Identification and characterization of M-Ras, a novel member of the Ras-superfamily of small GTPases

by

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ABSTRACT

Ras proteins have been implicated in up to 30% of all human cancers. However, in some tissues the incidence of mutations in p21 Ras proteins is very low. This prompted us to screen the EST-database for new Ras-family members. Here we describe the identification and characterization of M-Ras, a novel and highly conserved member of the Ras family. M-Ras is a widely expressed protein with an apparent molecular weight of 29 kDa. Activated mutants of M-Ras transformed NIH 3T3 fibroblasts and led to factor-independent growth of an IL-3-dependent cell line. A dominant negative mutant of M-Ras was able to block activation of the c-fos promoter by an activated Src Y527F. Importantly, M-Ras was recognized by the monoclonal anti-Ras antibody Y13-259, a widely used tool to assay p21 Ras activation. In contrast to p21 Ras, M-Ras interacted only weakly with the Ras binding domains of Raf-1 and RalGDS. This led us to screen a cDNA library prepared from NIH 3T3 cells for novel effectors of M-Ras using the yeast 2-hybrid system. Several known Ras effectors were identified, thus allowing us to establish a subset of p21 Ras effectors that also interacted with M-Ras. We also identified a novel effector protein that interacted strongly with activated mutants of M-Ras and p21 Ras. Sequence analysis of this novel protein revealed a new member of the RalGDS-family of exchange factors, which we termed RPM. Murine RPM mRNA was widely expressed. In contrast to other RalGDS proteins, which have been shown to synergize with p21
Ras in activating the c-fos promoter, RPM displayed a strong inhibitory effect on an Elk-1 dependent reporter system. This inhibitory activity was dependent on a second signal that could be provided by activated p21 Ras or by MEKK-1 but not by Raf-1. Furthermore, RPM also displayed a strong negative regulatory effect on the growth of NIH 3T3 fibroblasts that were transformed by an activated Src Y527F. Since RPM did not inhibit the MEKK-1-dependent activation of the MAP-kinases Erk, JNK and p38, we conclude that expression of RPM uncouples Elk-1-dependent gene induction from MAP-kinase activation.
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DH</td>
<td>dbl-homology</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GDS</td>
<td>guanine nucleotide dissociation stimulator</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemaglutinin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosomal entry site</td>
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JNK: c-Jun N-terminal kinase
kB: kilobasepairs
kDa: kilodalton
LPA: lysophosphatidic acid
MAP: mitogen activated protein
MAPK: mitogen activated protein kinase
MEK: MAP/Erk kinase
MEKK: MEK kinase
MKK: MAP kinase kinase
mRNA: messenger ribonucleic acid
MTT: 3,4,5-dimethylthiazole-2,5-diphenyltetrazolium bromide
NFκB: nuclear factor kappa B
nt: nucleotide(s)
PBS: phosphate buffered saline
PDGF: platelet derived growth factor
PH: pleckstrin homology
PI: phosphatidylinisitol
PKC: protein kinase C
PLD: phospholipase D
RACE: rapid amplification of cDNA ends
Rb: retinoblastoma
RBD: Ras binding domain
RT-PCR: reverse transcriptase polymerase chain reaction
scr: structurally conserved region
SLF: steel locus factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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</table>
Preface

Thesis format

This thesis is divided into 5 chapters. The first chapter starts with a brief historical introduction about the identification of the first Ras proteins and then goes on to introduce the regulation, functions and mutations of Ras proteins. Chapter II describes the methods and materials used in chapters III and IV. Chapters III and IV describe and discuss data obtained during the course of this study. Chapter V contains a summary of general conclusions that can be drawn from this study as well as future directions. The original search of the EST-database for novel Ras family members was performed by Dr. Kevin Leslie (Figure 3.1). Northern blot analysis (Figure 3.4, Figure 4.6) was performed by Dr. J.S. Wieler.

Publications obtained during the course of this thesis:


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There is a theory which states that if ever anyone discovers exactly what the universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states that this has already happened.

D. Adams
CHAPTER I: INTRODUCTION

1.1 History

In 1964, J.J. Harvey published the discovery of a virus that caused the rapid appearance of tumors in mice (Harvey, 1964). Subsequently, two more transforming viruses derived from rats were discovered, the Kirsten murine sarcoma virus (Kirsten and Mayer, 1967) and the Rasheed rat sarcoma virus (Rasheed et al., 1978), respectively. The oncogenic properties of these viruses were due to the expression of a protein of 21 kDa, which bound the guanine nucleotides GDP and GTP (Shih et al., 1979). Following a proposal for naming host cell-derived inserts in retrovirus genomes (Coffin et al., 1981), the genes encoding these proteins became known as 'ras'-genes, indicating their origin from rat sarcoma viruses. The nucleotide sequences of the transforming viral genes and their cellular homologues were determined 19 years later (Capon et al., 1983; Dhar et al., 1982; Tsuchida et al., 1982). The identification of the cellular counterparts of these viral genes was accompanied by the striking finding that activation of the proto-oncogene was the result of a single point mutation (Reddy et al., 1982; Tabin et al., 1982). A third member of this gene family was simultaneously identified by three groups and termed N-Ras (Hall et al., 1983; Murray et al., 1983; Shimizu et al., 1983). Ever since, the Ras family of small GTPases has been expanding continually and encompasses now 6 sub-families with more than 60 members. The discovery that activating mutations of ras-genes were found in over 30%
of all human tumors (Bos, 1989) made these proteins the subject of intense investigation over the last two decades.

1.2 The Ras-superfamily of small GTPases

The Ras-superfamily of small GTPases can be divided into the 6 subfamilies of the Ras- (Bos, 1997; Campbell et al., 1998), Rho- (Bishop and Hall, 2000; Zohn et al., 1998), Ran- (Melchior and Gerace, 1998; Moore, 1998), Arf- (Moss and Vaughan, 1998), Rab- (Schimmoller et al., 1998) and Kir/Rad/Rem-like GTPases (Maguire et al., 1994; Reynet and Kahn, 1993). These GTPases control diverse cellular functions including proliferation, differentiation, regulation of cell-shape, nuclear transport and vesicle trafficking. Each of these subfamilies is comprised of several proteins, with the Ras-subfamily consisting of the 'classical' p21 Ras proteins Harvey (H)-, Neuroblastoma (N)- and Kirsten (K)-Ras, which can be alternatively spliced and thus generate two additional proteins, K-Ras4A and K-Ras4B. In addition to the 'classical' p21 Ras proteins, the Ras-subfamily also includes the 'non-classical' Rap, Ral, R-Ras, TC21/R-Ras2, M-Ras/R-Ras3, Rin, Rit and Rheb proteins.

Ras proteins are small monomeric GTPases with conserved structure and mechanism. The crystal structure of the GTP-bound and GDP-bound proteins (de Vos et al., 1988; Milburn et al., 1990; Schlichting et al., 1990) reveals a core of six hydrophobic β-sheets connected by hydrophilic α-helices and loops (see Figure 1.1). Five highly conserved regions, designated...
G1-G5, are involved in binding the guanine nucleotide and in coordinating an essential Mg\(^{2+}\) cation (Bourne et al., 1991). Among those conserved regions, the first four are also easily identified in other GTPases, such as the \(\alpha\)-subunit of heterotrimeric G-proteins and the bacterial elongation factor EF-Tu (Boguski and McCormick, 1993).

![Ribbon diagram of H-Ras in the GTP-bound conformation.](image)

**Figure 1.1** *Ribbon diagram of H-Ras in the GTP-bound conformation.* Residues 1-166 are displayed. The switch I region (residues 32-38) and switch II region (residues 60-76) are indicated in green and red, respectively. Connecting loops (\(\lambda^x\)) are indicated. (Figure adapted from Pai et al., 1989).

Ras proteins function as molecular switches inside the cell. This remarkable feature results from a structural change of two regions of the protein relative to each other, depending on whether the protein is GTP- or GDP-bound. These regions are termed switch I and switch II and
correspond to residues 32-38 and 60-76 of p21 Ras, respectively. Changes in the guanine nucleotide loading status of the Ras protein lead to a conformational change spanning 40 Å of switch I and switch II (Milburn et al., 1990) and thus allowing Ras to assume either an 'on' or an 'off' position and to interact with different sets of proteins.

A crucial requirement for p21 Ras proteins to function is its location at the inner leaflet of the plasma membrane. This membrane-localization is achieved by carboxy-terminal lipid modifications of the protein. A 'CAAX'-motif, where 'C' is a cysteine, 'A' is an aliphatic residue and 'X' represents any residue, provides the recognition sequence for the post-translational protein modification. An isoprenyl group, either a geranylgeranyl moiety or a farnesyl moiety, is attached to the cysteine. Consequently, the residues 'AAX' are proteolytically removed and the now C-terminal modified cysteine is further methylated (reviewed in (Reuter et al., 2000)). In addition, cysteine residues amino terminal to the isoprenylated cysteine can be palmitoylated. Ras proteins lacking those additional cysteine(s), such as K-Ras4B and M-Ras, display a stretch of positively charged residues which are thought to support membrane localization. Recent evidence suggests that palmitoylated Ras proteins like H-Ras are transported to the plasma membrane via the Golgi apparatus whereas non-palmitoylated Ras proteins like K-Ras4B are transported to the plasma membrane with different kinetics by a different mechanism involving microtubules (Choy et al., 1999). Therefore, different post-translational modifications might result in subsets of Ras proteins that are localized in distinct microdomains of the plasma membrane.
1.3 Regulation of Ras family members

In order for Ras to function as a molecular switch inside the cell, the protein has to be able to shuttle between a GTP-bound and a GDP-bound state (see Figure 1.2).

![Diagram of Ras-protein regulation]

**Figure 1.2** Ras-proteins function as molecular switches. They shuttle between an active, GTP-bound conformation (shown in red), in which they are able to bind effector proteins, and an inactive, GDP-bound conformation. Examples of regulatory proteins are indicated.

Proteins belonging to the family of guanine nucleotide exchange factors (GEF) catalyze the exchange of GTP for GDP, which is accompanied by the transition of Ras from the inactive to the active conformation. On the other hand, Ras has to be able to hydrolyze GTP to GDP and inorganic phosphate at a rate much higher than its relatively low intrinsic GTPase activity. This
intrinsic GTPase activity is enhanced by several orders of magnitude by GTPase activating proteins (GAP) (Cherfils and Chardin, 1999; McCormick, 1998). Insights into the molecular mechanisms underlying these processes came from successful co-crystallization studies of Ras complexed with the catalytic domains of the exchange factor mSOS and the GAP p120 RasGAP, respectively.

The mechanism underlying the nucleotide exchange could be deduced from co-crystallization studies of Ras with the GEF-region of mSOS. Thus, the exchange factor binds GDP-loaded Ras at the switch II region and inserts a glutamic acid residue and a leucine into the phosphate- and Mg\(^{2+}\)-binding sites. In addition, two hairpin helices of SOS completely remove switch I from the nucleotide binding site. The result of these multiple interactions is a conformational change that leads to the release of GDP (Boriack-Sjodin et al., 1998). The 10 times more abundant GTP replaces GDP and a ternary complex of GTPase, nucleotide and exchange factor is formed. Finally, the exchange factor will be released from GTP-bound Ras (Lenzen et al., 1998).

Since the intrinsic GTPase activity of Ras is 0.02 min\(^{-1}\) (Manne et al., 1985) and therefore too low to allow it to effectively function as a molecular switch, GTPase activating proteins bind to activated Ras and ensure efficient hydrolysis of GTP to GDP and inorganic phosphate. Mechanistically, this is achieved by providing a critical arginine residue that neutralizes a partial negative charge during a nucleophile attack of a water molecule on the phosphate in the \(\gamma\)-position and thus stabilizing the transition state. In addition, this arginine residue also stabilizes
the switch II region, especially the crucial residue glutamine 61 of Ras, which positions the water molecule that serves as the attacking nucleophile (Scheffzek et al., 1997).

In unstimulated cells, Ras is predominantly in the inactive, GDP-loaded state. A great number of studies using Ras-activation assays based on the monoclonal anti-Ras antibody Y13-259 and the development of dominant negative mutants of Ras soon revealed that Ras is activated in response to a wide variety of stimuli. These include growth factors that activate receptor tyrosine kinases such as insulin, PDGF, EGF and SLF (Duronio et al., 1992; Porras et al., 1992; Satoh et al., 1993; Satoh et al., 1991) and factors that activate receptors without intrinsic tyrosine kinase activity such as the cytokines IL-2, IL-3, IL-5, GM-CSF, but not IL-4 (Duronio et al., 1992; Izquierdo et al., 1992; Satoh et al., 1991). The antigen-receptors of B- and T-lymphocytes, also with associated tyrosine kinase activity, have been linked to activation of Ras (Graves et al., 1991; Harwood and Cambier, 1993; Lazarus et al., 1993) as have cell adhesion molecules (Kapron-Bras et al., 1993) and ligands for G-protein coupled receptors such as thrombopoietin and lysophosphatidic acid (van Corven et al., 1993). Whereas activation of Ras by the cytokines IL-3, IL-5, GM-CSF and SLF has been shown to be dependent on tyrosine kinase activity (Duronio et al., 1992), activation of Ras by G-protein coupled receptors can be inhibited by pertussis toxin (van Corven et al., 1993). Evidence has been presented that Ras activation by G-protein coupled receptors involves the transactivation of the EGF-receptor (Daub et al., 1997).
Early work in lower eucaryotes had shown that the *S. cervisiae* Cdc25 gene product regulates the Ras/adenylate cyclase pathway by promoting the release of GDP and the uptake of GTP (Broek et al., 1987; Jones et al., 1991). Also, in an invertebrate system, the product of the 'Son of Sevenless' (SOS) gene was identified as a potential Ras activator and implicated in the *D. melanogaster* eye development (Bonfini et al., 1992).

Following the discovery of the mammalian homologues of the *S. cervisiae* GEF CDC25 and the *D. melanogaster* GEF 'Son of Sevenless' (SOS), CDC25Mm/RasGRF (Martegani et al., 1992; Shou et al., 1992) and mSOS1/2 (Bowtell et al., 1992), respectively, a large number of Cdc25-like GEFs have been described. These include CalDAG-GEF I (Kawasaki et al., 1998), CalDAG-GEF II/RasGRP (Ebinu et al., 1998; Kawasaki et al., 1998), CalDAG-GEF III (Yamashita et al., 2000), Epac1/cAMP-GEF I and Epac2/cAMP-GEF II (de Rooij et al., 1998; Kawasaki et al., 1998), PDZ-GEF and MR-GEF (Rebhun et al., 2000) as well as C3G (Tanaka et al., 1994). Whereas CDC25Mm/RasGRF, mSOS1/2 and CalDAG-GEF II activate p21 Ras as well as R-Ras, Tc21 and M-Ras (with mSOS1/2 being less efficient in activating R-Ras and Tc21), the remaining exchange factors are specific for Rap1 and Rap2, with C3G and CalDAG-GEF I also having moderate GEF-activity towards R-Ras and Tc21 (Ohba et al., 2000). A third group of Cdc25-like proteins consists of the Ral-specific exchange factors RalGDS (Albright et al., 1993), Rlf (Wolthuis et al., 1996), Rgl (Kikuchi et al., 1994), RPM/RGL3 (Ehrhardt et al., 2001; Shao and Andres, 2000), Rgr (D'Adamo et al., 1997) and RalGPS/RalGEF2 (de Bruyn et al., 2000; Rebhun et al., 2000). In addition to these Cdc25-like proteins, two proteins that show
no homology to the prototypical exchange factor Cdc25, SmgGDS (Kaibuchi et al., 1991; Kikuchi et al., 1992) and AND-34 (Cai et al., 1999; Gotoh et al., 2000), also display exchange factor activities. In contrast to Cdc25-like proteins, which are active only on members of the Ras family, these two proteins display exchange factor activities also on Rho-proteins.

