated the potential for SH3 binding (Prop). The mutation construct was still able to cause transformation. The hypothesis was contradicted and it was concluded that the proline rich region was not required for transformation. Deletion of the entire 3’ region up to and including the proline rich region (CA3) abolished transformation. Addition of prenylation signal onto this deletion construct (CA3/pr) was able to restore transforming ability. This data also supports the notion that membrane localization is a potentially important event required for proper RasGRP activity.

Hypothesis 7 and Prediction: *If the α-helical domain or the 5’ extension region provided a repressive function then addition of these domains onto the original cDNA would abolish transformation.*

This hypothesis was tested by the overexpression of a construct created by the addition of the 3’ PCR extension product isolated from the T28 library to the TL37-2A cDNA. This α-helical domain containing construct was named FL and it was able to cause transformation. It was therefore concluded that the α-helical region did not provided a repressive function to RasGRP. Addition of the 5’ extension onto RasGRP/FL and overexpression of this construct was also able to cause transformation. This data contradicted the hypothesis and it was concluded that the 5’ extension region was unable to provide a repressive function.
Hypothesis 8 and Prediction: *If the $\alpha$ helix can serve as an alternate to the Cl domain then deletion of the Cl domain from $\alpha$-helical domain-containing constructs should have no effect upon transformation.*

This last hypothesis was tested by the overexpression of the Cl deleted form of the 5’ and 3’ extended RasGRP cDNA.(CA4) Although this construct contained the $\alpha$-helical region it was unable to cause transformation. Therefore it was concluded that the $\alpha$-helix could not substitute for the Cl domain in permitting transformation via RasGRP.

**5.3 A Potential Model for Fibroblast Transformation by RasGRP**

The data generated from the deletion mutant analysis implicates the REM/GEF and the Cl domain as being essential for RasGRP function. These results also show that the basic amino acid cluster (KKRIK), the proline rich region, the EF hands, the $\alpha$-helical region, or the potential myristoylation signal are not necessary or inhibitory for RasGRP function as assayed by NIH 3T3 transformation. Based upon this and what is known about the different motifs found within RasGRP (See: Chapter IV) a potential model can be proposed to explain how RasGRP activation causes transformation in NIH 3T3 fibroblast cells. The model is based upon a hypothetical mechanism of RasGRP activation whereby exchange activity is regulated by a Cl-mediated translocation event. This activation model (See: Figure 17) can be broken down into the following steps:

First, cells can be stimulated by serum to produce high levels of DAG in membranes and this increase in DAG concentration attracts the Cl domain of RasGRP. Second, binding of the Cl domain to DAG translocates RasGRP to the plasma membrane and places the exchange factor in close proximity to its Ras target. Third, REM/GEF-dependent GDP
Figure 17. Model of fibroblast transformation by RasGRP overexpression

a) In unstimulated cells Ras is inactive in its GDP bound form and RasGRP is found in an area surrounding the nucleus, b) Stimulation of the cells produces DAG in membranes by the cleavage of PIP$_2$ or PC into DAG by PLC. The CI domain of RasGRP is attracted to DAG and translocates to the membrane, c) GDP exchange takes place on Ras and this leads to activation of Ras.
exchange takes place which leads to Ras activation. Finally, constitutive activation of Ras leads to the transformation of the NIH 3T3 fibroblast cells. We decided to test two different aspects of this model using three different experimental systems.

The first aspect of the model that was tested was whether or not RasGRP could activate Ras by serving as a guanine nucleotide exchange factor. This aspect of the model was based upon RasGRP’s high sequence similarity to the CDC25 family of GNEFs and the fact that an active exchange factor domain as well as the REM box were required for fibroblast transformation. In order to test this, Elk-1 and ERK phosphorylation experiments were performed on cells overexpressing various RasGRP constructs in order to indirectly assess the level of Ras activation. These results will be discussed in Chapter VI.

The second aspect of the model that was tested focused on the potential activation mechanism of RasGRP suggested by the requirement of its C1 domain for transformation. The ability of a PKC-C1 domain or a prenylation signal to substitute for RasGRP’s C1 domain in the transformation assay also alluded to this potential role. It is known that PKC-C1 domains function to translocate these proteins from the cytosol to membranes in response to PE/DAG stimulation. (Oancea et al, 1998) It is also known that protein prenylation signals tether molecules to the plasma membrane.(Seabra, 1998) Based upon the above information we decided to test whether or not the C1 domain of RasGRP was also capable of mediating protein translocation to the plasma membrane through binding to PE/DAG. The results from these experiments will be discussed in Chapters VII.
Chapter VI: Mechanism of Ras activation via RasGRP

6.1 Introduction

Assessing the Model: Can RasGRP stimulate Ras Activation?

In order to test whether RasGRP could activate Ras by serving as an exchange factor, we utilized an Elk-1 reporter gene assay which detects MAPK activity, as well as antibodies capable of detecting the activated forms of the MAPKs, ERK1 and 2. (See: below) These two different experimental systems were used to monitor the activation status of signalling molecules found downstream of Ras in order to indirectly assess the effects of RasGRP overexpression on Ras activation. Direct measurements of Ras activation levels can only be performed by GTP/GDP loading assays (Gibbs et al, 1990; Satoh et al, 1990; Gibbs et al, 1995; Satoh et al, 1995) or by methods which utilize the Ras binding domain (RBD) of Raf to immunoprecipitate GTP-bound Ras from cells. (Taylor and Shalloway, 1996) We chose to utilize these two indirect measuring systems because of their availability.

This first testable aspect of the model was broken down into a series of specific questions such as: Does RasGRP overexpression lead to the predicted activation of MAPK as determined by Elk-1 assays? If so, what are the domain requirements for this activation? What forms of Ras does RasGRP activate? Which MAPK is being activated? Is there a role for membrane localization in RasGRP activation? Is there a role for serum and/or PE stimulation in RasGRP activation?
Indirect Measurements of Ras Activation

One potential way of determining levels of Ras activation in cells is to monitor the activity of downstream targets of the Ras pathway. The two methods used in this study make use of the transcription factor Elk-1 and the MAP kinases, ERK 1 and 2. Elk-1 is one of a family of proteins that can form a ternary complex with the transcriptional activator, serum response factor (SRF), and therefore plays a key role in the regulation of fos gene expression. (Marais et al, Cell 1993) Elk-1 can be phosphorylated and activated by MAP kinases such as ERK1 and 2. (Waskiewicz and Cooper, 1995) ERK1 and ERK2 are activated by phosphorylation by MEK. MEK is activated by phosphorylation by Raf and Raf is activated by binding to GTP-Ras. (Dent et al, 1995; Marshall, 1996)

Therefore, Elk-1 activation and ERK1/2 phosphorylation are indirect indicators of Ras GTP loading. It should also be noted that other signalling pathways can also influence the levels of MAPK and Elk-1 phosphorylation independent of Ras activation. For example, PKC has been shown to activate Raf-1 in a PE dependent manner. (Cai et al, 1997) In addition, alternative MAP kinases such as JNK have been shown to phosphorylate Elk-1. (Treisman R., 1996; See: Figure 18a)

The Elk-1 phosphorylation assay utilized an artificial transcription factor that became activated only when phosphorylated in its Elk-1 portion by MAP kinases. In other words, this assay measured the level of MAPK activation by its ability to phosphorylate Elk-1. The artificial transcription factor consisted of a fusion of the Gal4 DNA binding domain and the transactivation domain and MAPK sites of Elk-1 (pGal4-Elk-1). A reporter construct was also used in these assays and consisted of the luciferase
Figure 18. Activation of the Ras signalling pathway and Elk-1 reporter gene assay

a) Raf/MAP kinase signalling cascade induced by Ras activation (Black). Alternative, Ras-independent ways to induce ERK and Elk-1 phosphorylation via PKC and JNK. (Grey) b) The Elk-1 reporter gene assay utilized a hybrid transcription factor made up of the Gal4 DNA binding domain (Gal4) and the Elk-1 transactivation domain (Elk-1). The reporter gene construct was made up of Gal4 binding sites (Gal4bs) and a promoter region (Promoter) fused to the luciferase coding region.
coding region driven by a minimal promoter and tandem Gal4 binding sites (pGal4-LUC). NIH 3T3 cells were co-transfected with: different RasGRP and/or Ras cDNAs in expression vectors, pGal4/Elk-1, and pGal4/LUC. The amount of Elk-1 phosphorylation was then determined as a measure of luciferase activity.

In the second experimental system, antibodies capable of detecting the phosphorylation of tyrosine 204 in the MAP kinases ERK1 and 2 were used to assess the activation status of these kinases in NIH 3T3 and BOSC23 cells overexpressing RasGRP.

