Identification of New Targets for the Rho and Rac GTPases

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Abstract

Rho and Rac are small GTP binding proteins which, as molecular switches, are involved in the co-ordinated regulation of the actin cytoskeleton and gene transcription. Rho controls the formation of actin stress fibres and focal adhesion assembly, while Rac regulates formation of lamellipodia and focal complexes. Both Rho and Rac have also been implicated in regulation of gene transcription through the Serum Response Factor, NFκB, and JNK MAP Kinase pathways. It is thought that Rho and Rac mediate these effects by interacting with a variety of cellular targets, but the molecular details of the signalling pathways involved are still unclear. In this thesis I describe the cloning and characterisation of ROKβ and POSH, two targets of Rho and Rac GTPases. ROKβ is a candidate target for Rho and Rac in controlling the actin cytoskeleton. POSH is a candidate target for Rac in controlling gene transcription through the NFκB and JNK pathways.
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CHAPTER 1

INTRODUCTION

1.1 THE RAS SUPERFAMILY OF SMALL GTP-BINDING PROTEINS

The Ras genes were first identified as the transforming principle of two related murine retroviruses, Harvey Sarcoma Virus and Kirsten Sarcoma Virus (Barbacid, 1987). Human cellular homologs of these genes were subsequently isolated (Ha- and Ki-Ras), along with a third member of the family, N-Ras (Bos, 1989). Ras proteins are expressed in all eukaryotic cells. They function as monomeric regulatory GTP (guanosine triphosphate)-binding proteins, cycling between an active GTP-bound and an inactive GDP (guanosine diphosphate)-bound form to control cell growth and differentiation (Hall, 1990). This GTPase cycle is controlled by GTPase Activating Proteins (GAPs), which accelerate the slow intrinsic rate of GTP hydrolysis, and Guanine nucleotide Exchange Factors (GEFs), which catalyse exchange of GDP for GTP (see figure.1.1). Thus, the activation state of these switches is tightly controlled.

Once activated, Ras proteins exert their cellular effects by interacting with effector or target molecules. The crystal structure of Ha-Ras has revealed that the region between amino acids 30-40, which had been shown by mutational analysis to be important for biological activity, undergoes a significant conformational change upon exchange of GDP to GTP (Wittinghofer and Pai, 1991). This confirmed the importance of this region of Ras for biological function. A major cellular role of Ras is to regulate the ERK1/2 (Extracellular Regulated Kinase) MAP (Mitogen Activated Protein) Kinase pathway by directly interacting with an upstream kinase, c-raf (Marshall, 1996). Other known mammalian Ras effectors include phosphatidylinositol-3-OH kinase (Rodriguezviciana et al., 1994, Kodaki et al., 1994), ralGDS (ral GDP Dissociation Stimulator) (Hofer et al., 1994, Spaargaren et al., 1994), Rin1 (ras
interaction/interference) (Han and Colicelli, 1995) and PKC\(\zeta\) (Diaz-Meco et al., 1994).

\[
\text{GTP} \xrightarrow{\text{GEF}} \text{GDP} \xrightarrow{\text{GAP}} \text{GTP} \xrightarrow{\text{Pi}} \text{GDP}
\]

**Figure 1.1 The GTPase cycle**

GEF: Guanine nucleotide exchange factor. GAP: GTPase activating protein.

Since the discovery of Ras, a large number of Ras-related GTP-binding proteins have been discovered. These proteins share around 50% amino acid homology with Ras and regulate a variety of cellular functions (see figure 1.2). Ras-related proteins have been grouped into seven subfamilies according to sequence homology. The Ras family controls cell growth and
differentiation, while the Rab, Sar and ARF families are implicated in vesicle transport and Ran is involved in nuclear transport (Hall, 1990, Balch, 1990). Members of the Rho family control the actin cytoskeleton and gene transcription (Van Aelst and D'Souza-Schorey, 1997)

<table>
<thead>
<tr>
<th>subfamily</th>
<th>members</th>
<th>biol.function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras</td>
<td>H-,Ki-,N-Ras, R-Ras, Rap 1,2, Ral, TC21</td>
<td>growth, differentiation</td>
</tr>
<tr>
<td>Rho</td>
<td>Rho A,B,C, Rac1,2, Cdc42, Rho E, RhoG, RhoH, TC10</td>
<td>actin cytoskeleton, gene Transcription, NADPH oxidase</td>
</tr>
<tr>
<td>Rab</td>
<td>Rab1-X</td>
<td>vesicle transport</td>
</tr>
<tr>
<td>ARF</td>
<td>ARF1-6</td>
<td>vesicle transport</td>
</tr>
<tr>
<td>Sar1</td>
<td>Sar1</td>
<td>vesicle transport</td>
</tr>
<tr>
<td>Ran</td>
<td>Ran1</td>
<td>nuclear protein import</td>
</tr>
</tbody>
</table>

**Figure 1.2 The Ras-superfamily**
The more distantly related Rad/Gem/Kir family is not included.
1.2 THE RHO SUBFAMILY

1.2.1 Introduction

1.2.1.1 Cloning, sequence and structure

The Rho gene was originally cloned in the marine mollusc *Aplysia californica*, where it was fortuitously isolated in a low stringency cDNA screen. The *Aplysia* sequence was used to clone the first human Rho family members: RhoA, B and C (Madaule and Axel, 1985, Yeramian et al., 1987). There are currently eight mammalian Rho subfamily members: Rho (A, B and C isoforms), Rac (isoforms 1 and 2), Cdc42 (Cdc42Hs and G25K isoforms), RhoE, RhoG, TC10 and RhoH/TTF (Van Aelst and D'Souza-Schorey, 1997). Most of these genes are ubiquitously expressed, with the exception of RhoB and G which are serum-inducible and Rac2 and RhoG/TTF, which are mostly present in myeloid cells (Didsbury et al., 1989, Moll et al., 1991, Shirsat et al., 1990, Vincent et al., 1992). Homologs of some of these proteins have now been identified in many other organisms including yeast (Van Aelst and D'Souza-Schorey, 1997).

Proteins of the Rho family each share around 30% overall homology with other Ras superfamily members and at least 55% homology with each other (see figure 1.3). Amino acids 140-153 of Rho GTPases correspond to a 14 amino acid insert region which is not found in Ras. Apart from this, the three dimensional structure of Ras and Rho proteins seems largely similar (Hirshberg et al., 1997, Rittinger et al., 1997).


Ha-Ras       MTEY...V..A.GV..SA.T.QLIQNH.VDE.D
rhoA         MAAIRKRLVIVGDGACGTKTLLIVFSKDFQPEVYV
rac1         .Q..  .C.V......V........SYTTNA..GE.I
G25K         .QT.  .C.V......V........SYTTNK..SE..
TC10         MAHGPG.LML.C.V......V......MSYAN.A...E..

Ha-Ras       .IES.D.RKQVV...ETCL.DIL......E.SAM.
rhoA         PTVFENVADIEVGKQVELALWDTAGQEDYDRLR
rac1         ....D..S.NVM....P.N.G.............
G25K         ....D..AVTVMIG.EPYT.G.F.............
TC10         ....DH.AVSVT.G...YN.G.Y.............

Ha-Ras       DQYMTGGEF.CV.A.NNTK.F.D.HQYREQIKRV
RhoA         PLSYPEDTVILMCFSIDSPDLENIEPKWTFEVK
Rac1         ....Q...F.I.....LV..A.F..VRA..Y...R
Cdc42        ....Q...F.V.....VV..S.F..VK...V..IT
TC10         ....M.....I.....VVN.A.FQ.VK.E.V..L.

Ha-Ras       KDSDD..MV.....C..AARTVES.QAQDLARSYG
RhoA         HFCPNVPILLVGNKKDLRNDEHTRRELAKMKQEPV
Rac1         ....H......T......T.L......D.KD.IEK.KEL.LT.I
Cdc42        ....H..KT.FL...TQI...D.PS.IEK...N.K.I
TC10         EYA.....FL.I.TQI...D.PK.LAR.ND..EK.I

Ha-Ras       I.        .I.T....RQ..EDA.YTL
RhoA         KPEEGRDAMNIGAFGMECSAOKTDGVREVVEFA
Rac1         TYPQ.LA..KE....VK.L....L.QR.LKT..DE.
Cdc42        T..TEAKL.RDLK.VK....L.QR.LKN...DE.
TC10         CV.Q.QKL.KE....CC.V.....L.QK.LKT..DE.

Ha-Ras       V.EIR.HKLR.LNPPDESFGPSCMSCK.VLS
RhoA         TRAAALQARRGKKKSG      CLVL
Rac1         I..V.CPPP.V..RKRK     ..L.
Cdc42        IL...EPPETQP.RK     .CIF
TC10         II.I.TFKKHTV.KRIGSRCIN C..IT

Figure 1.3  Sequence comparison of Ha-ras with members of the rho-subfamily
Cdc42: G25K isoform shown.
1.2.1.2 Post-translational modifications and localisation of Rho proteins

Like Ras, Rho proteins are post-translationally modified by addition of a lipid moiety to a cysteine residue four amino acids from the carboxy terminus, the so-called "CAAX box". For Rho (A, B, C) and Rac (1 and 2), "X" is a leucine, for Cdc42 (Cdc42Hs and G25K) and RhoH/TTF, X is a phenylalanine, and for TC10, X is a threonine. These proteins are therefore expected to be modified by addition of a C20 geranyl-geranyl moiety, although RhoB has been shown to be present in both geranyl-geranyl (C20) and farnesyl (C15)-modified forms. It is likely, therefore, that other signals besides the CAAX box are important for lipid modification (Adamson et al., 1992a, Katayama et al., 1991, Kinsella et al., 1991, Yamane et al., 1991). The lipids are thought to act as plasma membrane anchors.

The three Ras proteins have been shown to contain an additional plasma membrane targeting signal (Hancock et al., 1990). Most Rho subfamily members possess a polybasic region immediately amino terminal of the CAAX box similarly to Ki-ras, while RhoB and TC10 have two cysteine residues forming a potential palmitoylation signal, similarly to Ha- and N-Ras. Despite these signals and unlike Ras proteins, Rho subfamily members are not exclusively localised to the plasma membrane. RhoA and C are predominantly cytosolic, with only a small proportion at the plasma membrane, while most of RhoB localises to early endosomes and pre-lysosomal compartments (Adamson et al., 1992b). Rac is mainly cytosolic, while Cdc42 appears to be mostly present on the endoplasmic reticulum and the Golgi apparatus (Abo et al., 1991, Quinn et al., 1993, Erickson et al., 1997). RhoE was found both in the cytoplasm and in unidentified "worm-like" structures at the plasma membrane (Foster et al., 1996).

1.2.2 Regulators of Rho Proteins

1.2.2.1 GTPase Activating Proteins

Ras-related proteins have a relatively slow intrinsic rate of GTPase activity (Hall, 1990), but this can be stimulated by
GTPase activating proteins or GAPs. p50RhoGAP was the first characterised GAP for Rho proteins. It was originally identified as an activity present in spleen cytoplasm, then purified from spleen and platelets and finally cloned (Garrett et al., 1989, Garrett et al., 1991, Lancaster et al., 1994, Barfod et al., 1993). The RhoGAP domain consists of three blocks of homology of 30-40 amino acids each and spread over around 150-200 amino acids. Around 14 GAPs for Rho GTPases have subsequently been identified through sequence homology to p50RhoGAP (see figure 1.4). Apart from the GAP domain (usually 20-40% identity at the amino acid level), the structural characteristics of the rest of these proteins are very varied (figure 1.4).

Some GAPs have domains that suggest a connection with cytoskeletal regulation. Both MyosinIXb and Myr5 (fifth unconventional myosin from rat) possess a GAP domain, though GAP activity has only been demonstrated for Myr5 (Reinhard et al., 1995). Since formation of actin-myosin structures is an important aspect of Rho signalling, these myosins could well play a part in regulating the cytoskeleton. Graf (GTPase regulator associated with FAK) binds to Focal Adhesion Kinase (FAK) and could be involved in focal adhesion assembly/disassembly (Hildebrand et al., 1996). Interestingly, p85, the regulatory subunit of PI3Kinase, also has a GAP-like domain but shows no detectable GAP activity Nobes et al., 1995, Reif et al., 1996, Rodriguez-Viciana et al., 1997).

The substrate specificity of GAPs has been examined in vitro. Some, like p50RhoGAP or p190RhoGAP can catalyse GTPase activity on Rho, Rac and Cdc42, while the activity of chimaerin and BcrGAP appears to be restricted to Rac and Cdc42 (Lamarche and Hall, 1994). The specificity of GAPs in vivo has also been examined: microinjection of p190RhoGAP into cells, for example, inhibits formation of Rho-induced stress fibres, but not of Rac-induced membrane ruffles (Ridley et al., 1993). Both the regulation and the cellular role of GAPs in Rho signalling pathways are largely unknown.
Figure 1.4  Schematic representation of mammalian proteins containing a rhoGAP (GAP) homology domain

Regions with homology to rhoGAP are shown in black; P: proline-rich (SH3 binding) region; Cys: cysteine-rich (phorbol ester binding) region; DH: Dbl homology domain; SH2, SH3: Src-homology domains; IQ: IQ (calmodulin binding) motif.
1.2.2.2 Guanine nucleotide exchange factors (GEFs)

The Dbl protein was cloned in a screen for focus-inducing DNAs in NIH 3T3 fibroblasts (Eva and Aaronson, 1985). It shares 29% sequence identity with Cdc24, a budding yeast protein lying genetically upstream of the small GTPase Cdc42p. This homology provided the first clue to Dbl's function as a Rho family GTPase regulator (Ron et al., 1991). It was subsequently demonstrated that Dbl is able to catalyse exchange of GDP to GTP on human Cdc42 in vitro. Deletion analysis showed that exchange activity is due to a 200-250 a.a. domain termed Dbl Homology (DH) domain, which is also required for oncogenicity (Hart et al., 1991, Ron et al., 1991, Hart et al., 1994). Both Dbl and Cdc24 bear a Pleckstrin Homology (PH) domain. PH domains are approximately 100 a.a.-long motifs thought to mediate plasma membrane localisation, possibly by interacting with lipids such as PIP2 (Pawson, 1995).

Since then, over 20 proteins containing a DH/PH module have been identified, many in screens for novel oncogenes (see Cerione and Zheng, 1996 for a review). Although most of these proteins have been assumed to be Rho family protein exchangers, in vitro activity has not been demonstrated for all of them. In vivo specificity has been examined for some of these GEFs, such as Lbc (Rho but not Rac or Cdc42) or FGD1 (Cdc42 but not Rho or Rac) (Olson et al., 1996). Like GAP-domain-containing proteins, DH/PH-containing proteins have varied stuctures outside their DH/PH modules (see fig 1.5). Bcr even has a GAP domain and DH/PH domains. The Trio protein has two DH/PH modules, the N-terminal one being active on Rac and the C-terminal one on Rho. The signalling events that link plasma membrane receptors to Rho GTPases via GEFs are not well understood (Cerione and Zheng, 1996).

SmgGDS is not a DH domain-containing protein, but it has been shown to catalyse nucleotide exchange on Rho as well as Ras proteins (Kikuchi et al., 1992). The physiological function of SmgGDS is unclear.
### Figure 1.5 Schematic representation of mammalian proteins containing a dbl homology (DH) domain

<table>
<thead>
<tr>
<th>Protein</th>
<th>DH: Dbl homology domain; PH: Pleckstrin homology domain; GAP: rhoGAP homology domain; P: proline-rich (SH3 binding) region; C: cysteine-rich (phorbol ester-binding) region; SH2, SH3: Src-homology domains; Cdc25: region with homology to cdc25; S: Spectrin-like repeat; Coil: Coiled-coil.</th>
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<tbody>
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1.2.2.3 RhoGDI (Rho GDP Dissociation Inhibitor)

RhoGDI was purified from bovine brain cytosol and found to inhibit the dissociation of GDP from and the subsequent binding of GTP to Rho proteins (Ueda et al., 1990, Fukumoto et al., 1990). It interacts only with post-translationally modified Rho, Rac and Cdc42 (Ueda et al., 1990, Knaus et al., 1992, Leonard et al., 1992). Furthermore, RhoGDI interacts with GTP-bound Rho, Rac and Cdc42 and blocks intrinsic and GAP-stimulated GTPase activity in vitro (Hart et al., 1992, Chuang et al., 1993, Hancock and Hall, 1993). Confusingly, therefore, RhoGDI appears to lock GTPases in both their active and inactive conformation.

Two novel GDIs have recently been cloned. LY-GDI (also known as D4-GDI) is expressed only in hemopoietic cells and binds Rho, Rac and Cdc42 (Lelias et al., 1993, Scherle et al., 1993). RhoGDIγ (and its mouse homolog RhoGDI3) binds RhoB, RhoG, Cdc42 and possibly RhoA (Zalcman et al., 1996, Adra et al., 1993). D4-GDI can be cleaved by apoptotic proteases, though the significance of this finding is unknown (Na et al., 1996).

The most attractive model for GDI function is as a regulator of GTPase plasma membrane translocation. In unstimulated cells, RhoA is cytoplasmic and complexed with RhoGDI which, it is thought, masks the GTPase's C-terminal membrane anchor. Upon agonist stimulation, a fraction of the GTPase is released from GDI and tranlocates to the plasma membrane (Takai et al., 1995). Recently Takahashi et al showed that GDI interacts directly with ERM (Ezrin Moesin Radixin) proteins in vitro and that this interaction resulted in release of Rho from GDI (Takahashi et al., 1997). It is not yet known whether the ERM protein mediate GTPase release from GDI in vivo which would then allow guanine nucleotide exchange to occur.
1.3 FUNCTION OF MAMMALIAN RHO, RAC AND CDC42

1.3.1 Biological Functions of Rho

1.3.1.1 Rho, the actin cytoskeleton and adhesion

The bacterium *Clostridium botulinum* produces an exoenzyme, C3 transferase, which ADP-ribosylates Rho on asparagine 41, causing loss of function (Aktories, 1997). It is believed that modification of this residue interferes with the interaction between Rho and its cellular targets. C3 transferase has proved to be an invaluable tool in the study of Rho function. Its delivery to a variety of cell types induces dissolution of actin filaments and cell rounding within 15-30 min (Aktories *et al.*, 1989, Chardin *et al.*, 1989, Sekine *et al.*, 1989, Paterson *et al.*, 1990). Complementary experiments using a constitutively active form of Rho (G12V) revealed that when introduced into fibroblasts by microinjection, it triggered an increase in organised actin filaments (Paterson *et al.*, 1990). It was clear from these early studies that Rho had a critical function in the regulation of the actin cytoskeleton.

The actin cytoskeleton is found in all eukaryotic cells and is highly dynamic. It consists of rapidly polymerising and depolymerising filaments composed of actin monomers. Actin filament assembly and disassembly are key features of many cell processes including motility, chemotaxis, cell division, muscle contraction, axonal outgrowth and phagocytosis. The most thermodynamically favourable state for actin in the cell is the filamentous form, but in a typical cell, only 50% of the total actin is filamentous (F-actin), while 50% is monomeric (G-actin). The length and number of actin filaments is therefore tightly regulated. This is achieved by actin monomer sequestering proteins such as thymosin and filament capping proteins such as capZ or gelsolin which bind to the fast-growing (barbed) end of actin filaments to prevent addition of monomers. Both types of protein can be regulated by signal transduction events when an increase in polymerised actin is required, as will be discussed below. It is also known that nucleation of new actin filaments can occur but this is not yet well understood (Stossel, 1993).
The actin cytoskeleton is also intimately associated with cellular adhesion. Actin filaments make contact with extracellular matrix components such as fibronectin and vitronectin through focal complexes. These multi-molecular structures contain integrin receptors for the extracellular matrix which, through their cytoplasmic tail, interact with actin-binding proteins such as talin and α-actinin. Focal complexes contain many other actin-binding proteins, like tensin, as well as signalling molecules such as ILK (Integrin-Linked Kinase) and Focal Adhesion Kinase (FAK). They are thought to be important signalling centres (Clark and Brugge, 1995, Hannigan et al., 1996).

Actin filaments also interact with cadherin-containing cell-cell contact sites at adherens junctions. Like focal adhesions, these structures are based around transmembrane proteins called cadherins, which can interact homotypically with other cadherin molecules on neighbouring cells. Cadherins are linked to the cytoskeleton via α-catenin, which is also thought to be involved in intracellular signalling (Geiger and Ayalon, 1992, Grunwald, 1993).

The function of Rho GTPases in cytoskeletal regulation has been studied extensively in fibroblasts where at least four well defined types of filamentous actin structures can readily be seen: (a) Cortical actin: a thin band of filaments distributed at the cell cortex. (b) Lamellipodia: flat protrusions of the plasma membrane at the edge of the cell, containing arrays of short filaments tightly packed behind the membrane. Lamellipodia often detach and fold back into the cell in a process called membrane ruffling. (c) Filopodia or microspikes: thin, finger like structures that extend from the cell body and contain long actin bundles. These structures are very dynamic and constantly extend and retract. (d) Stress fibres: thick, well organised bundles of actin which traverse the cytoplasm. These structures attach the cell firmly to the substratum, as they terminate in large, arrowhead-shaped focal complexes.

Swiss 3T3 fibroblasts have provided a powerful model cell system to elucidate the role of Rho GTPases in cytoskeletal regulation. When these cells are grown to a confluent quiescent
state and subsequently starved of serum, they lose most of their polymerised actin structures (apart from a thin band of cortical actin). Microinjection of an activated form of Rho (G14V or Q63L) protein into these cells induces the rapid reassembly of thick parallel stress fibres, associated with large focal complexes (Ridley and Hall, 1992). Serum (whose active component is Lysophosphatidic acid (LPA)) also triggers stress fibre formation but its effect can be blocked by prior inactivation of endogenous Rho with C3 transferase. It has been concluded that Rho regulates a signal transduction pathway linking the LPA receptor to stress fibre and focal complex assembly. Since then, a similar function for Rho has been described in other cell types such as MDCK cells (Ridley et al., 1995a), macrophages (Allen et al., 1997) and neurons (Jalink et al., 1994).

There is some evidence that the assembly of stress fibres and focal adhesions might be a consequence of two separate activities of Rho. Pretreatment of Swiss 3T3 cells with cytochalasin D, for example, blocks Rho-induced stress fibre formation, but not focal complex assembly, while the serine/threonine kinase inhibitor staurosporine inhibits Rho-induced focal complex formation, but not some actin changes (Nobes et al., 1995). In any event, it has been observed that fibroblasts plated on poly-L-lysine reorganise their actin cytoskeleton in response to Rho or Rac in the absence of integrin clustering (Hotchin and Hall, 1995, Machesky and Hall, 1997).

A role for the Rho GTPase in the assembly of cell-cell contacts has been reported. Rho inactivation in the polarised intestinal epithelium cell line T84 resulted in increase in tight junction permeability, disruption of actin localisation and displacement of the tight junction component ZO-1 to the cytoplasm (Nusrat et al., 1995). More recently, Braga et al showed that both Rho and Rac are required for assembly of cadherin-containing cell-cell contacts in human keratinocytes (Braga et al., 1997). Since cell-cell contacts are associated with the actin cytoskeleton, the Rho-dependent pathways controlling their establishment are likely to have elements in common with the stress fibre/focal adhesion pathway. However, the precise molecular mechanisms have not yet been elucidated.
Other specialised functions for Rho include control of osteoclast cytoskeletal structures implicated in bone resorption and regulation of the assembly of an actin cable during embryonic wound repair (Zhang et al., 1995, Brock et al., 1996).

1.3.1.2 Rho and cell cycle progression

Several lines of evidence suggest that Rho is involved in G1 cell cycle progression. Microinjection of activated Rho has been reported to induce DNA synthesis in Swiss 3T3 cells, while microinjection of C3 transferase inhibits serum-induced DNA synthesis (Olson et al., 1995). This suggests that Rho plays a critical role in cell cycle progression through G1.

There have been conflicting reports as to whether Rho can act as an oncogene. The Rho gene from the marine snail Aplysia was reported to be tumorigenic when transfected into NIH3T3 cells (Perona et al., 1993). Self et al, on the other hand, detected rare foci when mammalian V14Rho cDNA was transfected into NIH3T3 cells, but these clones were not transformed (Self et al., 1993). This is surprising since Rho exchange factors such as Lbc were identified because of their oncogenic potential (Toksoz and Williams, 1994). It is possible that Lbc activates other Rho-independent pathways that contribute to transformation.

More recently, it has been reported that Rho plays a critical role in Ras-induced transformation (Qiu et al., 1995, Prendergast et al., 1995).Dominant negative Rho inhibited Rat1 cell transformation induced by Ras but not by v-Raf (Qiu et al., 1995, Prendergast et al., 1995, Khosravi-Far, 1995). Furthermore, even though Rho did not induce focus formation in these experiments, it collaborated with Raf-CAAX (a Raf construct activated by fusion to the Ki-Ras membrane localisation signal) for focus formation. (Qiu et al., 1995, Khosravi-Far, 1995). These results indicate that Rho plays a critical role in Ras-induced transformation. Rac and Cdc42 have also been shown to be required for Ras transformation (see below), leading to the suggestion that Ras transforms cells via a combination of the Rho GTPases and the ERK MAPK pathway (Khosravi-Far, 1995).
1.3.1.3 Rho in cytokinesis

Rho is also required later in the cell cycle during cytokinesis. At the end of mitosis, an actin/myosin contractile ring forms along the cleavage plane. Contraction leads to ingression of the cleavage furrow and separation of the daughter cells (Schroeder, 1990). Microinjection of C3 transferase into dividing sand dollar eggs disrupted organisation of the contractile ring and inhibited cytokinesis (Toksoz and Williams, 1994). Interestingly, cells transformed with oncogenic GEFs such as Lbc, Dbl or Vav are often prone to becoming multinucleate (Katzav et al., 1995; Katzav, 1995; Ron et al., 1991).