The link between receptor activation by growth factors and activation of p21 Ras was discovered in 1993, when several groups reported that Grb2, the mammalian homologue of the *D. melanogaster* protein Drk and the *C. elegans* protein Sem5 was found in a complex with mSOS. Whereas this interaction was mediated via the SH3-domains of Grb2, the SH2-domain of Grb2 would interact either with autophosphorylated tyrosine residues on the receptors or with phosphorylated tyrosine residues on the adapter protein Shc (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Lowenstein et al., 1992; Olivier et al., 1993; Rozakis-Adcock et al., 1993; Simon et al., 1993). These findings pointed to a mechanism, whereby Ras would be activated upon translocation of the exchange factor. Later studies with constitutively membrane localized exchange factors mSOS1 and Cdc25Mm confirmed this model (Aronheim et al., 1994; Quilliam et al., 1994). In addition to Shc, more adapter molecules have been identified that recruit the Grb2/SOS-complex to the plasma membrane, among them Gab1/2 (Gu et al., 1998; Holgado-Madruga et al., 1996; Zhao et al., 1999), LAT (Zhang et al., 1998), BLNK (Fu et al., 1998) and Cbl (Meisner et al., 1995; Odai et al., 1995). Besides phosphorylation events that are necessary for Ras activation by GEF proteins like SOS, other exchange factors like RasGRF1/2, and members of the CalDAG-family have
been shown to activate Ras in response to second messengers like Ca\textsuperscript{2+} and DAG (Ebinu et al., 1998; Kawasaki et al., 1998).

1.4 Mutations of Ras family members

Several mutations have been identified in Ras proteins that disrupt an orderly cycle between the GTP-and the GDP-bound state. Activating mutations in the classical p21 Ras proteins have been found in up to 30% of all human cancers (Bos, 1989). These mutations occur predominantly at positions 12, 13 or 61 and lead to an impairment of the intrinsic GTPase activity. As a result, the mutated Ras proteins are locked in their active, GTP-bound conformation. Clues to the mechanism of these activating mutations came from the successful crystallization studies of Ras complexed with RasGAP. Glycine 12 is in very close proximity to the catalytically important glutamine 61 and the conserved arginine provided by RasGAP in the Ras/RasGAP complex. Mutations of position 12 lead to a replacement of a glycine residue (with no side chain) with a consequently bulkier residue. This leads to spatial constraints where glutamine 61 of Ras and the catalytic arginine residue provided by RasGAP are no longer correctly positioned and thus to an impaired GTPase activity. One function of glutamine 61 is to position a water molecule that serves as the attacking nucleophile on the γ-phosphate of GTP. Replacement of this residue with any other residue leads to a loss of the correctly positioned nucleophile and thus to a GTP-bound Ras protein (Scheffzek et al., 1997).
Besides activating mutations, several studies demonstrate that Ras proteins with mutations at residues 15, 16 or 17 result in proteins with dominant negative characteristics (Feig and Cooper, 1988; Powers et al., 1989). The most commonly used mutant has the residue serine 17 replaced by an asparagine. Serine 17 is one of the residues that coordinates the Mg$^{2+}$ ion. Mutation of this residue results in significantly reduced affinity of the protein for GTP, while the affinity for GDP is reduced only moderately (Feig and Cooper, 1988). Dominant negative mutants of Ras are thought to inhibit activation of endogenous Ras proteins by sequestering exchange factors (Feig, 1999). Upon release of the nucleotide from Ras-GDP, the exchange factor binds the nucleotide-free Ras with relatively high affinity (4-5 nM). Release of the exchange factor from nucleotide-free Ras relies on the even higher affinity of nucleotides for nucleotide-free Ras ($10^{-11}$ M) (Lenzen et al., 1998). The 10-fold higher concentration of GTP over GDP inside the cell results in GTP-loading of Ras. Dominant negative mutants of Ras proteins would require even higher concentrations of intracellular GTP to successfully disrupt the high-affinity GEF/Apo-Ras complex.

Both constitutively active mutants and dominant negative mutants of Ras are very widely used tools to investigate signal transduction pathways involving Ras. In addition to the constitutively active mutants Ras G12V and Ras Q61L, double-mutants of Ras have been identified which, in addition to the G12V mutant, carry a second mutation in the effector loop (residues 32-40 of 21 Ras). Three of these mutants, Ras V12S35, Ras V12G38 and Ras V12C40 have become particularly valuable tools since they bind only Raf, RalGDS and PI3kinase,
respectively (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995). Dominant negative mutants of Ras have been widely used to implicate Ras in various cellular events. However, these mutants do not inhibit Ras directly. Instead, they sequester the GEF proteins necessary to activate endogenous Ras. Whereas much valuable information has been gained based on the use of these mutants, it is important to keep in mind that these mutants do not discriminate between various Ras proteins.

Figure 1.3 Mutants of p21 Ras. Numbers indicate the positions of the corresponding amino acids of p21 Ras. For single mutations (closed arrowheads), only the most commonly used amino acid substitutions are indicated. Double mutations that are introduced into a G12V background are indicated by open arrowheads. Positions of the switch I and switch II regions are indicated in green and red, respectively.
A method of directly assaying Ras activation is based on the monoclonal antibody Y13-259. This antibody recognizes a highly conserved epitope in the switch II region of Ras. Ras proteins from lysates of cells that have been radioactively labeled with ortho-phosphate are immunoprecipitated with this antibody and the ratio of radioactive GTP to GDP assessed by thin-layer chromatography. As for the use of dominant negative mutants of Ras, this assay also fails to discriminate between the various Ras isoforms (Feig, 1999).

1.5 Ras effectors

The fact that not only one but several signaling pathways are activated by Ras proteins results from their ability to specifically interact with various proteins when in their active, GTP-bound conformation. These proteins are defined as 'effector proteins'. The number of Ras effector proteins has been steadily increasing (see Figure 1.4) over the last few years, reflecting the pleiotropic effects of activated Ras. Ras effector proteins encompass protein kinases, lipid kinases, exchange factors, GAPs as well as proteins that lack a clearly defined catalytic activity, making the elucidation of their function difficult.
Ras interacts with multiple effector proteins. In cases where Ras interacts with several members of the same protein family (e.g., Raf, RalGDS) only one family-member is shown. Indicated downstream signaling proteins are not in all cases directly activated by the corresponding Ras effectors.

1.5.1 Protein kinases

The first Ras effector protein identified was the serine/threonine kinase Raf-1 (Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Similar to what was observed with the exchange factors Cdc25Mm and mSOS, constitutive membrane localization of Raf-1 could bypass the requirement for activated Ras (Leevers et al., 1994). This led to a linear model in which receptor mediated Ras activation leads to membrane translocation (and thus activation) of Raf-1, followed by sequential activation of the MAPK kinases MEK1/2 and the MAP kinases Erk1/2. Upon activation, these MAP kinases translocate into the nucleus and phosphorylate transcription factors such as Elk-1, which ultimately leads to gene induction (see Figure 1.5).
Figure 1.5  **Ras activates the Raf>Mek>Erk kinase cascade.** Translocation of the Grb2/SOS complex to receptors phosphorylated on tyrosine residues, either directly or via adapter molecules like Shc, leads to activation of Ras. Recruitment of Raf and activation of the Raf>Mek>Erk kinase cascade provides a link between receptor activation on the cell surface and the initiation of nuclear events. (Figure adapted from Campbell et al., 1998).

However, over the last years it became evident that, with the discovery of additional effector proteins, this linear model was overly simplistic. Closer investigation revealed also that translocation of Raf kinases is a crucial part of Raf activation, but full activation appears to involve conformational changes, as well as phosphorylation events and binding of other proteins such as 14-3-3 (reviewed in (Campbell et al., 1998)). The exact mechanism of Ras-mediated Raf activation is still not completely understood.
A second protein kinase that specifically interacts with GTP-bound Ras is the serine/threonine kinase MEKK-1. Originally identified as a kinase that can activate MEK1/2 and thus the MAP kinases Erk1/2, it was subsequently shown to potently activate the stress activated protein kinases JNK and p38 (Lange-Carter et al., 1993; Xu et al., 1996). In addition to activating MAP kinases, overexpression of MEKK-1 has been shown to activate the transcription factor NFκB. This finding correlates well with the purification of MEKK-1 as part of cytosolic IKK complexes (Lee et al., 1998). Apart from its function as a protein kinase, evidence has also been presented that suggests a role for MEKK-1 as a scaffolding protein, based on its ability to interact with M KK4 and JNK (Xu and Cobb, 1997; Yan et al., 1994).

A third protein kinase that is recognized by the active form of Ras is the ζ isoform of PKC (Diaz-Meco et al., 1994). Although other reports failed to confirm the interaction between Ras and PKCζ in a different cellular context (Williams et al., 1995), Ras-dependent activation of PKC could result in subsequent activation of Raf and MEK and thus have a pro-mitogenic impact on the cell.

### 1.5.2 Lipid kinases

In addition to protein kinases, the catalytic p110 subunit of the lipid kinase PI3-kinase has been demonstrated to bind to GTP-bound Ras (Rodriguez-Viciana et al., 1994). PI3-kinase is a heterodimeric protein, consisting of a catalytic subunit of 110 kDa and a regulatory subunit of
85 kDa. Upon membrane localization, it phosphorylates the membrane components phosphotidylinositol-4-phosphate (PI4P) and phosphotidylinositol-4,5-bisphosphate (PI4,5P₂) to phosphotidylinositol-3,4-bisphosphate (PI3,4P₂) and phosphotidylinositol-3,4,5-trisphosphate (PIP₃). PI3,4P₂ can also be generated by dephosphorylation of the phosphate group in the D-5 position of PIP₃, a reaction catalyzed by the inositol phosphatase SHIP (Damen et al., 1996). These phospholipids bind to pleckstrin homology (PH) domains of various proteins and play important roles in their activation (Leevers et al., 1999). PH-domains have been found in a number of important signal transduction molecules, among them exchange factors for the Rho-family of small GTPases Cdc42, Rac and Rho, which have been demonstrated to be activated by p21 Ras (Ridley and Hall, 1992; Ridley et al., 1992). Evidence presented by Bar-Sagi and co-workers suggested that the PH-domain of mSOS negatively regulates the exchange factor activity of the adjacent DH-domain towards Rac in the absence of PIP₃. Ras-mediated activation of PI3-kinase, by production of PIP₃ would release the autoinhibition of mSOS and thus point to a possible mechanism of Ras-mediated activation of Rac (Nimnual et al., 1998).

1.5.3 Guanine nucleotide exchange factors

A group of Ras effectors distinct from protein- and lipid kinases are the guanine nucleotide exchange factors for the small GTPase Ral. Initially demonstrated for the founding member of this group, RalGDS (Spaargaren and Bischoff, 1994), it has now been demonstrated that the other members of the RalGDS-family, Rgl, Rlf and RPM also interact with GTP-bound
Ras (Kikuchi et al., 1994; Wolthuis et al., 1996; Shao and Andres, 2000; Ehrhardt et al., 2001). All of these proteins have been shown to catalyze the exchange of GTP for GDP specifically for the small GTPase Ral. Whereas over-expressed RalGDS, Rgl and Rlf proteins synergize with activated mutants of Ras and Raf to activate the Ras-inducible c-fos promoter and to transform fibroblasts, overexpressed RPM has the opposite effect in that it inhibits gene induction and transformation. Although the only function of these proteins described thus far is the activation of the small GTPase Ral, several lines of evidence suggest that some of the effects of these proteins are independent of their exchange factor activity (see chapter IV).

1.5.4 GTPase activating proteins

GTPase activating proteins form a separate group of Ras effectors in that they not only bind to Ras proteins in their active conformation but also directly regulate Ras activity by increasing the rate of GTP-hydrolysis of Ras by several orders of magnitude (Gideon et al., 1992). Five mammalian GAPs have been described thus far, the first of which has been the prototypical p120GAP. In addition to its negative regulatory function on Ras, p120GAP displays several other functional signaling modules such as SH2-, SH3- and PH-domains and interacts with p56 Lck, Bcr-abl and p190 RhoGAP (Amrein et al., 1992; Druker et al., 1992; Ellis et al., 1990). Other Ras-specific GAPs are the gene products of the neurofibromatosis gene, NF-1 (Xu et al., 1990), mGAP1 and its close homologue GAPIII (Baba et al., 1995) and the inositol-4-
phosphate binding protein GAP1\textsuperscript{IP4BP} (Cullen et al., 1995), which in addition to Ras also stimulates the GTPase activity of Rap (Wittinghofer, 1998).

1.5.5 Proteins without defined enzymatic activity

A number of proteins qualify for Ras effectors in that they recognize only GTP-bound Ras. However, their lack of enzymatic activity precludes a definite functional characterization. This group of proteins includes Rin-1, Nore-1, AF-6 and the recently identified RasSF1. Rin-1 was originally identified as a protein that interferes with Ras-mediated heat-sensitivity in S. cervisiae (Han and Colicelli, 1995). Further studies revealed that Rin-1 associates with c-Abl via its SH3-domain and is phosphorylated by c-Abl. In addition, Rin-1 potentiates the oncogenic activity of Bcr-Abl (Afar et al., 1997; Han et al., 1997). Nore-1 was identified in a yeast 2-hybrid screen as a protein that interacts with GTP-bound H-Ras. The presence of a DAG-binding site and of several potential SH3-domain binding sites suggests that this protein is an adapter protein (Vavvas et al., 1998). Another Ras effector that was identified by means of the yeast 2-hybrid system is AF-6, the mammalian homologue of the D. melanogaster protein gene canoe which has been linked to Ras function in eye development (Matsuo et al., 1997). The most recently identified Ras effector RasSF1 is a protein with a high degree of similarity with Nore-1 (Dammann et al., 2000). The gene encoding RasSF1 is located in a 120 kb region on chromosome 3p21.3, a region frequently deleted in lung cancers (Kok et al., 1997). Re-introduction of RasSF1 in A549 lung cancer cells diminished anchorage independent growth and
tumor formation (Dammann et al., 2000). In a separate study, RasSF1 was found to induce apoptosis of Ras-transformed cells in a caspase-dependent fashion (Vos et al., 2000).

### 1.6 Ras functions

Ras proteins become activated in response to a wide variety of stimuli. These include stimuli that activate receptor tyrosine kinases (e.g. EGF receptor), receptors without intrinsic kinase activity (e.g. cytokine receptors), immunoreceptors (e.g. BCR, TCR), cell adhesion molecules (e.g. integrins) as well as serpentine receptors (see chapter 1.3). The large number of stimuli that activate Ras is reflected in the wide range of cellular functions, ranging from proliferation and differentiation to cell death, in which Ras proteins have been implicated.

Effects on cell proliferation are closely linked to the transforming abilities of p21 Ras. For example, overexpression of constitutively active mutants of Ras leads to transformation of fibroblasts, with the ability to grow under low serum conditions, the loss of anchorage-dependence and contact inhibition (Hurlin et al., 1987; Hurlin et al., 1989; Li et al., 1993; Zhan et al., 1987). Indeed, in many early studies Ras genes were identified as the transforming oncogenes from a variety of tumors based on their ability to form foci when introduced into NIH3T3 fibroblasts (Der et al., 1982; Hall et al., 1983; Parada et al., 1982; Shimizu et al., 1983). In addition, factor-independent growth of factor-dependent hematopoietic cell lines caused by activated Ras genes has been documented (Andrejauskas and Moroni, 1989; Boswell et al., 1990;
The pro-proliferative characteristics of Ras are also reflected in the induction of immediate-early genes such as c-fos (Hill and Treisman, 1995) and in the modification of the cell-cycle controlling protein machinery (Downward, 1997; Marshall, 1999). Micro-injection studies using the anti-Ras monoclonal antibody Y13-259 demonstrated the importance of Ras in cell-cycle entry and passage through G1-phase in response to serum stimulation (Dobrowolski et al., 1994). A critical step in cell-cycle progression is the activation of the cyclin-dependent kinases 4 and 6 by D-type cyclins, which in turn leads to hyperphosphorylation and inactivation of the Retinoblastoma protein Rb. Supporting a role for Ras in cdk4/6 activation is the Ras-responsiveness of the cyclin D1-promoter and the accumulation of cyclin D1 in response to Erk-signaling (Albanese et al., 1995; Gille and Downward, 1999; Lavoie et al., 1996). Also, in fibroblasts from Rb-knockout animals dominant negative Ras is no longer able to inhibit cell-cycle progression (Mittnacht et al., 1997; Peeper et al., 1997). Whereas sustained Erk-signaling was shown to be a crucial step for S-phase entry, other PI3-kinase mediated signals also appear to be required, as DNA synthesis in response to an inducible MEK construct can be abrogated by pharmacological inhibitors of PI3-kinase (Treinies et al., 1999). In a different cellular context however, microinjection of activated Ras did not result in DNA-synthesis in serum starved cells, due to the lack of a Rho-mediated signal to prevent upregulation of the cell-cycle inhibitor p21 CIP (Olson et al., 1998). In primary fibroblasts, overexpression of activated Ras resulted in upregulation of p53, as well as the cell cycle inhibitors p16 INK and p21 CIP and was accompanied by p53-dependent cell cycle arrest.
Experiments in which an activated mutant of Raf was conditionally expressed indicated a mechanism in which low levels of MAP kinase activation result in a proliferative response whereas high levels of MAP kinase activation is accompanied by cell cycle arrest (Woods et al., 1997). These data indicate that the effects of activation of Ras or of expression of activated mutants of Ras can result in different cellular responses, strongly dependent on cellular context.