6.2 Results

a) RasGRP can stimulate Elk-1 phosphorylation

RasGRP constructs were given to Ian Whitehead (Chapel Hill) and he generated the data depicted in Figure 19. Briefly, NIH 3T3 cells were co-transfected with the Gal4-Elk-1 artificial transcription factor construct, the Gal4-LUC reporter construct, and expression vectors containing the indicated cDNAs. (See: Figure 18b) The cells were switched to low serum conditions (0.5%FBS DMEM) 34 hours post transfection in order to minimize the background levels of Elk-1 activity. After an additional 14 hours in low serum, the cells were assayed for luciferase activity. The results of these experiments are expressed as a fold activation over vector alone (VECTOR).

Does RasGRP overexpression lead to the predicted activation of MAPK as determined by Elk-1 assays?

Overexpression of the original clone isolated from the library screen (RasGRP/TL37-2A) caused a 200 fold increase in Elk-1 activity. Furthermore this level of activation was comparable to that of overexpressing a prenylated version of the Ras-
Figure 19. Results of Elk-1 phosphorylation studies performed in NIH 3T3 cells overexpressing RasGRP

NIH 3T3 cells were co-transfected with pGal4-LUC and pGal4-Elk-1, along with expression vectors expressing RasGRP cDNAs, prenylated RasGRF-1 and normal or dominant negative (DN) forms of Ras family GTPases. Data is represented as fold activation over cells expressing vector alone and are representative of three separate experiments, with each point performed in triplicate in each experiment. It should also be noted that overexpression of H-, K-, or N-Ras alone had little or no effect on the level of Elk-1 activation. (Data not shown) Transfections and data collection performed by Ian Whitehead, Chapell Hill. (1998)
specific GEF, RasGRF1 (RasGRF/pr). From these results it was concluded that RasGRP expression could lead to the activation of MAPK.

**What are the domain requirements for this activation?**

The domain requirements for this activation were tested by overexpressing either a RasGRP/TL37-2A construct lacking the C1 domain (RasGRP-CΔ1) or a RasGRP/TL37-2A construct possessing a mutation of a residue within the exchange factor domain that has been shown to be critical for interaction with Ras (RasGRP-GEFμ; Lai et al., 1993; Fam et al., 1997). Both of these changes abolished any additional activation of Elk-1 above that of vector alone. It was concluded that the C1 domain and an active GEF domain were essential in order for RasGRP expression to cause Elk-1 activation. Additional information concerning the role of the C1 domain was generated by overexpressing a prenylated version of a C1 deletion mutant (RasGRP-CΔ3/pr). This protein localized to the plasma membrane. Overexpression of this construct led to a 700 fold increase in Elk-1 activation over vector alone and a 3.5 fold increase over RasGRP/TL37-2A alone. These results suggest that constitutive membrane localization can cause high levels of Elk-1 activation and that the C1 domain may serve a similar membrane localizing function in RasGRP.

**What forms of Ras does RasGRP activate?**

Cells were made to overexpress RasGRP/TL37-2A in conjunction with different Ras family members in order to get a sense of which RasGTPases were able to synergize with RasGRP in stimulating Elk-1 phosphorylation. The co-expression of H-, K-, or N-Ras with RasGRP/TL37-2A was able to cause 1.25 to 1.5 fold higher Elk-1 activation.
compared to expressing RasGRP/TL37-2A alone. In addition to this, the co-expression of dominant negative (DN) forms of either H- or K-Ras in conjunction with RasGRP/TL37-2A was found to decrease the level of Elk-1 activation when compared to overexpression of RasGRP/TL37-2A alone. Co-expression of either R-Ras or TC21 in conjunction with RasGRP/TL37-2A was unable to increase the level of Elk-1 activation over that of RasGRP/TL37-2A. Furthermore, overexpression of the dominant negative (DN) form of R-Ras was unable to suppress the levels of RasGRP/TL37-2A induced Elk-1 activation. From these results it was concluded that RasGRP is capable of synergizing with H, K, and N-Ras in stimulating Elk-1 activation, possibly by serving as a GEF for these three forms of Ras.

b) ERK Assays: Detection of MAP kinase phosphorylation in cells overexpressing RasGRP

The results generated by the Elk-1 activation studies indicated that a MAP kinase was being activated. Based upon this information we hypothesized that if Ras was being activated in RasGRP overexpressing cells then a likely candidate for the MAPK would be ERK. In order to test this hypothesis, a phosphospecific antibody to the MAP kinases, ERK1 and 2, was used to determine the levels of ERK phosphorylation in cells overexpressing RasGRP. These experiments were performed as a collaborative effort between Heather Kirk (Terry Fox Lab) and myself. The results of these experiments are depicted in Figure 20.

Are ERKs being activated? Is there a role for serum in RasGRP activation?
Figure 20. MAPK phosphorylation status of NIH 3T3 and BOSC23 cells overexpressing RasGRP

NIH 3T3 cells or BOSC23 cells were made to overexpress either: a control construct; RasGRP/TL37-2A (original clone isolated from the library screen); or RasGRP/CD3/pr (a prenylated version of RasGRP). Serum starved cells were either left untreated or were treated in serum-containing media for 15 minutes or with media containing 50ng/ml of PMA for 15 minutes. Equal amounts of protein were run out on acrylamide gels and phosphospecific MAPK antibodies were used in Western blot analysis in order to observe the activation status of ERK1 and 2. (H. Kirk and C. Tognon, 1998)
NIH 3T3 fibroblast cells or BOSC23 cells (a derivative of the 293T human epithelial cell line) were engineered to express either a control construct or a RasGRP/TL37-2A construct and grown in serum containing medium or in serum free medium for 2.5 hours. In NIH 3T3 cells, serum stimulation itself induced high levels of ERK phosphorylation (Control + serum) and these levels did not appear to increase with the overexpression of RasGRP (RasGRP/TL37-2A + serum). However, when these cells were cultured in serum free conditions the levels of ERK phosphorylation decreased (Control -serum) and in comparison, overexpression of RasGRP (RasGRP/TL37-2A - serum) was observed to cause a visible increase in ERK phosphorylation. From these results it was concluded that serum stimulation of NIH 3T3 cells, in itself, caused high levels of ERK phosphorylation and that overexpression of RasGRP in NIH 3T3 cells was incapable of increasing ERK phosphorylation under serum conditions. However, it was concluded that RasGRP was capable of weakly inducing ERK phosphorylation based on the experiments performed in serum free conditions.

A different set of results were obtained when the same experiments were performed in BOSC23 cells. In this case, serum appeared to induce very little ERK phosphorylation (Control + or - serum) and because of this low background level, overexpression of RasGRP in these cells was observed to induce high levels of ERK phosphorylation in both serum and serum-free conditions (RasGRP/TL37-2A + and - serum). No additive effects of serum stimulation were observed in cells overexpressing RasGRP. From these experiments it was concluded that RasGRP overexpression was capable of stimulating ERK phosphorylation in BOSC23 cells and that serum stimulation had little effect upon ERK phosphorylation. The differences in the results observed
between the two cell lines may be due to intrinsic differences in their ability to respond to serum.

**Is there a role for membrane localization in RasGRP activation?**

NIH 3T3 and BOSC23 cells were engineered to express a prenylated form of RasGRP which lacked a region containing the C1 domain (RasGRP-CA\_3/pr). These cells were either grown in serum containing medium (Serum +) or in serum free medium for 2.5 hours (Serum -). The most pronounced effect upon ERK phosphorylation occurred in the NIH 3T3 cells under serum free conditions (serum -). Under these conditions, the highest level of ERK phosphorylation was observed in RasGRP-CA\_3/pr overexpressing cells. RasGRP/TL37-2A overexpressing cells exhibited moderate levels of ERK phosphorylation and control overexpressing cells exhibited the lowest levels of ERK phosphorylation. Based upon this data it was concluded that prenylation alters serum independent activation of RasGRP and that this occurs as a result of tethering the protein to the plasma membrane.

In serum stimulated NIH 3T3 cells, overexpression of RasGRP-CA\_3/pr stimulated only slightly higher levels of ERK phosphorylation when compared to control and to RasGRP/TL37-2A overexpressing cells. From this data it was concluded that the effects of RasGRP-CA1/pr overexpression could not be observed because serum stimulation saturated ERK activation in NIH 3T3 cells.

In both serum and serum free conditions, overexpression of RasGRP-CA\_3/pr in BOSC23 cells produced only slightly higher levels of ERK phosphorylation when compared to cells overexpressing RasGRP/TL37-2A. Based upon this data, it was
concluded that the effects of prenylation could not be observed in the BOSC23 cells because overexpression of RasGRP/TL37-2A itself stimulated saturating levels of ERK phosphorylation.

Is there a role for PE stimulation in RasGRP activation?