Treatment of Xenopus eggs with C3 resulted in disruption of the cortical actin present at the membrane and prevented formation of the contractile ring (Drechsel et al., 1997). C3 did not, however, block specification of the cleavage furrow, or insertion of new membrane at the cleavage furrow. These results indicate that Rho regulates actin structures connected to cytokinesis, but not vesicle trafficking leading to insertion of new membrane.

1.3.1.4 Rho and the immune system

Cells of the immune system such as macrophages or T-lymphocytes are highly motile and contain a variety of specialised actin structures. It is likely, therefore, that Rho plays a part in immune system function. In agreement with this, C3 induces disorganisation of the actin microfilament network and inhibits spontaneous and chemoattractant-induced motility in bovine neutrophils (Stasia et al., 1991). In mast cells, Rho regulates assembly of actin fibres and is required for regulated secretion, though it is thought that these might represent two distinct activities of the GTPase (Norman et al., 1994). C3 blocks lymphocyte-mediated cytotoxicity when introduced into natural killer cells (Lang et al., 1992).

Integrin-mediated adhesion is also an important process in lymphocytes; chemoattractants, for example, induce recruitment of lymphocytes to sites of infection or injury where they adhere to the blood vessel surface. This adhesion process is
mediated by integrins such as LFA-1, fMLP, Interleukin-8 or PMA-induced leukocyte adhesion via integrins are blocked by C3 (Laudanna et al., 1996, Tominaga et al., 1993). These findings suggest a role for Rho in lymphocyte adhesion.

Transgenic mice carrying a C3 transgene targeted to the thymus had strikingly small thymi (Henning et al., 1997). Further analysis revealed that this phenotype was due to decreased proliferation in some cell types and increased cell death in others, but not to defects in T-cell differentiation (Henning et al., 1997, Galandrini et al., 1997).

1.3.1.5 Rho and neuronal cells

In PC12 and NIE-115 cells, C3 treatment induces neurite outgrowth (Nishiki et al., 1990, Takaishi et al., 1993). On the other hand, activation of Rho through addition of LPA causes rapid neurite retraction, an effect which can be blocked by C3 (Jalink, 1994). Thus, Rho may regulate contractile actin/myosin cables that prevent neurite outgrowth.

1.3.1.6 Rho and cell motility

Studies using microinjection of RhoGDI as a means of inhibiting Rho proteins demonstrated a role for these GTPases in motility of Swiss 3T3 cells (Takaishi, 1993). This study does not, however, address the contribution of the individual GTPases in motility. The same group subsequently showed that C3 treatment, but not dominant negative Rac, blocks Hepatocyte Growth Factor (HGF)-induced motility in keratinocytes (Takaishi et al., 1994). This shows that Rho but not Rac is required for motility in keratinocytes. In contrast, Ridley et al showed that C3 did not affect MDCK cell scattering induced by HGF, while overexpression of activated Rho blocked this process (Ridley et al., 1995b). C3 treatment blocked motility in sperm and chemotaxis in neutrophils (Hinsch et al., 1993, Stasia et al., 1991).

1.3.1.7 Rho and gene transcription

Regulation of gene transcription by small GTPases via multiple pathways has been a topic of intense interest over the
past few years. Rho was shown to stimulate gene transcription via the Serum Response Factor (SRF) (Hill et al., 1995). Furthermore, Rho is essential for SRF activation by LPA, serum and AIF4-. SRF usually associates with TCF (Ternary Complex Factor) proteins to stimulate transcription of genes containing SREs (Serum Response Elements) in their enhancer region such as the c-fos gene. However, Rho can stimulate SRE transcription in a TCF-independent pathway. One report also claimed that Rho stimulates gene transcription via NFkB (Perona et al., 1997).

1.3.1.8 Rho and vesicle trafficking

Activated mutants of RhoA and Rac have been reported to block endocytosis, though the physiological relevance of this observation is not clear (Lamaze et al., 1996).

1.3.2 Cellular Targets of Rho

Rho is believed to fulfill its biological functions by interacting with a range of cellular target (or effector) molecules (Van Aelst and D'Souza-Schorey, 1997). Since the GTP-bound form of Rho-related GTPases is the active form, it is expected that most cellular targets will bind preferentially to Rho-GTP. A vast amount of work over the past four years has gone into the isolation of targets for Rho and the study of their function in downstream signalling events (Van Aelst and D'Souza-Schorey, 1997).

1.3.2.1 Protein Kinase N (PKN)

PKN (also known as PRK1) and its close homolog PRK2 are 120 kDa serine/threonine kinases with a catalytic domain highly similar to that of PKC and a leucine zipper region (Mukai and Ono, 1994, Palmer et al., 1995). PKN was identified as a Rho target by affinity column chromatography and through homology to another Rho target, Rhophilin (Watanabe et al., 1996a, Amano et al., 1996b). PKN binds to Rho in a GTP-dependent manner and autophosphorylates upon Rho-GTP binding. Its kinase activity is modestly stimulated by cotransfection with activated Rho in COS cells using a PKC
peptide as substrate (Watanabe et al., 1996a, Amano et al., 1996b). PKN was also shown to translocate to the nucleus upon heat shock (Mukai et al., 1996). PRK2, on the other hand, has been reported to bind to and be activated by both GDP- and GTP-Rho (Vincent and Settleman, 1997).

There have been various reports describing potential substrates for PKN. The intermediate filament proteins vimentin and neurofilament were shown to be efficiently phosphorylated by PKN and interestingly this inhibited their ability to assemble into filaments (Mukai et al., 1996, Matsuzawa et al., 1997). It is possible, therefore, that PKN regulates intermediate filament assembly, though, to date, Rho has not been implicated in this process.

PKN has also been shown to bind the actin filament cross linking protein α-actinin in a PIP2-dependent manner, providing a possible connection to the actin cytoskeleton (Mukai et al., 1997). Another report demonstrated an interaction between PKN and the second SH3 domain of the adaptor molecule Nck, though the significance of this is unknown (Quilliam et al., 1996). When microinjected into fibroblasts, PKN has an effect similar to C3 transferase i.e. dissolution of stress fibres, and rounding up of the cells (Vincent and Settleman, 1997). Because of this dominant negative effect, it does not seem likely that PKN has a role in the Rho-dependent assembly of actin filaments.

1.3.2.2 Rho-kinase

Rho-kinase α (ROKα, also known as RhoA-binding kinase α and ROCKII) and its close relative Rho-kinase β (ROKβ also known as p160ROCK) are large (respectively 164 and 160 kDa) serine/threonine kinases with an N-terminal kinase domain related to that of Myotonic Dystrophy Kinase (MDK), a gene mutated in certain forms of Myotonic Dystrophy (Brook et al., 1992, Matsui et al., 1996a, Ishizaki et al., 1996, Ishizaki, 1996 ). Rho-kinase α and β also have a large coiled-coil region which contains the GTPase-binding domain, as well as a Pleckstrin Homology (PH) domain and a cysteine-rich stretch at the N-terminus. Both kinase isoforms are expressed in a wide
variety of tissues They were identified as Rho-binding proteins by ligand overlay assay, yeast two-hybrid, and column chromatography (Leung, 1995, Matsui et al., 1996a, Ishizaki et al., 1996). Rho-kinase binds to Rho in a GTP-dependent manner and there have been some reports that it can also interact with Rac (Matsui et al., 1996a, Ishizaki et al., 1996, Ishizaki, 1996, Lamarche et al., 1996, Watanabe et al., 1997). The function of the Rho-kinases will be discussed in detail in chapter 3.

1.3.2.3 p140mDia

p140mDia was identified in a yeast two-hybrid screen for Rho-interacting proteins (Watanabe, 1997). p140mDia binds to Rho, but not Rac or Cdc42, in a GTP-dependent manner and is a 140 kDa member of a family of formin-related proteins, which includes *Drosophila* Diaphanous and Capuccino and the budding yeast Bni1p. Diaphanous has been shown to be necessary for cytokinesis, whereas Capuccino is essential for egg polarity (Castrillon and Wasserman, 1994, Emmons et al., 1995). Bni1p is involved in yeast bud formation and has already been connected with GTPase signalling (discussed below). p140mDia co-localises with profilin and RhoA to membrane ruffles and co-precipitates with profilin from Swiss 3T3 cells. RhoA, profilin and p140mDia also co-cluster around fibronectin-coated beads. In addition, overexpression of p140mDia in Cos cells leads to an increase in disorganised filamentous actin staining; a potentially interesting result since profilin binds actin and is thought to catalyse its nucleotide exchange, thus promoting actin filament assembly. Finally, p140mDia staining was seen in some but not all cleavage furrows in dividing Swiss 3T3 cells suggesting a role in cytokinesis.

1.3.2.4 Rhophilin

This 71 kDa protein was identified as a Rho target using the yeast two-hybrid system (Watanabe et al., 1996b). The 130 N-terminal residues form the Rho-binding domain, which has 40% homology to PKN. Rhophilin also shares homology with the
C. elegans protein YNK-1 which, like Rhophilin, has no known function.

1.3.2.5 Rhotekin

Rhotekin is a 61 kDa protein isolated as a Rho-specific partner in the yeast two-hybrid system (Reid et al., 1996). Rhotekin has little in the way of identifyable structures, apart from two proline-rich stretches. It shares around 30% homology in its Rho-binding domain with Rhophilin and PKN, leading the authors to name this region REM-1 (Rho Effector Motif-1). No functional data have been published for Rhotekin.

1.3.2.6 Kinectin

Kinectin is thought to be a membrane-bound anchor molecule for the microtubule motor protein kinesin (Burkhardt 1996). Human kinectin comes as a 140 kDa or a 160 kDa protein. The 160kDa form has an N-terminal hydrophobic fragment thought to mediate membrane binding, and thus enable kinesin to move vesicles and organelles along microtubules (Hirokawa, 1996). Hotta et al have isolated human kinectin in a yeast two-hybrid screen for Rho-interacting proteins (Hotta et al., 1996). This is potentially an interesting link between Rho and motor proteins, but no in vitro or in vivo data currently support this interaction.

1.3.2.7 Citron

Identified through the yeast two-hybrid system, Citron binds Rho and Rac but not Cdc42 (Madaule et al., 1995). It is a 183 kDa protein with a PH domain, four leucine zippers and a putative zinc finger. A large coiled-coil region contains the Rho/Rac-binding domain which is similar to the ROK Rho-binding site. This different GTPase-binding motif is termed REM-2 (Rho Effector Motif-2). Citron has no known function.

1.3.2.8 p116RIP

p116RIP (Rho-Interacting Protein) is a Rho-binding protein that was isolated using the yeast two-hybrid system (Gebbink et al., 1997). It binds both Rho-GTP and Rho-GDP, at
least in vitro, and it is not clear, therefore, whether this protein acts as a Rho target. Like Citron, p116 has a large coiled-coil region and a PH domain, but the two proteins do not share significant sequence homology. Overexpression of p116 stimulates neurite outgrowth in N1E-115 cells, an effect similar to C3-induced Rho inactivation. The function of p116 is unknown.

1.3.2.9 Rho and Lipid kinases

One group has reported that Rho stimulates a phophatidylinositol 4-phosphate 5-kinase activity (PI4P5K) leading to the production of PIP2 (Phosphatidylinositol 4,5-bisphosphate) (Chong et al., 1994). The same laboratory reported that a 68 kDa type I PI4P5PK could be affinity purified using immobilised Rho (Ren et al., 1996). However, this binding was not GTP dependent and prior treatment of Rho with C3 enhanced rather than suppressed PI4P5K activity. It is still unclear whether or not the interaction between Rho and PI4P5K is direct or indeed significant since another group, using a similar approach to identify a Rac-linked PI4P5K activity, failed to find any Rho-linked activity (Tolias, 1995).

PIP2 production can potentially affect the actin cytoskeleton in several ways. It has been shown that several actin binding proteins such as profilin, as well as actin filament capping proteins such as gelsolin or capZ bind PIP2 and it has been postulated that PIP2 could promote actin polymerisation both by releasing actin monomers and by displacing capping proteins (Stossel, 1993). More recently, Gilmore and Burridge have shown that binding of PIP2 to vinculin elicited a conformational change that increased vinculin's affinity for actin and talin (Gilmore and Burridge, 1996). This raises the interesting possibility that Rho could mediate formation of focal adhesions and actin filaments at least in part through a PI4P5K. In agreement with this, the same study showed that an anti-PIP2 antibody blocks stress fibre and focal adhesion assembly in fibroblasts.

Two type I PI4P5Ks have now been cloned, called Iα (also known as Iβ1, has an alternatively spliced variant Iβ2) and Iβ
(also known as Iα1, has two splice variants Iα2 and Iα3) (Ishihara et al., 1996, Loijens and Anderson, 1996). PI4P5KIα is 68 kDa in molecular weight and could, therefore, be the protein identified in the Ren et al report (Ren et al., 1996). When transfected into Cos cells, Iα causes increased levels of polymerised actin, an effect which is not blocked by dominant-negative Rho (Shibasaki et al., 1997). However, PI4P5K-induced filaments were short and disorganised and focal complexes were smaller and more numerous than in control non-transfected cells. This is tentative evidence for an involvement of PI4P5KIα in actin rearrangement downstream of Rho, but the question of how Rho affects PI4P5KIα activity, if at all, remains to be addressed.

Several reports have also linked Rho to other enzymes involved in lipid metabolism. Firstly, treatment of Rat-1 cells with C3 blocked PDGF-induced Phopholipase D (PLD) activation (Hess 1997). Secondly, C3 was shown to inhibit GTPγS- or thrombin-induced Phosphatidylinositol 3-kinase (PI3K) activation in platelets (Zhang et al., 1993, Zhang et al., 1995). PLD activation leads to the formation of diacylglycerol and lysophosphatidic acid (via phosphatidic acid), which are important intra and extra cellular signalling molecules respectively. PI3K activation results in the production of PI3,4,5P3 and PI3,4P2. The role of these lipids is not yet fully understood, but it has been proposed that PI3K could play a role in cytoskeletal rearrangements (Rodriguez-Viciana et al., 1997).

1.3.2.10 The ERM proteins

Recently, the ERM (Ezrin Radixin Moesin) family of proteins has generated a lot of interest among groups studying Rho-mediated cytoskeletal rearrangement. These proteins are thought to anchor actin filaments to the plasma membrane by directly interacting with actin and membrane-spanning proteins such as CD44 (Tsukita et al., 1997). Binding of ERM proteins to CD44 in BHK cells was shown to be markedly reduced by treatment with C3 transferase (Hirao et al., 1996). More recently, our laboratory purified moesin as an activity capable of restoring the ability of Rho to induce stress fibres and focal
adhesions in digitonin-permeabilised Swiss 3T3 cells (Mackay et al., 1997). Furthermore, Rho activity appears to be necessary for normal localisation of ERM proteins in MDCK cells (Kotani et al., 1997). These findings make the ERM proteins attractive candidates for mediating some of the effects of Rho on the cytoskeleton. Precisely how Rho affects these proteins remains to be established, since moesin, at least, does not interact directly with Rho in vitro (Mackay et al., 1997).

1.3.2.1 Conclusion

As discussed above, Rho has many cellular functions. Due to intense research activity in the past four years, at least nine potential targets of Rho have now been identified. Despite this, the signalling events downstream of Rho are still poorly understood.

1.3.3 Function of Rac

1.3.3.1 Rac and the actin cytoskeleton

Microinjection of constitutively activated V12 or L61 Rac into confluent, serum-starved Swiss 3T3 cells results in actin polymerisation at the plasma membrane leading to lamellipodia and membrane ruffles (Ridley et al., 1992). Some growth factors, such as PDGF, EGF, thrombin and insulin, have a similar effect which can be blocked by microinjection of N17 Rac, a dominant negative form locked in a GDP-bound state (Ridley et al., 1992). This has led to the proposal that Rac controls a growth factor-mediated signal transduction pathway leading to lamellipodia formation. Lamellipodia are characteristic of motile cells and are thought to be required for cell movement, suggesting that Rac is an important player in cell movement (Machesky and Hall, 1997). Ruffles are derived from detached lamellae and are believed to play a part in pinocytosis.

The ruffling response to growth factor stimulation or to Rac injection is observed after 5 minutes. It was noted that at later time points (20-30 minutes), the cells start to develop stress fibres which can be blocked with C3. This suggested that,
as well as stimulating membrane ruffling, Rac also leads to the activation of Rho, albeit more weakly than direct activation by LPA. It has been reported that Rac-mediated leukotriene production accounts for the activation of Rho (Peppelenbosch et al., 1995).

Microinjection of V12 Ras has been long recognised to cause membrane ruffling (Bar-Sagi and Feramisco, 1985). It is now clear that this is also due to cross-talk between small GTPases and that Ras activates Rac (Ridley et al., 1992). In Swiss 3T3 cells, Ras is not required for Rac activation by PDGF, EGF or insulin (Ridley et al., 1992).

Activation of Rac by PDGF in Swiss 3T3 cells is dependent on PI3K activity, since PDGF receptor mutants that cannot interact with PI3 kinase can no longer induce ruffling. Also, the PI3 kinase inhibitor wortmannin blocks membrane ruffling in response to PDGF or insulin (Wennstrom et al., 1994, Kotani et al., 1994). However, wortmannin has no effect on membrane ruffling induced by microinjection of activated Rac, showing that PI3 kinase activity is required upstream of Rac (Nobes et al., 1995). Reports that PDGF stimulates the formation of Rac-GTP in a PI3 kinase-dependent manner confirm this model (Hawkins et al., 1995). Nobes et al reported that wortmannin did not inhibit Ras-induced membrane ruffling in Swiss 3T3 cells, but Rodriguez-Viciana et al have gone on to show complete inhibition of Ras ruffling with a dominant negative PI3K construct in Porcine Aortic Endothelial (PAE) cells (Nobes et al., 1995, Rodriguez-Viciana et al., 1997). This raises the possibility that Ras can activate Rac through a wortmannin-insensitive PI3K. An interaction between the p85 subunit of PI3 kinase and Rac, requiring the rhoGAP domain of p85, has been described (Zheng et al., 1994). This interaction is GTP-dependent and may activate the PI3 kinase activity.

Rac, like Rho, also induces the formation of integrin-based focal adhesion complexes at the plasma membrane (Nobes and Hall, 1995). These focal complexes are similar in composition to classical focal adhesions (e.g. they also contain vinculin, paxillin and pp125FAK), but they have a distinct morphology; they are smaller and distributed along the entire length of the
lamellipodia edge. They are formed independently of Rho and their function is unknown. However, like Rho, the actin and adhesion complex pathways appear to be separable, since Rac-mediated actin polymerisation still occurs under conditions where focal complexes cannot form (such as in cells plated on poly-L-lysine) (Hotchin and Hall, 1995, Machesky and Hall, 1997).

In addition to the reports describing Rac-induced lamellipodia formation in Swiss 3T3 cells, similar effects have been found in other cell types, for example the epidermal cell line KB (Nishiyama et al., 1994), in MDCK epithelial cells (Ridley et al., 1995b), in mast cells (Norman et al., 1994) and in macrophages (Allen et al., 1997). Rac, like Rho, is necessary for establishment of cadherin-based cell-cell contacts (Braga et al., 1997) and has also been reported to be involved in endocytosis (Lamaze et al., 1996) and regulated exocytosis (O'Sullivan et al., 1996).

1.3.3.2 Rac, stress kinases and control of transcription

Perhaps the most interesting development in the Rho GTPase field in the past couple of years has been the discovery that these proteins control gene transcription via several distinct pathways (Van Aelst and D'Souza-Schorey, 1997). Although it is not clear at this stage what genes are directly controlled by Rho, Rac and Cdc42, it seems that, in response to extracellular stimuli, they co-ordinately regulate long term changes involving gene expression with the short term cytoskeletal rearrangements described above. All of the studies on transcription control by mammalian Rho GTPases have so far involved transfection of tissue culture cells, though recent work in *Drosophila* has shown that Rho GTPases do indeed control gene transcription *in vivo* (see 1.3.6.4 for a detailed discussion).

**JNK/SAPK and p38/RK**

MAP Kinase pathways are conserved modular kinase cascades that mediate signal transduction events in all eukaryotes (Herskowitz, 1995, Marshall, 1994, Kyriakis and Avruch, 1996). The prototypical MAP Kinase pathway is the Ras-controlled ERK1/2 pathway of mammals. In addition
mammals have at least two other MAP Kinase pathways, the JNK (c-jun N-terminal Kinase)/SAPK (Stress activated protein kinase) and p38/RK (Reactivating Kinase) pathways, also known as stress response kinases (reviewed by Kyriakis and Avruch, 1996). Growth factors that stimulate the ERK pathway are mostly poor activators of the stress response kinases, though EGF has been reported to give a 15-fold activation of JNK in HeLa cells (Minden et al., 1995). In contrast, JNK and p38 are both commonly activated in response to cellular stresses such as ultraviolet irradiation or heat and inflammatory cytokines such as TNFα and interleukin-1 (IL-1). These kinase pathways are, therefore, thought to play a role in inflammation. Figure 1.6 summarises the kinases involved that have been cloned so far and their known transcription factor targets. (Treisman, 1995, Treisman, 1996, Hill and Treisman, 1995). It can be seen that, for the JNK and p38 pathways, a large number of isoforms of each kinase have been found and the overall picture is made more complex by functional redundancy and crosstalk between the different pathways.

While the Ras-controlled ERK pathway is well understood, until a few years ago, events linking stress and cytokines to the JNK and p38 pathways were unclear. Activated versions of Rac and Cdc42 have now been reported to stimulate both the p38 and JNK cascades in Cos, NIH 3T3 and HeLa cells (Coso et al., 1995, Minden et al., 1995, Bagrodia et al., 1995, Olson et al., 1995, Zhang et al., 1995). In 293T cells, Cdc42 and Rho, but not Rac, appear to stimulate JNK and p38 (Teramoto et al., 1996a). Thus it would appear that there are cell type differences in Rho GTPase regulation of stress kinases. All these experiments have been done with overexpressed GTPases and kinases.
Figure 1.6  Mammalian MAP kinase pathways
Comparison of the ERK, p38 and JNK/SAPK MAP kinase pathways (see text and Kyriakis and Avruch, 1996 for details). Some of the transcription factors regulated by JNK and p38 are shown.
Some experiments have been performed using dominant negative GTPases to block agonist-induced JNK and p38 activation. Several groups have reported a partial inhibition of IL-1-induced JNK and p38 activation by N17 Rac/Cdc42 in Cos cells (Zhang et al., 1995, Bagrodia et al., 1995). Another report described partial inhibition of both TNFα- and EGF-induced JNK activation by N17 Rac/Cdc42 in Cos cells (Coso et al., 1995). In HeLa cells, on the other hand, N17 Rac partially blocked EGF-induced JNK activation, but had no effect on TNFα (Minden et al., 1995). Using a reporter construct under the control of the Elk-1 promoter (which is a target of both the JNK and p38 pathways), Whitmarsh et al saw a partial blockage of IL-1-induced reporter activation by dominant negative Rho, Rac and Cdc42 in Chinese Hamster Ovary cells (Whitmarsh et al., 1997). All these studies indicate that Rho GTPases play a role in the regulation of JNK and p38, but they appear not to be absolutely required. It is clear that the regulation of these kinase cascades is a complex issue where both the type of assay used and the cell type have to be considered when interpreting results. Also most of these studies were carried out using JNK1/SAPKβ and it would be interesting to examine the regulation of the other isoforms.

Once active, JNK and p38 control gene transcription via a variety of proteins as shown in fig 1.6 (Treisman, 1995, Treisman, 1996, Hill and Treisman, 1995). JNK activates AP1 by directly phosphorylating Jun, which results in increased transcription of genes containing TREs (TPA Response Elements) in their enhancer region. Jun has also been shown to act as a repressor of some genes. Both p38 and JNK phosphorylate and activate the TCF (Ternary Complex Factor) protein Elk-1, though only p38 activates SAP-1, another TCF component. TCF is known to associate with SRF to induce transcription through SREs. In addition, p38 activates MAPKAP-K2 (MAPK Activated Protein Kinase 2) which itself phosphorylates CREB (cAMP Response Element Binding protein). Finally, both JNK and p38 activate the transcription factor ATF-2.

What are the functions of JNK and p38? Both are thought to play a role in inflammation and in stress response (Kyriakis and Avruch, 1996). However, an increasing body of evidence
suggests a role for these kinase pathways, particularly of JNK, in the control of Programmed Cell Death (PCD) or apoptosis. This will be discussed in more detail in chapter 5.

Using mutations in the effector region of Rac and Cdc42, several groups have shown that lamellipodia and filopodia formation, oncogenic transformation and cell cycle progression through G1 occur independently of JNK activation (Lamarche et al., 1996, Westwick et al., 1997, Joneson et al., 1996). An F37A mutant of Rac, for example, does not induce lamellipodia formation, transformation or DNA synthesis, but still activates JNK. Conversely, a Y40C mutation results in loss of JNK stimulation without affecting lamellipodia formation, transformation or DNA synthesis.