In addition to cell proliferation, a role for Ras in cell differentiation has been established, using the rat pheochromocytoma cell line PC12 as a model system. In PC12 cells, microinjection of activated p21 Ras leads to neuronal differentiation in the absence of NGF (Bar-Sagi and Feramisco, 1985). In addition to this in vitro differentiation system, transgenic mouse models, which overexpress dominant negative Ras proteins in the B- and T-cell compartments revealed the requirement for Ras in lympho-hematopoietic development. Perlmutter and co-workers demonstrated that Ras activation is required early in the development of B-lymphocytes since the dominant negative transgene inhibited the Ras-mediated Raf-activation necessary for the transition from a pre-pro-stage to the proB-cell progenitor (Iritani et al., 1997), according to the Hardy-scheme of B-cell development (Hardy et al., 1991). In contrast, Ras activation in the T-cell compartment is necessary at a later stage since the dominant negative transgene impaired positive selection but not negative selection of thymocytes (Swan et al., 1995). Using the same mouse model, Yamashita and co-workers provided evidence that Ras-mediated activation of the MAP kinase pathway is required for antigen-induced Th2-cell differentiation (Yamashita et al.,
1999). Given the wide variety of stimuli and cellular responses connected to Ras activation, targeted disruption of Ras genes was expected to result in severe phenotypes. Mice with homozygous deletion of the K-Ras gene died between day 12 and 14 of gestation with severe defects in liver and neuronal development as well as signs of anemia (Johnson et al., 1997; Koera et al., 1997). In contrast, targeted disruption of the H-Ras and N-Ras genes resulted in mice that developed normally, probably indicating a high degree of redundancy between the Ras isoforms (Ise et al., 2000; Umanoff et al., 1995).

Whereas most of the work on Ras has focused on its effects on proliferation and differentiation, more recent work is documenting pro-apoptotic characteristics. Under circumstances of cellular stress, like loss of adhesion or overexpression of c-myc (Kauffmann-Zeh et al., 1997; McGill et al., 1997), activated Ras functions in a pro-apoptotic manner in several systems. Activated Ras has also been shown to render TNF-resistant fibroblasts susceptible to TNF-induced apoptosis (Trent et al., 1996). Other studies have implicated Ras in Fas- and ceramide-induced apoptosis (Basu et al., 1998; Gulbins et al., 1995; Gulbins et al., 1996). The mechanisms behind Ras-mediated apoptosis are mostly unknown. Involvement of the recently identified pro-apoptotic Ras-effector RasSFI remains to be demonstrated. In different experiments Joneson and co-workers implicated the level of Ras signaling in the induction of apoptosis. Whereas low levels of activated Ras induced only low levels of apoptosis, increased amounts of microinjected constitutively active Ras induced apoptosis in an Erk- and JNK-
dependent manner that could be prevented by co-injection of activated Rac and which correlated
with NFκB activation (Joneson and Bar-Sagi, 1999).

1.7 The non-classical Ras family members

Whereas most of the work on Ras has focused on the classical p21 Ras proteins H-, N-
and K-Ras4A/B, relatively little is known about the non-classical Ras proteins Rap, Ral, R-Ras,
Tc21 and M-Ras. These proteins share significant sequence homology with the classical p21 Ras
proteins (40-60% sequence identity), including conserved characteristic features like the switch I
and switch II regions and the C-terminal CAAX-motif.

1.7.1 Rap

Rap1 was originally identified as a protein that reversed the transformed phenotype of
fibroblasts expressing an activated K-Ras and thus termed K-rev (Kitayama et al., 1989). In
contrast to Ras, Rap proteins are predominantly localized on endocytic and lysosomal vesicles
(Pizon et al., 1994). This intracellular localization may also explain why Rap is activated by
several second messenger molecules such as Ca^{2+}, DAG and cAMP (Franke et al., 1997; M'Rabet
et al., 1998; McLeod et al., 1998; Zwartkruis et al., 1998). The initial finding by Kitayama and
co-workers that Rap1 reverted the transformed phenotype of K-Ras transformed cells led to the
hypothesis that Rap acts as a Ras-antagonist by competing for effector proteins. Indeed, the
effector domains of Ras and Rap are highly conserved (Bos, 1998) and Ras effector proteins such as Raf-1 and RalGDS-family members have been shown to interact with GTP-bound Rap (Kishida et al., 1997; Spaargaren and Bischoff, 1994; Woltius et al., 1996). Further support for the 'competition-model' came from observations that constitutively active RapG12V inhibited LPA-induced, Ras-dependent Erk activation (Cook et al., 1993), and that insulin treatment of CHO cells overexpressing the insulin receptor resulted in a decrease of Raf-1 associated with Rap1 and a concomitant increase of Raf-1 associated with Ras (Okada et al., 1998). However, more recent studies suggest that endogenous Rap might have a function different from antagonizing Ras. For example, Rap is rapidly activated in response to several stimuli that activate Raf-1, such as EGF and PDGF, without compromising Erk-activation (Zwartkruis et al., 1998). In PC12 cells, Rap was found to be important for a sustained, B-Raf-mediated Erk-signal in response to NGF leading to differentiation, whereas Ras was involved in mediating rapid and transient Erk-activation in response to EGF, resulting in a proliferative response (York et al., 1998). In addition, a number of recent reports implicate Rap1 as an important component in the regulation of integrin affinity (Arai et al., 2000; Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Schmidt et al., 2001; Tsukamoto et al., 1999).

Whereas most of the data on Rap address the function of Rap1, only very little is known of Rap2. Rap1 and Rap2 share an identical set of effector proteins, GEFs and GAPs. However, Rap2 is relatively insensitive to GAPs and as a result has an exceptionally high ratio of GTP-bound versus GDP-bound proteins (>50%). Based on these data, Matsumoto and co-workers
suggest a model in which Rap1 and Rap2 function as the fast and slow molecular switches of the same signaling pathways (Ohba et al., 2000).

1.7.2 Ral

RalA and RalB are two closely related Ras-like GTPases with 58% amino acid homology (Zhao and Rivkees, 2000). Interest in Ral proteins heightened in 1994 when guanine nucleotide exchange factors for these GTPases were found to be effectors of p21 Ras (Kikuchi et al., 1994; Spaargaren and Bischoff, 1994). Exchange factors for Ral include the Ras-binding domain containing proteins RalGDS (Albright et al., 1993), Rgl (Kikuchi et al., 1994), Rlf/Rgl2 (Wolthuis et al., 1996), RPM/Rgl3 (Ehrhardt et al., 2001; Shao and Andres, 2000), the PH-domain containing RalGPS/RalGEF2 (de Bruyn et al., 2000; Rebhun et al., 2000) and the Rgr protein, the GEF portion of the Rsc fusion protein (D'Adamo et al., 1997). Unlike p21 Ras, constitutively active mutants of Ral are not transforming; however, they strongly enhance the transforming activities of activated mutants of Ras and Raf-1. In addition, dominant negative mutants of Ral strongly inhibit the transforming activities of activated mutants of Ras and Raf-1 (Urano et al., 1996).

How Ral mediates its effects remains largely unknown. Active, GTP-bound Ral interacts with RalBP1, a protein possessing GAP activity towards the Rho-family GTPases Rac and Cdc42 (Cantor et al., 1995). In other experiments, PLD could be co-immunoprecipitated with Ral from the lysates of src-transformed fibroblasts (Jiang et al., 1995). Later, this interaction was
demonstrated to be direct (Luo et al., 1997). Whereas the Ras-binding domains of RalGDS-like GEFs place Ral downstream of Ras, Ras-independent but Ca$^{2+}$-dependent Ral activation has also been demonstrated (Hofer et al., 1998).

More recent observations suggest the involvement of Ral in a variety of cellular functions such as chemotactic migration (Suzuki et al., 2000), receptor endocytosis (Nakashima et al., 1999) and regulation of the cell cycle (Henry et al., 2000). Interestingly, data have been presented that place Ral upstream of the src tyrosine kinase in response to EGF and insulin treatment of cells (de Ruiter et al., 2000; Goi et al., 2000). The mechanism by which Ral exerts this regulatory function remains to be established.

1.7.3 R-Ras and TC21/R-Ras2

R-Ras and TC21/R-Ras2 form a distinct branch of the Ras subfamily (see Figure 3.2). R-Ras was originally identified based on its high homology to p21 Ras (55%) (Lowe et al., 1987). Like p21 Ras, R-Ras interacts with Raf-1, RalGDS and PI3-kinase. However, activated R-Ras leads only to activation of Akt, but not to activation of Erk and Ral which are downstream of Raf and RalGDS, respectively (Marte et al., 1997; Urano et al., 1996). Activation of JNK by R-Ras has been reported, but this finding is controversial (Mochizuki et al., 2000). In addition to Raf1, RalGDS and PI3-kinase, R-Ras has also been reported to interact with Nore1 and Bcl-2 (Fernandez-Sarabia and Bischoff, 1993; Oertli et al., 2000). The identification of Bcl-2 as a protein binding to R-Ras prompted work investigating the involvement of R-Ras in apoptosis.
Contradictory results have been obtained in which R-Ras protected the IL-3 dependent cell-line Ba/F3 from growth factor deprivation (Suzuki et al., 1997), whereas it enhanced apoptosis in response to growth factor withdrawal in the IL-3 dependent cell line 32D (Wang et al., 1995). Similar to the controversy concerning R-Ras function in JNK activation and apoptosis are data concerning the transforming activities of R-Ras. Whereas Cox and co-workers reported transformation of NIH3T3 fibroblasts by an activated mutant of R-Ras in the absence of a transformed morphology (Cox et al., 1994), others noticed a transformed morphology of NIH3T3 cells expressing activated R-Ras indistinguishable from those expressing activated p21 Ras (Huff et al., 1997).

Although R-Ras function remains poorly understood, several characteristics distinguish it from p21 Ras. Thus, unlike p21 Ras, dominant negative mutants of R-Ras are not growth inhibitory in fibroblasts (Huff et al., 1997). Also, unlike p21 Ras which inhibits skeletal myogenesis, constitutively active mutants of R-Ras promote skeletal myogenesis (Suzuki et al., 2000). However, the most striking effects of R-Ras are directed towards cell adhesion. It has been shown that p21 Ras reduces integrin activity (Hughes et al., 1997). In contrast, R-Ras promotes integrin activity (Zhang et al., 1996). An intact effector loop as well as a proline-rich motif at the C-terminus of R-Ras that serves as a recognition sequence for the adapter molecule Nck was shown to be crucial for R-Ras mediated integrin regulation (Oertli et al., 2000; Wang et al., 2000).
TC21/R-Ras2 is most closely related to R-Ras (70% amino acid identity) (Drivas et al., 1990). Unlike R-Ras, constitutively active mutants of TC21 have strong transforming activity on fibroblasts and epithelial cells. Furthermore, constitutively active TC21 has been shown to inhibit skeletal myogenesis and to induce differentiation of PC12 cells, whereas dominant negative mutants of TC21 proved to be growth inhibitory in fibroblasts (Graham et al., 1994; Graham et al., 1999; Graham et al., 1996; Movilla et al., 1999). These functions are very similar to those of p21 Ras. Indeed, activated TC21 activates the MAP kinases Erk, JNK and p38 (Graham et al., 1999). Earlier findings that TC21 failed to bind to and activate Raf remain controversial (Graham et al., 1996) since more recent studies demonstrated an involvement of Raf in TC21 signal transduction (Rosario et al., 1999). A TC21-specific function has not been established thus far.

1.7.4 Other non-classical Ras-like GTPases

Besides the above mentioned non-classical Ras-like GTPases, this group also encompasses the proteins Rin, Rit, Ric (Lee et al., 1996; Wes et al., 1996), Kir (Cohen et al., 1994), Rad (Reynet and Kahn, 1993), Rem (Finlin and Andres, 1997), Rheb (Yamagata et al., 1994) and κB-Ras1/2 (Fenwick et al., 2000). Most of these monomeric GTP-binding proteins display a lower degree of homology in critical domains like the switch I and switch II regions to the classical p21 Ras proteins than the non-classical Ras proteins Ral, Rap, R-Ras, TC-21 and
M-Ras. Also, with the exception of Rheb, all of these proteins lack the C-terminal CAAX-motif. This group of proteins remains essentially uncharacterized.

1.7.5 M-Ras/R-Ras3

The observation that in some tumors, like those of the breast, and in a number of lymphoid disorders, mutations of p21 Ras proteins occur at an extremely low frequency led us to speculate that this might reflect the presence of a new thus far undescribed member of the Ras family. A homology search of the EST-database revealed the existence of such a protein, termed M-Ras. In chapter III, the identification and characterization of M-Ras will be described. A characterization of the effector proteins recognized by M-Ras, including the identification of a novel member of the RalGDS family, will be presented in chapter IV.
CHAPTER II: MATERIALS AND METHODS

2.1 Methods used for Chapter III

2.1.1 Materials.

Plasmids encoding the GST-fusion proteins GST-Raf-1-RBD and GST-RalGDS-RBD were kindly provided by Dr. D. Shalloway (Cornell University, New York, USA) and Dr. J. Bos (Utrecht University, Netherlands), respectively. Src Y527F was obtained from Dr. J. Cooper (Fred Hutchinson Centre, Seattle, USA) and N-Ras Q61K from Dr. R. Kay (The Terry Fox Laboratory, Vancouver, Canada). Biotinylated or native antibodies against the HA-epitope (12CA5) were purchased from Boehringer Mannheim (Boehringer Mannheim, Laval, Quebec, Canada) and anti-myc-epitope antibody 9E10 was kindly provided by Dr. H. Ziltener (The Biomedical Research Centre, Vancouver, Canada).

2.1.2 Cloning of M-Ras

The EST-database was searched for cDNAs encoding novel Ras family members using the highly conserved switch I and switch II regions as templates. A clone with an open reading frame of 627 nt encoding a novel Ras family member was discovered.
2.1.3 Generation of antisera

Rabbits were injected with peptides corresponding to residues 187 - 204 (KKKTKWRGDRATGTHKLQ, pep I) and 142-159 (KEMATKYNIPYIETSAKD, pep II) of M-Ras, respectively. Both peptides were fused to the C-terminus of the tetanus toxoid epitope QYIKANSKFIGITEL (Panina-Bordignon et al., 1989). For affinity-purification of the polyclonal antisera, the peptides were covalently bound to NHS-activated Sepharose (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québéc, Canada). Antibodies bound to the affinity-purification column were washed with 10 column volumes of PBS, eluted with 3 ml of 100 mM glycine (pH 2.5) and dialyzed against 2 l of PBS over night.