NIH 3T3 and BOSC23 cells were engineered to express either a control construct (Control) or a RasGRP construct (RasGRP/TL37-2A) and grown in serum free medium for 2.5 hours and then stimulated with 50ng/ml of phorbol myristate acetate (PMA) for 15 minutes (PMA +). In both cell types, PMA treatment resulted in a large increase in ERK phosphorylation in the absence of RasGRP expression. This effect was postulated to be due to the stimulation of PKC (See: Chapter IV for further information on PKCs) since it is known to be able to mediate the activation of Raf-1 in a Ras-independent way and it can also be activated by PE. (Cai et al, 1997; See: Figure 20) No further increase in ERK phosphorylation was induced in PMA stimulated cells by RasGRP activation. It was concluded that PMA stimulation induced high and possibly saturating levels of ERK phosphorylation in and of itself and it was therefore impossible to discern any effects of PE stimulation on RasGRP activation.

6.3 Discussion

The results obtained from the Elk-1 reporter gene assays and the MAP kinase phosphorylation studies suggest that overexpression of RasGRP in cells can stimulate the activation of both of these downstream targets of Ras. More specifically, results from the Elk-1 phosphorylation assays provide evidence for the requirement of an active exchange factor domain and the presence of the C1 domain in order for RasGRP overexpression to
stimulate Elk-1 activation. These results suggest that RasGRP may be mediating its effects through specific exchange on GTPases and that this activity requires the presence of the C1 domain. These results coincide with data generated from the transformation assay described in Chapter V. From those experiments it was determined that an active exchange factor domain as well as a C1 domain were absolutely required for transformation.

Additional results obtained from the Elk-1 phosphorylation assay indicate that RasGRP overexpression can synergize with H-, K-, and N- Ras but not R-Ras and TC21 overexpression to stimulate Elk-1 phosphorylation. H-, K-, and N-Ras are highly related protein members of the true Ras subfamily of RasGTPases (See: Chapter I) whereas R-Ras and TC21 exhibit only 55% sequence identity to the true Ras subfamily and have been classified as distinct Ras subfamilies in and of themselves. Therefore RasGRP has the potential to be an exchange factor for true Ras family members.

Since RasGRP overexpression was shown to induce Elk-1 phosphorylation it was hypothesized that the MAP kinases ERK 1 and 2 might be responsible for this phosphorylation event. Results obtained from the ERK phosphorylation assays confirmed this hypothesis.

The ERK phosphorylation studies were unable to generate any evidence which would suggest a role for serum or PMA in stimulating RasGRP activation. Serum and PE stimulated equivalent levels of ERK phosphorylation in control and RasGRP/TL37-2A overexpressing NIH 3T3 cells and they had no additive effects upon BOSC23 cells overexpressing RasGRP/TL37-2A. In all of these cases it is possible that the levels of ERK phosphorylation may have been saturated by either serum stimulation (See: NIH
3T3 data), RasGRP/TL37-2A overexpression (See: BOSC23 data), or PMA stimulation (See: NIH 3T3 and BOSC23 data). Therefore, these results do not preclude the possibility of serum or PE stimulation having effects upon RasGRP activation.

Data generated by the Elk-1 and ERK phosphorylation assays point towards a possible role for membrane localization in the activation of RasGRP. For example, deletion of the C1 domain (RasGRP-CA1) abolished Elk-1 activation while prenylation of RasGRP (RasGRP-CA3/pr) was capable of causing high levels of Elk-1 as well as ERK phosphorylation. Furthermore, in NIH 3T3 cells prenylation replaced the requirement for serum in achieving full RasGRP activation. This information coincides with results obtained from the fibroblast transformation assays (See: Chapter V). In these experiments it was determined that the C1 domain was required for fibroblast transformation induced by RasGRP overexpression and that a prenylation signal could substitute for the C1 domain. Taken altogether, these results point to a possible role for membrane association in regulating exchange activity of RasGRP.

All of the data presented above help to confirm the first aspect of the model tested which stated that if RasGRP was an exchange factor for true Ras proteins then it would be capable of activating these proteins. In addition to this, these results also begin to highlight a membrane localization requirement for RasGRP activation and suggest a potential role for the C1 domain in mediating this event. The next chapter will focus on addressing the question of whether RasGRP’s C1 domain is functioning in this capacity.
Chapter VII: Role of the RasGRP C1 domain and other domains in targeting RasGRP to membranes

7.1 Introduction

A model has been created in an attempt to explain how RasGRP overexpression could cause fibroblast transformation. (See: Chapter 5.3) Work described in Chapter VI tested one aspect of this model and confirmed that RasGRP had the potential to function as an exchange factor for Ras proteins. This chapter describes the work performed in testing another aspect of the model and describes experiments performed in order to determine whether the C1 domain of RasGRP could mediate protein translocation to the plasma membrane by binding to PE/DAG. This particular aspect of the model can be broken down into three testable predictions. First, that RasGRP is capable of becoming membrane localized in stimulated cells. Secondly, that this localization is due to the C1 domain. And lastly, that this process can be mediated by PE or DAG.

In order to test these predictions we set up an experimental system by stably overexpressing green fluorescent protein (GFP)-tagged constructs in fibroblast cells. The various constructs utilized in this chapter are illustrated in Figure 21. Changes in protein localization were monitored following treatment of cells with serum, PE, or a DAG generating phospholipase.

GFP has been widely used to track the movement of proteins in response to various stimuli. The most relevant example of this work was performed on the C1 domains of PKCs. (Oancea et al, 1998a/b) In vitro studies have shown that these domains bind to lipid membranes in the presence of DAG or PE. (Ono et al, 1989;
Figure 21: Constructs utilized in localization experiments
Names and corresponding line diagrams outlining domain boundaries for each construct.
GFP: green fluorescent tag; REM: Ras motif box; GEF: guanine nucleotide exchange factor domain; P: proline rich region; EF: EF hand motif; Cl(white): RasGRP C1 domain; Cl(shaded): PKC C1 domain; +: KKRIK sequence; bbbb: basic region; α: alpha helical domain or leucine zipper; *: stop codon.
Castagna et al, 1982; Hannun et al, 1985; Reviewed by Hurley et al, 1998) Studies performed in living cells on GFP-tagged forms of the first C1 domain of PKC-γ have demonstrated that these domains can translocate to the plasma membrane in response to G-protein or tyrosine kinase coupled receptor stimulation, diacylglycerol analogs such as DiC8, and the phorbol ester PMA. Full length GFP-tagged PKC-γ proteins were able to translocate to the plasma membrane in response to receptor or PMA stimulation.(Oancea et al, 1998a) In a similar fashion, we visualized proteins produced by the stable expression of various GFP-tagged RasGRP constructs in fibroblast cells using fluorescent microscopy and attempted to answer the following specific questions: What subcellular region(s) is RasGRP localizing to?; How do each of the domains influence the localization of the RasGRP protein under normal serum conditions?; Is the C1 domain capable of mediating translocation in response to PE and PC-PLC stimulation?; Are the EF hands capable of mediating or influencing translocation in response to calcium ionophore stimulation?; and What is the role of the α-helical domain in protein localization?

7.2 Results

a) RasGRP protein localization

The results of the following localization experiments are found in Figure 22. NIH 3T3 cells were engineered to stably overexpress either a GFP control construct or a construct containing the 3' extended form of RasGRP (RasGRP/FL) that was tagged with GFP at its C terminus. GFP was observed to be ubiquitously throughout the nucleus and the cytoplasm in serum stimulated cells.(See: b; GFP Control; Green) The fluorescence
Figure 22. Co-localization experiments performed in RasGRP overexpressing NIH 3T3 cells with ER-specific antibodies and Mitochondrial dyes

NIH 3T3 cells were made to overexpress: a GFP negative control construct (Control (-GFP)), a GFP control construct (GFP Control) or a GFP-tagged RasGRP/FL construct (RasGRP/FL). Cells were fixed and internal cellular structures representing either a) the endoplasmic reticulum (ER) or b) the mitochondria, were highlighted using an anti-BIP ER specific antibody or MitoTracker dye, respectively, as described in the material and methods. Photomicrographs were taken in both the green and red channels. Arrow heads point out the level of background staining observed in negative control cells.
a  
RasGRP/FL

Green  Red

b  
Control (-GFP)

Green  Red

GFP Control

RasGRP/FL
pattern of RasGRP/FL in the cells could be described as a diffuse lattice found within the cytoplasm surrounding the nucleus with some fluorescence often observed to one side of the nucleus. (See: a/b; RasGRP/FL; Green) Cytoplasmic structures known to have a lattice pattern are the endoplasmic reticulum (ER) and the mitochondria (Mt). The fluorescence pattern of RasGRP/FL raised the question of whether RasGRP was localizing to these structures.