**The Serum Response Factor**

Rac and Cdc42 have been shown to activate transcription via the SRF independently of TCF (Hill et al., 1995). This activation does not seem to correlate with activation of JNK and is not mediated by Rho. Thus Rac and Cdc42 mediate gene transcription via TCF/JNK/p38-dependent and independent pathways. The molecular details of this TCF independent pathway are not known.

**Nuclear Factor κB (NFκB)**

Recently, two reports have linked the Rho GTPases to NFκB activation. Perona et al reported that activated Rho, Rac and Cdc42 could stimulate NFκB-dependent transcription via phosphorylation of IκB using a transfection assay in Cos cells (Perona et al., 1997). The authors also claimed that dominant negative Cdc42 and Rho but not Rac inhibited TNF-induced NFκB activation. In another report, Rac but not Cdc42, activated NFκB-dependent transcription in transfected HeLa cells (Sulciner et al., 1996). Furthermore, dominant negative Rac inhibited IL-1β-stimulated NFκB activation. Interestingly, Rac-induced NFκB activation was blocked by treatment with the oxygen free-radical scavenger N-acetyl-cysteine (NAC) and transfection of activated Rac has been reported to produce reactive oxygen species (ROS).

The NFκB/Rel family of transcription factors is composed of five members (RelA/B, c-Rel, p52, p50) and the prototypical
family member is p65RelA/NFkB (reviewed by Verma et al., 1995). NFkB is regulated by association with an inhibitory IkB subunit which masks the NFkB nuclear localisation signal. Upon stimulation of the cell by cytokines such as IL-1 or TNFα, IkB becomes phosphorylated which triggers its polyubiquitination and degradation. NFkB is then free to translocate to the nucleus where it dimerises with other Rel subunits and activates transcription of genes that contains κB domains in their enhancer region. Such genes include MHC (Major Histocompatibility Complex) proteins, cytokines, interferons and viral proteins. NFkB is, therefore, thought to play a central part in regulation of many immune responses. Recently, the enzymes responsible for IkB phosphorylation have been cloned and called IKKa/β (IkB Kinase α/β) (DiDonato et al., 1997, Mercurio et al., 1997, Woronicz et al., 1997). These proteins are stimulated upon cytokine treatment and therefore provide a link between cell surface receptors and NFkB activation. These kinases form a complex with NIK, a kinase that was shown to be on the IL-1 and TNF pathways to NFkB activation (Regnier et al., 1997). Thus NIK and IKK appear to couple cytokine receptors to NFkB activation.

The function of NFkB in Rho/Rac/Cdc42 signalling is so far unclear. A potential connection between Rac/Cdc42 and NFkB is MEKK1 which binds to these GTPases and has been shown to activate IKK (Lee et al., 1997). Like the JNK pathway, the NFkB pathway has been connected with PCD (reviewed in Baichwal and Baeuerle, 1997). In many cases, NFkB appears to be induced as a protection mechanism against PCD, as in TNFα-mediated toxicity. However, in some cases, such as glutamate-induced PCD in neurons, NFkB seems to promote rather than inhibit PCD.

1.3.3.3 Rac and the NADPH oxidase

In phagocytic cells, Rac has been shown to regulate the NADPH oxidase (Segal and Abo, 1993). This membrane-bound enzyme complex uses NADPH as an electron donor to generate superoxide radicals. Superoxide plays a crucial role in the killing of phagocytosed pathogens. The complex consists of two membrane-bound cytochrome b558 catalytic subunits
(gp91phox and gp21phox) and two cytosolic regulatory proteins (p67phox, p47phox). Absence of one of these components results in Chronic Granulomatous Disease (CGD). CGD patients are immuno-compromised and suffer from a high frequency of opportunistic infections. An additional regulatory component of the oxidase complex was purified and found to be a Rac/RhoGDI complex (Abo et al., 1991). It has proved possible to reconstitute oxidase activity in a cell free assay using recombinant p67phox, p47phox, gp91phox, gp21phox and GTP-bound (but not GDP-bound) Rac (Abo et al., 1992).

Analysis of neutrophil activation has revealed that 10% of cellular Rac dissociates from RhoGDI and translocates to the plasma membrane from the cytosol (Abo et al., 1994, Quinn et al., 1993). p67phox and p47phox also translocate, though this is thought to be independent of Rac (Heyworth et al., 1994). The target of Rac in the oxidase complex is p67phox, with which it interacts directly (Diekmann et al., 1994). Binding of Rac to p67phox is necessary for oxidase activation, but the precise effect of this binding is unknown (Diekmann et al., 1994).

Although Rac, cytochrome b558, p67phox and p47phox are sufficient for superoxide production in vitro, the situation in vivo is probably more complex. Rap1A and another cytosolic component p40phox appear to be involved, though their precise role has not been discovered (Maly et al., 1994; Bokoch et al., 1991).

1.3.3.4 Rac, cell growth and cell transformation

Rac has been shown to stimulate cell cycle progression through G1 and to be necessary for serum-induced DNA synthesis in quiescent Swiss 3T3 cells (Olson et al., 1995). Furthermore, Rat1 cells overexpressing activated V12 Rac display many characteristics of cellular transformation, such as partial loss of contact inhibition, reduced serum dependence, anchorage independence and ability to form tumors in nude mice (Qiu et al., 1995). Dominant negative Rac, on the other hand, severely reduces activated Ras' (but not activated Raf-CAAX's) ability to induce focus formation in NIH3T3 cells (Qiu et al., 1995, Khosravi-Far, 1995). Furthermore, activated Rac
strongly synergises with Raf-CAAX for focus formation in NIH3T3 cells. This has led to the proposal that Ras transforms cells via both a Raf-dependent and a Rho/Rac-dependent pathway (Qiu et al., 1995, Khosravi-Far, 1995).

Dominant negative N17 Rac was also found to block cell proliferation induced by the non-receptor tyrosine kinase v-Abl (Renshaw et al., 1996). Expression of N17 Rac in v-abl-transformed cells reduced serum independence, but had no effects on morphological transformation or anchorage independence (Renshaw et al., 1996). Thus, v-Abl exerts some but not all of its transforming effects via Rac.

Another potentially important role for Rac in cancer is in tumour invasion. The GEF Tiam-1 was originally found in a screen for invasion-inducing genes (Habets et al., 1994). Further analysis revealed that, like Rac, Tiam-1 could cause membrane ruffling in fibroblasts, and that it could catalyse nucleotide exchange on Rac but not Rho or Cdc42 in vitro (Michiels et al., 1995). Tiam-1 and V12 Rac can both induce an invasive phenotype in a non-invasive T-lymphoma cell line (van et al., 1995). The downstream proteins involved in invasion are not yet known.

1.3.3.5 Rac and the nervous system

Microinjection of activated Rac and Cdc42 into N1E-115 neuroblastoma cells increased growth cone complexity (Kozma et al., 1997). Furthermore, dominant negative Rac and Cdc42 blocked acetylcholine-induced lamellipodia and filopodia formation, respectively. In another study, activated Rac was found to promote growth cone collapse and dominant negative Rac blocked collapsin-1-mediated collapse (Jin and Strittmatter, 1997). These studies show that Rac can activate or collapse growth cones depending on the context. Mice expressing activated V12 Rac in Purkinje neurons had disrupted axons and dendrites were more numerous and smaller than in wild type animals (Luo, 1996). This report demonstrates an in vivo function for Rac in nervous system development.
1.3.4 Effectors of Rac

1.3.4.1 Partner Of Rac1 (POR1)

This 34kDa protein was identified as a Rac partner in the yeast two-hybrid system (Van Aelst et al., 1996). POR1 interacts with Rac in a GTP-dependent manner, but not with Rho or Cdc42. Overexpressed POR1 synergizes with V12Ras to cause membrane ruffling but has no effect when expressed alone or with V12 Rac. A truncation of POR1 that does not bind Rac inhibits V12Rac-induced ruffling in NIH3T3 cells, while the F37A L61 Rac mutant which does not induce ruffling also fails to bind full-length POR1. These data are consistent with a role for POR1 in Rac-induced ruffling.

POR1 also interacts with the ARF-related protein ARF6. A POR1 truncation that does not bind ARF6 inhibits ARF6-induced actin polymerisation at the cell periphery (D'Souza-Schorey et al., 1997). This implicates POR1 in cytoskeletal control by ARF6 as well as Rac. POR1 has no known enzymatic activity or conserved motifs (apart from a putative leucine zipper) and its biochemical role in cytoskeletal rearrangements is unclear.

1.3.4.2 p21-Activated Kinase (PAK)

p65PAK, a serine/threonine kinase related to the budding yeast protein Ste20, was the first target to be identified for Rac. It was isolated using a ligand overlay assay by Manser et al (Manser et al., 1994). There are now three mammalian PAK proteins termed PAK1 (also known as PAKα), PAK2 (also known as PAKγ or hPAK65), and PAK3 (also known as PAKβ) (Fanger et al., 1997a) with molecular weights of, respectively, 68, 62 and 65 kDa. PAK2 is ubiquitous, while PAK1 is found in the brain, muscle and spleen and PAK3 is expressed predominantly in the brain. Upon binding to either activated Rac or Cdc42, PAK autophosphorylates and its kinase activity is activated approximately 50-fold using Myelin Basic Protein (MBP) as a substrate. In addition to their C-terminal serine/threonine kinase domain, PAKs have potential SH3-binding motifs. The adaptor molecule Nck, through its second SH3 domain, has been shown to interact with PAK, resulting in PAK relocalisation to
the plasma membrane and activation of its kinase activity (Lu et al., 1997).

Since Ste20 in yeast is known to regulate the pheromone response MAP Kinase-related pathway (see below), a number of groups have investigated the possibility that PAK mediates Rac-induced activation of the JNK/SAPK and p38 MAP kinase cascades in mammalian cells. It has been reported that overexpression of activated PAK in Cos-1 or Cos-7 cells leads to activation of both the JNK and p38 cascades (Zhang et al., 1995, Brown et al., 1996, Bagrodia et al., 1995, Frost et al., 1996). However, according to another report, co-expression of wildtype PAK diminished rather than increased Rac or Cdc42-induced JNK activation in Cos-7 cells. Thus, wildtype PAK acted as a dominant negative (Teramoto et al., 1996a). Moreover, a 43D Rac mutant, which was unable to activate PAK, was still able to induce JNK activation (Westwick et al., 1997).

A recent report showed that, while activated PAK (L83, L86) stimulated both the JNK and p38 cascades in Rat-1 cells, a triple mutant (L83, L86, R299), which is dead for both GTPase binding and kinase activity did not have a dominant negative effect upon Rac- or Cdc42-induced JNK or p38 activation (Tang et al., 1997). However, this triple mutant blocked oncogenic transformation of Rat1 cells induced by Ki-Ras and partially blocked Ki-Ras-induced (but not v-Raf-induced) ERK activation. The authors proposed that PAK has a role in Ras-induced transformation, possibly through the ERK cascade. In support of this idea, activated PAK, Rac and Cdc42 were reported to synergise with c-Raf1 for activation of the ERK MAPK pathway (Frost et al., 1997, Tang et al., 1997). Furthermore, activated PAK was able to directly phosphorylate MEK-1 on serine 298 in vitro. This phosphorylation did not increase MEK1's kinase activity, but appeared to increase its affinity for its upstream regulator, Raf (Frost et al., 1997). It is therefore possible that, via PAK, Rac and Cdc42 could control ERK activity in a "cross-cascade" fashion. The role of PAK in mediating Ras-, Rac- and Cdc42-induced MAP kinase pathway activation is still not clear, though it could be that PAK is responsible for this GTPase function in some cell types but not others.
A number of proteins bearing homology to PAK such as HPK1 (Hematopoietic Progenitor Kinase), NIK (Nck Interacting Kinase) or GCK (Germinal Center Kinase) have been shown to activate JNK when overexpressed (Su et al., 1997, Fanger et al., 1997a). HPK1 binds to MLK3 and NIK binds to MEKK1; these kinases could therefore directly be connected to JNK, though none have so far been shown to bind Rac/Cdc42.

Some reports have suggested a link between PAK and the cytoskeleton. One group claims that kinase-dead PAK (L83, L86, R299) leads to formation of filopodia and membrane ruffles when overexpressed in Swiss 3T3 cells (Sells et al., 1997). However, other groups reported that mutations of Rac and Cdc42 at codon 40, which abolish binding to PAK, did not affect Rac- or Cdc42-mediated cytoskeletal rearrangements in Swiss 3T3 cells (Lamarche et al., 1996, Joneson et al., 1996, Westwick et al., 1997). Another group demonstrated that introduction of activated PAK in HeLa cells resulted in loss of stress fibres and focal adhesions, and proposed that part of PAK's function is to antagonise Rho (Manser et al., 1997). Mammalian PAK, like Ste20 in yeast, was also shown to phosphorylate the Myosin I heavy chain, leading to an increase in its ATPase activity (Brzeska et al., 1997). Despite all these studies, the precise role of PAK in cytoskeletal regulation remains confusing and unclear.

A recent development in the study of PAK function is the finding that PAK2 is proteolytically cleaved by caspases during Fas-mediated apoptosis in Jurkat T-cells (Rudel and Bokoch, 1997). This event yields a 34 kDa C-terminal fragment which is catalytically active. Furthermore, the authors showed that overexpression of dominant negative PAK blocked some of the morphological changes that occur during Fas-mediated apoptosis. This raises the possibility that PAK is an important player in the apoptotic programme. Another report claimed that Xenopus PAK protected G2-arrested Oocytes against apoptosis (Faure et al., 1997). These two findings are not mutually exclusive, since it is thought that many proteins can both cause and prevent apoptosis depending on the cellular context (Baichwal and Baueuerle, 1997, Herdegen et al., 1997).
1.3.4.3 Mixed Lineage Kinase (MLK)

By taking the minimal Rac/Cdc42-binding domain of PAK and searching protein databases, Burbelo et al identified a 16 amino acid motif common to a large number of Rac/Cdc42-binding proteins (Burbelo et al., 1995). This so-called CRIB (Cdc42/Rac Interactive Binding) motif is found on MLK1/2/3, a family of kinases similar to MAPKKKs (Dorow et al., 1993, Katoh et al., 1995). In addition, MLKs have a serine/threonine kinase domain, an SH3 domain, a leucine zipper and a C-terminal divergent region. MLK2/3 were found to bind Rac and Cdc42 in a GTP-dependent manner and both are potent activators of the JNK and p38 MAP Kinase pathways (Burbelo et al., 1995, Teramoto et al., 1996a, Tibbles et al., 1996, Rana et al., 1996, Nagata et al. 1998, Hirai et al., 1997). MLK2/3 have been shown to activate JNK via SEK-1 and MLK3 was demonstrated to activate p38 via M KK3/6 (Tibbles et al., 1996, Hirai et al., 1997). MLKs appear to be constitutively active in transfection assays; how they are regulated in vivo is not clear.

Nagata et al recently reported that the MLK2/3 divergent region associates with 14.3.3e, a calcium-binding protein called hippocalcin, two kinesin-related proteins KIF3X and KIF3A and a KIF3-binding protein, KAP3A (Nagata et al. 1998). In these studies, MLK2 co-localised with KIF3A along microtubules, suggesting that the kinase might play a role in microtubule motor function. Rac has been reported to bind to tubulin directly in vivo, though the physiological significance of this interaction, if any, is unknown (Best et al., 1996). MSE55, another CRIB containing protein, has no known function (Burbelo et al., 1995).

1.3.4.4 MEKK1/4

MEKK4 is a CRIB containing member of the MAPKKK family. Both MEKK1 and 4 have been shown to co-immunoprecipitate from cell lysates with Rac and Cdc42 and to bind directly to these GTPases in vitro (Fanger et al., 1997b). MEKK1 and 4 both activate the JNK pathway, but only MEKK1 is able to give some ERK activation and neither activates p38 (Lange-Carter et al., 1993, Gerwins et al., 1997). MEKK1 is a 196 kDa protein with a proline-rich motif and two PH domains.
MEKK1 has been shown to bind Ras in a GTP-dependent manner (Gerwins et al., 1997). MEKK4 is 180 kDa, similar to MEKK1, but has only one PH domain, a proline-rich region, and a CRIB motif (Gerwins et al., 1997). Both of these proteins are good candidate effectors for Rac and Cdc42, but not Rho, in activating JNK.

1.3.4.5 S6 Kinase

There has been one report that pp70S6K binds to and is activated by GTP-bound Rac and Cdc42, though it is not clear whether the interaction is direct or not (Chou and Blenis, 1996). pp70S6K plays an essential part in G1-S transition since microinjection of neutralising antibodies against pp70S6K inhibits serum-induced DNA synthesis (Proud, 1996). Furthermore, pp70S6K is activated by many mitogenic stimuli, including growth factors, cytokines, phorbol esters as well as by oncogenes (Chou and Blenis, 1995). Dominant negative Rac/Cdc42 inhibited activation of pp70S6K by serum (Chou and Blenis, 1996). pp70S6K is therefore a candidate target for Rac/Cdc42 in mediating cell cycle progression through G1.

1.3.4.6 PRK2 and Citron

These are two Rho targets (see above) that have also been shown to bind Rac, though the physiological relevance of this binding remains to be proven (Vincent and Settleman, 1997; Madaule et al., 1995).

1.3.4.8 PI4P5K

It has been reported that thrombin and activated Rac mediate uncapping and severing of actin filaments in platelets (Hartwig, 1995). This is in agreement with the work of Machesky and Hall who found the Rac induced rapid incorporation of fluorescent actin monomers at areas of lamella formation (Machesky and Hall, 1997). Furthermore, Hartwig et al were able to inhibit actin filament uncapping using PIP2-binding peptides supporting the involvement of a PI4P5K as a mediator of Rac-induced actin polymerisation. Indeed, Tolias et al purified a PI4P5K activity by affinity chromatography with GTP/GDP-bound Rac, though the identity and function of the
enzyme remains to be investigated (Tolias, 1995). Rac was also shown to bind PI3K, but the significance of this finding is unknown (Tolias, 1995).

1.3.5 Function of Cdc42

1.3.5.1 Cdc42 and the cytoskeleton

Microinjection studies in Swiss 3T3 cells have recently revealed a function for Cdc42 in actin cytoskeleton rearrangements (Nobes and Hall, 1995, Kozma et al., 1995). Injection of Cdc42 into subconfluent serum-starved cells results in the formation of numerous filopodia or microspikes, which are highly dynamic, finger-like protrusions of the plasma membrane. These structures contain long bundles of filamentous actin and are associated with small focal complexes of a similar composition to Rac- and Rho-induced complexes. Furthermore, Kozma et al. found that the brain peptide bradykinin can induce Cdc42-dependent filopodia formation. In HeLa cells, Cdc42 transfection causes a similar phenotype. Cdc42-induced filopodia are usually accompanied by localised lamellipodia formation due to activation of Rac by Cdc42 (Nobes and Hall, 1995). Filopodia, often found associated with lamellae in highly motile cells and in growth cones, are thought to be important for sensing neighbouring cells and attractive/repulsive signals in the extracellular matrix.

The studies on Cdc42 led to the proposal that the Rho family proteins Rho, Rac and Cdc42 lie in a hierarchical cascade whereby Cdc42 can activate Rac, which in turn can activate Rho (see fig. 1.7.). Obviously, in vivo, the situation is likely to be more complicated, as the cell is subjected to many signals from neighbours and the extracellular matrix and these signals might modulate individual components of this cascade differently depending on different contexts. It has been found, for example, that in HeLa cells, an activated form of PAK (which is a target of both Cdc42 and Rac) promotes disassembly of stress fibres (Manser et al., 1997).
1.3.5.2 Other functions of Cdc42

A dominant negative form of Cdc42 has been reported to block cytokinesis without disturbing the actin/myosin contractile ring in *Xenopus* eggs (Drechsel et al., 1997), while in T-cells, Cdc42 regulates polarisation of the microtubule organizing center (MTOC) towards antigen-presenting cells (Stowers, 1995).
Cdc42 also regulates gene transcription. In one report, Rho and Cdc42 but not Rac activated the JNK pathway in the human fetal kidney cell line 293 (Teramoto et al., 1996b), otherwise, Cdc42 and Rac mostly appear to behave in a similar fashion for control of transcription, at least in tissue culture cells.

Rat1 fibroblasts expressing Cdc42 display some features of transformation such as anchorage independence and tumour formation in nude mice (Qiu et al., 1997). Cdc42 does not, however, induce serum-independence. Dominant negative Cdc42 causes reversion of Ras-induced transformation, while active Cdc42, like Rac and Rho, induces DNA synthesis when microinjected into Swiss 3T3 fibroblasts (Olson et al., 1995). Dominant negative Cdc42 blocks serum-induced DNA synthesis (Olson et al., 1995). Thus, Cdc42 appears to play a crucial role in cell cycle progression through G1 and cell transformation.

1.3.5.3 Targets of Cdc42

Many CRIB containing proteins appear to be shared targets between Rac and Cdc42, at least in vitro (see above). One notable exception is the Wiskott-Aldrich Syndrome protein (WASP) which contains a CRIB motif, but has a very marked preference for Cdc42 (Aspenstrom et al., 1996, Symons et al., 1996). WASP is mutated in Wiskott-Aldrich Syndrome, a disease affecting hematopoietic cells, particularly platelets and lymphocytes. Patients have a severely reduced platelet count, weakened immune response and are prone to infection (Kirchhausen and Rosen, 1996). At a cellular level, the cytoskeleton of T-cells appears to be affected in WAS patients. WASP interacts with the SH3-containing adaptor molecule Nck and the Fyn tyrosine kinase (Rivero et al., 1995, Banin et al., 1996). It has some restricted sequence similarity to VASP, a protein known to interact with actin filaments. Symons et al reported that overexpression of WASP in Jurkat cells induced the appearance of large cytoplasmic clusters of polymerised actin co-localized with WASP (Symons et al., 1996. This suggests that WASP could regulate cytoskeletal changes induced by Cdc42 in hematopoietic cells.
ACK is a 120 kDa tyrosine kinase that binds to Cdc42 in a GTP-dependent manner (Manser et al., 1993). Besides its kinase domain, ACK bears an SH3 domain and a proline-rich region. Recently, an ACK-like molecule, ACK2, has been cloned and found to bind Cdc42 (Yang and Cerione, 1997). There are no functional data on ACK or ACK2.

IQGAP (1 and 2) interacts with both Rac and Cdc42 and appears to localise to membrane ruffles (Brill et al., 1996, Hart, 1996). It is a large (190kDa) protein and has a number of recognisable domains including a functional calmodulin-binding domain, a WW motif (two tryptophans forming what is thought to be a protein-binding motif), a proline-rich region and, surprisingly, a RasGAP-like domain. However, so far, no GAP activity or indeed interaction with Ras has been found. IQGAP co-precipitates with Cdc42 and actin, but its function remains unclear (Erickson et al., 1997). Another recently isolated Cdc42-specific target is CIP4, a protein bearing homology to the non-kinase domain of the FER family of tyrosine kinase (Aspenstrom, 1997). The function of CIP4 is unknown.

1.3.6 Rho GTPases in genetic systems

1.3.6.1 Caenorhabditis elegans

*Caenorhabditis elegans* is proving to be a valuable model system for studying cell migration and axonal guidance. This is because this organism has a simple body plan, well characterised genetics and precise fate maps for every single cell (Ahringer, 1997). *C. elegans* equivalents of RhoA, Rac1 and 2, Cdc42, PAK, ROK, as well as a GAP called CeGAP have been cloned (Chen et al., 1993b, Chen et al., 1993a, Chen and Lim, 1994, Chen et al., 1996, Wissmann et al., 1997). CeRhoA is localised mainly at the plasma membrane and enriched in the developing nerve. CeGAP is related to Bcr and is active not only on Rho family members but also on Ras and Rab homologs. Both CeRac1 and CeCdc42 are expressed throughout development. Mutation of let-502, which is similar to Rho-kinase, blocks embryonic elongation, a process based on actin/myosin-driven contraction of hypodermal cells. CePAK colocalises with CeRac1 and CeCdc42 at hypodermal cell
boundaries during embryonic body elongation. Furthermore, mutations in a novel Rho-family member, mig-2 (most similar to Rac) blocked a variety of cell migration events, including axonal guidance (Zipkin et al., 1997). *C. elegans* is, therefore, likely to prove a useful model system for the study of cell migration and contraction in a developmental context.

1.3.6.2 *Saccharomyces cerevisiae*

Since all the budding yeast genome has now been sequenced, it is known that there are five distinct members of the Rho family in this organism: Rho1, 2, 3 and 4 and Cdc42. Interestingly, yeast has no Rac homolog. These Rho family proteins have been implicated in a variety of processes such as bud specification and growth, mating, pseudo-hyphal growth and cytokinesis (Leberer et al., 1997a, Herskowitz, 1995). In this section, I will concentrate on two of these processes, namely the role of Cdc42 in the control of the mating pheromone pathway, and the role of Rho in the budding process.