2.1.4 Cell Culture, Transfections and Retroviral Infections

All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 50 units/ml streptomycin (Stem Cell Technologies, Vancouver, Canada) and 10% fetal calf serum (R6-X cells, HEK293 cells and BOSC23 cells) or 10% calf serum (NIH3T3 fibroblasts). R6-X, an IL-3 dependent bipotential, mast-cell/megakaryocyte line (Schrader and Crapper, 1983) was passaged in the presence of medium conditioned by the cell-line WEHI-3B (2% of 10-fold concentrated medium, W3) as a source of IL-3. Retroviruses for retroviral infections of R6-X cells or NIH3T3 cells were produced by transient transfection of BOSC-23 cells with the corresponding retroviral constructs. After 36 hours, the medium on the transfected packaging cells was replaced and the fresh
medium taken off the cells after an additional 24 hours. This supernatant containing infectious retroviral particles was sterile filtered and applied to the target cells in the presence of 10 μg/ml polybrene. After 10 hours the R6-X cells were washed and resuspended in medium lacking IL-3. In the case of NIH3T3 cells, and in some experiments R6-X cells, infected clones were selected in puromycin at 1 μg/ml (Sigma, Mississauga, Ontario, Canada). Selection for drug resistant clones of R6-X cells was performed in the presence of 2% W3 as a source of IL-3. Analysis of growth and survival of polyclonal or clonal populations of virally infected R6-X cells involved cell-counting, assessment of uptake of 3,4,5-dimethyltiazole-2,5-diphenyltetrazolium bromide (MTT), or clonal growth in agar. For assessment of growth or survival using MTT, cells were plated at 5000 cells per well, in 96-well, flat-bottomed plates, in the absence of IL-3. MTT uptake was assessed 3 days later. Colony assays were performed by plating cells in medium and indicated growth factors in a final concentration of 0.3% agar (Difco, Mi, USA). R6-X cells were cloned by picking colonies from agar. In some cases populations were enriched in EGFP-expressing cells by fluorescence-activated cell sorting using a FACSCalibur instrument. Experiments were performed on retrovirally infected R6-X cells derived from 2 independent infections with consistent results. For cell counting experiments of NIH3T3 fibroblasts, 2000 cells/well were plated into 6-well dishes in triplicate. The cells were removed from the dishes at the indicated time points by treatment with 0.05% trypsin/0.53 mM EDTA and counted using an improved Neubauer hemacytometer.
2.1.5 Generation of Mutations by Site-Directed Mutagenesis

The M-Ras cDNA was subcloned into pBluescript for site-directed mutagenesis and sequencing. To generate the constitutively active mutants M-Ras G22V and M-Ras Q71L, as well as the dominant negative mutant M-Ras S27N, the codons 22 (GGT), 71 (CAG) and 27 (AGT) were mutated to 'GTT' (codon 22), 'TTG' (codon 71) and 'AAT' (codon 27) by standard PCR techniques. Constructs were cloned with N-terminal myc- or HA-epitope tags into the eukaryotic expression vectors pEF-BOS or pcDNA3.1, or, for retroviral infections, into pMX-PIE (a gift of Dr. Alice Mui, DNAX, San Francisco, USA). This vector drives expression of the enhanced green fluorescent protein (EGFP) from an IRES-element downstream of the Ras cDNA. Identities of all constructs were verified by DNA-sequencing. DNA sequencing was performed by the DNA sequencing laboratory (University of British Columbia, Vancouver, BC, Canada).

2.1.6 Northern Blot Analysis and RT-PCR

Northern blots on multiple tissue northern blots were performed according to the manufacturers instructions (CLONTECH, Palo Alto, California, USA) with a probe corresponding to the 120 carboxy-terminal amino acids of M-Ras. RT-PCR was performed on 2 µg of total RNA from the indicated cell lines. Oligonucleotides used were GTGAGTGGCCGGTGGCCCTGTCTCCCTCGC (RT-reaction), TTTGGTTGCCATTTCTTTTCCTTGGTCCC (PCR antisense),
ACCAGCGCTGTCCAAAGTGAACCTTCC (PCR sense),
CACATATAAACTAGTATGGGTGGGATGG (nested PCR sense),
CTTAGGTGATCAGATCCACCTTGTG (nested PCR antisense).

As negative control, the RT-reaction was performed on 2 μg of tRNA. The identities of the amplified fragments were verified by a restriction digest.

2.1.7 Reporter Gene Assays

HEK293 cells were plated at a density of 2.5x10^6 cells/6cm dish 24 hours prior to transfection using the Lipofectamine reagent (Canadian Life Technologies, Burlington, ON, Canada). A ratio of 5 μl Lipofectamine per 1 μg plamid DNA was used. The cells were co-transfected with the indicated plasmids plus 0.25-0.5 μg of the luciferase reporter gene under control of the full length c-fos promoter. Cells were harvested 40 hours after transfection and lysed in 200 μl of lysis buffer (Promega, Madison, Wisconsin, USA). Luciferase activity was determined with a luminometer (Dynex, Chantilly, VA, USA) and the values normalized to total protein using BCA Protein Reagent (PIERCE, Rockford, Illinois, USA).

2.1.8 Preparation of GST-fusion proteins

The Ras-binding domains of RalGDS and Raf-1 fused to glutathione S-transferase were expressed and purified by affinity chromatography. A 5 ml culture of the E. coli strain DH5α transformed with the plasmid encoding the GST-fusion protein was grown overnight in LB
containing 100 μg/ml ampicillin. This culture was used to inoculate a 1 liter culture of the same medium the next day. Following inoculation, this culture was grown until it reached an OD 600 of 0.3-0.4. The culture was then placed on ice for 30 minutes before addition of IPTG to a final concentration of 0.1 mM and further incubation at 26 °C for 2-3 hours. The cells were pelleted at 3,000 rpm for 15 min and resuspended in 10 ml of resuspension buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM 2-ME, 10 μg/ml soybean trypsin inhibitor, 40 μg/ml PMSF). The bacteria were then treated with 50 μl of lysozyme (10 mg/ml) and incubated on ice for 30 minutes, followed by addition of 1 ml of 10% NP-40 and three freeze-thaw cycles between liquid nitrogen and a 37 °C water bath. The bacterial DNA was disrupted by three brief sonication pulses, the bacterial lysate aliquoted into Eppendorf tubes and centrifuged for 10 minutes at maximum speed. The resulting supernatant was frozen at -70 °C. Aliquots were subsequently thawed and incubated with 300 μl of a 50% slurry of glutathione Sepahrose beads (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Quebéc, Canada) at 4 °C for 2 hours. The beads were washed extensively with ice-cold PBS to which an additional 350 mM NaCl and 5 mM 2-ME was added and finally resuspended in the corresponding affinity precipitation buffer.
2.1.9 Immunoblotting, immunoprecipitation, affinity precipitation and cell fractionation

Transiently transfected HEK293 cells were washed in PBS and lysed in 500 μl of 1x lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 5mM EDTA, 10 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor, 0.7 μg/ml pepstatin and 40 μg/ml PMSF). Proteins were denatured by adding 5x Laemmli's sample buffer (10% SDS, 50% glycerol, 200 mM Tris-HCl pH 6.8, bromphenol blue) and then boiling for 3 minutes. Denatured proteins were subjected to SDS-PAGE, run in 1x running buffer (25 mM Tris, 192 mM Glycine, 0.1 mM SDS) at 75 volts for 30 minutes, then at 150 volts until the bromphenol blue dye front had run off. Proteins were transferred to nitrocellulose (0.45 μm pore size, Schleicher & Schuell, Germany) at a constant amperage of 0.8 mA/cm² on a semi-dry transfer apparatus (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada) in 1x transfer buffer (39 mM Glycine, 48 mM Tris, 0.0375% SDS (w/v), 20% methanol (v/v). The membranes were stained with Ponceau S (Sigma, Oakville, ON) to visualize molecular weight markers (BioRad) followed by blocking in 5% BSA (Sigma, Oakville, ON) plus 1% ovalbumin (Sigma, Oakville, ON) in 1x Tris buffered saline (20 mM Tris, 150 mM NaCl, pH 7.5, TBS) for 1 hour at room temperature or overnight at 4°C. Primary antibodies 12CA5 (0.2 μg/ml), 9E10 (ascites, 1:1000 dilution), Y13-259, (10 μg/ml) and anti-Ras sera (1:125) were diluted in TBS / 1% BSA and incubated overnight at 4°C. Following three washes with TBS-N (TBS, 0.05% NP-40), secondary antibody coupled to horseradish peroxidase (DAKO, Denmark) was added at a 1:10000 dilution in TBS-N for 1 hour at room temperature. Following three more washes with TBS-N, the HRP-signal was detected using the
enhanced chemiluminescence (ECL) reagent according to the manufacturer's instructions (Amersham, Oakville, ON). To decrease the Ig light chain signal in Ras immunoprecipitations using Y13-259, we used secondary antibodies recognizing specifically the Ig heavy chain.

To immunoprecipitate endogenous p21 Ras and M-Ras, 15 μg of Y13-259 were incubated with 1.5 mg of the indicated tissue lysates or 500 μg of NIH3T3 lysate for 45 minutes at 4C° under constant rotation in the presence of protein G-sepharose beads (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Quebec, Canada). The beads were washed three times with lysis buffer before boiling in Laemmli's 1.5x sample buffer (2% SDS, 10% glycerol, 40 mM Tris-HCl, pH 6.8, bromphenol blue).

Affinity purifications to assay binding of M-Ras to the Ras-binding domains of Raf-1 and Ral-GDS were performed as described by Taylor et al. (Taylor and Shalloway, 1996). Briefly, GST-fusion proteins were affinity-purified with glutathione beads from lysates of E.coli. 15 μl of packed bead volume (approximately 10 μg) was mixed for 40 min with lysates of HEK293 cells expressing the indicated proteins in 25 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.25% sodium deoxycholate, 1% NP-40, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 1 mM NaVO₄, 10 μg/ml leupeptin and 10 μg/ml aprotinin. The beads were washed with lysis buffer, boiled in 1.5x Laemmli's buffer and the eluted proteins resolved on 15% denaturing polyacrylamide gels.

Cell fractionations were performed by suspending cells in homogenization buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 μg/ml each of leupeptin, aprotinin, soybean trypsin
inhibitor, 0.7 μg/ml pepstatin and 40 μg/ml PMSF). Cells were broken by brief sonication, the nuclei were pelleted and the supernatant subjected to ultra-centrifugation for 20 min at 150,000 x g. The resulting supernatant was designated cytosolic fraction (c) and the pelleted fraction membrane enriched fraction (m).

2.2 Methods used for Chapter IV

2.2.1 Materials

The cDNAs for Raf-1 and H-Ras were provided by Dr. I. Sadowski (University of British Columbia, Vancouver, Canada) and Dr. A. Ambrosini (DiBiT, H. San Raffaele, Milan, Italy), respectively. Plasmids encoding Rlf and the GST-fusion protein GST-RalGDS RBD were kindly provided by Dr. J. Bos (Utrecht, The Netherlands).

2.2.2 Tissue culture

HEK293 cells and NIH3T3 fibroblasts were cultured as described in 2.1.4. For MTT-assays, NIH3T3 fibroblasts were plated at 500/well in 96-well plates and 10 μl MTT (5 mg/ml) was added at the indicated time points. The cells were incubated with MTT for 4 hours at 37 °C before addition of an equal volume of lysis buffer (10% SDS, 50% dimethylformamide) and further incubation at 37 °C overnight. The assay was quantitated using an ELISA reader set at 550 nm.
2.2.3 Two-hybrid Screen

An oligo dT primed NIH3T3 library (Clontech, Palo Alto, CA) was screened with constitutively active M-Ras G22V lacking the C-terminal CAAX-motif. This cDNA was cloned into the ‘bait’ vector pAS2-1 downstream of the Gal-4 DNA binding domain. The screen was performed using the Matchmaker Two hybrid system (Clontech, Palo Alto, CA) according to the manufacturer’s manual. Briefly, 1 mg of M-Ras G22V in pAS2-1 was co-transformed with 0.5 mg of the library plasmids and 20 mg of salmon sperm carrier DNA and plated onto 15 cm plates lacking His/Leu/Trp. After 7 days, colony lift assays were performed to screen for colonies with LacZ-activity. Paper filters (VWR grade 410) were placed onto the plates for 3 minutes and subsequently immersed in liquid nitrogen for 30 seconds. Following that, the filters with attached yeast colonies were placed on blank filters soaked in Z-buffer (Na$_2$HPO$_4$ 60 mM, NaH$_2$PO$_4$ 35 mM, KCl 10 mM, MgSO$_4$ 1 mM) with added β-mercaptoethanol (40 mM final concentration) and X-Gal (1.67 ml of 20 mg/ml stock solution). The appearance of blue color as LacZ readout was monitored after overnight incubation at room temperature. 45 LacZ$^+/$/His$^+$ colonies were identified and the ‘prey’ plasmids of these colonies were isolated after growing the colonies on cycloheximide (1µg/ml) containing plates lacking Trp. After confirming that these clones interacted with active M-Ras G22V but not with dominant negative M-Ras S27N in a yeast mating assay, the inserts of the prey plasmids were analyzed by DNA sequencing. They contained (partial) cDNAs of several known Ras-effector proteins as well as the 3’ UTR and the C-terminus of one novel protein. From the full length cDNA of this protein (see 2.2.4), several
mutants were generated. A membrane localized mutant of RPM was generated by attaching the sequence coding for the C-terminal 27 amino acids of H-Ras and M-Ras, respectively, to the C-terminus of RPM and a mutant deficient in binding to Ras was generated by deleting the C-terminal 143 residues of RPM. The constructs were epitope-tagged at the N-terminus with the c-myc or the HA epitope and cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA).

2.2.4 5'RACE amplification of full length RPM

5' RACE was performed on an oligo dT-primed library of NIH3T3 fibroblasts using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer’s manual. Two rounds of nested PCR (30 cycles at 59 °C with 2 min extension at 72 °C) were performed using the adapter primers AP1 and AP2 and the gene-specific antisense primer pairs AS1, AS2 and AS4, AS5, respectively. The 5' RACE reactions were cloned into pBluescript, plated onto five 15 cm LB/amp plates (50 μg/ml ampicillin) and screened for RPM containing clones using a filter-lift assay (see 2.2.5) with PCR-generated probes using the primer pairs S1/AS3 for the first round of 5' RACE and S2/AS6 for the second round of 5' RACE. 20 clones that gave a strong signal were amplified, the plasmid DNA purified and 5 clones with the longest inserts were analyzed by DNA sequencing. Primer sequences used for 5'RACE reactions are:
2.2.5 Colony filter lift assay

For filter lift assays, bacteria transformed with plasmids containing the 5' RACE reactions were plated onto 15 cm LB/amp plates. Nylon membranes (Schleicher and Schuell, Dassel, Germany) were placed on the plates for 5 min. After removal of the membranes from the bacterial plates, the membranes were subsequently placed onto filter paper soaked with 10% SDS for 3 minutes, denaturing solution (500 mM NaOH / 1.5 M NaCl) for 5 minutes and neutralizing solution (500 mM Tris-HCl pH 7.0 / 1.5 M NaCl) for 5 minutes. The membranes were then air-dried and the DNA UV-crosslinked. For detection of positive clones, the membranes were incubated for 60 min at 65 °C in prehybridization buffer (500 mM Na₂HPO₄ / 7% SDS / 1 mM EDTA, pH 7.2) followed by overnight incubation under the same conditions with a ³²P-dCTP labeled, PCR-generated probe (nt 1592-2090 of RPM for first round of 5'RACE,
primers S1/AS3 and nt 891-1411 of RPM for second round of 5'RACE, primers S2/AS6).