GFP-tagged control cells and GFP-tagged RasGRP/FL cells were handled as described in the material and methods section (See: Chapter II/Co-localization Experiments). Briefly, structures corresponding to the endoplasmic reticulum (ER) were identified by using an antibody against BIP, a protein found only in the ER. Structures corresponding to the mitochondria were identified using Mitotracker, a fluorescent dye that specifically accumulated in mitochondrial membranes.

GFP-tagged RasGRP/FL proteins appeared to co-localize to some extent with the ER since both staining patterns highlighted a similar diffuse lattice within the cytoplasm. (See: a; Green/Red) However, the complete staining pattern for RasGRP/FL also included areas found to one side of the nucleus as well as some areas within the cytoplasm that were not highlighted with the ER antibody staining. Background from the red fluorescent ER staining was minimal since non-GFP labeled cells exhibited little or no fluorescence in the green channel. (See: arrow, a; Green)

Mitochondria were observed as an extensive tubular network running throughout the cytoplasm. (See: b; All constructs; Red) The background levels of fluorescence produced by the Mitotracker dye in the green channel could be observed in non-GFP control cells. (See: arrow, b; Control -GFP, Green) The localization pattern of GFP-
tagged RasGRP/FL did not coincide with this mitochondrial staining pattern indicating that RasGRP/FL was not localizing to the mitochondria. (See: b; RasGRP/FL; Green and Red) It was concluded that RasGRP/FL is localized to at least a portion of the ER. In addition, the observed fluorescence pattern was compatible with localization to other membranes since the protein could also be observed in areas ringing the nucleus and in a bright spot found to one side of the nucleus in some cells. It was also concluded that RasGRP/FL did not co-localize to the mitochondria. From this point on, the term “ER+” will be used to describe localization to portions of the ER as well as to areas ringing the nucleus and to one side of the nucleus and thus will be used to describe any fluorescence pattern which is similar to that described for RasGRP/FL.

b) Domain contribution to RasGRP protein localization

Since RasGRP/FL protein localized predominantly to the ER+ we were interested in determining how each of the domains influenced RasGRP protein localization in serum stimulated cells. This question was answered by overexpressing different GFP-tagged constructs in NIH 3T3 cells and growing the cells in serum containing media. The fluorescence patterns were observed for all constructs. (See: Figure 23)

The role of the α-helical domain was tested by overexpressing the form of RasGRP isolated from the cDNA library screen (TL37-2A). TL37-2A lacked the α-helical region and was observed to localize to the ER+. (See: RasGRP/TL37-2A) In many cases, an area of concentrated fluorescence was also observed in a region found to one side of the nucleus. (See: arrow head). It should also be noted that the pattern produced by TL37-2A overexpression was more diffuse than that produced by
Figure 23. Localization of GFP-tagged RasGRP constructs under serum conditions
NIH 3T3 cells were made to overexpress the following GFP tagged constructs: a control construct (Control), the original clone isolated from the library screen (TL37-2A), the 3' extended full length clone (RasGRP/FL), the 5' and 3' extended full length clone (RasGRP/X), the isolated EF hands (RasGRP-EFH), the isolated EF hands plus Cl domain (RasGRP-EFH/C1), the isolated Cl domain (RasGRP-C1), the isolated Cl domain of PKC-δ (PKC-C1), the Cl deleted form of RasGRP/FL (RasGRP/FL-CA1), and the Cl deleted form of RasGRP/X (RasGRP/X-CA1). The cells were grown in serum containing media, fixed, and then photographed as described in the material and methods. Arrowheads highlight regions of concentrated fluorescence.
RasGRP/FL overexpression. It was concluded from this that the \(\alpha\)-helical domain was not required for localization to the ER+.

The localizing role played by the 5’ extension was determined by observing the fluorescence pattern of the full length protein, RasGRP/X. RasGRP/X exhibited a similar fluorescence pattern to RasGRP/TL37-2A. It was concluded that addition of the 5’ extension region did not impart any alternative localizing characteristics to the RasGRP protein.

In order to assess whether the C1 domain had any influence on protein localization, the fluorescence patterns of C1 deleted forms of RasGRP/FL (RasGRP/FL-CA1) and RasGRP/X (RasGRP/X-CA1) were compared to those of their wild type counterparts. Both of the C1 deleted constructs exhibited a fluorescence pattern comparable to the GFP control. (See: Control, RasGRP/FL-CA1, RasGRP/X-CA1) Fluorescence in all three cases was observed to be throughout the nucleus and cytoplasm. Based upon this data it was concluded that the C1 domain was required for the localization of FL and X forms of RasGRP to the ER+.

Since it appeared as though the C1 domain was responsible for localization to the ER+ we were interested in determining whether it could function by itself outside of the context of the full length protein. In order to test this, constructs containing the isolated C1 domain alone were overexpressed in fibroblast cells. This protein was observed to localize to the ER+. It should be noted that the fluorescence pattern produced by RasGRP-C1 overexpression was more diffuse than that produced by either RasGRP/FL or RasGRP/X and appeared very similar to that of TL37-2A overexpression. Based upon
this data, it was concluded that the isolated C1 domain was capable of acting on its own to localize protein to the ER+ and did not require any other region of RasGRP. This fluorescence pattern was identical to that observed when the isolated C1 domain of PKCδ was overexpressed in the fibroblast cells. (See: PKC-C1) From these observations, it was concluded that RasGRP’s C1 domain functioned like the C1 domain of PKCδ with respect to localizing proteins to the ER+.

The role of the EF hands on the localization of RasGRP was tested using two different constructs. One contained both the EF hands and the C1 domain (See: RasGRP-EFH/C1) and the other contained the isolated EF hands alone. (See: RasGRP-EFH) RasGRP-EF/C1 protein was observed to localize to the ER+ while RasGRP-EFH protein was distributed evenly throughout the cytoplasm and the nucleus. It was concluded that the EF hands did not possess a localizing function on their own and did not influence the localizing effect of the C1 domain.

c) Effects of serum and PMA stimulation on RasGRP localization

DAG is generated rapidly and transiently in cellular membranes in response to stimulation with serum, growth factors, hormones, and neurotransmitters. (Reviewed by Wakelam et al, 1998) DAG is produced by the phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylcholine (PC) or phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₂ cleavage also produces inositoltrisphosphate (IP₃) which stimulates the release of intracellular calcium stores and the opening of calcium channels within the membrane. Therefore, PIP₃ cleavage results in the generation of DAG in membranes and an increase in calcium concentration within the cells.
C1 domains have been shown to bind with high affinity to analogs of DAG known as phorbol esters (PE). (Ono et al, 1989) Phorbol myristate 13-acetate (PMA) is a lipophilic, long chained phorbol ester that binds to C1 domains with much higher affinity than that of DAG. PMA has been shown to activate PKCs in a manner very similar to that of DAG. (Kikkawa et al, 1989; Bell and Burns, 1991; Newton, 1997)

Studies performed on PKC molecules have shown that the interaction between the C1 domain and PE/DAG does not significantly alter the conformation of the protein. It does however, alter its surface by forming a hydrophobic cap which then allows the protein to embed itself in the membrane. (Newton, 1995; Zhang et al, 1995) The most significant consequence of PE/DAG binding to PKC-C1 domains is the translocation of the protein to membranes. The second consequence of PE/DAG binding, which couples with PKC phosphorylation, is the activation of the kinase domain. This is achieved through a conformational change which removes the pseudosubstrate domain from the kinase pocket. (Reviewed by Newton, 1995)

We were interested in finding out whether the C1-mediated localization of RasGRP described in the previous section was dependent upon serum stimulation and whether this localization could be altered by PE stimulation. We predicted that serum stimulation could cause localization by generating DAG in membranes and conversely, that serum withdrawal would decrease DAG production and cause de-localization from membranes. In order to test this, NIH 3T3 cells overexpressing various GFP-tagged constructs were serum starved for 3.5 hours. The serum starved cells were then either stimulated with serum for 15 minutes or incubated with media containing 50ng/ml of PMA for 15 minutes. The exact details of the stimulations are described in the material
and methods section. (See: Chapter II) The localization of the GFP-tagged proteins were monitored using immunofluorescence and the results of these experiments are compiled in Figure 24.

GFP (See: Control) was evenly distributed throughout the cytoplasm and the nucleus under all three conditions. GFP-tagged 3' extended RasGRP proteins (RasGRP/FL) remained localized to the ER+ in serum starved cells (Nil) and in serum stimulated cells (Serum). Based upon this data, it was concluded that serum starvation had no effect upon the localization of this protein. In most cases, when NIH 3T3 cells were stimulated with PMA they appeared to flatten and produce membrane ruffles. RasGRP/FL proteins appeared to localize to these membrane ruffles as well as to the ER+ in most PMA stimulated cells. From this data it was concluded that the RasGRP/FL could respond to PMA stimulation by re-localizing to membrane ruffles.