**Cdc42 and the mating pheromone pathway**

Mating in budding yeast is controlled by a MAPK cascade which is activated by mating pheromone and triggers expression of genes necessary for formation of the mating projection or "shmoo" (Reviewed by Leberer et al., 1997a). Mating pheromones are small peptides that bind to seven-pass receptors (Ste2p for α and Ste3p for α) on the surface of yeast cells of the opposite mating type. Like mammalian seven-pass receptors, Ste2/3p are coupled to a heterotrimeric G-protein made up of an α (Gpa1p), a β (Ste4p) and a γ (Ste18p) subunit. Pheromone binding causes release of Ste4p/Ste18p. Ste4p then interacts with the PAK homolog, Ste20p, which is thought to act as a MAPKKKK in the pheromone MAPK cascade. The components of this cascade are most similar to mammalian JNK pathway proteins. The MAPKKK is Ste11p, the MAPKK is Ste7p and the MAPK is Fus3p or Kss1p. These MAPKs then activate the transcription factor Ste12p which switches on transcription of genes necessary for mating and cell cycle arrest. The MAPKs also activate Far1p which is responsible for cell cycle arrest.
All the components of the pheromone response MAPK cascade are associated in a complex with an adaptor or scaffold molecule called Ste5p which binds directly to Ste4p and all the components of the MAPK cascade, including Ste20p. The role of Cdc42p in the activation of the pheromone cascade is still not clear. Early reports showed that temperature-sensitive alleles of Cdc42p affected pheromone signalling, leading the authors to postulate that Cdc42p controls the pheromone pathway by activating Ste20p (Simon et al., 1995, Zhao et al., 1995). However, recent studies have shown that the Ste20p-Cdc42p interaction is dispensible for MAPK activation in the pheromone response (Peter et al., 1996, Leberer et al., 1997b). From these studies, it seems that Cdc42p does not activate Ste20p, but instead is required for proper localisation of Ste20p and the MAPK/Ste5p signalling complex to the shmoo tip.

Another adaptor protein is necessary for mating. Bem1p was originally identified as a protein important for budding (Chenevert et al., 1992). This protein contains SH3 domains and binds directly to the cell cycle arrest protein Far1p, the adaptor Ste5p and Cdc24p, a GEF for Cdc42p (Lyons et al., 1996). Bem1p does not interact directly with Cdc42p, but the fission yeast Bem1p equivalent, Scd2 does bind to fission yeast Cdc42 (Chang et al., 1994). It has been reported that Bem1p co-precipitates with actin, though probably not via a direct interaction. (Leeuw et al., 1995). It is therefore thought that Bem1p plays a role in co-ordinating the pheromone response MAPK pathway with changes in the actin cytoskeleton required for shmoo outgrowth towards the pheromone source (Leberer et al., 1997a). Other proteins required for shmoo outgrowth are Cdc42p, Far1p and Ste20p. Ste20p has recently been shown to phosphorylate myosin and it is therefore thought to be important for cytoskeletal changes (Wu et al., 1996). Perhaps the most promising target for Cdc42p in cytoskeletal rearrangements is Bni1p (Evangelista et al., 1997). This formin family protein, which has also been implicated in Rho1p signalling, is now known to interact with Cdc42p and to be required for cytoskeletal reorganisation during shmoo outgrowth. Bni1p interacts with two actin-binding proteins: profilin and Bud6p.
**Rho1p in budding**

*rho1* mutants are deficient for bud formation (Yamochi *et al*., 1994) and Rho1p localises to the bud growth site, suggesting that Rho1p mediates some aspect of budding. In agreement with this, three target molecules for Rho1p have been identified by genetic and biochemical analysis. Firstly, 1,3-β-glucan synthase, which is important for cell wall synthesis (Qadota *et al*., 1996, Drgonova *et al*., 1996). Secondly, Bni1p, which is involved in regulation of the actin cytoskeleton (Imamura *et al*., 1997). Thirdly, Pkc1, which triggers a MAPK pathway that is essential for cell wall integrity (Kamada *et al*., 1996, Herskowitz, 1995).

Thus, it is clear from all these studies in yeast that Rho family GTPases are central players in the regulation of MAPK pathways that control various aspects of the life cycle and in regulating the organisation of the actin cytoskeleton (See Herskowitz, 1995 for a review). It is likely that by studying cytoskeletal and morphogenesis control in yeast, we may derive useful information for the study of these processes in mammalian cells.

**1.3.6.4 Drosophila melanogaster**

*Drosophila* is a powerful organism for dissecting signalling pathways. Several groups are now using *Drosophila* to study the role of Rho GTPases in different developmental contexts. Rho1, the RhoA equivalent, as well as DRac1, DRac2 and DCdc42 have been cloned (Hariharan *et al*., 1995, Luo *et al*., 1994) and each has been implicated in a variety of morphogenetic processes involving the actin cytoskeleton and gene transcription.

Expression of dominant negative or activated DRac1 in neuronal cells interfered with axonal outgrowth, but did not appear to affect dendrites. This phenotype is similar, though not identical, to the effect of activated Rac on neurons in transgenic mice (Luo, 1996). Though the active and dominant negative mutants of Rac result in similar axonal growth defects, they affect the actin cytoskeleton in different ways. Activated Drac1-expressing cells still show actin accumulation in growth cones, but the timing and localisation are affected. Expression of dominant negative DRac1, on the other hand, prevents actin
accumulation altogether. Consistent with a role for Drac1 in axonal outgrowth, Sone et al recently identified a putative exchange factor most similar to Tiam-1 called Still life (Sone et al., 1997). Interfering with this protein affects axonal outgrowth as well as synapse formation. When injected into human cells, Still life caused membrane ruffling suggesting that it is a regulator of DRac in controlling the actin cytoskeleton during nerve cell development.

Expression of mutants of DCdc42 in the nervous system has more widespread effects, interfering with neuronal position and dendritic and axonal outgrowth, consistent with a role for DCdc42 in cell migration (Luo et al., 1994). Thus, DRac1 and DCdc42 are crucial regulators of Drosophila nervous system development. Genetic analysis will hopefully allow the identification of upstream and downstream components of this complex process.

The Drosophila compound eye has been the system of choice in the study of the Ras pathway and the role of Rho proteins is just starting to be investigated in this system (Hariharan et al., 1995). Flies overexpressing wild type Rho1 in the eye display a dose-dependent rough eye phenotype due to effects on morphological changes late in development rather than perturbation of early cell-fate specification (Hariharan et al., 1995). More recently, strong negative alleles of Rho expressed in the eye result in failure to proliferate due to effects on the cell cycle or viability, while weaker alleles had planar polarity defect similar to frizzled (fz) alleles (frizzled encodes for a putative serpentine receptor molecule). This study placed Rho downstream of Fz and upstream of Basket (Bsk) the JNK equivalent in this signalling process (Strutt et al., 1997).

The Drosophila wing is covered by an array of distally pointing hairs. These form from an extension of a single process from polarised epithelial cells which then give rise to one hair per cell (Eaton et al., 1996). The extension is preceded by actin and microtubule accumulation, which extend into the growing hair as it is formed. Cells expressing dominant negative Cdc42 fail to accumulate actin at the site of hair growth. Cells expressing dominant negative Rac, on the other hand, produced
multiple but morphologically normal hairs. This suggests a role for Cdc42 in hair outgrowth and a role for Rac in hair growth site selection (Eaton et al., 1996). Interestingly, Rho loss of function alleles in the wing displayed a similar multiple hair phenotype to that of Rac (Strutt et al., 1997). Fz and Bsk also appear to be part of this pathway.

It has been reported that DRac, DCdc42 and RhoL (a novel Rho-like GTPase) play different roles in various cytoskeletal events during oogenesis (Murphy and Montell, 1996). For example, activity of all three GTPases were required for transfer of the nurse cell cytoplasms into the oocyte, an actin/myosin-driven event known as "dumping". DRac1 and DCdc42 have also been reported to play crucial roles in muscle development (Luo et al., 1994).

Dorsal closure (DC) is perhaps the most promising system for the study of the in vivo function of DRac and DCdc42. During DC, two symmetrical sheets of epidermal cells migrate over the amnioserosa to fuse at the dorsal midline (Martin-Bianco, 1997). The cells at the leading edge (LE) of the advancing epidermis first elongate and then transmit a signal to the rest of the epidermal cells which then also elongate. An important part of this signal is thought to be Decapentaplegic, the BMP-4 (Bone Morphogenetic Protein) homolog, which is expressed at the LE (Martin-Bianco, 1997). Once the advancing epithelia meet, they fuse at the dorsal midline. DC, which involves both cell migration and elongation, is thought to be driven by extensive cytoskeletal rearrangements. Indeed, actin and myosin are localised towards the dorsal midline in all the LE cells.

The first clues for an involvement of Rac in DC was the finding that expression of dominant negative (N17) Rac led to the disruption of actin/myosin accumulation at the LE and a dorsal open (DO) phenotype (where DC is not completed and the epidermis has not closed properly) (Harden et al., 1995). Such phenotypes are easy to screen and therefore a number of genes that affect DC have now been identified. Several of these genes encode cytoskeletal molecules like the non-muscle myosin heavy chain equivalent Zipper, or extracellular matrix components, such as the integrin-like Lethal(1)myospheroid
(Young et al., 1993, MacKrell et al., 1988). The other category of genes displaying a DO phenotype are signalling molecules like the Drosophila JNK-kinase, Hemipterous (Hep), or the DJNK basket (Bsk) which can be phosphorylated by Hep in vitro (Glise et al., 1995, Riesgo-Escovar et al., 1996, Sluss et al., 1996).

Recently, an important step forward in understanding the signalling events involved in DC came with four reports describing different aspects of this process using epistatic analysis (Glise and Noselli, 1997, Hou et al., 1997, Riesgo-Escovar and Hafen, 1997b, Kockel et al., 1997). Figure 1.8 describes the proposed model inferred from these studies. Firstly, loss of function mutation of the DJun transcription factor resulted in a DO phenotype and a disruption of actin/myosin distribution at the LE (Hou et al., 1997). However, ectopic expression of wildtype DJun resulted in expression of both dpp, and puckered (puc, see below), a phenotype which was suppressed by Bsk (JNK) deficiency. This proved that transcriptional activation through DJNK and DJun leads to expression of dpp (TGFβ) and puc in the LE cells. Glise and Noselli then demonstrated that expression of activated DRac1 or DCdc42 resulted in dpp/puc transcriptional activation, thereby implicating these GTPases in the JNK DC pathway (Glise and Noselli, 1997). Further epistatic analyses confirmed the following hierarchy: Rac/Cdc42->Hep (JNKK)->Bsk (JNK)->DJun->Dpp/Puc.

This pathway is under tight control from proteins such as the MAPK phosphatase Puckered and the Ets domain repressor Aop (Glise and Noselli, 1997). This is presumably to restrict Dpp production to the LE cells. What is the function of the Dpp (BMP-4) signal? The Dpp receptors thickveins (tkv) and punt (put) also display a DO phenotype when disrupted (Hou et al., 1997). The signalling events downstream of the Dpp receptor have not yet been elucidated, but are known to involve gene transcription via DFos, DJun and Schnurri (Riesgo-Escovar and Hafen, 1997a).
Figure 1.8 A model for signalling events during Drosophila dorsal closure (see text for details)
These studies have provided the first clear indication that Rac and Cdc42 control gene transcription via the JNK pathway in a living organism. However, many questions remain to be addressed. Firstly, what is signal that activates Rac/Cdc42 at the leading edge? Secondly, how do Rac and Cdc42 activate Hep (JNKK)? Interestingly, DPAK disruption also gives a DO phenotype (Harden et al., 1996). Since some groups have reported that mammalian PAK activates JNK, this could be an interesting clue. However, in this system, DPAK was localised with the actin/myosin at the leading edge and could therefore be important mainly for cytoskeletal rearrangement (Harden et al., 1996). Thirdly, how do the shape changes take place? Given the role of Rho, Rac and Cdc42 in controlling the actin cytoskeleton in mammalian cells and Drosophila, it is probable that they will be involved in the shape changes themselves as well as in transcriptional activation. The study of DC should, therefore, yield more fascinating insights into the function of Rho-related proteins in the next few years.
CHAPTER 2

MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY

2.1.1 Molecular Cloning

2.1.1.1 Restriction digests

Plasmid DNA (2-3μg) was normally digested in a volume of 30μl with New England Biolabs enzymes (using the manufacturer's buffers) for 1-2h at 25-37°C. Fragments were separated by agarose gel electrophoresis.

2.1.1.2 Klenow treatment

Klenow polymerase was used to fill protruding DNA ends after digestions. The DNA was incubated with 1mM dNTPs and 2 units DNA Polymerase I (Klenow fragment, New England Biolabs), in high salt buffer (100mM NaCl, 50mM Tris-HCl (pH7.5), 10mM MgCl₂, 1mM DTT) for 10min at 16°C followed by 10min at 37°C. As Klenow Polymerase is heat stable, the DNA was purified using phenol/chloroform extraction.

2.1.1.3 Phosphatase treatment

To avoid religation of linearised vectors, DNA was treated with 1 unit of Calf Intestinal Phosphatase (New England Biolabs) in phosphatase buffer (10x buffer: 0.5M Tris-HCl (pH9.0), 10mM MgCl₂, 1mM ZnCl₂, 10mM Spermidine) for 15-30min at 37°C to remove 5' phosphates. The enzyme was removed using phenol/chloroform extraction.

2.1.1.4 Kinase treatment

Oligonucleotides were 5'-phosphorylated before ligation. The DNA was treated with 5 units T4 polynucleotide kinase (Boehringer Mannheim) in 5mM Tris-HCl (pH7.4), 1mM MgCl₂, 5mM DTT and 1mM ATP. After 30 minutes at 37°C, the kinase was inactivated at 70°C for 15min.
2.1.1.5 Agarose gel electrophoresis

For fragments between 500 and 5000 basepairs (bp), a 1% agarose gel was used. Fragments of less than 500bp were separated on a 1.5-2% gel. For analytical purposes normal agarose (Gibco BRL) was boiled in 50ml 1xTBE (10xTBE: 162g Trizma base, 27.5g borate, 9.5g EDTA in 1L water), ethidium bromide (0.5mg/ml) was added and the solution left to solidify in a gel plate. If the DNA fragments were to be used directly for ligation, low melting point agarose (sea plaque, FMC BioProducts, Cat-No. 50102) was used. The DNA sample was loaded in 0.1 volume of 10x loading buffer (40% glycerol, 0.4% bromophenol blue) and electrophoresis was carried out at 120-130mA for about 1h.

2.1.1.6 Ligation of DNA fragments

Ligations were usually carried out in low melting point agarose, after melting and addition of 10x ligation buffer (200mM Tris-HCl (pH7.4), 100mM MgCl2, 100mM DTT, 100mM ATP). The molar ratio of vector to insert was between 1:5 and 1:10 (typically 10-20ng vector : 200ng insert). Ligations were performed for 6-15h at 16°C for sticky ends and at room temperature for blunt ends. The ligation mixes were "cleaned up" by dilution in 400µl of TE (pH7.5) (10mM Tris-HCl (pH7.5), 0.4mM EDTA) followed by extraction first with an equal volume of Phenol/Chloroform/Isoamyl Alcohol (24/24/1), then with chloroform/Isoamyl alcohol (24/1). The DNA was ethanol precipitated and resuspended in sterile double distilled water.

2.1.1.7 Preparation of competent cells

Electrocompetent stocks of DH5α and XL-1 blue cells were made using the following protocol. Cells were streaked from a glycerol stock onto an LB-Agar (L-broth + 1.5% agar) plate and incubated overnight at 37°C. One colony was picked from the plate and grown overnight in 10ml of LB in a 37°C shaker. The overnight culture was diluted in the morning into 1L of LB, and grown at 37°C until an OD600 of 0.6 was reached. The cells were transferred to four 250 ml sterile Corning centrifuge tubes, left
to cool on ice for 5min, and pelleted at 3000rpm in a refrigerated Beckman JA-20 centrifuge at 4°C for 30min. The cell pellet was resuspended in 1L of ice-cold sterile water. The procedure was repeated first with 500ml of water, then with 20ml 10% glycerol in water and finally the cells were resuspended in 2.5ml of ice-cold 10% glycerol. 80μl aliquots were snap-frozen in liquid nitrogen, and stored at -80°C for up to 6 months. Transformation efficiencies of 10^8 colonies/μg of plasmid DNA were usually achieved.

2.1.1.8 Transformation

1-10μl of DNA to be transformed were added, on ice, to 40μl of electrocompetent cells in a microfuge tube, mixed gently and transferred to a 0.2cm electroporation cuvette (Biorad). The cells were electroporated on a Biorad instrument at 2.5Kv, 200 Omhs and 25μF. The cells were transferred to a 5ml universal tube with 1ml of LB and incubated in a 37°C shaker for 1h. Cells were pelleted at 13000rpm, resuspended in 100μl LB and plated on an LB-Agar plate containing 100μg/ml Ampicillin. The cells were incubated overnight and minipreps made from individual colonies to analyse DNA incorporation.

2.1.2 DNA Purification

2.1.2.3 Minipreps

A colony of *E.coli* was grown overnight in 4ml LB in a round bottom 15ml tube (Falcon) at 37°C with vigorous agitation. 1.5ml of the culture were pelleted at 13000rpm for 5min and resuspended in 100μl of STET buffer (8% sucrose, 5% Triton X-100, 50mM Tris-HCl (pH8.0), 50mM EDTA). 10μl of a freshly made solution of lysozyme (Sigma, 10mg/ml in 10mM Tris-HCl (pH8.0)) were added to the tube, which was then boiled for 45s. Following centrifugation at 13000rpm for 15min, the pellet was removed with a toothpick and discarded. 100μl of ice-cold isopropanol were added and the tube was centrifuged for 8min at 13000rpm. The DNA pellet was washed with 400μl of 80% ethanol, dried and resuspended in 50μl of TE (pH7.5). 5μl were digested and analysed by agarose electrophoresis.
2.1.2.2 DNA purification using PEG

The following protocol was used for making DNA for sequencing. A 100ml overnight culture was centrifuged and cells resuspended in 5ml 10mM EDTA and left on ice for 5min. The cells were lysed with 10ml lysis solution (1% SDS, 0.2M NaOH) and 7.5ml of 3M Na Acetate (pH5.2) were added. After 5min on ice, the tubes were centrifuged at 4200rpm for 10min and the supernatant was collected in a fresh tube. An equal volume of ice-cold isopropanol was added, the tube was inverted several times and left on dry ice for 10min. DNA was collected by centrifugation at 4200rpm for 10min. The DNA pellet was resuspended in 5ml of TE pH7.5 and an equal volume of PEG solution (20% PEG mw8000, 2.5M NaCl) was added, the tube was inverted several times and then left on ice for 5min. DNA was collected by centrifugation for 10min at 4200rpm. The pellet was resuspended in 400µl of TE (pH7.5) and 2µl of RNAse solution (2u/µl, Stratagene) were added. The solution was incubated at 37°C for 1h, proteins were removed by Phenol/Chloroform/Isoamyl Alcohol (25/24/1) extraction followed by Chloroform/Isoamyl alcohol (24/1). The DNA was precipitated using 0.1vol. 3M NaAc and 2vols. ethanol and the pellet rinsed with 70% ethanol before being resuspended in 200-300µl of TE (pH7.5). The quality and concentration of the DNA was assessed on a gel and by measuring optical density at 600 nm on a Beckman DU7400 instrument.

2.1.2.3 Caesium chloride gradient plasmid purification.

This method was used to generate highly pure DNA for microinjection. A 500ml overnight culture of bacteria was centrifuged at 4000 rpm, the pellet was resuspended in 10ml resuspension buffer (25mM Tris-HCl (pH8.0), 10mM EDTA, 50mM glucose, 10mg/ml lysozyme) and left on ice for 15min. 20ml of lysis solution (as for PEG preps) were added followed by 15ml cold 3 M NaAc. The solution was gently inverted six times and left on ice for 10 min to allow for full lysis, then centrifuged at 4200rpm for 12 min. The supernatant was put in a fresh tube, the DNA was precipitated with 0.6 volumes of cold
isopropanol and centrifuged at 4200 rpm for 12 min. The pellet was resuspended in 10ml of 10x TE pH8.0 and the DNA was re-precipitated with 10ml of 5M ammonium acetate and 20ml of ethanol. The pellet was resuspended in 3.9ml 10x TE pH8.0 and 4g CsCl were added together with 200μl ethidium bromide (10mg/ml). This solution was transferred to a heat-sealable tube (Beckman) and centrifuged for 15h at 100,000 rpm in an ultracentrifuge (Beckman).

The supercoiled plasmid DNA (lower band) was removed with a wide-bore syringe, diluted to 4ml with water and ethidium bromide extracted with 4x 8ml of water-saturated isobutanol. The DNA was precipitated with 2 volumes of ethanol, resuspended in 500ml TE, then precipitated again with 40ml of 3M NaAc and 1ml ethanol (ice cold), and washed with 70% ethanol. The pellet was resuspended in 300-500μl of water or TE (pH7.5) and the quality and concentration of the DNA was analysed by gel electrophoresis and optical density.

2.1.3 cDNA Library Screening.

2.1.3.1 Preparation of radiolabelled probe.

cDNA fragments were generated by restriction enzyme digestion for 2 h at 37°C and electrophoresed on a 0.8% Seaplaque Low Melting agarose gel (FMC Bioproducts). Fragment bands were excised using a razor blade, loaded onto a gel purification spin-column (Millipore) and centrifuged at 3500rpm in a microfuge for 4min. A desalting column (Pharmacia) was prespun at 2500rpm for 1min in a microfuge. The DNA sample was loaded on the prespun column and centrifuged at 2500rpm for 1min. The volume was adjusted to 300μl with TE (pH7.5) and the DNA precipitated with 40μl of 3M NaAc (pH5.2) and 800μl of 100% ethanol. Following 5min incubation on dry ice, the sample was centrifuged at 13000 rpm in a microfuge, the pellet was washed with 800μl of 80% ethanol and re-centrifuged at 13000 rpm for 2min. The ethanol was discarded and the DNA pellet dried in a speedvac (Savant) and resuspended in 20μl TE (pH7.5). DNA concentration was
measured using spectrophotometry and its purity checked on an agarose gel.

Radioactive labelling of the probe was performed using the multiprime kit (Amersham). 25ng of cDNA fragment in a volume of 10μl of water were boiled for 3min and the solution then chilled on ice for 2min. 10μl of 5x multiprime buffer and 5μl of primer/BSA mix were added on ice, followed by 10μl of [α-32P]dCTP (Amersham nbr PB10475, specific activity 6000 Ci/mmol, less than one week old) and 2μl of Klenow DNA polymerase (NEB). The mixture was left overnight at room temperature. After overnight incubation, the reaction was stopped by addition of 2μl of 500 mM EDTA (pH8.0) and the probe purified from unincorporated nucleotides on a ChromaSpin 400 column (Clonetech). The column was prespun at 2800rpm for 2min. 1.5μl of boiled carrier DNA (10mg/ml) were added to the probes and the mixture loaded on the column, taking care not to break to matrix surface. The column was centrifuged for 2min at 2800rpm in a microfuge and the eluant put in a fresh tube. A 2 μl aliquot was mixed with 8ml of scintillation fluid (Packhard 6013389) and counted in a Beckman instrument. The specific activity of the probe was calculated using the following equation:
Specific activity = (total cpm X volume X 20) cpm/μg of DNA.
Specific activities of 10⁹ cpm/μg could typically be achieved using this method. The probe could be stored at -20°C for a few days prior to use.

2.1.3.2 Screening procedure
15ml of XL-1 Blue cells (Stratagene) were grown overnight at 37°C in L-Broth with 10mM MgSO₄. 1 and 4μl of mouse whole embryo (days 11/12/13) cDNA library in λZAP (kind gift of Dr P.Burbelo) were put at the bottom of 15ml Falcon tubes, 200μl of bacterial culture were added and the tubes incubated for 10min at 37°C. 10ml of L-Top agar (0.7% Agarose, 10mM MgSO₄) were added to each Falcon tube and the mixtures plated on a 150mm dish with L-Agar containing 10mM MgSO₄. The plates were left to dry at 37°C before being
inverted and left overnight at 37°C. The day after, the bacteriophage plaques were counted and the titre calculated. Approximate 10,000 to 20,000 plaques per dish were plated on eight 150mm dishes as described above. After overnight incubation, the bacteriophage were transferred to two 137mm nitrocellulose membrane circles (Stratagene 420107). The first membrane was left on the plate for 3min and the second for 7 min. Membranes were marked with a syringe and pencil, soaked in 1M NaOH for 30s, neutralised in 1.5M NaCl, 0.5M Tris-HCl (pH8.0) for 30s and rinsed in 2x SSC for 30s (from 20x SSC: 0. M NaCitrate, 3M NaCl, pH adjusted to 7.0 with 10M NaOH). These three steps were carried out by placing membranes (phage side up) on large pieces of Whatman number 1 paper soaked with the relevant solutions. The membranes were placed on a fresh piece of Whatman paper to dry at room temperature and then baked at 80°C (under vacuum) for 2h. Baked membranes were prehybridised in 200 ml of prehybridisation mix (10x Denhardtts, 6x SSC, 0.1% SDS) for 5h at 65°C. The radiolabelled probe was boiled for 5min and added to 50ml of hybridisation mix (2x SSC, 0.1% SDS, 1x Denhardtts) to 2.5x10^6 cpm/ml and incubated with membranes at 65°C for 5h. The membranes were then washed 3x with 200ml of 2x SSC, 0.1% SDS and 2x with 200ml of 0.2x SSC, 0.1% SDS at room temperature, then wrapped in Saran wrap and exposed overnight to Kodak X-AR film. Radiactive spots present on both duplicates were marked for further analysis.