Following hybridization, the membranes were washed two times 10 minutes at 65 °C in 500 mM Na$_2$HPO$_4$ / 1% SDS, and subsequently in 250 mM Na$_2$HPO$_4$ / 1% SDS, 100 mM Na$_2$HPO$_4$ / 1% SDS and 50 mM Na$_2$HPO$_4$ / 1% with a final high stringency washing step in 1x SSC / 0.01% SDS. The hybridized probe was detected by autoradiography after 6 hours exposure at room temperature. Primer sequences used for generation of radiolabeled probes are:

S1  5'-CCGAGAGAAGAACTCATCTCCTCTCCTG-3'

AS3  5'-AAGCAAGAAGTCACCAGGGGCAG-3'

S2  5'-ACCGTAGCCCAGTTCAATACGGTG-3'

AS6  5'-TGGCAGCGTCTGCTGTAGCTGCTGG-3'

2.2.6 Northern Blot Analysis

A multi-tissue northern blot (Clontech, Palo Alto, CA) of 2 μg of polyA-purified mRNA of various murine tissues was probed with a radiolabeled probe corresponding to nt 1584-2060 of the RPM coding sequence according to the manufacturer’s manual. The hybridized probe was detected by autoradiography after overnight exposure at -80 °C using an intensifying screen.
Transient Transfection and Reporter Gene Assays

For transient transfections, HEK293 cells were plated at a density of 2x10⁶ cells per 6 cm dish 24 hours prior to transfection. The cells were transfected with a total of 3.5 μg of DNA using the Lipofectamine reagent (Gibco BRL, Burlington, ON). For luciferase assays, HEK293 cells were transfected with 0.5 μg of the reporter gene expression plasmids Gal-4/Elk and 5xGal4/luciferase (PathDetect, Stratagene, La Jolla, CA) at a ratio of 1:2 plus 3 μg of the indicated constructs. Empty vector (pcDNA3.1) was added where necessary to transfect equal amounts of DNA. Cells were harvested 40 hours after transfection and lysed in 250 μl lysis buffer (Promega, Madison, WI). Luciferase activity was determined using a luminometer (Dynex, Chantilly, VA) and the values normalized to total protein using the BCA Protein Reagent (Pierce, Rockford, IL). Protein expression was verified by western blotting. In some experiments, 100 ng of the GFP-expressing vector pEGFP-C1 (Clontech, Palo Alto, CA) was included as control to examine potential cytotoxic effects of RPM.
2.2.7 Western blots and immunoprecipitations

Transiently transfected HEK293 cells expressing myc-tagged RPM constructs and HA-tagged Ras constructs were lysed in 750 μl of 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10 mM MgCl₂, 1 mM EDTA in the presence of a protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada). Western blots were performed as described in 2.1.8. For immunoprecipitations, 2.5 μg of anti-HA antibody 12CA5 was added and the lysate incubated for 30 minutes at 4°C under constant rotation in the presence of protein G Sepharose (Amersham Pharmacia Biotech Inc., Baie d’Urfé, Quebec, Canada). The beads were washed three times with lysis buffer before boiling in Laemmli’s 1x sample buffer (2% SDS, 10% glycerol, 40 mM Tris-HCl, pH 6.8, bromphenol blue). Denatured proteins were subjected to SDS-PAGE and immunoblotted with the indicated antibodies as described in 2.1.8.

2.2.8 Affinity precipitation assays

For affinity precipitation assays, GST-fusion constructs were generated by PCR-amplifying the Ras-binding domains of Nore1 (nt 570-1245), AF-6 (nt 1-646), and RPM (nt 1750-2127) and cloning them into the procaryotic expression vector pGEX 4T2 (Amersham Pharmacia Biotech Inc., Baie d’Urfé, Quebec, Canada). Affinity precipitations to assay the binding of M-Ras and p21 Ras to various Ras-binding domains were essentially performed as described in 2.1.8 with the difference that the GST-fusion protein was kept as the limiting
component (1 µg recombinant GST-fusion protein of various Ras-binding domains pre-coupled to glutathione beads).
CHAPTER III: IDENTIFICATION AND CHARACTERIZATION OF M-Ras, A NOVEL MEMBER OF THE RAS-SUPERFAMILY OF SMALL GTPASES

3.1 Cloning of M-Ras

Activating mutations of p21 Ras proteins have been implicated in about 30% of all human cancers. Whereas some cancers like pancreatic carcinomas have an incident rate of close

GTGGGAAGGGCCCATAGCAGGCCGTTAGACAGACTCCTTTGATTTGAGACATCTCGCTGGGCCGGGCTACTACCAAGAAC
ATGCCGACCAGCGCTTTGCTTCCAATGAGGTCCCCCTACTATATCAACATCTAGTGTGCTGCTGATGGAGATGGTGGTGTGGGCAGAGTGCGCTCACT
MATSAVPSENLPTYKVVGDGGVGKSALT

IQQFFQKIFVDPYDPTIEDIYSYLKHTEIDNQW

GCCATTTTGATGTTCGGACACAGCCGGAGAGTGCACTGGGACACATCCACCTTACATTCAAGGAGAGAAGGAGTGGCTCACTTCCCA
VYSVTDKASFEHVDRFHQLILRVRKDRESFP

ATGATCTCTGTGGGAACTCGGACACAGCCGGAGAGTGCACTGGGACACATCCACCTTACATTCAAGGAGAGAAGGAGTGGCTCACTTCCCA
MILVANKVDLMHLRKVTRDGKATKYYN

Figure 3.1 DNA and protein sequence of M-Ras. The initiating methionine is underlined and in-frame stop codons are shown in bold. Sequences corresponding to the switch I and switch II regions are shaded light grey and medium grey, respectively. The C-terminal CAAX-motif is boxed and sequences encoding the poly-lysine stretch are double underlined.
to 100%, others, like carcinomas of the breast and prostate have an extremely low rate of activating mutations of p21 Ras (Bos, 1989; Sweetenham, 1994). This prompted us to search the EST-database for novel Ras family members, which might compensate for the apparent absence of mutated p21 Ras proteins in these tissues. Using the highly conserved switch I and switch II regions of Ras in a search of the EST database, we identified a novel Ras family member, termed M-Ras (Ehrhardt et al., 1999).

We also identified a homologous protein in the *C. elegans* database. M-Ras exhibits a high degree of homology with other members of the Ras family, with 47% identity with H-Ras and 52% with R-Ras2/Tc21. While it is identical in the switch I region (residues 42-48) it displays 3 conserved exchanges in the switch II region (residues 70-86). Significantly, none

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**Figure 3.2** M-Ras is a highly conserved small GTPase. The dendrogram was generated using a Clustal V alignment of the indicated GTPases and the Phyllips Distance Methods Algorithm. Sequences are those of murine proteins except where otherwise indicated.
of the residues in switch II, which have been shown to be involved in binding of Y13-259 to p21 Ras (Sigal et al., 1986), differed between H-Ras and M-Ras. Analysis of the evolutionary relatedness of M-Ras and its *C. elegans* homologue with other members of the Ras family showed that M-Ras formed a discrete branch of this phylogenetic tree.

Comparison of the carboxy-terminal sequences of the murine and *C. elegans* M-Ras homologues with those of p21 Ras, R-Ras and R-Ras2, indicated that M-Ras had distinctive characteristics. Figure 3.3 shows alignments of the translated protein sequences of the C-termini of M-Ras and its *C. elegans* homologue with those of other members of the family of small GTPases. In contrast to R-Ras and R-Ras2, M-Ras and its *C. elegans* homologue lack the

<table>
<thead>
<tr>
<th></th>
<th>183 C-terminus</th>
<th>208</th>
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<tbody>
<tr>
<td>M-Ras (Ce)</td>
<td>SMASVPRTKKRDKG----KCLIS</td>
<td></td>
</tr>
<tr>
<td>M-Ras</td>
<td>NQKKKKKTKWRGDRATGTHKLOCVIL</td>
<td></td>
</tr>
<tr>
<td>R-Ras</td>
<td>QEQLPPSAPRKKDDGGCVL</td>
<td></td>
</tr>
<tr>
<td>R-Ras2 (Hs)</td>
<td>QEQECPPSPEPTEKDKGCCHCVIF</td>
<td></td>
</tr>
<tr>
<td>H-Ras</td>
<td>KLRKLNPPPDESGPGCMS---KCVLS</td>
<td></td>
</tr>
<tr>
<td>N-Ras</td>
<td>RLKKNSSDDGTQQCMS---PCVLM</td>
<td></td>
</tr>
<tr>
<td>K-RasA</td>
<td>RLKIK SKEETPGCVIKK---PCVLM</td>
<td></td>
</tr>
<tr>
<td>K-RasB</td>
<td>K-EKM-KDGKKKKSRT--PCVLM</td>
<td></td>
</tr>
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</table>

**Figure 3.3** Comparison of the amino acid sequences of the C-termini of various Ras proteins. All sequences are murine with the exception of R-Ras2/Tc21 (human) and the *C. elegans* orthologue of M-Ras. Gaps are indicated by a hyphen (-). The CAAX-motifs are boxed. Shaded areas indicate regions of homology between M-Ras and its *C. elegans* ortholog (dark gray), the proline-rich R-Ras box (medium gray) and conserved regions in H-Ras, N-Ras and K-RasA (light gray). Poly-lysine motifs in M-Ras and K-RasB are underlined. Cysteine residues that might be palmitoylated are double underlined. *C. elegans* sequence accession number is CE00958.
cysteine-residues (double-underlined in Figure 3.3) close to the C-terminus that are necessary for palmitoylation. Moreover, murine and C. elegans M-Ras lacks a proline–rich motif characteristic of the C-termini of R-Ras and R-Ras2, which we term the R-Ras box (shaded medium gray in Figure 3.3). Instead, M-Ras and its C. elegans homologue exhibit a distinctive motif, rich in basic residues (shaded dark gray in Figure 3.3). It includes a threonine residue at position 190 of murine M-Ras which, as noted by Matsumoto and co-workers (Matsumoto et al., 1997), might serve as a target for phosphorylation. The C-terminus of murine M-Ras displays a stretch of lysine residues (underlined in Figure 3.3) which resembles that occurring at the C-termini of K-Ras4B.
3.2 M-Ras mRNA and protein are widely expressed

To assess the pattern of expression of M-Ras mRNA, RT-PCR and Northern blot analysis were performed. Northern Blot analysis revealed two major transcripts of approximately 1.5 kb and 4.6 kb that were expressed highly in brain and heart tissues with lesser but significant levels in liver, lung, pancreas, placenta and kidney (Figure 3.4). Thus, in contrast to other reports (Kimmelman et al., 1997; Matsumoto et al., 1997), we found that M-Ras was expressed widely. Using RT-PCR we observed expression of M-Ras mRNA in all cell lines examined, including hematopoietic cell lines, fibroblasts and epithelial cells derived from breast, skin, and kidney (Table 1.1).

\[ \text{Figure 3.4 Tissue distribution of M-Ras mRNA. A northern blot with 2 \mu g of poly A+ RNAs from the indicated tissues were probed with the C-terminal portion of M-Ras cDNA.} \]
Table 1.1  Cell lines of multiple lineages express M-Ras

<table>
<thead>
<tr>
<th>Cell line</th>
<th>species</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDMAC</td>
<td>murine</td>
<td>myeloid progenitor</td>
</tr>
<tr>
<td>BAC 1.2F5</td>
<td>murine</td>
<td>macrophage</td>
</tr>
<tr>
<td>Ba/F3</td>
<td>murine</td>
<td>myeloid progenitor</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>murine</td>
<td>macrophage</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>murine</td>
<td>fibroblast</td>
</tr>
<tr>
<td>Rat embryonic fibroblasts</td>
<td>rat</td>
<td>fibroblast</td>
</tr>
<tr>
<td>A431</td>
<td>murine</td>
<td>epidermal carcinoma</td>
</tr>
<tr>
<td>HEK 293</td>
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</tr>
<tr>
<td>MCF-7</td>
<td>human</td>
<td>breast carcinoma</td>
</tr>
<tr>
<td>MDA.MB 231</td>
<td>human</td>
<td>breast carcinoma</td>
</tr>
</tbody>
</table>

a RT-reactions were performed as described above on 2 µg of total RNA with t-RNA as a negative control.

To examine the levels of expression of the endogenous M-Ras protein, we generated preparations of polyclonal antibodies that were specific for M-Ras (α pep I, Figure 3.5A) or that recognized both M-Ras and, with approximately 3-fold less efficiency, p21 Ras (α pep II, Figure 3.5A). Immunoprecipitations were performed on whole cell lysates of spleen, thymus and various other tissues and cell lines using Y13-259. The immunoprecipitates were resolved on SDS-PAGE and immunoblotted either with the M-Ras specific antibodies (α pep I) or the
Figure 3.5  M-Ras protein is widely expressed. (A) Lysates of HEK293 cells transiently expressing HA-tagged M-Ras or N-Ras were immunoblotted with anti-HA antibody 12CA5. The same blot was stripped and immunoblotted with Y13-259, the M-Ras specific α pep I antibodies and the cross-reactive α pep II antibodies. (B) Lysates of the indicated tissues and of NIH 3T3 fibroblasts were subjected to immunoprecipitation with 15 μg Y13-259. The immunoprecipitates were resolved on SDS-PAGE and immunoblotted with M-Ras specific antibodies (α pep I, top panel) or antibodies that cross-reacted between M-Ras and p21 Ras (α pep II, bottom panel).
antibodies recognizing both M-Ras and p21 Ras (α pep II). M-Ras was detected as a discrete band of an apparent molecular weight of 29 kDa in all tissues and cell lines examined (Figure 3.5 B, upper panel). Use of the cross-reactive α pep II antibody permitted comparisons of relative levels of expression of M-Ras and p21 Ras. Immunoblotting of these immunoprecipitates revealed levels of expression of M-Ras in the spleen and thymus that respectively exceeded or equaled those of p21 Ras. M-Ras protein was also detected in heart, in brain and in NIH3T3 fibroblasts (Figure 3.5 B, lower panel).

3.3 M-Ras is recognized by the monoclonal anti-Ras antibody Y13-259

The monoclonal anti-p21 Ras antibody Y13-259 has been used extensively to monitor activation or function of p21 Ras. On the basis of the similarity of the switch II regions of M-Ras and p21 Ras, we next investigated the reactivity of M-Ras with the monoclonal anti-p21 Ras antibody Y13-259.

Y13-259 recognized M-Ras and N-Ras with equivalent efficiency in immunoblotting of lysates of HEK293 cells expressing HA-tagged M-Ras or N-Ras (Figure 3.6). Importantly, for the interpretation of assays of the activation of p21 Ras, Y13-259 immunoprecipitated M-Ras and N-Ras from those cell lysates with equal efficiency.
Figure 3.6  M-Ras is recognized by the monoclonal anti-Ras antibody Y13-259. Lysates of HEK293 cells transiently expressing myc-tagged M-Ras or HA-tagged N-Ras were subjected to immunoprecipitation with 10 μg of Y13-259. The immunoprecipitates, in parallel with samples of the same lysates (WCL), were resolved on SDS-PAGE and immunoblotted with Y13-259 as described.

3.4 Expression of activated M-Ras leads to activation of the c-fos promoter

Previously it has been shown that the c-fos promoter is a Ras-inducible (Hill and Treisman, 1995). The c-fos promoter contains binding sites for the serum response factor (SRF) and ternary complex factor (TCF) transcription factors. In addition, it also displays a cAMP-responsive element (CRE) and a sis-inducible element (SIE) (for reviews see (Treisman, 1995; Treisman, 1994)). We therefore tested the ability of M-Ras and mutants of M-Ras to activate the c-fos promoter using the luciferase reporter gene assay in a transient transfection system. As
expected, expression of wild-type M-Ras did not activate the \textit{c-fos} promoter (Figure 3.7). However, expression of the constitutively active G22V mutant of M-Ras resulted in 3-fold activation of the \textit{c-fos} promoter. This was reproducibly less than the 9-fold activation of the \textit{c-fos} promoter induced by constitutively active N-Ras Q61K (Figure 3.7).

\textbf{Figure 3.7}  \textbf{Activation of the \textit{c-fos} promoter by constitutively active M-Ras.} HEK293 cells were transiently transfected with 3 µg of the indicated constructs and 0.5 µg of the luciferase reporter gene under control of the \textit{c-fos} promoter. All samples were normalized to total protein and shown as fold-activation over 'empty vector'. Values indicate mean +/- SD (n=5).
3.5 Expression of dominant negative M-Ras S27N completely inhibits activation of the *c-fos* promoter by activated Src Y527F

To investigate the possibility that M-Ras shares exchange factors with p21 Ras proteins, we generated a S27N mutant of M-Ras, analogous to the S17N dominant inhibitory mutants of p21 Ras. Expression of M-Ras S27N itself failed to activate transcription of the *c-fos* promoter (Figure 3.7). However, when co-expressed it completely suppressed activation of the *c-fos*

![Bar graph showing the relative luciferase units (RLU) for different conditions.](image)

**Figure 3.8** Dominant negative M-Ras S27N inhibits activation of the *c-fos* promoter by activated Src Y527F. HEK293 cells were transfected with 0.25 µg of the *c-fos* luciferase reporter plasmid and 0.25 µg of a Src Y527F plasmid and the indicated amount in (µg) of dominant negative M-Ras S27N and H-Ras S17N, respectively. Empty vector was added where necessary to ensure equal transfection efficiency. All samples were normalized to total protein. Shown are relative luciferase units expressed as the mean +/- SD of 3 independent experiments.
promoter induced by expression of constitutively active Src Y527F (Figure 3.8). Thus, M-Ras S27N, like H-Ras S17N, acted as a dominant inhibitor of the Src activation of the c-fos promoter.