In serum free conditions, the form of RasGRP lacking the C terminal regions (RasGRP/TL37-2A) was observed throughout the cytoplasm and the nucleus. Upon stimulation with serum and PMA, RasGRP/TL37-2A was observed to localize to the ER+. From this data it was concluded that this protein, unlike RasGRP/FL, lost its ability to localize to the ER+ when grown in serum free conditions. In addition, RasGRP/TL37-2A proteins responded to serum or PMA stimulation by localizing to the ER+.

RasGRP lacking the C1 domain (RasGRP/FL-CΔ1) was distributed throughout the cytoplasm and the nucleus in unstimulated, serum stimulated or PMA stimulated cells. This data reinforced the fact that the C1 domain is absolutely necessary for
Figure 24. Translocation of RasGRP in response to serum or PMA stimulation
NIH 3T3 cells were made to overexpress the following GFP tagged constructs: a control construct (Control), the original clone isolated from the library screen (TL37-2A), the 3′ extended full length clone (RasGRP/FL), or the C1 deleted form of RasGRP/FL (RasGRP/FL-CΔ1). The cells were grown in serum free conditions for 3.5 hours (Nil) and then stimulated with either 10FCS/DMEM for 15 minutes or 50ng/ml of PMA for 15 minutes. The cells were fixed and then photographed as described in the material and methods. Arrowhead highlights the concentration of GFP labeled protein in membrane ruffles.
RasGRP/FL to localize to the ER+ and that it is also necessary for the ability of this protein to relocalize to membrane ruffles in response to PMA stimulation.

**d) Effects of serum and PMA stimulation on the localization of isolated C1 domains**

Based upon the data presented above we were able to conclude that the C1 domain was required for localization of the protein to the ER+. This domain also appeared to be required for re-localization of RasGRP/FL proteins to membrane ruffles in response to PMA stimulation. We were interested in answering the following questions: Is the isolated C1 domain capable of de-localizing under serum free conditions? And is the isolated C1 domain capable of responding to PMA stimulation by re-localizing to membranes?

In order to answer these questions, NIH 3T3 cells were engineered to overexpress either GFP alone (Control), or GFP-tagged C1 domains of RasGRP (RasGRP-C1) or PKCδ (PKC-C1). These cells were grown in serum-containing media, serum free media for 3.5 hours, in serum free media for 3.5 hours and then stimulated with 50ng/ml PMA for 1 minute or 15 minutes. The results of these experiments are compiled in Figure 25.

As witnessed previously, serum starvation or PMA stimulation had no effect upon the localization of GFP control proteins.(See: Control: Nil, PMA 1’ or 15’) The overexpression of C1-containing constructs in serum stimulated fibroblast cells produced an ER+ fluorescence pattern.(See: RasGRP-C1 or PKCδ-C1: Serum) When these cells were cultured in serum free media for 3.5 hours (Nil) no change in the fluorescence pattern was observed. When these cells were serum starved and then stimulated with PMA for 1 minute a portion of the C1 domains localized to the PM.(See: arrowheads,
Figure 25. Translocation of isolated C1 domains in response to serum or PMA stimulation

NIH 3T3 cells were made to overexpress the following GFP tagged constructs: a control construct (Control), the isolated C1 domain from RasGRP (RasGRP-C1), or the isolated C1 domain from PKC-δ (PKC-C1). The cells were grown in serum containing media (Serum), serum deprived for 3.5 hours (Nil), serum deprived for 3.5 hours and then stimulated for 1 minute (PMA 1') or 15 minutes (PMA 15') with 50ng/ml of PMA. The cells were fixed and then photographed as described in the material and methods. Arrowheads highlight the localization of GFP-tagged proteins at the plasma membrane.
PMA 1'). When these cells were stimulated for 15 minutes with PMA the protein was observed to revert to being localized to the ER+. The PKC-C1 domain responded in an identical manner to the RasGRP-C1 domain in all respects.

The following is a possible interpretation of these results based upon a hypothesis which states that RasGRP's C1 domain is capable of binding to DAG with moderate affinity and to PMA with higher affinity. First, we speculated that RasGRP's C1 domain exhibits an ER+ pattern of localization because it binds to DAG produced in these structures, both in the presence or absence of serum. Second, we attempt to explain the inability of the C1 domain to localize to the PM following serum stimulation by assuming that serum stimulation does not establish a sufficiently high concentration gradient of DAG to drive the relocalization of these proteins from the ER+ to the PM. To explain the differences observed in the localization pattern of isolated C1 domains in cells stimulated for short (1 minute) or long (15 minutes) times with PMA we speculate that by 1 minute, PMA would have diffused into the plasma membrane but would have not yet reached the internal membranes. This high concentration gradient of PMA, in conjunction with the greater affinity of the C1 domain for PMA versus DAG would then result in the migration of a detectable portion of the C1 domain from the ER+ to the PM. After 15 minutes, PMA would have been evenly distributed throughout the cell's membranes. If this were the case, then these results suggest that RasGRP's C1 domain has the potential to respond to PMA stimulation in a rapid and transient manner by localizing to the PM. After which, the C1 domain reverts to an ER+ distribution. The hypothesis that the RasGRP C1 domain binds directly to PMA or DAG is supported by
the observation that it was functionally identical to the PKC-C1 domain in all respects tested by these experiments.

e) **Re-localization of RasGRP and C1 domains in response to a DAG-generating phospholipase**

Since it appeared that some C1 domain-containing RasGRP proteins were capable of responding to PMA stimulation by re-localizing to the PM we were interested in determining whether RasGRP C1 domains are capable of responding in a similar manner to the production of DAG at the plasma membrane.

Phosphatidylcholine specific phospholipase C (PC-PLC) is an enzyme that generates DAG by cleaving phosphatidylcholine in the outer leaflet of the plasma membrane, with DAG flipping to the inner leaflet where it exerts its biological function. (Besterman et al, 1986; Larrondera et al, 1990) This enzyme generates a large quantity of DAG at the PM which then rapidly redistributes to internal structures in the cell. (Larrodera et al, 1990) Stimulation with PC-PLC does not induce changes in calcium concentrations since IP₃ is not generated during the cleavage of PC.

NIH 3T3 cells were engineered to overexpress the following GFP-tagged constructs: control, TL37-2A, RasGRP/FL, RasGRP-C1, PKC-C1, and RasGRP/FL-CA1. These cells were grown in serum free media for 3.5 hours and then stimulated with 4U/ml of PC-PLC for 45 minutes. The results of these experiments are compiled in Figure 26.

Although PC-PLC stimulation had no effect on the distribution of GFP control proteins, C1 domain-containing proteins (TL37-2A, RasGRP/FL, RasGRP-C1, or PKCδ-
Figure 26. Localization of C1 domain-containing proteins in response to PC-PLC stimulation
NIH 3T3 cells were made to overexpress the following GFP tagged constructs: a control construct (Control), the original clone isolated from the library screen (TL37-2A), the 3’ extended full length clone (RasGRP/FL), the isolated C1 domain from RasGRP (RasGRP-C1), the isolated C1 domain from PKC-δ (PKC-C1), or the C1 deleted form of RasGRP/FL (RasGRP/FL-CΔ1). The cells were grown in serum free conditions for 3.5 hours and then stimulated with 4U/ml of PC-PLC for 45 minutes. The cells were fixed and then photographed as described in the material and methods.
C1) were observed to localize to small spherical “dots” found distributed throughout the cytoplasm. A diffuse fluorescence surrounding the nucleus could also be detected in these cells. Cells overexpressing RasGRP/FL C1-deleted constructs (RasGRP/FL-CΔ1) did not exhibit a punctate pattern of fluorescence in response to PC-PLC stimulation. A similar pattern of fluorescence is observed when fibroblasts are treated with fluorescently tagged phosphatidic acid, which is metabolized to DAG and then to triacylglycerol once inside the cell. (Pagano et al, 1983) Therefore, the localization of RasGRP and its C1 domain to these punctate structures may represent binding of the C1 domains to the lipid droplets which became highly enriched in DAG following PC-PLC treatment. The identical localization pattern of the PKCδ-C1 domain, which is known to bind specifically to DAG (Zhang et al, 1995), is further evidence that the RasGRP C1 domain is relocating via DAG binding. However, since the exact kinetics of DAG production and internalization are unknown, firm conclusions about the ability of the C1 domain to bind to DAG cannot be made.

f) Effects of calcium ionophores on the localization of EF hand-containing proteins

It was previously determined that in serum stimulated cells, RasGRP’s EF hands were not capable of conferring any specific localization to proteins either on their own or in conjunction with other regions of RasGRP. (See: Figure 27) Failure of the EF hands to direct detectable localization could be due to insufficient calcium mobilization in response to serum stimulation. Therefore, we were interested in determining whether localization of RasGRP or its EF hands could be affected by a calcium ionophore.