In order to purify positive bacteriophage, plaques were subjected to two additional screens. First round positives were picked using the thick end of a pasteur pipette. These agar "plugs" were suspended in 500μl of phage buffer (100mM NaCl, 10mM MgSO4, 50mM Tris-HCl (pH7.5)) and the remaining bacteria killed by addition of a drop of chloroform. The agar plugs were left to soak for 1h at room temperature with intermittent vortexing to resuspend the phage. The suspensions were kept at 4°C and titrated. For each putative positive clone, two 150mm dishes were prepared containing approximately 50 and 450 plaques. The plaques were transferred to nitrocellulose filters, denatured and screened as described above. After this
second round, individual positive plaques could be seen. To ensure purity of the bacteriophage stocks, a third round of screening was carried out. After the third round, homogeneous bacteriophage stocks were obtained.

2.1.3.3 Recovery of plasmids from λZAP-based libraries

10 ml cultures of XL-1 Blue (Stratagene) and SOLR (Stratagene) cells were grown in LB overnight in a 37°C shaker. The next morning, the cultures were diluted 1/100 in a volume of 50 ml of LB. At OD$_{600}$ of 0.2-0.5, the XL-1s centrifuged at 1500 x g and resuspended to an OD$_{600}$ of 1.0. 200 μl of XL-1 Blue cells, 200 μl of phage stock to be rescued (containing less than 1 x 10$^5$ phage particles) and 1 μl of ExAssist helper phage stock at 1 x 10$^6$ pfu/μl (Stratagene) were added to a 50 ml Falcon tube, mixed gently and left at 37°C for 10 min. Meanwhile, at OD$_{600}$ of 1.0, the SOLR cells were put in a 37°C incubator. 2 ml of LB were added to the XL1/phage mix and the tube was put in a 37°C shaker for 4 h. The tube was heated at 70°C for 15 min and centrifuged at 4000 x g for 15 min. The supernatant containing the excised plasmid packaged into phage particles was decanted into a fresh tube and 10 μl were added to 200 μl of SOLR cells in a microfuge tube. After 15 min incubation at 37°C, 20 μl of this mix were plated on an LB-Agar plate containing 100 mg/ml ampicillin (Sigma). After overnight growth, colonies containing the rescued cDNAs in the vector pBluescript SK- were visible and glycerol stocks were made. The inserts were analysed by DNA sequencing.

2.1.4 The Polymerase Chain Reaction

2.1.4.1 Standard PCR protocol.

This was used for introducing new restriction sites at the end of cDNAs or for generating truncations of cDNAs. Reactions were usually performed in a total volume of 50 μl, containing 2.25 mM MgCl$_2$, 6 mM KCl, 15 mM Tris-HCl (pH 8.8) and 1 μl of 10 mM dNTPs. 10 pmol of each primer (about 30 ng) and 20-50 ng template DNA were added. 2 u Taq Polymerase
(Stratagene) were used per reaction and the sample overlaid with mineral oil (Sigma) to prevent evaporation. The following cycles were repeated 30 times: 94°C for 30-40s, 50-55°C for 30s, 72°C for 1-1.5 min and a final extension at 72°C for 5 min. 5μl were loaded on an analytical agarose gel to confirm the size and purity of the PCR product.

2.1.4.2 RACE PCR protocol.
This method is used to clone full-length cDNAs from partial clones. A mouse whole embryo Marathon-Ready cDNA library was purchased (Clonetech number PT1156-1) and used according to the manufacturer's instructions with the Advantage cDNA PCR kit (Clonetech number K1905-1).

2.1.5 DNA Sequencing

2.1.5.1 Manual sequencing.
3-5 μg of double-stranded DNA per reaction were denatured in 0.2M NaOH and 0.2mM EDTA in a total volume of 20μl. 2μl 2M NH₄Ac (pH 4.6) and 60μl ethanol were added and the solution left on dry ice for 5min. The DNA was pelleted in a microfuge at 4°C and resuspended in 6 μl water. Sequencing reactions were carried out (within four hours of denaturation) using the dideoxy chain termination method with a Sequenase V2.0 kit (United States Biochemical Corporation). DNA was labelled with [³⁵S] ATP (Amersham) and reactions performed as described in the kit instructions. Samples were loaded onto a 6% polyacrylamide wedge gel, containing 7 M Urea in TEE buffer and electrophoresed at 80W. The gel was transferred onto Whatman number1 paper and dried under vacuum at 80°C for 1h. Sequences were visualised after overnight exposure at -80°C on a Kodak Biomax-MR film (Sigma).

2.1.5.2 Automated sequencing.
Automated sequencing was performed on an ABI Prism instrument at Eisai Laboratories, University College, London, using the ABI prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). This method was used for
sequencing large inserts, to check PCR-amplified cDNAs and to sequence PCR products.

PCR products were first purified on an Amersham desalting column according to the manufacturer's instruction. For each sequencing reaction, the following mix was prepared: 8 μl Terminator Ready Reaction Mix, 2μl template (at 0.2 mg/ml for plasmids DNA, or 40 μg/ml for PCR products), 1 μl of diluted primer (at 3.2 pmole/μl) and 9μl of water. This mix was subjected to 25 cycles of PCR as follows: 96°C for 30s, 50°C for 15s, 60°C for 4min. The PCR reaction was transferred to a tube containing 2μl 3M sodium acetate (pH4.6) and 50μl 100% ethanol, vortexed and placed on ice for 10min. The tube was centrifuged for 20min, the supernatant carefully aspirated and the pellet was washed with 80% ethanol before being dried in a speedvac (Savant). The sample was processed at Eisai according to the Perkin-Elmer manual.

2.1.6 Multiple Tissue Northern Blots

The radioactive probe was generated as described in 2.1.3.1. The probe obtained was roughly 10⁹ cpm/μg and approximately 10⁷ cpm were added for a reaction volume of 5ml. The probe was first denatured at 100°C for 5min in a microfuge tube placed in a beaker of boiling water, then chilled on ice and finally added to 5ml of ExpressHyb (Clonetch) at 65°C. Prehybridisation and hybridisation of the Northern Blot (Mouse MTN, Clonetech number7762-1) were carried out according to the manufacturer's instructions at 65°C. Washing and stripping were also carried out according to the instructions. The blots were generally exposed for 6h and overnight, then stripped immediately in 0.1% SDS in water at 100°C for 30min, care being taken to avoid the membrane drying out.
2.2 PROTEIN BIOCHEMISTRY

2.2.1 Purification of GTPases

All recombinant proteins used in these studies were produced in E.coli as glutathione-S-transferase (GST) fusion proteins using pGEX vectors. 100ml of LB medium containing 100μg/ml ampicillin were inoculated with E.coli containing the plasmid of choice, incubated overnight at 37°C, then diluted in 1L of LB with ampicillin and grown for a further 3h. Expression of fusion protein was then induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 0.2mM and the culture was incubated for another 2h at 37°C. Cells were pelleted at 4000rpm for 10min and resuspended in 5ml cold lysis buffer (50mM Tris-HCl (pH7.5), 50mM NaCl, 5mM MgCl₂, 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF)). The bacteria were lysed by sonication on ice (4-6x 30s) and the lysate centrifuged for 10min at 10,000rpm. The supernatant was incubated on a rotating wheel at 4°C with 300μl glutathione agarose beads (1:1 suspension; Sigma), which had been prewashed with several volumes of lysis buffer. After 30min, the suspension was centrifuged at room temperature in a bench top centrifuge and the beads were washed 5-6 times with several volumes of cold buffer (lysis buffer, but with 100 mM NaCl and no PMSF) to remove unbound proteins.

GTPases were recovered by resuspending the beads in 300μl thrombin resuspension buffer (50mM Tris-HCl (pH8.0), 150mM NaCl, 5mM MgCl₂, 2.5mM CaCl₂, 1mM DTT) and incubating overnight with 5u bovine thrombin (Sigma). The beads were pelleted by brief centrifugation, the supernatant transferred to a fresh microfuge tube and the beads washed once with 300μl thrombin buffer. Thrombin was removed from the supernatant by mixing with 10μl p-aminobenzamidine-agarose beads (Sigma) for 30min at 4°C. Proteins were concentrated on a centricon 10 filter unit (Amicon) by centrifugation at 6500rpm on a fixed-angle rotor, aliquoted, snap-frozen and kept in liquid nitrogen. Concentration and
purity were estimated on an SDS-polyacrylamide gel and using a GTP filter binding assay.

2.2.2 Preparation of Target Proteins and GAPs

A modified form of the protocol described above was used. Inoculation, growth and induction were as above, except in the case of p65PAK, where these steps took place at 30°C instead of 37°C. Following washing of the beads, the protein was eluted using 3x 500 µl thrombin resuspension buffer containing 1.5mM reduced glutathione (Sigma) for 10min at 4°C. The eluate was pooled, concentrated on a centricon 10 column and treated as above.

2.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were boiled for 3min with 2x loading buffer (80mM Tris-HCl (pH6.8), 0.1mM DTT, 10% glycerol (v/v), 2% SDS (v/v) 0.1% bromphenol blue (w/v)) and loaded onto a polyacrylamide gel (4% stacking gel with 1% SDS in 0.375M Tris-HCl (pH6.8) and 10-13% separating gel with 0.1% SDS in 0.125M Tris-HCl (pH8.8). Running buffer was 0.2M glycine, 0.1% SDS and 25mM Tris base. Electrophoresis was performed at 150-200V. Proteins were visualised using 1% Coomassie blue in 10% acetic acid/12% methanol and the gel destained in 10% acetic acid/12% methanol. Alternatively, proteins were transferred from the gel to nitrocellulose in a Western blot apparatus.

2.2.4 Western Blotting

Electrotransfer was performed at 200mA with transfer buffer (250mM Tris-HCl (pH7.5), 200mM glycine, 20% ethanol) for 2h to overnight. In a Biorad submerged apparatus with a Biorad PowerPak 300. The nitrocellulose was incubated for 1-3h in 5% (w/v) dried powdered milk in PBS, then for a further 1h with the antibody of choice diluted in PBS containing 0.1%
Tween 20. After washing for 3x 5min with PBS/Tween, the membrane was incubated with an HRP-conjugated secondary antibody (rat anti mouse and donkey anti rabbit were from Sigma and both used at 1/10,000 dilution) for 30min and then washed in PBS/Tween 3x 5min and 1x 15min. The blot was developed using the ECL kit (Amersham) according to the manufacturer's instructions.

2.2.5 GTP-Binding Assay

A simple GTP-binding filter assay was used to measure protein concentrations of each new preparation of GTPase. 0.1µl, 0.3µl and 0.5µl of concentrated protein were incubated in a total volume of 40µl binding buffer (50mM Tris-HCl (pH7.5), 1mM DTT, 5mM EDTA) with 0.2µl [3H] GTP (Amersham, 10 Ci/mmol, 1 mCi/ml) for 10min at 30°C. The reaction was stopped on ice with 1ml cold wash buffer (50mM Tris-HCl (pH7.5), 50mM NaCl, 5mM MgCl2) and then filtered through prewetted nitrocellulose filters (Schleicher & Schuell, NC 45, 0.45m). The filters were washed with 10ml wash buffer, air-dried and radioactivity measured by scintillation counting.

If one mole of p21 binds one mole of [3H] GTP, then 1 µg p21 should give 10^6 dpm. The concentration of protein was calculated using the following equation:

\[
\text{conc. p21 [mg/ml]} = \frac{\text{cpm/ml}}{10^6} \times \frac{100}{\text{counting efficiency (ca.20%)}}
\]

The concentration of active protein calculated from this assay was usually about 3-5 fold lower than that estimated from a coomassie blue stained SDS polyacrylamide gel. All concentrations reported in the results section are based on GTP binding assays.
2.2.6 Dot Blot Assay

This method was used as a fast and sensitive way of detecting interactions between GTPases and proteins of interest in vitro. 2-5μg of recombinant protein (2-10μl) were spotted onto strips of nitrocellulose (Schleicher&Schuell, BA 85) and the membranes then incubated for 2h at room temperature with blocking buffer (1M glycine, 5% Marvel, 1% ovalbumin and 5% fetal calf serum). 100ng of p21 were loaded with 10μCi [γ-32P] GTP (6000Ci/mmol) for 10' at 30°C in 200μl of 50mM Tris (pH7.5), 5mM EDTA and 0.5mg/ml BSA. Nucleotide exchange was stopped on ice by addition of MgCl₂ to 10mM. The strips were washed twice with buffer A (50mM Tris-HCl (pH7.5), 100mM NaCl, 5mM MgCl₂, 0.1mM DTT) and incubated in 2.5ml of the same buffer (ice cold) containing 1mM cold GTP together with the radioactively labelled GTPase. After 5min at 4°C the strips were washed three times with 5ml cold 1x TBS + 0.1% Tween and autoradiographed to visualise bound radiolabelled GTPase. The relative intensities of the signals were estimated by densitometry.

2.2.7 GTP Dependence

Target proteins were tested for their ability to bind to GTP- or GDP-bound Rho proteins using a modified dot blot assay. The spotting and blocking steps were as described above, but [α-32P] was used GTP instead of [γ-32P] GTP. After the GTPase loading reaction was stopped, the aliquot was split into two tubes, 10ng of RhoGAP were added to one tube which was incubated at 30°C for 10min, while the remainder of the reaction was left on ice for 10min. The GTPases were then used as for a normal dot blot. The addition of a small amount of RhoGAP causes rapid hydrolysis of GTP to GDP, but since the nucleotide used is α-labelled, the GDP-bound protein remains radioactive, thus GDP and GTP-bound proteins can be compared.
2.2.8 Exchange Assay

The following protocol was used to determine whether or not a protein of interest is able to catalyse exchange of nucleotide on Rho GTPases. 1µg of bacterially-produced wild type Rac was added on ice to each of three tubes containing 250µl of buffer A and 2µl of [³H] GTP (Amersham, 10Ci/mmol). To the first tube, 6 µl of 500mM EDTA were added as a positive control, as this causes rapid exchange. To the second tube, 1µg of GST was added as a negative control, and to the third tube, 1µg of the protein to be tested was added. The tubes were then incubated at 30°C, and 50µl aliquots were taken after 0, 5, 10, 20 and 30min and filtered through prewetted nitrocellulose circles as for GTP binding assays. Bound radioactivity was measured by scintillation counting.

2.2.9 JNK1 assays

Cos-1 cells were transfected as described in 2.4.3. At 9-10pm on day 4 of the transfection, following overnight serum deprivation, transfected Cos-1 cells were harvested using 250µl/dish of lysis buffer (20mM Tris (pH8.0), 40mM sodium pyrophosphate, 50mM NaF, 5mM MgCl₂, 100µM Na vanadate, 10mM EGTA, 1% Triton X-100, 3mM PMSF, 20µg/ml Leupeptin/Aprotinin) and a cell scraper (Falcon). The lysates were snap-frozen in aliquots, and kept at -80°C.

20µl of each of the lysates were mixed with 8µl of 3x sample buffer, loaded onto a 12% SDS PAGE gel and transferred to nitrocellulose. In this assay, it is critical to have equal amounts of the kinase in each condition and in order to accurately quantitate the amounts of JNK1 in each transfection, [¹²⁵I]-labelled protein A (Amersham) was used to detect and quantitate the transferred proteins. Blocking and washes between antibodies were performed as described in 2.2.4. Primary antibodies were anti-myc (kind gift from D. Drechsel) used at 1/300 to detect myc-tagged Rac, POSH and PAK proteins and anti-FLAG (ISI) used at 1/400 to detect FLAG-JNK1. After washing, an unconjugated rat anti-mouse antibody (Pierce) was
applied at 1/1000 for 45min to amplify the signal. The blot was then incubated for 2h in 10ml of TBS/Tween containing 0.1μCi/ml [\(^{125}\text{I}\)]-labelled protein A (Amersham, 0.1 mCi/ml). After washing, the radioactivity was visualised by overnight exposure on Biomax-MS film (Kodak) and quantified using a Biorad model GS-250 PhosphorImager.

Using the quantification data, the amounts of lysate required to give equal amounts of kinase in each immunoprecipitation were calculated. The activity of FLAG-JNK1 in each lysate was assayed on immunocomplexes as follows. The appropriate amounts of each lysate (typically 100-300μl) were put in 1.5ml screw-cap microfuge tubes and the volumes adjusted to 1ml with immunoprecipitation buffer (20mM Hepes pH7.6, 50mM NaCl, 2.5mM MgCl\(_2\), 0.1mM EDTA, 0.05 % Triton X-100, 40mM Na pyrophosphate, 50mM NaF, 100 μM Na vanadate, 3 mM PMSF, 20 μg/ml aprotinin and leupeptin). 6μg of anti-flag antibody (ISI) were added to these immunoprecipitations (IPs), which were then incubated at 4°C for 1h on a rotating wheel. 30μl of protein G-separose beads (Sigma) previously washed in several volumes IP buffer were added to each tube. The tubes were incubated for a further 1h at 4°C. The beads were pelleted at 13000rpm for 1min, and carefully washed with 3x 1ml of IP buffer.

After the last wash, all of the IP buffer was carefully removed and the beads were resuspended in 30μl of kinase buffer (20mM Hepes (pH7.6), 20mM MgCl\(_2\), 4mM NaF, 20mM para nitrophenyl-phosphate, 0.1mM Na vanadate, 2mM dithiothreitol, 20mM cold ATP) containing 1μl/reaction of [\(\gamma\)-\(^{32}\text{P}\)] ATP (Amersham, 3,000Ci/mmol) and 1-2μg/reaction of substrate (recombinant c-jun protein). The mixture was incubated for 30min at 30°C, the reactions were stopped by addition of 8μl of 5x sample buffer and boiling for 3min loaded onto an 8% SDS PAGE gel and after electrophoresis transferred to nitrocellulose. Phosphorylated c-jun was visualised on Biomax MR film (Kodak), and quantified on a PhosphorImager. The levels of JNK1 in each IP was checked by western analysis using a rabbit anti-JNK1 antiserum (Santa Cruz), an HRP conjugated anti rabbit secondary antibody (Sigma) and the ECL.
kit (Amersham). In general, the $^{125}$I-labelled protein A step guaranteed reliable IP results.

### 2.3 YEAST TWO-HYBRID

#### 2.3.1 Plasmids and Materials

The protocols described here were modification of the methods described by Aspenström and Olson (Aspenstrom and Olson, 1995). pYTH9 was used as bait plasmid in these studies and was a kind gift of Dr Robin Brown (Glaxo-Wellcome, Stevenage). This plasmid cannot replicate autonomously and is therefore integrated in the yeast genome by homologous recombination in the Trpl gene. To facilitate the integration process, the plasmid was linearised with XbaI prior to being transformed into yeast. Once integrated in the genome, constructs were far more stable than non-integrated vectors and glycerol stocks, containing a variety of baits, could be kept without any problems at -70°C. GTPase baits were generated by PCR and the cysteine in the "CAAX box" was mutated to a serine in order to prevent targeting of the fusion proteins to the plasma membrane. The prey plasmids used in these studies were pACTII (kind gift from Dr Steve Elledge, Baylor College, Houston), pGAD-GH (Clonetech), and pGAD-10 (Clonetech). The recombination-deficient bacterial strain DH5α was used for plasmid amplification and maintenance since all the plasmids were large (over 7kbp), and prone to deletions when kept in recombination-competent bacteria. The yeast strain was Y190 [MATα, gal5-542, gal80-538, his3, trp1-901, ade2-101, ura3-52, leu2-3, URA3::GAL1-lacZ, LYS2::GAL1-HIS3cyh]$^7$ (a kind gift from Dr Steve Elledge).

Plates used for selection were as follows: YPD (rich medium): 20g/l peptone (Difco), 10g/l yeast extract (Difco), 20g/l agar (Difco), 2% (w/v) D-glucose. SC-Trp: 6.7g/l yeast nitrogen bases w/o amino acids (Difco), 2g/l of drop-out mix (1g each of all the essential amino acids except tryptophan, leucine and histidine, with 1g of inositol, 0.25g of adenine, 1g of uracil,
and 0.1g of para-aminobenzoic acid, mixed on a wheel for 2h
and kept in a foil-wrapped bottle at room temperature), 20g/l
of agar, 0.1g/l of histidine, 0.2g/l leucine. SC-Leu-Trp: 6.7g/l
yeast nitrogen bases, 2g/l of drop-out mix, 20g/l of agar, 0.1g/l
of histidine. 3-AT (most selective medium) 6.7g/l yeast
nitrogen base , 2g/l of drop-out mix, 20g/l of agar, 25mM 3-
amino triazole (from a 1M stock dissolved in water and filter-
sterilised).

2.3.2 Transformation of Bait Plasmids

One colony of the *Saccharomyces cerevisiae* strain Y190
was picked from a non selective YPD plate (20g/l peptone,
10g/l yeast extract, 20g/l agar, 2% (w/v) D-glucose) and grown
in 10ml of YPD medium (same as plate without agar) overnight
at 30°C with vigorous agitation. The culture was diluted in 50ml
of fresh medium, and grown for 4h. The yeast were harvested
by centrifugation at 2500rpm for 2min, then washed in 20ml of
sterile TE (pH7.5) and resuspended in 1ml of 0.1M LiAc in TE
(pH7.5). After two further washes in 1ml of LiAc/TE, the yeast
were resuspended in 400μl of LiAc/TE. 20μl of boiled carrier
DNA (Herring testis, Sigma) were added to a 1.5ml microfuge
tube containing the DNA to be transformed (3-5μg, digested
with XbaI for 1h at 37°C, then heated for 10min at 70°C to
inactivate the enzyme). 100μl of the yeast cell suspension and
700μl of 44% polyethylene glycol mw3350 in 0.1M LiAc were
added to the tube, which was then kept at 30°C for 30min (with
several inversions). The cells were heat-shocked at 42°C for
20min, harvested by centrifugation, plated on a selective SC-
Trp plate, and grown for 2-3d at 30°C. Glycerol stocks were
made by pelleting a 10ml SC-Trp overnight culture and
resuspending cells in 1ml of YPD medium/1ml of 40% glycerol.
Stocks were kept at -80°C.

2.3.3 Transformation of "Prey" Plasmids

The method is similar to bait transformation apart from
the following differences. The overnight culture containing bait
plasmid of interest is grown in SC-Trp (not YPD since the bait plasmid bears the $Trpl$ marker). The plasmid to be transformed was not digested. After the transformation, yeast were plated on SC-Trp-Leu plates to select for both plasmids. Reporter gene expression was not selected at this stage to allow the negative controls to grow. After two days at 30°C, the yeast were tested using the plate lift and growth on 3-AT assays as described below.

2.3.4 "Plate lift" Assay for $\beta$-galactosidase

The $LacZ$ gene is under the control of the GAL4 promoter and is therefore only expressed when two plasmids encoding interacting proteins are present in the yeast cells. Yeast cells were transferred from a plate to a Whatman number 1 filter disc (9cm) which was then dropped in liquid nitrogen for 30s for lysis. Once thawed, the discs were transferred to a 10cm dish containing a filter disc soaked with 2.5ml of buffer Z (60mM Na$_2$HPO$_4$·7 H$_2$O, 40mM NaH$_2$PO$_4$·H$_2$O, 10mM KCl, 1mM MgSO$_4$·H$_2$O) with 50mM mercaptoethanol and 0.35mg/ml 5-bromo-4-chloroindolyl-$\beta$-D-galactoside (X-Gal). Positive yeast became blue after 30min-6h, but were generally left overnight at 30°C in a sealed container (later dried to stop the reaction). This assay is not quantitative, but gives an indication of the strength of the interaction, as the kinetics of the reaction are dependent on the level of expression of the reporter gene. Targets of GTPases were generally compared to RhoGAP as a positive control and empty vector as a negative control.

2.3.5 Growth on 3-amino triazole (3-AT)

$HIS3$ is the second reporter gene placed under the control of the GAL4 promoter. However, the $his3$ mutant genetic background in the yeast strain used is leaky and a low level of functional imidazoleglycerol-phosphate dehydratase (IGPD, the product of the $HIS3$ gene) is present, even without reporter gene activation. In order to test for $HIS3$ reporter activation, it is therefore necessary to add 3-AT, an inhibitor of IGPD, which
increases the threshold of IGPD activity necessary for cell growth. When cells are restreaked on a 25mM 3-AT plate, only the ones containing interacting proteins will be able to grow. Cells were first transformed as described above, equal sized colonies of yeast containing the protein to be tested, as well as positive and negative controls were streaked in squares on a fresh 3-AT plate. The cells were grown at 30°C for 3-4d. The plates were then image-grabbed using Adobe Photoshop.

2.3.6 Library Screening

The great strength of the yeast two-hybrid is the ability to isolate novel interacting partners to a protein of interest by screening cDNA libraries in the "prey" vector against a bait containing the gene of interest.