3.6 M-Ras G22V transforms NIH3T3 fibroblasts

Figure 3.9 NIH3T3 fibroblasts expressing constitutively active M-Ras G22V display a transformed morphology. NIH 3T3 fibroblasts were retrovirally transduced with (a) empty vector, (b) N-Ras Q61K, (c) M-Ras wt or (d) M-Ras G22V. Shown are phase-contrast photomicrographs of cells grown in medium containing 10% calf serum.
We next used retroviral vectors to express wild-type M-Ras, M-Ras G22V or N-Ras Q61K in NIH-3T3 fibroblasts. As shown in Figure 3.9, NIH3T3 cells expressing M-Ras G22V, but not those expressing wild-type M-Ras, exhibited a refractile, transformed morphology, strongly resembling that of the cells expressing N-Ras Q61K. Cells expressing M-Ras G22V, but not those expressing wild-type M-Ras, also resembled cells expressing N-Ras Q61K in that they were able to grow to higher saturation densities. In cell counting experiments, cells expressing activated mutants of M-Ras or N-Ras reached saturation densities exceeding those of cells expressing either M-Ras wild-type or vector alone approximately 4-fold (Figure 3.10).

**Figure 3.10** NIH3T3 fibroblasts expressing activated mutants of M-Ras or N-Ras grow to higher saturation densities. Cell-densities reached by polyclonal populations of NIH3T3 cells expressing vector alone (○), M-Ras wt (□), M-Ras G22V (●) and N-Ras Q61K (▲). Cells were grown in 10% calf serum and figures represent data from two independent experiments, each performed in triplicate, and are shown as means +/- SD.
3.7 High levels of expression of active M-Ras G22V result in factor-independent growth of an IL-3-dependent cell line

To investigate the effects of M-Ras expression on hemopoietic cells, activated M-Ras G22V was cloned into a retroviral expression vector which co-expressed the enhanced green fluorescent protein (EGFP) downstream of an IRES element. In addition, the retroviral vector also contained the puromycin resistance gene. IL-3-dependent R6X cells were retrovirally transduced with activated M-Ras G22V or 'empty vector', drug-selected and cultured in the presence or absence of IL-3. A sub-population of M-Ras G22V-expressing cells failed to die in the absence of IL-3 and slowly increased in number. Plating of polyclonal M-Ras G22V-expressing R6X cells in soft agar in the absence of IL-3 for 7 days resulted in growth of small colonies at low frequency (0.2%) (Figure 3.11 A). Randomly selected clones of R6X M-Ras G22V from colonies grown in the presence or absence of IL-3 were then expanded in IL-3-containing medium, washed and their survival compared in the absence of IL-3. Cells derived from clones selected in the absence of IL-3 displayed strong metabolic activity compared to cells derived from clones selected in the presence of IL-3, which exhibited significant cell death when cultured without IL-3 (Figure 3.11 B). FACS analysis of these cells showed a strong EGFP signal for cells selected in the absence of IL-3, indicating high levels of M-Ras G22V expression. This was confirmed directly by assessing levels of M-Ras G22V by immunoblotting cell lysates with anti-HA epitope tag antibodies (Figure 3.11 C).
Figure 3.11  Expression of constitutively active M-Ras G22V in an IL-3-dependent cell line results in factor-independent growth. (A) Polyclonal, puromycin-resistant R6-X M-Ras G22V cells (open bars) or empty vector cells (closed bars) were plated in agar in triplicate in the absence of IL-3 at 2.5 x 10^4 / ml or 2.5 x 10^3 /ml. Colonies were counted at day 7. (B) Clones of R6-X M-Ras G22V cells derived from colonies grown in medium alone ( □ ) or in the presence of IL-3 ( ● ) were expanded in IL-3, washed and incubated in medium alone for 3 days, when survival and growth were assessed by uptake of MTT. The uptake of MTT (expressed as OD units) was plotted against the mean intensity of fluorescence (MFI) of EGFP assessed by flow cytometry of the same clones grown in the presence of IL-3. (C) 20 μg of whole-cell-lysates of four of the clones shown in (B), two exhibiting high EGFP expression (M.4, MFI 1775, and M.6, MFI 2271) and two with low EGFP expression (F.1, MFI 216, and F.3, MFI 474), were immunoblotted using a monoclonal antibody specific for the HA-tag present on the M-Ras G22V. Also shown are lysates of parental R6-X cells, of 'vector alone' infected cells and M-Ras G22V-infected cells, selected by fluorescence activated cell-sorting ('sorted').
M-Ras G22V interacts weakly with the Ras-binding domains of Raf-1 and RalGDS

We investigated whether constitutively active M-Ras interacts directly with two known effectors of p21 Ras, Raf-1 and Ral-GDS. The binding of M-Ras G22V to the Ras binding domain (RBD) of Raf-1 was readily detectable, although the amounts of M-Ras G22V present in eluates were significantly less than those of Q61K N-Ras observed in parallel experiments (Figure 3.12). This indicated that M-Ras G22V interacted with the Raf-1-RBD with lower affinity than did N-Ras Q61K. Likewise M-Ras G22V bound detectably to RalGDS-RBD. Again, comparison of the amount of M-Ras G22V bound with that of N-Ras Q61K indicated that

Figure 3.12  M-Ras interacts only weakly with the Ras binding domains (RBD) of Raf-1 and Ral-GDS. Lysates of HEK293 cells transiently transfected with HA-tagged M-Ras wt, M-Ras G22V, N-Ras Q61K or vector alone were mixed as indicated with GST fusion proteins of the RBD of Raf-1 or Ral-GDS precoupled to glutathione beads. Proteins were eluted from the beads by boiling and, in parallel with samples of the original lysates (WCL), were subjected to SDS-PAGE and immunoblotting with anti-HA antibody 12CA5. Closed triangles indicate HA-M-Ras and open triangles HA-N-Ras.
M-Ras G22V had a lower affinity for Ral-GDS. As expected, neither the RBD of Raf-1, nor that of RalGDS bound to wild-type M-Ras, which in the absence of activating stimuli is predominantly in its inactive, GDP-bound conformation.

3.9 Discussion of Chapter III

Our data show that M-Ras is a 29 kDa protein that is widely expressed, including in lymphohemopoietic cells, breast epithelial lines and fibroblasts. In the spleen, (and in NIH3T3 fibroblasts) the levels of expression of M-Ras protein exceed those of members of the p21 Ras family whereas this ratio was reversed in the thymus. Importantly, M-Ras could not be distinguished from p21 Ras in assays based on the use of the anti-Ras monoclonal antibody Y13-259 or dominant negative Ras mutants. Our observations that expression of a constitutively active mutant of M-Ras in an IL-3-dependent mast cell/megakaryocyte cell line increased survival and resulted in factor-independent growth, and in NIH3T3 fibroblasts in transformation, indicate that mutants of M-Ras may have a role in oncogenesis. Collectively these data suggest that M-Ras and p21 Ras are activated by common exchange factors and can regulate growth via both shared and distinctive effectors.

Comparison of the C-termini of M-Ras and those of other members of the Ras family show that, although it shares the terminal CAAX-motif, M-Ras lacks the cysteine residues characteristic of p21 Ras, R-Ras and R-Ras2/Tc21, which are involved in palmitoylation. M-Ras also lacks the proline-rich region characteristic of R-Ras and R-Ras2, which we term the R-Ras
box, as well as a motif found in the C-termini of the classical p21 Ras proteins. Instead M-Ras, in both mammals and C. elegans, exhibits a conserved carboxy terminal motif that is rich in basic residues and has a conserved threonine. These features may govern the interaction of M-Ras with other molecules or with other structures, such as subregions of the membrane.

The best characterized signaling pathway downstream of p21 Ras involves activation of the kinase cascade Raf-1, MEK 1/2 and Erk 1/2, which ultimately leads to transcriptional activation of genes such as c-fos (Hill and Treisman, 1995). We observed that constitutively active M-Ras was able to activate transcription of a reporter gene under the control of the c-fos promoter. The moderate degree of activation of the c-fos promoter induced by co-expression of M-Ras G22V relative to that of N-Ras Q61K, correlated with their relative affinities for the Ras-binding domain of Raf-1 and their relative abilities to induce activation of Erk2 (Kimmelman et al., 1997). It remains to be established whether M-Ras activates the Raf/MAPK pathway under physiological conditions. The presence of homologues of both p21 Ras and M-Ras in C. elegans suggests that these molecules are likely to have distinct functions and to utilize distinct effectors. The assignment of functions to M-Ras, however, is complicated by the fact that characterization of the function of p21 Ras proteins in their various physiological and pathological processes has relied heavily on the monoclonal antibody Y13-259, and dominant inhibitory mutants such as H-Ras S17N. As discussed below these two experimental tools fail to discriminate between M-Ras and p21 Ras.
Y13-259 binds to a highly conserved epitope on the switch II region of p21 Ras (Sigal et al., 1986) and blocks interaction of Raf-1 with p21 Ras. The demonstration that Y13-259 recognizes M-Ras and N-Ras with equivalent efficiency, is consistent with the overall similarities in the sequences of M-Ras and p21 Ras in the switch II region, and the fact that those residues known to comprise the epitope (Glu-63, Ser-64, Ala-66, Met-67, Gln-70 and Arg-73 of p21 Ras) are identical. Given our evidence that M-Ras is expressed ubiquitously, and is more abundant than p21 Ras in tissues like spleen and in cells like NIH3T3 fibroblasts (which have been used widely to study p21 Ras), it will be necessary to re-evaluate many published experiments to determine whether the experimental observations reflected the function of p21 Ras, M-Ras or both. For example, the biological effects of micro-injection of Y13-259 into 3T3 cells expressing oncogenes (Gibbs et al., 1990; Mulcahy et al., 1985), which have been interpreted as reflecting interference with the function of p21 Ras, could equally reflect inhibition of M-Ras function. Moreover, experiments designed to assess the activation of p21 Ras by measuring ratios of GTP:GDP in Y13-259 immunoprecipitates in various cell types (Porras et al., 1992; van Corven et al., 1993) and in hemopoietic cells stimulated by hemopoietic growth factors (Duronio et al., 1992; Satoh et al., 1991) or antigens (Downward et al., 1990; Lazarus et al., 1993) may have been also monitoring the activation of M-Ras.

Dominant-negative p21 Ras mutants such as H-Ras S17N (Feig and Cooper, 1988) act by sequestering GEFs involved in the activation of p21 Ras proteins (Boriack-Sjodin et al., 1998; Stacey et al., 1991) and have been widely used to implicate p21 Ras in paths downstream of cell-
surface receptors and oncogenes (de Vries-Smits et al., 1992; Feig and Cooper, 1988; Lange-Carter and Johnson, 1994; Okuda et al., 1994; Satoh et al., 1990) and in the development of cells of hemopoietic origin (Iritani et al., 1997; Swan et al., 1995). The demonstration that M-Ras S27N also acts as a dominant inhibitor of activation of the c-fos promoter by an activated Src Y527F suggests that M-Ras shares GEFs with p21 Ras. Recent studies by Matsumoto and co-workers confirmed that M-Ras and p21 Ras are activated by the same set of GEFs, including mSOS1/2, Cdc25Mm and CalDAG GEF II (Ohba et al., 2000). Thus, M-Ras is likely to be activated by most stimuli that translocate GEFs to the plasma membrane and have been thought to activate p21 Ras. This failure of dominant negative mutants to discriminate between p21 Ras and M-Ras and the high levels of expression of M-Ras in tissues such as the spleen, means that the conclusions of experiments based upon their use, for example concerning the role of p21 Ras in the action of hemopoietic growth factors (Okuda et al., 1994) or in the development of the thymus (Swan et al., 1995) or B-lymphocytes (Iritani et al., 1997), will require re-evaluation. It will be important to determine the frequency of activating mutations of M-Ras in leukemia and other neoplasias of lymphohematopoietic origin. Likewise given evidence that activated mutants of M-Ras can transform NIH3T3 fibroblasts, it will be important to determine whether mutations of M-Ras occur in solid tumors, particularly those involving tissues such as breast or prostate, where mutations in p21 Ras occur at a relatively low frequencies (Bos, 1989).

In summary, evidence is presented that M-Ras is widely expressed, with the spleen and thymus being major sites of expression, interacts with at least some of the GEFs acting on p21
Ras, and when constitutively active can result in enhanced survival and factor-independent growth of a hemopoietic cell-line and transformation of NIH3T3 fibroblasts. Collectively, these observations suggest that in many cells, including those of hematopoietic origin, M-Ras and p21 Ras will be activated coordinately and will activate parallel, but distinct signaling pathways. Future work will determine the molecular nature and degree of overlap of these paths and their role in the growth and differentiation of the lymphohemopoietic system. Finally, some of the functions of the M-Ras pathway may have been incorrectly attributed to the classical p21 Ras pathway on the basis of the use of Y13-259 and S17N mutants of p21 Ras and it will be important to develop more selective reagents.
CHAPTER IV: IDENTIFICATION AND CHARACTERIZATION OF RPM, A NOVEL MEMBER OF THE
RALGDS-FAMILY OF GUANINE NUCLEOTIDE EXCHANGE FACTORS FOR THE
SMALL GTPASE RAL

4.1 Cloning of RPM

In Chapter III, we described the cloning and characterization of a novel member of the
Ras family, M-Ras. Overexpression of activated M-Ras led to factor-independent growth of an
IL-3-dependent cell line and to transformation of NIH3T3 fibroblasts. The fact that M-Ras
recognizes the Ras effectors Raf-1 and RalGDS only weakly suggested that M-Ras may utilize a
set of effector proteins distinct from those employed by p21 Ras. To investigate the effector
proteins involved in M-Ras signaling, we employed the yeast 2-hybrid system to screen a cDNA
library prepared from NIH3T3 fibroblasts. The yeast 2-hybrid system is based on in vivo direct
protein-protein interactions. It allows the identification of these protein-protein interaction based
on their ability to form an active transcription factor complex. Yeast cells that are auxotrophic
for the amino acids leucine, tryptophan and histidine are co-transformed with a plasmid encoding
the gene of interest (M-Ras G22V) fused to the transactivation domain of the gal4-transcription
factor as well as a plasmid encoding the unknown protein (library) fused to the DNA-binding
domain of the gal4-transcription factor. Whereas the information for leucine and tryptophan is
encoded on both of these plasmids, biosynthesis of histidine requires interaction between the
protein of interest ('bait') and the unknown protein ('prey'). Interaction of these two fusion
partners brings the two domains of the gal4-transcription factor into close proximity and thus forms a functional transcription factor (Figure 4.1) (Mendelsohn and Brent, 1994).

**Figure 4.1** The yeast 2-hybrid system. Constitutively active M-Ras G22V was cloned into the "bait" vector pAS2-1 to screen a library prepared from NIH3T3 fibroblasts cloned into the "prey" vector pACT2.
Ras effector proteins recognize a conformational epitope that is present in GTP-loaded Ras but not in GDP-loaded Ras and that does not require post-translational modifications such as phosphorylation. This characteristic makes the yeast 2-hybrid system particularly well suited for identification of Ras effector proteins. Since we have shown previously that levels of M-Ras protein exceed those of p21 Ras in NIH3T3 cells, we screened a library prepared from NIH3T3 fibroblasts. 45 His'/LacZ' colonies were identified. Sequencing of the cDNA plasmids derived from these clones revealed 6 known Ras effectors and one clone with a novel sequence (Table 2.1).