Experiments were performed on cells overexpressing various EF hand-containing proteins...
Figure 27. Localization of EF hand containing proteins in response to ionomycin stimulation

NIH 3T3 cells were made to overexpress the following GFP tagged constructs: a control construct (Control), the original cDNA isolated from the library screen (RasGRP/TL37-2A), the isolated EF hands plus C1 domain (RasGRP-EFH/C1), and the isolated EF hand alone (RasGRP-EFH). The cells were grown in serum free conditions for 3.5 hours (Serum Free) or grown in serum free media for 3.5 hours and then stimulated with 100ng/ml of ionomycin for 15 minutes (SF+Ionomycin). The cells were fixed and then photographed as described in the material and methods.
constructs. The cells were serum starved for 2.5 hours or serum starved and then stimulated with 100ng/ml of the calcium ionophore, ionomycin. Ionophores are molecules that facilitate entry of calcium into cells by binding to calcium and creating a neutralized and hydrophobic molecule capable of crossing the membrane. The results from these experiments are compiled in Figure 27.

Serum starvation and ionomycin stimulation had no effect upon the localization of GFP control proteins. (See: Control GFP; Serum free, SF + ionomycin) As observed previously, serum starvation of cells overexpressing RasGRP/TL37-2A exhibited a ubiquitous fluorescence pattern. (See: RasGRP/TL37-2A; Serum Free) No changes were observed when these cells were then stimulated with ionomycin. (See: RasGRP/TL37-2A; SF+ionomycin) NIH 3T3 cells were also engineered to overexpress either a construct containing both the EF hands and the C1 domain (RasGRP-EFH/C1) or a construct containing the EF hands alone (RasGRP-EFH). This was done in order to determine whether an obvious effect of calcium stimulation on localization could be observed in proteins containing only the EF hands. In addition we were interested in determining whether calcium stimulation had any effect on C1 domain mediated localization. As observed previously, serum starvation had no effect on the localization of RasGRP-EFH/C1 containing proteins to the ER+. No changes in this fluorescence pattern were observed upon ionomycin stimulation of these cells. Cells overexpressing the EF hands alone (RasGRP-EFH) exhibited a ubiquitous fluorescence pattern under serum starvation and no effects were observed when these cells were stimulated with ionomycin. Based upon these experiments it was concluded that the EF hands had no influence on RasGRP localization under the conditions tested.
g) Role of the basic region and the $\alpha$-helix in protein localization

Unlike RasGRP/TL37-2A proteins, RasGRP/FL proteins were unable to de-localize in serum free conditions. This discrepancy appears at first glance, to be solely attributable to the 3' end extension region found in RasGRP/FL. However, isolated C1 domains are also unable to de-localize and they do not possess this 3' extension. Nonetheless, these results suggest that the C terminal region might be able to influence how RasGRP is localized.

In order to determine if the C terminal region had an independent localization function or whether it could affect the localization function of the C1 domain, a number of RasGRP constructs were created covering a region between the EF hands and the stop codon. This series of constructs was produced by systematic deletion of the 5' end. The following nomenclature was used to describe distinct sub-regions found within these deletion constructs: **EFH**: EF hand; **C1**: C1 domain; **+:** KKRIK sequence 3' of the C1 domain; **bbbb**: region 5' of the $\alpha$ helix containing a number of basic residues; **$\alpha$:** alpha helical domain. For the sake of clarity, diagrams outlining the composition of each construct are found to the right of each micrograph in Figure 28.

Surprisingly, the C terminal region lacking the C1 domain was very specifically localized to the nucleus. (See: RasGRP-+/$\alpha$) One question that arose out of this observation was whether the KKRIK cluster (+) or basic region (b) were capable of serving as nuclear translocation signals. The RasGRP-/$\beta/\alpha$ construct (lacking the KKRIK sequence) was still observed to be completely nuclear, while the RasGRP-/$\alpha$ construct (lacking both the KKRIK and the basic regions) was observed in both the
GFP

RasGRP-+/α

RasGRP-b/α

RasGRP-α
Figure 28. Localization of \( \alpha \)-helical domain-containing proteins under serum conditions

NIH 3T3 cells were made to overexpress a number of different GFP tagged constructs. Line diagrams to the right of the photographs represent the various deletion constructs. The following nomenclature was used to describe distinct sub-regions found within the deletion constructs: **EFH**: EF hand; **b**: basic residues found 5' of the C1 domain; **C1**: C1 domain; **+**: basic residues found 3' of the C1 domain (KKRIK); \( \Delta^{+} \): removal of the KKRIK cluster; **\( \alpha \)**: alpha helical domain. The cells were grown in serum containing media, fixed, and then photographed as described in the material and methods.
nucleus and the cytoplasm. Therefore, the basic region seems to contribute to, but not completely determine nuclear localization with the α-helical region on its own being capable of partial nuclear localization.

Since full length forms of RasGRP have never been observed to significantly localize to the nucleus (See: RasGRP/FL) we attempted to determine what was preventing these proteins from localizing to the nucleus. In order to answer this question, the EF hands and/or the C1 domain were added back to the RasGRP-+/α construct. When the C1 domain alone was added back it produced a fluorescence pattern similar to that produced by the full length protein.(See: RasGRP-C1/α) Addition of a region containing both the EF hand and the C1 domain produced a similar fluorescence pattern. (See: RasGRP-EFH/α) Based upon these results it was concluded that the presence of the C1 domain overrides the localization effects of the basic/α helical region and prevented these proteins from localizing to the nucleus. Furthermore, it was concluded that the presence of the EF hands had no additional effect upon the localization of these proteins. It should be noted that the proposed nuclear localizing effects of the isolated basic/α- helix region appear to be only partially functional in the context of the full length RasGRP protein as full length proteins lacking the C1 domain are both nuclear and cytoplasmic.(See: Figure 23; RasGRP/FL-CΔ1)

7.3 Discussion

The results presented in this chapter outline the domain requirements for the localization of RasGRP and for its ability to localize in response to different stimuli. Based upon this data it was determined that RasGRP localizes to the ER as well as other
membranes and that this localization requires the C1 domain. It was also determined that the C1 domain was responsible for re-localization in response to PMA or PC-PLC stimulation and that this was most likely due to the direct binding of the C1 domain to PMA or DAG, respectively.

Although the response to PC-PLC treatment was identical for full length RasGRP proteins and their isolated C1 domains, these proteins did not respond in an identical manner to serum or PMA stimulation. In fact, differences in responses were observed even between RasGRP/TL37-2A and RasGRP/FL proteins. For example, RasGRP/TL37-2A proteins were capable of de-localizing in response to serum starvation but did not respond to PMA stimulation. RasGRP/FL proteins, on the other hand, were unable to respond to serum starvation but were capable of responding to PMA stimulation by localizing to membrane ruffles in some but not all cells. Isolated C1 domain-containing proteins were unable to respond to serum starvation but localized to the PM upon PMA stimulation. A number of possible explanation can be given in order to attempt to explain these results.

First, it was assumed that serum starvation would produce a decrease in DAG concentration in membranes. DAG is produced in all cellular membranes including the plasma membrane as well as those found in the ER. (Wakelam, 1998) It is possible that growing cells in serum free media for 3.5 hours is not sufficient to decrease the concentration of DAG in the ER. If this were the case, then it might explain why RasGRP/FL proteins were incapable of de-localizing in serum free media. This reasoning does not explain why RasGRP/TL37-2A proteins are capable of doing so.
Another possible explanation for why RasGRP/FL proteins are unable to de-localize in serum free media involves the C terminal extension found in these proteins. This additional region may provide some tethering function and its absence in RasGRP/TL37-2A proteins could allow for the de-localization under serum free conditions. It could also explain RasGRP/TL37-2A's diffuse localization under serum conditions when compared to proteins containing the C terminal region, such as RasGRP/FL and X. (See: Figure 23) Alternatively, the increase in size of the RasGRP/FL protein may influence its ability to de-localize. These explanations are not sufficient to explain why isolated C1 domains are unable to de-localize under serum free conditions since they also lack this C terminal region.