On day one, a tube containing 10ml of SC-Trp was inoculated with a colony of yeast bearing an integrated copy of the gene of interest and left to grow overnight at 30°C with vigorous agitation. In the morning of day 2, the 10ml culture was diluted in 100ml of SC-Trp in a 250ml conical flask and left to grow until the next day. On day 3, the saturated culture was diluted in 1L of YPD medium (to an OD600 of 0.3-0.4) and grown to an the OD600 of 0.9-1.0. Cells were harvested by centrifugation at 3000rpm in sterile 250ml corning flasks and resuspended in 500ml of TE (pH7.5). After a second centrifugation, the cells were resuspended in 20ml of 0.1M LiAc in TE and incubated at 30°C for 10min. 4ml of freshly boiled carrier DNA mixed with 100-200µg of library DNA and 100ml of 40% polyethylene glycol/0.1M LiAc were added to the cells. This transformation was gently mixed, incubated at 30°C for 30min, then heat shocked at 42°C in a 2L flask for 20min, with occasional mild agitation. The cells were harvested by centrifugation, resuspended in 1L of YPD, transferred to a 2L conical flask and grown for 1h in a 30°C shaker. Finally, the cells were harvested, washed in 250ml of TE, resuspended in 15ml of TE and plated on 10 24x24 cm dishes (Nunc) containing SC-Trp-Leu-His with 25mM 3-AT. 1 and 10µl of the mix were
plated on SC-Trp-Leu plates in order to calculate the efficiency of transfection and the total number of colonies screened.

The plates were sealed in Saranwrap and incubated at 30°C for 10-15d. "Bipps" (big pink positives) were restreaked on 3-AT plates and tested for β-galactosidase activity. Plasmid DNA was extracted from clones that were positive in both assays after replating. The plasmids were amplified in bacteria and retransformed into the yeast strain used for the screen to eliminate false positives. Plasmids that were positive after the second transformation were sequenced and further characterised.

2.3.7 Plasmid DNA Extraction from Yeast

This was used in order to rescue library plasmids after a screen. Cells from an overnight culture in SC-Trp-Leu or a colony growing on a 3-AT plate were resuspended in 400μl yeast lysis buffer (2.5M LiCl, 50 mM Tris-HCl (pH8.0), 4% Triton X-100, 62.5mM EDTA). 400μl of phenol/chloroform/isoamyl alcohol and 200μl of glass beads (0.40mm, BDH) were added to the tube, which was then vortexed vigorously for 2min to lyse the cells and centrifuged at 13000rpm in a microfuge. The aqueous phase was put in a fresh tube and mixed with 1ml of DNA-binding resin suspension form the Wizard clean-up kit (Promega). The sample was then processed according to the kit instructions and the DNA eluted from the beads with 3x 50μl of 70°C TE (pH7.5). DNA was precipitated with sodium acetate and ethanol, resuspended in 10μl of water and 2-5μl were used to transform competent DH5α as described above.

2.4 CELL BIOLOGY

2.4.1 Fibroblast Culture and Injection

Swiss 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) in the presence of 10% fetal calf serum (FCS, Sigma) and antibiotics. For microinjection, cells
were plated on acid-washed, round 13 mm coverslips at 6x10^4 cells/coverslip in four-well dishes. Seven to ten days after plating, the confluent quiescent cells were serum-starved for 16h in DMEM containing 2 g/l NaHCO₃ and antibiotics. The cells were injected using an Eppendorf micromanipulator on a Zeiss Axiovert 135M microscope in an atmosphere maintained at 10% CO₂ and 37°C. DNA constructs (diluted in PBS at 0.03-0.1 mg/ml) were injected into the nucleus using FITC-conjugated dextran (Sigma) as a marker. The coverslips were put back into their wells after injection and fixed in 4% paraformaldehyde at the indicated times.

NIH 3T3 cells were maintained in DMEM with 10% Donor Calf Serum (Stratech) and antibiotics. Cells for microinjection were plated on acid washed coverslips at 5x10^4 per coverslip. For serum starved cells, the medium was replaced 6h after plating, and the cells were left for 16h prior to injection. The cells were microinjected and fixed as Swiss cells.

2.4.2 Cos1 Cells: Maintenance and Transfections

Cos1 cells were maintained in DMEM with 10% FCS and antibiotics. Transfections were performed as follows. On day 1, a flask of Cos1 cells was trypsinised and cells resuspended in 10ml of DMEM containing 10% NuSerum typeV (Stratech). 1ml of this cell suspension was added to 10ml of DMEM with NuSerum in 90mm dishes (Falcon). The dishes were gently shaken to ensure even spreading of the cells and cells left to settle overnight. On day 2, 5ml of transfection medium per plate were made up in 5ml universals (Falcon) for each of the plates to be transfected, containing 5ml of DMEM with 10% NuSerum, 40μl of 5% DEAE-Dextran, 5μl of chloroquine phosphate solution (0.26g of powder dissolved freshly into 5ml of DMEM with NuSerum). The DNA was then added to this mixture, with empty vector so that all the transfections had an equal amount of DNA in total. The medium on the plates was aspirated and replaced with the tranfection medium and incubated cells at 37°C for 4h. The transfection mix was then replaced with 5ml of 10% DMSO in PBS-A for precisely 2
minutes at room temperature. The DMSO was then removed and 10ml of DMEM with 10% FCS and 10µM BOC-D (Enzyme Systems Products, from a 10mM stock in DMSO) were added to each plate, which were put back in the 37°C incubator. On day 3 between 5 and 7 pm, the medium was aspirated and replaced with 6 ml of serum-free medium containing BOC-D. On day 4, the cells were harvested using a cell scraper (Falcon).

2.4.3 Immunofluorescence Microscopy

The cells were microinjected as described and incubated at 37°C for the indicated times. The coverslips were rinsed in PBS and fixed for 10min with 4% (w/v) paraformaldehyde in PBS. Coverslips were extensively rinsed in PBS between every step of the staining procedure. After fixing, the cells were permeabilised for 5min in 0.2% Triton X-100 in PBS (10min for NFκB staining). Free aldehyde groups were reduced by treatment with 0.5mg/ml sodium borohydride for 10min. Primary antibodies were applied for 1h diluted in PBS at the following dilutions: anti-myc (9F10) antibody (kind gift of Dr D. Drechsel) was diluted 1/200 in PBS, anti-NFκB (Santa Cruz) was diluted 1/200 in PBS.

After washing, the coverslips were incubated for 30min with secondary antibodies: goat anti mouse FITC (Pierce), donkey anti rabbit TRITC (Jackson), diluted 1/100 in PBS. In some cases, the second incubation also contained 1/400 Hoescht reagent (Sigma) or 1/400 Rhodamine phalloidin (Sigma). Coverslips were mounted on moviol mountant containing p-phenyl-enediamine as an antibleaching agent. After 1h at 37°C, the coverslips were examined and the cells counted on a Zeiss axiophot microscope using Zeiss 40x 1.3 and 63x 1.4 oil-immersion objectives. Pictures were taken on Kodak T-MAX 400ASA film.

2.4.4 Time-lapse microscopy

A CCD camera (Panasonic BL-22) and time-lapse controller (Eos electronics) were attached directly to a Zeiss Axiovert 135M microscope. Cells were microinjected in serum-free
medium, incubated for 1h in a 37°C incubator, then sealed in a flask (Falcon) containing 4ml of serum-free medium and incubated at 37°C on the time-lapse microscope. Pictures were taken under phase-contrast at 10 frames/min on a Sony Betacam videotape recorder. Images were processed using Adobe Photoshop.
CHAPTER 3

CLONING AND CHARACTERISATION OF A NEW RHO TARGET

3.1 INTRODUCTION

Rho regulates the formation of actin stress fibres and focal adhesions in fibroblasts in response to agonists such as LPA or bombesin (Ridley and Hall, 1992). Rho also controls a variety of other processes that involve the actin cytoskeleton, such as cytokinesis and vesicle trafficking (Van Aelst and D’Souza-Schorey, 1997). In addition, it is thought that Rho co-ordinates cytoskeletal regulation with changes in gene transcription via the transcription factors SRF and NFκB and by the JNK MAPK pathways (Van Aelst and D’Souza-Schorey, 1997). With this variety of functions, it might be anticipated that Rho interacts with a wide range of cellular target molecules, but in 1994, when this project was initiated, no effectors for Rho had yet been identified. I therefore made use of the yeast two-hybrid selection system to identify Rho targets.

The yeast two-hybrid system (YTHS), devised by Fields and Song as a means of detecting protein-protein interactions (Fields and Song, 1989), takes advantage of the modular structure of transcription factors. For example, the protein of interest (the bait) is expressed in yeast as a fusion protein with the DNA-binding domain of the yeast transcription factor GAL4. A second protein (the prey) is then introduced as a fusion with the activation domain of GAL4. An interaction between the bait and prey leads to the reconstitution of a functional GAL4 transcription factor. This can be measured by expression of reporter genes (HIS3 and LacZ) contained within the yeast host. Using the YTHS, it is also possible to screen a cDNA library for sequences encoding proteins that interact with the bait. The YTHS has been successfully used in many fields and many target proteins for small GTPases have subsequently been isolated in this way (See 1.3.4).
My strategy in using the YTHS to screen for Rho targets was to isolate proteins that interact specifically with the GTP-bound form. In order to achieve this, I have used L63 Rho as a bait. This mutant is unable to hydrolyse GTP and is consequently constitutively in a GTP-bound state (Lamarche et al., 1996). In this chapter, I describe the use of the YTHS in the isolation of a candidate cellular target of Rho.

3.2 RESULTS

3.2.1 Yeast Two Hybrid cDNA Library Screen

A commercial oligo-dT-primed HeLa cDNA library in the GAL4 activation domain plasmid pGAD-GH (Clonetech) was screened in a yeast strain containing an integrated plasmid expressing L63 Rho fused to the GAL4 DNA-binding domain (as described in the materials and methods). Approximately 10^6 clones were screened. The original screen was carried out by Dr Pontus Aspenström who identified 45 (fastest-growing) potential positive clones. These were picked, restreaked on fresh SC-Leu-Trp plates and tested for β-galactosidase activity in a plate lift assay. 29 of the 45 clones turned blue and were processed further.

In YTHS screens, it is critical to eliminate false positives at an early stage since processing many clones is time consuming. I rescued plasmids from the 29 yeast strains and transferred them to bacteria. The plasmids were re-transformed back into the yeast strain containing integrated L63 Rho. This second round of transformations normally reduces the number of positives. After the second round, five clones were positive both in β-Galactosidase plate lift assays and for growth in the presence of 25mM 3-AT (a test for HIS3 reporter expression). These five clones were later tested in vitro (see 3.2.2) and only one, clone 38, interacted strongly with L63 Rho. Clone 38 was therefore selected for further analysis.

In order to test its specificity, clone 38 was transformed into strains of yeast containing integrated L63 Rho, L61 Rac, L61 Cdc42 and L87 R-Ras. Colonies from these transformations were picked and restreaked on SC-Trp-Leu-His + 25mM 3-AT
plates in order to select for expression of the HIS3 reporter (figure 3.1). As can be seen, in the YTHS, clone 38 interacts with the constitutively activated forms of Rho, Rac and Cdc42, but not with R-Ras.

Figure 3.1 Interaction of clone 38 with GTPases in the yeast two-hybrid system
Yeast strains containing integrated L63 Rho, L61 Rac, L63 Rho and L87 R-Ras in pYTH6 were transformed with pGAD-GH-clone 38, or with pACTII-Raf as a positive control for R-Ras (pACTII-Raf was a kind gift from Prof C. Marshall, ICR, London). Transformations were grown for 3d, colonies of equal size were picked and restreaked on SC-Trp-Leu-His+25mM 3-AT plates and grown for four days.
3.2.2 In vitro interactions between clone 38 and Rho GTPases

Clone 38 contained a 0.5 kbp cDNA insert which was isolated and transferred from pGAD-GH into pGEX-KG by insertion into Smal/XhoI sites. In this way, clone 38 was expressed as a GST fusion protein in *E. coli* which, after purification on glutathione agarose, could be analysed by SDS PAGE gel. As can be seen in figure 3.2, 38-GST migrates at approximately 46 kDa and since GST has a molecular weight of 29 kDa, the protein product of clone 38 is about 17 kDa (consistent with an insert size of 500 base pairs). It can be seen in figure 3.2, that the clone 38-GST fusion protein is sensitive to proteolysis in *E. coli* and gives rise to numerous degradation products.

Figure 3.2 Production of recombinant clone 38

GST-Clone 38 was purified from bacteria as described in the methods. 1/20 of the total sample volume was loaded on a 10% SDS PAGE gel. Proteins were visualised with coomassie blue. Molecular weights (MW) are indicated in kDa and correspond to prestained Rainbow markers (Amersham).
The interaction of clone 38 with recombinant GTPases was examined \textit{in vitro} using a dot blot protocol (see materials and methods). Firstly, L63 Rho, L61 Rac and L61 Cdc42 were used (Figure 3.3). Consistent with the yeast two-hybrid result, clone 38 interacted strongly with activated Rho, Rac and Cdc42 in vitro. Next, the binding of clone 38 to wildtype GTPases was examined (figure 3.4). Surprisingly, clone 38 interacted with wildtype Rho and Rac, but not significantly with Cdc42. The amounts of bound radiolabelled GTPases were measured from three independent experiments by scintillation counting. Using this assay, I estimate the interaction of clone 38 with wildtype Rho to be two or three times as strong as its interaction with wildtype Rac. This is consistent with clone 38 being a candidate effector for both Rho and Rac but not Cdc42.

\textbf{Figure 3.3} Clone 38 interacts with activated Rho, Rac and Cdc42 \textit{in vitro}

GST-clone 38 (10µg) or GST-RhoGAP (10µg, a.a. 198-439) were spotted onto strips of nitrocellulose and probed with [γ32P]GTP-bound GTPases (100ng each). The filters were washed and exposed for 30min to Kodak X-AR film.
Figure 3.4 Clone 38 binds to wildtype Rho and Rac, but not Cdc42 \textit{in vitro}.

Different amounts of GST-clone 38 were spotted onto nitrocellulose as indicated. WASP (10 μg) was spotted as a positive control for Cdc42 and GST (10 μg) as a negative control. The nitrocellulose strips were probed with [γ32P]GTP-labelled wildtype GTPases. Bound radioactivity was visualised by autoradiography (1.5h exposure on X-AR film).
Effector molecules for Ras-related proteins are expected to show preferential binding to the GTP-bound form of the GTPase. The binding of clone 38 to GTP- or GDP-bound Rho was compared in a modified dot blot assay. In this assay, Rho was labelled with $[^α-32P]GTP$ instead of $[^γ-32P]GTP$. The loaded GTPase was split into two aliquots and a small amount of RhoGAP was added to one to hydrolyse the GTP to GDP. Thus, radiolabelled GTP- and GDP-bound Rho were used in the standard dot blot assay. It can be seen in figure 3.5 that clone 38 interacted strongly with GTP-bound Rho, but not with GDP-bound Rho, as expected for a target molecule.

![Figure 3.5 Clone 38 binds to Rho in a GTP-dependent manner](image)

GST-clone 38 (10μg) and GST (10μg) were spotted onto nitrocellulose strips. $[^α 32P]$-labelled GTP- and GDP-bound wildtype Rho were generated as described in the methods and used to probe the nitrocellulose strips. Bound radioactivity was visualised by autoradiography (1.5h exposure on X-AR paper).
The effector region of Ras proteins, between amino acids 30 and 40, is important for binding to target molecules. The analysis of point mutations in this region has proved an effective way of dissecting the different signalling pathways downstream of Ras and Rho-related GTPases (White, 1995, Van Aelst and D'Souza-Schorey, 1997). When this work was carried out, effector mutants of Rho were not yet available. However, in collaboration with Dr Nathalie Lamarche, I examined binding of clone 38 to Rac effector mutants in dot blot assays. As can be seen in figure 3.6, clone 38 binds to Y40C L61 Rac, but not to F37A L61 Rac. This is in contrast with the Rac target PAK which binds F37A L61 Rac but not Y40C L61 Rac. Dr Nathalie Lamarche showed that F37A L61 Rac is unable to induce lamellipodia formation or DNA synthesis, while Y40C L61 Rac is unimpaired in these Rac functions (Lamarche et al., 1996). This raises the possibility that clone 38 might be involved in cytoskeletal rearrangement downstream of Rac.

From these biochemical studies, I conclude that clone 38 is a candidate effector for Rho and perhaps Rac, but not Cdc42.
Figure 3.6 Binding of clone 38 to Rac effector mutants

GST-RhoGAP (10μg, a.a. 198-439), GST-PAK (10μg), GST-clone 38 (10μg), GST-p67phox (10μg, a.a. 1-199) and GST (10μg) were spotted onto strips of nitrocellulose. L61 Rac, F37A L61 Rac and Y40C L61 Rac were labelled with [γ32P]GTP and used to probe the nitrocellulose strips. Bound radioactivity was visualised by autoradiography (1.5h exposure on X-AR paper). This dot blot was carried out by Dr Nathalie Lamarche.
3.2.3 Sequence analysis of clone 38

Full-length DNA sequence of both strands of clone 38 was obtained by manual sequencing (as described in the materials and methods) and is shown in figure 3.7. This DNA sequence encodes an ORF (Open Reading Frame) of 168 amino acids (figure 3.8). When this sequence was entered in the protein databases, low homology was found throughout with the rod domain of myosin. Using the Chou-Fasman method, the sequence is predicted to be mostly α-helical; typical of coiled-coil containing proteins. Using the paircoil program (Berger et al., 1995), the clone 38 sequence is indeed predicted to adopt a coiled-coil conformation. Coiled-coils are found in structural molecules such as myosin where they form long rods. Shorter stretches of coiled-coils have also been found in leucine zipper transcription factors where they are thought to act as dimerisation domains (see Lupas, 1996 for a review).

No Kozak consensus could be found anywhere in the clone 38 sequence suggesting that the clone was not complete at the 5' end. Furthermore, no stop codon was found in-frame at the 3' end of the ORF sequence. This resulted in the ORF of clone 38 terminating at sequences in the vector. Interestingly, the end of the cDNA consisted of eighteen adenosines. Since the library used in the two hybrid screen was oligo-dT primed, it is likely that this internal stretch of adenosines accounts for clone 38 lacking the 3' end of the message. Internal priming events of this kind (as illustrated in figure 3.9) are relatively frequent during cDNA library construction.
Figure 3.7 DNA sequence of the clone 38 insert

Figure 3.8 Predicted protein sequence of clone 38
Figure 3.9 Schematic representation of an internal priming event during synthesis of the first strand of an oligo-dT-primed cDNA library leading to a truncated cDNA

3.2.4 Screening for full-length clone 38

In order to isolate a full-length cDNA of clone 38, a bacteriophage lambda mouse whole embryo cDNA library was screened. Library phage stock was a kind gift of Dr Peter Burbelo, MRC Laboratory for Molecular Cell Biology, London. The clone 38 insert was isolated from pGAD-GH using Smal and XhoI on a low melting agarose gel. The fragment was purified from the gel slice and radiolabelled as described in the methods. This radiolabelled probe was used to screen approximately $2 \times 10^6$ phage plaques immobilised on duplicate
nitrocellulose filters. Two phage plaques were positive on both duplicates on the first screen. These cDNAs were taken through two more rounds of screening in order to obtain homogeneous phage stocks.

The cDNAs, 38.1 and 38.2, were excised from phage by using helper phage (see methods) and the cDNA clones in pBluescript SK+ were transferred to DH5α E.coli. DNA stocks were made for restriction digestion and manual sequencing. The plasmids were digested with EcoRI to remove the inserts and visualised on an agarose gel. 38.1 and 38.2 inserts were 2.5 kbp and 1.4 kbp respectively. DNA sequence from both strands was obtained for clone 38.1 and from one strand for the smaller clone 38.2. Sequence analysis of 38.1 (figure 3.10) revealed that the 18 adenosine residues found at the end of clone 38 are in fact present within an ORF that extends further. Clone 38.2 was in fact derived from internal priming at the same place as clone 38 during synthesis of the mouse phage library. The larger clone 38.1 gave additional sequences both 5' and 3' to the original clone 38 as shown in figure 3.10. The ORF continued through the adenosine-rich region giving 321 amino acids of additional sequence until an in-frame stop codon was reached. The cDNA contained a polyA stretch at the 3' end. Sequence analysis of the 38.1 insert revealed an ORF of 886 amino acids (see figure 3.11). This protein comprises a large N-terminal coiled-coil domain, a C-terminal PH domain and a putative zinc finger (see figure 3.12 (top) for a schematic representation).

The 38.1 DNA sequence did not have a good Kozak sequence near the 5' end and had no in-frame stop codon, suggesting that this clone was unlikely to encode the full-length ORF. At this point in the work, the sequences of two closely related targets of Rho, Rho-kinase α (ROKα) and Rho-kinase β (ROKβ or p160ROCK) were published (Leung et al., 1995, Matsui et al., 1996b, Ishizaki, 1996). Analysis of these sequences revealed that 38.1 was the mouse homologue of Rho-kinase β (Ishizaki, 1996). This confirmed that the 38.1 clone contained an incomplete cDNA and in fact lacked 468 amino acids encoding a serine/threonine kinase domain (see figure 3.12).
The sequence corresponding to the original clone 38 is underlined. The stop codon is shown in bold.

Figure 3.10  The DNA sequence of the clone 38.1 ORF
Figure 3.11 Predicted protein sequence of clone 38.1
The sequence corresponding to the original clone 38 is underlined.
Figure 3.12 Schematic representation of the 38.1 (top) and Rho-kinase α/β (bottom) sequences.

S/T kinase: serine/threonine kinase domain.
Leu: putative leucine zipper
PH: Pleckstrin Homology domain
Cys: cysteine rich region (putative zinc finger).

3.2.5 Cell biological analysis of Rho-Kinase function

A full-length clone of bovine Rho-kinase (ROKα), in a mammalian expression vector was obtained from Dr Kozo Kaibuchi (Nara Institute of Science and Technology, Japan) and transferred to the mammalian expression vector pRK5myc by Dr David Drechsel (Laboratory for Molecular Cell Biology, London). A construct encoding the catalytic domain of Rho-Kinase α (ROK-CAT, a.a. 6-553) was also made in pRK5myc by Dr David Drechsel.

I microinjected myc-tagged pRK5 constructs encoding activated L63 Rho, full-length Rho-kinase α and ROK-CAT into serum-starved, confluent quiescent Swiss 3T3 fibroblasts. The cells were left to express the constructs for 3 hours, fixed and
permeablis’d. Expression of the microinjected constructs was visualised with anti-myc antibody and filamentous actin was visualised with rhodamine-phalloydin (figure 3.13).

**Figure 3.13** Effects of overexpression of Rho, Rho-kinase α and ROK-CAT on the actin cytoskeleton

Serum-starved Swiss 3T3 cells were microinjected in the nucleus with pRK5myc-full-length Rho-kinase α (a, b), pRK5myc-ROK-CAT (c, d) or pRK5myc-L63 Rho (e, f) all at 0.1mg/ml, incubated for 3h and fixed. Expression of the constructs was visualised using anti-myc antibody (a, c, e) and filamentous actin was visualised with rhodamine-phalloydin (b, d, f). Scale bar: 20μm.
Full-length Rho-kinase was found to induce reorganisation of the actin cytoskeleton to form actin filaments (figure 3.13: a, b). Although many of the actin filaments induced by full-length Rho-kinase were of a similar length to stress fibres induced by Rho, they were much less numerous, more disorganised and thinner. With ROK-CAT, the result was very spectacular: very thick actin bundles were seen, often radiating from one or two points within the cell (figure 3.13: c, d). These ROK-CAT-induced filaments were very different from Rho-induced stress fibres, being less numerous, much thicker and differently distributed (figure 3.13, compare d and f). The ROK-CAT-injected cells were often smaller than Rho-injected cells and had a contracted appearance.

3.3 DISCUSSION

This chapter describes the identification of a cDNA encoding a new target molecule for Rho using the yeast two-hybrid system. The ORF of clone 38 encodes a 166 amino acid protein which was found to interact with activated mutants of Rho, Rac and Cdc42, but only with wildtype Rho and Rac. Clone 38 interacted with GTP-bound, but not GDP-bound Rho. Furthermore, clone 38 interacted with the Y40C L61 Rac effector mutants, but not with F37A L61 Rac. A longer version of clone 38 containing an 886 amino acid ORF was isolated form a phage library and found to encode the mouse homolog of a newly published serine/threonine kinase, ROKβ (Ishizaki, 1996). A full-length clone of Rho-kinase α (Rho-kinase β's close relative) was obtained and microinjected into serum-starved Swiss 3T3 cells where it was shown to induce the formation of actin/myosin filaments bundles. These results implicate clone 38 as a potential cellular target of Rho and Rac (but not Cdc42) contributing to their effects on the actin cytoskeleton.

3.3.1 The structure of the Rho-Kinases

Rho-Kinase β (see figure 3.12) is a 160 kDa protein with an N-terminal serine/threonine kinase domain related to that of MDK (Myotonic Dystrophy Kinase, Brook et al., 1992). Rho-
kinase β shares around 74% amino acid identity with MDK, but MDK itself does not appear to be a Rho effector (Ishizaki, 1996, Matsui et al., 1996b). In addition to its kinase domain, Rho-kinase β possesses a large coiled-coil region that contains the Rho/Rac binding domain, a PH domain and a putative zinc finger. Rho kinase β has a close relative called Rho-Kinase α (Leung et al., 1995, Matsui et al., 1996b). The two Rho-kinases share 64% amino acid identity overall, with 90% in the kinase domain, 55% in the coiled-coil region and 70% at the C-terminus.