Table 2.1  M-Ras interacts with different Ras-effectors in the yeast 2-hybrid system

<table>
<thead>
<tr>
<th>clone</th>
<th>LacZ-activity</th>
</tr>
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<tbody>
<tr>
<td>novel 'RPM' (nt 1537-stop)</td>
<td>+++</td>
</tr>
<tr>
<td>Nore1 (nt 382-stop)</td>
<td>+++</td>
</tr>
<tr>
<td>AF-6 (full length)</td>
<td>+++</td>
</tr>
<tr>
<td>Rin1 (nt 205-stop)</td>
<td>+++</td>
</tr>
<tr>
<td>RalGDS (nt 528-stop)</td>
<td>++</td>
</tr>
<tr>
<td>Raf-1 (nt 71-stop)</td>
<td>+</td>
</tr>
<tr>
<td>A-Raf (full length)</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* LacZ-activity was assayed by intensity of the LacZ signal and by the time the signal appeared. +++ strong interaction, ++ medium interaction, + weak interaction, numbers indicate the most 5' nucleotide of the coding sequence, stop indicates the termination signal.
This cDNA encoded the 3' untranslated region (UTR) and 590 nucleotides (nt) of a novel protein. To obtain a full-length clone, we used a PCR-based approach, 5’RACE (Figure 4.2). After two rounds of 5’RACE, a full-length clone encoding an open reading frame of 2127 nt, which translated into a 709 amino acid protein with a predicted molecular weight of 78 kDa, and 58 nt of 5’ UTR was identified.

Figure 4.2 5’RACE-strategy to amplify full-length RPM. Two rounds of nested PCR-reactions were performed using the primer combinations AP1/AS1, AP2/AS2 and AP1/AS3, AP2/AS4, respectively. Vertical open arrowheads indicate the positions of the 5’ end of the amplified transcript, the start codon (ATG), the stop codon (TGA) and the 3’ end of the transcript. Closed vertical arrowheads indicate the positions of the 5’ ends of the cDNA clone obtained from the 2-hybrid screen (2HY) and the first RACE reaction (RACE-1). Horizontal arrows indicate the positions of the sense (open arrowheads) and antisense primers (closed arrowheads). The sizes of the domains of RPM, the intervening regions and the untranslated regions are not depicted to scale.
Three independently amplified clones contained 58 nt upstream of an in-frame ATG that was preceeded by a Kozak-consensus sequence and a fourth clone contained 52 nt upstream of this ATG. Attempts to identify additional sequence information yielded no results. Although we did not detect an in-frame stop-codon upstream of the predicted initiating ATG, the overall length of this cDNA, including 5' UTR and 3' UTR corresponded to the size of the signal obtained from northern blotting of polyA mRNA (Figure 4.6). Thus we believe the cloned cDNA represented a full-length clone (Figure 4.3).
Figure 4.3  DNA and protein sequence of RPM. The initiating methionine is underlined and the stop codon is shown in bold. Regions corresponding to the N-terminal scr-0 domain are shaded light grey and regions corresponding to the predicted catalytic scr1, scr-2 and scr-3 domains are shaded medium grey. Residues corresponding to the C-terminal Ras binding domain are boxed. A potential profilin binding site is indicated by the dashed underlining.
4.2  **RPM is a highly conserved, novel member of the RalGDS-family**

Analysis of the translated cDNA sequence indicated that it belonged to the RalGDS-family of guanine nucleotide exchange factors, with 31-36% identity and 45-51% homology with the RalGDS-family members RalGDS, Rlf and Rgl. We termed this protein RPM (for Ras Pathway Modulator). Analysis of the evolutionary relatedness of the different RalGDS-family members suggested that RPM is most closely related to Rlf (Figure 4.4).

![Dendrogram](image)

**Figure 4.4  Evolutionary relatedness of RalGDS-family members.** The dendrogram was generated using a Clustal V alignment and the Phylips distance method algorithm. (m) indicates murine sequences, (Ce) indicates *C. elegans*. Accession number for the *C. elegans* orthologue is CE04456.

The regions of RPM exhibiting the most amino acid similarity with the other RalGDS-family members were the Ras-binding domain (RBD) and the structurally conserved regions (scr) scr1, scr2 and scr3 which are conserved in the catalytic regions of guanine nucleotide exchange factors.
of the cdc25 family. The N-terminal scr-0 region also displayed a high degree of sequence
conservation. Unlike other RalGDS-family members, RPM is distinguished by multiple prolines
between residues 539 and 591, which form potential binding sites for SH3 domains (Figure 4.5).
In addition, RPM displays a stretch of 10 prolines preceded by a leucine at residues 114-124
which form a consensus binding site for profilin (see Figure 4.3).

Figure 4.5  Alignment of the protein sequences of the C-termini of RPM, RalGDS, Rif
and Rgl. Double underlining indicates the Ras-binding domains. Conserved residues are shaded
light gray and identical residues medium gray. Prolines in a proline-rich stretch of RPM that
form potential SH-3 domain binding sites are indicated by an asterisk. Gaps are indicated by a
hyphen (-).
4.3 Tissue distribution of RPM mRNA

To investigate the pattern of expression of RPM, we performed northern blot analysis of multiple murine tissues using a probe corresponding to nucleotides (nt) 1584-2060 of RPM. We observed a signal corresponding to 3 kb in kidney, liver, lung, heart and brain tissues. The size of the observed signal corresponded well with the overall length of the cloned RPM cDNA, including 5' UTR and 3' UTR. Additional signals corresponding to approximately 1.4 kb and 7.2 kb were detected in heart and in kidney. These may reflect the existence of alternatively spliced isoforms of RPM (Figure 4.6).

Figure 4.6 Tissue-distribution of RPM mRNA. A murine multiple tissue northern blot of 2 μg of polyA⁺-purified mRNA for each indicated tissue was probed with a radiolabeled probe corresponding to nt 1584-2060 of RPM. The signal corresponding to RPM mRNA is indicated by an arrowhead.
4.4 Mutants of RPM

To investigate the biological properties of RPM, we generated a number of mutants of RPM (Figure 4.7 A). To facilitate detection of these proteins, all mutants had the c-myc epitope

A

<table>
<thead>
<tr>
<th>RPM</th>
<th>scr-0</th>
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<th>RBD</th>
</tr>
</thead>
<tbody>
<tr>
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<td>m</td>
<td>scr-0</td>
<td>scr-1</td>
<td>scr-2</td>
<td>scr-3</td>
<td>PRR</td>
</tr>
<tr>
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<td>m</td>
<td>scr-0</td>
<td>scr-1</td>
<td>scr-2</td>
<td>scr-3</td>
<td>PRR</td>
</tr>
<tr>
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<td>m</td>
<td>scr-0</td>
<td>scr-1</td>
<td>scr-2</td>
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</tr>
</tbody>
</table>

B

IB: α myc

<table>
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<tr>
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<th>RPM CAAX</th>
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<tr>
<td>[Western blot for RPM]</td>
<td>[Western blot for RPM CAAX]</td>
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</table>

Figure 4.7  Schematic and subcellular localization of the overexpressed RPM proteins. (A) Scr-0-3 indicate the structurally conserved regions 0-3, PRR indicates the proline rich region, RBD the Ras binding domain, CAAX the 22 C-terminal residues including the CAAX-motif of H-Ras and 'm' the N-terminally fused c-myc epitope tag. (B) Cell-fractionation of wt and membrane localized RPM. Cell-fractionations were performed as described and equal volumes of cytoplasmic (c) and membrane (m) fraction were separated by SDS-PAGE. RPM proteins were detected by western blotting using the α-myc antibody 9E10.
tag fused at their N-termini. It has been shown for a number of Ras effectors (Raf-1, PI3-kinase and RalGDS), that permanent membrane localization by attachment of a Ras CAAX-motif leads to a constitutively active form of the corresponding protein (Didichenko et al., 1996; Leevers et al., 1994; Murai et al., 1997). To verify that attachment of a CAAX-motif indeed led to a membrane localized protein, we performed cell fractionation studies. Whereas RPM wt was predominantly in the cytosolic fraction, the RPM-CAAX mutant was predominantly in the membrane-enriched fraction (Figure 4.7 B)

4.5 The Ras-binding domain of RPM is necessary for interaction with M-Ras and p21 Ras

To confirm and extend our observation of an interaction between M-Ras G22V and RPM in the yeast two-hybrid system, we investigated the ability of full length RPM to co-immunoprecipitate with wild-type M-Ras or with the constitutively active mutants of M-Ras or H-Ras. Transiently overexpressed RPM was co-precipitated with M-Ras G22V and, more efficiently, with a second constitutively active mutant of M-Ras, M-Ras Q71L. It also co-precipitated with H-Ras G12V (Figure 4.8). As expected, RPM did not co-precipitate with M-Ras wt. A mutant of RPM that was membrane localized by addition of a CAAX-motif at the C-terminus (RPM CAAX) could also be co-precipitated with constitutively active mutants of M-Ras and H-Ras. This ability to co-precipitate was abolished by deleting the 143 C-terminal
RPM binds to constitutively active M-Ras and H-Ras. HEK293 cells were co-transfected with the indicated myc-tagged RPM constructs and HA-tagged Ras- and Cdc42-constructs. The Ras- and Cdc42-constructs were immunoprecipitated with 2 µg of the monoclonal anti-HA antibody 12CA5. The immunoprecipitates (IP) and whole cell lysates (wcl) were separated by SDS-PAGE and blotted against the myc-epitope on RPM using the monoclonal antibody 9E10 (top and middle panel) or the HA-epitope on the indicated Ras constructs using the antibody 12CA5 (bottom panel), respectively.
residues of RPM (Figure 4.8). Also, neither RPM nor any of the mutants of RPM co-precipitated with an activated mutant of the small GTPase Cdc42 (Figure 4.8). Thus, RPM bound selectively to GTP-bound Ras proteins and the C-terminal 143 residues encoding the putative Ras binding domain (RBD) were necessary for this binding.

4.6 The Ras-binding domain of RPM is sufficient for interaction with M-Ras and p21 Ras

Next, we investigated whether the Ras-binding domain (RBD) of RPM was sufficient for binding to GTP-bound Ras. We also compared the binding of M-Ras and H-Ras to the RBDs of Nore1, AF-6 and RalGDS, which interacted with activated M-Ras in the yeast two-hybrid system (see Table 2.1). Lysates of HEK293 cells overexpressing mutants of M-Ras and H-Ras were incubated with the various GST-RBD-fusion proteins, thus allowing us to directly compare the relative affinities of the co-expressed mutants of M-Ras and H-Ras for a given RBD. Both, M-Ras G22V and H-Ras G12V bound to the RBDs of RPM, Nore1 and AF-6, indicating that the RBDs of RPM, Nore1 and AF-6 were sufficient for binding to M-Ras or H-Ras (Figure 4.9 A and B). Since, in the absence of a stimulus, only a very small proportion of Ras is in the active GTP-bound form, only a very weak signal for Ras wt was detected after prolonged exposure (Figure 4.9 and B, lane Nore 1*). We had noted that M-Ras Q71L bound more strongly to RPM than did M-Ras G22V (Figure 4.8). Therefore we used the M-Ras Q71L mutant to directly compare the strength of the binding of activated M-Ras and activated H-Ras to these effector
proteins. Analysis of the signals obtained within each lane using these Ras mutants indicated that the RBD of RPM, like those of Nore1 and AF-6, bound with comparable strength to M-Ras Q71L and H-Ras G12V. In contrast, the RBD of RalGDS, despite its sequence similarities with the RBD of RPM (see Figure 4.5) bound activated H-Ras G12V much more strongly than activated M-Ras (Figure 4.9 C).

Figure 4.9   The Ras-binding domains of RPM, Nore1, RalGDS and AF-6 are sufficient for the binding of constitutively active M-Ras and p21 Ras. (A) Whole cell lysates of transiently transfected HEK293 cells expressing H-Ras wt (myc) and M-Ras G22V (myc), (B) H-Ras G12V (myc) and M-Ras wt (myc) and (C) H-Ras G12V (myc) and M-Ras Q71L (myc) were incubated with 1 µg GST-fusion protein of the indicated Ras-binding domain. Ras proteins binding to the GST-fusion proteins were eluted off the beads by boiling in SDS, separated by SDS-PAGE, transferred onto nitrocellulose membranes and blotted against the myc-epitope with the monoclonal antibody 9E10. M-Ras proteins are indicated by an open arrow head and H-Ras proteins by a closed arrow head. The lane labeled "Nore-1*" represents a longer exposure of lane "Nore-1".
4.7 RPM inhibits activation of the Elk-1 transcription factor mediated by p21 Ras- and MEKK1-, but not Raf-1

RalGDS, Rgl and Rlf have been reported to synergize with constitutively active p21 Ras proteins in activating the c-fos promoter (Murai et al., 1997; Okazaki et al., 1997; Wolthuis et al., 1997). To investigate whether RPM displayed the same properties, we co-transfected HEK293 cells with an Elk-1-dependent reporter gene, H-Ras G12V and RPM. We also investigated the activity in these assays of mutants of RPM (Figure 4.7 A) with a C-terminal CAAX motif, mimicking their recruitment to the membrane by activated Ras. As a control, we replaced the RPM constructs with an expression vector encoding Rlf. When expressed alone, neither Rlf nor any of the RPM constructs induced activation of the reporter gene (Figure 4.10). Whereas co-expression of Rlf and H-Ras G12V resulted in synergistic activation of the Elk-1 dependent reporter gene, co-expressed RPM or the membrane-localized RPM mutants not only failed to synergize with H-Ras G12V, but dramatically inhibited induction of reporter gene activity (Figure 4.10). The ability of membrane-localized RPM mutants to block H-Ras G12V mediated reporter gene induction was consistent with the notion that this inhibitory activity was a genuine effector function, normally initiated by translocation of RPM to activated Ras and thus the membrane. Western blotting of the cell lysates revealed that the observed decrease in reporter gene activity was not caused by lower expression levels of the activating protein in the presence of RPM (Figure 4.10).
Figure 4.10  RPM inhibits Ras-induced activation of the Elk-1 transcription factor. HEK293 cells were transiently transfected with 0.5 μg of the reporter gene (5x gal4-luciferase/gal4-elk-l), 0.2 μg of the indicated RPM constructs and 50 ng of H-Ras G12V. Empty vector (pcDNA3.1) was added where necessary to ensure equal transfection efficiency. Cells were harvested after 40 hours and the luciferase activity determined. All samples were normalized to total protein and values are shown as fold-induction over 'reporter-gene alone'. All experiments were repeated at least 4 times. Error bars indicate standard deviation.
In further experiments we observed that RPM exerted a similar inhibitory effect on MEKK-1-mediated activation of the reporter gene, not only with the membrane bound mutants of RPM but also the non-modified RPM proteins (Figure 4.11). In contrast, co-transfected Rlf synergized strongly with MEKK-1 to activate the reporter gene. In some of the reporter gene assays described above, we co-transfected an expression vector encoding the green fluorescent protein (GFP) under control of the CMV-promoter. Analysis of an aliquot of the cells for GFP-expression by flow cytometry indicated that expression of the RPM mutants did not affect the percentage of GFP expressing cells or the levels of GFP-expression in the transfected cells (data not shown). Thus the observed inhibitory function of RPM was not merely a result of cytotoxic effects.

RalGDS has also been reported to synergize with activated Raf-1 in activating the c-fos promoter (Okazaki et al., 1997), whereas Rlf appears to have no synergistic effects in this system (Wolthuis et al., 1997). To investigate the effects of RPM on Raf-1 mediated activation of the Elk-1 transcription factor, we co-transfected HEK293 cells with constitutively active Raf-1-CAAX, various mutants of RPM and a luciferase reporter gene under control of the Elk-1 transcription factor. Co-expression of RPM or the membrane localized mutants of RPM had no effect on Raf-1-CAAX mediated activation of the reporter gene (Figure 4.11). As it is conceivable that the CAAX-motifs of M-Ras and H-Ras could direct the RPM mutants to different localizations within the plasma membrane, we investigated whether the CAAX-motif of M-Ras or H-Ras would have a different influence on RPM. However, we did not observe any
Figure 4.11  RPM inhibits MEKK-1 induced activation of the Elk-1 transcription factor, but does not affect Raf-1 induced Elk-1 activation. HEK293 cells were transiently transfected with 0.5 µg of the reporter gene (5x gal4-luciferase/gal4-elk-l), 0.2 µg of the indicated RPM constructs and 3 µg of MEKK-1 or 3 µg of Raf-1 CAAX. Empty vector (pcDNA3.1) was added where necessary to ensure equal transfection efficiency. Cells were harvested after 40 hours and the luciferase activity determined. All samples were normalized to total protein and values are shown as fold-induction over 'reporter-gene alone'. All experiments were repeated at least 4 times. Error bars indicate standard deviation.
difference between RPM fused to the CAAX-motifs of M-Ras or of H-Ras (data not shown) in any of the described experiments.