Isolated C1 domains were capable of transient localization to the PM in response to PMA stimulation (1 minute) and no obvious plasma membrane relocalization was detectable for RasGRP/FL or TL37-2A. The differences observed between full length proteins and isolated C1 domain-containing proteins may reflect differences in the rate of movement of larger, bulkier, full length proteins in comparison to smaller proteins produced by the isolated C1 domains. Work performed on full length PKC molecules and their isolated C1 domains has shown that PE induced translocation of full length proteins requires more than 100s compared to a few seconds for translocation of the isolated C1 domains alone. (Oancea et al, 1998) In this case, the translocation kinetics differences were explained by stating that additional time was required for the full length proteins because the DAG binding site was initially inaccessible. It is possible that translocation of RasGRP in response to PMA may also require that the C1 domain become accessible before translocation can take place.
Since the full length RasGRP proteins have not been observed to predominantly localize to the plasma membrane in response to PMA stimulation and C1 domain-containing proteins are observed to localize to presumptive lipid droplets upon PC-PLC stimulation, one of the questions that arises is whether the plasma membrane is actually the site of action for RasGRP. The plasma membrane would be a good candidate if RasGRP were a Ras exchange factor, as Ras proteins are tethered there by prenylation. Moreover, prenylation of RasGRP has been previously shown to increase Elk-1 activation indicating that targeting RasGRP to the plasma membrane has the potential to increase its activity. (See: Chapter VI) In addition, work performed by two independent groups has been able to show that PE stimulation of cells overexpressing RasGRP produced an increase in: the percentage of GTP bound Ras, the levels of activated ERKs, the ability to become transformed under low serum conditions, and the ability of RasGRP to localize to membranes in these cells. (Ebinu et al, 1998; Kawasaki et al, 1998) These results, in addition to the data presented in this chapter, help to support the proposed model of RasGRP activation which states that PMA stimulation or the production of DAG is capable of inducing RasGRP translocation to the PM where it is then able to exchange on Ras.

One of the questions that arises from these experiments is whether the C1 domain is the only region of RasGRP that possesses a localizing function. Results from localization experiments performed in serum conditions did not suggest any role for the EF hands in localizing proteins to the ER+. In addition, results from the ionomycin stimulation experiments do not implicate the EF hands as being capable of mediating re-localization in response to calcium ionophores. A potential role for the EF hands has
been highlighted by experiments performed on the rat homologue of RasGRP. Data obtained from Ras GTP binding experiments have suggested that a slight enhancement of H-Ras activation was achieved upon stimulation with the calcium ionophore, A23187. (Kawasaki et al, 1998) These results point towards a role for calcium signalling in GEF activation but do not necessarily suggest a role for the EF hands in localization. For example, calcium binding to the EF hands may be responsible for causing important conformational changes within RasGRP that are necessary for full GEF activity.

Experiments performed on isolated C terminal regions implicate the basic region, in conjunction with the α-helix, as being capable of localizing to the nucleus. The presence of the C1 domain appears to override the nuclear localization capabilities of the isolated C terminal region. In the full length protein, the presence of the C terminal region is not enough to push localization to the nucleus even when the C1 domain has been deleted. These results suggest two possibilities. First, that the nuclear localization observed with the isolated C terminal regions is artifactual and occurs only because these regions have been taken out of their full length context. The second possibility is that a real function for nuclear localization mediated by the C terminal region exists. If this were true then a mechanism would be required to override protein localization as determined by the C1 domain. This could be achieved by the induction of conformational changes within the RasGRP protein that suppress C1 domain localizing functions. Alternatively, the C terminal region could be cleaved off of the full length molecule and could be functioning on its own in the nucleus. The actual role of the α-helical domain remains to be elucidated.
Chapter VIII: General Discussion

Although our understanding of the Ras pathway has evolved in the past few years, there remain a number of gaps that need to be filled in order to more fully understand how it works to control and regulate different cellular functions. Ras plays a fundamental role in the transformation of cancer cells, with over 30% of all human malignancies having been found to contain mutations of the Ras molecule. (Reviewed in Bos, 1989; and Brandt-Rauf and Pincus, 1998) Knowledge of the different proteins that play a part in controlling this pathway will lead to a fuller understanding of the process behind Ras-mediated transformation. The main purpose of this project was to identify and characterize a novel gene involved in the Ras signal transduction pathway. This primary goal was fulfilled by the identification of RasGRP. RasGRP encoded a novel cDNA whose overexpression in fibroblast cells produced transformation phenotypically similar to that of Ras overexpression.

Work presented in Chapters III and IV describes the isolation and sequence analysis of the full length protein. Based upon this data, it appears as though RasGRP is the newest member of the CDC25 family of Ras exchange factors. To date, only two types of Ras-specific GEFs have been identified, they are: Sos and RasGRF. (Botwell et al, 1992; Chardin et al, 1992; Shou et al, 1992; Wei et al, 1992; Martegani et al, 1992) RasGRP contains high sequence similarity to these true Ras exchangers throughout the Ras Exchange Motif (REM) box and the three structurally conserved regions (SCR) of the GEF domain. In order to determine whether RasGRP had the ability to exchange on Ras, the activation status of downstream molecules found in the Ras pathway were
monitored in cells overexpressing RasGRP. (Chapter VI) ERK and Elk-1 phosphorylation was elevated in cells overexpressing RasGRP. This activation was stimulated by serum in some cases and prenylation of RasGRP caused constitutive activation of the Ras pathway. RasGRP also synergized with the overexpression of H-, K-, and N- Ras but not TC21 or R-Ras to increase Elk-1 activation. Based upon these indirect measures of Ras activity it appears as though RasGRP is an activator of the Ras pathway. These results have been confirmed by Kawasaki and co-workers (1998), who measured the percentage of GTP-bound Ras molecules in cell overexpressing the rat homologue of RasGRP (rRasGRP) They were able to show that rRasGRP is capable of active exchange on H-Ras but not Rap1A or RalA. However, this group was able to observe considerable exchange activity on R-Ras. Similar experiments, performed by a second group working on the rat homologue (Ebinu et al, 1998), measured Ras-GTP loading in cells overexpressing rRasGRP and observed specific exchange on H-Ras. Taken altogether these results suggest that RasGRP overexpression is capable of stimulating exchange on the true Ras proteins H-Ras, K-Ras, and N-Ras. In addition, RasGRP may also be capable of exchange on some but not all other Ras Family members such as R-Ras.

In addition to the REM/GEF domains, RasGRP contains a potential myristoylation signal, a pair of EF hands, a C1 domain, a proline rich region, and an α-helical domain. Work presented in Chapter V describes the results obtained from the overexpression of RasGRP deletion and mutation constructs in NIH 3T3 cells. Based upon this data, it was determined that in order for RasGRP to fully transform the fibroblast cells it had to possess both the REM/GEF domains and the C1 domain. All
other domains were found to be non-essential for transformation. It was also determined that the C1 domain of PKCδ or the addition of a membrane targeting prenylation signal could functionally replace RasGRP’s C1 domain which suggested a role for membrane localization in the activation of RasGRP. It is a well known fact that all the Ras proteins capable of being activated by RasGRP are found tethered to the plasma membrane by prenylation. Based upon this information and the results obtained from the fibroblast transformation assays it was hypothesized that RasGRP’s C1 domain might function to target the protein to the membrane in response to DAG or PE stimulation.

Results presented in Chapter VII confirmed this hypothesis. It was shown that the isolated C1 domain of RasGRP (RasGRP-C1) was capable of responding to phorbol ester stimulation by causing the protein to translocate to the plasma membrane. This appeared to be a rapid and dynamic process since longer PMA stimulations did not produce constitutive localization and the protein returned to its native area surrounding the nucleus. At the same time, full length RasGRP constructs (TL37-2A and RasGRP/FL) did not exhibit the same rapid and transient translocation in response to PMA stimulation. Maximal translocation in these cells was observed only after 15 minutes of PMA stimulation and only in the case of RasGRP/FL. These differences observed between the isolated C1 domain and the full length proteins could be partially explained by differences in the translocation kinetics or tethering ability of larger versus smaller proteins. Alternatively, conformational changes in other regions of RasGRP may be required for the C1 domain to have full access to DAG in the context of the full length protein. However, these explanations do not help to explain the differences observed
between RasGRP/TL37-2A and RasGRP/FL proteins in their ability to de-localize in response to serum starvation and PMA stimulation. RasGRP-C1 domain containing proteins were also able to respond to PC-PLC treatment by localizing to presumptive lipid droplets found in the cytoplasm. Taken altogether these results confirm the first part of the hypothesis and indicate that RasGRP's C1 domain is capable of localizing to the plasma membrane in response to PE stimulation. In addition, in the context of the full length protein the C1 domain is capable of mediating a localization in response to increases in DAG produced by PC-PLC stimulation.