PH domains are found in many kinases such as PKB/Akt or βARK (β-Adrenergic Receptor Kinase) and are thought to mediate membrane association via lipids such as PIP2 (Lemmon 1997 review). Rho-kinase α has been reported to translocate to the plasma membrane in HeLa cells upon cotransfection with activated Rho (Leung et al., 1995). However, the mechanism and functional significance of this translocation are unknown.

3.3.2 Function of Rho-Kinase downstream of Rho

Microinjection of mammalian expression constructs encoding Rho-Kinase α/β and the Rho-Kinase α/β catalytic domain has been reported to induce formation of stress fibres and focal adhesions in fibroblasts (Leung et al., 1996, Amano et al., 1997, Chihara et al., 1997). It is clear from my experiments, however, that the actin filaments induced by full-length Rho-kinase α and ROK-CAT overexpression are distinct both in distribution and organisation from stress fibres induced by Rho. Although full-length Rho-kinase-injected cells consistently had actin bundles of similar length to stress fibres, these were thinner and somewhat disorganised compared to the parallel arrays of stress fibres induced by Rho injection. ROK-CAT-injected cells, on the other hand, contained very thick and short actin cables often radiating from one or two points within the cell.

It has been reported that, Rho-kinase-induced actin filament assembly is insensitive to the Rho inhibitor C3 transferase, suggesting that Rho-kinase lies downstream of Rho in the control of actin organisation (Amano et al., 1997). Furthermore, a recently characterised Rho-kinase β inhibitor, Y-
was reported to block stress fibre and focal complex formation induced by activated Rho in HeLa cells (Uehata et al., 1997). My results and those published by others suggest that Rho-kinase is necessary but not sufficient for Rho-mediated formation of stress fibres and focal complexes.

David Drechsel in the Hall laboratory has recently analysed the effects of point mutations in the Rho effector region on binding of Rho to its targets and on Rho's biological activity. An F39A L63 Rho mutant was unable to bind to Rho-kinase and to two other known Rho targets, PKN and Rhotekin, both in vitro and in the YTHS (Drechsel and Hall, unpublished observations). However, when microinjected into serum-starved Swiss 3T3 cells, F39A L63 Rho is completely unimpaired in its ability to mediate stress fibre and focal adhesion assembly. This result indicates that the interaction between Rho-kinase and Rho is not the trigger for stress fibre assembly. One possibility is that Rho interacts with a scaffold-like protein which then forms a complex with several important proteins including Rho-kinase. This idea is supported by the fact that Rho-mediated stimulation of Rho-kinase activity in vitro is modest (5 fold at best), especially when compared with activation of PAK by Rac or Cdc42 (typically 50-75 fold) (Leung et al., 1996). A search for proteins that bind to F39A L61 Rho is currently in progress and could provide further important insights into Rho-mediated cytoskeletal reorganisation.

3.3.3 Rho-kinase and the control of Myosin Light Chain phosphorylation

Kaibuchi and his colleagues have recently published several elegant studies shedding some light on why Rho-kinase activity might be important for stress fibre assembly. First, Rho-kinase α can directly phosphorylate Myosin Binding Subunit (MBS), the regulatory subunit of Myosin Light Chain (MLC) phosphatase and this results in a downregulation of phosphatase activity (Kimura et al., 1996). Furthermore, Rho-Kinase can directly phosphorylate MLC on serine 19. Phosphorylation of MLC is thought to increase MLC ATPase activity and binding to actin (Amano et al., 1996a). Thus, it
appears that Rho-kinase can control MLC binding to actin both by directly phosphorylating MLC and by downmodulating MLC phosphatase. Consistent with this hypothesis, the same group reported that transfection of cells with V14 Rho or with the catalytic domain of ROKα resulted in an increase in the cellular level of phosphorylated myosin (Kimura et al., 1996, Chihara et al., 1997). Bundling of diffuse actin filaments via MLC could account, at least in part, for Rho-kinase's effect on the cytoskeleton as shown in figure 3.14.

3.3.4 Other functions of Rho-kinase

Rho has been reported to be involved in calcium ion sensitivity of smooth muscle contraction (Hirata et al., 1992). Addition of the Rho-kinase α catalytic domain to Triton-X-100 permeabilised smooth muscle cells resulted in contraction, concurrently with an increase in the phosphorylated MLC content of the cells (Kureishi et al., 1997). Furthermore, a novel smooth muscle relaxant was shown to be a specific inhibitor of Rho-kinase β and blocked activated Rho-induced actin rearrangements in HeLa cells (Uehata et al., 1997). These reports demonstrate that Rho-kinase is a critical target of Rho in mediating smooth muscle contraction, most probably via actin-myosin regulation. The study of this pathway could provide insights into diseases such as arteriosclerosis. Rho-Kinase α was also implicated as a potential regulator of gene transcription via the SRF pathway (Chihara et al., 1997).
Figure 3.14 A model for Rho-kinase mediated assembly via activation of myosin

Signalling by extracellular agonists such as LPA via Rho triggers activation of Rho-kinase. Use of Rho effector site mutants suggests that Rho interacts with an unknown cellular target (X) which somehow leads to activation of Rho-kinase. Rho-kinase directly phosphorylates MLC (Myosin Light Chain) and inhibits MLC phosphatase activity by phosphorylating its regulatory subunit, MBS (Myosin Binding Subunit). This leads to an increase in cellular levels of phosphorylated MLC which results in an increase in MLC's affinity for actin. Thus, Rho-kinase is believed to cause bundling of diffuse actin filaments.
3.3.5 Rho-Kinase and Rac

In this chapter, I have also shown that Rho-kinase β interacts with Rac as well as Rho, both in vitro and in the YTHS. Using different biochemical assays, other groups that isolated the Rho-kinases failed to detect an interaction with Rac (Leung et al., 1995, Matsui et al., 1996b, Ishizaki, 1996). However, one other group has also observed a Rac-Rho-kinase α interaction in the YTHS (Joneson et al., 1996, Westwick et al., 1997). In collaboration with Dr Nathalie Lamarche (MRC Laboratory for Molecular Cell Biology), I showed that Rho-kinase β interacts with Y40C L61 Rac, but not with F37A L61 Rac (Lamarche et al., 1996). Since F37A L61 Rac does not induce lamellipodia or focal complex formation, this finding raises the possibility that Rho-kinase might have a function in cytoskeletal control downstream of Rac. Similar conclusions have been made by other groups (Joneson et al., 1996, Westwick et al., 1997).

So far, however, there is no direct evidence to suggest a role for Rho-kinase in Rac signalling. In fact, lamellipodia formation induced by Rac is not blocked by the Rho-kinase inhibitor Y-27632 (Uehata et al., 1997). The role of Rho-kinase downstream of Rac, if any, is therefore unclear. One way to clarify this issue would be to generate a mutation in Rho-kinase that restores its binding to the F37A mutant of L61 Rac. If co-expression of both the Rac mutant and the Rho-kinase mutant resulted in rescue of the F37A L61 Rac loss of phenotype, this would confirm Rho-kinase's role in Rac signalling.

Recently, a novel Cdc42 target called Genghis Khan (Gek) has been identified in Drosophila (Luo et al., 1997). This protein is closely related to the mammalian Rho-kinases, but does not bind Rho or Rac. Gek mutants have a disrupted actin organisation in the egg chamber. This raises the possibility that a family of kinases, including the Rho-kinases and Gek, are involved in cytoskeletal regulation downstream of the Rho-related GTPases. The hunt for Rho-kinase relatives with different GTPase specificities will no doubt generate a lot of interest over the next few years.
CHAPTER 4

CLONING AND BIOCHEMICAL CHARACTERISATION OF A NEW EFFECTOR FOR RAC

4.1 INTRODUCTION

The Rac GTPase regulates rearrangement of the actin cytoskeleton leading to the formation of lamellipodia, membrane ruffles and associated focal complexes in fibroblasts and a variety of other cell types (Ridley et al., 1992, Van Aelst and D'Souza-Schorey, 1997). Rac has also been implicated in growth control and cell transformation and has been reported to regulate gene transcription via the SRF, NFκB and the JNK and p38 MAPK pathways (Van Aelst and D'Souza-Schorey, 1997).

Like other Ras-related GTPases, Rac mediates its downstream effects by interacting with cellular target molecules and, as with Rho, it is expected that numerous Rac targets exist in mammalian cells. Consequently, much research activity has been focussed on identifying target proteins for the Rac GTPase. The serine/threonine kinase PAK was the first identified Rac target molecule (Manser et al., 1994) but, since then, around 8 Rac targets have been reported (see 1.6). Studying the function of these downstream molecules has not proved easy. PAK itself has been reported by some groups to activate the JNK cascade, though others have argued against this (Zhang et al., 1996, Brown et al., 1996, Bagrodia et al., 1995, Frost et al., 1996, Tang et al., 1997, Teramoto et al., 1996a, Teramoto et al., 1996b). MLKs and MEKK1/4 are also potential mediators of gene transcription downstream of Rac since they are members of the MAPKKK family of proteins (Van Aelst and D'Souza-Schorey, 1997). Other targets such as POR-1 and PI4P5K have been implicated in Rac's effect on the cytoskeleton (Van Aelst and D'Souza-Schorey, 1997). Amino acid substitutions at codon 40 (Y40C or Y40H) in the effector region of Rac interfere with its ability to activate PAK and JNK, but do not affect lamellipodia formation, cell growth or oncogenic
transformation. A mutation at codon 37 (F37A), on the other hand, results in loss of lamellipodia formation, transformation or cell growth without affecting PAK and JNK activation. These observations would appear to exclude PAK and JNK from playing a role in cytoskeletal organisation and cell growth downstream of Rac. However, there has been one report that a kinase-dead mutant of PAK can induce changes in the organisation of the actin cytoskeleton when overexpressed in cells. This chapter describes the cloning of a new Rac effector using the yeast two-hybrid system.

4.2 RESULTS

4.2.1 Isolation of a Rac-interacting protein in the yeast two-hybrid system

A Ras-transformed NIH 3T3 cell mixed (random-primed and oligo-dT-primed) cDNA library (kind gift from Dr Chandra Kumar, Schering-Plough, USA) in the prey vector pGAD-10 was screened using activated L61 Rac in the integrated vector pYTH6 as a bait. Approximately $10^7$ yeast colonies were screened for their ability to grow in the presence of 25mM 3-AT. The 30 fastest growing clones were replated on SC-Trp-Leu plates, grown for 3d and tested for expression of the LacZ reporter gene using the plate-lift assay. All 30 clones were positive for β-galactosidase activity. Clones were rescued from yeast, transferred to DH5α and plasmid DNA transformed back into the yeast strain containing the integrated L61 Rac. After this second round of transformations, only one clone, clone 4, was positive for expression of both the LacZ and HIS3 reporter genes.

In order to test the specificity of clone 4, pGAD-10-clone 4 was transformed into yeast strains containing L61 Rac, L63 Rho, L61 Cdc42, as well as the Rac effector mutants, F37A L61 Rac and Y40C L61 Rac. Colonies were picked from these transformations and replated on SC-Trp-Leu-His+25mM 3-AT to test for expression of the HIS3 reporter gene (figure 4.1). This experiment showed that clone 4 interacts with activated Rac, but not activated Rho or Cdc42. Furthermore, clone 4 interacts
with the F37A L61 Rac mutant, but not detectably with the Y40C L61 Rac mutant. Since the Y40C L61 Rac mutant is still able to induce actin rearrangements as well as DNA synthesis and cellular transformation (Lamarche et al., 1996, Joneson et al., 1996, Westwick et al., 1997), it is unlikely that Rac's interaction with clone 4 is the trigger for these processes. However, Y40C L61 Rac does not activate the JNK MAP Kinase pathway. As clone 4 does not bind to this Rac mutant, I concluded that it is a potential target for Rac in activating JNK.

Figure 4.1 Interaction of Clone 4 with GTPases and Rac effector mutants
Clone 4 in the pGAD-10 prey vector was transformed into yeast strains containing integrated L61 Rac, L63 Rho, L61 Cdc42, F37A L61 Rac, and Y40C L61 Rac in pYTH6. Colonies of equal size were replated in the presence of 25 mM 3-aminotriazole and allowed to grow for 3 days.
4.2.2 Biochemical analysis of clone 4

The clone 4 insert was 2.4 kbp in length and was subcloned from the yeast library vector pGAD-10 into pGEX-4T3 using BamHI and HindIII. Clone 4 protein was generated as a GST fusion in *E. coli*, purified as described in the methods and an aliquot was electophoresed on an SDS PAGE gel (figure 4.2). Several bands could be seen after coomassie staining, the largest of which had an apparent molecular weight of approximately 96 kDa. Therefore, the clone 4 ORF must encode a protein of around 67 kDa, which would correlate with the 2.4 kbp size of the insert.

![MW](image)

**Figure 4.2** Expression of GST-clone 4 in *E. coli*

GST-clone 4 was prepared as described in the method. 1/40 of the prepared volume was electophoresed on a 12% SDS PAGE gel. Proteins were visualised using coomassie blue. Molecular weight (MW) are prestained Rainbow markers (Amersham).
The clone 4 protein was tested in dot blot assays for binding to L61 Rac and the effector mutants Y40C L61 Rac and F37A L61 Rac (figure 4.3). In agreement with the yeast two-hybrid results (figure 4.1), clone 4 binds to L61 Rac and F37A L61 Rac, but not to Y40C L61 Rac. As discussed in 3.2, targets are expected to bind to GTPases in a GTP-dependent manner. Binding of clone 4 to GTP- and GDP-bound Rac was therefore tested as described in the methods (figure 4.4). Like PAK, another known Rac target, clone 4 interacted strongly with the GTP-bound form of Rac, while no binding could be detected in this assay with GDP-bound Rac.

**Figure 4.3** Clone 4 binds to F37A but not Y40C Rac *in vitro*

GST-clone 4 (10µg), GST-p65PAK (8µg), GST-RhoGAP (10µg, a.a. 198-439), and GST (10µg) were spotted on nitrocellulose and probed with [γ³²P]GTP-labelled Rac mutants. Radioactivity was visualised by 40min exposure to X-AR film.

POSH: Clone 4
GST-clone 4 was found not to have detectable GAP activity towards wildtype Rac (not shown). Clone 4 was tested for exchange activity as described in the methods (see figure 4.5). In this assay, GST-clone 4 displayed no exchange activity and is therefore unlikely to be a GEF. From these biochemical studies, I concluded that clone 4 is a good candidate effector for the Rac GTPase.

Figure 4.4 Clone 4 binds to Rac in a GTP-dependent manner
GST-clone 4 (10μg), GST-p65PAK (8μg) and GST (10μg) were spotted onto strips of nitrocellulose which were probed with radiolabelled GTP or GDP-bound wildtype Rac. Radioactivity was visualised by 1.5h exposure to X-AR film.
Figure 4.5  Clone 4 does not encode an exchange factor
A nucleotide exchange assay was performed using radiolabelled wildtype Rac (1μg) as described in the methods with GST-clone 4 (1μg) or 10mM EDTA as a positive control and GST (1μg) as a negative control.

4.2.3 DNA sequence analysis of clone 4
The clone 4 insert was sequenced on both strands using the automatic sequencing protocol as described in the methods. This revealed that the clone 4 protein is an ORF of 600 amino acids with a predicted molecular weight of 67 kDa (see figure 4.6 and 4.7). This predicted molecular weight correlated with the result obtained from expression of clone 4 as a GST-fusion as described in 4.2.
Figure 4.6 The DNA sequence of the clone 4 ORF
The stop codon is shown in bold.
Figure 4.7 Predicted protein sequence of clone 4
SH3 domains are underlined.

The clone 4 protein sequence was compared with known protein sequences in the databases at NCBI (National Center for Biotechnology Information) using the blastp program (Altschul et al., 1990). This revealed that clone 4 bears two SH3 domains between a.a. 165-219 and 546-600. In addition, clone 4 contains a region relatively rich in proline (a.a. 76-111) including three potential SH3-binding sites (pxxp). Another interesting region of the clone 4 sequence is a.a. 125-139 which contains 13 alanines, possibly forming a flexible hinge region. The first methionine of clone 4 (a.a. 18) is surrounded by a Kozak sequence (AGGATGG) and clone 4 might, therefore, be a full-length cDNA.

4.2.4 Isolation of a full-length clone 4 cDNA

Since no stop codons were present upstream of clone 4's first methionine, the possibility remained that clone 4 was not a full-length cDNA. 5' RACE PCR experiments were undertaken in an attempt to isolate sequences lying upstream of the two-
hybrid clone 4 sequence. RACE is a technique that allows isolation of sequences both 5' and 3' of known sequences using PCR. cDNAs are made from the desired mRNA source and ligated to a double stranded anchor oligomer of known sequence. Primers corresponding to this anchor are then used in combination with gene-specific primers for PCR.

Gene-specific PCR primers were synthesised corresponding to the minus strand of the clone 4 sequence bp 223-247 (RACE2) and 312-339 (RACE1) (5'-GTCACCGGCTGCTAGGGGGTGGGG-3' and 5'-CCAGGAGAGGGGGTGGGGGAAGTGGAGG-3'). A PCR-ready mouse whole embryo RACE cDNA library was purchased (Clonetech). The standard RACE PCR program (see methods) was performed using anchor primer 1 (Clonetech) and RACE1. 1/10 volume of this first reaction was used as template in a second reaction using the same PCR program with the nested anchor primer and RACE2 as primers. See figure 4.8 for a schematic representation of the RACE strategy. After the second reaction, 4μl of the PCR mix were electrophoresed on an agarose gel and the PCR product was visualised under UV light. A major RACE product appeared at 1.4kbp, which was not present when the gene-specific primer was omitted from the first PCR reaction.

![Diagram](image)

**Figure 4.8  Strategy for performing 5' RACE PCR**
The RACE fragment was digested using NotI and NcoI and subloned into pYTH9 for automated sequence analysis. The overlap between the RACE product and the two-hybrid clone was identical. This RACE experiment confirmed that the original clone 4 was not a full length cDNA. The ORF spanned a further 292 amino acids in the N-terminal direction to give a total ORF of 892 amino acids, with a predicted molecular weight of 93kDa (see figures 4.9 and 4.10 for full-length DNA and protein sequences of the ORF). The first methionine was surrounded by a Kozak sequence (AAGATGG). Furthermore, this methionine was preceded by an in-frame stop codon.

Analysis of the RACE fragment sequence revealed two additional SH3 domains (a.a. 137-190 and 199-254). Since the full-length cDNA contains a total of four SH3 domains, I decided to call it POSH (Plenty Of SH3s). The four POSH SH3 domain sequences (POSH1-4) were analysed in detail with the help of Dr Sarah Courtneidge (Sugen, USA). These SH3 domains are closely related to each other and, in addition, POSH1 and 4 have high homology with the second p67phox SH3 domain (51.9% and 42.6% similarity at the amino acid level respectively). An alignment of the POSH SH3 domains and some of their close relatives is shown in figure 4.11. In addition to the four SH3 domains, the POSH sequence contains a putative zinc finger (a.a. 12-82). See figure 4.12 for a diagram of the full-length POSH structure.
Figure 4.9 The DNA sequence of the POSH ORF
Stop codon is shown in bold and start codon is underlined.
Figure 4.10 Predicted protein sequence of POSH
Dotted line: Rac-binding domain. SH3 domains are underlined.

POSH1 pckakalmyegkepd lkskgldtiil rrvqdenwhygevs gvhgffmnopvqtyii
POSH2 pggklckalydfekdkealclplfakddvltl vrr vdenaegmla dkgifpsyvefn
POSH3 psv yvaiyptrkelelrgemf1 vfercgqd wykgtsmtskigvpgnyvapv
POSH4 vcerhrvvsyppqseaelelk egdivf vhkkredg wfgtqlqrngktlpgsfvendi
p67-2 sqvealfsytaped lefqegd iilvl skvnewlegeck gkvgifpkvfvedc
Grb2-2 pccralydfepenege lgfkegdiitlnqi denwyegmlh shsffpynyveil

Figure 4.11 Protein alignment of the POSH (1-4), p67phox (2nd) and Grb2 (1st) SH3 domains
Alignments were constructed using Sequence Alignment and Modelling Software System (U.C. Santa Cruz).
4.2.5 Identification of the clone 4 Rac-binding domain

In order to determine the region of POSH important for binding to Rac, various truncations were made, expressed as GST-fusion proteins in E. coli and tested using the dot blot assay. Truncations were generated by PCR using Pfu polymerase. The primers were designed to introduce a BamHI site at the 5’ end and an EcoRI site at the 3’ end of the PCR product (see table 4.1). PCR products were digested with BamHI and EcoRI and ligated with pGEX-4T3 cut with the same enzymes. See figure 4.13 for schematic representation of the truncations that were generated in this way. Dot blot analysis revealed that amino acids 292-362 of POSH were sufficient for binding to Rac (see figure 4.13).

<table>
<thead>
<tr>
<th>POSH truncations</th>
<th>PCR Primers</th>
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<tbody>
<tr>
<td>POSH T1</td>
<td>5’ sense</td>
</tr>
<tr>
<td></td>
<td>gcgggatcccccaccccctctctcttgccggcc</td>
</tr>
<tr>
<td></td>
<td>3’ antisense</td>
</tr>
<tr>
<td></td>
<td>gcggaatccggaccaactttaactctcta</td>
</tr>
<tr>
<td>POSH T2</td>
<td>5’ sense</td>
</tr>
<tr>
<td></td>
<td>gcgggattcctcgaccgaccaagagagaaac</td>
</tr>
<tr>
<td></td>
<td>3’ antisense</td>
</tr>
<tr>
<td></td>
<td>gcggaatccgtactccctctgcagctccag</td>
</tr>
<tr>
<td>POSH T3</td>
<td>5’ sense</td>
</tr>
<tr>
<td></td>
<td>gcgggattcctcgacaccaagagagaaac</td>
</tr>
<tr>
<td></td>
<td>3’ antisense</td>
</tr>
<tr>
<td></td>
<td>gcggaatccgtacagtttattagatgggtg</td>
</tr>
</tbody>
</table>

Table 1  PCR primers used for constructing POSH truncations
The Rac-binding domain of POSH was screened for homologies with other proteins known to bind to Rac using the Macaw program. POSH a.a. 292-362 contains no CRIB motif, nor does it have homology to p67phox. I concluded from this that POSH belongs to a new class of Rac effector. No homologies between the POSH Rac-binding domain and known sequences were found using the blastp program at the NCBI.

![Diagram of Rac-binding domain of POSH with truncations]

**Figure 4.13 Identification of the Rac-binding site of POSH**

10μg of GST-fusion proteins of clone 4 and truncations were spotted onto nitrocellulose and tested for interaction with L61 Rac. TH: clone 4 from two hybrid screen.

- T1: Truncation 1 (a.a. 352-892)
- T2: Truncation 2 (a.a. 292-398)
- T3: Truncation 3 (a.a. 292-362)

Pro: Proline-rich region
RBD: Rac-binding domain
4.2.6 Tissue distribution of POSH

Rac is expressed in most cell types (Moll et al., 1991). Some Rac effectors are tissue-restricted, such as p67phox which is expressed only in hematopoietic cells. It was important to determine the tissue distribution of POSH in order to know if it could potentially be involved in Rac signalling in a wide variety of tissues. A cDNA fragment corresponding to bp 1-325 of full-length POSH was generated by restriction digest and radioactively labelled with $[\alpha\text{-}^{32}\text{P}]dCTP$. This fragment was used to probe a mouse multiple tissue northern blot (Clonetech). The blot was washed and the remaining radioactivity was visualised using Kodak X-AR film (Figure 4.14).

![Figure 4.14 Tissue expression of POSH](image)

A mouse multiple tissue Northern blot was probed as described in the methods. Radioactivity was visualised by 14h exposure to X-AR film. Lane 1: Testis, 2: Kidney, 3: Skeletal Muscle, 4: Liver, 5: Lung, 6: Spleen, 7: Brain, 8: Heart.
POSH mRNA was present as a single band at approximately 5 kilobases in most tissues. On overexposure, bands could be seen in all lanes. Therefore, POSH is expressed ubiquitously. However, kidney and brain appeared to contain relatively high levels of transcript, while spleen and skeletal muscle had low levels.

As described in 4.2.4, the first methionine of the two hybrid clone 4 is surrounded by a strong Kozak consensus, raising the possibility that the POSH message might be alternately spliced. In order to investigate this possibility, the northern blot was stripped and reprobed with a radioactive cDNA generated from the 3' end of POSH (bp 1747-2472). An identical pattern of expression and size of transcript was obtained as in figure 4.14. I therefore concluded that POSH is transcribed as a single mRNA species.

4.3 DISCUSSION

This chapter describes the cloning and biochemical characterisation of POSH, a new target molecule for Rac. POSH binds Rac in a GTP-dependent manner and is expressed ubiquitously. Interaction of POSH with Rac effector mutants suggests that POSH might mediate JNK activation, but not actin polymerisation, downstream of Rac. Finally, POSH is composed of multiple protein-interaction domains, indicating that it might function as a scaffold molecule.