Finally, we investigated the effects of RPM on M-Ras-mediated activation of the Elk-1 transcription factor. It was shown previously that activated mutants of M-Ras weakly activated a co-expressed luciferase reporter gene under control of the c-fos promoter or the serum response element (SRE) (Ehrhardt et al., 1999; Kimmelman et al., 1997). M-Ras G22V-mediated activation of the Elk-1 dependent reporter gene was partially inhibited by RPM and completely inhibited by membrane localized RPM. However, co-expression of the mutants of RPM lacking the RBD failed to inhibit reporter gene activation (Figure 4.12).
Figure 4.12 Inhibition of M-Ras induced Elk-1 activation by RPM is dependent on the Ras binding domain of RPM. HEK293 cells were transiently transfected with 0.5 μg of the reporter gene (5x gal4-luciferase/gal4-elk-1), 0.2 μg of the indicated RPM constructs and 3 μg of M-Ras G22V. Empty vector (pcDNA3.1) was added where necessary to ensure equal transfection efficiency. Cells were harvested after 40 hours and the luciferase activity determined. All samples were normalized to total protein and values are shown as fold-induction over 'reporter-gene alone'. All experiments were repeated at least 4 times. Error bars indicate standard deviation.
4.8 RPM inhibits anchorage-independent growth of NIH3T3 fibroblasts expressing an activated Src Y527F

Since RPM had a strong influence on Elk-1 dependent reporter gene expression, we were interested to see if this effect was reflected on cell growth. To investigate the effects of RPM in vivo, we transduced NIH3T3 fibroblasts that expressed an activated Src Y527F with retroviral constructs that encoded the various mutants of RPM upstream of an IRES-element and the GFP protein. The infected cells were sorted to greater than 99% purity and plated into semi-solid medium to assay anchorage independent growth. Whereas cells that were infected with the

![Graph showing the effect of RPM on anchorage-independent growth of NIH3T3 fibroblasts expressing activated Src Y527F.](image)

**Figure 4.13** RPM inhibits anchorage-independent growth of NIH3T3 fibroblasts expressing activated Src Y527F. Cells were infected with the indicated constructs that expressed in addition to RPM the EGFP-protein downstream of an IRES-element. 1000 GFP-positive cells were plated into 1.1% methyl-cellulose in triplicate and incubated for 9 days. The experiment was repeated 3 times. Values represent mean +/- SD.
empty vector gave rise to colonies in semi-solid medium, this colony formation was greatly reduced in cells expressing the various mutants of RPM (Figure 4.13). This reduced ability to form colonies correlated well with a reduced cell growth of these cells as assayed by their ability to metabolize MTT (Figure 4.14).

Figure 4.14  RPM inhibits growth of NIH3T3 fibroblasts expressing activated Src Y527F. 1000 GFP-positive cells were plated into 96 well plates (10 replicates per construct) and MTT was added at the indicated times for 4 hours. Cells infected with empty vector are indicated by open squares (□), cells infected with RPM wt by closed diamonds (●), cells infected with RPM ΔRBD-CAAX by closed circles (○) and cells infected with RPM-CAAX by closed triangles (▲). Values represent mean +/- SD.
4.9 RPM does not affect the phosphorylation state of the Erk, JNK and p38 MAP kinases

MEKK-1 is an activator of Erk- and JNK-kinases, and, more weakly, p38 MAP-kinase (Lange-Carter et al., 1993; Minden et al., 1995; Winzen et al., 1999). We had observed a strong inhibitory effect of RPM on MEKK-1 mediated activation of the Elk-1 reporter gene.

![Figure 4.15](image)

Figure 4.15 RPM does not inhibit activation of the Erk, JNK and p38 MAP-kinases. HEK293 cells were transiently transfected with the indicated constructs, the cell lysates separated by SDS-PAGE and immunoblotted with the indicated phospho-specific antibodies or the α-myc antibody 9E10. Blots were stripped and re-probed with non-phospho-specific antibodies (bottom panels) to verify equal loading.
(Figure 4.11). To investigate if this observed inhibition of reporter gene activity was caused by a loss of MAP-kinase activation, we transiently transfected HEK293 cells with MEKK-1 with or without RPM and used phospho-specific antibodies to detect activated MAP-kinases in from these cells. Despite strong inhibition of reporter gene activity, expression of RPM had no effect on the phosphorylation state of the Erk, JNK and p38 MAP-kinases (4.15).

4.10 Discussion of Chapter IV

We have identified a novel potential effector protein of M-Ras and p21 Ras we termed RPM. RPM was most closely related to Rlf. The regions of highest conservation between RPM and other members of the RalGDS-family were the C-terminal Ras binding domain (RBD), the scr1, 2 and 3 regions that form the catalytic core of Cdc25 family members of GEFs and the N-terminal scr-0 domain. Consistent with these structural features, Shao et al., who independently identified the same protein based on its ability to interact with an activated mutant of Rit, demonstrated that RPM is a specific GEF for the small GTPase Ral (Shao and Andres, 2000). Although the RBDs of RalGDS and RPM were very similar, the RBD of RPM bound comparably to constitutively active mutants M-Ras Q71L and H-Ras G12V, whereas the RBD of RalGDS showed strong preference for constitutively active H-Ras G12V and bound only weakly to constitutively active M-Ras Q71L. These data obtained from in vitro precipitations of Ras proteins from cell lysates using the Ras-binding domains of various Ras effectors are consistent with the data obtained from our yeast two-hybrid screen (Table 1.1). They suggest that Nore1,
AF-6, Rin1 and RPM form a subset of potential p21 Ras effectors, which are also used by M-Ras. They also indicate that the C-terminal 143 residues of RPM are necessary and sufficient for Ras-binding.

In other experiments we confirmed the interaction of full length RPM with constitutively active M-Ras. Again, full length RPM co-precipitated equally well with M-Ras Q71L or H-Ras G12V. We noted however that the binding of RPM to M-Ras G22V was much weaker than to M-Ras Q71L. This might reflect conformational differences between the two mutants or differences in their residual intrinsic GTPase activity. In separate experiments we have shown that RPM binds to GTP-bound wild-type M-Ras after activation by co-expression of an exchange factor (Korherr et al., manuscript in preparation). We have also demonstrated the binding of RPM to activated but not inactive forms of M-Ras or H-Ras by three different means; the yeast two-hybrid system, co-immunoprecipitation and affinity-purification. Collectively, these data indicate that RPM is a potential effector of both M-Ras and p21 Ras.

The previously described members of the RalGDS-family RalGDS, Rlf and Rgl synergize with p21 Ras in activating the \textit{c-fos} promoter whereas membrane localization of these proteins by fusing them to the CAAX-motif of p21 Ras rendered them constitutively active (Murai et al., 1997; Okazaki et al., 1997; Wolthuis et al., 1997). Likewise, RalGDS and Rgl synergize with Raf-1 in activating the \textit{c-fos} promoter (Murai et al., 1997; Okazaki et al., 1997) although Rlf, which is most closely related to RPM, does not (Wolthuis et al., 1997). These synergistic effects of RalGDS and Rlf on activation of the \textit{c-fos} promoter are not solely related to
their GEF-activity for Ral, since a constitutively active Ral G23V could not substitute for RalGDS or Rif in these experiments (Okazaki et al., 1997; Wolthuis et al., 1997). It remains possible that Ral has to be able to shuttle between a GTP-bound state and a GDP-bound state in order to synergize with Ras in gene induction. A constitutively active mutant of Ral, which remains locked in its GTP-bound configuration might thus be unable to replace the exchange factor in this experimental setting. However, the fact that constitutively active Ral G23V is able to activate transcription from a NFκB-controlled reporter gene (Henry et al., 2000) argues against this possibility. Our data indicate that RPM differs markedly from the other RalGDS-family members in this respect. Thus, RPM not only failed to synergize with H-Ras G12V and MEKK-1 in activating the Elk-1 transcription factor, but potently inhibited this activation. This observed inhibition was not the result of lack or decrease of expression of the activating construct. Moreover, it was not due to toxic effects of overexpression of RPM, as overexpression of RPM or any of the RPM mutants had no effect on Raf-1 mediated reporter gene activation and did not influence expression of co-transfected GFP. Nor was the inhibitory effect the result of blockade of p21 H-Ras by the formation of inactive RPM-Ras complexes. The mutant of RPM that lacked the RBD and was thus no longer able to bind to Ras, still strongly inhibited H-Ras-dependent activation of Elk-1. In contrast, the presence of the RBD of RPM was necessary for inhibition of M-Ras-mediated Elk-1 activation. This suggests that the inhibition of M-Ras-mediated reporter gene activation was solely due to blockade of M-Ras by the formation of RPM / M-Ras complexes that cannot activate Elk-1. There was compelling evidence that a second
signal, provided by activated p21 Ras or MEKK-1, but not by Raf-1 or activated M-Ras, was required for RPM to exert its negative effects on Elk-1 activation. Our data indicate that this signal might be provided by either MEKK-1, or proteins downstream of MEKK-1, which lead to the activation of the stress activated protein kinases (SAPK/JNK) and p38. The fact that M-Ras mediated activation of Elk-1 could not be inhibited by RPM that lacked the RBD argues that PI3-kinase could not provide this second signal as M-Ras interacts with and activates PI3-kinase (Kimmelman et al., 2000). Interestingly, transformation suppressor activity that was independent of exchange factor activity has also been reported for the GEF C3G (Guerrero et al., 1998).

Recently, another inhibitory Ras effector, RASSF1, has been described (Dammann et al., 2000). RASSF1 acts as tumor suppressor and induces apoptosis (Vos et al., 2000). In contrast, the inhibitory effects of RPM on cell growth and Ras-mediated gene induction occur without inducing cell death.

The mechanism of this inhibitory action of RPM requires further investigation. Our data demonstrate that the phosphorylation state of the Erk, JNK and p38 MAP kinases is not affected. It remains possible that activation of RPM uncouples Elk-1 activation from MAP kinase phosphorylation. Inhibition of Elk-1-dependent reporter gene activity without inhibition of MAP kinase phosphorylation has recently been reported. In particular, the Kinase Suppressor of Ras (KSR) inhibits EGF-induced Elk-1 phosphorylation without affecting Erk activity (Sugimoto et al., 1998). Inhibition of activation of Elk-1 or the c-fos promoter without affecting Erk activity has also been reported as a result of an overexpressed Gab2 mutant in Ba/F3 cells stimulated
with IL-3 (Gu et al., 1998). The fact that RPM not only inhibited reporter gene activity but also led to decreased colony formation of src-transformed fibroblasts in semi-solid medium suggests that RPM might have important regulatory roles in cell physiology.

The clones we obtained from our yeast two-hybrid screen with M-Ras G22V fell into three groups, based on the strength of their interaction with M-Ras G22V. The group most strongly interacting with M-Ras G22V was comprised of RPM, AF-6, Norel and Rinl. These studies define a subset of p21 Ras effectors as candidate effectors for M-Ras. Our results obtained from the yeast two-hybrid system contrast with those of Quilliam et al. (Quilliam et al., 1999), who failed to see any interaction in the yeast two-hybrid system between constitutively active M-Ras Q71L and RalGDS, A-Raf and particularly Rin1. These differences could be related to the different yeast two-hybrid systems, as we used a Gal4-based two-hybrid system whereas Quilliam et al. used a LexA-based system (Mendelsohn and Brent, 1994). Co-immunoprecipitation studies using the full-length proteins in addition to affinity precipitations should resolve these discrepancies.

Interestingly, we noted that all of the potential effectors which showed strong binding to constitutively active mutants of M-Ras in both yeast two-hybrid system and co-precipitation assays were distinguished by the presence of proline rich regions in which a four proline 'PPPP'-motif (residues 31-34 in Nore1, residues 1396-1399 in AF-6, residues 287-290 in Rin1, residues 563-566 in RPM) is flanked by other 'PxxP'-motifs that could form potential SH3-domain
binding sites. The significance of these poly-proline motifs in regulating interactions with other signaling molecules or scaffolds is currently under investigation.
CHAPTER V: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The p21 Ras proteins have been subjects of intense investigation over the last two decades. In the first part of this study we describe the identification of a novel member of the Ras family and demonstrate that the tools that have been used to assess activation of p21 Ras do not discriminate between M-Ras and p21 Ras, implicating that data hitherto ascribed to p21 Ras activity might in fact reflect activation of both, p21 Ras and/or M-Ras. Development of tools that do discriminate between p21 Ras and M-Ras will help to clarify this situation. In chapter IV we present two such tools, namely the Ras binding domains of RPM and of Norel fused to GST, and demonstrate that affinity purification assays with these reagents can address the states of activation of the different Ras proteins.

One of the most critical questions regarding M-Ras, which remains largely unanswered, is whether there is a specific function associated with this protein. Most of the biochemical evidence for p21 Ras proteins suggest a high degree of redundancy, although the lethal phenotype of mice with a targeted disruption of the K-Ras gene clearly indicates also non-redundant functions. The high degree of phylogenetic conservation of M-Ras allows one to speculate that this particular Ras protein might have a specific function. Recent evidence suggests that M-Ras might be regulated at the transcriptional level (Louahed et al., 1999; Wang et al., 2000). Therefore, M-Ras function must be assessed only in physiological settings. Targeted disruption of the M-Ras gene, development of M-Ras-specific, intracellularly expressed
single-chain antibodies (scFv) or M-Ras antisense constructs will help to answer questions concerning physiological functions of M-Ras. Using polyclonal antisera that were specific for M-Ras or that crossreacted with p21 Ras, we were able to demonstrate a ubiquitous pattern of expression of M-Ras. Unfortunately, the antisera could not be used for FACS-analysis or immunohistochemistry. Valuable information might be gained if M-Ras specific antibodies can be used in FACS-analysis to determine which subsets of cells in spleen and thymus do express M-Ras.

The observation that M-Ras interacts specifically but only very weakly with the Ras-binding domains of Raf-1 and RalGDS suggests that M-Ras recognizes an overlapping but distinct set of downstream effectors. Based on data derived from our yeast 2-hybrid screen, we could place the Ras effectors RPM, Nore1, Rin1 and AF-6 into a group of proteins that might constitute (part of) the set of M-Ras effector proteins. Since M-Ras has been shown to be upregulated in Con A-treated lymphocytes (Louahed et al., 1999), screening a library prepared from these cells might place additional members into this selected group of M-Ras effector proteins.

The discovery and initial characterization of RPM led to a number of questions that remain to be investigated. We demonstrated that RPM, in contrast to other RalGDS-family members, negatively affects Elk-1 transcriptional activity initiated by activated p21 Ras or MEKK-1. Whereas phosphorylation of MAP kinases was not impaired, immunohistochemistry with anti-phospho MAP kinase antibodies will address the question of whether translocation of
activated MAP kinases into the nucleus is inhibited by RPM. Further possibilities such as RPM expression leading to dephosphorylation of Elk-1, exclusion of Elk-1 from the nucleus, or inhibition of DNA binding of Elk-1, remain to be investigated. Although RPM mRNA was widely expressed, the pattern of expression of RPM protein needs to be investigated. Given the dramatic negative effect of RPM on cell growth and gene induction in response to stimuli that elicit a pro-mitogenic cellular response, one could expect a mechanism of transcriptional regulation of RPM. Identification and characterization of the promoter region of RPM will be important to address this possibility. Data on RalGDS family published thus far hint at a dual function. One function is the activation of Ral whereas an additional, Ral-independent function leads to the observed synergistic effect on gene induction (or inhibition in the case of RPM) in the presence of activated p21 Ras. Mutants of RPM that are incapable of activating Ral might thus be still able to inhibit gene induction, thereby clearly demonstrating an additional function besides activating Ral.

Finally, the question of the physiological role of RPM remains to be addressed. RPM requires a second signal to exert its negative effects on cell growth and gene induction, which can be provided by p21 Ras. Possible scenarios in which a cellular response to a Ras signal is not of mitogenic nature might be in situations of cell stress or in differentiation processes. Targeted disruption of the RPM gene will help to answer these questions. Since the initial characterization of RPM revealed strong inhibition of transformed cells, a RPM 'knock-out' mouse model might help to establish RPM as a novel tumor suppressor.
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