A correlation between translocation and GEF activation was implied by the fibroblast transformation results since only prenylated or C1 domain containing RasGRP proteins have been found to be functionally active and C1 deleted constructs have been found to be functionally inactive. These experiments do not address the question of whether PE stimulation is capable of mediating changes in RasGRP activity. In order to test this, the level of ERK phosphorylation was monitored in RasGRP overexpressing cells before and after PE stimulation. Data produced in these experiments (Chapter V) was unable to identify any significant increase in ERK phosphorylation in RasGRP expressing BOSC23 or NIH 3T3 cells upon PMA stimulation. It should be noted that both control and RasGRP overexpressing cells exhibited saturating levels of ERK phosphorylation upon PE stimulation presumably to be due to PLC activation. Experiments performed on rRasGRP (Kawasaki et al, 1998) have been able to correlate an increase in GTP exchange activity upon PMA stimulation of 293T cells overexpressing rRasGRP and these results provide evidence for the ability of translocation to cause GEF activation.
In addition to the catalytic region and the C1 domain, RasGRP possesses a pair of calcium binding motifs known as EF hands. Comparison of this region to the consensus revealed that RasGRP had the potential to bind calcium. Ebinu and coworkers (Ebinu et al, 1998) were able to determine that the second EF hand of rRasGRP was capable of high affinity binding to calcium, suggesting that perhaps only one EF hand out of the pair was functional. Deletion of the EF hands from RasGRP had no effect on fibroblast transformation suggesting that they are not absolutely required for exchange activity. (Chapter V) The EF hands could hypothetically work in conjunction with the C1 domain to translocate RasGRP to the membrane. Alternatively, they could also cause calcium induced conformational changes in the protein thereby providing a potential mechanism for the liberation of the C1 domain that was described previously. Work in Chapter VI, detailing the effects of ionomycin stimulation on the localization of EFH containing constructs suggests that the EF hands do not play a role in protein translocation under these conditions. It should also be noted that no synergistic or negative effects of ionomycin stimulation have ever been witnessed in cells simultaneously stimulated with PMA and ionomycin. However, a direct effect on the activation of RasGRP by calcium has been observed by Kawasaki and coworkers (1998). They described a slight increase in H-Ras activation in response to the calcium ionophore A23187 in cells overexpressing RasGRP. We have not witnessed any effects of calcium stimulation on MAP kinase activation or Elk-1 activation in cells overexpressing RasGRP. (Tognon et al, 1998) Therefore, the exact consequence of calcium binding on the behavior of RasGRP has yet to be determined. If calcium binding does have the
ability to affect the GEF activity of this molecule it would provide evidence for an additional convergence point between calcium signalling and the Ras pathway.

The presence of other motifs within RasGRP, such as the proline rich domain (containing the potential MAP kinase phosphorylation site and SH3 binding site) and the \( \alpha \)-helical domain, suggested the potential for additional mechanisms to regulate the activity of the exchange factor. As stated previously, deletion of these domains from the full length protein had no effect on its ability to cause fibroblast transformation suggesting that they were not absolutely required for activation of the molecule. The \( \alpha \)-helical domain bears a resemblance to proteins found in the bZIP family of transcription factors such as \textit{jun} and \textit{fos}.(Chapter IV) This, coupled with the fact that RasGRP constructs containing the conserved leucine repeat and the upstream basic region localize to the nucleus (Chapter VII) suggests the potential for interaction with other \( \alpha \)-helical containing proteins. The exact function of the \( \alpha \)-helical region in the context of the full length protein has yet to be determined as full length C1 domain-, \( \alpha \)-helical domain-containing proteins (i.e. RasGRP/FL or X) have never been observed to localize to the nucleus.

All the data presented above can be synthesized into a simple model of how RasGRP causes fibroblast transformation via Ras activation.(See: Chapter V) Briefly, increases in DAG concentration attracts the C1 domain of RasGRP and it translocates from a region surrounding the nucleus to the plasma membrane. Once at the plasma membrane RasGRP is able to exchange on true Ras molecules which are tethered there by
prenylation. In this manner, RasGRP overexpression causes constitutive Ras activation which eventually results in fibroblast transformation.

In this particular model, the C1 domain plays an important role in activation via plasma membrane translocation of RasGRP. However, it should be noted that RasGRP is not normally expressed in fibroblast cells and therefore alternative or additional regulatory mechanisms could function to control exchange activity when RasGRP is expressed at physiological levels in its native cell type. These mechanisms could include such things as interaction with receptor-regulated adapter molecules via the proline rich region, response to increases in intracellular calcium via the EF hands, or binding to other \( \alpha \)-helical domain containing proteins via the \( \alpha \)-helical domain.

RasGRP is expressed in hematopoietic tissues such as thymus, spleen, and bone marrow, some lymphoid derived cell lines and brain. (Chapter III) This pattern of expression is unique when compared to other Ras GNEFs and suggests the potential for RasGRP to play a role in Ras activation in these cell types. One of the major questions that remains to be determined is under what context does RasGRP become activated or alternatively, what cellular activity does RasGRP help to control?

Ras can be activated in lymphocytes by stimulation of the antigen receptor in mature peripheral T and B cells (Downward et al, 1990; Li et al, 1996; Saxton et al, 1994), by the stimulation of high affinity receptors for IgE (FceR1) in mast cells (Turner and Cantrell, 1997), and by cytokine or co-receptor stimulation (Satoh et al, 1991; Graves et al, 1992; Gomez et al, 1996). Many of these extracellular signalling events also result in the production of DAG in membranes which suggests the potential for RasGRP
involvement in Ras activation. (Henning and Cantrell, 1998) Up until now the main mechanism for Ras activation was thought to involve the binding of phosphorylated receptor complexes by Grb2/Sos. (Simon et al, 1993; McCormick, 1995; Reif et al, 1994). In B cells this is further complicated by the presence of an additional adapter molecule known as Shc which is thought to link the Grb2/Sos complex to the activated receptor. Recently, a number of additional lymphoid-specific adapter proteins have been identified, such as LAT (linker for activation of T cells; Zhang et al, 1998; Finco et al, 1998) which appears to provide an alternative docking site for Grb2, and 3BP2, a syk kinase interacting protein that has been shown in vitro to bind to LAT and Grb2. (Deckert et al, 1998) The existence of these alternative adapter proteins increases the potential complexity of the membrane proximal signalling events that link extracellular signals to Ras activation. Using the yeast two hybrid system, Sos has also been shown to bind to the SH3 domain of the protein tyrosine kinases lck, src and fyn which suggests an adapter-less mechanism for Sos translocation. (Park et al, 1998) The existence of RasGRP not only adds to the growing family of Ras specific GTPases but also provides a unique way to activate Ras which does not appear to require adapter molecules for membrane translocation and is responsive to the second messenger, DAG.

Some of the more well known consequences of Ras activation in lymphocytes are: the induction of growth factors such as IL-2, IL-4, GM-CSF, and TNF-\(\alpha\) via regulation of NFAT-1 (nuclear factor of activated T cells) activity (Satoh et al, 1991; Graves et al, 1992; Genot et al, 1996; Turner et al, 1997); the induction of IL-2 mediated T cell proliferation (Gomez et al, 1996); and involvement in the selection of thymocytes. (Swan
et al, 1995; Fields et al, 1996; Li et al, 1996) It is possible that RasGRP could function to coordinate Ras activation in response to DAG production in the aforementioned situations. For example, if RasGRP were inactivated in lymphoid cells they might be incapable of proliferating in response to cytokine stimulation. Another possible effect of RasGRP inactivation might be the production of a population of entirely anergic mature T cells since it has been shown that Ras and ERK remain inactive in anergic T cells during the process of selection of immature T cells in the thymus. (Fields et al, 1996)

A number of different approaches could be utilized in order to begin to determine the function of RasGRP in lymphocytes. For example, B and T cell lines could be engineered to overexpress various RasGRP constructs and they could then be assayed for differences in their ability to respond to various stimuli. Cells could be tested for a number of different properties such as: their ability to respond to phorbol ester and calcium ionophore stimulation, their rate of apoptosis induced by this stimulation, their rate of proliferation in serum and serum free conditions, or their ability to upregulate their levels of surface molecules (i.e. IL-2 receptor) in response to stimulation. Differences observed between control and RasGRP overexpressing cells could indicate that RasGRP might play a role in the normal functioning of these cells. In addition, the use of deletion and mutant RasGRP constructs could help to determine which regions are required for the proper functioning of RasGRP in these assays.

It would also be of interest to knock out RasGRP expression in lymphocytes. One potential way to achieve this would be to utilize the DT40 cell line in order to make a RasGRP knock out cell line. The DT40 cell line is an avian leukemia virus (AVL)-induced chicken B cell lymphoma that undergoes continuous diversification of its Ig
genes in vitro by gene conversion via an unknown mechanism. (Buerstedde and Takeda, 1991) This activity results in an increase of homologous to random integration frequency following transfection. (Kim et al, 1990; Buerstedde and Takeda, 1991) RasGRP DT40 knock out cell lines could be used to determine whether RasGRP is required for Ras activation and apoptosis in response to phorbol esters, calcium, or BCR ligation.

Alternatively, the effects of RasGRP overexpression or disruption could be observed within the context of a whole organism. RasGRP transgenic or knockout mice could be generated in order to determine the effects of overexpression or lack of expression on mouse development, on the production of lymphocyte populations, and on the ability of mature lymphocytes to respond normally to stimuli, to name a few. The actual function of RasGRP in lymphocytes remains to be elucidated and will provide an interesting area for further study.
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