4.3.1 The role of SH3 domains

SH3 domains are protein modules of approximately 60 amino acids that are thought to form discrete domains within proteins and mediate protein:protein interactions (Pawson and Gish, 1992). SH3 domains have been found in a large number of signalling molecules, such as kinases (Fyn, Src, Abl), adaptor molecules (Grb2, Crk, Nck) or cytoplasmic components of the NADPH oxidase (p67phox, p40phox, p47phox). An increasing body of evidence supports the idea that proline-rich regions are the ligands for SH3 domains (Pawson, 1995). PXXP appears to be a basic requirement for SH3-binding sites, but additionnal
prolines are common. SH3 domains are present in all eukaryotes including yeast (unlike SH2 domains) and they are thought to be crucial for the assembly of protein complexes during signal transduction. Given its four SH3 domains, it seems likely that POSH is a scaffolding protein responsible for bringing components of a signalling cascade together.

4.3.2 SH3 domain-containing proteins in Rho GTPase-dependent pathways.

p67phox is a cytoplasmic component of the phagocytic NADPH oxidase enzyme complex (Segal and Abo, 1993). This membrane-bound complex is responsible for generation of superoxide which forms a part of the pathogen-killing mechanism of professional phagocytes. Rac was shown to be critically involved in regulating the activity of the NADPH oxidase through direct interaction with its effector p67phox, although the precise mechanism by which Rac mediates an increase in enzyme activity is not yet understood (Diekmann, 1995). p67phox has two SH3 domains which show close homology to the first and fourth SH3 domains of POSH (51.9% with POSH 1, 42.6% with POSH 4). This raises the possibility that POSH might fulfill a similar function to that of p67phox in nonphagocytic cells and in fact, Rac has been reported to induce the production of reactive oxygen species in HeLa cells (Sulciner et al., 1996). To date, NADPH oxidase components have not been described in nonphagocytic cells.

The SH3-containing protein Bemlp plays a crucial role in signalling mediated by Cdc42p in budding yeast (reviewed by Leberer et al., 1997a). Bemlp contains two SH3 domains, though these show no special degree of homology to POSH. Interestingly, Bemlp is known to play a role in the regulation of the MAP Kinase pathway that controls mating pheromone response (Leberer et al., 1997a). Bemlp interacts directly with Cdc24p, the exchange factor for Cdc42p, as well as with Ste20, a target for Cdc42p (the first kinase on the pheromone MAP kinase pathway and a PAK homolog). In fission yeast, the Bemlp homolog (Scd2) interacts directly with Scd1, an exchange factor.
for Cdc42 and with Cdc42 itself (Chang et al., 1994). No Bem1p/Scd2 equivalents have been found in higher eukaryotes.

Although POSH does not have homology to Bem1p or p67phox outside the SH3 domains, it is tempting to think that, like these two proteins, POSH might act as a scaffold molecule, bringing together various components of a Rac signalling pathway. Attempts to isolate POSH-binding proteins are currently under way in the Hall laboratory to address this possibility.
CHAPTER 5

FUNCTIONAL CHARACTERISATION OF POSH

5.1 INTRODUCTION

Although many cellular targets for Rho GTPases have been identified, most of these molecules have, at best, poorly characterised functions (with the notable exception of Rho-kinase, see chapter 3). This may be due to the fact that multiple activities are required to induce cellular responses similar to the GTPases themselves. In the previous chapter, I described the identification of a new Rac effector, POSH. POSH does not interact with Y40C L61 Rac \textit{in vitro} or in the yeast two-hybrid system. This Rac effector mutant is functional in assays for actin rearrangement, DNA synthesis and transformation but is unable to activate the JNK MAP Kinase pathway (Lamarche \textit{et al.}, 1996, Joneson \textit{et al.}, 1996, Westwick \textit{et al.}, 1997). This raises the possibility that POSH might mediate Rac's effect on the JNK pathway. This chapter describes the analysis of the cellular function of POSH.

5.2 RESULTS

5.2.1 POSH induces apoptosis

In order to carry out the functional analysis of POSH, various POSH constructs were made in the mammalian expression vector pRK5myc (figure 5.1). The original two-hybrid clone (TH, lacking the first two SH3 domains) was transferred from pGEX-4T3 using BamHI and HindIII. A truncation that did not bind Rac \textit{in vitro} (T1) was transferred using the same enzymes. Full length POSH was reconstituted by introducing a BamHI site at the 5' end of the RACE product and inserting it into a construct already containing the original two-hybrid clone in pRK5myc using BamHI and NcoI. These constructs will be referred as POSH TH, POSH T1 and POSH FL respectively (see figure 5.1 for a schematic representation).
Although the binding characteristics of POSH to Rac effector mutants (both in yeast and \textit{in vitro}) appeared to exclude it from cytoskeletal regulation, it was still important to verify that POSH overexpression could not induce actin polymerisation. POSH DNA constructs were microinjected into serum-starved quiescent Swiss 3T3 cells which were then incubated for various times (1h, 3h, 5h, 8h, 14h). Filamentous actin was visualised using TRITC-conjugated phalloidin. As shown in figure 5.2, overexpression of POSH FL or POSH TH did not induce changes in the actin cytoskeleton after 3h (panels c and e respectively). No effect was seen at any of the other time points (not shown). POSH itself was visualised in these cells using anti-myc antibody and was present mainly as dense clusters (see figure 5.2).
Figure 5.2  Overexpression of POSH does not cause reorganisation of the actin cytoskeleton
Confluent, quiescent, serum-starved Swiss 3T3 cells were microinjected with POSH FL (b,c) or POSH TH (d,e) in the myc-tagged pRK5 vector at a concentration of 0.1mg/ml. (a) is a control non-injected cell. Cells were incubated for 3h at 37°C then fixed and stained with anti-myc for POSH (b,d) and rhodamine-phalloidin for F-actin (a,c,e). Scale bar is 20μm.
During the course of these microinjection studies, it became apparent that the majority of POSH FL-injected cells were not staying on the coverslips at the later time points (8h-12h). Furthermore, POSH TH and POSH T1 did not have this effect. In an attempt to determine whether POSH FL was causing cell death, NIH 3T3 cells were injected with POSH FL, POSH T1 and L61 Rac, left to express for 14h in the presence of 10% serum and the surviving cells were counted. Nuclear morphology was examined using Hoechst staining. As shown in figure 5.3, POSH FL induced cell death in 90% of injected cells, while Rac or POSH T2 had no effect. Most of the remaining POSH FL-injected cells had shrunken and condensed (pyknotic) nuclei (figure 5.3 bottom, middle panel) that are typical of cells undergoing apoptosis (Jacobson et al., 1997). The POSH-FL injected cells were often rounded up and on a different plane of focus from healthy uninjected cells (seen as halos in figure 5.3 bottom, middle panel). Rac- or POSH T1-injected cells had normal nuclear morphologies (figure 5.3 bottom). Similar results were obtained in primary Rat Embro fibroblasts (not shown, REFs were a kind gift from Dr C. Nobes, MRC LMCB)

In order to further characterise the effect of POSH FL overexpression, NIH 3T3 cells were injected with POSH FL and visualised using time-lapse video microscopy (figure 5.4). In the absence of serum, POSH FL-injected cells appeared normal for the first 2h, then rapidly retracted and rounded up. Rounding up was rapidly followed by intense membrane blebbing. These morphological events are also typical of the apoptotic programme (Jacobson et al., 1997). Cells microinjected with L61 Rac on the same coverslip as a control were still alive after 16h (not shown). In the presence of 10% DCS (Donor Calf Serum), the cells survived for 5h, then underwent the same sequence of events as shown on figure 5.4. From these experiments, I conclude that POSH overexpression induces apoptotic cell death in primary or immortalised fibroblasts.
Figure 5.3 Full-length POSH induces apoptosis in NIH 3T3 cells

NIH 3T3 cells were replated on glass coverslips and left for 24 hours in DMEM containing 10% DCS and the nuclei of 50 cells were injected with an expression vector containing POSH FL, POSH T1 (a.a. 352-892) or L61 Rac at a concentration of 0.04mg/ml. The cells were fixed and stained after a 14h incubation in the presence of serum. Bottom panel shows typical nuclear morphology of injected cells (arrows) using Hoechst. In the middle panel, non-injected cell nuclei are in a different plane of focus from the rounded up POSH injected cells. Scale bar: 20μm. The error bars represent standard deviation over three independent experiments.
Figure 5.4 Morphological aspects of POSH-induced cell death

NIH 3T3 cells were serum-starved overnight and then microinjected with POSH FL (0.1mg/ml). Cells were photographed at the indicated times after microinjection.
5.2.2 POSH activates the JNK pathway

As discussed in 4.3, POSH is a potential mediator of Rac-induced JNK activation. In an attempt to define a role for POSH in JNK activation, POSH DNA constructs were cotransfected with FLAG-tagged JNK1 into Cos-1 cells as described in the methods. However, as in fibroblasts, POSH FL appeared to prove toxic to Cos-1 cells, particularly when coexpressed with JNK. This was apparent in the fact POSH FL expression was barely detectable when overexpressed with JNK (figure 5.5, lane 2). Again the POSH truncations did not have such an effect (figure 5.5, lane 3).

Figure 5.5 The caspase inhibitor BocDfmk restores POSH expression in transfected Cos-1 cells
Cos-1 cells were cotransfected with pCMV5FLAG-JNK1 (5μg) and pRK5myc-POSH FL (Lanes 1 and 2), pRK5mycPOSH T1 (Lane 3) (all 3μg) in the presence (+) or absence (-) of BocDfmk at 20μM. Cells were harvested in 300μl of 3x protein sample buffer, and 1/10 of the lysates were analysed by western blotting with anti-myc and anti-JNK1 antibodies.
In order to try and overcome this problem, the caspase inhibitor BocD-fmk (Boc-Aspartic acid-fluoromethylketone) (Weil et al., 1997, Deshmukh et al., 1996) was added to the media used to maintain the transfected cells. Caspases (such as ICE-like proteases) are important mediators of apoptosis and peptide inhibitors of these proteins have been shown to block cell death (Weil et al., 1997, Deshmukh et al., 1996). As shown in figure 5.5, lane 1, treatment of transfected cells with BocD-fmk largely overcame the toxic effects of POSH FL+JNK cotransfection in Cos-1 cells.

Under these conditions, JNK assays could be performed (figure 5.6). These experiments revealed that POSH FL overexpression induced a 5.6 fold stimulation of JNK activity, compared with 11.0 fold for L61 Rac. POSH T1, on the other hand, did not stimulate JNK activity (figure 5.6). When co-transfected with L61 Rac, the POSH T1 fragment did not inhibit L61 Rac-induced JNK activation (figure 5.7).
Figure 5.6 POSH activates JNK in Cos-1 cells

pCMV5FLAG-JNK1 was cotransfected with empty pRK5myc vector (lane 1), pRK5myc-L61 Rac (lane 2), pRK5myc-POSH T1 (a.a. 352-892) (lane 3), or pRK5myc POSH FL (lane 4) into Cos-1 cells, all in the presence of 20μM BocDfmk. Aliquots of each transfection were electrophoresed on an SDS PAGE gel and expression levels of transfected constructs were visualised on western blots using anti-myc (for POSH, Rac), and anti-FLAG (for JNK1) antibodies followed by [\(^{125}\)I]-labelled protein A (top panel). JNK1 activity was assayed on immune complexes using GST-c-jun as a substrate and quantified on a Biorad Molecular Imager (middle panel). Levels of JNK1 in the immunoprecipitates were visualised using an anti JNK1 antibody (bottom panel).
Figure 5.7 POSH T1 does not inhibit L61 Rac-induced JNK activation

Cos-1 cells were cotransfected with pCMV5FLAG-JNK1 and either pRK5myc-L61 Rac plus pRK5myc POSH T1 (lane 1), pRK5myc POSH T1 (lane 2), or pRK5myc-L61 Rac (lane 3). JNK1 activity was assayed as for figure 5.6.

5.2.3 POSH stimulates NFkB translocation to the nucleus

Two reports have shown that the transcription factor NFkB can be activated by Rac (Sulciner et al., 1996, Perona et al., 1997). Furthermore, agonist-induced JNK activation is often accompanied by NFkB activation (Lee et al., 1997). To examine whether POSH might affect NFkB regulation, DNA constructs were microinjected into serum-starved Swiss 3T3 cells and
translocation of NFκB into the nucleus was visualised by immunofluorescence 5h later. As shown in figure 5.8, both POSH FL and POSH T1 are potent activators of NFκB translocation. L61 Rac and F37A L61 Rac also stimulated NFκB translocation, but Y40C L61 Rac did not. Since POSH binds to F37A L61 Rac, but not to Y40C L61 Rac, these results are consistent with POSH being an effector for Rac in NFκB activation.

It was noted that NFκB translocation often occurred in neighbouring, non-injected cells (figure 5.9, bottom, see arrow). This effect correlated with NFκB translocation in injected cells. It is likely that nuclear translocation of NFκB induced by Rac and POSH is dependent on induced autocrine/paracrine factors.
Figure 5.8  POSH induces nuclear translocation of NFκB in Swiss 3T3 cells
Myc-tagged pRK5 plasmids (0.04mg/ml) encoding POSH FL, POSH T1 (a.a.352-892), L61 Y40C Rac, L61 F37A Rac, L61 Rac, or empty vector (coinjected with FITC-dextran) were microinjected into serum-starved, confluent Swiss 3T3 cells and the cells fixed 5h later. POSH/Rac expression was visualised using anti-myc antibody, while NFκB localization was visualized using an anti-NFκB antibody (Santa Cruz). The percentage of myc-positive cells in which clear nuclear fluorescence of NFκB was seen were scored as positive. Results were averaged over four independent experiments and between 28 and 85 myc-expressing cells were analyzed per experiment. Error bars represent standard deviation over four independent experiments.
Figure 5.9  **NFκB is activated in neighbouring, non injected cells**

The right panel shows a typical phenotype of a myc-positive, nuclear NFκB positive cell after injection with POSH T1. The arrow points to a neighbouring, non-injected cell that is nuclear NFκB positive. The left panel shows a typical phenotype of a myc-positive, nuclear NFκB negative cell after injection with L61 Y40C Rac.
5.3 DISCUSSION

5.3.1 POSH and the JNK pathway

In this chapter I have shown that overexpression of full-length POSH leads to JNK activation in Cos-1 cells. Together with POSH's binding characteristics to Rac effector mutants as described in chapter 4, this suggests that the interaction of Rac with POSH could be the trigger for JNK activation. Cotransfection of Rac and a dominant negative version of POSH would confirm that this is the case. However, the C-terminal fragment of POSH (POSH T1) did not show a dominant negative effect. Construction of N-terminal fragments of POSH is currently in progress in the laboratory and should hopefully address this question.

The fact that overexpression of an SH3-containing protein with no catalytic activity can activate JNK is perhaps surprising. However, Lyons et al reported that Bem1p overexpression in yeast could substantially potentiate pheromone-stimulated Fus3 (the last kinase in the pheromone pathway) activation in a wildtype genetic background (Lyons et al., 1996). Furthermore, in a STE11-4 genetic background, Bem1p overexpression alone was able to activate Fus3 independently of pheromone treatment. STE11-4 is a mildly activated allele of the MAPKKK Ste11p and gives a slightly elevated level of basal kinase activity. This might well resemble the situation in a Cos cell transfection where the transfected cells may have high endogenous basal activities of numerous signalling cascades through autocrine/paracrine loops. It is also becoming clear that the assembly of multimolecular signalling complexes is required for activation of MAPK pathways (Leberer et al., 1997a). It is possible that POSH overexpression is able to trigger the formation of such a complex leading to activation of the JNK pathway. Alternatively, POSH might cause JNK activation by sequestering an inhibitor of the JNK pathway. Identification of POSH-binding proteins should resolve this issue. Rac has been reported to activate the p38 MAPK cascade as well as the JNK cascade (Van Aelst and D'Souza-Schorey,
It would, therefore, be interesting to test whether POSH can also activate p38.

5.3.2 POSH and NFκB

In this chapter, I have also shown that overexpression of POSH in Swiss 3T3 cells results in NFκB translocation to the nucleus. Furthermore, L61 Rac and F37A L61 Rac, but not Y40C L61 Rac, also stimulated NFκB translocation. Since POSH binds F37A L61 Rac but not Y40C L61 Rac, this suggests that POSH might mediate NFκB activation downstream of Rac.

NFκB and JNK activation are often co-activated by stimuli such as TNFα or IL-1 (Kyriakis and Avruch, 1996). It is clear from the experiments described here that POSH activates JNK and NFκB via distinct pathways, since the C-terminal truncation POSH T1 still stimulates NFκB translocation but has no effect on JNK. How Rac and POSH exert their effect on NFκB is not clear. It has been shown that transfection of activated Rho, Rac and Cdc42 into Cos-7 cells triggered an increase in IκB phosphorylation (Perona et al., 1997). Furthermore, the microinjection assay used in these studies suggests that an autocrine/paracrine factor is released, since stimulation of NFκB is observed in neighbouring non-injected cells. It is also possible that this factor might be responsible for NFκB translocation in the injected cells. Sulciner et al have reported that Rac-induced NFκB activation in HeLa cells can be blocked by the reactive oxygen species (ROS) scavenger NAC (Sulciner et al., 1996). Furthermore this group and others have shown that Rac can mediate ROS production in non-phagocytic cells (Sulciner et al., 1996, Irani et al., 1997). Since POSH has some homology to p67phox, it is tempting to speculate that POSH might fulfill a similar function as a regulator of a non-phagocytic oxidase. Further studies involving measurement of ROS release from POSH-transfected cells should resolve this issue.

5.3.3 POSH and cell death

An unexpected result of this work was POSH's ability to induce apoptosis in several cell types even in the presence of
serum. Since Rac does not have this effect, it is possible that Rac triggers other signalling events that protect the cell against POSH-induced cell death. Interestingly, some very recent studies have established a connection between Rho GTPases and cell death. Chuang and colleagues showed that stable transfection of Cdc42 into Jurkat T cells caused extensive cell death and went on to demonstrate that this effect could be blocked both by caspase inhibitors and dominant negative components of the JNK/SAPK pathway (Chuang et al., 1997). Moreover, a transgenic mouse expressing a thymus-directed L61 Rac2 transgene had highly increased levels of apoptosis in its thymus. (Lores et al., 1997). These data provide a potentially interesting connection between Rho GTPases, JNK activation and apoptosis. Indeed, other upstream components of the JNK/SAPK pathway, such as MLK2/3, or ASK1 have also been shown to induce cell death (Nagata et al. 1998, Ichijo et al., 1997, Herdegen et al., 1997). JNK is also known to be activated as part of the apoptotic response to the Fas-binding protein Daxx, or NGF withdrawal in neurons (Yang et al., 1997, Xia et al., 1995).

In my NIH 3T3 cell death assay, dominant negative SEK1 (SEK AL) did not block POSH-induced apoptosis (data not shown). However, since there are numerous intermediates in the JNK pathway, it is possible that POSH might mediate its effect on JNK via other MAPKKs (such as M KK7 or MEK5). Therefore, I cannot conclude that POSH-induced cell death is independent of JNK activation. A detailed analysis of POSH truncations might reveal whether or not POSH can induce JNK activation independently of apoptosis.
CHAPTER 6

FINAL DISCUSSION

Rho and Rac are two members of the Rho GTPase family that have been implicated in the regulation of the actin cytoskeleton. Rho has been shown to trigger the formation of stress fibres, while Rac induces lamellipodia and membrane ruffles. In addition, there is mounting evidence that Rho and Rac have important regulatory functions in controlling cell growth, transformation and gene transcription. It is thought that Rho and Rac control these various pathways by interacting with a variety of effector molecules. In the past three years, many groups have identified and analysed the function of new effectors for Rho and Rac. The methods of choice in these studies have been affinity chromatography, ligand overlay assays and the yeast two-hybrid system. This thesis describes the identification of two novel target molecules, one for Rho and one for Rac, using the yeast two-hybrid system.

Rho:

A yeast two-hybrid library was screened with activated L63 Rho in order to find proteins that interact with GTP-bound Rho. The Rho-binding region of the serine/threonine kinase Rho-kinase β was identified using this strategy. A partial cDNA (lacking the kinase domain) was isolated from a bacteriophage library and biochemical analysis revealed that Rho-kinase β interacts with Rho and Rac \textit{in vitro}. Rho was shown to bind to Rho-kinase β in a GTP-dependent manner.

Mammalian expression constructs encoding full-length Rho-kinase α (Rho-kinase β's close relative) and the Rho-kinase α catalytic domain were obtained from Dr Kozo Kaibuchi and microinjected into serum-starved Swiss 3T3 cells. Overexpression of these proteins triggered the formation of actin:myosin filaments though these appear to be distinct from Rho-induced stress fibres. Furthermore, David Drechsel, in the Hall laboratory, showed that an F39A L63 Rho mutant which
does not bind Rho-kinase is completely unimpaired in its ability to form stress fibres. Finally, it has been reported by others that Rho-induced stress fibre formation is blocked by a Rho-kinase β mutant deficient for both kinase activity and for Rho-binding (Ishizaki et al., 1997). Taken together, these experiments indicate that Rho-kinase is required for Rho-induced stress fibre formation, but that the binding of Rho to Rho-kinase is not the trigger for actin rearrangement. Other proteins in addition to Rho-kinase are, therefore, required to form stress fibres. It will be of great interest to identify proteins that are still able to interact with F39A L63 Rho. Such a protein might, for example, bind to both Rho and Rho-kinase, or be required for Rho-kinase activation. Many other Rho effectors such as Rhotekin, Rhophilin or Citron have been identified, but not functionally characterised, and there is a possibility that these might also play a role in stress fibre formation.

In these studies, I have also shown that Rho-kinase interacts with Rac in vitro and in yeast. Furthermore, in collaboration with Dr Nathalie Lamarche in the Hall laboratory, I have demonstrated that Rac-Rho-kinase binding is disrupted by an F37A mutation in Rac. This mutation results in the loss of Rac's ability to trigger lamellipodia formation, suggesting that Rho-kinase might play a role in Rac signalling. However, a dominant negative form of Rho-kinase β and an inhibitor of Rho-kinase β activity failed to block Rac-induced ruffling, which would argue against a role for Rho-kinase in Rac signalling (Ishizaki et al., 1997). Recently, a Cdc42-specific Rho-kinase-related protein called Genghis Khan has been cloned in Drosophila and shown to be involved in control of actin (Luo et al., 1997). This raises the interesting possibility that a Rac-specific Rho-kinase-related protein may exist in mammals.

**Rac:**

In the second part of this work, a yeast two-hybrid library was screened using L61 Rac as a bait. This led to the isolation of POSH, a protein that contains four SH3 domains. POSH binds to Rac in a GTP-dependent manner, but not to Rho or Cdc42. A plasmid encoding POSH was introduced into fibroblasts and
found to be a potent inducer of apoptosis, while a truncation lacking the first two SH3 domains did not have such an effect. Rac itself does not induce cell death in fibroblasts and the reason for this discrepancy is not clear.

A Y40C L61 Rac mutation was shown to disrupt the interaction between POSH and Rac and since this mutation blocks Rac's ability to activate the JNK pathway, POSH is a potential effector for Rac in the JNK pathway. In agreement with this, overexpression of POSH leads to activation of JNK in Cos-1 cells. A truncation lacking the first two SH3 domains and the Rac-binding domain of POSH did not activate JNK. These results suggest that POSH is an effector of Rac in mediating JNK activation. However, in order to formally prove that this is the case, a dominant negative version of POSH will be required (which should block Rac-mediated JNK activation). Various truncations of POSH are currently being constructed in the Hall laboratory (the C-terminal two SH3 domains of POSH did not act as a dominant negative) and it is hoped that this will answer that question. How POSH feeds into the JNK pathway is unclear, but it is tempting to postulate that it might interact with one or more of the kinases in this pathway. Candidates include other Rac targets such as PAK, MLK or MEKK4 which have been implicated in JNK signalling.

Overexpression of full-length POSH or truncated POSH in Swiss 3T3 cells caused NFkB translocation to the nucleus. As reported by two other groups, Rac also induced NFkB translocation (Sulciner et al., 1996, Perona et al., 1997). Y40C L61 Rac which does not bind POSH, was unable to cause NFkB translocation suggesting that POSH is also implicated in Rac-induced NFkB activation. Activation of NFkB by Rac and POSH was often accompanied by activation of NFkB in neighbouring non-injected cells, suggesting the involvement of an autocrine factor. It is not clear how POSH or Rac activate NFkB. One possibility is through the generation of superoxide radicals, since POSH has some sequence homology to another Rac effector, p67phox; a regulator of the phagocytic oxidase complex.

From the experiments described above, I conclude that POSH is a target that may mediate some of Rac's effects on gene
transcription and in particular transcriptional events regulated by JNK and NFkB. Given that POSH contains multiple protein interaction modules, it is likely that it functions as a scaffold-like protein, bringing together different components of a signalling cascade. Isolation of POSH binding proteins is currently in progress and should provide further insight into POSH function.
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Additions:

On page 85 after the first paragraph:
The Leucine substitutions (L63 for Rho, L61 for Rac, L61 for Cdc42 and L87 for R-Ras) are equivalent to a naturally occurring oncogenic mutation in Ha-Ras (Hall, 1990). The result of these mutations is to block the intrinsic and GAP-stimulated GTPase activity and thus the GTPases are locked in the active (GTP-bound conformation).

On page 99 after the first paragraph:
All constructs in pRK5 were myctagged at the N-terminus as described (Lamarche et al., 1996).